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Activation of the Silent *psbA1* Gene in the Cyanobacterium *Synechocystis* sp Strain 6803 Produces a Novel and Functional D1 Protein

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The photosystem II reaction center protein D1 in *Synechocystis* sp strain 6803 is encoded by the *psbA2* and *psbA3* genes of the three-membered *psbA* gene family. The silent and divergent *psbA1* copy of the *psbA* gene family was activated by exchanging part of its upstream region with a corresponding fragment of the *psbA2* copy. The light-regulated expression of the activated *psbA1* gene showed that the inserted *psbA2* segment contains the information necessary for light-dependent as well as high-light–stimulated transcription. The activated *psbA1* gene expressed a novel D1 protein, D1′. A mutant strain containing *psbA1* as the only active *psbA* gene grew photoautotrophically at a rate comparable to that of the wild type. This finding demonstrates that despite its unusual amino acid sequence, D1′ is exchangeable for D1 in the photosystem II complex, at least under normal laboratory conditions. The D1′ protein was found to have a degradation rate similar to that of the D1 protein under low- or high-light conditions. Another mutant containing the activated *psbA1* gene together with the *psbA2* and *psbA3* genes produced both the D1 and D1′ proteins.

INTRODUCTION

The photosystem II (PSII) reaction center protein D1 is highly conserved throughout the plant kingdom, with amino acid identities of ~80% between such diverse organisms as angiosperms and cyanobacteria (Jansson and Mäenpää, 1997). D1 is encoded by the *psbA* gene. In plants and algae, *psbA* typically exists as a single-copy gene, whereas in cyanobacteria it belongs to small gene families (Mohamed et al., 1993; Golden, 1994). In the cyanobacterium *Synechocystis* sp strain 6803, the *psbA* gene family contains three members: *psbA1*, *psbA2*, and *psbA3* (Jansson et al., 1987). The *psbA2* and *psbA3* coding regions share 99% nucleotide identity, and they encode an identical D1 protein (Ravnikar et al., 1989; Metz et al., 1990). Both genes are expressed in a light-regulated manner (Mohamed and Jansson, 1989, 1990, 1991), with *psbA2* accounting for >90% of the produced *psbA* transcripts (Mohamed et al., 1993).

The presence of the *psbA1* gene in *Synechocystis* sp 6803 is enigmatic. There are no indications that the gene is expressed (Mohamed and Jansson, 1989, 1991; Mohamed et al., 1993). In the 5′ noncoding region, *psbA1* is only 40 to 45% identical to *psbA2* and *psbA3* (Mohamed et al., 1993). However, the open reading frame of the *psbA1* gene is intact, with a nucleotide and amino acid identity of 75 and 85%, respectively, to the coding regions of *psbA2* and *psbA3* (Osiewacz and McIntosh, 1987; Ravnikar et al., 1989; Mohamed et al., 1993). If the *psbA1* gene in *Synechocystis* sp 6803 were expressed, it would encode a D1 polypeptide that differs from that produced by the *psbA2* and *psbA3* genes in 56 of 360 amino acids. As shown in Figure 1, most of these amino acids would be clustered in the aqueous N-terminal and C-terminal tails. From a compilation of 45 D1 amino acid sequences (Jansson and Mäenpää, 1997), we found that of the 56 amino acid substitutions in the deduced amino acid sequence of the *psbA1* gene, eight are at positions that otherwise are conserved in all D1 proteins. One of these substitutions would exchange the phenylalanine at position 186 for a leucine (Figure 1). Molecular modeling studies have placed Phe-186 in the protein environment around the redox-active tyrosine residue Tyr-161 (Tyr_Z) (Svensson et al., 1991), and it has been implicated in electron transfer between Tyr_Z and P680 (Svensson et al., 1990). Mutating Phe-186 to Tyr-186 in *Synechocystis* sp 6803 abolished oxygen-evolving activity (Mäenpää et al., 1995).

On the other hand, the codon changes in the *psbA1* gene do not affect several other amino acids or amino acid sequences that have been suggested to be of structural or functional importance from site-directed mutagenesis studies (for a recent review, see Jansson and Mäenpää, 1997). These include amino acids that are putative Mn or Ca ligands, such as Asp-59, Asp-61, Glu-65, Asp-170, His-332, Glu-333, His-337, Asp-342, and Ala-344 (Debus, 1992; Nixon and Diner, 1992; Barry et al., 1994; Babcock, 1995; Chu et al., 1995a, 1995b). Also, the His-190 residue that might serve as a hydrogen bond acceptor for Tyr_Z (reviewed

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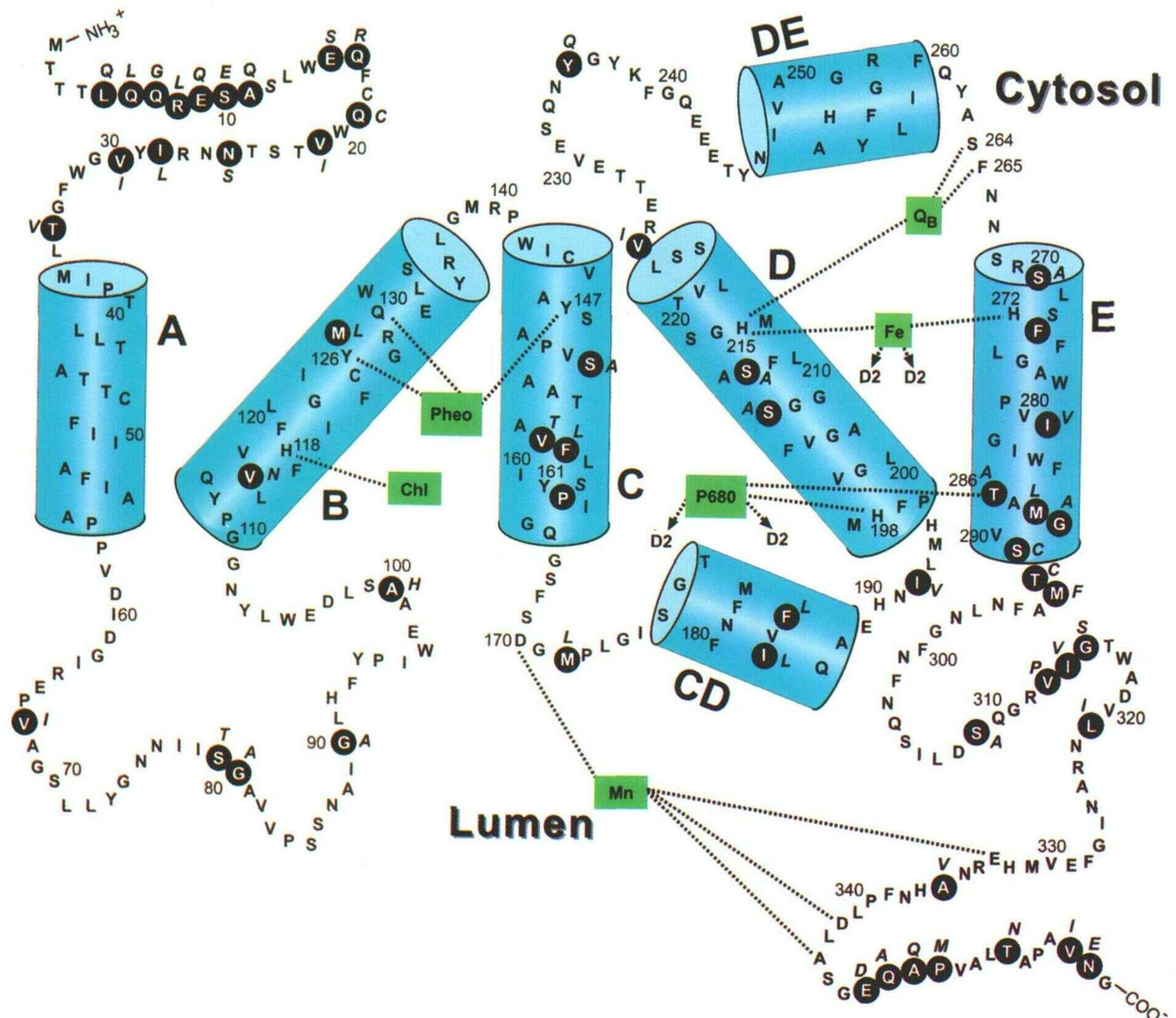

Figure 1. Sequence and Folding Pattern of the D1 Protein in *Synechocystis* sp 6803.

The D1 protein is shown with five α helices (A, B, C, D, and E) that span the thylakoid membrane and two short α helices (CD and DE) that are parallel to the membrane. Positions that are changed in the *psbA1* coding sequence are marked with filled circles, and the substituting amino acid is shown in italics. Amino acid residues that serve as potential ligands to various cofactors are indicated. Chl, accessory chlorophyll; Fe, non-heme iron; Mn, manganese cluster; P680, the reaction center chlorophyll dimer; Pheo, pheophytin; Q_B, secondary quinone electron carrier.

in Debus, 1992; Babcock, 1995) or be involved in the electron transfer between Tyr_Z and P680 (Svensson et al., 1996) is conserved. Other examples are Asp-59 to Asp-61, Asp-103 to Gly-109, and Asn-325 to Glu-333, which are crucial for D1 translocation or stability (Salih et al., 1996b); Tyr-254, Phe-255, Leu-271, and Glu-226 to Ser-232, which are required for optimal binding properties of the Q_B site (Ohad and Hirschberg, 1992; Nixon et al., 1995); and Glu-229, Glu-243, and Glu-242 to Glu-244, which influence the recovery capacity of the D1 protein after photoinhibition (Mäenpää et al., 1993; Tyystjärvi et al., 1994).

Also, amino acids that have been predicted from modeling work to bind various cofactors are conserved in the deduced amino acid sequence of the *psbA1* gene (Figure 1). These amino acids include putative ligands to P680, pheophytin, the non-heme Fe, an accessory chlorophyll (reviewed in Svensson et al., 1996), and to Q_B (Tietjen et al., 1991). One exception to this list, Thr-286, is postulated to be hydrogen bonded to an ester oxygen in the P680 dimer (Svensson et al., 1996) and is Ala-286 in the *psbA1* sequence (Figure 1). However, even apart from the *psbA1* gene, the threonine at position 286 is not totally conserved because it is replaced

with an alanine also in the amino acid sequence of the *psbA2* gene from the cyanobacterium *Synechococcus elongatus naegli* (EMBL accession number P358777; see Jansson and Mäenpää, 1997). The significance of this observation is not certain until it is known to what extent the *psbA2* gene from *S. e. naegli* is active.

To address the question of whether a D1 protein encoded by the *psbA1* gene in *Synechocystis* sp 6803 would be structurally and functionally active in the PSII complex, we have used site-directed mutagenesis to activate the *psbA1* gene and follow its expression.

RESULTS

No conditions have been found thus far under which the *psbA1* gene in *Synechocystis* sp 6803 is expressed. Sequence analyses of the *psbA* genes in *Synechocystis* sp 6803 show that both *psbA2* and *psbA3* have −35 and −10 elements characteristic of bacterial σ^{70} promoters (Mohamed et al., 1993; Nixon and Jansson, 1996). The transcription start points for *psbA2* and *psbA3* have been mapped to position −49 and −88, respectively, relative to the ATG site (Mohamed et al., 1993). Expression analyses have led us to conclude (Mohamed and Jansson, 1989, 1991; Mohamed et al., 1993) that the −35 and −10 motifs of *psbA2* constitute a strong light-inducible promoter in *Synechocystis* sp 6803. No expression of the *psbA1* gene has been detected (Mohamed and Jansson, 1989), and alignment of the upstream regions of the three *psbA* genes (Figure 2) has shown that the two putative *psbA1* promoters (Osiewacz and McIntosh, 1987; Mohamed et al., 1993; Nixon and Jansson, 1996) differ significantly from the *psbA2* and *psbA3* promoters, particularly in the −35 element, which is identical in *psbA2* and *psbA3*.

The distal promoter-like region of *psbA1* is positioned far upstream of the ATG site (Figure 2). However, without an identified transcription start, the probable location for a *psbA1* σ^{70} promoter is uncertain. The presence in *psbA1* of a basal promoter other than σ^{70} cannot be discounted, and bipartite regions with significant similarity to consensus sequences for both σ^{54} and σ^{32} promoters can be found (Figure 2). Attempts to activate the *psbA1* gene by shifts in nitrogen, phosphorus, sulfur, or iron status or by altering temperature or light intensity were unsuccessful (J. Eriksson and C. Jansson, unpublished data), and the identity of *psbA1* promoters, if any, remains unknown. The *psbA1* gene also lacks a Shine–Dalgarno sequence (Figure 2), and although such a sequence is not an absolute prerequisite for translation (Schmetterer, 1990), its absence adds to the deviant nature of *psbA1* compared with the active *psbA2* and *psbA3* genes.

To learn which *cis*-acting elements are required to activate the *psbA1* gene and whether a D1 protein encoded by this gene could be a functional component of PSII, we used site-directed mutagenesis to modify the upstream region of *psbA1* stepwise (Figure 3). Because *psbA2* is highly expressed under normal growth conditions (Mohamed and Jansson, 1989), we first tried to activate the *psbA1* gene by engineering −35 and −10 promoter elements identical in

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psbA1 TCTTGTTTTTTTAAAGAAATATTATTAATCTGAAATTCAAGGGAAGTTAATCAATGCC -135
psbA2 ---------------------------------------------------------- -126
psbA3 ---------------------------------------------------------- -133

psbA1 AATAATTATCTCGCATTATTAATC|CC|T|TA|CTATCTGGTTG||T|GG|TTT|G||  -77
psbA2 -----------------------AACTTCCTGTTA.......CAAAGCTTTACAAAACT  -75
psbA3 AGCAAGCTATTTACAAATTGTT|CAA||T||||TATTACT||T|TACA||ACCGG||    -75

psbA1 GAT|GTT|ATCA||AAA|T|AC|||CAA|A|||AA|||||G||T||ACC|A|A|TC|T  -19
psbA2 CTCA...TTAATCCTTTAGACTAAGTTTAGTCAGTTCCAATCTGAACATCGACAAATA  -20
psbA3 |C||...AGC|AAGAAATCT||G||C||GAGGCCAAATCC|T|||||||T||||||||  -20

psbA1 AC|TC|T||||||.T|GCTATG                                       +1
psbA2 CATAAGGAATTATAACCAAATG                                       +1
psbA3 ||||||||||||||||||||ATG                                       +1
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Figure 2. Alignment of the 5′ Noncoding Regions of the *psbA1*, *psbA2*, and *psbA3* Genes in *Synechocystis* sp 6803.

The *psbA1* sequence is from Osiewacz and McIntosh (1987), the *psbA2* sequence is from Mohamed et al. (1993), and the *psbA3* sequence is from Metz et al. (1990) and Mohamed et al. (1993). Distances are given relative to the ATG start codon (italics). The −35 and −10 promoter elements of the *psbA2* and *psbA3* genes as well as the two putative σ^{70} promoters in *psbA1* are shown as black boxes. Regions in the *psbA1* gene resembling consensus sequences for *Escherichia coli* σ^{54} and σ^{32} promoters (Wolfe, 1993) are overlined with solid and dashed lines, respectively. Shine–Dalgarno sequences are shown as open boxes. The mapped transcription start points for *psbA2* and *psbA3* (Mohamed et al., 1993) are underlined. Nucleotides identical to the *psbA2* sequence are shown as vertical bars, and introduced gaps are indicated by dots. Sequences upstream of the *psbA2* and *psbA3* genes are indicated by dashes.

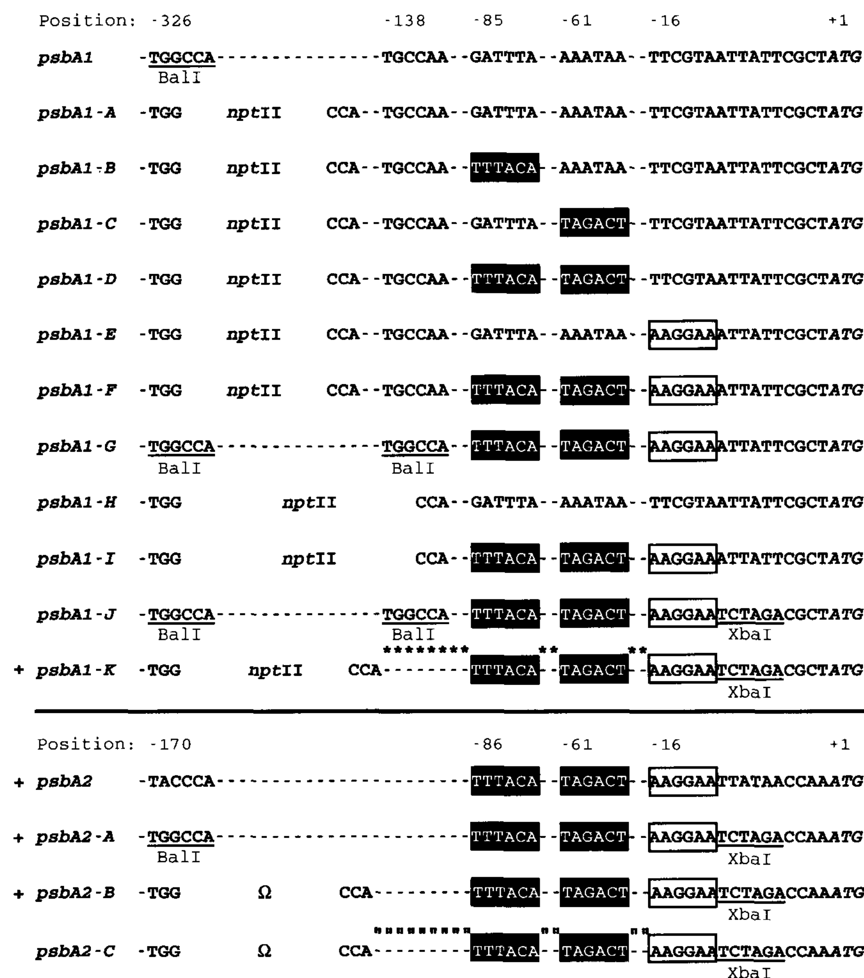

Figure 3. Construction of Mutations in the 5′ Region of *psbA1* and *psbA2*.

Distances are given relative to the ATG start codon. Promoter and Shine–Dalgarno sequences are indicated as given in Figure 2. Restriction sites are underlined. In *psbA1-A*, the kanamycin resistance gene (*nptII*) was inserted in the BalI site at position −323 relative to the ATG site. In *psbA1-B*, *psbA1-C*, and *psbA-E*, the −35, −10, and Shine–Dalgarno sequences, respectively, of *psbA2* were inserted at the corresponding positions. In *psbA1-D*, both promoter elements are present, and *psbA1-F* contains the promoter elements plus the Shine–Dalgarno sequence. A second BalI site was generated in *psbA1-G*. In *psbA1-H*, the region between the two BalI sites was deleted and replaced with the *nptII* gene. The same region is deleted in *psbA1-I*, which contains the promoter and Shine–Dalgarno elements of *psbA1-F*. The *psbA1-J* construct was obtained by generating an XbaI site downstream of the Shine–Dalgarno sequence in *psbA1-G*. Replacing the 360-nucleotide-long upstream BalI-XbaI fragment of *psbA1-J* with the 160-nucleotide-long upstream BalI-XbaI fragment of *psbA2-A* and reinserting the *nptII* gene in the BalI site yielded *psbA1-K*. The *psbA2-A* construct was derived by generating a BalI site at position −170 and a XbaI site at position −10 in the *psbA2* gene. Ligation of the Ω fragment into the BalI site generated *psbA2-B*. The BalI-XbaI fragment of *psbA1-J* was then exchanged for the corresponding fragment of *psbA2-B*, and the Ω fragment was inserted in the BalI site, resulting in the *psbA2-C* construct. A (+) in front of the constructs denotes an active gene. Asterisks over the active *psbA1-K* gene indicate inserted *psbA2* sequences that are not present in the inactive *psbA-J* gene. Conversely, quotation marks over the inactive *psbA2-C* gene indicate inserted *psbA1-J* sequences that are not present in the active *psbA2-B* gene. Dashes indicate unspecified nucleotides.

sequence and position (relative to the ATG site) to those of *psbA2*. Also, a Shine–Dalgarno sequence was added in the appropriate position.

Expression analyses (Figure 4) showed that introduction of the −35 signal, the −10 signal, or both could not induce transcription. Thus, it was obvious that the presence of a promoter alone does not suffice to render the *psbA1* gene active (Figure 3, *psbA1-D* and *psbA1-F*). To determine the possibility that upstream sequences repress or otherwise inhibit transcription of the *psbA1* gene from either the puta-

tive downstream native promoter or the introduced *psbA2* promoter, we removed a 189-nucleotide fragment from the 5′ region of the gene (Figure 3, *psbA1-H* and *psbA1-I*). We found that this deletion had no effect on *psbA1* activity (Figure 4).

Although the *psbA1-J* gene is identical to the *psbA2* gene with respect to sequence and position of the −35 and −10 promoter elements and of the Shine–Dalgarno site, it differs in intervening and flanking sequences. Therefore, in the next attempt to force transcription of the *psbA1* gene, we exchanged the 320-nucleotide-long upstream BalI-XbaI fragment of *psbA1* for an upstream fragment of *psbA2*. The modifications of the *psbA2* 5′ region required for this shift, that is, engineering of restriction sites (Figure 3, *psbA2-A*), did not affect the activity of the gene. The *psbA1* gene *psbA1-K*, obtained by using this technique (Figure 3), was transcribed, and a full-length *psbA1* transcript was produced (Figure 4). To show further that the upstream region of *psbA1-J* is insufficient to drive transcription, we replaced the BalI-XbaI fragment of the active *psbA2-A* with that of *psbA1-J*. As indicated in Figure 3, the resulting *psbA2* gene, *psbA2-C*, was inactive.

Transcript Analyses

The *Synechocystis* sp 6803 mutant containing *psbA1-K* as the only active *psbA* gene, hereafter referred to as strain A1-K, grows photoautotrophically. RNA gel blot analyses using *psbA1*- and *psbA2/3*-specific probes (Figure 5) confirmed that the *psbA1* transcript is specific for the A1-K mutant and that this strain contains no other full-length *psbA* transcripts. The short *psbA* fragment in A1-K that hybridized with the *psbA2/3* probe corresponds to a truncated *psbA2* transcript produced by the inactivated *psbA2* gene (Mohamed and Jansson, 1989, 1990, 1991; Mohamed et al., 1993). The *psbA1-K* gene was amplified by polymerase chain reaction (PCR) from A1-K cultures at three independent times and sequenced over the entire coding region and the 250-nucleotide flanking 5′ end. The sequence agreed fully with the *psbA1-K* construct. These results, together with DNA gel blot and PCR experiments (data not shown), also served to demonstrate the stability of the genotype in A1-K.

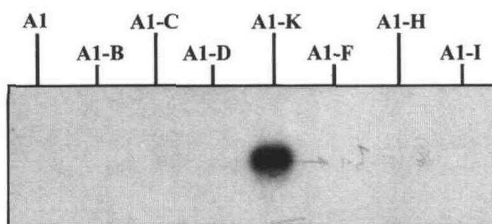

Figure 4. RNA Gel Blot Analyses of Various *psbA1* Mutants.

RNA was isolated from the recipient mutant strain A1 containing *psbA1* as the only intact *psbA* gene and from mutants A1-B, A1-C, A1-D, A1-F, A1-H, A1-I, and A1-K containing, respectively, *psbA1-B*, *psbA1-C*, *psbA1-D*, *psbA1-F*, *psbA1-H*, *psbA1-I*, and *psbA1-K* as the only intact *psbA* gene. The RNA gel blots were probed with a *psbA1*-specific probe. The band in the A1-K lane corresponds to a full-length 1.2-kb *psbA* transcript.

From the expression analyses with the A1-K mutant (Figure 5A), it is evident that the *psbA1-K* gene is light controlled in a fashion similar to what was found for *psbA2* and *psbA3* (Mohamed and Jansson, 1989; Mohamed et al., 1993), that is, no steady state levels of *psbA1-K* transcripts could be detected in dark-cultured cells, and the levels in light-grown cells increased with light intensity. Although the difference in *psbA1-K* transcript levels between high-light–grown and low-light–grown cells as displayed in Figure 5A is not striking, it was consistently observed in all analyses. Studies with light-controlled transcription in mutant A1 (the recipient strain for introduction of the *psbA1-K* construct that thus contains the same genetic background as the A1-K mutant) have also been conducted with the *psbD2*, *rbcL*, *rbcS*, and *rrn* genes. These genes encode, respectively, the PSII reaction center protein D2, the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase, and rRNA. The transcription pattern for these genes was the same in mutant A1 as in the wild type, with increased levels of *psbD2* and *rbcS* transcripts and decreased levels of *rbcL* transcripts when shifted from low-light to high-light conditions (Mohamed and Jansson, 1989). No obvious light-dependent effects were observed for *rrn* transcription (Mohamed and Jansson, 1989).

We found previously (Mohamed et al., 1993) that when the *psbA2* gene is inactivated, the steady state levels of transcripts from the otherwise poorly expressed *psbA3* gene increase eightfold. We were interested in determining whether the presence of the active *psbA1-K* gene in *Synechocystis* sp 6803 would have the effect of downregulating the *psbA2* and *psbA3* genes. By substituting *psbA1-K* for *psbA1* in the *Synechocystis* sp 6803 wild-type strain, we generated *Synechocystis* sp 6803 strain K, in which *psbA1*, *psbA2*, and *psbA3* are all active genes. Evaluation of results from RNA gel blot experiments indicated that steady state levels of *psbA2* plus *psbA3* transcripts were approximately the same for the *Synechocystis* sp 6803 wild type and for strain K (Figure 5C). Also, levels of *psbA1-K* transcripts were approximately the same in strains A1-K and K (Figure 5B). This finding suggests that there is no major adjustment of transcript production when the *psbA* gene dosage is increased by introducing the activated *psbA1* gene in a genetic background in which the *psbA2* and *psbA3* genes remain active. Consequently, strain K should contain higher levels of total *psbA* transcripts than either the wild-type strain or strain A1-K (Figure 5).

A heterogeneous appearance was often observed for the *psbA1* mRNA in strain A1-K (Figure 5A). This could be

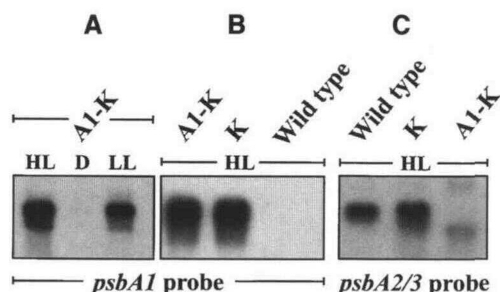

Figure 5. RNA Gel Blot Analyses of the Wild Type and Mutants A1-K and K.

RNA was isolated from the wild type, mutant A1-K (containing *psbA1-K* as the only active *psbA* gene), and mutant K (containing *psbA-K*, *psbA2*, and *psbA3* as active *psbA* genes) that had been incubated in high-light (HL) conditions (1200 μE m^{-2} sec^{-1}), low-light (LL) conditions (50 μE m^{-2} sec^{-1}), or darkness (D).

(A) and **(B)** RNA probed with a *psbA1*-specific probe.

(C) RNA probed with a *psbA2/3*-specific probe.

The major bands in **(A)** to **(C)**, with the exception of lane A1-K in **(C)**, correspond to a 2.1-kb transcript.

due to degradation and might indicate that the *psbA1* transcript is less stable than is the *psbA2* trancript. We previously reported on the differential stability of the *psbA2* and *psbA3* transcripts (Mohamed et al., 1993) and mapped the transcription termination points for *psbA2* and *psbA3* (Eriksson et al., 1995). Given the divergence between the *psbA1* and *psbA2/3* genes in the noncoding 3′ region (data not shown), it is possible that they yield transcripts with different transcription termination signals and therefore different stabilities.

Protein Analyses

The photoautotrophic growth of *Synechocystis* sp strain A1-K clearly reveals that this mutant synthesizes a novel, functional D1 protein. Production of the novel D1 form, named D1′, was further demonstrated by protein gel blot analyses (Figure 6). The weaker signal obtained for D1′ compared with D1 (Figure 6A) could indicate that production of D1′ in the A1-K strain is low. However, the weaker signal could also, and possibly solely, reflect the different antigenicity of the two protein forms toward the antibody. To illustrate the different antigenic properties of the two proteins, we used an antibody raised specifically against the N-terminal tail of the *Synechocystis* sp 6803 D1 protein. In this region, the identity between D1 and D1′ is only ~51% (Figure 1), and as expected, the N-terminal antibody recognized D1 but not D1′ (Figure 6B). When thylakoid membranes of *Synechocystis* sp 6803 were incubated with the lysine-specific protease endoproteinase Lys-C, a 21-kD N-terminal and a 14-kD C-terminal fragment were produced by cleavage at the unique Lys-238 site (Salih et al., 1996b). As is also demonstrated in Figure 6B, the N-terminal antibody is specific for the 21-kD N-terminal proteolytic fragment.

The electrophoretic mobility of the D1′ protein on SDS-PAGE gels was faster than for the D1 protein (Figure 6A). To examine whether this is due to a difference in size or to some other phenomenon, we compared the sizes for the

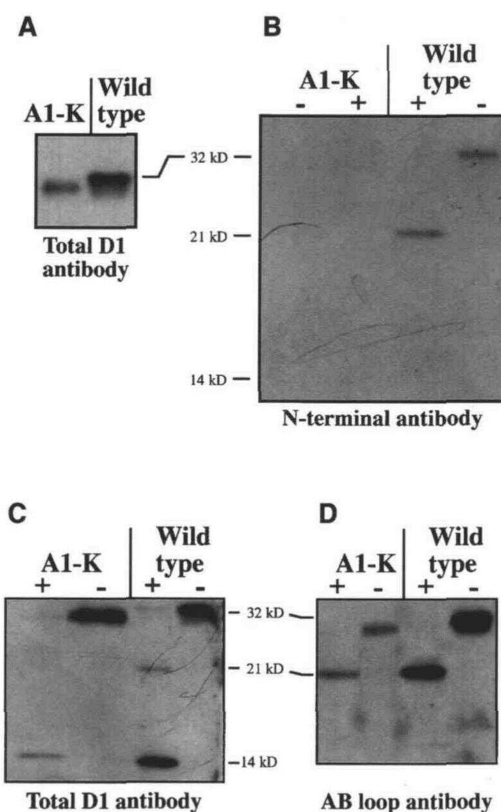

Figure 6. Protein Gel Blot Analyses of the Wild Type and Mutant A1-K.

(A) Thylakoid membranes were isolated from cells of the wild type (producing D1) and mutant A1-K (producing D1′) and probed with an antibody directed against the major portion of the spinach D1 protein (Total D1 antibody). Sizes are indicated in kilodaltons.

(B) to **(D)** Thylakoid membranes were isolated from cells of the wild type (producing D1) and mutant A1-K (producing D1′) and probed with an N-terminal–specific antibody **(B)**, an antibody directed against the major portion of the spinach D1 protein (Total D1 antibody) **(C)**, or an AB loop-specific antibody **(D)**. The thylakoids were incubated in the presence (+) or absence (−) of the lysine-specific protease endoproteinase Lys-C before electrophoresis. The A1-K lanes in **(B)** to **(D)** were overloaded compared with those of the wild-type lanes. Sizes are indicated in kilodaltons.

N-terminal and C-terminal proteolytic fragments after digestion with endoproteinase Lys-C. Our general D1 antibody did not recognize the N-terminal fragment in D1′ (Figure 6C). To detect this fragment, we instead used an antibody raised against the AB loop in which the identity between D1 and D1′ is relatively high (Figure 1). A true size difference between D1 and D1′ should show up as a clear downshift for either the C-terminal fragment (Figure 6C) or the N-terminal fragment (Figure 6D). This was not found to be the case; therefore, we concluded that the different migratory behavior of the two D1 forms, during electrophoresis, does not reflect a difference in size. The signal obtained for the proteolytic 14-kD C-terminal fragment of D1′ with the general D1 antibody was very faint compared with that of the undigested protein (Figure 6C). This difference probably reflects the fact that many of the antigenic epitopes are located in the central portion of D1, where the similarity between the two D1 forms is high (Figure 1).

To date, we have found no appreciable difference in the photoautotrophic growth rate of *Synechocystis* sp 6803 wild-type strain and strain A1-K. Preliminary results indicate that the oxygen-evolving activity under continuous illumination is approximately the same for both strains. Thus, despite its atypical amino acid sequence (Figure 1), D1′ can successfully replace D1 in an operational PSII complex. It has been amply documented that the stability of the D1 protein is affected by its amino acid sequence. This sequence dependence has been demonstrated both by the presence of the two different D1 forms naturally occurring in *Synechococcus* sp strain 7942 (Schaefer and Golden, 1989a, 1989b; Clarke et al., 1993; Kulkarni and Golden, 1995) and by results obtained from site-directed mutagenesis experiments (Tyystjärvi et al., 1994). We compared the stability of D1′ and D1 by protein gel blot analyses of thylakoid membranes from cultures incubated in low-light (Figure 7A) or high-light (Figure 7B) conditions and by pulse–chase experiments with high-light–incubated cells, using labeled ^{35}S-methionine (Figure 7C). We made no attempt in this study to determine the half-life of the two D1 forms. However, our interpretations of the results in Figure 7 suggest that there is no dramatic difference in stabilities between the D1′ and D1 proteins. The overall lower incorporation of the label in D1′ (Figure 7C) might be explained by the fewer methionine residues in this form compared with D1 (nine versus 12; Figure 1).

To determine whether the *psbA1-K* gene was also expressed at the protein level in *Synechocystis* sp 6803 strain K and whether this strain could maintain simultaneous synthesis of both the D1 and D1′ proteins, we studied the D1 content of the *Synechocystis* sp 6803 wild-type strain, strain K, and strain A1-K by protein gel blot analyses (Figure 8). When the samples were challenged with our general D1 antibody, both D1 and D1′ were detected in the K strain. With the N-terminal antibody, only the D1 protein was visible in *Synechocystis* sp strain 6803-K. Thus, it is clear that both D1 and D1′ are synthesized in strain K and that both forms exist together in the thylakoid membrane.

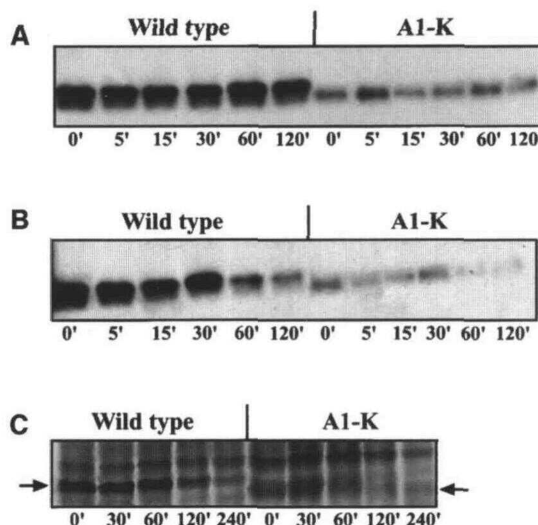

Figure 7. Protein Gel Blot and Pulse–Chase Analyses of the Wild Type and Mutant A1-K.

(A) and **(B)** Thylakoid membranes were isolated from the wild type and mutant A1-K that had been incubated in the presence of the translation inhibitor lincomycin either in low light (50 μE m^{-2} sec^{-1}) or high light (1200 μE m^{-2} sec^{-1}), respectively. Samples were collected and processed at intervals between 0 and 120 min (0′ to 120′).

(C) Pulse–chase experiments with the wild type or mutant A1-K labeled in vivo with ^{35}S-methionine for 30 min and then chased with cold methionine. Samples were collected and processed at intervals between 30 and 240 min (30′ to 240′). Both pulse and chase were at 1200 μE m^{-2} sec^{-1}. Positions of the bands corresponding to D1 (wild-type lanes) and D1′ (A1-K lanes) are indicated by arrows.

DISCUSSION

Whether the *psbA1* gene in *Synechocystis* sp 6803 plays a role under very specific circumstances or is merely an evolutionary remnant has not been determined. No laboratory conditions have been found under which the gene is expressed. It is possible that such conditions exist in nature, where fluctuations in nutrient supply, agitation, temperature, and light can differ significantly from the laboratory environment. It is also possible that the wild-type strain of *Synechocystis* sp 6803 used in various laboratories has become genotypically distinct from the original isolate and that *psbA1* is an active gene in cells of *Synechocystis* sp 6803 in their natural habitats. The wild-type strain used in most laboratories is *Synechocystis* sp strain 6803-G, which has enhanced glucose tolerance and originally was obtained as a spontaneous mutant from the laboratory of L. McIntosh (Michigan State University, East Lansing; Williams, 1988). We have analyzed the original strain of *Synechocystis* sp 6803 from the Pasteur Cyanobacteria Collection (Paris,

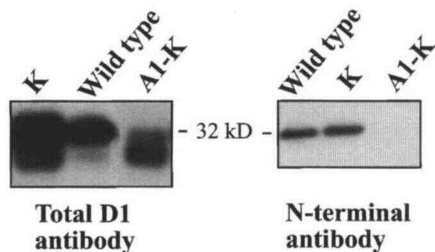

Figure 8. Protein Gel Blot Analyses of the Wild Type and Mutants A1-K and K.

Thylakoid membranes were isolated from the wild type and mutants A1-K or K and probed with either an antibody directed against the major portion of the spinach D1 protein (Total D1 antibody) or an antibody specific for the N-terminal tail of the D1 protein in *Synechocystis* sp 6803 (N-terminal antibody). The position corresponding to a protein of a relative molecular mass of 32 kD is indicated.

France). In this strain, the *psbA1* gene was also found to be inactive under typical laboratory conditions.

The lack of obvious promoter elements as well as a ribosome binding site (Figure 2) tend to argue against *psbA1* being a functional gene. Also, the high degree of divergence in amino acid sequence between *psbA1* on the one hand and *psbA2* and *psbA3* on the other (Figure 1) suggests that *psbA1* is a pseudogene. Yet virtually all amino acids implied as ligands to various cofactors are conserved in the *psbA1* coding sequence (Figure 1). This might indicate a selection pressure during evolution. In this report, we have demonstrated that the D1 protein produced by an activated *psbA1* gene can indeed function in the PSII complex.

Activation of the Cryptic *psbA1* Gene

Engineering a σ^{70} promoter and deleting upstream sequences in *psbA1* did not induce transcription. However, an active *psbA1-K* gene was constructed by fusing the 160-nucleotide, immediate 5′ region of *psbA2* with *psbA1* (Figure 3). The light responsiveness of *psbA1-K* gene transcription was similar to that of the *psbA2* and *psbA3* genes (Figure 5). Thus, the 160-nucleotide-long *psbA2* fragment in *psbA1-K* harbors all of the necessary elements required for both light-dependent and high-light–stimulated transcription. This agrees well with the situation in *Synechococcus* sp 7942, for which it has been demonstrated (Li et al., 1995) that a high-light–responsive element is located between the transcription start site and the ATG site of the *psbA*II and *psbA*III genes. It is not known which *cis*-acting elements are required for the light-dependent transcription of the *psbA* genes, but comparative mapping and mutagenesis of the 5′ ends of *psbA2*, *psbA1*, and *psbA1-K* in *Synechocystis* sp 6803 should provide helpful information.

The Novel D1′ Protein

The D1′ protein produced in *Synechocystis* sp 6803 strains A1-K and K differs in primary structure from D1 in 56 of 360 positions (Figure 1). Perhaps the most striking difference in the two proteins is Leu-186 in D1′ versus Phe-186 in D1. The apparent unimpaired photoautotrophic growth of the A1-K mutant is difficult to reconcile with a model in which electron transfer between Tyr-161 and Phe-186 is imperative for PSII photochemistry (Svensson et al., 1996). Furthermore, we have shown (Mäenpää et al., 1995) that mutating Phe-186 to Tyr-186 in *Synechocystis* sp 6803 completely abolishes oxygen-evolving activity. One possible explanation for these seemingly contradictory results would be that although Phe-186→Leu-186 is permissive for PSII activity, Phe-186→Tyr-186 is not. It might also be important to consider the total number of changes in the vicinity of Tyr-161 in D1′, which, according to the model by Svensson et al. (1996), would also include the substitution Pro-162→Ser-162. Mutants of *Synechocystis* sp 6803 with a D1 protein containing either the single Phe-186→Leu-186 substitution or the double Phe-186→Leu-186/Pro-162→Ser-162 substitutions are currently being made in our laboratory.

The reason for the apparent lower molecular weight of D1′ compared with D1 is not known. There are two reasons why the difference is unlikely to be due to proteolytic degradation. First, we saw no effects resulting from the presence of protease inhibitors during thylakoid preparations. Second, when the proteins were cleaved by endoproteinase Lys-C, the proteolytic fragments of the two proteins did not differ in size (Figure 6). Another possibility could be that the C-terminal processing of D1′ differs from that of D1. However, this is also not in agreement with the results from the endoproteinase Lys-C experiments because differences in processing would yield different sizes for the C-terminal fragments. Instead, a plausible explanation is that the large number of amino acid substitutions between the two proteins results in differences in SDS binding and unfolding during electrophoresis and hence different mobilities. A faint band in the same position as that corresponding to the D1′ protein was also visible in samples from the wild-type strain (Figures 6 to 8). This is a typical electrophoretic heterogeneity observed for the D1 protein, and the reason is not understood (Greenberg et al., 1987; Kettunen et al., 1996).

We have shown that the D1 and D1′ proteins can be synthesized simultaneously in *Synechocystis* sp 6803 strain K (Figure 8). To what extent PSII can differentiate between the two forms in the assembly process remains to be determined.

METHODS

Synechocystis sp strain 6803 cells were cultured as described previously (Mohamed et al., 1993; Salih et al., 1996b). The strains *Synechocystis* sp 6803-G (Williams, 1988) and *Synechocystis* sp 6803-AR

(Mäenpää et al., 1993; Salih et al., 1996b) were used interchangeably as control strains and are referred to as the wild type.

Oligonucleotide-directed mutagenesis was as given in Salih et al. (1996b), using the appropriate primers. The mutated *psbA1* constructs replaced the corresponding regions of vector pGR1 (Salih et al., 1995) and were introduced by homologous recombination into the photosystem II (PSII)–deficient strain A1 of *Synechocystis* sp 6803 in which the *psbA2* and *psbA3* genes had been inactivated (Mohamed and Jansson, 1989, 1990). By using vector pGS3-A (Salih et al., 1995), we likewise introduced the mutated *psbA2* constructs into strain A2 of *Synechocystis* sp 6803 in which the *psbA1* and *psbA3* genes had been inactivated (Mohamed and Jansson, 1989, 1990). Preparation of *Synechocystis* sp 6803 chromosomal DNA was performed as described by Salih et al. (1996a). Polymerase chain reaction (PCR) amplification, DNA gel blot hybridization, and DNA sequencing were as given in Salih et al. (1996b). RNA isolation was performed as given in Mohamed and Jansson (1989), and RNA gel blot hybridization was as given in Mohamed et al. (1993). The *psbA1*-specific probe was a BalI-StyI fragment encompassing 750 bases of the 5′ region. The *psbA2/3*-specific probe was a KpnI-KpnI fragment from the 5′ end of the *psbA2* gene (Salih et al., 1996b).

Thylakoid preparations, protein gel blot analyses, and protease mapping with endoproteinase Lys-C were according to Salih et al. (1996b). The general D1 antibody was raised against the major portion of the spinach D1 protein and was a kind gift of M. Ikeuchi (Solar Energy Research Group, Institute of Physical and Chemical Research, Wako, Japan) and was used at a 500 times dilution. An antibody raised against the AB loop of the *Synechocystis* sp 6803 D1 protein was a kind gift from B. Andersson and H. Salter (Department of Biochemistry, Stockholm University, Sweden) and was used at a 500 times dilution. An antibody raised against the N-terminal tail of *Synechocystis* sp 6803 was produced by custom antibody production (Research Genetics, Inc., Huntsville, AL) and used at a 10,000 times dilution. Pulse–chase experiments were as described previously (Salih et al., 1996b), except that both the pulse (30 min) and the chase were performed at 1200 $\mu E\ m^{-2}\ sec^{-1}$. Incubation with the translation inhibitor lincomycin at either 50 or 1200 $\mu E\ m^{-2}\ sec^{-1}$ was as described previously (Tyystjärvi et al., 1994). PSII-mediated oxygen evolution was measured according to Styring et al. (1990).

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