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Enhancer activity of light-responsive regulatory elements in the untranslated leader regions of cyanobacterial *psbA* genes

(photosynthesis/photosystem II/promoter/reporter gene/*Synechococcus*)

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ABSTRACT Three *psbA* genes encoding the D1 protein of the photosystem II reaction center are differentially expressed under different light intensities in the cyanobacterium *Synechococcus* sp. strain PCC 7942. Two of the three *psbA* genes, *psbAII* and *psbAIII*, are induced rapidly when light intensity is increased from 125 × 10^{-6} mol·m^{-2}·s^{-1} to 750 × 10^{-6} mol·m^{-2}·s^{-1}. A recombinational cloning vector that carries a transcriptional *lacZ* reporter gene was used to characterize the controlling elements responsible for light induction. At least three distinct cis elements are present in the regulatory regions of *psbAII* and *psbAIII*: basal promoters, comparable to *Escherichia coli* σ^{70} promoters in position and sequence, confer constitutive expression of the genes under both low and high light intensities; negative elements upstream of the promoters down-regulate the expression of the corresponding gene; and sequences downstream of the promoters that correspond to the untranslated leader regions of the mRNAs (+1 to +41 in *psbAII* and +1 to +39 in *psbAIII*) are responsible for increased expression under high light. When these light-responsive elements were combined with an *E. coli* promoter (*conII*) in different positions and orientations, the expression of the *lacZ* gene was induced 4- to 11-fold. The induction of gene expression under high light by these enhancers was position independent but orientation dependent. When the elements were combined with the *conII* promoter in the correct orientation, they also conferred a small but reproducible level of light-responsive expression on this *E. coli* promoter.

Cyanobacteria are photosynthetic prokaryotes that carry out oxygenic photosynthesis through a series of two reaction centers (photosystems) which are homologous to those of higher plants (1). Photosystem II undergoes a photochemical reaction which results in charge separation across the thylakoid membrane and the oxidation of water to provide electrons for the oxidized chlorophyll. The reaction center core of photosystem II contains a dimer of two proteins, D1 and D2, which are encoded by the *psbA* and *psbD* genes, respectively. D1 and D2 house the photoreactive chlorophyll, primary and secondary electron acceptors, and other cofactors involved in electron flow through photosystem II (2).

Synechococcus sp. strain PCC 7942 (hereafter referred to as *Synechococcus*) has three *psbA* genes which encode two distinct forms of the D1 protein (3). These three *psbA* genes are differentially expressed in response to changes in light intensity (4). Under light intensities lower than 250 μE·m^{-2}·s^{-1} [1 μE (microeinstein) = 10^{-6} mol of photons], the *psbAI* transcript dominates the *psbA* mRNA pool (3), but when light intensity increases, *psbAII* and *psbAIII* transcript levels increase rapidly while the *psbAI* transcript level decreases (5). Both transcriptional induction of *psbAII* and *psbAIII* and accelerated degradation of the *psbAI* and *psbAIII* messages contribute to this response (6).

In prokaryotes, several classes of positive and negative regulatory systems that influence transcription have been studied. Promoters of two basic kinds, those recognized by RNA polymerase containing either σ^{70} or σ^{54} subunits, have specific conserved basal elements and distinctive additional types of cis-regulatory elements: the former, when subject to repression or activation, usually has a control site located near the promoter to allow direct communication between specific trans-acting factors and RNA polymerase; the latter, however, is regulated solely by activation (7, 8). Enhancers, whose function is position and orientation independent, are associated with several σ^{54}-type promoters, and their involvement in gene regulation through DNA looping has been established (9, 10).

We report here the identification of cyanobacterial promoters and their accessory elements which drive light-responsive expression of the *Synechococcus psbAII* and *psbAIII* genes. The regulatory regions have several novel features: they combine basal promoters of the σ^{70} type, which do not require activation, with stimulatory elements that have the properties of enhancers; the enhancer elements are normally located immediately adjacent to the promoter but are also active when moved ≈200 bp away; the enhancer elements stimulate expression in response to high light intensity when present in their native orientation; and the native position of the enhancer elements is downstream of the transcription start sites.

MATERIALS AND METHODS

Strains. Wild-type *Synechococcus* sp. strain PCC 7942 (previously referred to as *Anacystis nidulans* R2, Pasteur Culture Collection no. 7942) was the genetic background into which reporter gene fusions were introduced by transformation (11). Transformed reporter strains were selected on modified BG-11 (12) agar plates containing spectinomycin (40 μg/ml) and were grown in liquid modified BG-11 medium containing the same antibiotic concentration. They are designated in the text by their accession numbers in a laboratory culture collection.

Escherichia coli DH10B (Bethesda Research Laboratories) was the host for all plasmids constructed in this study. Ampicillin (100 μg/ml) and spectinomycin (50 μg/ml) were added to LB growth medium (13) for selection of plasmids in *E. coli*.

Plasmids. All plasmids used as recombination substrates for *Synechococcus* were derivatives of pAM990 (Fig. 1*A*). This plasmid is based on pBR328 (14) and replicates only in *E. coli*. It contains a random segment of the *Synechococcus* chromosome (termed a neutral site), which allows homologous recombination between the transforming plasmid DNA

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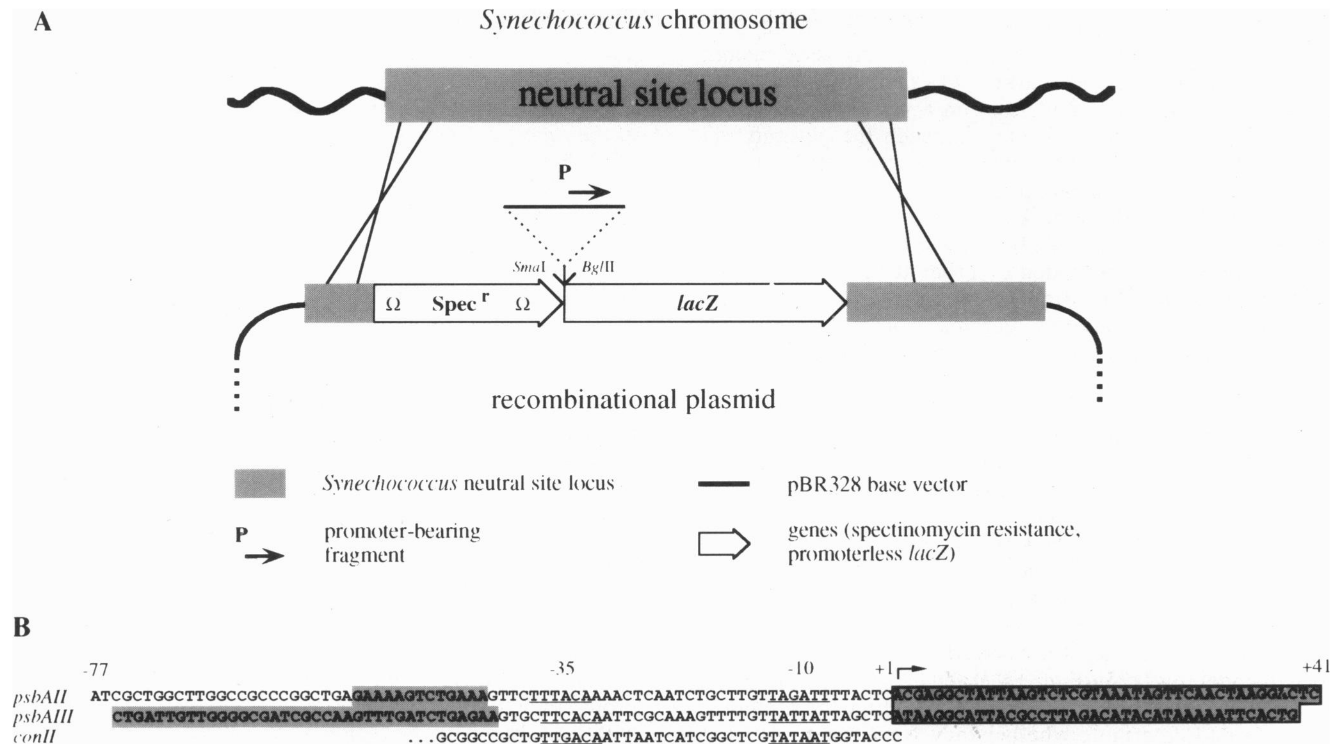

FIG. 1. (*A*) Schematic representation of the insertion of transcriptional *lacZ* reporter constructs into a neutral site of the *Synechococcus* chromosome. The pBR328-based recombinational plasmid pAM990 cannot replicate in the cyanobacterium. It contains a spectinomycin-resistance cassette and a promoterless *lacZ* gene flanked by *Synechococcus* neutral-site sequences (see *Materials and Methods*). Putative promoters and other cis-regulatory elements can be cloned at *Sma* I and *Bgl* II sites preceding the *lacZ* gene. Transformation of *Synechococcus* with the plasmid results in spectinomycin-resistant colonies in which homologous recombination has transferred the reporter construct to the cyanobacterial chromosome. (*B*) Promoter and cis-controlling elements inserted into pAM990. Sequences are shown for the fragments of *psbAII* and *psbAIII* present in the recombinant plasmids used to generate strains AMC190 and AMC192. Only the promoter region is shown for the *conII* fragment present in strain AMC209; the fragment extends ≈200 bp upstream. The σ^{70}-type promoter elements are underlined and marked as −35 or −10. The transcriptional start sites are aligned and marked by a rightward arrow and +1. Negative elements of *psbAII* and *psbAIII* are highlighted in gray; enhancers containing the light-responsive elements are boxed and shaded gray.

and the recipient cyanobacterial chromosome (15). A spectinomycin-resistance cassette flanked by strong transcription terminators (16) was cloned into the plasmid neutral site to confer selection for transformants. We added the following downstream of the spectinomycin-resistance gene to create a promoterless reporter gene: two unique cloning sites, a synthetic Shine–Dalgarno sequence (17), a translational start codon, and the *Bam*HI *lacZ* fragment from pMC1871 (18). During transformation of *Synechococcus* with pAM990 and its derivatives, the recombination event transfers cloned segments that are flanked by the neutral site sequence to the cyanobacterial chromosome, but the pBR328 portion of the plasmid is lost. pAM990 was the recombination substrate for AMC181, a strain used as a promoterless control for the reporter gene. Other recombinational plasmids were constructed by cloning either a restriction fragment or a PCR-generated DNA segment into the unique cloning site(s) of pAM990. The *E. coli conII* promoter was a *Not* I–*Sma* I fragment from pNN396 (19), the −77 to +41 segment of *psbAII* was a *Pvu* I–*Hin*fI fragment from pSG201 (3), and the −75 to +44 region of *psbAIII* was a *Pvu* II–*Hin*fI fragment from pSG302 (3). Other *psbAII* and *psbAIII* fragments used for cloning were generated by PCR using synthetic oligonucleotide primers with defined endpoints. PCRs were performed according to Sambrook *et al.* (13) with *Taq* DNA polymerase (Promega) in a MiniCycler (MJ Research, Cambridge, MA). DNA restriction endonucleases and modifying enzymes were purchased from Boehringer Mannheim and Bethesda Research Laboratories. DNA manipulations were as described (13). The nucleotide sequences of cloned regulatory regions and junctions with *lacZ* were confirmed for all plasmids by using the Sequenase 2.0 kit (United States Biochemical).

Culture Conditions and β-Galactosidase Assays. For controlled light-shift experiments, each reporter strain was inoculated from a 3-day-old shaking flask to an OD_{750} of about 0.2 in 50-ml Pyrex tubes. The tubes were submerged in an aquarium at a constant temperature of 30°C, bubbled with 1% CO_2 in air, and maintained at a light intensity of 125 $\mu E \cdot m^{-2} \cdot s^{-1}$ (15). When the OD_{750} of the culture was between 0.4 and 0.5, two samples were taken 2 hr apart and the remaining culture was shifted to 750 $\mu E \cdot m^{-2} \cdot s^{-1}$ (approximately one-third the intensity of full sunlight). A third sample was taken 2 hr after the shift to high light. We chose the 2-hr intervals after determining that induction of β-galactosidase is maximal by this time following the shift to high light. The time point at −2 hr, prior to the light shift, served as a control to ensure that there was no time-dependent increase in β-galactosidase in the absence of a change in light intensity.

Synechococcus cells were lysed as described (4). The concentration of protein from the aqueous phase was determined by a modified Lowry procedure (20). β-Galactosidase activity was measured by colorimetric assay using the method of Miller (21) and expressed as specific activity (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein).

RESULTS

Recombination of Transcriptional *lacZ* Reporter Genes at a Neutral Site on the *Synechococcus* Chromosome. Activity of *psbA* regulatory regions under different light intensities was

measured in *Synechococcus* by using transcriptional gene fusions to the *E. coli lacZ* gene. Each *lacZ* gene fusion, together with a selectable marker, was flanked by *Synechococcus* genomic DNA sequences on a nonreplicating plasmid. Homologous recombination between the plasmid and the *Synechococcus* chromosome at the neutral-site locus (15) results in antibiotic-resistant transformants with normal morphology and growth rate. By this strategy, the *in vivo* expression of each *lacZ* gene fusion was under the control of transcription signals provided by promoter elements cloned upstream of *lacZ*; expression was monitored by determining the β-galactosidase activity derived from *lacZ*. Fig. 1*A* depicts the insertion of a transcriptional *lacZ* gene fusion at the neutral site of the *Synechococcus* chromosome. Strain AMC181, containing the promoterless *lacZ* gene in the neutral site, produced a background level of β-galactosidase activity of 16–18 units. This strain served as a control for all other reporter strains, and its background β-galactosidase activity was subtracted from all other values.

Promoters and Other Cis-Acting Regulators of *psbAII* and *psbAIII*. A series of *Synechococcus* reporter strains was constructed in which *lacZ* gene expression was driven by fragments from the upstream regions of the three *psbA* genes. β-Galactosidase activity was assayed from each strain during growth under low (125 $\mu E \cdot m^{-2} \cdot s^{-1}$) and high (750 $\mu E \cdot m^{-2} \cdot s^{-1}$) light. The purpose of the analysis was to define the promoter elements and determine whether they were separable from signals that conferred light-responsive expression.

The search for promoters and other cis-regulatory elements of the three *psbA* genes was initiated with ≈1-kb segments from upstream of each gene, including the transcribed but untranslated leader regions of the genes (<55 bp) and about 60 bp of each open reading frame. The expression of *lacZ* driven by these fragments showed the expected patterns when the light intensity of the culture was changed from low to high: i.e., *psbAI–lacZ* expression decreased slightly while *psbAII–* and *psbAIII–lacZ* expression increased (data not shown). Additional fragments were generated for *psbAII* and *psbAIII* to remove sequences upstream or downstream of putative promoter regions centered at −35 and −10 relative to the transcription start sites (ref. 3; Fig. 1*B*). Fig. 2 summarizes the data from *psbAII–lacZ* reporter strains. The minimal fragment required for basal expression of the reporter extends from −39 to +12 (AMC203); neither AMC200 nor AMC201 showed β-galactosidase activity higher than the promoterless control strain. All strains that contain promoter fragments whose upstream ends correspond to −77 or −52 (strains AMC190, AMC197, AMC199, and AMC200) showed no more than 25% of the β-galactosidase activity of AMC203, indicating the presence of a negative element between −52 and −39 that down-regulates *psbAII–lacZ* expression under both low and high light. Strains AMC204, AMC206, and AMC207, containing the basal promoter and successively longer transcribed but untranslated sequences, showed progressively higher β-galactosidase activities under low light; the presence of sequences extending to +29 or +41 gave a reproducible induction under high light up to 80%. This suggests that a positive regulatory element, which contains the region responsible for light induction, is located downstream of the *psbAII* basal promoter and includes the region from +12 to +41.

Similar experiments were performed with *psbAIII* regulatory regions (Fig. 3). As seen for *psbAII*, this region of *psbAIII* can be dissected into three distinct elements. Basal promoter activity, unaffected by light intensity, was detected with *lacZ* driven by the region from −38 to −1 relative to the transcription start site (AMC219). Thus, unlike the *psbAII* promoter, the *psbAIII* promoter shows no requirement for sequences downstream of +1 (compare Fig. 3, AMC219, with Fig. 2, AMC201). Increased low-light expression and light-responsive expression were acquired by the extension of the sequences downstream of the basal promoter to +39, and the extent of induction by high light was about 2-fold. Inclusion of sequences upstream of the promoter had the effect of decreasing overall expression from *lacZ* under both low and high light intensities.

The Light-Responsive Cis-Controlling Elements of *psbAII* and *psbAIII* Have Enhancer Activity. To characterize further

Strains	Cloned *psbAII* Regulatory Regions (−77, −35, −10, +1, +41, ATG)	β-Galactosidase Activities -2 h	0 h	+2 h
AMC190	-77 to +41	30.9±1.7	30.4±1.6	56.7±3.9
AMC197	-52 to +18	36.4±3.7	36.4±4.8	38.8±2.8
AMC199	-52 to +12	35.8±2.7	35.6±4.8	36.8±2.4
AMC200	-52 to +7	0	0	0
AMC201	-39 to +7	0	0	0
AMC203	-39 to +12	142±8	142±4	143±7
AMC204	-39 to +18	180±4	180±9	185±7
AMC206	-39 to +29	253±18	258±16	354±10
AMC207	-39 to +41	315±23	317±23	572±21
AMC208	+1 to +41	11.1±2.0	11.1±1.1	11.4±0.9

FIG. 2. Effect of upstream regions of *psbAII* on *lacZ* expression under low and high light intensities. Regulatory fragments having the indicated endpoints relative to the transcriptional start site of *psbAII* were fused individually to the *lacZ* gene in pAM990; the resulting plasmids were used to transform wild-type *Synechococcus* to generate reporter strains, which are identified by AMC culture collection numbers. Expression of each *psbAII–lacZ* fusion gene was assayed by measuring β-galactosidase specific activity from samples of cells grown under low (−2 and 0 hr) and high (+2 hr) light intensity. The specific activities are the means of measurements from three independent light-shift experiments, with standard deviations indicated. Values were corrected by subtracting the background β-galactosidase activity produced by the promoterless control strain AMC181, which was assayed in parallel for each experiment.

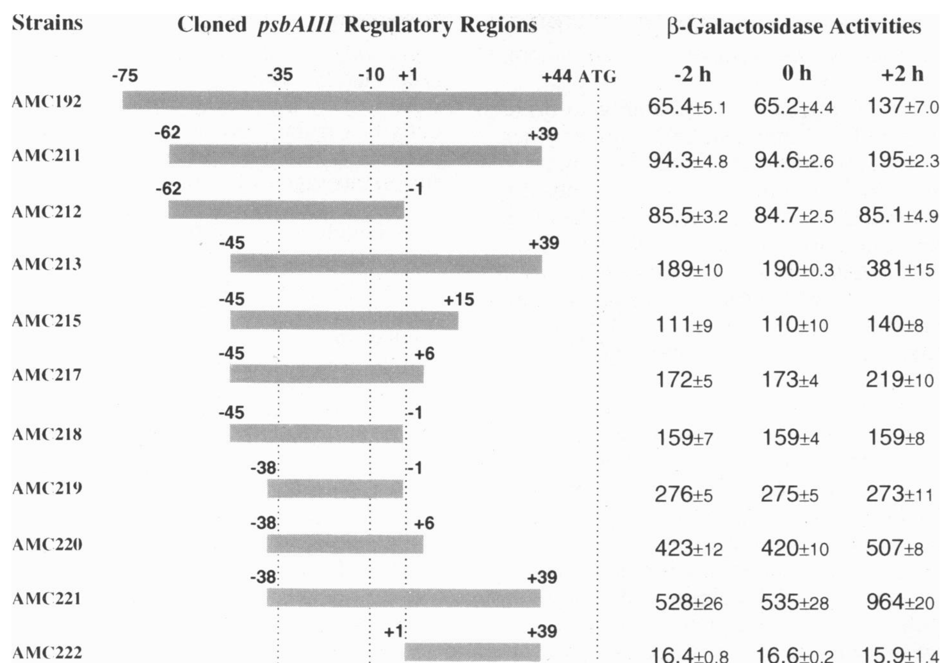

FIG. 3. Effect of upstream regions of *psbAIII* on *lacZ* expression under low and high light intensities. Regulatory fragments having the indicated endpoints relative to the transcriptional start site of *psbAIII* were fused individually to the *lacZ* gene in pAM990. Other experimental conditions, presentation, and data processing are the same as for Fig. 2.

the light-responsive elements of *psbAII* and *psbAIII*, we designed experiments involving a heterologous *E. coli* promoter, *conII* (ref. 19; Fig. 1*B*), which by itself was constitutively expressed under both low and high light in *Synechococcus* (strain AMC209 in Fig. 4). When the sequence from +1 to +41 of *psbAII* was cloned downstream of the *conII* promoter in both orientations, it enhanced the *conII*-driven *lacZ* expression 11- and 7.5-fold, respectively (compare AMC226 and AMC227 with AMC209). In strain AMC228, in which +1 to +41 was cloned approximately 200 bp upstream of the *conII* promoter element, the β-galactosidase activity was 5.7-fold higher than for *conII–lacZ*. When this sequence was moved upstream of the native promoter in strain AMC238, it enhanced the *psbAII* basal promoter activity by nearly 3-fold (compare with AMC203 in Fig. 2). This series of strains showed clearly that the +1 to +41 element of *psbAII* can enhance gene expression in a position- and orientation-independent manner. When the element was cloned in its native orientation (strains AMC226 and 228 in Fig. 4), it also conferred a 12–17% induction under high light on the *conII* promoter. Although this increase is small, it was observed in every experiment, and no increase at high light was ever observed in the absence of the +1 to +41 element or when it was present in the opposite orientation relative to its native configuration. When +1 to +39 of *psbAIII* was fused with the *conII* promoter in different positions and orientations, it enhanced *lacZ* expression 4- to 6-fold (strains AMC224, -225, and -239 in Fig. 4); strains in which the element was cloned in its native orientation showed 28–38% induction at high light (AMC224 and AMC225). These results show that the upstream untranslated region of *psbAIII* also exhibits enhancer activity and mediates light-responsive gene expression.

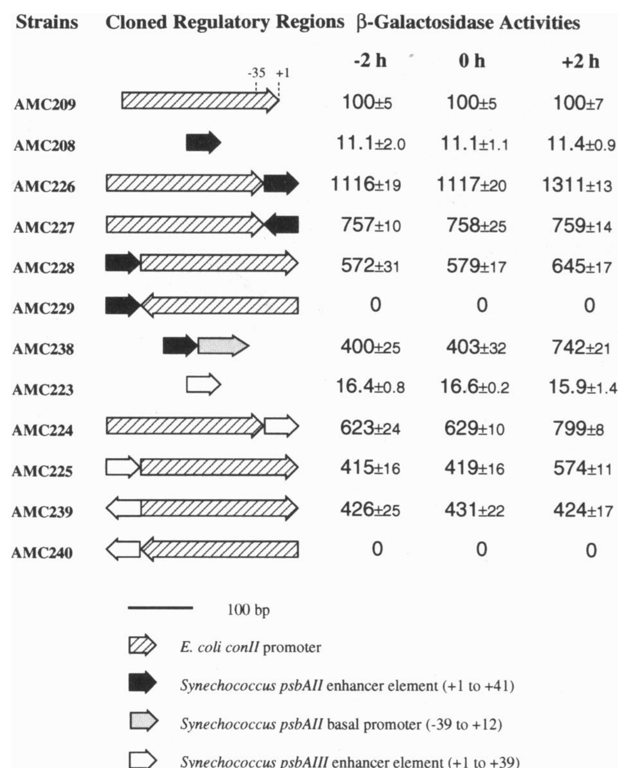

FIG. 4. Enhancer activity of light-responsive regulatory elements of *psbAII* and *psbAIII*. The *E. coli conII* promoter alone or in combination with either +1 to +41 of *psbAII* or +1 to +39 of *psbAIII* was cloned upstream of *lacZ* in pAM990. The *E. coli conII* fragment was from pNN396, and its promoter sequence is at the extreme right end as indicated (ref. 19; Fig. 1*B*). AMC238 was constructed by cloning the *psbAII* basal promoter sequence, −39 to +12, into the *Bgl* II site of the recombinational plasmid used to produce AMC208. Experimental conditions, presentation, and data processing are the same as for Fig. 2.

DISCUSSION

Recombination of transcriptional *lacZ* fusions at the neutral site of *Synechococcus* allowed us to characterize promoters and cis-controlling elements responsible for induced expression of *psbAII* and *psbAIII* under high light. Insertion of all reporter genes into the chromosome at a specific site removed the influence of copy number, which can affect values from plasmid-borne reporter genes (22). This approach, and

highly stereotyped procedures for culturing cells prior to the assays, yielded precise data with very small variations among experiments.

The basal promoters, identified by their ability to drive *lacZ* expression under both low and high light without other cis-acting elements, show structural and functional similarity to the well-studied *E. coli* σ^{70}-type promoter. These elements were previously identified as putative promoters on the basis of nucleotide sequence and position relative to the transcript 5′ ends (ref. 3; Fig. 1*B*). Their position is also consistent with that of the *Synechococcus psbDII* promoter, as mapped by exonuclease deletion analysis (12), and the *cpeBA* promoter in *Calothrix* sp. strain PCC 7601, as identified by DNase I footprinting with RNA polymerase (23).

We also showed that light-responsive elements located downstream of the basal promoters of *psbAII* and *psbAIII* have enhancer activity. Previously reported prokaryotic enhancer elements are solely associated with σ^{54}-type promoters (7). In the well-defined *Salmonella* NtrC (9) and *Caulobacter* flagellar gene systems (24), enhancer elements activate transcription in a position- and orientation-independent manner and contain symmetrical binding motifs for their target proteins. DNA looping between the enhancer and promoter has been visualized via electron microscopy (10). The *psbAII* and *psbAIII* enhancers are normally located immediately downstream of their basal promoters, making it physically impossible to form a DNA loop. Direct contact of DNA-binding proteins with RNA polymerase would be possible in this configuration but is unlikely to explain the enhancement we observe when the elements are placed upstream of the 250-bp *conII* fragment, which carries the promoter at its extreme right end. Other structural changes besides looping could be involved (25). Although *psbAII* and *psbAIII* enhancers share similar physical location and function, they do not share sequence similarity (ref. 3; Fig. 1*B*), and neither contains an obvious protein-binding motif. Specific binding of *Synechococcus* proteins to the upstream region of *psbAII* has been demonstrated (26), and we have observed binding to the enhancer element (unpublished data).

Strains that carried only the *psbAII* or *psbAIII* enhancer element upstream of *lacZ* (AMC208 and AMC223) produced β-galactosidase activities that were higher than the values for AMC181, the promoterless control strain. We believe that this results from enhancement of the cryptic transcription that gives rise to the background level of β-galactosidase in the promoterless strain. Primer extension analysis of the *lacZ* messages from AMC224 and AMC225 showed that the initiation site for transcription from the *conII* promoter was unaffected by the presence of the *psbAIII* enhancer (data not shown). The element must affect gene expression by acting at the DNA level rather than by affecting posttranscriptional events, because it is not present on the AMC225 *lacZ* message.

Light induction is mediated by the enhancers in an orientation-dependent manner. It is likely that two trans-acting factors are involved in interacting with the enhancer sequences, only one of which confers light induction. This is suggested by gel mobility-shift assays using a series of *psbAII* upstream fragments and partially purified protein extracts from *Synechococcus*, in which two specific protein–DNA complexes were formed with the sequences downstream of the transcriptional start site (unpublished data). Deletion of protein-binding sites in the untranslated leader region of *psbDII* affects overall expression of the gene without abolishing induction by high light (12). However, a light-regulatory element near the translational start codon would not have been identified in that analysis.

With our assay system, the maximum induction of *psbAII* and *psbAIII* expression under high light was 85% and 110%, respectively. These inductions are much lower than those observed by measuring transcript levels or β-galactosidase activities from translational fusions of *lacZ* to *psbAII* and *psbAIII* (4, 5, 27). This may be due to low stability of the fusion messages (4) combined with inefficiency of translation in the transcriptional reporter system. The putative ribosomal binding site and translation initiation signals in front of the *lacZ* gene of pAM990 may not be recognized well in *Synechococcus*. We cannot rule out the possibility that sequences beyond those we studied are also involved in regulation.

Inclusion of sequences immediately upstream of the *psbAII* and *psbAIII* basal promoters significantly decreased overall expression of the *lacZ* gene. The nature of these negative elements is unknown. However, this reminds us that *in vivo*, overlapping effects of activation and repression of particular sets of genes allow flexibility in the regulatory apparatus to optimize adaptation to an ever-changing environment. This complexity may be the key to survival and evolution over the billions of years of cyanobacterial existence.

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