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cis-Acting Sequences Required for Light-Responsive Expression of the *psbDII* Gene in *Synechococcus* sp. Strain PCC 7942

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We analyzed the sequences required for promoter activity and high-light responsiveness of the *psbDII* gene in the cyanobacterium *Synechococcus* sp. strain PCC 7942 by using transcriptional fusions to a *lacZ* reporter gene. The basal promoter drives high constitutive expression, although no canonical −35 element is evident. The smallest fragment that showed clear light-responsive expression extends from −38 to +160, which includes 52 bp of the *psbDII* open reading frame. Sequences downstream from the promoter, within the untranslated leader region from +11 to +24, were required for high-light induction.

The cyanobacterium *Synechococcus* sp. strain PCC 7942 expresses three *psbA* genes and two *psbD* genes which encode the proteins of the heterodimeric photosystem II reaction center (6, 8, 9). Both gene families have members that respond to an increase in incident light intensity. The *psbDI* gene is expressed constitutively as a dicistronic transcript with *psbC*, which encodes a chlorophyll *a*-binding apoprotein of photosystem II named CP43 (2, 8). The *psbDII* gene, which is not adjacent to *psbDI*, is expressed as a monocistronic message (8) which is induced up to fivefold by an increase from moderate to high light intensity (4). The two *psbD* genes encode identical D2 polypeptides (8, 9). Thus, the effect of light-regulated *psbDII* expression is a quantitative change in D2 synthesis: a strain defective for *psbDII* is growth impaired, and its thylakoids are deficient in D2, at high light (4).

Studies with *psbDII-lacZ* translational fusions previously showed that the information required for light-responsive expression lies downstream of the promoter (3). Three sites within the untranslated leader region of the *psbDII* gene are bound by proteins isolated from PCC 7942 cells shifted to high light intensity. Small deletions in these binding sites dramatically reduce expression of the reporter gene but failed to pinpoint the elements involved in light-regulated expression. This strategy posed some limitations in narrowing regulatory elements because of the need to preserve *psbDII* translation signals. The current investigation used transcriptional fusions to a promoterless *lacZ* gene to further dissect the *cis* sequences required for regulated expression of *psbDII*. We demonstrated that, in contrast to our earlier conclusions, a minimal promoter fragment that lacks untranslated leader sequences drives high constitutive expression of the reporter gene. However, the presence of untranslated leader (UTR) sequences was required for light-responsive regulation, and each previously identified protein-binding site influenced expression. Sequences within the open reading frame (ORF) were also necessary for full induction by high light. Although UTR and ORF sequences were not required for promoter activity, if present, the ORF sequences had to be translated to allow detectable expression of the reporter.

Sequences required for basal promoter function of the *psbDII* gene. We used a strategy that previously identified promoter sequences of the *psbAII* and *psbAIII* genes in this organism (16). DNA fragments containing the *psbDII* promoter and endpoints in the UTR or *psbDII* ORF were generated using PCR (Fig. 1). Reaction mixtures (25 μl each) contained the following: 10 to 20 ng of DNA template, 1.5 mM $MgCl_2$, approximately 0.04 to 0.07 μM primers, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 0.25 U of *Taq* DNA polymerase (Promega Corporation, Madison, Wis.). DNA templates were either PCC 7942 genomic DNA (100 ng) or the plasmid pAM737 (3), which contains a 250-bp *Rsa*I-*Taq*I fragment (Fig. 1), encompassing the promoter and upstream region of the *psbDII* gene, cloned into pBluescript KS+ (Stratagene, La Jolla, Calif.). Thirty cycles of amplification were performed as follows: denaturation at 92°C for 30 s, annealing of the primer to template at 50°C for 5 min, and elongation at 70°C for 1 min in a Minicycler (MJ Research, Watertown, Mass.). Promoter fragments thus generated were cloned into the unique *Sma*I site of the vector pAM990 to produce transcriptional fusions with a promoterless *lacZ* gene (16). The nucleotide sequence of each DNA fragment was verified by using a SequiTherm kit (Epicentre Technologies, Madison, Wis.) as recommended by the manufacturer. All promoter fusions containing sequences within the *psbDII* ORF were constructed out of frame with the translational start codon of the *lacZ* gene in pAM990 (Fig. 2). *Escherichia coli* DH10B (*mcrA mcrB*) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), used as the host for all plasmids, was propagated in Luria-Bertani broth (1a) and Terrific Broth (21) media in the presence of antibiotics as needed: ampicillin (100 μg/ml), spectinomycin (50 μg/ml), and chloramphenicol (17 μg/ml).

Wild-type *Synechococcus* sp. strain PCC 7942 was transformed as detailed elsewhere (7) by plasmid derivatives of pAM990 (16). Each promoter construct, together with a selectable antibiotic resistance marker, recombined into the cyanobacterial chromosome by homologous recombination at a locus designated neutral site I (4; GenBank database accession no. U30252). Integration at the expected site was confirmed by Southern blot analysis (data not shown). Cyanobacterial *lacZ* reporter strains are described in Table 1. Nucleotides relevant for the construction of plasmids are numbered relative to the transcriptional start site (+1).

The first set of *psbDII-lacZ* transcriptional fusion constructs was designed to define the smallest fragment of the *psbDII* gene that exhibits basal promoter activity. Wild-type *psbDII* fragments containing upstream endpoints at either −96 or −38 relative to the transcriptional start site, but with differing

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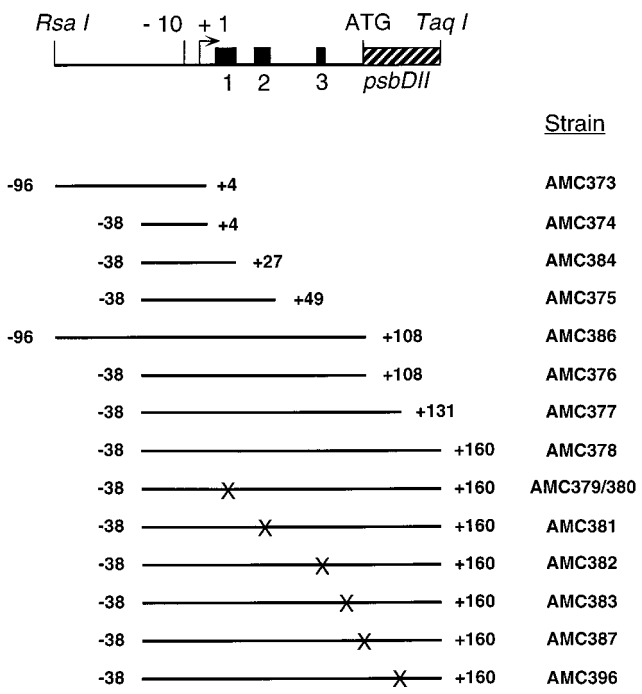

FIG. 1. Schematic representation of the promoter-bearing fragments used to create *psbDII-lacZ* transcriptional reporter gene fusions. A map of relevant features of the *psbDII* upstream and N-terminal coding regions is shown at the top. Restriction sites are shown for *Rsa*I and *Taq*I, which correspond to the endpoints of the longest PCR products used to create gene fusions. Closed boxes represent the three protein-binding sites of the UTR. The ATG and hatched box represent the beginning and N-terminal coding region of the *psbDII* ORF. The transcriptional start site is indicated by +1 and a rightward-bent arrow. The position of a consensus *E. coli* promoter element is indicated by −10. Horizontal lines below the map show the endpoints of the regulatory region of *psbDII* that is present in each construct, and the corresponding reporter strain is named at the right. Nucleotide positions (relative to the transcription start site) of leftward and rightward endpoints are given. X, regions where site-specific nucleotide changes were created in the *psbDII* region of certain constructs.

downstream endpoints, were generated by PCR (Fig. 1). In vivo *psbDII* promoter activity was monitored by determining the β-galactosidase activity of the respective cyanobacterial reporter strains under low (125 μE m^{-2} s^{-1}) and high (750 μE m^{-2} s^{-1}) light conditions as previously described (16). A strain (AMC181) that carries the promoterless reporter gene was assayed in parallel with all other strains, and its β-galactosidase value, ranging from 9 to 12 U, was subtracted from all other values.

The data in Fig. 3A indicate that the −38 to +4 region of the *psbDII* gene has strong promoter activity under both low- and high-light conditions (AMC374). Inclusion of sequences up to −96 did not alter the β-galactosidase values (AMC373), confirming that sequences upstream of −38 do not contribute to basal promoter activity or confer high-light regulation (3). The promoter of the *psbDII* gene contains a −10 region that matches the canonical *E. coli* Pribnow box (17, 18), but sequences resembling the bacterial −35 region of a typical σ^{70} promoter are absent (Fig. 2). Certain bacterial promoters that are recognized by alternative sigma factors, e.g., σ^{54}, require upstream activating sequences for transcription (14). Our analysis revealed that such upstream activating sequences are not necessary for *psbDII* promoter activity.

Some bacterial promoters that lack typical −35 regions are transcribed by a σ^{70}-containing RNA polymerase holoenzyme (13). These promoters contain an "extended −10" region with the consensus sequence TGNTATAAT, and all of the contacts between the σ^{70} subunit and the promoter occur at this extended −10 region. The sequence of the *psbDII* promoter near −10 contains an extra nucleotide compared to the canonical extended −10-type promoter (Fig. 2) and therefore cannot be assumed to use this strategy to recruit sigma factors. *Synechococcus* contains at least four genes that encode different σ^{70}-like factors, *rpoD1* to *-4* (20). The *rpoD1* gene is thought to encode the major sigma factor and is essential in this organism (19). Inactivation of the *Synechococcus rpoD2* gene affects the circadian expression of a subset of genes including *psbAI* and *ndhD* (22). To test the possibility that transcription at the *psbDII* promoter is carried out by one of the minor σ^{70} factors, reporter gene fusions carrying the *psbDII* minimal promoter region or the −38 to +160 region were used to transform strains that had *rpoD2*, *rpoD3*, or *rpoD4* or combinations of two of these genes, inactivated. The β-galactosidase values obtained from the fusions in the mutant genetic backgrounds were similar to values obtained from the corresponding wild-type reporter strains, although slightly elevated (data not shown), indicating that none of these sigma factors is essential for recognition of the promoter.

Sequences essential for high-light expression are contained within the UTR and ORF. Our second objective was to determine sequences required for high-light induction of this gene. Strains containing the basal promoter and successively longer regions of transcribed but untranslated leader sequences showed lower overall β-galactosidase activity than did AMC373 and 374, indicating either that the leader region contains one or more negative regulatory elements or that increased spacing between the promoter and the reporter adversely affects expression (Fig. 3A and B). Strains with reporter gene fusions that carry one (AMC384), two (AMC375), or all three (AMC376) protein-binding sites in addition to the promoter region did not show strong high-light induction of reporter gene activity. However, the low-level activity in AMC384 increased approximately twofold in each of the three replicates; AMC 386 and 376 showed a twofold induction in two of the three experiments. High-light induction of β-galactosidase activity of the magnitude seen for the *psbDII* transcript (4), and in the range of values obtained with translational gene fusions (3), was observed only in strain AMC378 (Fig. 3B); this reporter fusion has a promoter-distal endpoint at +160, which includes 52 bp of the *psbDII* ORF (Fig. 1 and 2). Strain AMC377, whose reporter carries a promoter-distal endpoint in the ORF at +131, did not exhibit high-light induction of the magnitude seen for AMC378. These data suggest that sequences within the *psbDII* ORF from +131 to +160 are necessary for full high-light regulation of this gene. Expression of *lacZ* driven by an *E. coli* consensus promoter (16), or the promoter for *psbDI*, which is not regulated by light (4), was not affected by light intensity changes under these experimental conditions (data not shown).

Analysis of *cis*-acting elements of the leader region involved in high-light expression. If promoter-reporter spacing influences overall expression, then variations in expression from fragments with variable endpoints might obscure the contributions of individual regulatory elements. Therefore, we used pAM1439, the recombinational substrate for AMC378, as a starting point for PCR-based mutagenesis to create specific nucleotide substitutions in the *psbDII* UTR or ORF (10, 11; Table 2). The resulting constructs have identical endpoints but carry modifications in each of the three regions of the *psbDII* UTR (Fig. 2, bs-1, bs-2, and bs-3) that previously showed protein protection from copper phenanthroline cleavage (3). Strikingly, mutations in bs-1 completely abolished high-light

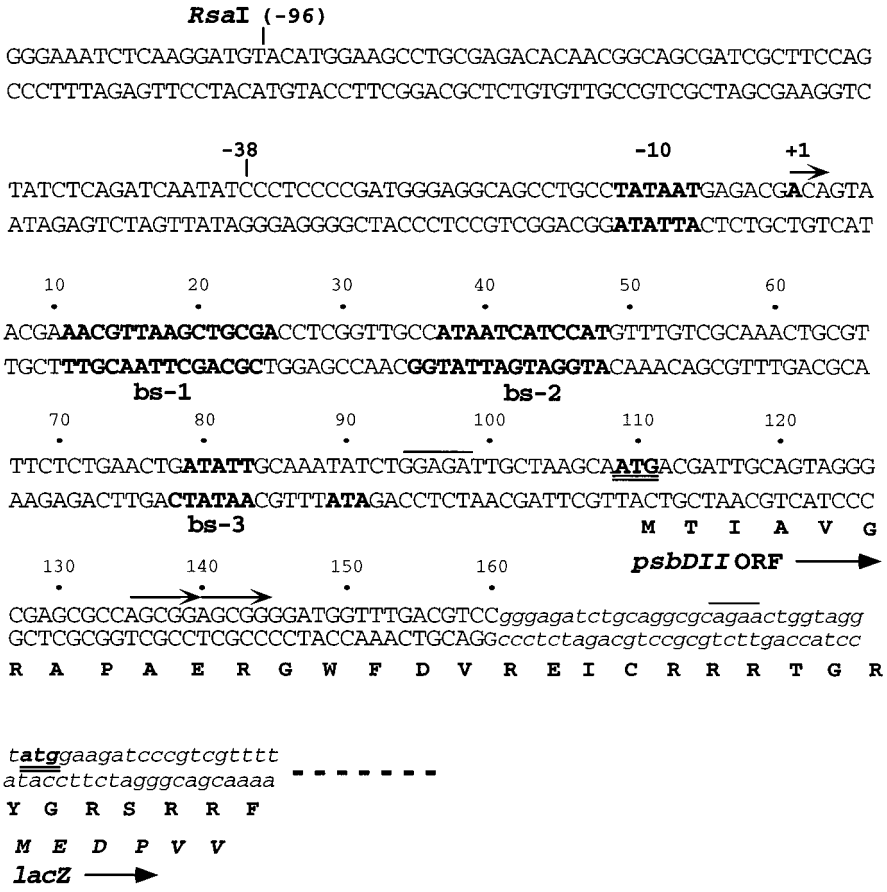

FIG. 2. Nucleotide sequence of *psbDII* regulatory regions and a fusion to *lacZ* sequences. Nucleotide positions are numbered for transcribed sequences from the *psbDII* gene and relevant upstream positions; numbers are relative to the transcription start site (+1, boldface letter, rightward arrow). Sequences characteristic of an *E. coli* promoter element are in bold and labeled −10. Three protein-binding sites within the untranslated leader region, determined previously (3), are shown by bold letters and labeled bs-1, bs-2, and bs-3. Potential ribosome binding sites for the *psbDII* and lacZ genes are overlined and labeled. Two forward arrows overline a direct repeat sequence. Italicized lowercase letters represent sequences from the *lacZ* reporter vector pAM990. The translational start sites of the *psbDII* and *lacZ* ORFs are double underlined and shown in bold letters. Single-letter amino acid sequences are shown for the N-terminal coding regions of both genes.

responsiveness of the reporter gene (Fig. 4, AMC379). Two different sets of site-specific changes were made in this region (Table 2). One set (positions +20, +22, +24, and +25; AMC380) mutated the right end of bs-1, a change similar to that in the 4-bp deletion mutant AMC099 analyzed in a previous study (3). In AMC099, the deletion dramatically reduced expression but did not completely abolish high-light responsiveness. However, AMC380 showed higher low-light levels than the wild-type reporter strain (AMC378) and no induction at high light. The second strain, AMC379, with changes at positions +16, +17, and +19, was created to inactivate the left end of the first binding site upstream from the *Alu*I site used as an endpoint in the previous study (Fig. 2 and Table 2). AMC379 had low-light expression reduced by half and showed no high-light induction. We concluded that the entire first binding site functions as a light regulatory element. These results are in contrast to those obtained with AMC099, where the altered spacing caused by the 4-bp deletion is likely to account for overall poor expression, which in turn made changes in response to high light difficult to discern.

AMC381, which carries three nucleotide substitutions in bs-2 (Table 2), showed slightly less high-light induction than the control strain AMC378 (threefold versus fourfold) in each of the replicates. Base changes at three positions in bs-3 (AMC382; Table 2) increased β-galactosidase activity relative to the wild type, suggesting that this *cis*-acting element functions as a negative element within the *psbDII* leader region (Fig. 4). Clearly, mutations within the second and third protein binding sites did not eliminate the high-light induction of *psbDII* gene expression (Fig. 4). We concluded that normal high-light regulation of the *psbDII* gene requires at least the promoter, the first binding site, and sequences within the ORF from +131 to +160.

To determine whether we could identify a region within the ORF (+131 to +160) that influences high-light regulation, we changed the sequence of a discernible motif, a direct repeat sequence located between positions +135 to +144 (Fig. 2; strain AMC396). Nucleotide substitutions were made such that the direct repeat was abolished but the identity of the amino acids encoded by this region of the ORF remained unchanged (Table 2). β-Galactosidase activity in AMC396 was consistently higher than that in the strain carrying the wild-type construct, AMC378 (Fig. 4). However, this mutant strain showed normal high-light regulation of the reporter gene, indicating that the direct repeat sequence did not function as a light regulatory element of *psbDII* but may function as a negative regulatory element. Therefore, sequences within the ORF involved in high-light induction of *psbDII* are limited to a small region of DNA from +131 to +160, but it is, as yet, unclear how these 29 nucleotides function in high-light responsiveness.

TABLE 1. *Synechococcus lacZ* reporter strains and plasmids

Strain[a]	Plasmid[b]	*psbDII* promoter region[d]
AMC181	pAM990[c]	None
AMC373	pAM1434	−96 to +4
AMC374	pAM1435	−38 to +4
AMC375	pAM1597	−38 to +49
AMC376	pAM1437	−38 to +98
AMC377	pAM1438	−38 to +131
AMC378	pAM1439	−38 to +160
AMC379	pAM1591	−38 to +160 with base changes at positions +16, +17, and +19
AMC380	pAM1592	−38 to +160 with base changes at positions +20, +22, +24, and +25
AMC381	pAM1593	−38 to +160 with base changes at positions +39, +44, and +46
AMC382	pAM1594	−38 to +160 with base changes at positions +81, +82, and +84
AMC383	pAM1595	−38 to +160 with base changes at positions +94, +95, and +96
AMC384	pAM1596	−38 to +27
AMC386	pAM1436	−96 to +98
AMC387	pAM1598	−38 to +160 with base changes at positions +109 and +110
AMC396	pAM1599	−38 to +160 with base changes at positions +138 and +141

[a] Carries a *lacZ* reporter gene integrated at the neutral site locus (16).
[b] Plasmid used to transform *Synechococcus* sp. strain PCC 7942 to introduce reporter gene.
[c] From reference 16.
[d] Endpoints of DNA fragment, numbered according to *psbDII* transcription start site, cloned upstream of the promoterless *lacZ* gene of pAM990.

Effect of *psbDII* translation signals on expression. It was evident from our analysis of wild-type promoter constructs that the region upstream of the *psbDII* ORF corresponding to the UTR of the mRNA plays a role in expression of this gene. To further test this observation, we constructed strains containing the −38 to +160 fragment in which either the translational start codon or the ribosome binding site of the *psbDII* gene was abolished (Table 2). Although the Shine-Dalgarno sequence and translational start codon of the *lacZ* gene of pAM990 were left untouched, *lacZ* is out of frame with the *psbDII* ORF (Fig. 2), and other fusions demonstrate that the *lacZ* translation signals are functional in the cyanobacterium (e.g., AMC373, Fig. 3A), reporter gene activity was completely eliminated in the strains in which either the *psbDII* ribosome binding site or translational start codon were changed (AMC383 and AMC387; Fig. 4). We suggest that this observation reflects dramatic destabilization of the reporter gene transcript when the *psbDII* UTR near the ribosome binding site, or the ORF, is not covered by ribosomes.

Loading of ribosomes at the *psbDII* Shine-Dalgarno sequence may contribute to the stability of *psbDII-lacZ* mRNA by masking potential RNase cleavage sites present on the 5′ end of the transcript. Mutations in *lamB-lacZ* fusions that alter the translational initiation frequency by sequestering the Shine-Dalgarno sequence within a secondary structure increase the degradation of the fusion messages (24). Posttranscriptional message stability also plays a key role in the expression of the *psbAI* and *psbAIII* genes in high light, and the leader regions of these genes have been implicated in determining mRNA stability under high-light conditions (12). This may be a common strategy used to regulate transcript abundance, and thus gene expression, for the *psbA* and *psbDII* genes.

We attempted to compare the stability of the *lacZ* fusion mRNAs in strains AMC378, AMC383, and AMC387, using Northern blot analysis of total RNA extracted from *Synechococcus* cells grown in the presence or absence of the transcriptional inhibitor rifampin. The results of these Northern blots were inconclusive because we could not detect full-length fusion mRNAs in any of the strains.

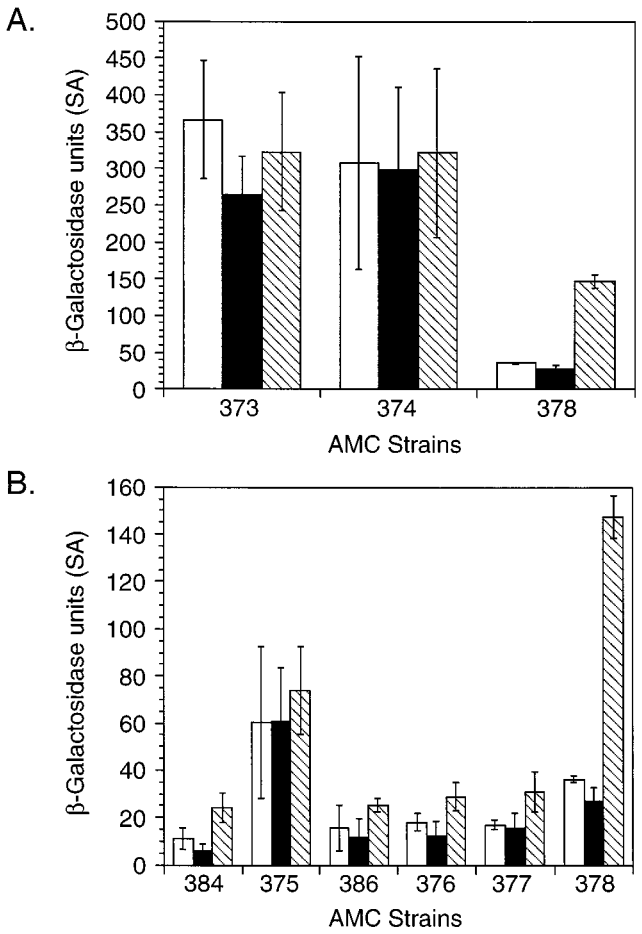

FIG. 3. Expression of *lacZ* fusions that carry different *psbDII* regulatory elements at standard and high light intensities. Specific activity (SA) (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein) was determined for each of the *psbDII-lacZ* reporter strains in light shift experiments. Histograms show β-galactosidase levels for each strain 2 and 0 h before (white and black bars, respectively) and 2 h after (hatched bars) the shift to high light intensity. β-Galactosidase levels were corrected by subtracting background levels from a promoterless *lacZ* strain, AMC181. The culture collection number for each reporter strain is indicated. The graphed values represent the means of triplicate experiments with the standard deviations indicated. (A) Strains carrying the minimal promoters and the wild-type fragment capable of light induction. (B) Strains containing fusions with DNA fragments extending out from the *psbDII* promoter region into the UTR.

We have characterized *cis*-acting sequences within the UTR of the *psbDII* gene that are required for expression. The results suggest that three *cis* elements within the UTR function synergistically to regulate transcription from the *psbDII* promoter itself, together with sequences carried within the ORF. The level of induction of expression obtained at high light with both the translational (3) and transcriptional (this study) reporter gene fusions are similar and parallel the increase in mRNA levels observed at high light intensity for this gene (4). Thus, we have seen no evidence for translational control governing the expression of the *psbDII* gene. β-Galactosidase activity of transcriptional reporter gene fusions of the *psbAII* gene is induced only twofold in high light intensity (16). In contrast,

TABLE 2. Site-specific changes made in *psbDII* UTR and ORF

Region	Position	Sequence	Mutated sequence	Strain
Binding site 1 (bs-1)	+11 to +25	AACGT**TAAG**CTGCGA	AACGT**CG**A**C**CTGCGA	AMC379
Binding site 1 (bs-1K)	+11 to +25	AACGTTAAG**CTGCGA**	AACGTTAAG**GTACCT**	AMC380
Binding site 2 (bs-2)	+37 to +48	GC**C**ATAAT**C**ATCCAT	GC**G**ATAA**G**C**T**TCCAT	AMC381
Binding site 3 (bs-3)	+79 to +83	GA**TATT**	GA**AT**T**C**	AMC382
Ribosomal binding site	+94 to +98	**GGA**GA	**ATC**GA	AMC383
Translational start codon	+109 to +111	**ATG**	**TA**G	AMC387
Direct repeat	+135 to +144	AGC**GGAG**CGG	AGC**C**GA**A**CGG	AMC396

the expression of translational gene fusions of this gene increase 10-fold or more under high-light conditions (15), implying that translational control mechanisms are involved in its expression. Preliminary results indicate that the increase in β-galactosidase activity of *psbAII* translational gene fusions in high light requires photosynthetic electron transport (1).

The intriguing question remains as to how the binding of protein factors to the *psbDII* UTR modulates high-light expression of this gene. The *psbAII* gene in *Synechococcus* also shows an increase in transcriptional activity in high light (16). A 210-bp DNA fragment containing the upstream region of the *psbAII* gene forms DNA-protein complexes when incubated with the same heparin-Sepharose fraction that contains *psbDII*-binding proteins (3). Moreover, in gel mobility shift assays this unlabeled *psbAII* fragment efficiently competes for the formation of two of the three DNA-protein complexes of the *psbDII* untranslated region (3). The two genes, therefore, share binding sites for similar if not identical regulatory proteins. Sequence comparison of the upstream regions of the *psbDII* and *psbAII* genes reveals only a six-nucleotide segment, TTAAGC, whose sequence and position relative to the promoter and transcriptional start site is conserved in these genes. In *psbDII*, this sequence lies within the +11 to +25 region of the first binding site and, when changed, abolished high-light responsiveness of this gene (Table 2, AMC379 and AMC380). Sharing of a common high-light-specific protein factor would ensure coordinate expression of the *psbAII* and *psbDII* genes under high-light conditions. A genetic selection method is currently being used in this laboratory to isolate the protein factor(s) responsible for high-light responsiveness of the *psbAII* gene (5, 23). Characterization of the transcriptional factors involved in high-light expression of the *psbAII* and *psbDII* genes will elucidate their role in modulating gene expression and further our understanding of the high-light-driven signal transduction pathway in this cyanobacterium.

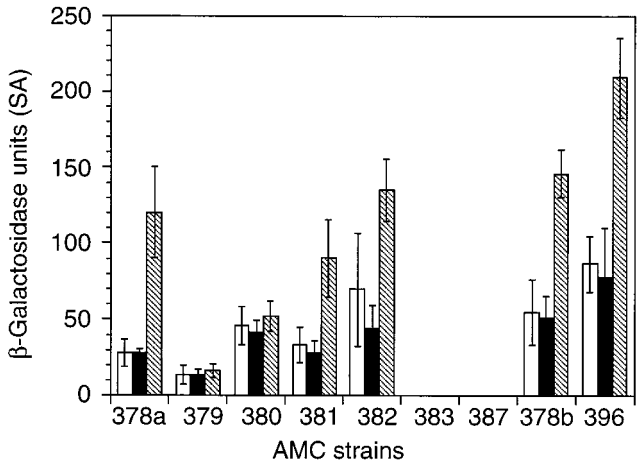

FIG. 4. Effect of site-specific nucleotide changes in the upstream region of *psbDII* on β-galactosidase activity. Specific activity (SA) was determined for each of the *psbDII-lacZ* reporter strains in light shift experiments as described in the legend to Fig. 3. Values for strains AMC383 and AMC387 were below those determined from the promoterless *lacZ* control strain AMC181. Strain AMC378 was assayed in triplicate experiments in parallel with strains AMC379, -380, -381, -382, -383, and -387; these values are labeled 378a. Additional triplicate experiments paired this strain with AMC396 at a later date; these values are labeled 378b.

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