Light-responsive psbA Transcription Requires the \(-35\) Hexamer in the Promoter and Its Proximal Upstream Element, UPE, in Cyanobacteria

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We characterized the function of the \(-35\) hexamer in the promoter and an element just upstream, UPE, in the expression in a unicellular cyanobacterium, Microcystis aeruginosa K-81, of the light-responsive gene psbA2, which encodes a reaction center key protein for photosynthesis. A series of mutants with mutations at the \(-35\) hexamer (\(-35\) to \(-30\)) and a novel conserved upstream element (UPE: \(-45\) to \(-36\), +1 referring to the transcription start point) were constructed. Expression of the mutants was examined in vivo and in vitro by analyses using a \(\beta\)-galactosidase assay, primer extension, and a DNA-mobility shift assay with RNA polymerases. Results indicated that the \(-35\) hexamer and its proximal UPE act as effective cis-elements for the light-responsive and/or basal transcription, respectively. A model of the 5\(^{'\prime}\)-upstream region with cis- and possible trans-acting factors is presented for the psbA regulatory system.

Key words: transcription; RNA polymerase; sigma factor; curved DNA; photosynthesis

It has generally been accepted that the ancestors of cyanobacteria gave rise to plant chloroplasts through endosymbiotic events, thereby conferring the ability for photosynthesis on algae and plants. Members of the genus Microcystis, categorized as of the same group as Synechocystis, are cyanobacteria (blue-green algae) which perform oxygenic photosynthesis involving two photosystems, PS I and PS II, as do higher plants. One such member, the unicellular colony forming Gram-negative cyanobacterium Microcystis aeruginosa strain K-81 (hereafter referred to as K-81) contains a psbA2 gene encoding a D1 homolog as a core protein in PS II.1) In higher plants, a psbA gene is encoded in the chloroplast genome. In contrast, cyanobacteria have multi-gene families of psbA in chromosomal DNA, the transcription of which is differentially expressed in response to changes in light intensity (photon flux density). For example, the transcript of the psbAI gene, which encodes the Form I type of D1 protein in a unicellular cyanobacterium, Synechococcus elongatus PCC 7942 (hereafter referred to as PCC 7942), is predominantly expressed under low light intensity, whereas transcripts of the psbAII and III genes, which encode the Form II type of D1 protein, rapidly increase in number upon a shift to high light intensity along with a correlative decrease in the psbAII transcript.2–5) The K-81 strain also has multiple psbA homologs.6) The psbA2 gene is one of them, which encodes the Form II type (as defined for PCC 7942) of the D1 homolog.7,8)

Although the light-dependent and circadian-rhythmic gene expressions have been studied in cyanobacteria, the functions of DNA architectures with cis-elements and response regulators for the transcription have not been sufficient. Important 5\(^{'\prime}\)-upstream cis-acting elements of the K-81 psbA2 gene, involving its unique promoter and an AU-box, have been characterized for basal, light-responsive, and circadian-rhythmic transcription.9–11) The light-dependent expression may occur through basal transcription modified by cis- and trans-acting factors. Previous studies also showed that the AU-box motif (psbA2, \(+26\) to \(+33\), UAAAUAACA) in the 5\(^{'\prime}\)-upstream untranslated leader region (5\(^{'\prime}\)-UTR, \(+1\) to \(+52\)) confers mRNA instability in darkness for the light-dependent expression.12) The light-dependent expression is therefore controlled at the post-transcriptional level at least. It has been reported that a deletion mutant of the \(-35\) hexamer produces abundant psbA2 transcripts with an almost constant pattern in light/dark conditions.13) This implies that light-dependent expression is also modified at the transcriptional level. On the other hand, it is accepted that the tertiary structures of DNA affect gene expression, and sequence-directed (intrinsic, static) or protein-induced DNA bends refer to changes in the DNA double helix conformation. We have found and characterized a novel curved DNA, CIT (Changeable bending-center sites of an Intrinsic curvature under Temperature conditions) located in the 5\(^{'\prime}\)-upstream region of psbA2.14) We have reported the

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universality of the curved DNA structure in psbA genes of another cyanobacterium, red alga, and in plants, and also showed the roles of the curvature in basal transcription in cyanobacteria. Potential factors contributing to the basal transcription of psbA2 with the CIT curvature were also found.10)

The bacterial RNA polymerase holoenzyme, composed of a σ subunit and a core enzyme containing the major subunits α2 (RpoA), β (RpoB), and β′ (RpoC), plays a central role in gene transcription.13) In cyanobacteria, the β' subunit has diverged into γ (RpoG or RpoCl) and β'' (RpoC or RpoC2), corresponding to β' (RpoCl) and β'' (RpoC2) in higher plant chloroplasts.14) The core enzyme functions in RNA polymerization but requires the sigma subunit for specific transcription initiation at the promoter.15,16) General switching in transcription is mainly due to the modulated promoter selectivity of multiple RNA polymerase holoenzymes combined with the replacement of a common core enzyme with several different sigma factors, responding to environmental or internal cellular change. Heterogeneous sigma factors are classified into three groups.17–21) The cyanobacterial group 1 comprises principal sigma factors that recognize Escherichia coli σ70 (RpoD) type promoters of housekeeping genes, and are essential for cell viability. The cyanobacterial group 1 sigma subunits have been cloned and characterized.17–21) In the K-81 cyanobacterium, σ35 (rpoD1 gene product) is the principal factor and can recognize specifically the psbA2 promoter,17,18,19) which has an E. coli consensus sequence, TTTACA at the −35 hexamer and TAGTAT at the −10 hexamer, with a 17-bp spacer.

Recent experiments with mutants also revealed that some positive trans-acting factor associated with the AT-rich sequence (−60 to −40) might effectively recruit the RNAP holoenzyme to the −35 hexamer, or the binding of a negative trans-acting factor to a −35-like sequence could be prevented in the −35 hexamer mutant.10,11) However, transcription with these cis-elements upstream from the −35 hexamer in the promoter have not been well characterized in cyanobacteria. In this article, we analyzed the function of the −35 hexamer in the promoter and its proximal upstream element, which is novel and conserved in the cyanobacterial Form II type of psbA (defined as psbA2/3 or psbAII/III), in light-responsive transcription.

Materials and Methods

Bacterial strains and growth conditions. E. coli strain DH5αMCR, routinely used for recombinant DNA manipulation, was grown in 2×TY liquid medium22) or on plates containing the appropriate antibiotics ampicillin (75 µg/ml) and spectinomycin sulfate (40 µg/ml), if necessary. Wild-type and recombinant PCC 7942 were cultivated in BG11 liquid medium23) without and with spectinomycin (40 µg/ml), respectively, under continuous white-light illumination (35 µE m⁻² s⁻¹).

Construction of plasmids. Oligonucleotides were designed to create a series of mutagenized UPEs (or promoters) of psbA2, and are listed in Table 1. A set (F and R) of oligonucleotides were annealed in

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The linker (small characters) involving the SmaI, BglII, or EcoRI sites (underlines) and mutagenized sequences (bold) are shown.
buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA·2Na] at 70°C. The resultant double-strand DNA was isolated, digested with SmaI and BglII, and inserted into the SmaI-BglII site of pAM990, which is a promoter-probe transcriptional fusion vector,7 to yield pIY reporter plasmids. Nucleotide sequences of the insert DNA of pIY were verified and their plasmids were used for transformation and an in vitro assay.

Transformation. The pIY plasmids were introduced into host E. coli DH5αMCR or PCC 7942 cells by previously reported methods.3,24,25 Transformants were selected on a 2×TY plate for E. coli or on a BG11 plate for PCC 7942, supplemented with 75 μg/ml of ampicillin and 40 μg/ml of spectinomycin sulfate or 40 μg/ml of spectinomycin sulfate only, respectively. Spectinomycin-resistant cells of PCC 7942 were isolated. Recombination was confirmed by Southern hybridization using a 2.5-kb PCR fragment, which contains the PCC 7942 genome sequence derived from pAM990, as a probe. The recombinants were eventually designated as IY strains.

β-galactosidase activities. The activities of transcription from psbA2-lacZ fusion constructs in E. coli DH5αMCR or a cyanobacterium PCC 7942 were measured by a procedure described previously.26

Total RNA isolation. Total RNA was isolated from 50 ml of cell culture of recombinant PCC 7942 strains, grown under conditions of continuous white-light illumination (35 μE m⁻² s⁻¹) or darkness, to the mid-log phase (OD750 = 0.4 to 0.5), by a hot phenol method described previously.7

Primer extension. Primer extension analyses were done with the total RNA and lacZ internal primers of lacZ-R1 (Table 1) at 50°C79 or an M4 primer (Table 1) at 42°C, as described previously.7 The annealing sites of each primer are partially overlapped and located downstream approximately 40 bp from the initiation codon of lacZ. Products of the extension were finally dissolved in 7 μl of dye and denatured at 95°C for 5 min. A sample of 3 μl was resolved on a 6% polyacrylamide sequencing gel containing 8 M urea. In vitro primer extension was done as described above, following in vitro mRNA synthesis (see the section below). The experiments were done independently at least two or three times. Each electrophoresis was basically done on a polyacrylamide gel supplying the sample of wild type construct (pIY1 or IY-1), of which the pattern has been evaluated by an internal control, and the signals intensities were also evaluated on an X-ray film, as reported previously.12

RNA polymerase (RNAP) and in vitro mRNA synthesis. The homologous RNAP holoenzymes of E. coli Erα20, heterologous E+σA1 [reconstituted with E. coli core enzyme (E) and the purified K-81 principal sigma factor, σA1], and partially purified RNAP fraction, *EσA1 (isolated from the K-81 cells grown under light), were prepared as described previously.7,18 A multiple-round run-off reaction for in vitro synthesis was done as follows. First, 40 μl of assay mixture [50 mM Tris-HCl (pH 8.0), 0.05 mM EDTA·2Na, 0.5 mM DTT, 10 mM MgCl₂, 0.1 mM dNTPs, template supercoiled DNAs of pIY (2 μg) of a series for the psbA2 promoter mutant, and E. coli Erα (1 μl, 1 pmol), heterologous E+σA1 (E: 0.5 μl, 1.45 pmol; σA1: 1 μl, 2.92 pmol), or partially purified RNAP of K-81 *EσA1 (1.5 μl, 1 pmol)] was incubated at 30°C for 15 min, and then the reaction was stopped by the addition of 60 μl of stop solution (40 mM EDTA·2Na, 300 μg/ml of E. coli RNAse, and 300 mM LiCl) and precipitated with 2-propanol. The resultant total mRNAs were put through in vitro primer extension.

Gel mobility shift assay. A template DNA carrying the psbA2 upstream region (WT, −47 to +14, Table 1 and Fig. 5) was amplified by PCR with the plasmid pIY1 plus the primers IY13-UPER and IY13-UPER (Table 1). This PCR fragment was digested with EcoRI, then a 164-bp segment was isolated. This fragment was end-labeled by [α-32P] dCTP (Muromachi, Tokyo, Japan) and used as a template, i.e. free DNA, in the assay. Binding of RNAP to DNA was done in 20 μl of a reaction mixture [10 mM Tris-HCl (pH 7.9), 50 mM KCl, 1 mM EDTA·2Na (pH 8.0), 5% (v/v) glycerol, 0.0001% (w/v) salmon sperm DNA (0.01 μg), and 0.0001% (w/v) bovine serum albumin (0.01 μg)] containing 150 fmol of the labeled DNA (IY-1) fragments and Erα70 (0.5 pmol), or 20 fmol of the labeled DNA (IY-1) and *EσA1 (1 pmol). Competitor DNA fragments were also amplified by PCR with each plasmid, pIY1, pIY2, or pIY10, and the IY13-UPER/R primers, and prepared as described above. The labeled DNA template and competitors were added to the reaction mixture on ice and incubated at 30°C for 5 min. RNAP was then added, and incubation was continued at 30°C for 10 min. After the incubation, 3 μl of sample buffer [50% (v/v) glycerol, 0.1% (w/v) xylene cyanol FF, and 0.1% (w/v) bromophenol blue] was added. This mixture was then immediately resolved on a 4% polyacrylamide gel [40 mM Tris-HCl (pH 7.9), 5 mM Na-acetate acid (pH 7.0), 1 mM EDTA·2Na (pH 8.0), and 4% BIS-acrylamide (38:2)]. Electrophoresis was done at 150 V and 4°C for 1.5 to 2 hours with TBS buffer [40 mM Tris-HCl (pH 7.9), 5 mM Na-acetate acid (pH 7.0), and 1 mM EDTA·2Na (pH 8.0)]. The gel was autoradiographed using Fuji RX film.
In contrast, low activity levels of *Microcystis aeruginosa* K-81 to date, the K-81 *psbA* form II type in the *Synechocystis* strain PCC 7942 in our laboratory.9–11) First of all in this study, the heterologous system using recombinant *PCC 7942* and cyanobacteria. The result is shown in Fig. 1.

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**Fig. 1.** Alignment of the *Upstream Elements* (UPEs) from the –35 Sequence of Cyanobacterial *psbA* Families.

The nucleotide sequences from DDBJ or GenBank are shown in parentheses: *Microcystis aeruginosa* K-81 *psbA* (D84228/AB092628), *Synechocystis* sp. strain PCC 6803 *psbA2* (X13547/X56000), *Synechococcus elongatus* PCC 7942 *psbAllIII* (X04617/X04618), *Anabaena* sp. strain PCC 7120 *psbAIIIV* (U21334), respectively. S = G or C, Y = T or C.

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**Table 1** shows a significant function of the UPE and −35 hexamer for the basal transcription in *E. coli* or cyanobacteria

In previous experiments, a common regulatory mechanism for the *psbA* basal (=non light-responsive) transcription with RNA polymerases has been found in *E. coli*, cyanobacteria K-81 and PCC 7942, even when different species of Gram-negative bacteria were used.7,11) In addition, although homologous recombination has not been identified in *Microcystis aeruginosa* K-81 to date, the K-81 *psbA* transcripts with clear patterns of light-responsive and circadian-rhythmic expression have been well characterized in the heterologous system using recombinant PCC 7942 in our laboratory.9,13) First of all in this study, we examined the basal transcription of K-81 *psbA* in non-photosynthetic or photosynthetic bacteria using *E. coli* or PCC 7942 cells. The β-galactosidase activity was very weak or absent in *E. coli* strains carrying pLY2 (Δ−35), pLY4 [−35 (AG→TC)], and pLY5 [−38 to −35 (CT→GA), see Table 1] but not in other constructs (Fig. 2A). This shows a significant function of the −35 hexamer (−35 to −30) for the basal transcription in *E. coli*. In contrast, low activity levels of β-galactosidase were observed in the cyanobacterial mutants of not only the −35 hexamer (IY-2 and IY-4) but also UPE (IY-9 and IY-10) (Fig. 2B). This means that both the −35 hexamer and UPE apparently contribute to the basal transcription.

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**Results**

A novel consensus sequence, UPE, upstream of *psbA*

To characterize the upstream cis-elements of the form II type in the *psbA* family, we aligned the nucleotide sequences. The result is shown in Fig. 1. We found a consensus motif, TyAsAASSyyC, just upstream from the −35 hexamer in the promoter, TTTACA.

**Effects of the UPE and −35 hexamer for the basal transcription in *E. coli* or cyanobacteria**

In previous experiments, a common regulatory mechanism for the *psbA* basal (=non light-responsive) transcription with RNA polymerases has been found in *E. coli*, cyanobacteria K-81 and PCC 7942, even when different species of Gram-negative bacteria were used.7,11) In addition, although homologous recombination has not been identified in *Microcystis aeruginosa* K-81 to date, the K-81 *psbA* transcripts with clear patterns of light-responsive and circadian-rhythmic expression have been well characterized in the heterologous system using recombinant PCC 7942 in our laboratory.9,13) First of all in this study, we examined the basal transcription of K-81 *psbA* in non-photosynthetic or photosynthetic bacteria using *E. coli* or PCC 7942 cells. The β-galactosidase activity was very weak or absent in *E. coli* strains carrying pLY2 (Δ−35), pLY4 [−35 (AG→TC)], and pLY5 [−38 to −35 (CT→GA), see Table 1] but not in other constructs (Fig. 2A). This shows a significant function of the −35 hexamer (−35 to −30) for the basal transcription in *E. coli*. In contrast, low activity levels of β-galactosidase were observed in the cyanobacterial mutants of not only the −35 hexamer (IY-2 and IY-4) but also UPE (IY-9 and IY-10) (Fig. 2B). This means that both the −35 hexamer and UPE apparently contribute to the basal transcription.

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**Fig. 2.** Transcription Activities in *E. coli* and Cyanobacteria.

*E. coli* DH5αMCR or PCC 7942 transformants carrying the K-81 mutagenized promoter region were grown in 2×TY liquid medium until log-phase for *E. coli* (A) or grown in BG11 liquid medium under continuous white-light illumination (35 μE m⁻² s⁻¹) for PCC 7942 (B). The cells were harvested and β-galactosidase activities were measured. Relative average values (%) are shown for three independent experiments with error bars.

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psbA2 expression in the UPE or −35 hexamer mutants of cyanobacteria

To obtain direct evidence of the transcripts, and to elucidate further the function of the UPE and −35 hexamer, UPE and −35 box mutants were examined in a cyanobacterium, PCC 7942 (Fig. 3). In this work, we used a functional K-81 *psbA* upstream fragment containing the region from −47 to +14, which has been known as an essential cis-element for *psbA* transcription in cyanobacteria.9,11) Since this region does not have the AU-box sequence (+26 to +33) at the 5'-UTR, we thought that the −47 to +14 fragment [core promoter (−38 to +14)] is a good candidate for light-responsive transcription analysis without the cis-elements (−47 to −39) is a good candidate for light-responsive transcription analysis without the cis-elements contributing to the post-transcriptional regulation. A pattern of partial light-responsive *psbA* transcription was observed in the wild-type cells (IY-1, −47 to +14) under light/dark light (L/D/L) conditions, compared with that in the AG429 (−38 to +14) strain which has an almost non light-responsive expression, as reported previously.12) This means that the region from −47 to −39 containing UPE contributes to the light-responsive transcription (see Fig. 7). When the −35 hexamer was deleted (IY-2) or mutagenized (IY-4), abundant transcripts were observed under darkness (16 h) as reported previously.13) This constant pattern under the L/D/L condi-
tion means that the $-35$ hexamer is required for the light-responsive transcription. In previous experiments, we have used a deletion mutant of the $-35$ sequence (AG447), which has a long upstream region from $-404$ to $+113$. This time, we could confirm the function of the $-35$ hexamer in the promoter, using only the region from $-47$ to $+14$. In addition, transcription at 4 h light was increased in the delta $-35$ mutant (IY-2). This might indicate that the change of the $-35$ sequence to UPE boosts the basal transcription. However, relative amounts of the transcripts decreased in IY-4 ($-35$ to $-39$, AC$\to$TG) and IY-9 (UPE $\times$ 2: a ratio of L28/WD16 = 2.6) compared with that in IY-1 (WT: L28/WD16 = 2.31), suggesting that a core sequence, AAAC, in UPE has some effect on the transcription.

We could observe slightly increased light-responsive patterns in IY-8 (consensus UPE like: a ratio of L28/WD16 = 2.5) and IY-9 (UPE x 2: a ratio of L28/WD16 = 2.6) compared with that in IY-1 (WT: L28/WD16 = 2.31). Furthermore, the pattern of light-responsive transcription was destroyed under dark (16 h) to light (28 h) conditions in IY-10 (delta UPE), indicating that this UPE sequence functions as a positive element for light-responsive transcription in cyanobacteria. Notably, we could also observe low activities when the upstream region from $-47$ to $-36$ was mutagenized in IY-3, -6, -8, -9, and -10 under the continuous light condition (L4), indicating that this region with UPE also contributes to not only the light-responsive but also to the basal transcription (see Fig. 7).

**In vitro psbA2 transcription on the UPE or $-35$ hexamer mutant constructs**

We tested in vitro transcription using RNA polymerase holoenzymes of *E. coli* Es70, heterologous E + $\sigma^A$ [reconstituted with *E. coli* core enzyme (E) and the purified *M. aeruginosa* K-81 principal sigma factor, $\sigma^A$], and a partially purified fraction, *Es$\sigma^A$ (K-81 RNAP with $\sigma^A$ isolated from K-81 cells), respectively (Fig. 4). Specific psbA transcription was driven from the wild-type DNA template (pIY1) by each RNAP. The levels of transcripts were relatively decreased in the $-35$ deletion construct (pIY2), indicating that the $-35$ promoter sequence contributes to the in vitro transcription using each RNAP. The reason why transcripts did not increase in the case of *Es$\sigma^A$ on the IY-2 DNA template will be discussed later. When the UPE deletion construct (pIY10) was used, the transcripts also decreased in each case, however, the order was $E\sigma^A > E+\sigma^A >$ *Es$\sigma^A$, while that in the case of the $-35$ deletion construct (pIY2) was $*Es\sigma^A > E+\sigma^A > E\sigma^A$. These findings show a similar function of each RNAP in psbA2 basal transcription, whereas the UPE sequence is required more for the transcription with cyanobacterial RNAP (*Es$\sigma^A$) than *E. coli* (E$\sigma^A$). In contrast, the $-35$ promoter sequence contributes more to the

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<td>53.2 ± 13.8</td>
<td>40.6 ± 4.1 26.2 ± 9.5</td>
</tr>
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Fig. 3. UPE Mutants and Their Expression in Cyanobacteria.

PCC 7942 transformants carrying the K-81 mutagenized UPE promoter region were grown under continuous white-light illumination (35 µE m$^{-2}$ s$^{-1}$) and then exposed to a 12 h-light/12 h-dark/12 h-light cycle. The cells were harvested at specific times (4 h, 16 h, and 28 h). Total RNA (10 µg) was prepared from the cells and analyzed by primer extension with the M4 primer (Table 1). Relative signal intensities (+1 as the transcription start point, TSP) on an X-ray film are presented for the psbA transcription, with a 100% value referring to that after 4 h-light in the wild-type strain (WT, IY-1). Mutagenized nucleotide sequences are also shown in Fig. 6. Relative average values (%) are shown with standard deviations for two or three independent experiments.
In vitro Transcription from the Mutagenized UPE or −35 Promoters.

Transcripts synthesized with various RNAPs were analyzed by primer extension with the lacZ-R1 primer (Table 1). *EσA1, partially purified authentic K-81 RNAP fraction containing the K-81 principal sigma factor (σA1); E+σA1, heterologous RNAP = E. coli core + K-81σA1; Eσ70, E. coli RNAP holoenzyme. Relative average values (%) are shown with standard deviations for three independent experiments.

Fig. 5. RNAP Binding to the Mutagenized Upstream Regions.

Gel mobility shift assay was done using RNAP of E. coli Eσ70 (A) or cyanobacterial K-81 *EσA1 (B). 32P-end-labeled IY-1 (wild type) and unlabeled competitor fragments (IY-1, IY-2, and IY-10) are shown (Materials and methods). Positions of the free labeled DNA or DNA-RNAP complex are also indicated. The competitor was added at a ×1 (=150 fmol)-, ×10 (=1,500 fmol)-, and ×20 (=3,000 fmol)-fold excess to the reaction with Eσ70, or ×1 (=150 fmol)-, ×8 (=1,200 fmol)-, and ×16 (=2,400 fmol)-fold excess to the reaction with *EσA1, respectively.

Upstream Elements for psbA Transcription

basal transcription with Eσ70 than *EσA1. These phenomena seem not to be contradictory to those results in Fig. 2.

Binding of RNA polymerase to the mutagenized UPE or −35 promoter region

We also examined the binding of RNAP to fragments containing the mutagenized UPE or −35 promoter element (Fig. 5). When we used E. coli Eσ70 (left panel), signal intensities of DNA-RNAP clearly decreased with an increase in the wild-type competitor (IY-1), as in the case with the ΔUPE competitor (IY-10). In contrast, the signal intensities were almost constant if the Δ−35 hexamer competitor (IY-2) was added. The binding strength of Eσ70 for templates is therefore WT = ΔUPE > Δ −35. This shows that the −35 promoter sequence is specifically required for the binding of Eσ70 to the promoter region, although the UPE sequence is not important. On the other hand, when cyanobacterial K-81 *EσA1 was used (right panel), a similar phenomenon was observed using wild-type and Δ−35 competitors. Interestingly, the signal intensity with *EσA1 was partially decreased by adding the ΔUPE competitor. The binding strength of *EσA1 for templates is WT > ΔUPE > Δ −35. This shows that the −35 promoter sequence is also important for the binding of *EσA1 to the promoter. The UPE sequence apparently contributes to the binding of cyanobacterial *EσA1 for the transcription.

Discussion

The findings of this study are summarized in Fig. 6. We previously identified the region from −38 to +14 as a minimal promoter sequence for psbA2 basal (= non light-responsive) transcription.12) In this study, we used the region from −47 to +14 (UPE + minimal promoter sequence) and confirmed the light-responsive transcription (IY-1, Fig. 3). The phenotype of UPE mutants of IY-3 (Δ−38 to −36, ΔCCC), IY-6 (−42 to −36, AC→TG), and IY-10
(ΔUPE) had a reduction of basal- and light-responsive \( \text{psbA2} \) transcription in cyanobacteria. The binding of cyanobacterial RNAP \(*E\text{a}^A1\) to the ΔUPE fragment was also reduced as presented in Fig. 5. These findings apparently indicate that the K-81 UPE sequence, TTAAAACCCC, is required as a positive \( \text{cis} \)-element for the basal- and light-responsive transcription. As a general approach for the scanning mutagenesis, we designed the sequence of IY-5 (−38 to −35, CCCT→GGGA; Table 1), IY-6 (−42 to −39, AAAC→TTTG), and IY-7 (−46 to −43, CTTA→GAAT), corresponding to transversions (\( R \) [A, G] to \( Y \) [T, C] and vice versa) rather than transitions (\( R \) to \( R \) and \( Y \) to \( Y \)). Although we could observe a slight reduction of pattern for light-responsive transcription in IY-6 (a core sequence, AAAC, to TTTG: Fig. 3), the thymidine and \( W \) or guanine nucleotides of TTTG in IY-6 still remain in the consensus UPE sequence, TyAsAAAsyyC. Therefore, another change of the nucleotide sequence at the AAAC position may have more influence on the transcription. A stringent control involving the K-81 UPE sequence, or the direction, length, and phase of the DNA helix might affect the transcription. Of note, we tried to make mutants, IY-5 (−38 to −35, CCCT→GGGA; Table 1) and IY-7 (−46 to −43, CTTA→GAAT), for 4-bp scanning mutagenesis with IY-6 (−42 to −39, AAAC→TTTG). In this experiment, however, we did not succeed in constructing IY-5 and IY-7 mutants of cyanobacteria. Unfavorable modifications of the original UPE might not be tolerated by the PCC 7942 cell. However, the results in \( E. \text{coli} \) cells harboring pIY5 and pIY7 (Fig. 2) were interesting and might not be contradictory to those results in Fig. 3. On the other hand, we could confirm the importance of the −35 hexamer for the binding of the cyanobacterial RNAP to the promoter (IY-2, Figs. 5 and 6) and light-responsive transcription (IY-2 and IY-4, Fig. 3). In recent studies, the RNAP was able to bind to a mutant promoter lacking the −10 sequence, though this did not lead to actual transcription and an arrhythmic circadian pattern for the transcription was observed in mutants lacking the −35 region in AG447 (using a −404 to +113 fragment, Δ−35, +AU-box). It may be of most interest why transcripts are constantly abundant in the −35 promoter mutants under darkness. This question leads us to a negative regulatory system involving some \( \text{trans} \)-acting factor, which can specifically bind to the −35 promoter and hinder transcription in the dark.

A model for light-responsive \( \text{psbA} \) transcription is presented in Fig. 7 based on this study and previous results. Certain functional \( \text{cis} \)-elements and the unique architecture of the \( \text{psbA2} \) upstream region have been characterized. In this study, the UPE and −35 promoter sequences were found to be effective \( \text{cis} \)-elements for the \( \text{psbA2} \) transcription. The −10 hexamer is essential for the basal transcription whereas the −35 hexamer is not. The basal transcription, which is conserved even in non-photosynthetic bacteria, might occur both in light and in darkness. The instability of \( \text{psbA2} \) mRNA was caused by the AU-box element with \( \text{trans} \)-acting factors, which is located just upstream from an SD-sequence in the 5′-UTR.\(^{11,12}\) We have found and characterized a novel curved DNA, CIT, located in the 5′-upstream region of \( \text{psbA2}, \)\(^{8}\) the evolutionally conserved structure of which is present in another cyanobacterium, red alga, and in plants. Potential factors in the RNAP fraction of *E*a\(^A1\) contributing to the basal transcription of \( \text{psbA2} \) with the CIT curvature are also found in the K-81 cyanobacterium (Fig. 7).

Effective \( \text{cis} \)-elements upstream from promoters can...
regulate the transcription associated with the trans-acting factor, by bending or looping DNA.\(^{26,27}\) It is still not clear whether the positive trans-acting factor with the CIT curvature associates with the UPE or not. The K-81 RNAP fraction of *E* \(\sigma\)^31 seems to involve some trans-acting factor, which acts on the UPE sequence, since a reduction of transcription by *E* \(\sigma\)^31 was observed in the \(\Delta UPE\) mutant (Fig. 4).

Cyanobacterial core enzymes as well as K-81 \(\sigma\)^31 (the amino acid residues of conserved region 1.2, 2, 3, and 4 in \(\sigma\)^31 were 64% identical to those of \(\sigma\)^30) have a high degree of structural similarity to those of *E. coli* RNAP subunits.\(^{14,18,28}\) We therefore can not exclude the possibility of intramolecular interaction between the UPE and RNAP, because, for example, the \(\alpha\)-subunit of core RNAP prefers interaction involving an AAAG (AAAC in the case of K-81) sequence proximal upstream from the −35 promoter, similar to that in UPE, for effective transcription in *E. coli*.\(^{20}\) From an overview of this experiment on in vitro mRNA synthesis and DNA-binding using cyanobacterial RNAP of *E* \(\sigma\)^31, heterologous RNAP of Ec+\(\sigma\)^31, and *E. coli* RNAP of \(\sigma\)^30, the function for specific transcription by these RNAPs is essentially the same (Figs. 4 and 5). Differences between cyanobacterial RNAP and *E. coli* RNAP were also found in mRNA synthesis or DNA-binding to the UPE sequence in this study (Figs. 2, 4, and 5). These phenomena are not inconsistent with our previous results.\(^{10,11}\) It might be of interest to elucidate why K-81 psbA2 transcription was dramatically increased in vivo but not in vitro on the −35 deletion constructs (Figs. 3 and 4). This suggests that cyanobacterial RNAP of *E* \(\sigma\)^31, prepared from the K-81 cyanobacterium grown under light, might not contain the negative trans-acting factor which can bind to the −35 hexamer and/or the RNAP sigma factor in the dark. A recent study found a light-responsive sigma factor in a cyanobacterium.\(^{21}\) Some negative trans-acting factors, e.g., anti-sigma factors, have been identified and their homolog genes also exist in cyanobacterial genomes (http://www.kazusa.or.jp/cyano/).\(^{28,30}\) This regulatory system with negative factors is being examined in our laboratory. We are also interested in differential activities in the −35 mutants (IY-2: the position of UPE is shifted to the −35 hexamer site by the deletion of the hexamer), observed in vivo between \(\beta\)-galactosidase assay (Fig. 2B) and primer extension (Fig. 3, Light-4 h). Although the nucleotide sequences of the *psbA2* (+1 to +14)-lacZ transcripts must have been the same in each cyanobacterial mutant, the −35 hexamer sequence had some effects on the transcription. For example, there is a possibility that the accelerated *psbA2* transcription occurred with partial premature termination at the downstream of the M4 primer-anealing site in lacZ. In this particular case, abundant transcripts at the 5′-end region are detected by primer extension (Fig. 3, IY-2) but a reduction of \(\beta\)-galactosidase activities are observed (Fig. 2B, IY-2).

We have found two distinct DNA curvatures at the *psbA2* upstream, one is CIT (the centers of intrinsic curvatures are the positions from −184 to −141) and another is RIB (RNA polymerase-induced DNA bending, the center is approximately +10).\(^{8,10,11}\) It is unclear whether there is an interference between the *psbA2* promoter region and lacZ will be preferable for understanding of *psbA2* expression. Functional analyses of cis- and trans-acting factors will also provide clues as to the role of *psbA* light-responsive gene expression.

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**References**


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