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In Vivo and In Vitro Footprinting of a Light-Regulated Promoter in the Cyanobacterium *Fremyella diplosiphon*†

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Certain filamentous cyanobacteria, such as *Fremyella diplosiphon*, modulate the components of their light-harvesting complexes, the phycobilisomes, and undergo complex morphological changes in response to the wavelength of incident light, or light quality. The operon encoding the subunits of phycoerythrin, *cpeBA*, is transcriptionally activated in green light and is expressed at very low levels in red light. To begin elucidating the signal transduction pathway between the detection of specific light wavelengths and changes in gene expression, we have used in vivo footprinting to show that a protein is bound to the region upstream of the *cpeBA* transcription start site in both red and green light: two guanosine residues at −55 and −65 bp are protected from dimethyl sulfate modification in vivo. Using DNA mobility shift gel electrophoresis, we have shown that partially purified extracts of *F. diplosiphon* from both red and green light contain DNA-binding activity specific for the *cpeBA* promoter region. Using in vitro footprinting with dimethyl sulfate and DNase I, we have defined a binding site for this putative transcription factor, designated PepB (phycoerythrin promoter-binding protein), that extends from −67 to −45 bp on the upper strand and from −62 to −45 bp on the bottom strand, relative to the transcription start site. The binding site includes two hexameric direct repeats separated by 4 bp, TTGTTAN$_4$TTGTTA. We conclude from these results that PepB is bound to the region upstream of the *cpeBA* promoter in *F. diplosiphon* in both red and green light. Therefore, additional factors or protein modifications must be required to allow light-regulated transcription of this operon.

Cyanobacteria are a morphologically diverse group of oxygenic photosynthetic prokaryotes with widely varying habitats. Phylogenetic analyses indicate that these disparate cyanobacterial genera form a closely related group in the eubacterial kingdom; included within this group is the lineage of higher plant chloroplasts (13). This evolutionary relationship between cyanobacteria and higher plant chloroplasts is particularly evident in the structural and functional conservation of the two primary photosystems involved in electron transport and oxygen evolution (4). The primary source of diversity among organisms exhibiting oxygenic photosynthesis is in the light-harvesting antenna complexes associated with photosystem II. The primary light-harvesting structures in cyanobacteria, the phycobilisomes (15), are peripherally associated with the thylakoid membranes and function efficiently in transferring a wide spectrum of light energy to the photosynthetic reaction centers. The phycobilisomes fulfill the role of the chlorophyll a/b-binding proteins of higher plant chloroplasts (4). The pigmented proteins of the phycobilisome (phycobiliproteins) are hydrophilic polypeptides covalently associated with linear tetrapyrrole chromophores which are closely related to the chromophore found in the plant photoreceptor, phytochrome (14, 23). The phycobiliproteins constitute ~85% of the mass of the complex, with the remainder consisting of nonpigmented linker proteins (41). In most cyanobacteria, the phycobilisome is a hemidiscoidal structure containing two distinct substructures: the core complex of allophycocyanin and associated linker proteins and six rods radiating from the core, composed of phycocyanin with its linkers (proximal to the core) and phycoerythrin with its linkers (distal to the core) (14).

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Certain filamentous cyanobacteria, such as *Fremyella diplosiphon*, modulate the components of their light-harvesting complexes and undergo morphological and developmental changes in response to the wavelength of incident light, or light quality, a phenomenon known as complementary chromatic adaptation (3, 39, 40). Differential expression of the phycobiliproteins and the associated linker proteins is due to changes in the transcription of these genes (8, 9, 16, 30, 42). Synthesis of new transcripts can be detected within 30 min of a change in light conditions (8, 30). Some studies indicate that these responses exhibit photoreversibility in red and green light with characteristics similar to those of the photoreceptor phytochrome in higher plants (31).

Most of the genes encoding components of the phycobilisome from *F. diplosiphon* have been cloned and sequenced previously (6, 8, 9, 16, 20, 21, 24, 28, 42). One of these operons, which is expressed at high levels in green light, *cpeBA*, encodes the α and β subunits of phycoerythrin (16, 28). The *cpeCDE* operon, encoding the phycoerythrin-associated linker proteins, is also transcriptionally activated in green light and expressed at very low levels in red light (8, 9). Coordinate expression of *cpeBA* and *cpeCDE* must be regulated by a similar mechanism that results in the proper stoichiometry of components for phycobilisome assembly.

We are interested in defining the signal transduction pathway between the detection of specific wavelengths of light by a photoreceptor and subsequent changes in gene expression in *F. diplosiphon*. We have begun analyzing the *cis*-acting DNA elements and *trans*-acting factors which are involved in the expression of the phycoerythrin operon, *cpeBA*. We have utilized in vivo footprinting to examine the status of the *cpeBA* promoter in cultures of *F. diplosiphon* growing in red and green light. In addition, we have partially purified a DNA-binding protein, PepB, found under both growth conditions and examined its specificity by using in vitro footprinting procedures. Up to now, no molecular

analysis of red- or green-light-regulated cyanobacterial promoters has been reported, and this is the first report regarding any regulatory proteins which may be involved in this signal transduction pathway.

MATERIALS AND METHODS

Materials. Restriction enzymes, Klenow fragment of DNA polymerase I, T4 DNA ligase, Sequenase, T4 polynucleotide kinase, and Ultrapure-grade urea were from U.S. Biochemicals Corporation. DNase I (ribonuclease free) was obtained from Worthington. Radioactive nucleotides ([γ-^{32}P]ATP [>6,000 Ci/mmol], [α-^{32}P]dCTP, dATP, and dTTP [>3,000 Ci/mmol]) were obtained from New England Nuclear. Deoxyribonucleotide triphosphates were obtained from Pharmacia. Dimethyl sulfate (DMS) and piperidine were purchased from Aldrich; the piperidine was redistilled and stored in aliquots at −80°C. All other chemicals were reagent or molecular biology grade.

Bacterial strain. *F. diplosiphon* 33 (a strain of *Calothrix* sp., PCC 7601 and UTEX 481) was grown at 32 to 35°C in red or green light at ~15 microeinsteins/m^2/s in BG-11 medium buffered with 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0) and aerated with a mixture of 1 to 3% CO_2 in air as previously described (9).

General methods. Difference spectra of cultures of *F. diplosiphon* were obtained on a Perkin-Elmer Lambda 3B spectrophotometer interfaced to an IBM 55SX computer with PECSS software, by the method described by Zhao and Brand (43). RNA was prepared from cultures of *F. diplosiphon* grown in red or green light by the method described by Mazel et al. (28), except that LiCl precipitation was used prior to ethanol precipitation. Electrophoresis of RNA (2 μg) was performed on denaturing formaldehyde agarose gels; RNA was transferred to nitrocellulose membranes and hybridized to radioactive probes under standard conditions (2). Radioactive probes for hybridizations were prepared by the random oligonucleotide priming method described by Feinberg and Vogelstein (10). Restriction fragments for mobility shift gel analysis and in vitro footprinting were labeled with appropriate α-^{32}P-deoxynucleoside triphosphates (dNTPs) by filling in the 3′ ends with the Klenow fragment of DNA polymerase I (2); the footprinting probes were labeled on only one strand by using a linearized plasmid containing the desired restriction fragment as the substrate for the fill-in reaction and then by releasing the labeled fragment with a second restriction enzyme. Primers were labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (2).

In vivo footprinting. In vivo methylation of DNA was carried out with DMS by modifying the method described by Sasse-Dwight and Gralla (36). Cyanobacterial cultures were grown in 1-liter cylinders under red or green light to mid-log phase. Each culture was split in half; the cells were harvested by centrifugation and resuspended in 10 ml of BG-11 medium, and the suspension was reincubated under starting light conditions for another 2 h. During subsequent manipulations, the tubes were covered with red or green acetate when transported outside the light chambers. DMS was added to one tube in each light treatment to a final concentration of 6 mM; the tubes were vortexed vigorously for 15 s and incubated under starting light conditions for 5 min. The reaction was stopped by rapidly chilling the tubes in an ice water bath for ~5 min. Both treated and untreated cells were harvested by centrifugation and resuspended in 5 ml of 10 mM $MgSO_4$, and the suspensions were frozen at −80°C. The same results have been obtained by adding DMS directly to ~250 ml of mid-log-phase cyanobacterial culture growing in red or green light. Chromosomal DNA was isolated by modifications of standard procedures (2). The thawed cell pellets were resuspended in 5 ml of TES buffer (10 mM Tris-HCl [pH 7.5], 100 mM EDTA, 150 mM NaCl), 40 mg lysozyme was added, and the suspensions were incubated for at least 2 h at 37°C. If lysis was not complete after this time, as determined microscopically, an additional 40 mg of lysozyme was added, and the incubation was continued for another 1 h at 37°C. Standard procedures of proteinase K digestion in the presence of sodium dodecyl sulfate, phenol-chloroform extractions, RNase treatment, and precipitation from isopropanol were then followed. The final DNA pellets were resuspended in 0.5 to 1 ml of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). The control DNA samples (200 μg) which were not treated with DMS in vivo were modified in vitro as described elsewhere (36). All methylated DNA samples (200 μg) were cleaved with 1.0 M piperidine at 90°C for 15 min as described by Maxam and Gilbert (27). After removal of the piperidine by repeated lyophilizations from water, 40 μg of each DNA sample was analyzed by primer extension with 5′ end-labeled primers and the Klenow fragment of DNA polymerase I, separation on a sequencing gel with dideoxy sequencing ladders (34) with the same primers, and autoradiography at −80°C.

Preparation of protein extracts from *F. diplosiphon*. One-liter cultures of *F. diplosiphon* were grown in red or green light to mid-log phase as described above. Filaments were collected on GF/A filters and briefly frozen in liquid nitrogen; all subsequent steps were performed at 4°C. After thawing, the cell paste was resuspended in 8 ml of 0.65 M sodium-potassium phosphate, pH 7.0, containing 1 mM phenylmethylsulfonyl fluoride and 1 mM NaF and was lysed by two passes through a French pressure cell at 18,000 lb/in^2. Cell debris was removed by centrifugation at 10,000 × *g*. The supernatants were centrifuged at 100,000 × *g* for 90 min; the pellets were washed with 5 ml of lysis buffer and recentrifuged as before. The two high-speed supernatants were pooled and fractionated by ammonium sulfate precipitation. Saturated ammonium sulfate solution was added to the supernatant fractions to 25, 37, 50, and 62% saturation. The pellets were resuspended in a minimal volume of 50 mM potassium phosphate buffer, pH 7, containing 50 mM KCl and 1 mM NaF, and dialyzed twice against 1 liter of the same buffer. The fractions were made 20% in glycerol and stored at −80°C.

DNA mobility shift assays. DNA mobility shift gel electrophoresis (11) was used to detect binding of proteins from *F. diplosiphon* extracts to restriction fragments containing the *cpeBA* promoter region. Appropriate restriction fragments were end labeled by filling in the 3′ ends with the Klenow fragment of DNA polymerase I and [α-^{32}P]dNTPs (2); radioactive fragments were purified on polyacrylamide gels and eluted overnight at 37°C in a minimal volume of 50 mM Tris-Cl (pH 8)–50 mM NaCl–5 mM EDTA. In the standard binding reaction, protein extracts were incubated at room temperature for 20 min with labeled DNA fragments (~1 ng) in 10 mM Tris-Cl, pH 7.5, with 1 mM EDTA, 50 mM KCl, 0.1 mM dithiothreitol, 5% glycerol, 50 μg of bovine serum albumin per ml, and 1 μg of sonicated pUC9 as a nonspecific competitor. Loading buffer was then added, and the reaction mixtures were electrophoresed at 150 V on 4% minipolyacrylamide gels (prerun for 1 h) in 45 mM Tris–45 mM borate–1 mM EDTA (0.5× TBE) buffer at 4°C. The gels were dried, and the mobilities of the DNA fragments were visualized by autoradiography. In competition assays, the pUC9

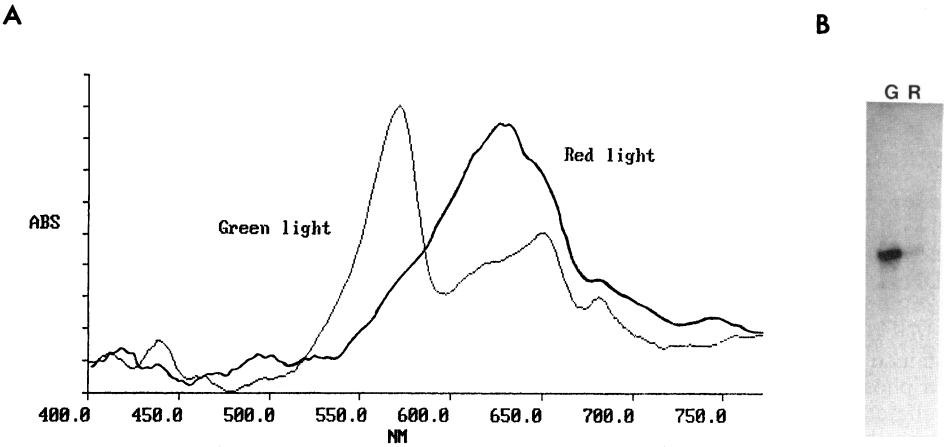

FIG. 1. (A) Whole-cell difference absorption (ABS) spectra of *F. diplosiphon* cultures grown in red or green light. For each light regimen, the absorption spectrum of the untreated culture was recorded. The same sample was then heated for 10 min at 65°C as described elsewhere (43) to denature the phycobiliproteins; the absorption spectrum of the heated culture was recorded and subtracted from the original spectrum to generate the curves shown. The bold line represents cultures grown in red light. The peak at 562 nm represents phycoerythrin. (B) Northern (RNA) blot of RNA purified from *F. diplosiphon* cultures grown in red (R) or green (G) light. Two micrograms of RNA was electrophoresed on a formaldehyde-agarose gel, transferred to nitrocellulose, and probed with fragment d (Fig. 2). The lanes were scanned with a densitometer, and the area under the main band at ~1,500 nucleotides was measured in both R and G lanes; there is ~16-fold more *cpeBA* transcript in the cultures grown in green light.

was eliminated from the binding reactions; instead, differing amounts of specific or nonspecific restriction fragments or oligonucleotides were incubated with the protein extracts for 10 min, the radioactive DNA probe was added, and the incubation was continued for another 20 min prior to electrophoresis.

In vitro footprinting. Regions of the phycoerythrin promoter which were protected by binding to factor(s) in *F. diplosiphon* extracts were identified by in vitro footprinting with either DMS or DNase I. Under limiting conditions, DNase I will produce single-strand nicks and DMS will methylate the DNA, leaving the starting fragment intact during nondenaturing electrophoresis. In both cases, preformed protein-DNA complexes were treated with the modifying reagent and then immediately electrophoresed on a native mobility shift gel as described above to separate and purify bound from unbound DNA fragments (33, 38). The unbound DNA fragment served as the control against which the bound DNA fragment was compared. Details of each treatment are given below.

DMS footprinting. Restriction fragments labeled on only one strand were incubated with protein extracts under standard conditions. After 20 min, DMS was added to a final concentration of 6 mM, and the solution was incubated for 5 min at 37°C; loading dye was added, and the samples were immediately electrophoresed on a 4% native polyacrylamide gel as described above. Protein-bound and free DNA fragments were visualized by autoradiography of the wet gel for several hours at 4°C; both the protein-bound DNA band and the unbound DNA fragment were excised, and both DNA fragments were separately eluted as described above. The DNAs were precipitated with ethanol, dried, and cleaved with piperidine. After removal of the piperidine, the samples were electrophoresed on a 10% denaturing polyacrylamide gel along with appropriate Maxam-Gilbert sequencing reactions as markers. DNA fragments were visualized by autoradiography of the dried gel at −80°C.

DNase footprinting. Restriction fragments labeled on only one strand were incubated with protein extracts under standard conditions. After 20 min, $MgCl_2$ and $CaCl_2$ were added to 10 mM final concentration; an appropriate concentration of DNase I was added, and the mixture was incubated for 30 s at room temperature. A 10-fold excess of poly(dI-dC) was added along with loading dye; the samples were chilled on ice and then electrophoresed as described above. The wet gel was autoradiographed at 4°C for several hours to localize the positions of the bound and free DNA fragments, which were both excised and eluted separately overnight as described above. After ethanol precipitation, the samples were electrophoresed on a 10% denaturing polyacrylamide gel along with appropriate Maxam-Gilbert sequencing reactions as markers. DNA fragments were visualized by autoradiography of the dried gel at −80°C.

RESULTS

Phycoerythrin protein and transcript levels in *F. diplosiphon* grown in red and green light. Growth of cultures of *F. diplosiphon* in red light results in very low levels of expression of the phycoerythrin operon *cpeBA*, resulting in phycobilisomes containing barely detectable levels of phycoerythrin subunits. Conversely, in green light, transcription from the *cpeBA* promoter is dramatically increased, resulting in high amounts of phycoerythrin assembled into phycobilisomes (Fig. 1) (8, 16, 28). Under our growth conditions, steady-state levels of the *cpeBA* transcript are increased ~16- to 19-fold in green light compared with red light.

In vivo footprinting of the *cpeBA* promoter in red and green light. To obtain information regarding the binding of regulatory proteins to the *cpeBA* promoter, we performed in vivo methylation protection studies on *F. diplosiphon* cells growing under the two different light regimens. The small organic molecule DMS readily diffuses into cells and rapidly methylates exposed guanosine residues in DNA. However, if a region of DNA is protected by the binding of a protein, certain guanosine residues in contact with the protein may remain unmodified. DNA isolated from samples treated in vivo can be compared with naked DNA modified in vitro to

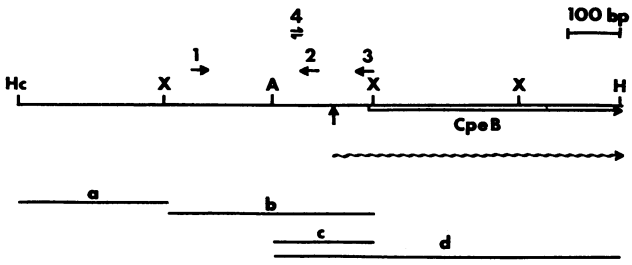

FIG. 2. Partial restriction map of the *cpeBA* operon. The vertical arrow indicates the approximate start site of transcription (8). The open box represents the coding region for CpeB, which extends further in the 3′ direction. Fragments a through d are different restriction fragments used in this work as probes, competitor DNA, or in in vitro footprinting. The small arrows above the map refer to oligonucleotides used as primers in in vivo footprinting or for sequencing (no. 1 to 3) and to a double-stranded oligonucleotide (no. 4) used as a specific competitor in DNA mobility shift gel analysis. The sequences and exact locations of these oligonucleotides are given in Table 1. A, *Ava*II; H, *Hin*dIII; Hc, *Hin*cII; X, *Xba*I.

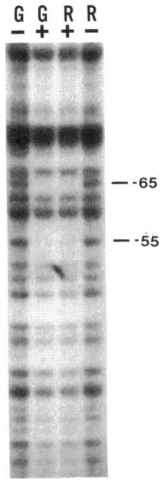

FIG. 3. In vivo footprinting of the *cpeBA* promoter. *F. diplosiphon* cultures growing in red (R) or green (G) light were treated with DMS; genomic DNA was isolated, cleaved with piperidine, and analyzed by primer extension (with primer no. 3; see Table 1), followed by separation on a DNA sequencing gel. The inner lanes (+) were samples from cultures modified in vivo with DMS; the outer lanes (−) are control reactions, in which genomic DNA was purified from parallel cultures and modified with DMS in vitro. The arrows indicate the two guanosine residues at −65 and −55 bp (relative to the start of transcription) which are protected from DMS modification in vivo in both red and green light.

detect protected sequences which may be important in gene regulation.

F. diplosiphon cultures grown in red or green light were briefly treated with DMS, and then genomic DNA was isolated. Control cultures were not treated in vivo, but the naked genomic DNA was modified with DMS after purification. Using primer extension with radiolabeled primers, we analyzed both the top and the bottom strands in the upstream region of *cpeBA* from approximately −300 to +50 (the start of the coding region of CpeB is +65). A partial restriction map of this region is shown in Fig. 2, including the positions of the primers used; the sequences of these primers are given in Table 1. Comparison of the cleavage patterns of the control DNA samples, modified in vitro, with the patterns of the DNA samples modified in vivo shows that two guanosine residues are protected in *F. diplosiphon* cells growing in both red and green light (Fig. 3). These residues are on the top strand at −55 and −65 bp upstream of the transcription start site. In addition, the guanosine residues are within two direct hexamer repeats, TTGTTA; the 10-bp separation of the protected guanosine residues would place them on approximately the same face of the DNA helix. Two guanosine residues located between these hexamers at −59 and −60 bp are not protected; these would lie on the opposite face of the DNA helix. These results imply that a factor is bound to the region upstream of the start of *cpeBA* transcription during growth in both light regimes.

Partial purification of a DNA-binding protein specific for the *cpeBA* promoter. To begin characterization of the factor(s) responsible for the in vivo footprint and light-regulated expression of the *cpeBA* promoter, we analyzed partially purified extracts of *F. diplosiphon* for specific DNA-binding activity. *F. diplosiphon* cultures grown in red or green light were harvested and lysed by passage through a French pressure cell; the cells were lysed in a high-ionic-strength phosphate buffer to minimize dissociation of the phycobilisomes from the thylakoid membranes. The soluble fractions (supernatants from centrifugation at 100,000 × *g*) were fractionated by the addition of saturated ammonium sulfate and assayed for specific binding to the *cpeBA* promoter region by DNA mobility shift gels. A 199-bp *Ava*II-*Xba*I fragment (fragment c in Fig. 2) and a 423-bp *Xba*I fragment (fragment b in Fig. 2; data not shown) were both used as binding probes in these assays. Although DNA-binding activity was found in several of the fractions (Fig. 4), the activity which was pelleted in 62% saturated ammonium sulfate (designated P4) in both red and green light extracts was highly specific for the region 5′ of the transcription start site for *cpeBA* (see below). Gel filtration chromatography through Superose 12 was used to further fractionate the specific binding activity; however, the activity eluted across a wide size range and did not result in effective purification

TABLE 1. Oligonucleotides used in footprinting and competition analyses

Oligonucleotide identification no.	Sequence	Location	Use
1	5′GTGCAGCTCATCCGTAAGG3′	−275 to −257 (top strand)	In vivo footprinting
2	5′CTATCTCAGTCTCAGTTATG3′	−22 to −41 (bottom strand)	In vivo footprinting
3	5′TCTAGAAAAAGCATC3′	+85 to +70 (bottom strand)	In vivo footprinting
4	5′TTTGTTAAGGATTGTTACTT3′ 3′AAACAATTCCTAACAATGAA5′	−67 to −49 (both strands)	Competition
5	5′CGAGTATTAATTCCGAGATGG3′ 3′GCTCATAATTAAGGCTCTACC5′	Pea DNA chitinase gene	Competition

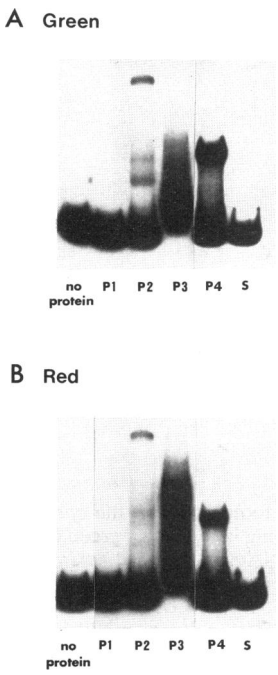

FIG. 4. DNA mobility shift gel electrophoresis of *F. diplosiphon* protein extracts. Lysates of *F. diplosiphon* cultures grown in green (A) or red (B) light were fractionated with ammonium sulfate as described in Materials and Methods. Protein-DNA complexes were formed and electrophoresed in a native 4% polyacrylamide gel. The gels were dried and exposed to X-ray film at −80°C. The radioactive DNA probe was fragment c (Fig. 2), the 199-bp *Ava*II-*Xba*I fragment.

(data not shown). Some of the assays discussed below were carried out with fractions from the Superose column, while others were done with the P4 fraction. The binding activities in these separate fractions were very similar.

The specificity of factor binding to the *cpeBA* promoter region was shown by competition assays, in which unlabeled competitor DNA was added to the protein fractions prior to incubation with labeled promoter fragments. As shown in Fig. 5A, a 100-fold molar excess of unlabeled fragment c virtually abolished binding to the labeled ligand, whereas a 290-bp *Xba*I fragment originating further 5′ of the *cpeBA* operon (fragment a in Fig. 3) did not compete effectively for factor binding. To further show the binding specificity of this factor, a double-stranded oligonucleotide which encompassed the region defined by the in vivo footprint was synthesized (Table 1 [oligonucleotide no. 4]). Figure 5B shows that oligonucleotide no. 4 is an effective competitor of factor binding to fragment c, compared with an oligonucleotide with a similar size and an unrelated sequence (Table 1 [oligonucleotide no. 5]). The data shown were obtained by using the protein fraction isolated from red-light-grown cells; very similar results were seen with protein fractions isolated from green-light-grown cells (data not shown). Thus, the binding activity specific for the *cpeBA* promoter appears to be the same in red or green light. We are designating the factor responsible for this binding activity PepB (phycoerythrin promoter-binding protein).

In vitro footprinting of the *cpeBA* promoter. (i) DMS protection. The partial purification of PepB, a DNA-binding protein specific for the *cpeBA* promoter, is consistent with the data from our in vivo footprinting experiments, in that PepB is present in both red and green light extracts and binds to the predicted region. To confirm that the partially purified PepB exhibits the same ability to protect the *cpeBA* promoter that was observed in vivo, we performed in vitro footprinting with DMS on the 199-bp *Ava*II-*Xba*I fragment (fragment c in Fig. 3) labeled at the 3′ end of either the top or the bottom strand. As shown in Fig. 6A, the same two guanosine residues on the top strand at −55 and −65 bp were protected from methylation in vitro and in vivo (Fig. 6C). Extracts from both red and green light gave equivalent levels of protection. On the bottom strand, the guanosine residue at −51 was strongly protected with extracts from both red and green light (Fig. 6B); protection of this nucleotide could not be detected in our in vivo footprints. In addition, background levels of cleavage at residues other than guanosine can be detected in some regions; two cytidine residues at −59 and −60 were protected from this nonspecific modification with both red and green light extracts. These residues are complementary to the two guanosine residues on the top strand between the direct hexameric repeats which were not protected in vivo or in vitro. No other protected residues on either the top or the bottom strand were observed in this assay.

(ii) DNase protection. DMS is a small organic molecule which can theoretically modify residues in close proximity to a DNA-binding protein; in addition, DMS has relatively high specificity for methylation of guanosine residues, which are not randomly distributed throughout the promoter region. Cleavage of DNA in the presence of DNA-binding proteins with the less-specific enzymatic activity of DNase I usually identifies a larger protected region because of steric hindrance between the proteins. To further define the binding site for the putative transcription factor PepB, we performed in vitro DNase I protection assays on fragments b and c of the *cpeBA* promoter, labeled at the 3′ end of either the top or

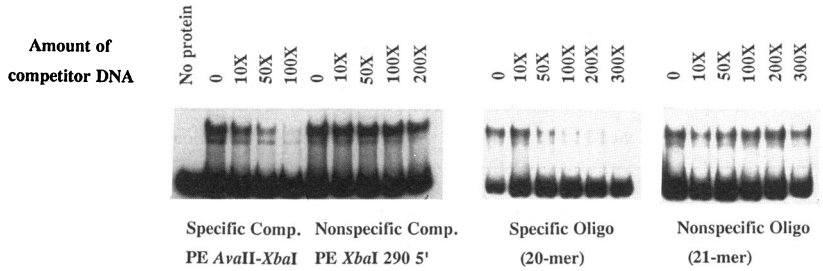

FIG. 5. Competition assays. Partially purified *F. diplosiphon* extracts from red light were preincubated with specific or nonspecific competitor (comp.) DNA as indicated. The incubations were continued for another 20 min with the labeled restriction fragment (fragment c), and the samples were electrophoresed on a 4% native polyacrylamide gel. The gels were dried and exposed to X-ray film at −80°C. PE, phycoerythrin.

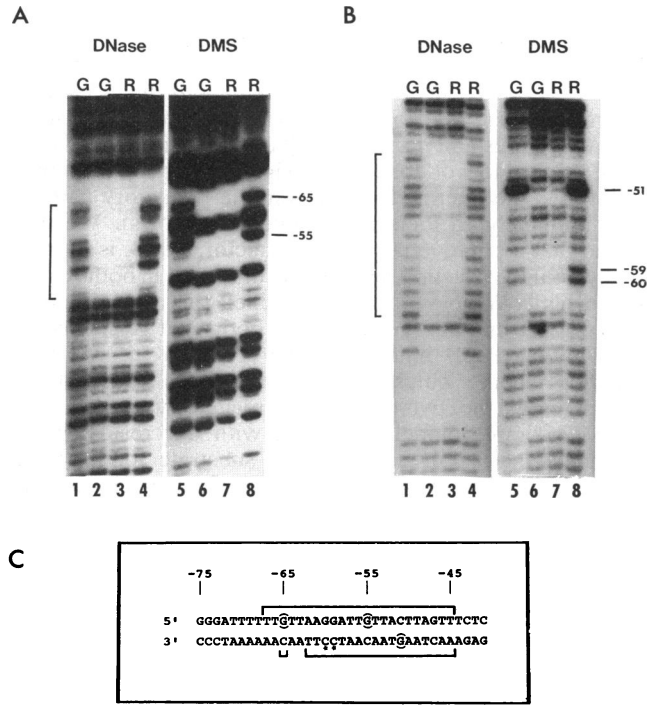

FIG. 6. In vitro footprinting experiments. The DNA probe used is the 199-bp *Ava*II-*Xba*I fragment (fragment c in Fig. 2) labeled at the 3′ end of the top strand (A) or the bottom strand (B). (C) Summary of in vivo and in vitro footprinting data. The brackets indicate the regions defined by DNase I protection, while the half circles identify the residues protected from DMS modification. The asterisks indicate the cytidines at −59 and −60 bp which were protected from background levels of DMS modification. Only the guanosines at −55 and −65 were protected from methylation by DMS in vivo. G, green; R, red.

the bottom strand. With fragment c, extracts from both red- and green-light-grown cultures produced nearly identical footprints (Fig. 6A and B). A footprint extending from −67 to −45 bp is seen on the upper strand, which encompasses the two guanosine residues at −65 and −55 bp protected from DMS modification both in vitro and in vivo. On the bottom strand, a region is protected from −62 to −45, with an additional cytidine protected at −65. When the larger fragment b was used for footprinting, similar footprints were obtained, with no other protected regions visible (data not shown). Because of the limited sequence specificity exhibited by DNase I, some sequences in the *cpeBA* promoter were cleaved by DNase I at low frequency. These data thus do not precisely define the boundaries of binding of the putative transcription factor PepB. The sequences protected by all of the in vivo and in vitro footprinting protocols are summarized in Fig. 6C.

DISCUSSION

In *F. diplosiphon*, growth in green light results in the accumulation of high steady-state levels of transcripts from the *cpeBA* and *cpeCDE* operons, whereas growth in red light leads to very low transcript levels (Fig. 1B). These genes encode the α and β subunits of phycoerythrin and its associated linker proteins (8, 9, 28). When these proteins are assembled into the rods of the phycobilisomes, the cells are able to maximally absorb the available energy from green light (Fig. 1A). In considering the mechanisms underlying light-activated gene expression of the *cpeBA* operon, we hypothesize that differential recognition of the promoter and upstream sequences by transcription factors will play an important role. Consequently, as an initial step, we defined the transcription start sites of both green-light-activated operons, *cpeBA* and *cpeCDE*, by primer extension and S1 analyses (8). Extending those studies, we report here the analysis of the in vivo state of the promoter region of the *cpeBA* operon in both red and green light by a methylation protection assay to reveal potential protein-binding sites. We found two guanosine residues at −55 and −65 bp upstream of the start of transcription which were strongly protected in both red and green light (Fig. 3). The location of this binding site would potentially place this putative regulatory protein upstream of RNA polymerase binding to the promoter. Using the knowledge of the binding site for the putative transcription factor and of its presence in both red and green light, we began purification of this DNA-binding activity from *F. diplosiphon* cultures grown in both light regimens. Using a DNA mobility shift gel assay with a labeled fragment containing the region upstream of *cpeBA*, we found that several ammonium sulfate fractions of cell extracts from red or green light contain DNA-binding activity (Fig. 4). However, the pellet from a 50 to 62% ammonium sulfate cut contained a factor, PepB, which binds specifically to fragment c as shown by competition studies (Fig. 5). In vitro methylation of fragment c with DMS in the presence of either the red or the green light extract resulted in protection of the same two guanosine residues (−55 and −65 bp) on the top strand that were protected in vivo; an additional protected guanosine (−51) on the bottom strand was protected in vitro (Fig. 6). The binding site for PepB was further defined by using in vitro DNase I footprinting as the region from −45 to −67 on the top strand and from −45 to −62 on the bottom strand. Again, extracts from both red- and green-light-grown cultures gave essentially identical footprints. Together with the in vivo footprinting data, these results imply that the factor PepB is bound to the region upstream of the transcription start site for *cpeBA* under all light conditions. Thus, to achieve light-regulated transcription of this operon, differential modification of PepB and/or interaction with other transcription factors must be required. Coordinate regulation of *cpeBA*, encoding the α and β subunits of phycoerythrin, and *cpeCDE*, encoding the associated linker proteins, should be achieved by PepB binding to the regulatory regions of both operons. Previously, we had noted the presence of a 17-bp sequence which is common to the upstream regions of both *cpeBA* and *cpeCDE* (8); this element is upstream of the PepB-binding site defined in the present work, and its significance is still under investigation. A search of the region upstream of the *cpeCDE* transcription start site (8) for a sequence homologous to that of the PepB-binding motif revealed two copies of hexameric repeats (TTGTAA) with homology (5 of 6 bp) to those found upstream of *cpeBA* (TTGTTA); however, the spacing between these repeats is 52 bp for *cpeCDE*, and they are located on the opposite strand (i.e., opposite orientation) to that of *cpeBA*. The genes encoding the linker proteins are expressed at lower levels than the phycoerythrin subunits (8), which reflects the stoichiometry in the phycobilisome. Thus, lower levels of expression could be due to lower affinity of PepB for the putative binding site upstream of *cpeCDE* because of its altered sequence and/or because of the increased distance between the repeats. We are currently

investigating the binding interactions between the regulatory region of *cpeCDE* and PepB.

There are two noteworthy features of the data reported in the present work. First, the putative regulatory factor PepB is continuously bound to the region upstream of the phycoerythrin transcription start site under both activating and repressing conditions and, second, the binding site identified is composed of directly repeated sequences instead of containing a region of dyad symmetry. There are many precedents in both prokaryotic and eukaryotic systems for transcription factors which are constitutively expressed and continuously bound to their target DNA sequences. One of the best characterized in *Salmonella typhimurium* and *Escherichia coli* is the protein NtrC, which regulates transcription of *glnA* (encoding glutamine synthetase) in response to the nitrogen status of the cell (22). With the specialized σ^{54} subunit of RNA polymerase, the phosphorylated form of NtrC activates transcription from the *glnA* promoter by binding to an upstream enhancer-like element both in vivo and in vitro. The unphosphorylated form of NtrC can still bind its cognate sequence but does not activate transcription. Consequently, NtrC continuously bound to the *glnA* enhancer sequence is poised to activate transcription in immediate response to a change in nitrogen availability.

The majority of transcription factors whose recognition sequences have been characterized bind to sequences with dyad symmetry, i.e., to inverted repeats. In contrast, the motif to which PepB binds, $TTGTTAN_4TTGTTA$, consists of hexameric direct repeats. The central guanosine residues which are protected from methylation by DMS both in vivo and in vitro are separated by 10 bp, approximately one turn of the DNA helix, and are located upstream of the presumed promoter region. A limited number of other prokaryotic transcription factors with similar binding specificities have been identified. The *E. coli* phage λ transcriptional activator protein cII (17, 19) and the analogous *S. typhimurium* P22 protein C1 (18) both regulate lysogenic development and recognize tetrameric direct repeats, $TTGCN_6TTGC$, located at the −35 region of phage promoters. Other characterized examples include the *E. coli* PhoB (26) and OmpR (25) proteins and *Vibrio cholerae* ToxR (29). PhoB, ToxR, and OmpR, as well as the NtrC discussed above, are all members of two-component regulatory protein pairs involved in sensing and responding to different environmental signals (1).

Changes in gene expression in response to light intensity have been studied in another cyanobacterium, *Synechococcus* sp. strain PCC7942, by Bustos and coworkers (5, 37). They have found that multigene families encode the photosystem II reaction center polypeptides D1 and D2 and that light intensity affects the expression of some of these genes. Sequences within the untranslated leader region of the *psbDII* gene (encoding the D2 protein) are required for efficient expression and may be involved in the induction by high light intensity. Light-responsive genes of higher plants contain multiple upstream DNA elements which are recognized by diverse transcription factors (for a review, see reference 12). The activity of some of these factors appears to be modified by phosphorylation (7, 35).

One of the long-term goals of the present work is the elucidation of the signal transduction pathway by which *F. diplosiphon* detects differing light qualities and responds to them by alterations in gene expression. Biological responses regulated by light are the final result of a sensory perception process triggered by the absorption of specific wavelengths of light by a photoreceptor. The nature and number of photoreceptors responsive to red and green light in this organism have not yet been defined, and the steps between the detection of the initial light stimulus and the final transcriptional changes are completely unknown. We have chosen to start with a highly expressed light-regulated operon, *cpeBA*, and to characterize the required *cis*-acting DNA elements and *trans*-acting factors. Using this information, we plan to work back in the signal transduction chain to determine the intermediate steps in the regulatory pathway and ultimately to identify the photoreceptor(s). We have found that a putative transcription factor, PepB, is continuously bound to the upstream region of *cpeBA* in both red and green light. We have partially purified this factor from *F. diplosiphon* cultures grown under both light regimens and shown that PepB binding to the region 5′ of *cpeBA* is identical whether obtained from red or green light. With the paradigm of the two-component regulatory system as a basis, a simple model consistent with our results would postulate that PepB could be the substrate for a protein kinase. When the photoreceptor detects green light, it could undergo a conformational change which then activates the kinase, stimulating it to phosphorylate PepB. The phosphorylated form of PepB might then be capable of interacting productively with RNA polymerase to activate transcription of *cpeBA*. Other evidence suggests that this simple model will not be sufficient to encompass the entire signal transduction pathway. It has been reported that protein synthesis is required for transcription of the *cpeBA* operon when cultures of *F. diplosiphon* previously grown in red light are shifted to green light (32). Since we have shown that PepB is present continuously in red and green light, the synthesis of another protein factor would be required in response to the switch to green light prior to activation of *cpeBA* transcription. This additional factor could be a light-regulated sigma factor or another transcription factor which interacts with PepB. We are continuing our purification of PepB and analysis of the DNA-binding activities of the other ammonium sulfate fractions of *F. diplosiphon* extracts. The determination of the binding specificity of PepB for the upstream region of *cpeCDE* will provide additional data as to the requirements for regulation of gene expression by green light. Further delineation of other components involved in this signal transduction chain will add to our understanding of the common and diverse mechanisms used by organisms to respond to environmental stimuli.

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