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Isolation and characterization of a gene for a major light-harvesting polypeptide from *Cyanophora paradoxa*

(phycobilisome/β-phycocyanin/immunological screening/chloroplast genes)

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ABSTRACT **Antibodies raised against mixtures of phycobilisome polypeptides from the eukaryotic alga *Cyanidium caldarium* were used in an immunological screen to detect expression of phycobiliprotein genes in an *Escherichia coli* library containing segments of plastid (chloroplast, cyanelle) DNA from another eukaryotic alga, *Cyanophora paradoxa*. The four candidate clones obtained were mapped by restriction analysis and found to be overlapping. The clone with the smallest insert (1.4 kilobases) was partially sequenced and a coding region similar to the carboxyl terminus of the phycobiliprotein subunit β-phycocyanin was found. The coding region for the β-phycocyanin gene in *C. paradoxa* has been mapped to the small single copy region on the cyanelle genome, and its orientation has been determined. A short probe unique to a conserved chromophore binding site shared by at least two phycobiliprotein subunits has now been generated from the carboxyl terminus of the β-phycocyanin gene. This probe may be useful in identifying specific phycobiliprotein subunit genes, β-phycocyanin, β-phycoerythrocyanin, and possibly β-phycoerythrin, in other eukaryotic algae and in prokaryotic cyanobacteria.**

Phycobilisomes are macromolecular complexes found in prokaryotic cyanobacteria (blue-green algae) and in eukaryotic red algae (1) that harvest light energy and funnel it to the photosynthetic reaction centers (2, 3). Functionally analogous to the light-harvesting chlorophyll–protein complex in higher plants (4, 5), phycobilisomes can provide 30–50% of the light-harvesting capacity and may comprise 60% of total soluble protein (6). The synthesis of phycobilisome polypeptides is regulated in many algae so that the levels of the individual components are adjusted according to the quantity and quality of light. The complex contains both pigmented and nonpigmented polypeptides with ≈85% being made up of three groups of pigmented proteins or phycobiliproteins—namely, allophycocyanin (APC), phycocyanin (PC), and phycoerythrin (PE). The remainder of the phycobilisome is composed of nonpigmented linker polypeptides, which hold the phycobiliproteins in an ordered array (7), and a high molecular weight, pigmented polypeptide, the anchor protein, which attaches the phycobilisome to the photosynthetic membranes (8). Each of the phycobiliproteins contains an α and β subunit and each subunit covalently binds one or more tetrapyrrole chromophores (1). Amino acid sequence data (9–17) have shown amino acid conservation within the α subunits as a group and the β subunits as a group and among the various α and β subunits. This had led to speculation that individual polypeptide subunits of the different phycobiliprotein groups may have evolved from duplications of a single progenitor gene (11).

Recent work has demonstrated that the subunits of APC, PC, and PE from specific eukaryotic algae are translated within and probably encoded by the chloroplast (18–20). Based on these studies, an approach was devised for cloning the phycobiliprotein subunit genes from the small cyanelle (plastid, chloroplast) genome of the eukaryotic alga *Cyanophora paradoxa*. Antibodies were raised against phycobiliprotein subunits from *Cyanidium caldarium* and, after demonstrating antigenic cross-reactivity to *C. paradoxa* phycobilisomes, were used to immunologically detect expression of phycobiliprotein genes in an *Escherichia coli* library of cyanelle sequences.

MATERIALS AND METHODS

Materials. All chemicals used were of reagent grade. Alkaline phosphatase and DNA polymerase I were obtained from Boehringer Mannheim; polynucleotide kinase, from P-L Biochemicals; deoxyribonuclease I, from Sigma; T4 DNA ligase, from Bethesda Research Laboratories; and restriction enzymes, from Bethesda Research Laboratories and New England Biolabs. [γ-^{32}P]ATP (5000 Ci/mmol; 1 Ci = 37 GBq), [α-^{32}P]dCTP (800 Ci/mmol), and ^{125}I (carrier-free) were obtained from Amersham.

Vectors, Strains, and Growth Conditions. The pBR322 derivative pUC8, used for library construction, was transformed into the *E. coli* strain JM83 (21). *C. paradoxa*, a subculture of UTEX LB555, was grown in Schenk's medium (22) at 22°C. *C. caldarium* was grown as described (18). All algal cultures were bubbled continuously with 5% CO_2/95% air and illumination was from fluorescent tubes (100 microeinsteins $m^{-2}\cdot s^{-1}$).

Isolation of Cyanelle DNA and Construction of *E. coli* Library. Cyanelle DNA was isolated from logarithmically growing algae by the procedure of Mucke *et al.* (23), except that the cells were resuspended in 50 mM Tris·HCl (pH 8.0) and passed through a chilled French pressure cell at 400 pounds per square inch (1 pound per square inch = 6845 Pa). Cyanelle DNA was prepared for the construction of the library by mixing aliquots taken at various times during a *Sau*3A digestion and separating the DNA by electrophoresis in a 1% agarose/TBE buffer (24) gel. Fragments, 1.1–1.5 kilobase pairs (kb), 4–5 kb, and 5–6 kb in size, were electroeluted, purified by passage over an NACS 52 column (Bethesda Research Laboratories), and ligated (manufacturer's specifications) into a pUC8 vector that had been cut by *Bam*HI and treated with alkaline phosphatase (manufacturer's specifications). The ligated DNA was transformed into competent JM83 (25) and resultant colonies, containing inserts (lac$^-$ phenotype), were chosen for representative libraries.

Isolation of Phycobilisomes and Preparation of ^{125}I-Labeled Antibodies. Intact *C. caldarium* phycobilisomes were isolated from sucrose step gradients (26), applied to preparative 12–18% polyacrylamide gradient gels, and stained as described (18). Bands corresponding to mixtures of phycobiliprotein subunits were excised from preparative gels, elec-

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Abbreviations: kb, kilobase pair(s); SSC, small single copy; APC, allophycocyanin; PC, phycocyanin; PE, phycoerythrin.

troeluted, and combined with Freund's adjuvant. Approximately 500 μg of protein was used for the primary injection into rabbits with booster injections of 50–100 μg given at 2-wk intervals. Antiserum was purified by the procedure of Harboe and Ingild (27) and, to remove anticoliform IgG, was incubated with Affi-Gel 15 (Bio-Rad), which had been coupled (according to manufacturer's specifications) to proteins from an *E. coli* extract (28). The antibodies were characterized by Ouchterlony plates (29) and crossed immunoelectrophoresis (30) prior to iodination (31). Iodinated antibody had a specific activity of 10^6 to 10^7 cpm/μg.

Immunlogical Screening of the *E. coli* Library. The screening method was a modification of published procedures (28, 32–34). Aminophenylthioether-paper was prepared (35), diazotized (according to the specifications of Schleicher & Schuell), and reacted with unlabeled antibody (50 μg of IgG per ml, not freed of anticoliform antibodies) in 25 mM phosphate buffer (pH 6.5) by sealing individual filters in SaranWrap and incubating at 4°C for 16 hr. Uncoupled diazotized sites were inactivated at 37°C for 2 hr in 0.5 M glycine/0.1% bovine serum albumin/0.1 M phosphate buffer, pH 7.0. Bacterial colonies (75 colonies per 10-cm diameter plate), grown overnight on nitrocellulose filters overlaid on nutrient agar plates, were lysed by placing them on Whatman 3MM paper saturated with 0.25 mg of lysozyme per ml and 0.025% $NaDodSO_4$ in 0.025 M phosphate buffer at pH 6.5 (30 min, 37°C) and then exposing them to chloroform vapors (10 min, room temperature). The coupled paper was appressed to the lysed colonies, sealed in SaranWrap, and incubated at 4°C for 2 hr and at room temperature for 1 hr. The filters were separated and the aminophenylthioether-paper was washed vigorously in cold 0.15 M phosphate-buffered saline at pH 7.4 (P_i/NaCl) containing 0.5% bovine serum albumin and 0.2% Triton X-100 (P_i/NaCl wash buffer) for ≈30 min, partially dried, and incubated at 4°C for 16 hr with ^{125}I-labeled antibody (5×10^6 cpm/ml) in P_i/NaCl wash buffer. Filters were washed in P_i/NaCl wash buffer at 4°C with gentle shaking for 1–3 hr and exposed for autoradiography to Kodak XAR5 film.

Restriction Mapping and Southern Hybridizations. Mapping of *E. coli* clones was done by classical restriction analysis of total plasmid DNA and labeled insert fragments. Relationships among the clones were determined by Southern hybridizations (36) using cloned DNA labeled by nick-translation (37) to probe other candidate clones. To analyze the location of the clones on the cyanelle genome, labeled DNA from the clones was used to probe restricted cyanelle DNA by Southern hybridization. Hybridizations were at 65°C (12–18 hr) in 0.5 M NaCl/0.1 M NaH_2PO_4/0.1 M Tris base/2 mM EDTA/0.1% $NaDodSO_4$, using labeled DNA that had been fragmented by boiling for 10 min in 0.5 M NaOH. Following hybridization the filters were washed three times (10 min each) in 10 mM phosphate buffer, pH 7.0/2 mM EDTA/0.1% $NaDodSO_4$ (room temperature) and then (30 min) in 50 mM phosphate buffer, pH 7.0/2 mM EDTA/0.1% $NaDodSO_4$ (room temperature). The washed filters were exposed to Kodak XAR5 film.

Sequencing of pCPC2368. Plasmid pCPC2368 was cut with *Eco*RI and *Hin*dIII, treated with alkaline phosphatase, labeled with polynucleotide kinase (38), and then recut with *Pst* I before applying to a preparative 6% polyacrylamide gel run in TBE buffer. The labeled insert fragments were electroeluted, purified by passage over a Sephadex G-50 column, and sequenced by utilizing five different overlapping chemistries (38). The sequence was read from multiple loadings on 8% and 20% gels.

RESULTS

Identification and Characterization of Phycobiliprotein Antibody-Reactive Clones. Cyanelle DNA isolated from *C. paradoxa* was partially restricted with the enzyme *Sau*3A and various-sized fragments (ranging from 1.1 to 6 kb) were ligated into the *Bam*HI site of pUC8. Appropriate numbers of clones containing inserts were selected to insure a high probability (P = 99%) of finding any specific plastid gene in a given library (600 for 1.1- to 1.5-kb size, 150 for 4- to 5-kb size, and 125 for 5- to 6-kb size) (39).

Colonies were screened for expression of phycobiliprotein subunit genes by covalently coupling phycobiliprotein antibody to cellulose paper, exposing this to lysed bacterial colonies, and reacting it with homologous ^{125}I-labeled phycobiliprotein antibody. Antisera were raised to phycobiliprotein polypeptides of isolated *C. caldarium* phycobilisomes (bands 1 and 2, Fig. 1). Each of these bands probably contains more than one phycobiliprotein polypeptide, and therefore the antisera raised to each may not be specific for a single phycobiliprotein subunit. These antisera cross-react with phycobilisomes isolated from *C. paradoxa* (unpublished data). Analyses by crossed immunoelectrophoresis showed an approximate 10% cross-reactivity of antiserum 1 with band 2 and antiserum 2 with band 1 (unpublished data). This could have resulted from physical contamination of the bands in preparative gels or from slight cross-reactivity of these antisera with denatured phycobiliprotein subunits (or both).

Clones synthesizing phycobiliprotein subunits were identified by autoradiography (Fig. 2*A*) and positive clones were placed in an array with known negative clones and rescreened (Fig. 2*B*). In general, the positive clones were equally reactive to both antisera with the exception of the smallest clone, pCPC2368, which reacted strongly with the antibody to band 2 and only weakly, or not at all, with the antibody to band 1.

Restriction enzyme analysis of the four clones isolated from three different-sized libraries led to the restriction maps shown in Fig. 3. Based on the coincidence of restriction sites and results of Southern hybridizations among the various candidates (unpublished data), it was concluded that all four clones physically overlap.

Sequencing of Clone pCPC2368. Analysis of a sequence from the pCPC2368 insert revealed the probable coding region for the carboxyl terminus of the β subunit of PC (β-PC) (Fig. 4). This identification was based on the similarity of the predicted amino acid sequence of the *C. paradoxa* polypeptide to the carboxyl terminus of β-PC from *C. caldarium*

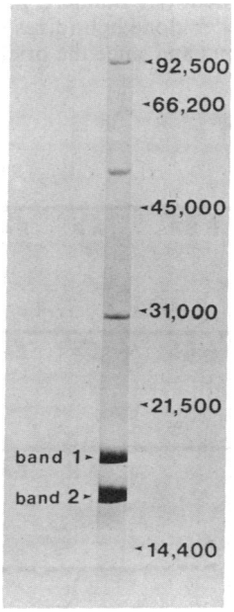

FIG. 1. Separation of phycobilisome polypeptides of *C. caldarium* on polyacrylamide gels. Molecular weight markers were phosphorylase b (M_r 92,500), bovine serum albumin (M_r 66,200), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400). Bands 1 and 2, the two bands containing pigmented phycobiliprotein subunits used to generate independent antisera.

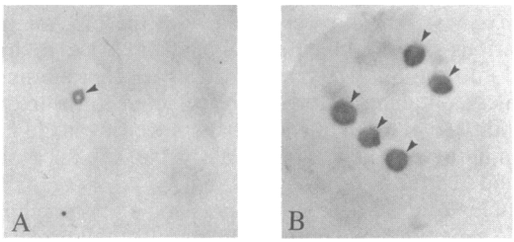

FIG. 2. Autoradiogram of immunological screens of an *E. coli* cyanelle DNA library. (*A*) Primary screen of *Sau*3A partial library of cyanelle DNA containing fragments 5–6 kb. The arrowhead indicates positive clone pCPC3029 after a 24-hr exposure. (*B*) Second screen of candidate pCPC3029. Positive clones found in the primary screen were replated as five independent colonies in an array with 20 negative clones and rescreened. Arrowheads indicate pCPC3029 after a 24-hr exposure.

(16). The α and β subunits of APC and PC [*C. paradoxa* contains PC and APC, but no PE (40)] have a chromophore binding site near the middle of the polypeptide (15, 16, 41). However, only β-PC contains a chromophore binding site near the carboxyl terminus. The region surrounding this chromophore binding site in *C. paradoxa* has seven conserved amino acids compared to the analogous site in *C. caldarium*. Three conserved amino acids, found at the carboxyl terminus in all subunits of APC and PC in *C. caldarium*, are also present in β-PC of *C. paradoxa*. Most of the differences in amino acid sequence of β-PC between the two algal species are neutral changes. Although based on this analysis it seems likely that this is the β-PC gene, it is possible that the sequence is for some minor phycobiliprotein component not yet characterized (42) or an unexpressed pseudogene. Further analysis should distinguish these possibilities. Fig. 4 also shows that the β-PC gene has been interrupted by cloning and is not in translational reading frame with the β-galactosidase gene in the vector.

Mapping of the β-PC Gene on the Cyanelle Map. Hybridizations of cyanelle DNA cut by *Sma* I with DNA from the largest and smallest antibody-reactive clones immediately placed the β-PC gene in the SSC region (17 kb) of the cyanelle genome (Fig. 5). Of the fragments generated by this enzyme, only the 20-kb fragment, which spans the SSC region (45, 46), hybridizes to both clones. Hybridization of both clones to a 25-kb *Xho* I fragment, which also maps to the SSC region (H. Bohnert, personal communication), confirmed this location. The larger clone hybridizes to three *Bgl* II fragments, including a 2.2-kb fragment (H. Bohnert, personal communication). Since the smaller clone hybridizes to only one of these three *Bgl* II fragments and since the orientation of the clones to each other (Fig. 3) and the position and direction of transcription of the β-PC gene on the clones (Fig. 4) are known, the location and direction of transcription of this gene on the cyanelle genome could be deduced (Fig. 5).

DISCUSSION

Since chloroplast genes can be expressed in *E. coli* from their own promoters (47), we used an approach employing immunological screening to identify clones expressing phycobiliprotein subunits. The source of plastid DNA for the library was a primitive eukaryotic alga, *C. paradoxa*, which may represent an evolutionary link between cyanobacteria and red algae (48). Because it contains little carbohydrate and its plastid is relatively resistant to lysis (due to a thin peptidylglycan cell wall), the isolation of pure plastid DNA was made easier. Furthermore, a restriction map for this DNA has already been established (45, 46).

Phycobilisomes from *C. paradoxa* contain the α and β subunits of APC and PC (42). We have located, by sequence analysis of clone pCPC2368, only the carboxyl terminus of the β-PC gene, but this clone may contain one additional phycobiliprotein subunit gene. If the antibody-reactive material in this clone resulted from the β-PC gene, it must be synthesized from a translational fusion with the β-galactosidase gene or from a translational restart. Since the amino acid sequence of the β-PC polypeptide is not in translational reading frame with the β-galactosidase polypeptide (Fig. 4) and since no new ribosomal binding sites or initiator codons were created by the fusion, it seems unlikely that this gene is being expressed. A possible explanation for the synthesis of antibody-reactive material by this clone is the presence of an additional gene on the cloned fragment. Such a gene could be expressed from its own promoter or from a fused message initiated from the β-galactosidase promoter.

Hybridizations at lower stringencies, with the clones from the SSC region, were used to try to locate other regions of the cyanelle genome that might encode phycobiliprotein subunits. Although amino acid conservation between either the α or β subunits of a single phycobiliprotein or between the β subunits of APC and PC in *Cyanidium* is only in the range of 30% (16), certain subregions of these polypeptides have up to 75% amino acid homology. Therefore, hybridization among the different subunit genes may occur under conditions of lower stringency. Preliminary experiments utilizing such an approach indicate that there is another region of the cyanelle genome that does hybridize to the largest clone (unpublished data). This region can be analyzed for the presence of genes coding for phycobiliprotein subunits.

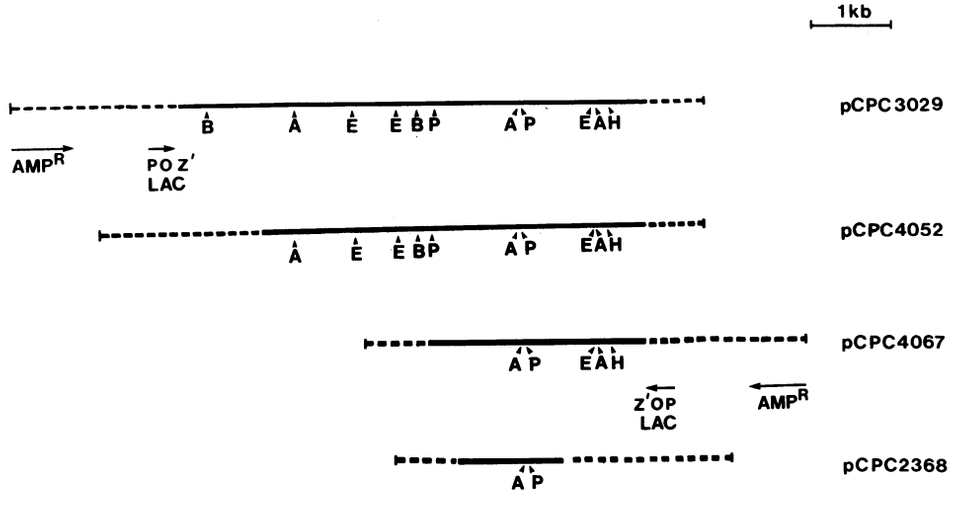

FIG. 3. Restriction maps of immunologically reactive clones from different-sized libraries. pCPC4052 and pCPC4067 were selected from the same library; pCPC3029 and pCPC2368 came from two other independently derived libraries. The dashed lines indicate vector sequences. The AMP^R and LAC P O Z′ designate the β-lactamase gene and the regulatory and amino terminus of the β-galactosidase structural gene, respectively. The direction of transcription of these genes is shown by arrows. The sizes of the inserts were: pCPC3029, 5.5 kb; pCPC4052, 4.5 kb; pCPC4067, 2.6 kb; and pCPC2368, 1.4 kb. Restriction enzyme abbreviations are A, *Ava* II; B, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; and P, *Pst* I.

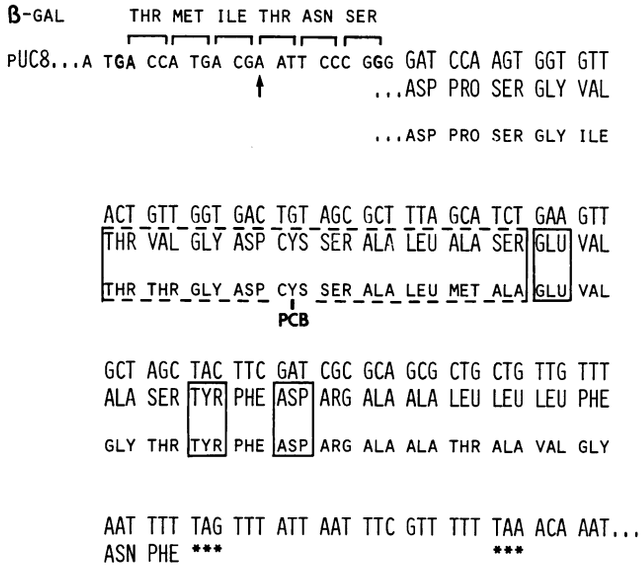

FIG. 4. Nucleotide sequence of the carboxyl terminus of the β-PC gene from pCPC2368. The upward arrow indicates the beginning of our sequence data. Lowercase nucleotide designations represent the sequence of the vector and the amino acid abbreviations above this portion indicate the reading frame and amino acid sequence of the portion of the β-galactosidase gene in pUC8 upstream from the insert. Uppercase nucleotide and amino acid designations represent the sequence obtained from the *C. paradoxa* β-PC gene. The lowercase amino acid abbreviations below this represent the sequence obtained for the β-PC gene in *C. caldarium* (16). The amino acids surrounding the conserved phycocyanobilin binding site (PCB) are encased in a dashed line. Additional amino acids conserved in all phycobiliprotein polypeptides from *C. caldarium* are designated by solid boxes. Terminator codons are indicated by ***.

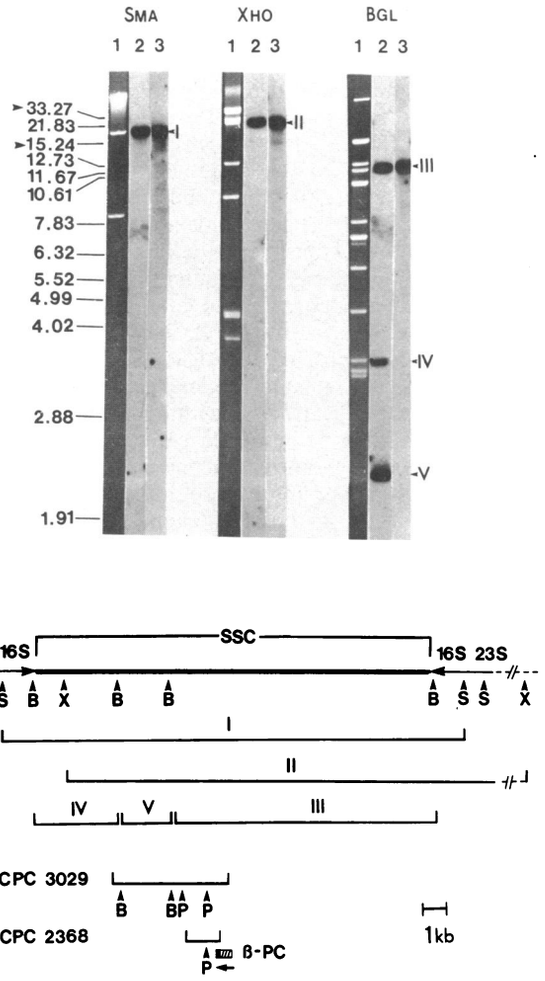

FIG. 5. Southern hybridization of clones to cyanelle DNA and positioning of the clones on the cyanelle map. (*Upper*) Cyanelle DNA was isolated, restricted, and run on a 1% agarose/TEA buffer (43) gel; 1–2 × 10^6 Cerenkov cpm per filter were used. Molecular weight standards (left side) are in kb and were from an *Eco*RI digestion of pRR12 (44) and a *Sal* I digestion of λ (indicated by arrowheads). For all enzymes, lane 1 shows a photograph of a stained gel; lane 2, an autoradiogram of a Southern hybridization to ^{32}P-labeled probe from pCPC3029; and lane 3, the autoradiogram using pCPC2368 as the probe. All autoradiograms were 18-hr exposures. The roman numeral designations I–V refer to the specific cyanelle DNA fragments depicted in *Lower*. (*Lower*) Restriction map of the SSC region of the cyanelle DNA. The SSC region is indicated by the heavy black line; the inverted repeats, by a thin black line. The brackets below the restriction map demarcate the predicted fragments for each restriction enzyme. The roman numeral designations correspond to those in *Upper* and their sizes in kb are I, 20.0; II, 25.0; III, 11.5; IV, 3.7; and V, 2.3. The relative sizes and locations of the largest (pCPC3029) and smallest (pCPC2368) inserts from antibody-reactive clones are indicated by brackets. The position of the β-PC gene is indicated by a box; the solid portion represents the region sequenced, whereas the hatched portion represents the projected size of the remainder of the gene. The direction of transcription of the gene is shown by an arrow. Restriction enzyme designations are B, *Bgl* II; P, *Pst* I; S, *Sma* I; and X, *Xho* I. *Pst* I sites are shown on the inserts for orientation purposes only.

If all genes for the phycobiliprotein subunits are not contained in the SSC region, there are several possible reasons why expression of other subunit genes was not detected. First, the promoters for the rest of the genes may not function well in *E. coli* and, hence, expression may be undetectable. Since PC is present at higher levels in the phycobilisome than APC (49–51), the promoter for the PC subunits might be more efficient in the cyanelle and in *E. coli*. Second, the remaining genes for phycobiliprotein subunits might contain introns and hence do not express antigenically reactive material. Finally, reactivity to the different antibodies may vary reflecting differential avidity of the various antibodies, varying antigenicity of the different subunits, or fluctuations in antibody titers to individual phycobiliprotein subunits. Higher titer antibody toward the PC subunits might result from the fact that these subunits are in greater abundance in the preparations used to generate the antisera.

The data and arguments presented here lead to certain predictions. First, sequencing of the entire insert of pCPC2368 may reveal a second phycobiliprotein gene. Second, sequencing of the appropriate region of clone pCPC4067, pCPC4052, or pCPC3029 will yield the amino-terminal portion of the β-PC gene and most likely the regulatory region(s). Since the level of synthesis of antigenic material from all clones is approximately equal (unpublished data) and since the sizes and orientations of the inserts are different among the clones, it is likely that some of the subunit genes are transcribed from a promoter on the chloroplast DNA (possibly a phycobiliprotein gene promoter). One end of the three largest clones is the same to within 50 base pairs (unpublished data, Fig. 3) and this may be indicative of the need for this region in expression. Another potential explanation for conservation of this region is that *Sau*3A has a preference for this site during partial digestions.

A unique DNA probe for the chromophore binding site located at the carboxyl terminus of the β-PC gene has been generated. Clone pCPC2368 was digested with *Sau*3A and a 63-base-pair fragment containing the conserved chromophore binding site has been cloned into pUC8 (unpublished data). This cloned fragment will be used for identifying specific genes, both in other eukaryotic algae and in prokaryotic cyanobacteria. Immunological relationships (52, 53) and

amino acid sequence data (13, 14, 16, 17, 41, 54, 55) have shown that individual phycobiliprotein subunits from eukaryotic algae are strongly related to those in prokaryotes. For instance, the region surrounding the chromophore binding site of β-PC in *C. paradoxa* has seven of nine amino acids conserved relative to β-PC in a prokaryotic cyanobacterium *Mastigocladus laminosus* (17). In addition, the β subunit of phycoerythrocyanin (56), a phycobiliprotein spectrally similar to PE, has six of the same nine amino acids surrounding its carboxyl-terminal chromophore binding site (17). The 63-base-pair β-PC probe has been found to hybridize with DNA from cyanobacteria (unpublished data) and can be used for identifying and isolating the β-PC, the β-PEC, and possibly the β-PE genes in prokaryotic algae. Sequence analysis of the structural and regulatory regions among various phycobiliprotein subunit genes, both within a single organism and between the same gene in a eukaryote and prokaryote, will provide evolutionary information and may eventually lead to a better understanding of the structural, functional, and regulatory relationships among these genes.

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