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Characterization of the Light-Regulated Operon Encoding the Phycoerythrin-Associated Linker Proteins from the Cyanobacterium *Fremyella diplosiphon*†

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Many biological processes in photosynthetic organisms can be regulated by light quantity or light quality or both. A unique example of the effect of specific wavelengths of light on the composition of the photosynthetic apparatus occurs in cyanobacteria that undergo complementary chromatic adaptation. These organisms alter the composition of their light-harvesting organelle, the phycobilisome, and exhibit distinct morphological features as a function of the wavelength of incident light. *Fremyella diplosiphon*, a filamentous cyanobacterium, responds to green light by activating transcription of the *cpeBA* operon, which encodes the pigmented light-harvesting component phycoerythrin. We have isolated and determined the complete nucleotide sequence of another operon, *cpeCD*, that encodes the linker proteins associated with phycoerythrin hexamers in the phycobilisome. The *cpeCD* operon is activated in green light and expressed as two major transcripts with the same 5′ start site but differing 3′ ends. Analysis of the kinetics of transcript accumulation in cultures of *F. diplosiphon* shifted from red light to green light and vice versa shows that the *cpeBA* and *cpeCD* operons are regulated coordinately. A common 17-base-pair sequence is found upstream of the transcription start sites of both operons. A comparison of the predicted amino acid sequences of the phycoerythrin-associated linker proteins CpeC and CpeD with sequences of other previously characterized rod linker proteins shows 49 invariant residues, most of which are in the amino-terminal half of the proteins.

Cyanobacteria are oxygenic photosynthetic procaryotes whose primary light-harvesting apparatus is the phycobilisome (12, 17, 22). These structures are peripherally associated with the thylakoid membranes and efficiently transfer a broad spectrum of light energy to the photosynthetic reaction centers. In most cyanobacteria, the phycobilisome is a hemidiscoidal structure that is composed of two distinct substructures: a core complex and six rods radiating from the core (21). The pigmented proteins of the phycobilisome (phycobiliproteins) are hydrophilic polypeptides with covalently attached tetrapyrrole chromophores. These proteins constitute ~85% of the mass of the complex, with the remainder comprising nonpigmented "linker" proteins (48). Both the pigmented and nonpigmented constituents of the phycobilisome rods can vary depending on growth conditions, such as temperature, light quality and quantity, and the nutritional status of the growth medium (2, 5, 25, 35, 38, 47, 49).

The phycobilisome contains three classes of phycobiliproteins. Allophycocyanin is the major component of the core complex (21). Phycocyanin constitutes the portion of the rods adjacent to the core complex, while phycoerythrin or phycoerythrocyanin, when present, is found at the periphery of the rods. Each type of phycobiliprotein monomer is composed of equimolar amounts of two subunits, α and β, which assemble into trimers and hexamers. Assembly into higher order structures is mediated by association with particular linker proteins that determine the assembly pathway of the macromolecular complex and optimize energy transfer from the periphery of the rods to the phycobilisome core and ultimately to the photosynthetic reaction center (56).

Many cyanobacteria alter the composition of their phycobilisomes in response to light quality, or wavelength, a phenomenon known as complementary chromatic adaptation (7, 48). The altered phycobilisome composition results in optimal absorption of prevalent wavelengths of light and is a consequence of de novo synthesis of the appropriate phycobiliproteins and linker proteins (9). In *Fremyella diplosiphon*, phycoerythrin is synthesized in response to green light but not to red light (6, 18, 51), whereas increased levels of phycocyanin are found in red light but not in green light (26, 53). These responses appear to be regulated primarily at the transcriptional level (13, 33, 36).

In *F. diplosiphon*, there are three operons encoding phycocyanin subunits that are expressed under different conditions. The *cpc1* operon encodes the β and α subunits of a phycocyanin which is expressed at similar levels in green and red light (constitutive phycocyanin) (14). The *cpc2* operon encodes phycocyanin subunits and associated linker proteins that are expressed in red, but not in green, light (inducible phycocyanin) (14, 32). In phycobilisomes from cells grown in both red and green light, the constitutive phycocyanin probably occupies the portion of the rods proximal to the core; the rod substructure is completed in red light-grown cells by the inducible phycocyanin hexamers with associated linker proteins. The third phycocyanin operon, *cpc3*, encodes a set of phycocyanin subunits and linker proteins which replaces the red light-inducible phycocyanin and its associated linkers under conditions of sulfur deprivation (34, 35).

In contrast to the multiple *cpc* operons, there is only one set of genes encoding the β and α subunits of phycoerythrin in *F. diplosiphon*. This operon, *cpeBA*, is expressed at high

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levels in green light and at very low levels in red light (33, 36). In green light, the phycoerythrin hexamers occupy the distal portions of the phycobilisome rods in place of phycocyanin hexamers (17). Also, unlike many of the phycocyanin operons, the genes encoding the linker proteins that associate with phycoerythrin are not linked to *cpeBA*. These linker proteins are highly expressed in green light and are required for the proper assembly of phycoerythrin into the rods of the phycobilisome (19, 20). No genes encoding phycoerythrin-associated linker proteins have previously been described for any organism, although a partial amino acid sequence of a linker protein associated with phycoerythrocyanin from *Mastigocladus laminosus* has been published (16).

Here, we describe the isolation and characterization of the *cpeCD* operon encoding the phycoerythrin-associated linker proteins in *F. diplosiphon*. The *cpeC* and *cpeD* genes are cotranscribed at high levels in green light and at barely detectable levels in red light. We have examined changes in steady state transcript levels from both of the green light-regulated operons (*cpeBA* and *cpeCD*) after changing conditions of illumination during growth from green to red light and vice versa. In addition, we have determined the sites of transcription initiation for both operons. A comparison of the amino acid sequences of the two linker proteins CpeC and CpeD to each other and to published sequences of linker proteins associated with other phycobiliproteins defines regions of sequence conservation which may have both structural and functional importance.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., Boehringer Mannheim Biochemicals, U.S. Biochemicals Corp., and Pharmacia, Inc. Klenow fragment of DNA polymerase I, T4 DNA ligase, Sequenase, calf intestinal alkaline phosphatase, and urea were purchased from U.S. Biochemicals; T4 polynucleotide kinase, reverse transcriptase, and S1 nuclease were from Pharmacia. Radioactive nucleotides ([γ-^{32}P]ATP [>5,000 Ci/mmol], [α-^{32}P]dCTP [>3,000 Ci/mmol], and [α-^{35}S]dATP [>600 Ci/mmol]) were obtained from Amersham Corp.

Phycobilisome preparation and analysis. *F. diplosiphon* 33 (a strain of *Calothrix* sp., PCC 7601; a subculture of UTEX 481) was grown at 30°C in BG-11 medium with 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 8.0) in an atmosphere of 5% CO_2 in air) in red or green light at 15 microeinsteins/cm^2 as described by Bruns et al. (8). Phycobilisomes were isolated from red light- and green light-grown cells as previously described (2). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli (31) and as modified by Anderson et al. (2). The linker polypeptides specifically expressed in green light (CpeC and CpeD) were isolated from a preparative polyacrylamide gel and were injected as a mixture into rabbits to raise polyclonal antibodies. Proteins were transferred from polyacrylamide gels to nitrocellulose by the method of Towbin et al. (51) and were stained immunologically with the polyclonal antibodies and protein A-conjugated horseradish peroxidase (Boehringer Mannheim) (3).

Preparation of an expression library. Genomic DNA from *F. diplosiphon* 33 was digested with *Sau*3A in four separate reactions ranging from ~10 to 90% completion. DNA fragments 1 to 3 kilobases (kb) in size were excised after electrophoresis in 0.7% agarose. The DNA was isolated from the gel by the freeze-squeeze method (50) and precipitated with ethanol. λZAP (Stratagene [46]) DNA was digested to completion with *Xho*I. Klenow fragment and the appropriate nucleotides were used to fill in the first two bases of the *Sau*3A and *Xho*I sites, generating a two-base compatible sequence. The DNA was ligated with T4 DNA ligase, plated on *Escherichia coli* BB4, and screened with the antibodies raised against CpeC and CpeD as described above. One immunopositive λ clone was obtained, and the internal Bluescript SK$^-$ plasmid containing a 1.6-kb insert was rescued in vivo from this λ clone in *E. coli* XL-1 Blue (46). Larger clones containing 5' and 3' sequences were obtained by screening an EMBL3 library and the λZAP library with fragments of the 1.6-kb immunopositive clone. Single-stranded template DNA was prepared from Bluescript, pUC118, and pUC119 plasmids containing fragments of the *cpeCD* genes by using the helper phage M13KO7 (52). DNA sequencing was performed by the dideoxy-chain termination method (41) on single-stranded and double-stranded templates by using Sequenase, according to the protocols of the manufacturer, with the M13 primer, reverse primer, and other appropriate primers synthesized on a Biosearch 8600 oligonucleotide synthesizer. DNA sequence analyses and protein homology alignments were performed by using the IBI Pustell Sequence Analysis Programs version 2.02. Predictions of RNA folding were obtained by using PCFOLD version 3.0 by Michael Zuker (57).

RNA isolation and analyses. RNA was isolated from *F. diplosiphon* 33 grown in red or green light by lysis in guanidinium chloride and CsCl centrifugation as described previously (13, 24). Electrophoresis of RNA (2 μg) was performed on 1% agarose-formaldehyde gels in 20 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 8.0); the RNA was transferred to nitrocellulose and probed with fragments labeled with [α-^{32}P]dCTP by the random oligonucleotide priming method (15). Primer extension analysis (54) was used to determine the 5' end of the mRNA encoding CpeC and CpeD. A 24-base oligomer complementary to nucleotides 63 through 86 of the coding region for CpeC was synthesized, end labeled with [γ-^{32}P]ATP, and used as a primer for reverse transcriptase with 10 μg of RNA isolated from cells grown in red or green light. The products were analyzed on a denaturing polyacrylamide gel with a sequencing ladder generated by using the same primer as a standard. The position of the transcription start site was confirmed by S1 nuclease protection (44) by using as probe the 890-base-pair (bp) *Ava*II-*Pst*I fragment labeled at the 5' end at the *Ava*II site. The probe was hybridized to 50 μg of RNA at 30 and 40°C in a total volume of 30 μl; digestion of the hybrids with 38 U of S1 nuclease per 330 μl at 37°C gave optimal results.

S1 nuclease protection was also used to determine the transcription start site for the phycoerythrin operon (*cpeBA*). A 270-bp *Xba*I-*Hae*III fragment starting 20 nucleotides (nt) within the coding region for CpeB was labeled at the *Xba*I site, and ~0.25 pmol was annealed to 50 μg of RNA at 35°C overnight in a total volume of 50 μl. The hybrids were digested for 60 min at room temperature with 2,000 U of S1 nuclease per 550 μl.

RESULTS

Cloning and characterization of the phycoerythrin-associated linker genes (*cpeCD*). Preparations of phycobilisomes from cultures of *F. diplosiphon* 33 grown in red or green light can be resolved by SDS-polyacrylamide gel electrophoresis

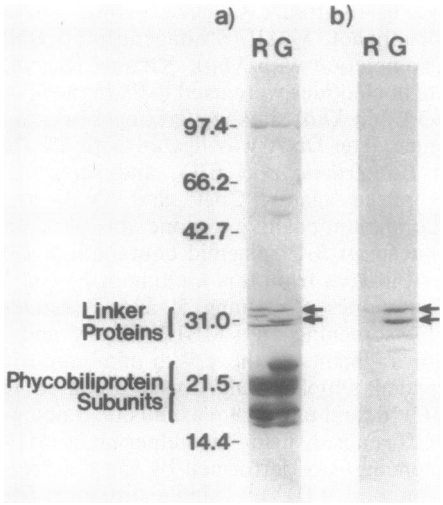

FIG. 1. Polyacrylamide gel electrophoresis of phycobilisomes isolated from *F. diplosiphon* 33 cultures grown in red (lanes R) or green (lanes G) light. (a) The gel was stained with Coomassie blue; size standards are in kilodaltons, and the linker proteins associated with phycoerythrin are marked with arrows. (b) Western blot of a duplicate gel as described for panel a developed with antibodies raised to a mixture of the phycoerythrin-associated linker proteins.

(Fig. 1). Differences in both the phycobiliproteins and the linker proteins are evident in phycobilisome preparations isolated from different light regimens as previously described (9, 32). The phycoerythrin-associated linker proteins (CpeC and CpeD) are present at high levels in green light (Fig. 1a) and can be detected by specific antibodies on Western blots (immunoblots) (Fig. 1b). The low levels of CpeC and CpeD detected by the antibodies in red light are consistent with the levels of phycoerythrin (CpeA and CpeB) which are observed under these growth conditions (8). The two smaller bands detected on the Western blot below the predominant linker protein bands are probably degradation products of the linker proteins and cannot be detected on the Coomassie blue-stained gel. No cross-reactivity is seen with the phycocyanin-associated linker proteins expressed in red light or with the constitutive 29-kilodalton (kDa) linker protein.

The antibodies raised to the phycoerythrin-associated linker proteins were used to screen an expression library of *F. diplosiphon* 33 DNA constructed in λZAP; one immunopositive clone containing an insert of ~1.6 kb was obtained and is designated in Fig. 2 as clone 1. This clone contained the entire coding region for CpeC and the amino-terminal half of CpeD. Fragments of this clone were used to screen an EMBL3 library and to rescreen the λZAP library to obtain clones containing flanking regions. A 3.0-kb *Hin*dIII-*Sau*3A fragment (Fig. 2, clone 2) subcloned from an EMBL3 phage added both the region upstream of *cpeC* and the remainder of the coding region of CpeD, including ~200 bp downstream. A 3.0-kb *Cla*I fragment (Fig. 2, clone 3) was obtained from another λZAP clone which extended further in the 3′ direction. Restriction mapping, Southern blotting, and DNA sequencing were used to construct the composite map shown in Fig. 2. The two open reading frames of the *cpeCD* operon have predicted amino acid sequences that are highly homologous to each other and to the phycocyanin-associated linker proteins (CpcH2 and CpcI2) from *F. diplosiphon* (32); Table 1 gives the nomenclature used for the various linker proteins (30). The nucleotide sequence for this region, including ~450 bp upstream of the presumed start codon for CpeC, and the derived amino acid sequences for CpeC and CpeD are presented in Fig. 3.

In *F. diplosiphon*, the phycoerythrin-associated linker genes (*cpeCD*) are not closely linked to the phycoerythrin genes (*cpeBA*); an EMBL3 clone containing *cpeBA* approximately centered within a 15-kb insert did not hybridize on a Southern blot to a fragment containing the linker genes (data not shown). The first gene in the *cpeCD* operon, *cpeC*, is predicted to encode a protein of 31.8 kDa with a pI of 9.2. A consensus ribosome binding site (AGGAG) (23, 45) precedes the initial methionine codon by 9 nt. The second gene (*cpeD*) encodes a protein of 27.9 kDa with a pI of 8.7 and is separated from *cpeC* by 40 nt, with a consensus ribosome binding site (AGGAG) 11 nt upstream of the initial methio-

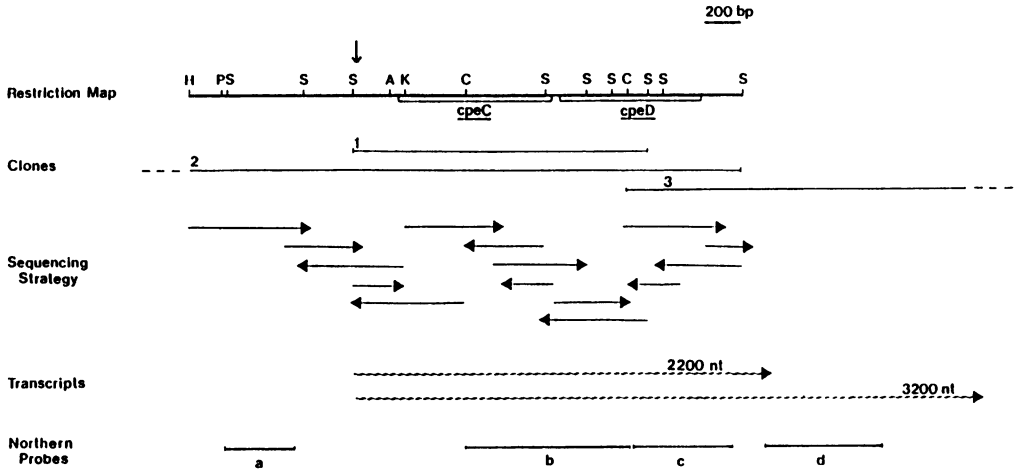

FIG. 2. Partial restriction map and sequencing strategy for the *cpeCD* operon encoding the phycoerythrin-associated linker proteins. Templates were derived from the following clones: 1, a 1.6-kb *Sau*3A fragment isolated from a λZAP clone; 2, a 3.0-kb *Hin*dIII-*Sau*3A fragment isolated from an EMBL3 clone; 3, a 3.0-kb *Cla*I fragment obtained from a λZAP clone. The initiation site for transcription is indicated by the arrow above the restriction map. The regions encoding the two linker proteins CpeC and CpeD are indicated by open boxes on the restriction map. The fragments indicated in the bottom line were used as probes of Northern blots as shown in Fig. 4. The indicated restriction sites are as follows: H, *Hin*dIII; P, *Pst*I; S, *Sau*3A; A, *Ava*II; K, *Kpn*I; C, *Cla*I.

TABLE 1. Cloned cyanobacterial phycobilisome linker genes and characteristics of light-regulated expression

Cloned gene	Organism	Protein product and regulation	Reference
cpeCD	*F. diplosiphon*	CpeC and CpeD; expressed in green light with phycoerythrin (*cpeBA*)	This report
cpcH2I2D2	*F. diplosiphon*	CpcH2, CpcI2, and CpcD2 (formerly known as $L_R^{37.5}$, L_R^{39}, and $L_R^{8.9}$); expressed in red light with inducible phycocyanin (*cpcB2A2*)	32
cpcH3I3D3	*F. diplosiphon*	CpcH3, CpcI3, and CpcD3; induced under sulfur stress with phycocyanin (*cpcB3A3*)	35
cpcCD	*Anabaena* strain 7120	CpcC and CpcD; expressed in white light with phycocyanin (*cpcBA*)	5
cpcCD	*Synechococcus* strain 7002	CpcC and CpcD; expressed in white light with phycocyanin (*cpcBA*)	10
apcC	*Synechococcus* strain 6301	ApcC; associated with the core ($L_c^{7.8}$); expressed in white light with allophycocyanin (*apcAB*)	29
apcC	*F. diplosiphon*	ApcC; associated with the core ($L_c^{7.8}$); expressed in white light with allophycocyanin (*apcA1B1*)	28

nine codon. In the ~275 bp 3′ of *cpeD*, there are no open reading frames encoding peptides longer than 27 amino acids. However, there are several small repeated sequences in this 3′ region; for example, TTACGAA is directly repeated four times (Fig. 3) and TAATTCGT is repeated twice. These sequences may form secondary structures, which could be involved in determining the ratio of the different transcripts in this region of the operon (see below).

Transcription of the *cpeCD* operon. Restriction fragments within and flanking the *cpeCD* genes were used to probe Northern blots (RNA blots) of RNA isolated from *F. diplosiphon* grown in red or green light. In green light, the *cpeCD* operon expresses a major 2,200-nt transcript and a less abundant 3,200-nt transcript, while in red light, very low levels of both transcripts can be detected (Fig. 4). The 2,200-nt transcript is large enough to encode the two linker proteins, while the 3,200-nt transcript extends an additional ~1 kb in the 3′ direction (see below). A probe 5′ to the predicted start of transcription (fragment a in Fig. 2) does not hybridize to any transcripts (data not shown), while probes from within the coding regions of *cpeC* and *cpeD* (fragments b and c in Fig. 2) hybridize to both the 2,200- and 3,200-nt transcripts (Fig. 4a and b). A probe from the region downstream of *cpeD* (fragment d in Fig. 2) hybridizes to the 3,200-nt transcript and to several smaller transcripts which may be processing intermediates or degradation products or both (Fig. 4c). In addition, on long exposures, a larger transcript of ~3,800 nt can be detected with both coding region and 3′ probes. A smaller band (~1,500 nt) may be an artifact on the basis of its position immediately below the small rRNA band.

Regulated expression of *cpeBA* and *cpeCD* by green light. Transcription of the *cpeBA* and *cpeCD* operons is coordinately regulated. When cultures of *F. diplosiphon* are grown in green light and then transferred to red light, the levels of transcripts encoding phycoerythrin and the phycoerythrin-associated linkers decline to low levels within 4 h (Fig. 5); however, the phycoerythrin transcripts are maintained at high levels somewhat longer than are the transcripts encoding the linker proteins. Conversely, when red light-grown cultures are transferred to green light, the initially low levels of *cpeBA* and *cpeCD* transcripts increase rapidly, reaching a maximum between 4 and 8 h. Both *cpeCD* transcripts accumulate at similar rates, although the steady state level of the 3,200-nt *cpeCD* transcript is ~20% of the level of the 2,200-nt transcript in green light (as determined by densitometry); the level of the 3,200-nt transcript is too low in red light to quantitate (Fig. 4 and 5b).

By using probes specific for the *cpeCD* operon, primer extension and S1 nuclease protection analyses of RNA from *F. diplosiphon* predicted a single transcription start site 187 bases upstream of the presumed AUG initiation codon of *cpeC* (data not shown); the start site is indicated in Fig. 3. The primer extension analysis established this same transcription start site in both green and red light. Since only one transcription start site was observed, both the 2,200- and 3,200-nt transcripts must have the same 5′ end and differ in their 3′ ends. The locations of the two transcripts relative to the *cpeCD* coding regions are shown in Fig. 2. The long leader sequence of the transcripts for the linker genes contains several small (14 to 21 amino acids) overlapping open reading frames that have potential ribosome binding sites and that start with methionine. At this time, the regulatory significance of this observation is unknown.

Although the DNA sequence of the coding region of the phycoerythrin operon (*cpeBA*) has been previously reported (33), only a small region of DNA sequence 5′ of the coding region has been published (25). The sequence of a 423-bp *Xba*I fragment containing the 5′ region of *cpeBA* including the first seven codons is shown in Fig. 6. The start of transcription of this operon was determined by S1 nuclease protection to be 62 to 64 bases 5′ of the translation initiation codon, as indicated in Fig. 6 (data not shown).

Having defined the transcription start sites for the *cpeBA* and *cpeCD* operons, we examined the upstream regions for DNA sequence homologies that might be involved in their coordinate regulation. A 17-bp element is found 83 bp 5′ of the transcription start site for the *cpeBA* operon (5′-TC CCCAGTCCCCAATCC) and is also found as the reverse complement 195 bp upstream of the transcription start site for the *cpeCD* operon (5′-GGATTGGGGACTGGGGA) (Fig. 3 and 6). This element is located in both promoters within stretches of DNA which contain multiple small repetitive sequences. We are currently investigating the significance of this sequence motif in the light-regulated expression of these operons.

As described above, transcription of the *cpeCD* operon results in the accumulation of two major transcripts with the same 5′ end but with different lengths of 3′ sequences. It is not known whether transcriptional termination or RNA processing is responsible for this differential transcript accumulation. However, examination of the sequence 3′ of the translation stop codon for CpeD revealed a potential RNA secondary structure with a ΔG^0 of −101.4 kcal/mol (1 cal = 4.184 J). The precise endpoint of the 2,200-nt transcript has not yet been mapped, but S1 nuclease protection analysis

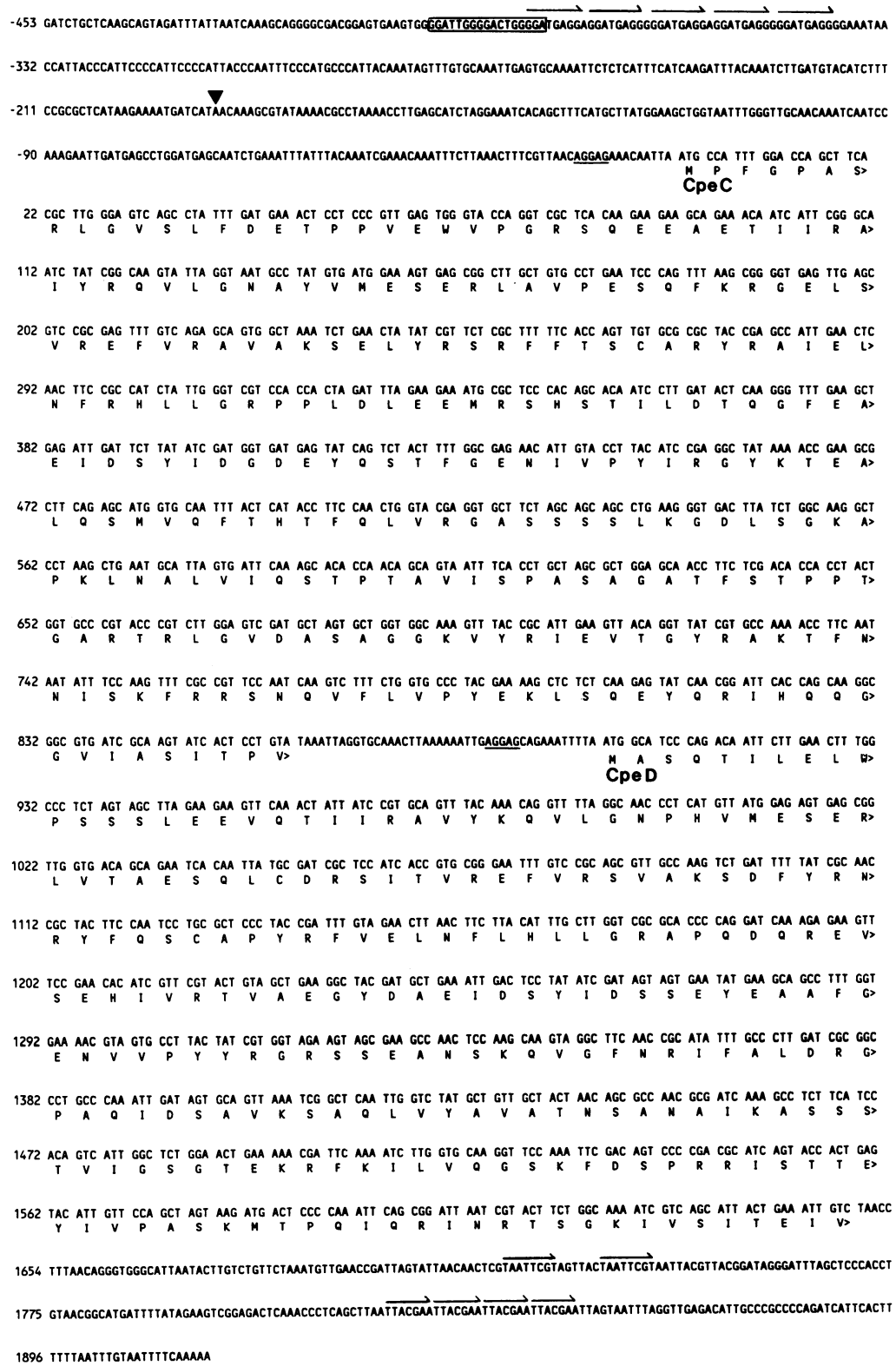

FIG. 3. DNA sequence of the *cpeCD* operon encoding the phycoerythrin-associated linker proteins, beginning with the second *Sau*3A site shown in the restriction map in Fig. 2. The boxed 17-bp sequence is found as the reverse complement in the promoter of the *cpeBA* (phycoerythrin) operon (Fig. 6). The underscored sequences are potential ribosome binding sites. The horizontal arrows above the DNA sequence indicate repetitive sequences. The arrowhead indicates the nucleotide at which transcription starts. The predicted amino acid sequence for the two linker proteins is shown beneath the DNA sequence. The GenBank accession number is M33832.

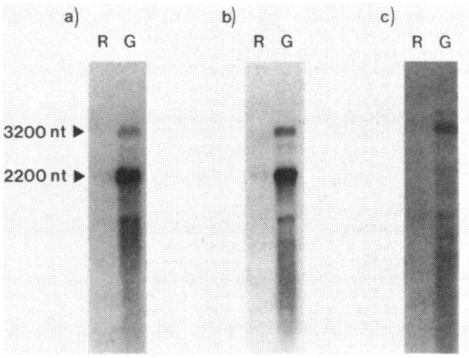

FIG. 4. Northern blots of RNA from *F. diplosiphon* grown in red (lanes R) or green (lanes G) light. The filters shown in panels a through c were probed with fragments b, c, and d, respectively, as shown in Fig. 2. The sizes of the two predominant transcripts are indicated.

indicates that it extends past the *Sau*3A site at the 3′ end of the map (Fig. 2; data not shown).

Comparison of polypeptide sequences of linker proteins. The predicted amino acid sequences of the two linker proteins associated with phycoerythrin, CpeC and CpeD, are closely related to each other. When the BestFit program is used to achieve maximal sequence alignment, an overall similarity of 62% is found with 45% identity (Fig. 7). Most of the sequence conservation occurs in the amino-terminal half of the proteins, while the carboxyl-terminal regions exhibit substantial divergence. These two phycoerythrin-associated linker proteins are also closely related to other characterized linker proteins (Fig. 7; Table 1). The limited regions of sequence conservation in the extreme carboxyl termini of these rod linker proteins can also be aligned with the family of small linker proteins (CpcD), which may be involved in stabilizing the terminal phycocyanin rod hexamers, and with the small core linker proteins (ApcC) (Fig. 7).

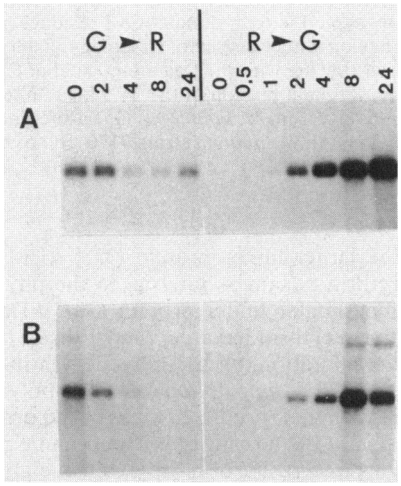

FIG. 5. Steady state transcript levels following changes in the growth conditions from green (G) to red (R) light and vice versa. Cultures of *F. diplosiphon* were started in red or green light and then switched to the opposite light treatment at time zero. Cells were harvested at the indicated times (given in hours), and RNA was isolated. Electrophoresis of the RNA was performed on a denaturing agarose gel, followed by transfer to nitrocellulose. The filter was probed with a 340-bp *Xba*I fragment internal to the coding region of the β subunit of phycoerythrin (A). After this probe had decayed, the same filter was reprobed with a 620-bp *Cla*I-*Sau*3A fragment encoding the carboxyl terminus of CpeD (B). Exposures were adjusted to give approximately equal intensities of the major transcripts for each operon.

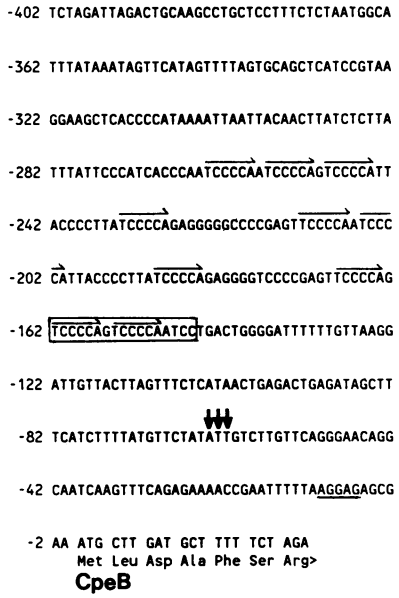

FIG. 6. DNA sequence of the 5′ region of the *cpeBA* phycoerythrin operon, including the first seven codons of the β subunit. The vertical arrows indicate the start of transcription. The boxed sequence is also present as the reverse complement in the 5′ region of the *cpeCD* operon (Fig. 3 and Results). The underscored sequence is a potential ribosome binding site. The horizontal arrows above the DNA sequence indicate repetitive elements.

DISCUSSION

In this report, we have described the isolation and characterization of an operon encoding the phycoerythrin-associated linker genes, *cpeCD*. This is the first description of these genes and the proteins that they encode. The identification is based on three criteria. First, the original λZAP clone (Fig. 2, clone 1) was isolated from an *E. coli* expression library constructed from *F. diplosiphon* genomic DNA by using an antibody preparation specific for the linker proteins expressed in green light (CpeC and CpeD; Fig. 1). Second, transcripts homologous to this clone are present at high levels in *F. diplosiphon* cells grown in green light and at very low levels in cultures grown in red light (Fig. 4b); this type of differential transcript accumulation is consistent with the linker protein levels found under the two light conditions and is similar to that of the phycoerythrin operon, *cpeBA*. Third, the protein sequences derived from these genes are closely related to each other and to linker proteins associated with phycocyanin from *F. diplosiphon* and other cyanobacteria (Fig. 7).

The masses of the phycoerythrin-associated linker proteins predicted from the DNA sequence are 31.8 kDa (286 amino acids) for CpeC and 27.9 kDa (249 amino acids) for CpeD. The apparent mass of CpeC, based on its mobility on an SDS-polyacrylamide gel, is 33.1 kDa, while that of CpeD is 30.9 kDa (Fig. 1a). These sizes are somewhat larger than the amino acid sequences would indicate; however, the mobility of the linker proteins on SDS-polyacrylamide gels is highly dependent on the electrophoretic buffer and salt

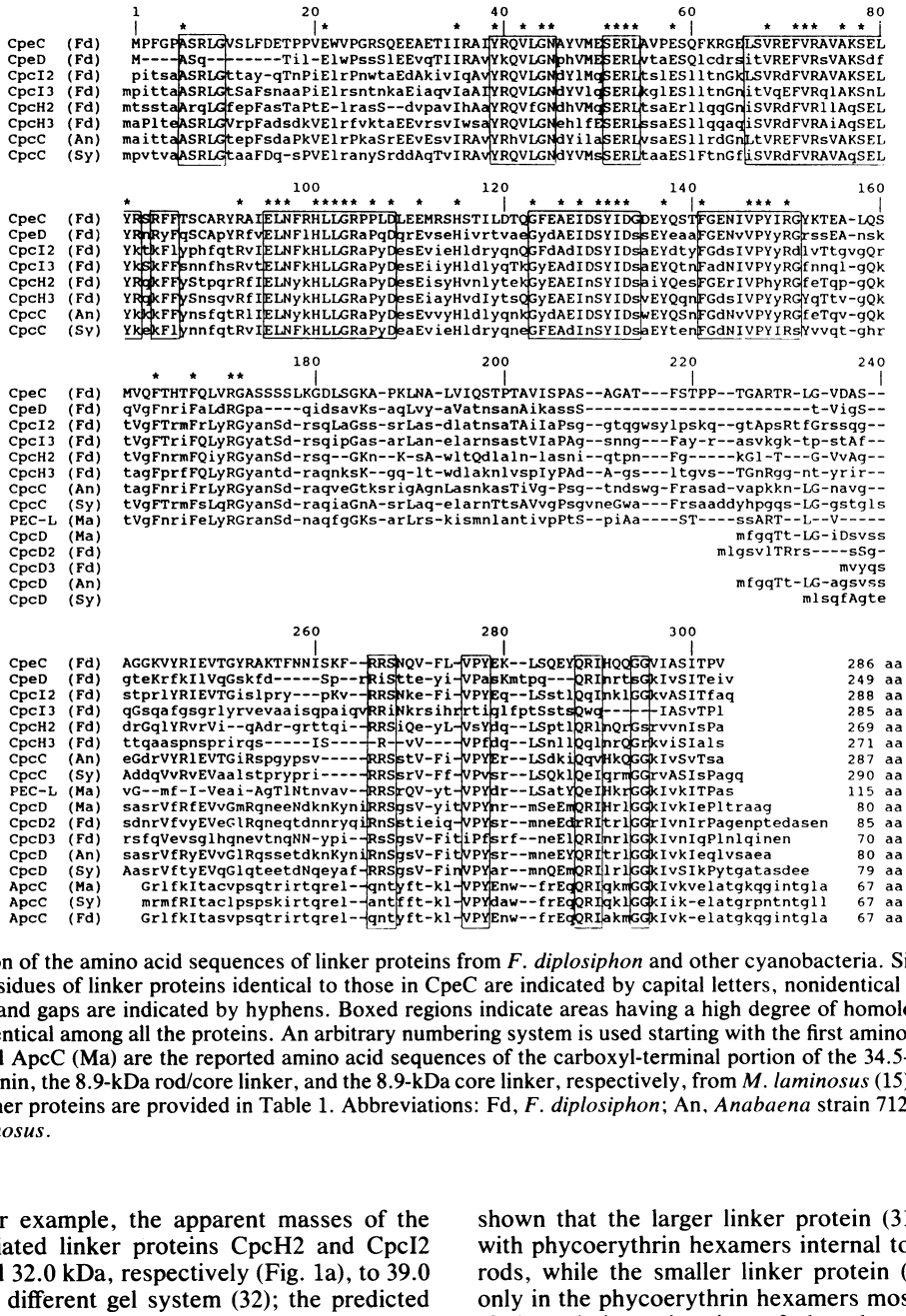

FIG. 7. Comparison of the amino acid sequences of linker proteins from *F. diplosiphon* and other cyanobacteria. Single-letter amino acid symbols are used. Residues of linker proteins identical to those in CpeC are indicated by capital letters, nonidentical residues are indicated by lowercase letters, and gaps are indicated by hyphens. Boxed regions indicate areas having a high degree of homology; asterisks indicate residues which are identical among all the proteins. An arbitrary numbering system is used starting with the first amino acid of CpeC. PEC-L (Ma), CpcD (Ma), and ApcC (Ma) are the reported amino acid sequences of the carboxyl-terminal portion of the 34.5-kDa linker associated with phycoerythrocyanin, the 8.9-kDa rod/core linker, and the 8.9-kDa core linker, respectively, from *M. laminosus* (15). References and brief descriptions of the other proteins are provided in Table 1. Abbreviations: Fd, *F. diplosiphon*; An, *Anabaena* strain 7120; Sy, *Synechococcus* strains; Ma, *M. laminosus*.

concentrations. For example, the apparent masses of the phycocyanin-associated linker proteins CpcH2 and CpcI2 range from 33.3 and 32.0 kDa, respectively (Fig. 1a), to 39.0 and 37.5 kDa on a different gel system (32); the predicted masses of these two proteins based on the DNA sequence are 32.5 and 30.5 kDa (32). This behavior may be due to the basic nature of these proteins, resulting in anomalous SDS binding. In addition, phycocyanin-associated linker proteins from *Anacystis nidulans* have been reported to contain covalently linked, glucose-containing polysaccharides (39, 40). By analogy, the linker proteins from *F. diplosiphon*, which are associated with both inducible phycocyanin and phycoerythrin, may also be glycosylated, consistent with their larger apparent molecular masses on SDS-polyacrylamide gels than were predicted from their amino acid compositions.

The structural roles of the individual linker proteins CpeC and CpeD in the assembly of phycobilisomes in *F. diplosiphon* are still undefined. However, reconstitution studies with isolated phycoerythrin subunits and the two associated linker proteins from *Synechocystis* strain 6701 (19, 20) have shown that the larger linker protein (31.5 kDa) associates with phycoerythrin hexamers internal to the phycobilisome rods, while the smaller linker protein (30.5 kDa) is found only in the phycoerythrin hexamers most distal to the core. If the relative migration of the phycoerythrin-associated linker proteins of *F. diplosiphon* on SDS-polyacrylamide gels correlates with their predicted sizes based on amino acid sequence (that is, if the mobilities of CpeC and CpeD are not reversed on gels as a result of more extensive glycosylation of the smaller protein), then the larger linker protein (CpeC) would correspond to the internal rod linker, while the smaller linker protein (CpeD) would be the linker associated with phycoerythrin hexamers at the ends of the rods in *F. diplosiphon*.

When the predicted amino acid sequences of CpeC and CpeD are compared with each other and with the sequences of previously characterized rod linker proteins, 49 amino acid residues (~17 to 20%) are invariant among all eight rod linker proteins, with 115 invariant amino acids between CpeC and CpeD (45%). While there is very little similarity at the amino-terminal end of the proteins, except for a cluster

of five amino acids (residues 6 through 10 in CpeC), a large block of conserved domains starts near residue 35 for most of the linker proteins and continues approximately through residue 175 (Fig. 7). Analysis of the amino acid distribution within the conserved regions does not provide any obvious insights into possible relationships between structure and function. For CpeC and CpeD, these regions exhibit a slightly lower percentage of polar amino acids and a slightly higher ratio of acidic amino acids than is represented in each protein as a whole, while the content of basic and nonpolar amino acids in these regions closely reflects their overall composition in each protein. The carboxyl-terminal regions of the rod linker proteins are much more divergent, with many more deletions, insertions, and substitutions than are found in the amino-terminal domains. Interestingly, it is these less conserved carboxyl-terminal regions that have limited homology to the family of small linker proteins such as CpcD2 and CpcD3 (Fig. 7), which have been proposed to function in the formation and assembly of the terminal phycocyanin rod hexamers (10). This similarity had been previously described between the $L^{8.9}$ linker protein from *M. laminosus* and the phycoerythrocyanin-associated linker $L^{34.5}$ (CpcD and PEC-L in Fig. 7, respectively); additionally, another 8.9-kDa protein (ApcC in Fig. 7) was described which is believed to be associated with the phycobilisome core (16). The genes encoding ApcC have recently been cloned from *Synechococcus* strain 6301 and *F. diplosiphon* (28, 29). The degree of sequence conservation among linker proteins which have different modes of regulation, which associate with different phycobiliproteins, and which derive from different organisms, implicates the conserved regions in important functional or structural interactions or both.

Schirmer et al. (42, 43) have determined the crystal structure of phycocyanin hexamers from *Agmenellum quadruplicatum* and phycocyanin trimers from *M. laminosus* in the absence of linker proteins. The phycocyanin complexes are arranged in a toroidal structure with an internal cavity with a maximum diameter of ~4 nm (43). The crystal structure of the phycocyanin hexamers predicts that some negatively charged residues protrude into the internal channel; these residues may be involved in specific interactions with the basic linker proteins. Evidence regarding the site of binding of the linker proteins to the hexamers was provided by studies of the susceptibility of *Anabaena variabilis* phycocyanin-linker protein aggregates to proteolysis (55). Those authors showed that the 32.5-kDa linker protein was digested to a 28-kDa polypeptide when present in phycocyanin trimers or hexamers but was completely protected from degradation in higher order aggregates. This implies that a 28-kDa domain of the linker protein may be bound within the internal cavity of the phycocyanin hexamers, while the 4.5-kDa domain is involved in the stacking interactions between hexamers. Another interesting point derived from the phycocyanin structure is that there is remarkable similarity in three-dimensional structure between the α and β subunits, even though only 46 residues are conserved (28 and 27%, respectively) (42). This characteristic of conservation of structure in spite of sequence divergence may also be a feature of the linker proteins associated with both phycocyanin and phycoerythrin because of the constraints imposed by the functional assembly of the phycobilisome.

We have shown that the *cpeCD* operon is expressed on two major transcripts which have the same 5′ ends but which differ in the 3′ ends. The smaller, 2,200-nt transcript is of sufficient length to encode both CpeC and CpeD, while the larger, 3,200-nt transcript extends another ~1 kb in the 3′ direction. These transcripts are abundant in *F. diplosiphon* cells grown in green light but are barely detectable in cultures grown in red light, which correlates with the observed levels of the phycoerythrin-associated linker proteins in these cultures. The precise 3′ end of the 2,200-nt transcript has not yet been determined; however, it probably lies 3′ of the very stable hairpin which is predicted to form just downstream of the coding region of CpeD. This potential structure is followed by a string of uridine residues, constituting a classic procaryotic simple terminator as defined in *E. coli* (37). Similarly, a smaller hairpin is predicted to occur in the *cpc2* operon of *F. diplosiphon*, between the genes encoding the β and α subunits of the inducible phycocyanin (*cpcB2A2*) and the genes encoding the associated linker proteins (*cpcH2I2D2*) (14). If the predicted secondary structure in the *cpeCD* operon acts as a simple terminator, the ratio of the 2,200- and 3,200-nt transcripts could be determined primarily by the percentage of transcriptional readthrough past the hairpin. In addition, this structure could act as a barrier to degradation from the 3′ end of the operon-length transcript. Such segmental stability of transcripts has been observed for the polycistronic *puf* operon in *Rhodobacter capsulatus* (4, 11). At this point, we have no evidence regarding these alternative mechanisms (which are not mutually exclusive) of regulating the levels of the two major *cpeCD* transcripts. We are currently continuing the sequence analysis of the region 3′ of *cpeD* to define any additional genes which might be encoded in this operon. No other proteins have yet been identified in *F. diplosiphon* which are known to be expressed preferentially in green light, but possibilities include other phycobilisome components or factors required for assembly, chromophorylation enzymes involved in phycobiliprotein modification, determinants of morphological changes mediated by green light, or regulatory factors involved in the signal transduction pathway.

Unlike the red light-inducible phycocyanin subunits and associated linker proteins (*cpc2*) (14, 32), the linker proteins associated with phycoerythrin are not closely linked in the *F. diplosiphon* genome to the phycoerythrin operon (*cpeBA*). This makes their coordinate light-regulated expression more complex. While the ratio of the phycocyanin-associated linker proteins to phycocyanin subunits is apparently maintained by lower levels of full-length transcripts relative to transcripts encoding only the phycocyanin subunits (as the result of transcriptional termination, readthrough, and/or processing, as discussed above), regulation of the ratio of phycoerythrin-associated linker proteins to phycoerythrin subunits must be achieved by independent transcriptional events. We have determined the transcriptional start sites for both green light-activated operons; transcription initiates 62 to 64 bp upstream of the first AUG in the phycoerythrin operon *cpeBA*, while the start of the *cpeCD* transcripts encoding the linker proteins is 187 bp 5′ of the putative translation start site for CpeC. The short leader sequence of the *cpeBA* transcript contains no open reading frames prior to the start of the CpeB coding region; in contrast, the long leader sequence of the *cpeCD* transcripts contains several small (14 to 21 amino acids) overlapping open reading frames with potential ribosome binding sites upstream of the AUG initiating translation of CpeC. We have no evidence at this time suggesting whether or not the short peptides are synthesized and involved in translational control of this operon.

The steady state level of the *cpeBA* transcript appears to be approximately 5- to 10-fold higher than that of the

2,200-nt *cpeCD* transcript, on the basis of Northern hybridizations with probes of similar specific activity; however, since these transcripts do not share any common sequences (unlike the transcripts of the inducible phycocyanin operon and its linker proteins), it is difficult to make very accurate estimates of relative transcript abundance. The kinetics of transcript accumulation in response to changes in light quality are very similar between the two operons (Fig. 5a and b), implying that similar features in the 5′ regulatory regions of these genes allow for their coordinate regulation. However, there is no obvious homology in the regions immediately upstream of the predicted transcription start sites for these two operons. In contrast, there is a high degree of sequence conservation in the regions immediately 5′ of the transcription start sites for the *cpeBA* operon in *F. diplosiphon* and in *Synechocystis* strain 6701 (1). Thus, in comparing these green light-regulated operons, it is still unclear what constitutes a basic promoter recognition sequence.

The sequences upstream of the transcription start sites for the *cpeBA* and *cpeCD* operons in *F. diplosiphon* contain a high number of repetitive elements. For example, in the 5′ region of *cpeCD*, the sequence GGATGAGG is found five times in a much larger region of high strand asymmetry containing predominantly guanosine residues (49 of 80 = 61% G versus 3 of 80 = 4% C, from −339 to −418 bp relative to the translation start site) (Fig. 3). This is followed by a region containing a high ratio of thymidine and cytosine residues, including elements such as the palindrome CCAT TACC, which is repeated directly and in slightly variant forms several times. Numerous other elements are found as direct or inverted repeats and as regions of dyad symmetry. Similarly, numerous repetitive elements can be found in the 5′ region of *cpeBA*, such as the sequence TCCCCA repeated 10 times (Fig. 6). Both promoter regions possess significant dyad symmetry over several hundred base pairs, and the possibility exists that cruciform DNA structures could play a role in the regulation of these operons (27). Embedded in these repetitive structures (which in general are not the same between the two operons) is one 17-bp element which is common to both operons, TCCCCAGTCCCCAATCC. This motif is found 83 bp 5′ of the transcription start site for the *cpeBA* operon and is found as the reverse complement 195 bp upstream of the transcription start site for *cpeCD*. This element is contained within the *cpeBA* promoter region in a 190-bp fragment which binds to a factor in extracts from *F. diplosiphon* cultures grown in green light, but not in extracts from cultures grown in red light, as determined by DNA mobility shift gel assays (N. A. Federspiel, unpublished results). If this element is important in green light activation of transcription, the lower steady state levels of transcripts from *cpeCD* may be due to the distance of this element from the transcription start site, its orientation, or the sequences flanking the element. However, this element is not found in the ~450 bp which have been reported 5′ of the *cpeBA* operon in *Synechocystis* strain 6701 (1). Further investigations of the specific DNA sequences and protein factors involved in the transcriptional activation of these operons by green light will lead to a fuller understanding of the signal transduction pathway between perception of light quality and changes in gene expression in cyanobacteria.

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