Characterization of High-light-responsive Promoters of the psaAB Genes in Synechocystis sp. PCC 6803

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In cyanobacteria, transcription of genes encoding subunits of PSI is tightly repressed under high-light conditions. To elucidate the molecular mechanism, we examined the promoter architecture of the psaAB genes encoding reaction center subunits of PSI in a cyanobacterium Synechocystis sp. PCC 6803. Primer extension analysis showed the existence of two promoters, P1 and P2, both of which are responsible for the light intensity-dependent transcription of the psaAB genes. Deletion analysis of the upstream region of the light intensity-dependent transcription of the two promoters, P1 and P2, both of which are responsible for the light response of these promoters is achieved in a totally different manner. The cis-element required for the light response of P1, designated as PE1, was located just upstream of the –35 element of P1 and was comprised of AT-rich sequence showing significant homology to the upstream promoter (UP)-element often found in strong bacterial promoters. PE1 activated P1 under low-light conditions, and the down-regulation of P1 was achieved by rapid inactivation of PE1 upon the shift to high-light conditions. On the other hand, the cis-element required for the light response of P2, designated as HNE2, was located upstream of the P1 region, far from the basal promoter of P2. The down-regulation of P2 seemed to be attained through the negative regulation by HNE2 activated only under high-light conditions. DNA gel mobility shift assays showed that at least five regions in psaAB promoters were responsible for the binding of putative regulatory protein factors.

Keywords: Cyanobacteria — High light — Promoter — PSI — psaAB — Synechocystis sp. PCC 6803.

Abbreviations: DIG, digoxigenin; DTT, dithiothreitol; HL, high light (250 μmol photons m⁻² s⁻¹); LL, low light (20 μmol photons m⁻² s⁻¹); UP-element, upstream promoter element

Introduction

Photosynthetic organisms must acclimate to their light environment by changing themselves. In response to changing light intensity, they modulate their capacities for light harvesting, photosynthetic electron transport, CO₂ fixation (Anderson 1986, Melis 1991, Anderson 1995, Walters 2005) and the system for scavenging reactive oxygen species (Grace and Logan 1996, Niyogi 1999). Under low-light (LL) conditions, the amount of light-harvesting antenna complexes is maintained at a high level, because maximal capture of light energy is required to fulfill the energy demand of cells. Under high-light (HL) conditions, however, energy supply tends to exceed its consumption, and photosynthetic electron transport components become relatively reduced. This may result in excess production of reactive oxygen species leading to severe damage to many cellular processes (Asada 1994). Thus, absorption of excess light energy must be avoided under HL conditions by decreasing the amount of light-harvesting antenna complex per reaction center or the amount of reaction center complexes per se.

In cyanobacteria, the decrease of photosystem content is typically observed under HL conditions, and the main target of down-regulation is not PSII but PSI (Murakami and Fujita 1991, Hihara et al. 1998). The physiological significance of the decrease in PSI content during HL acclimation has been demonstrated by the characterization of the two mutants of Synechocystis sp. PCC 6803, disruptants of pmgA (sll1968) and sll1961, both of which have a defect in decreasing their PSI content during HL acclimation (Hihara et al. 1998, Fujimori et al. 2005). The phenotype of these mutants could not be distinguished from that of the wild-type strain under LL conditions, and a normal decrease in the amount of PSI was observed just after the shift to HL conditions. However, after 12 h of HL exposure, the selective repression of PSI content was lost in these mutants. They grew better than the wild-type cells during a short-term exposure (e.g. 24 h) to HL because higher amount of PSI accelerated the rate of photosynthetic electron transport (Hihara et al. 1998). Under long-term exposure to HL, however, growth of the mutants was severely inhibited (Hihara et al. 1998, Sonobe et al. 2001, Fujimori et al. 2005), presumably due to the generation of reactive oxygen species at the acceptor side of PSI. These observations strongly suggest that the repression of PSI
content is indispensable for growth under continuous HL conditions.

In contrast to accumulating data showing the physiological importance of down-regulation of PSI content during HL acclimation, the molecular mechanisms controlling the process have been poorly understood. However, some recent reports have implied the involvement of transcriptional control in the decrease of PSI content during HL acclimation. In *Synechocystis* sp. PCC 6803, coordinated down-regulation of genes encoding subunits of PSI (PSI genes) was observed upon the shift to HL conditions preceding the decrease of PSI content (Hihara et al. 2001, Huang et al. 2002, Muramatsu and Hihara 2003, Tu et al. 2004). Herranen et al. (2005) showed by a pulse-labeling experiment that light-induced modulation in the translation rate of a reaction center subunit of PSI, PsaA protein, closely parallels the transcription rate of the *psaAB* genes. Furthermore, the defect in repression of the transcript levels of the *psaAB* genes is likely to cause the aberrant accumulation of PSI in a *pmgA* disruptant under prolonged HL conditions (Muramatsu and Hihara 2003). These results indicate that the decrease in transcript levels of PSI genes, in particular that of the *psaAB* genes, should be one of the prerequisite factors for down-regulation of PSI content under HL conditions. Since the stability of the *psaAB* transcripts does not decrease so much upon the shift to HL (Muramatsu and Hihara 2003, Herranen et al. 2005), the decrease in the *psaAB* transcripts under HL seems largely dependent on the regulation of promoter activities. Therefore, in this study, as the first step for elucidation of the light intensity-dependent mechanism of transcriptional regulation of PSI genes, we analyzed the promoter architecture of the *psaAB* genes using *luxAB* reporter genes. We show that the *psaAB* genes have two light-responsive promoters, P1 and P2. Although the activity of both promoters is coordinately down-regulated under HL, the mechanisms used to achieve the light response differ between them.

**Results**

**Mapping of the transcriptional start points of the psaAB genes**

The transcriptional start points of the *psaAB* genes were determined by primer extension analysis. When the $\gamma^{32}$P-labeled psaA-13 primer (Table 1) that primed 35 bp downstream of the *psaA* start codon was used, three 5' ends of transcript were detected and they were designated ep (end point) 1, ep2 and ep3 (Fig. 1A). The location of ep1 corresponded to 144 nucleotides upstream of the *psaA* start codon. There existed putative −35 and −10 elements upstream of ep1 (Fig. 1B). These elements, TTGCCT and TATTAT, were separated by a 16 bp spacer and deviated from the consensus −35 (5'-TTGACA-3') and −10 (5'-TATAAT-3') elements of *Escherichia coli* σ$^70$-type promoters (Harley and Reynolds 1987) in three out of 12 positions. The location of ep2 corresponded to 45 nucleotides upstream of the *psaA* start codon. The putative −35 and −10 elements located upstream of ep2, TTCCCT and TACACT, were separated by an 18 bp spacer and deviated from the consensus −35 and −10 elements in five out of 12 positions (Fig. 1B). The spacing between the putative −35 and −10 regions of both ep1 and ep2 was consistent with the 17±1 bp spacing reported for canonical *E. coli* σ$^70$ promoters (Harley and Reynolds 1987). In general, a conserved −10 element (5'-TANNNT-3') is found in cyanobacterial promoters, whereas the −35 element is weakly conserved or totally absent (Curtis and Martin 1994, Vogel et al. 2003). Thus, the structure of the *psaAB* upstream region seems atypical for cyanobacterial promoters in that a conserved −35 element is found at the appropriate position relative to both ep1 and ep2. This feature may partly contribute to active transcription of the *psaAB* genes under normal growth conditions.

We observed that both of two independent upstream regions including ep1 or ep2 could yield bioluminescence when they were fused to promoterless bacterial luciferase genes (*luxAB*), as described later. This indicates that the *psaAB* genes have at least two promoters, and ep1 and ep2 are transcriptional start points originating from these promoters. Thus, the upper and the lower promoters were designated as P1 and P2, respectively. As regards ep3, corresponding to 16 nucleotides upstream of the *psaA* start codon, it probably originated from the processed product, since the cells harboring the *luxAB* reporter fused to the fragment covering just upstream of ep3 exhibited only a background level of bioluminescence (data not shown) and putative promoter elements were not found in the upstream region of ep3.

Under HL conditions, the *psaAB* genes were transcribed from the same residues as those under LL conditions (Fig. 1A). The amount of the products from ep1 and ep2 greatly decreased after 12 h exposure to HL, indicating that both promoters of the *psaAB* genes can respond to changes in light intensity.

**In vivo reporter assay for identification of cis-elements required for the light intensity-dependent transcription of the psaAB genes**

Previously, we showed that the amounts of *psaAB* transcripts drastically decrease upon the shift from LL to HL conditions (Muramatsu and Hihara 2003). Considering that the amount of the products from ep1 and ep2 was low in HL-incubated cells compared with that in LL-acclimated cells (Fig. 1A), both P1 and P2 should contribute to this regulation. To identify cis-promoter elements required
for the light response, a series of 5'- and/or 3'-deleted fragments of the upstream region of the psaAB genes was cloned into pPT6803-1 carrying promoterless luxAB genes. These constructs were transformed into Synechocystis cells and inserted into the neutral site of the chromosome by homologous recombination.

First, we assessed the correlation between the transcript level of the endogenous psaAB, that of luxAB, and the level of bioluminescence under LL and HL conditions in the A0 strain harboring the full-length intergenic region between psaAB and the divergently transcribed open reading frame, sll1730. As shown in Fig. 2A, the amount of the endogenous psaAB transcripts decreased drastically upon the shift to HL conditions, and gradually increased by further incubation under HL. This response could be traced through analyzing the change in the luxAB transcript levels (Fig. 2B, A0). However, we found that the change in bioluminescence levels was inconsistent with that of psaAB and luxAB transcript levels, i.e. the extent of the decrease in the bioluminescence level in A0 strain 1 h after the shift to HL conditions (Fig. 2C, A0) was far smaller than that in the psaAB and luxAB transcript levels (Fig. 2A, B, A0). This might be ascribed to higher stability of LuxAB protein compared with that of the luxAB transcripts as shown by Liu et al. (1995). Another discrepancy is observed after 3 h of HL exposure; the bioluminescence level continued to decrease (Fig. 2C, A0) although the transcript level began to increase (Fig. 2A, B, A0). Such a discrepancy was more clearly observed when the A1 strain harboring a 5'-deleted promoter fragment (–176 to +144) was used for analysis. In this strain, a continuous decrease in bioluminescence levels was observed after the shift to HL conditions (Fig. 2C, A1), in spite of an almost constant level of the luxAB transcripts for the light response, a series of 5'- and/or 3'-deleted fragments of the upstream region of the psaAB genes was cloned into pPT6803-1 carrying promoterless luxAB genes. These constructs were transformed into Synechocystis cells and inserted into the neutral site of the chromosome by homologous recombination.

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Recently, Koga et al. (2005) reported that the activity of LuxAB expressed under the control of a lacUV5 promoter in *E. coli* declined upon entry into the stationary phase irrespective of the constant amount of LuxA and LuxB proteins. They demonstrated that this phenomenon, ADLA (abrupt decline of luciferase activity), was due to the decline in the supply of intracellular FMNH2 required for luciferase activity. The aberrant decrease of the bioluminescence level shown in Fig. 2C might be also due to the loss of reducing power during HL incubation.

Thus, in this study, we assessed the changes of promoter activities upon the shift from LL to HL conditions by monitoring the level of the luxAB transcripts in reporter strains. However, when reporter strains were incubated under LL conditions, the level of bioluminescence and that of luxAB transcripts showed good correlation (Fig. 2D). Hence, for LL-acclimated cells, we employed the measurement of bioluminescence to compare the promoter activities among reporter strains.

**The architecture of P1**

As mentioned above, the transcriptional start point of P1 corresponded to 144 nucleotides upstream of the *psaA* start codon (noted as +1), and P1 had conserved −35 and −10 elements (Fig. 1B). Strain A62, which possessed the (−46 to +2) fragment including the −35 and −10 elements, showed very low but substantial bioluminescence (1.5 × 10^6 relative units/OD730) compared with that from the control cells having promoterless luxAB genes (1.0 × 10^5 relative units/OD730) (Fig. 3B). Thus, in this study, we assigned the (−46 to +2) region to the minimal sequence for P1. The promoter activities of A62 (−46 to +2) and A63

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**Fig. 1** Mapping of the 5′ ends of the *psaAB* transcripts. (A) Total RNA (10 μg) was extracted from cells acclimated to LL and those exposed to HL for 12 h, and used for primer extension analysis. Detected 5′ ends of transcripts (ep: end point) are indicated by arrowheads and shown with dideoxy sequencing ladders. (B) Nucleotide sequence upstream of ep1 and ep2 of the *psaAB* genes. The positions of nucleotides are shown relative to that of ep1 denoted as +1.
(–46 to +19) were constitutively low irrespective of light intensity (Fig. 3C). In contrast, strain A61 (–69 to +2), which carries the sequence (–69 to –47), in addition to the minimal sequence, exhibited a typical light response for PSI genes. Thus, the (–69 to –47) region is a positive regulating element working under LL conditions and alone is sufficient to confer light response to P1. This positive regulating element, designated as PE1, seems to be inactivated just after the shift to HL conditions, and reactivated after 3 h of HL exposure. To identify the nucleotide sequences critical for the light response, base substitutions were carried out within the PE1 region of A61 (Fig. 4A). When the sequence TTTTT (from –61 to –57) or TTATT (from –54 to –50) was replaced with the sequence, CGCGC (strain A61mt2 and A61mt3, respectively), the bioluminescence levels under LL conditions were drastically decreased (Fig. 4B). This indicates that these AT-rich pentamers, TTTTT and TTATT, are the core sequences for the light response of P1. It should be noted that the nucleotide sequence of PE1 showed significant homology to the upstream promoter (UP)-element often found in strong bacterial promoters (Ross et al. 1993, Estrem et al. 1998). This feature will be discussed later.

Successive elongation at the 3′ end from +19 to +51, while maintaining the 5′ end at –91 (Fig. 3, compare strain A6 and A7), decreased P1 activity under both LL and HL conditions, although further elongation at the 3′ end to +83 (A8) did not affect P1 activity very much. This indicates that the (+20 to +51) region harbors a negative regulating element (NE1), which is not involved in the light response. Further elongation at the 3′ end from +83 to +115 (compare strain A8 and A9) increased promoter activity under both LL and HL conditions, which was probably due to the addition of the –10 element of P2 to the reporter fragment.

In summary, the analysis of P1 architecture suggests that P1 was controlled by at least two cis-elements. The basal promoter (–46 to +2) was up-regulated by PE1 located just upstream of the –35 element under LL conditions and probably under prolonged HL conditions. On the other hand, the negative element (NE1) located downstream of the basal promoter decreased P1 activity irrespective of light intensity.

The architecture of P2

The transcriptional start point of P2 corresponded to 45 nucleotides upstream of the psa4 start codon (noted as +100), and P2 had conserved –35 and –10 elements (Fig. 1B). A region required for the basal activity of P2 was found to be located within the (+58 to +101) region (Fig. 3B, A42). Elongation at the 3′ end from +101 to +123, while maintaining the 5′ end at +58 (compare strain A42 and A43), decreased P1 activity irrespective

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Fig. 2 Validation of the bacterial luciferase reporter assay under different light conditions. (A) Northern blot analysis of the psaAB genes upon the shift to HL conditions in wild-type (WT) cells. A 2 μg aliquot of total RNA was loaded in each lane. A DIG-labeled psaA-specific probe was used for detection. (B) Northern blot analysis of the luxAB reporter genes upon the shift to HL conditions in A0 and A1 reporter strains harboring the full-length (–271 to +144) and 5′-deleted (–176 to +144) fragment of the psaAB promoter, respectively. A 2 μg aliquot of total RNA was loaded in each lane. A DIG-labeled luxA-specific probe was used for detection. (C) Changes in bioluminescence levels from A0 and A1 strains upon the shift to HL conditions. Error bars represent the standard deviation obtained from three independent measurements. (D) Relationship between transcript levels of luxAB and bioluminescence levels in reporter strains acclimated to LL (filled circles) and those exposed to HL for 6 h (open circles). The luxAB transcript level was examined by Northern blot analysis and quantified with Scion Image software.
of light intensity. This indicates that the (+102 to +123) region works as a negative regulating element for P2. This element was designated as NE2B. Further elongation of the 3' end from +123 to +144 increased the luxAB expression under both LL and HL conditions (compare strain A43 and A44), indicating that there exists a positive regulating element in the (+124 to +144) region. This region was designated as PE2B.

Next, the 5' end was elongated from +58 to +20, while maintaining the 3' end at +144. The (+20 to +57) region seems to work as a positive regulating element of P2, because the expression level of the luxAB genes in A4 was higher than that in strain A44 under both LL and HL conditions. Thus, the (+20 to +57) region was designated as PE2A. Considering that the A4 strain which possessed PE2A, NE2B and PE2B did not show a light response, these elements are unlikely to be involved in the light response. Further elongation of the 5' end from +20 to −46 decreased luxAB expression under both LL and HL conditions (compare strain A4 and A3), in spite of inclusion of P1 in the fragment. This indicates that there exits a negative regulatory element for P2 in the (−46 to +19) region, designated as NE2A. NE2A was also not involved in the light-induced decrease of P2 activity. A2 and A1 strains showed constitutive luxAB expression although both strains possessed PE1, the cis-element required for the light response of P1. It is likely that the light response of P1 was masked by the constitutively high activity of P2 in these strains. However, total promoter activities of psaAB after 1 h of HL exposure gradually decreased by the successive elongation of the 5' end from −176 to −271, with a fixed 3' end at +144 (compare strain A1, A13, A12 and A0). This indicates that P2 can respond to changes in light intensity when the (−271 to −177) region is added. Thus, we designated the region as HNE2 because it is the HL condition-specific negative regulating element for P2.

Fig. 3  Deletion analysis of the psaAB promoter region. (A) A schematic representation of a series of 5'- and/or 3'-deleted psaAB promoter fragments in reporter strains (A0–A9). The numbers above the promoter fragments refer to the nucleotide positions relative to the transcriptional start point of P1 (tsp1), marked as +1. The arrangement of positive (PE) and negative (NE, HNE) regulatory elements in two promoters of the psaAB genes, P1 and P2, is shown underneath. (B) Bioluminescence levels from reporter strains and the control strain having promoterless luxAB genes, grown under LL conditions. Error bars represent the standard deviation obtained from at least four independent measurements. (C) Changes in levels of the luxAB transcripts of the reporter strains shown by dot blot analysis. Total RNA was isolated from reporter strains incubated under HL conditions for the indicated period. A 5 μg aliquot of total RNA for each dot was spotted onto the nylon membrane and hybridized with the DIG-labeled luxAB probe.
It is noticeable that HNE2 is located very far from the core of P2. HNE2 might be in contact with P2 by means of the change in the secondary structure of DNA. Whether the upstream region of \textit{psaAB} can bend in response to a change in light intensity to allow the interaction between HNE2 and P2 should be tested in the future.

In conclusion, P2 was also controlled by multiple \textit{cis}-elements, three NEs and two PEs. While HNE2 functioned to repress P2 activity under HL conditions, the other two NEs and two PEs affected the activity of the basal promoter (\(+58\) to \(+101\)) in a light intensity-independent manner.

**Protein factors bound to the promoter region of the \textit{psaAB} genes**

To detect the binding of putative regulatory proteins to the promoter region of \textit{psaAB}, DNA gel mobility shift assays were performed with the crude extract from \textit{Synechocystis} cells grown under LL conditions. As summarized in Fig. 5A, at least five regions (\(-271\) to \(-242\), \(-161\) to \(-126\), \(-125\) to \(-92\), (around \(+20\)) and \(+80\) to \(+123\)) were found to be responsible for the binding of protein factors, and were designated as BR (Binding Region) 1, BR2, BR3, BR4 and BR5, respectively. When the crude extract from cells grown under HL conditions was used, the observed DNA–protein complexes were exactly the same as those obtained with the crude extract from LL-grown cells (data not shown). However, we could not exclude the possibility that the result was due to the loss of the intracellular redox environment of HL-grown cells during preparation of the crude extract. To determine whether the redox environment affects the binding activity of putative regulatory proteins, DNA gel mobility shift assays were carried out under reduced conditions with dithiothreitol (DTT) using protein extract prepared in the presence of DTT or under oxidized conditions without DTT using protein prepared without DTT. In Fig. 5B–E, the results of DNA gel mobility shift assays with F/29, 27/23, 14/9 and 32/20, which are DNA fragments covering five BRs, are shown. When F/29 or 27/23 was used as a probe, a large shifted band was detected only under the oxidized condition (Fig. 5B, C, lane 3). As the extent of the band shift observed with F/29 and 27/23 was similar, we checked whether these shifts were ascribed to the same protein. By addition of a 300-fold excess of unlabeled 27/23 fragments to the reaction mixture, formation of the large shifted band of F/29 was completely prevented (Fig. 5B, lane 4). This indicates that the same protein factor is bound to BR1 and BR2. The nucleotide sequences of BR1 and BR2 are shown in Fig. 5F. As common features between them, AT-rich sequences, T(C/G)AAAAT(C/G)C and TT(C/T)(T/G)TAACA, were found.

Interestingly, when the 5/8 or 27/23 probe was used, only a small shifted band was detected under the reduced condition (Fig. 5C, lane 2) instead of the large shifted band observed under the oxidized condition (Fig. 5C, lane 3). The protein factor responsible for the small shifted band must be bound to BR3, considering that the shifted band was not observed with the 27/23 probe.

When 14/9 or 6/11 was used as a probe, a single shifted band was detected irrespective of the addition of DTT (Fig. 5D). On the other hand, the shifted band was not detected with either the 6/8 or the 7/18 probe. This indicates that a protein factor could be bound to BR4.

When 7/13, 32/20 or 32/13 was used as a probe, a clear shifted band was observed irrespective of the addition of DTT (Fig. 5E). Since no shifted band was observed with the 7/18 or 19/13 probe, the protein factor must be bound to BR5. Noticeably, the amount of the crude extract required for the band shift at BR5 was very low compared with that required for the band shift at the other BRs, indicating that the protein factor bound to BR5 may exist abundantly in \textit{Synechocystis} cells, or may have higher affinity compared with other protein factors bound to other BRs.
Discussion

The two light-responsive promoters in the psaAB genes

In this study, to elucidate the mechanism of light intensity-dependent regulation of psaAB gene expression in Synechocystis sp. PCC 6803, we analyzed psaAB promoter architecture using transcriptional fusions of a series of psaAB upstream regions to the promoterless luxAB reporter genes (Fig. 3). We found that two promoters (P1 and P2) were responsible for transcription of the psaAB genes. As for the psaB gene, it does not appear to have its own promoter as suggested by Herranen et al. (2005), since the intergenic region between psaA and psaB did not yield luciferase reporter activity (data not shown).

Although the activities of both P1 and P2 decreased upon the shift to HL conditions (Fig. 1), the mechanisms of achieving light response were completely different between them, as shown in Fig. 6. The key element for the light response of P1 is PE1 involved in positive regulation under LL conditions. P1 activity under LL is highly up-regulated by PE1. Upon a shift to HL conditions, PE1 is rapidly inactivated, which resulted in the drastic decrease of P1 activity. As for P2, the key element for the light response is HNE1 involved in negative regulation under HL conditions. Under LL where HNE1 is inactivated, P2 shows high activity. When shifted to HL, HNE1 becomes active and the decrease of P2 activity is observed. As a result, the activity of both P1 and P2 is greatly suppressed under...
HL conditions. Although the sensing mechanism of changing light intensity has remained unsolved, the over-reduction of the photosynthetic electron transport chain or imbalance in cellular metabolism caused by the HL shift may be a possible candidate for the HL signal controlling gene expression. Such an HL signal responsible for the down-regulation of P1 and P2 seems to be lost in the course of HL acclimation considering that the psaAB transcripts accumulated again about 3 h after the shift to HL conditions. It is noted that the amount of PSI complex decreased during HL incubation in spite of the gradual increase of protein complexes. It is noted that the amount of PSI complex accumulated again about 3 h after the shift to HL, and the decreased level of PSI was maintained for HL acclimation considering that the down-regulation of P1 and P2 seems to be lost in the course of HL incubation in spite of the gradual increase of protein complexes. The activity of P2 is down-regulated by HNE2. Enhancement of PE1, the activity of P2 is down-regulated by HNE2. (B) Upon the shift to HL conditions, up-regulation of P1 activity is lost, presumably through the inactivation of PE1. The activity of P2 is down-regulated by HNE2.

**The mechanism of the light response of P1**

The light response of P1 was achieved by PE1 located in the (–69 to –47) region just upstream of the –35 element (Fig. 3). By base substitution analysis of the PE1 region, we found that the AT-rich sequence of PE1 was indispensable for the light response of P1 (Fig. 4). In Synechococcus sp. PCC 7942, the psbAI promoter, whose activity is known to decrease upon the shift from LL to HL conditions, also possessed an AT-rich sequence, 5'-AGCTAAAAATTATTTTAA-3', located in the (–54 to –43) region just upstream of the –35 element (Nair et al. 2001). This segment was implicated in both promoter activation per se and light-responsive regulation of psbAI, just like PE1 for P1 of psaAB in Synechocystis sp. PCC 6803. In eubacterial promoters, AT-rich sequences of about 20 bp located just upstream of the –35 element, called UP-elements, play a role in increasing promoter activity by up to 300-fold (Ross et al. 1993, Estrem et al. 1998). The UP-element can interact with the α-subunit of RNA polymerase and has been referred to as the third promoter recognition element besides the –35 and –10 hexamers. (Ross et al. 1993, Rao et al. 1994, Czarniecki et al. 1997, Ross et al. 1998, Tagami and Aiba 1999, Ozoline et al. 2000). The UP-element is suggested to function in two parts, the distal subsite extending from –59 to –46, and the proximal subsite extending from –46 to –38. The consensus sequence of the distal subsite is 5'-AAAAAATTTTTT-3' and that of the proximal subsite is 5'-AAAAAARNR-3', each of which is sufficient for binding the α-subunit and can increase promoter activity by itself (Estrem et al. 1998, Gourse et al. 2000). In fact, numerous *E. coli* promoters, driving expression of a wide array of gene products, contain one of these subsites of the UP-element (Estrem et al. 1998). The –62 to –49 region of PE1, 5'-TTTTTTATTATTAG-3', matches the sequence of the distal site (12 out of 14), and also its location is suitable as the distal site, suggesting that PE1 comprises the UP-element and might work as a platform for the interaction with the α-subunit of RNA polymerase. The AT-rich sequence of psbAI in Synechococcus PCC 7942 may also belong to the UP-element, since the sequence of the (–51 to –43) region matches the consensus of the proximal subsite in seven out of nine positions. These findings implicate that P1 of psaAB in Synechocystis and the promoter of psbAI in Synechococcus possess a common mechanism to exhibit a light response although the mechanism of inactivation of these putative UP-elements upon the shift to HL remains to be solved. One explanation is that some *trans*-factors might affect these activities. It should be noted that a protein factor bound to a UP-element was reported in *E. coli*. Methylated Ada protein binds to a UP-like element in ada and aidB promoters to stimulate transcription by modifying the nature of the RNA polymerase–promoter interaction.
interaction (Landini and Volkert 1995). Also, it has been reported that the UP-element of the deoP2 promoter of E. coli overlaps with the DNA-binding site of CylR, a repressor of deoP2 (Shin et al. 2001).

The mechanism of the light response of P2

The light response of P2 is found to be regulated by the region designated as HNE2 located far from the core sequence of P2 (Fig. 3). HNE2 repressed P2 activity under HL conditions. Although how HNE2 modulates P2 activity is involved in this regulatory mechanism considering the distance between HNE2 and the core sequence of P2. There are some reports of participation of DNA bending in transcriptional regulation in both eukaryotes and prokaryotes. For example, in the gal operon of E. coli, DNA bending is known to be involved in the negative control of promoter activity (Irani et al. 1983). In the case of cyanobacteria, the occurrence of DNA bending is reported in psbAB in Microcystis aeruginosum K-81. This DNA bending, called CIT, is suggested to affect the basal activity of psbA2 transcription (Asayama et al. 2002).

Protein factors bound to the psaAB promoter region

DNA gel mobility shift assay revealed that at least four different protein factors were bound to five regions of psaAB promoters (Fig. 5). We showed that the same protein factor could be bound to BR1 and BR2 under oxidized conditions. Under reduced conditions, however, the binding activity to BR1 and BR2 was not detected. Taking into consideration that BR1 was located within HNE2, the protein factor bound to BR1 might be involved in the regulation of the light response of P2 through perception of the change in the cellular redox state. Regarding BR2, we could not assign any regulatory roles to this region by deletion analysis. However, there remains a possibility that BR2 works as a cis-element involved in the light response because BR2 bound the same protein factor as BR1. To ascertain this possibility, mutational analyses in BR2 will be required. As was the case for BR2, we could not assign any regulatory roles to the BR3 region. However, considering that the binding of a protein to BR3 was only observed in the oxidized condition where no binding activity was detected at BR1 and BR2, the proteins bound to BR3 and BR1/2 might work competitively in response to the cellular redox state. In contrast to the above-mentioned binding factors, the binding activities observed with BR4 and BR5 were not affected by the presence or absence of DTT. This feature is consistent with the fact that NE2A, PE2A, NE1 and NE2B affected promoter activity regardless of light intensity (Fig. 3, 5A).

Based on the information obtained in this study, we are going to isolate putative regulators that bound to psaAB promoters. Characterization of null mutants of these regulators will provide further information on the direct role of these proteins in the regulation of light-responsive gene expression. In addition, analysis of the promoter architecture of other PSI genes is now in progress. This analysis will lead to understanding of the mechanism of the coordinated light response of PSI genes scattered throughout the whole genome.

Materials and Methods

Strains and culture conditions

A glucose-tolerant wild-type strain and reporter-transformed strains of Synechocystis sp. PCC 6803 were grown at 31°C in BG-11 liquid medium (Stanier et al. 1971) with 20 mM HEPES-NaOH, pH 7.0. Unless stated otherwise, cultures were grown under continuous illumination provided by fluorescent lamps at 20 μmol photons m⁻² s⁻¹. To maintain reporter strains, spectinomycin (20 μg ml⁻¹) was added to cultures. Cells were grown in volumes of 50 ml in test tubes (3 cm in diameter) and bubbled with air. Cell density was estimated at OD₇₃₀ using a spectrophotometer (model UV-160A; Shimadzu, Kyoto, Japan). HL shift experiments were performed by transferring cells at the exponential growth phase (OD₇₃₀ = 0.1–0.2) from LL (20 μmol photons m⁻² s⁻¹) to HL conditions (250 μmol photons m⁻² s⁻¹).

Escherichia coli and DNA manipulation

XL1-Blue MRF' (Stratagene, La Jolla, CA, USA) was the host for all plasmids constructed in this study. When required, ampicillin (100 μg ml⁻¹) or spectinomycin (20 μg ml⁻¹) was added to Terrific Broth medium for selection of plasmids in E. coli. Procedures for the growth of E. coli strains and for the manipulation of DNA were as described in Sambrook et al. (1989). Sequencing of plasmids was carried out by the dideoxy-chain termination method using a dye terminator cycle sequencing ready reaction kit (ABI PRISM; Applied Biosystems, Foster City, CA, USA).

Primer extension analysis

Primer extension analysis of the psaAB genes was carried out using 10 μg of total RNA as the template. The primer, psaA-10 or psaA-13 (Table 1), was labeled with [γ⁻³²P]ATP (ICN Biomedicals, Irvine, CA, USA) using T4 polynucleotide kinase (USB, Cleveland, OH, USA). Reverse transcription was performed as follows. Total RNA was incubated at 70°C for 10 min in a 15 μl reaction mixture containing 5 x First Strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl and 15 mM MgCl₂) and 2 pmol of labeled primer. Then, 2 μl of 0.1 M DTT and 2 μl of 10 mM dNTPs were added and reaction mixture was further incubated at 42°C for 5 min. After addition of 1 μl of Superscript II (Invitrogen, Carlsbad, CA, USA), the reverse transcription reaction was performed at 42°C for 60 min. The extension products were ethanol precipitated, resuspended in loading buffer [98% formamide (v/v), 0.5 mM EDTA, 0.3% xylene cyanol (w/v), 0.3% bromophenol blue (w/v)] and denatured at 95°C for 3 min. Samples were electrophoresed together with DNA ladders on a gel containing 6% polyacrylamide and 8 M urea. DNA ladders were created using a T7 sequencing kit (USB) with the same end-labeled primer as that used for reverse transcription.
RNA isolation and RNA blot analysis

RNA isolation and Northern blot analysis were performed as described previously (Muramatsu and Hihara 2003). For dot blot analysis, 5 µg of total RNA per each dot was spotted onto the nylon membrane (Hybond N+; Amersham Biosciences, Uppsala, Sweden). To generate gene-specific probes, the psaA and luxA genes were amplified by PCR from genomic DNA of Synechocystis and from pPT6803-1 vector (see below), respectively. The following primers were used: psaA forward (5’TCCCCCTCCCCAGTGGT-3’), psaA reverse (5’-ACCCGGCCCTAGGATGAG-3’), luxA forward (5’-ACTTATCAGGACTTTCT-3’) and luxA reverse (5’-TATCTTTGCTCTATTTG-3’). To use PCR products directly as templates for in vitro transcription, the T7 polymerase recognition site was added to the reverse primers at their 5′ termini. The in vitro transcription reaction was carried out with a digoxigenin (DIG) RNA labeling kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions.

Construction of luxAB reporter strains

All plasmids used for transformation of Synechocystis cells were derivatives of pPT6803-1, which is a recombinatorial plasmid having the promoterless luxAB genes, the neutral site of Synechocystis sp. PCC 6803 (the downstream region of the ndhB gene) and the spectinomycin resistance cassette (Aoki et al. 2002, Muramatsu and Hihara 2003).

Each promoter fragment was generated by PCR with primers containing the BoWl site at their 5′ termini, and cloned into the unique BoWl site of pPT6803-1 to produce transcriptional fusions with the promoterless luxAB gene. All primers used in this study are shown in Table 1. The nucleotide sequence and direction of the promoter region in the reporter constructs were verified by sequencing. Wild-type Synechocystis was transformed with the pPT6803-1 derivatives, and transformants were selected and propagated in liquid BG-11 with spectinomycin. All the reporter strains used in this study are shown in Table 2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description of promoter fragment</th>
<th>Insert size</th>
<th>Primer combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>luxAB reporter vector (pPT6803-1) without promoter fragment</td>
<td>415 bp</td>
<td>PpsA-A-F/PpsA-A-R</td>
</tr>
<tr>
<td>A0</td>
<td>(-271 to +144) region of psaAB</td>
<td>385 bp</td>
<td>PpsA-28/PpsA-A-R</td>
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<tr>
<td>A12</td>
<td>(-241 to +144) region of psaAB</td>
<td>355 bp</td>
<td>PpsA-24/PpsA-A-R</td>
</tr>
<tr>
<td>A13</td>
<td>(-211 to +144) region of psaAB</td>
<td>320 bp</td>
<td>PpsA-5/PpsA-A-R</td>
</tr>
<tr>
<td>A1</td>
<td>(-176 to +144) region of psaAB</td>
<td>235 bp</td>
<td>PpsA-3/PpsA-A-R</td>
</tr>
<tr>
<td>A2</td>
<td>(-91 to +144) region of psaAB</td>
<td>190 bp</td>
<td>PpsA-6/PpsA-A-R</td>
</tr>
<tr>
<td>A3</td>
<td>(-46 to +144) region of psaAB</td>
<td>125 bp</td>
<td>PpsA-7/PpsA-A-R</td>
</tr>
<tr>
<td>A4</td>
<td>(+20 to +144) region of psaAB</td>
<td>44 bp</td>
<td>PpsA-17/PpsA-A-18</td>
</tr>
<tr>
<td>A42</td>
<td>(+58 to +101) region of psaAB</td>
<td>66 bp</td>
<td>PpsA-17/PpsA-A-20</td>
</tr>
<tr>
<td>A43</td>
<td>(+58 to +123) region of psaAB</td>
<td>87 bp</td>
<td>PpsA-17/PpsA-A-R</td>
</tr>
<tr>
<td>A44</td>
<td>(+58 to +144) region of psaAB</td>
<td>71 bp</td>
<td>PpsA-14/PpsA-A-15</td>
</tr>
<tr>
<td>A61</td>
<td>(-69 to +2) region of psaAB</td>
<td>48 bp</td>
<td>PpsA-6/PpsA-A-15</td>
</tr>
<tr>
<td>A62</td>
<td>(-46 to +2) region of psaAB</td>
<td>65 bp</td>
<td>PpsA-6/PpsA-A-8</td>
</tr>
<tr>
<td>A63</td>
<td>(-46 to +19) region of psaAB</td>
<td>110 bp</td>
<td>PpsA-3/PpsA-A-8</td>
</tr>
<tr>
<td>A6</td>
<td>(-91 to +19) region of psaAB</td>
<td>142 bp</td>
<td>PpsA-3/PpsA-A-9</td>
</tr>
<tr>
<td>A7</td>
<td>(-91 to +51) region of psaAB</td>
<td>174 bp</td>
<td>PpsA-3/PpsA-A-10</td>
</tr>
<tr>
<td>A8</td>
<td>(-91 to +83) region of psaAB</td>
<td>206 bp</td>
<td>PpsA-3/PpsA-A-11</td>
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<tr>
<td>A9</td>
<td>(-91 to +115) region of psaAB</td>
<td>71 bp</td>
<td>PpsA-61mt1/PpsA-A-15</td>
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<tr>
<td>A61mt1</td>
<td>pA61 with GGGGC (–68)→CCCCG</td>
<td>71 bp</td>
<td>PpsA-61mt1/PpsA-A-15</td>
</tr>
<tr>
<td>A61mt2</td>
<td>pA61 with TTTTT (–61)→GCCGC</td>
<td>71 bp</td>
<td>PpsA-61mt2/PpsA-A-15</td>
</tr>
<tr>
<td>A61mt3</td>
<td>pA61 with TTATT (–54)→CGCGC</td>
<td>71 bp</td>
<td>PpsA-61mt3/PpsA-A-15</td>
</tr>
</tbody>
</table>

Each insert DNA was amplified by PCR using primers indicated and cut with BoWl prior to cloning in the BoWl site of pPT6803-1.
adding 0.4 g of solid (NH₄)₂SO₄ to 1 ml of extract. After resuspension in 1 ml of extraction buffer, the extract was dialyzed twice for 12 h against 11 of the extraction buffer. All manipulations were carried out at 4°C. Protein concentration was determined using Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions.

**DNA gel mobility shift assays**

DNA fragments used as probes or competitors in gel mobility shift assays were generated by PCR. To prepare probes for assays, the 3′ end of DNA fragments were labeled with DIG-ddUTP by terminal transferase using the DIG Gel Shift Kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Assays were performed using a DIG Gel Shift Kit (Roche). Cell extract from *Synechocystis* was incubated with 30 fmol DIG-labeled DNA fragment in a 20 µl reaction mixture containing 1 µg of poly d(I-C), 0.1 µg of poly-L-lysine, 20 mM HEPES-KOH, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 0.2% Tween-20 (v/v), 30 mM KCl and 1 mM DTT. DTT was not added to the reaction mixture when the binding reaction was performed under oxidized conditions. After overnight incubation at 4°C, 5 µl of gel loading buffer consisting of 60% of 1× TBE (v/v) and 40% glycerol (v/v) was added to the reaction mixture. Samples were then applied onto a 6% polyacrylamide gel and subjected to electrophoresis at 95 V for 2.5 h at 4°C. DNA and protein were transferred to a nylon membrane (Hybond N+) by capillary transfer method and fixed by baking at 80°C for 2 h. Detection of the DIG-labeled probe was performed according to the standard protocol for the DIG Luminescent Detection Kit (Roche).

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**References**


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