JOURNAL OF BACTERIOLOGY, Mar. 1990, p. 1289–1296
0021-9193/90/031289-08$02.00/0

Vol. 172, No. 3

Genes for Phycocyanin Subunits in *Synechocystis* sp. Strain PCC 6701 and Assembly Mutant UV16†

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Received 20 July 1989/Accepted 3 December 1989

The cyanobacterial phycobilisome is a large protein complex located on the photosynthetic membrane. It harvests light energy and transfers it to chlorophyll for use in photosynthesis. Phycobilisome assembly mutants in the unicellular cyanobacterium *Synechocystis* sp. strain 6701 have been characterized. One such mutant, UV16, contains a defect in the assembly of the biliprotein phycocyanin. We report the cloning and sequencing of the phycocyanin genes from wild-type *Synechocystis* strain 6701 and demonstrate an alteration in the gene for the phycocyanin α subunit in UV16. Possible consequences of the lesion on phycobilisome assembly were assessed from its position in the phycocyanin tertiary and quaternary structures. The UV16 phenotype is complex and includes a reduced level of phycocyanin relative to that in the wild type. To determine whether the lower phycocyanin content results from lower transcript levels, a fragment of *cpcBA* was used as a probe for quantitating phycocyanin mRNA. Both the wild type and UV16 contained two phycocyanin transcripts of approximately 1.4 and 1.5 kilobases that were equal in abundance and that did not vary with light quality during cell growth. Equal levels of these transcripts in the wild type and UV16 suggest that the lower phycocyanin content in the mutant may be due to posttranscriptional events. The 5′ ends of the two phycocyanin mRNAs were mapped at 100 and 223 base pairs upstream of the *cpcB* initiation codon. Homologous regions upstream of the putative transcription initiation sites may be important for maintaining high levels of transcription from the *Synechocystis* strain 6701 phycocyanin gene set.

Cyanobacteria are photosynthetic procaryotes that generate energy from light by oxygenic photosynthesis in a manner similar to that in plants and eucaryotic algae. The predominant cyanobacterial light-harvesting complexes, called phycobilisomes, are multiprotein structures located on the surface of the photosynthetic membranes (19, 20). The phycobilisome is a very effective antenna complex, harvesting light in the 500- to 650-nm range and transferring this energy to chlorophyll at better than 95% efficiency (34). The transfer of light energy through the phycobilisome is mediated by a network of bilin chromophores that are covalently attached to the major phycobilisome structural components, the biliproteins. In the unicellular cyanobacterium *Synechocystis* sp. strain 6701, there are three classes of biliproteins, differentiated by their spectral properties, that are organized into two structural domains in the phycobilisome, the core and the rods. The allophycocyanin biliproteins are in the core, which contains the terminal acceptors of the phycobilisome energy transfer pathway and the membrane attachment sites (3, 26–28). The six rods attached to the core contain the phycocyanin (PC) and phycoerythrin (PE) biliproteins, with the PC biliprotein being proximal to the core. Phycobilisomes also contain nonchromophoric linker proteins that interact with the biliproteins to direct assembly and fine tune the energy transfer process (29).

Phycobilisome biosynthesis includes many events that can be examined by using our extensive knowledge of the structure and biochemistry of the complex combined with genetic and molecular techniques. Posttranslational modifications are numerous in the phycobilisome biosynthetic pathway. Sites on each class of biliprotein must be recognized for the covalent attachment of an appropriate bilin chromophore (19). Several biliprotein subunits contain a γ-*N*-methyl asparagine modification at the same conserved residue in many organisms (23). The linker proteins in the rods and the large anchor protein in the core are modified by glycosylation, a relatively rare phenomenon in procaryotes (35). These posttranslational modifications are part of an in vivo assembly pathway that depends on the repertoire of phycobilisome components that are available, and thus can change as the cyanobacterium modulates biliprotein and linker gene expression in response to light wavelength (known as chromatic adaptation) (10, 12, 13, 21, 44, 45) or nutrient availability (1, 43).

One approach for dissecting the events that occur during phycobilisome biosynthesis involves the characterization of mutants that are blocked at specific stages in the assembly pathway. UV16 is a mutant strain of the unicellular cyanobacterium *Synechocystis* strain 6701 that is defective in the assembly of the phycobilisome rod substructures (5, 6). The basic component of the rod is a biliprotein hexamer, $(\alpha\beta)_6$, that is complexed with a single copy of a specific linker protein (29). In the mutant UV16, assembly of PC does not proceed beyond the formation of a trimer, $(\alpha\beta)_3$, and reconstitution experiments suggested that the PC α subunit was defective (6). To help define the specific lesion that is responsible for defective PC assembly in UV16, we characterized the PC genes from wild type (WT) and UV16 strains of *Synechocystis* strain 6701. The data presented here confirm the presence of a mutation in the PC α subunit of UV16. Possible consequences of this mutation for PC structure and assembly are discussed in the context of the tertiary and quaternary structures of PC, as determined in recent crystallographic studies (38–40).

A block in the formation of PC hexamers could have significant effects on the stoichiometries of the different phycobilisome components, the activities of genes encoding

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† This is Carnegie Institution of Washington publication no. 1044.

these components, and the phycobilisome assembly pathway. The UV16 phenotype is complex and includes reduced levels of PC and PE relative to levels in the WT, accretion of PE hexamer-linker complexes into large, insoluble aggregates, and a significantly altered ratio of PE-associated linker polypeptides (6). The decreased levels of biliproteins in UV16 could result from altered transcriptional activity of biliprotein genes or posttranscriptional processes. Characterization of biliprotein transcript levels in the WT strain and in UV16 will help to delineate both the direct and the indirect consequences of a specific assembly block for phycobilisome biosynthesis.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The WT *Synechocystis* strain 6701 (PCC 6701, ATCC 27170) has been described by Rippka et al. (36). The UV16 mutant was obtained by UV mutagenesis of WT, and its phenotype has been previously described (5, 6). The UV16-40 mutant resulted from mutagenesis of UV16 and contains a mutation that affects PE assembly (6) as well as the PC assembly defect. Cyanobacteria were grown in BG-11 buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; final concentration, 0.02 M; pH 8.0) at 30°C and bubbled with 3% CO_2 in air in red or green illumination (10). The *Escherichia coli* strains used in this study were JM101 (46), for transformation with M13 vectors and the isolation of sequencing templates; DH5α, for propagation of plasmids (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); and NM538 and NM539, for constructing and screening a library of *Synechocystis* strain 6701 genomic DNA in the λEMBL3 cloning vector (Promega, Madison, Wis.).

Library construction, cloning, and sequencing. Chromosomal DNA was isolated from *Synechocystis* strain 6701 WT, UV16, and UV16-40 by published methods (2). By using a 3.8-kilobase-pair (kbp) *Hin*dIII fragment containing a PC gene set from *Fremyella diplosiphon* (14) as a hybridization probe (24), the *Synechocystis* strain 6701 PC gene set was shown to be on a single 17-kbp *Bgl*II fragment by Southern hybridizations. Complete *Bgl*II digests of chromosomal DNA from WT and UV16-40 were ligated to λEMBL3 arms (Promega) that were generated by digestion with *Bam*HI. UV16-40 DNA was used for the construction of the initial genomic library, since this mutant has both PE and PC assembly mutations (6), affording us the opportunity to define both lesions using clones from a single library. Ligation products were packaged in Gigapak Plus extracts (Stratagene, La Jolla, Calif.), and plaques were screened at low to medium stringency (54°C, as described previously [24]) by using a 800-bp *Eco*RI-*Pst*I ^{32}P-labeled probe that contained most of the *cpcB* gene from *Cyanophora paradoxa* (24). Phage DNA was isolated from positive plaques, mapped by Southern hybridizations by using *cpcB* and *cpcA* genes from *F. diplosiphon* as specific probes, and subcloned into the vectors mp18 and mp19 (31). Single-stranded sequencing (30) was done by the dideoxy method (37) by using the Klenow fragment of DNA polymerase I (Pharmacia, Inc., Piscataway, N.J.) or Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). A fragment containing most of the PC gene set from UV16 was cloned by amplification of 3 μg of chromosomal DNA by using the polymerase chain reaction as described by the manufacturer (Cetus, Emeryville, Calif.). Synthetic oligonucleotides (synthesized on an oligonucleotide synthesizer [Biosearch 8600]) were used to prime the polymerase chain reaction 90-bp 5′ of the *cpcB* translation initiation codon and 100 bp 3′ of the *cpcA* translation initiation codon (designated oligonucleotides 1 and 2, respectively; see Fig. 2). Cloning of the amplified product was achieved by restriction with *Nsi*I and ligation into mp19 cut with *Pst*I-*Sma*I.

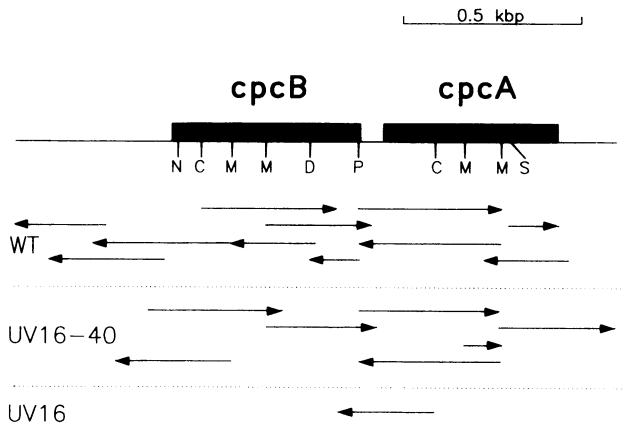

FIG. 1. Restriction map and sequencing strategy for *cpcB* and *cpcA* from *Synechocystis* strain 6701 WT, UV16-40, and UV16 strains. The restriction sites indicated on the map are defined as follows: N, *Nsi*I; C, *Cla*I; M, *Msp*I; D, *Dra*I; P, *Pst*I; S, *Sca*I. Arrows indicate the direction and the region of the DNA that was sequenced.

Transcription mapping and mRNA analyses. RNA was isolated from cultures of red light-grown *Synechocystis* strain 6701 (4), and the 5′ ends of the PC transcripts were determined by primer extension and S1 nuclease protection experiments. Primer extension reactions (16) were modified by increasing the incubation temperature to 42°C and using an end-labeled oligonucleotide (see Fig. 2) to prime the reaction. The S1 nuclease protection experiments were performed as described previously (7) with some modifications. The PC gene set was cloned into the pBluescriptSK$^-$ phagemid vector (Stratagene), making the plasmid pPC02. Infection of DH5α (pPC02) cells with the helper phage M13K07 (46) yielded a single-stranded template for generating the S1 nuclease probe. An end-labeled synthetic oligonucleotide that hybridized immediately downstream of the *cpcB* translation start site of pPC02 (see Fig. 2) was used to prime chain elongation by using the Klenow fragment of DNA polymerase I, and the product was cut with *Dra*II, which cleaved only in the pBluescriptSK$^-$ polylinker. The resultant 900-base, end-labeled probe was isolated on a denaturing 6% polyacrylamide–urea gel, eluted, precipitated, and suspended in water. The hybridizations, which included 10 μg of RNA and 100,000 cpm (Cerenkov) of probe in a 20-μl volume, were incubated overnight at 30°C. S1 nuclease (Pharmacia) was added to 700 U/ml. After 30 min at 37°C, the protected fragments were precipitated with 1 μg of glycogen (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as carrier, and one-half of the material was resolved on a sequencing gel (30) and sized by comparison with a sequence ladder that was generated with the same primer-template combination used to make the labeled S1 nuclease probe. Northern analyses (4) were performed by using a PC-specific 900-bp *Nsi*I-*Sca*I fragment (see Fig. 1) as a hybridization probe.

RESULTS AND DISCUSSION

PC genes in *Synechocystis* strain 6701. There are now at least five different cyanobacteria in which the sequences for

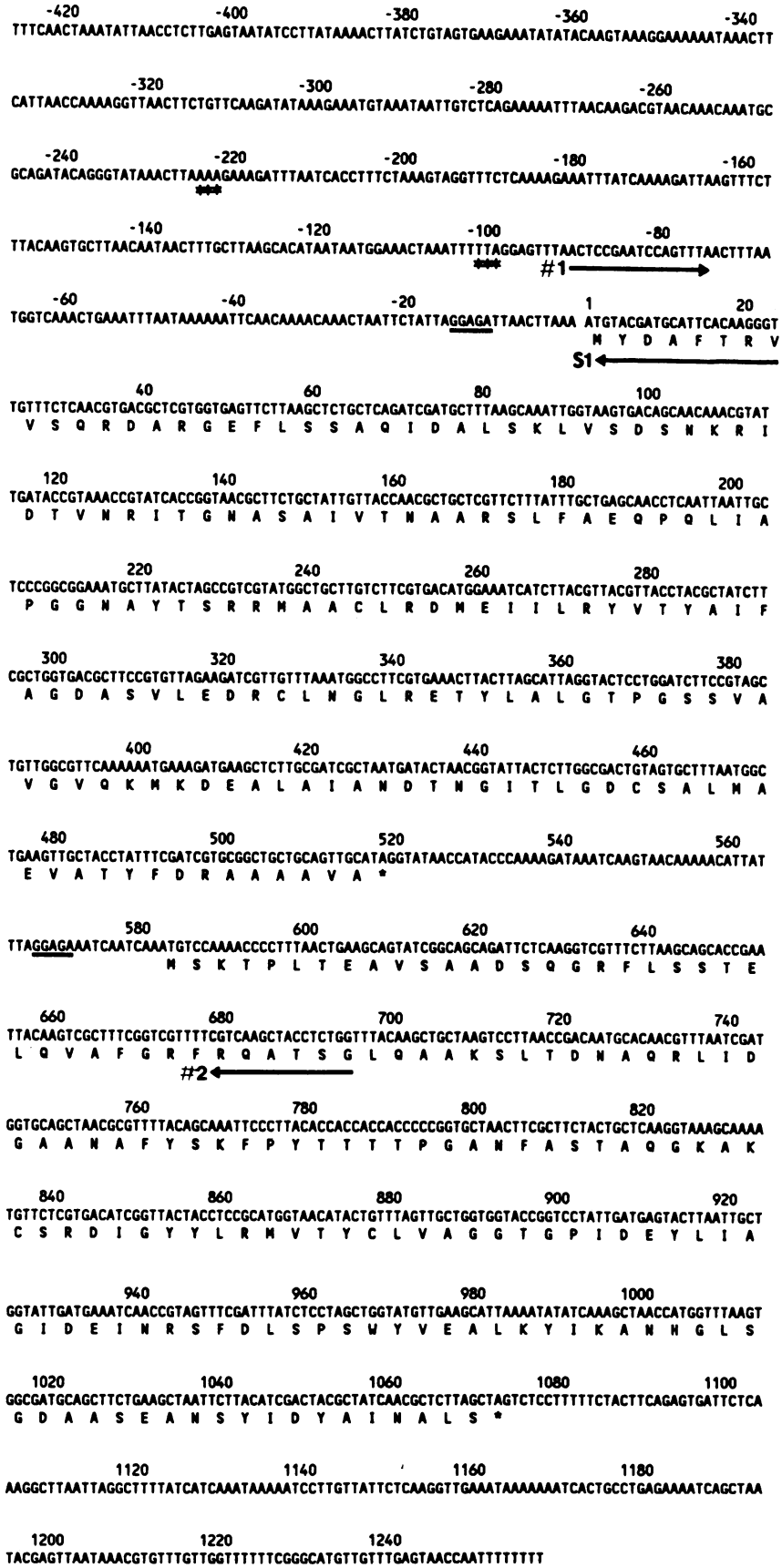

FIG. 2. Nucleotide sequence of *cpcBA* and the deduced amino acid sequence of PC β and α subunits from *Synechocystis* strain 6701 WT. Probable ribosome-binding sites (37) are underlined. Synthetic oligonucleotides are shown beneath the region of DNA to which they correspond. The primers used to amplify part of the UV16 gene set by the polymerase chain reaction are indicated as 1 and 2. The oligonucleotide used in primer extension reactions and to generate probe for S1 nuclease reactions is indicated by S1. The two potential transcription start sites for *cpcBA* are marked by asterisks.

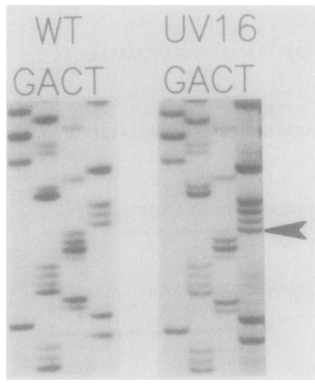

FIG. 3. Site of UV16 mutation in *cpcA* gene. Regions of sequence ladders for 6701 WT and UV16 strains are shown. The C to T transition in UV16 is indicated by the arrowhead.

PC genes have been determined, comprising at least eight β-α gene sets. Some of these cyanobacteria contain single PC gene sets (*Agmenellum quadruplicatum* [17, 33], *Anabaena* sp. strain 7120 [8]), while others have multiple gene sets (*F. diplosiphon* [14, 15], *Synechococcus* sp. strain 6301 [22]). Based on Southern hybridization studies, we found evidence for a single β-α PC operon in *Synechocystis* strain 6701 (data not shown). Genes for PC-associated linker proteins have been characterized in all four cyanobacteria listed above, and in each case they are cotranscribed with the genes that encode the PC subunits (8, 11, 22, 25). In *Anabaena variabilis* and *F. diplosiphon* a small, abundant transcript encodes the PC β and α subunits while a larger, less abundant transcript encodes the PC subunits plus linker polypeptides (8, 13, 25). There is no indication in *Synechocystis* strain 6701 of a larger PC transcript that could also encode linker polypeptides (see Fig. 6), suggesting that the *Synechocystis* strain 6701 PC-associated linker genes are transcribed independently of the PC genes. Southern hybridization studies of EMBL3 clones containing the *Synechocystis* strain 6701 *cpcBA* operon showed no signal when they were probed with heterologous PC-associated linker genes (the 1.8-kbp *Hin*dIII fragment from *F. diplosiphon* [25]), demonstrating a minimum 2.8 kbp of DNA between *cpcBA* and a linker gene. Since it is likely that expression of both PC and PC linkers is coordinately regulated in *Synechocystis* strain 6701 as it is in other cyanobacteria, mechanisms other than cotranscription probably ensure the proper stoichiometry of these components.

A restriction map of *cpcBA* from *Synechocystis* strain 6701 WT, UV16-40, and UV16 and the strategy used to sequence these genes are shown in Fig. 1. The sequence covers 1.7 kbp of DNA that includes 427 bp upstream of *cpcB*, 519 bp of *cpcB*, a 62-bp intergenic region, 492 bp of *cpcA*, and 184 bp downstream of *cpcA*. At least 90% of the *cpcBA*-coding regions were sequenced on both strands of the WT genes. The remaining 10% of the WT-coding region (the N terminus of *cpcB*) was sequenced on one strand but was confirmed by sequencing both strands of the UV16-40 PC genes. The nucleotide sequence of *cpcBA* and the deduced amino acid sequences of the PC β and α subunits are shown in Fig. 2. The UV16-40 sequence was identical to the WT sequence except for one site near the 5′ end of *cpcA* (discussed below). Since the PC assembly defect in UV16-40 was derived from its parent, UV16, it was necessary to confirm the presence of the *cpcA* lesion in UV16. This was accomplished by amplification of UV16 *cpcBA* in a polymerase chain reaction directed by primers 1 and 2 (Fig. 2). The nucleotide sequence of the amplified fragment was identical to the corresponding region from the UV16-40 sequence. The genetic lesion in UV16 and UV16-40 *cpcA* is referred to as the UV16 mutation.

Consequences of a mutation in the *cpcA* gene of UV16. A comparison of the nucleotide sequences located at the beginning of the coding region of *cpcA* in *Synechocystis* strain 6701 WT and UV16 shows a C-to-T transition in the mutant (Fig. 3). The deduced amino acid sequence for the UV16 PC α subunit differed from that of WT at position 5, where a leucine replaced a proline residue (Fig. 4). The boxed amino acids in Fig. 4 are residues that are identical among PC α subunits in several organisms (*Agmenellum quadruplicatum*

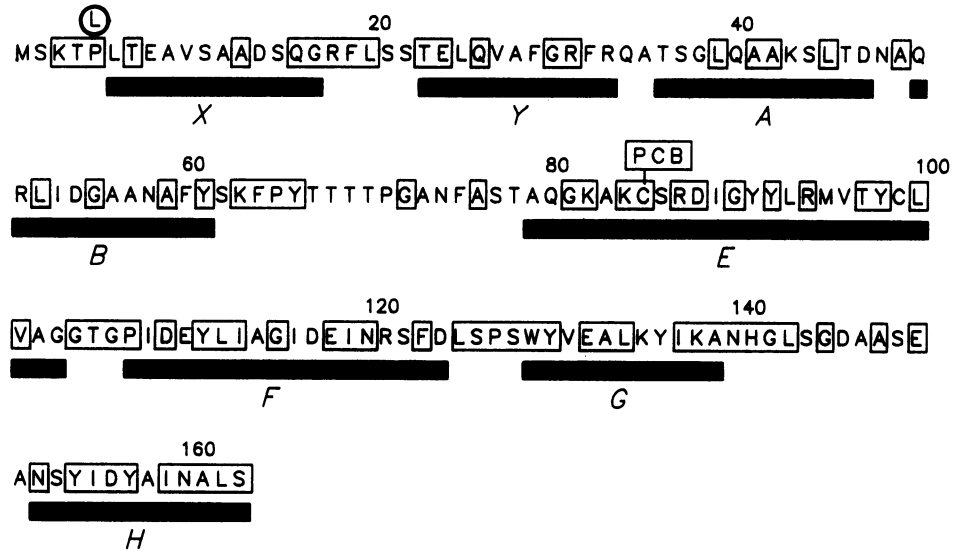

FIG. 4. Deduced amino acid sequence of the 6701 PC α subunit. The UV16 mutation is depicted by the circled L above Pro-5. Residues conserved between six different PC α subunits are boxed. The PC α-subunit secondary structure of eight α helices is indicated by the solid boxes marked X, Y, A, B, and E through H. The single chromophore-binding site in PC α is shown as PCB.

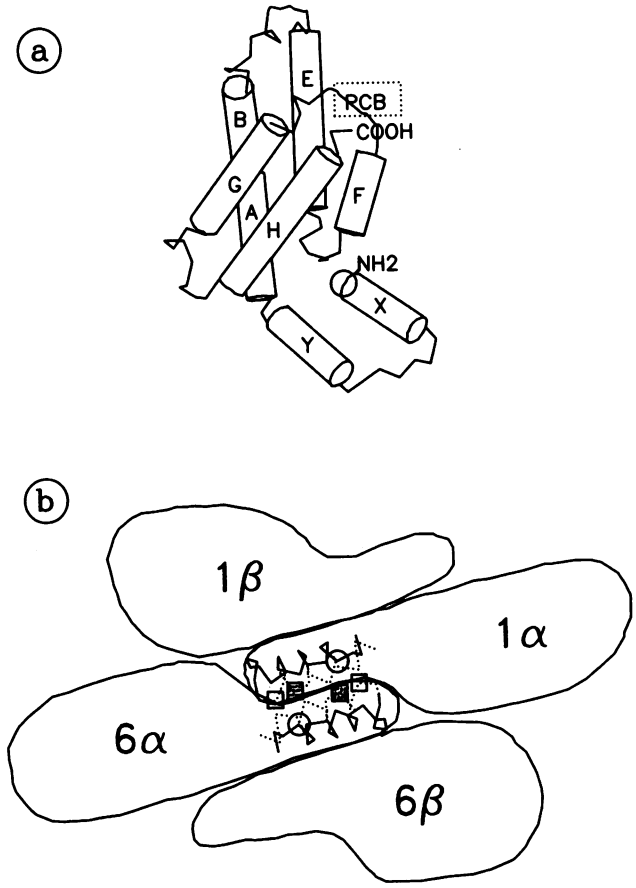

FIG. 5. Mutation in the UV16 PC α subunit and hexamer structure. (a) Tertiary structure of the PC α subunit. The site of UV16 mutation (Pro to Leu) is marked by the circle. The eight α helices of the secondary structure are labeled X, Y, A, B, and E through H. (b) Model of subunit interactions in the PC hexamer showing contacts between monomers 1 and 6. The Pro-Leu sites from panel a are indicated by circles that are centered on the α carbon of the amino acids. The α carbons for neighboring Thr and Gln residues are indicated by open and stippled squares, respectively (see text for explanation). The Y helices of the α-subunit structure are dotted lines and are behind the solid-line X helices. This figure was generated from information presented previously (38–40).

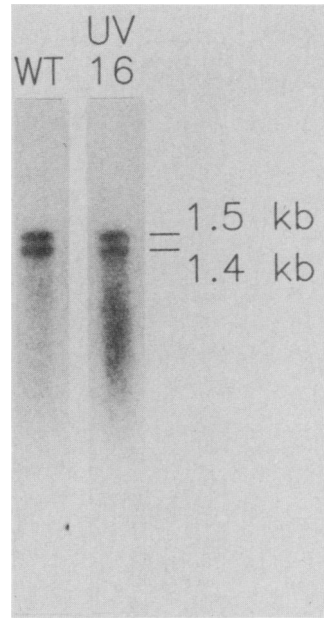

FIG. 6. Northern blot analysis of WT and UV16 PC mRNAs.

[33], *Anabaena* strain 7120 [8], *Mastigocladus laminosus* [18]; *Cyanidium caldarium* [32], and two of the genes from *F. diplosiphon* [14]) and show that the Pro-5 residue is part of a conserved triad of amino acids (Lys-Thr-Pro) near the N terminus. Since PC subunits from two taxonomically unrelated cyanobacteria are nearly identical in their tertiary structures (38), conserved residues are likely to have similar structural interactions in all PC biliproteins.

The three-dimensional structure of PC from *M. laminosus* and *Agmenellum quadruplicatum* (38–40) may help to explain the assembly-defective phenotype of the *Synechocystis* strain UV16 mutant. The PC α-subunit structure consists of eight α helices (X, Y, A, B, and E through H) that are shown aligned with the amino acid sequence in Fig. 4. The mutation in the PC α subunit of UV16 (Pro-5 to Leu) is at the start of the X helix. A three-dimensional representation of the PC α-subunit structure (Fig. 5a) shows that the X and Y helices form a platform, while the remaining helices constitute a more globular domain. The UV16 mutation site (indicated by a circle in Fig. 5a) is located in a region of contact between trimers when the hexamer is formed (Fig. 5b). We used the set of coordinates for the structure of the *Agmenellum quadruplicatum* hexamer (obtained from R. Huber [38]) and Promodeler I software (New England BioGraphics) to assess potential consequences of the Pro-Leu substitution on PC assembly. The close packing of neighboring α subunits in the hexamer can result in steric problems when the Pro-4 residue is replaced with the Leu side chain from the library of amino acid structures in the Promodeler system (note that the *Agmenellum quadruplicatum* residue numbers are offset minus one from *Synechocystis* strain 6701 residue numbers because of the different N termini of MKTP and MSKTP, respectively). Center-to-center measurements show that one δ carbon of Leu-4 is less than 0.22 nm from the γ carbon of Thr-22, while the Leu-4 δ carbon is less than 0.17 nm from the Gln-25 ε nitrogen. These distances are less than the minimum 0.25 nm at which steric hindrance is presumed to occur (38). Figure 5b shows the relative positions of the α carbons for residues Pro-4–Leu-4, Thr-22, and Gln-25 in the context of the *Agmenellum quadruplicatum* PC hexamer structure. Since both Thr-22 and Gln-25 are conserved residues in all known PC α-subunit primary structures, there is very good correlation between the potential steric problems of the UV16 PC mutation and the observed PC assembly defect in UV16.

Since UV16 was the product of a random mutagenesis, we cannot eliminate the possibility that other mutations may be responsible for the PC assembly defect. The PC-related linker proteins are potential sites for alternative mutations that block hexamer formation, but data from in vitro reconstitutions and mutant analyses argue against this possibility (see Discussion in reference 5). The mutation in the UV16 PC α subunit can result in incorrect assembly during in vitro reconstitution since the altered PC α subunit was less effective in assembling with the β subunits (6). These considerations, combined with the potential consequences of the lesion as inferred from the crystal structure, make it likely that the Pro-5–Leu substitution in the UV16 PC α subunit blocks PC assembly in vivo.

Analysis of PC mRNA in *Synechocystis* strain 6701 WT and

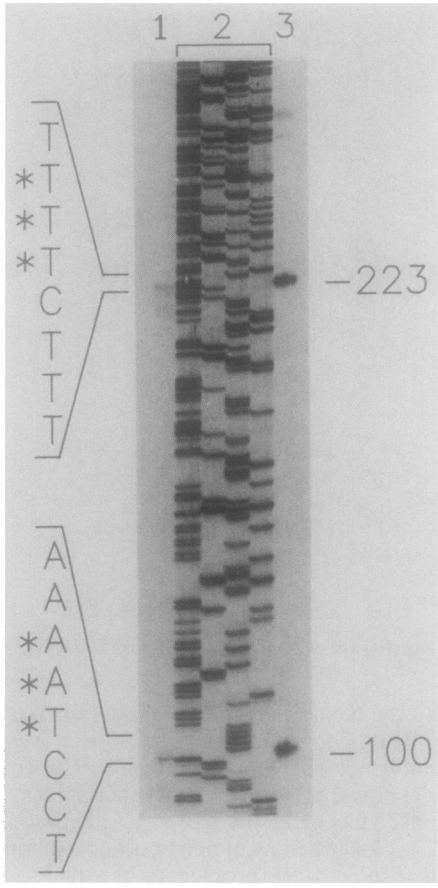

FIG. 7. Determination of PC mRNA 5′ ends by primer extension and S1 nuclease protection analyses. Lane 1, S1 nuclease reaction; lanes 2, the T, C, A and G sequencing reactions (left to right, respectively) that were used to size the fragments; lane 3, primer extension reaction. The nucleotide sequences at −100 and −223 bp from the *cpcBA* translation start are shown on the left, with the putative transcription initiation sites marked by asterisks.

UV16. The UV16 phenotype is complex, involving most components of the phycobilisome rod substructure (6). One facet of the phenotype is that the PC content in UV16 is approximately 30% of that in the WT. The decreased PC level could be due to the degradation of unassembled subunits or reduced rates of transcription or translation. We examined the possibility of transcriptional differences in WT and UV16 by measuring the accumulation of PC mRNA (Fig. 6). Two transcripts, 1.4 and 1.5 kb in size and approximately equal in abundance, hybridized to the *cpcBA*-specific probe. Similar levels of these transcripts were present in RNA preparations from WT and UV16, suggesting that the lower PC content of UV16 results from posttranscriptional events. Proteolysis of unassembled PC could be a major factor, given that assembly blocks in other systems can result in the degradation of unassembled components (9, 41). The PE content in UV16 is also reduced to 55% of the WT level, and the chromatic adaptation response may be abnormal (6). Differences in PE transcript accumulation between WT and UV16 grown in red or green light are described elsewhere (4) as part of our characterization of the PE gene set in *Synechocystis* strain 6701.

Potential transcription start sites for the *cpcBA* mRNA were localized by primer extension and S1 nuclease protection experiments by using RNA from WT cultures. Two major products of nearly equal abundance were common to the primer extension and S1 nuclease reactions (Fig. 7). These mapped at −223 and −100 bp relative to the initiation codon of *cpcB* and are marked by asterisks in the nucleotide sequence (Fig. 2). A third, larger product was observed only in the primer extension reactions. Since the S1 nuclease experiments never showed this signal and its appearance in the primer extension reactions was variable, it is likely an artifact. The 123-bp distance between the sites at −223 and −100 bp corresponds to the difference in size between the two PC transcripts seen in Northern hybridizations (Fig. 6). Significant regions of homology were found starting at 40 bp upstream of the site at −223 bp and 60 bp upstream of the site at −100 bp (Fig. 8). These common structural features could represent similar upstream regulatory sequences that ensure high levels of both PC transcripts in *Synechocystis* strain 6701, although we have not ruled out the possibility that the smaller transcript is a consequence of RNA processing. One of the PC gene sets in *Synechocystis* strain 6301 also has two putative transcription start sites, separated by about 100 bp, that correspond with two equally abundant PC transcripts (22).

Conclusion. This analysis of the *Synechocystis* strain 6701 UV16 mutant suggests that the replacement of Pro-5 by Leu in the PC α subunit affects the structure and assembly of phycobilisome components. Although there is excellent correlation between the mutation, its possible effect, and the UV16 phenotype, we cannot rule out the possibility of other mutations that prevent the formation of PC hexamers. The best proof of cause and effect between mutation and assembly defect would be a genetic one, in which the introduction of a mutant PC gene conferred the complete UV16 phenotype on the WT strain. We are trying to establish gene transfer in *Synechocystis* strain 6701 to perform such experiments.

ACKNOWLEDGMENTS

We thank Joel Bellinson and Glenn Ford for excellent technical assistance. Pamela Conley and Peggy Lemaux were very generous with advice and probes for biliprotein genes. Eugenio de Hostos, Nancy Federspiel, Dave Laudenbach, and Robert Sweet provided helpful discussions. We are grateful to R. Huber for the PC trimer and hexamer coordinate sets.

This study was supported by a plant biology postdoctoral fellowship from the National Science Foundation (to L.K.A.) and by

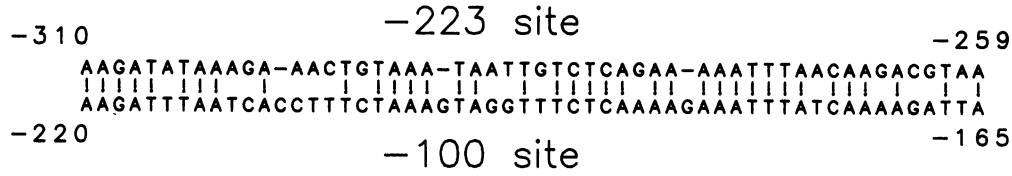

FIG. 8. Homologous regions upstream of putative *cpcBA* transcription initiation sites. The bases are numbered relative to the translation initiation codon of *cpcB*.

Public Health Service grant GM-334336-01 from the National Institutes of Health (to A.R.G.).

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