JOURNAL OF BACTERIOLOGY, May 1987, p. 1830–1835
0021-9193/87/051830-06$02.00/0
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Vol. 169, No. 5

Functional Expression of Plastid Allophycocyanin Genes in a Cyanobacterium†

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Received 7 November 1986/Accepted 22 January 1987

In *Cyanophora paradoxa*, the allophycocyanin apoprotein subunits, α and β, are encoded in the cyanelle (plastid) genome. These genes were transferred to the cyanobacterium *Synechococcus* sp. PCC 7002 on a plasmid replicon. Phycobilisomes isolated from transformed cyanobacteria were found to contain *C. paradoxa* allophycocyanin subunits. Thus, these plastid genes are expressed in the cyanobacterium as polypeptides which become linked to a chromophore and are incorporated into the light-harvesting apparatus.

The phycobiliproteins comprise a large fraction of the photosynthetic light-harvesting capacity in red algae and cyanobacteria. These polypeptides are linked covalently to linear tetrapyrrole chromophores and are organized into supramolecular complexes called phycobilisomes (for a review, see reference 9). Allophycocyanin (AP) is the major phycobiliprotein found in the core substructure of all phycobilisomes. It consists of two subunits, designated α and β, each approximately 17 kilodaltons in mass and each with a single phycocyanobilin chromophore bound to a cysteine residue of the apoprotein. Attached to the core and radiating outward from it are rod substructures composed of phycocyanin (PC) and often other phycobiliproteins. Light energy absorbed by components of the peripheral rods is transmitted to the core and thence to the photosystem II reaction centers in the thylakoid membrane.

In addition to cyanobacteria and red algae, phycobilisomes are found in cyanelles. These are plastids which resemble cyanobacteria in traits that include the presence of both an outer peptidoglycan layer and phycobiliproteins (20). The most extensively studied cyanelle, that of the dinoflagellate *Cyanophora paradoxa*, has a genome size and genetic content similar to those of chloroplasts in higher plants (12, 13). Hence, cyanelles might resemble the evolutionary precursors of chloroplasts, especially those of red algae.

In *C. paradoxa* the cyanelle, rather than the cytoplasm, has been identified as the site of phycobiliprotein synthesis (11). Recently the genes for *C. paradoxa* PC and AP subunits have been cloned, characterized, and mapped for the cyanelle genome (2, 13–15). These genes are denoted *cpcA* and *cpcB* for the α and β subunits, respectively, of PC; likewise, *apcA* and *apcB* represent the α and β subunits of AP. The *apc* and *cpc* loci are widely separated, but for each locus the α and β subunit genes are adjacent and cotranscribed (15). *apcA* is 5′ to *apcB*, with 39 base pairs separating the two (see Fig. 1). We have also cloned and sequenced the *apc* genes of the cyanobacterium *Synechococcus* sp. PCC 7002 (1, unpublished data). In this organism there is also a unique AP-encoding locus in which *apcA* is 5′ to *apcB*. We sought to transfer cyanelle AP genes into this cyanobacterium to determine whether the cyanelle genes would be expressed, and if so, whether the cyanelle apoprotein subunits would be linked with phycocyanobilin and incorporated into phycobilisomes.

MATERIALS AND METHODS

Microbial strains and culture. *Synechococcus* sp. PCC 7002 (*Agmenellum quadruplicatum* PR-6) was cultured as previously reported (19). The wild-type strain is referred to herein as PR6000. Plasmid transformation of PR6000 was performed by the method of Buzby et al. (5). The *C. paradoxa* strain and its growth conditions were described by Lambert et al. (13). Frozen cells were kindly supplied by David H. Lambert. *Escherichia coli* RDP145 (5) was used for molecular cloning of recombinant plasmids.

Phycobiliprotein purification and electrophoresis. Phycobilisomes were prepared from exponential-phase *Synechococcus* sp. PCC 7002 as described by Bryant et al. (4). AP-enriched phycobiliproteins were prepared by hydroxyapatite chromatography (7). AP from frozen *C. paradoxa* was partially purified by the method of Cohen-Bazire et al. (7). The product contained some PC. AP from wild-type *Synechococcus* sp. PCC 7002 (strain PR6000) was purified by the same method, with an additional DEAE-cellulose chromatography step. The product contained no electrophoretically detectable contaminants. Isoelectric focusing (IEF) and IEF-sodium dodecyl sulfate two-dimensional gel electrophoresis were performed by the method of O'Farrell (16), but Triton X-100 was omitted. IEF gels were run in 8 M urea in the pH range of 4 to 7. The SDS gel dimension was a 10 to 20% acrylamide gradient (acrylamide/bisacrylamide = 30:0.8). Gel staining was with Coomassie brilliant blue R-250.

Immune serum and immunodiffusion. Purified AP from *Synechococcus* sp. PCC 7002 was mixed with Freund complete adjuvant and used to immunize a New Zealand albino rabbit as previously described (10). Whole serum was brought to 33% saturation with ammonium sulfate. The precipitate was dissolved in phosphate-buffered saline, dialyzed against the same, filter sterilized, and stored at −20°C. Immunodiffusion was carried out by the method of Glazer et al. (10), except that the test wells contained IEF gel slices. These were cut as narrow sections from IEF gels, soaked in 0.05% SDS–10 mM Tris hydrochloride (pH 7) for 1 h, and placed in the test wells.

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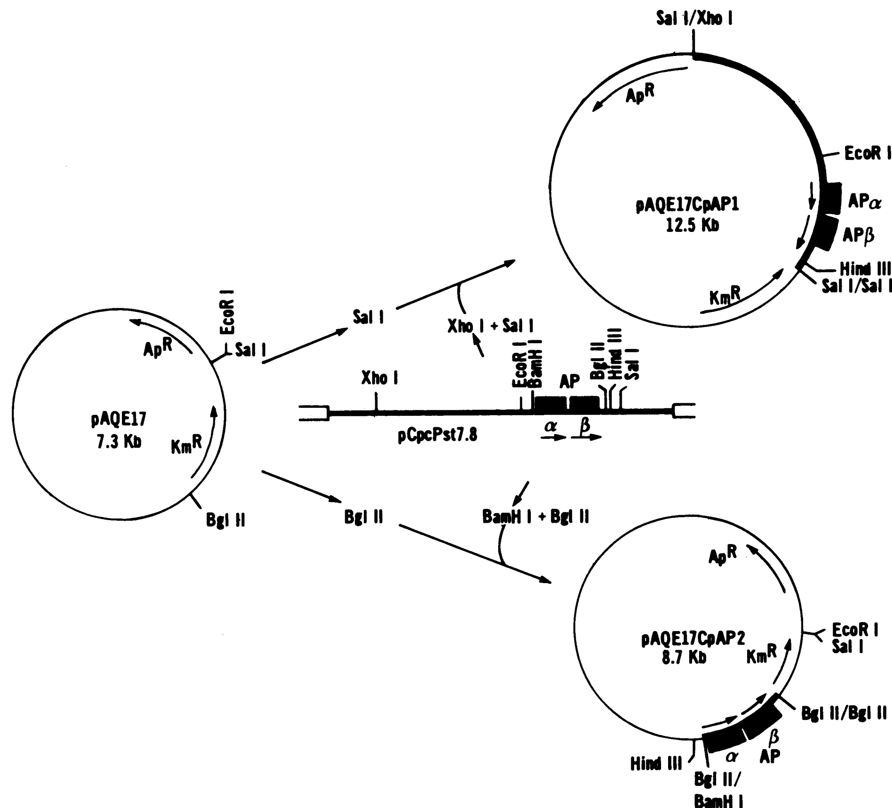

FIG. 1. Construction of plasmids containing *C. paradoxa* cyanelle *apc* genes for transformation into *Synechococcus* sp. PPC 7002. Thickened lines represent *C. paradoxa* cyanelle DNA. Ap^R denotes the β-lactamase gene and Km^R indicates the aminoglycoside phosphotransferase gene of the shuttle vector pAQE17. Arrows show the approximate bounds and direction (5′ to 3′) of coding sequences.

DNA manipulations. DNA from *Synechococcus* sp. PCC 7002 strains was purified as previously reported (8) except that plasmid and chromosomal DNA fractions were collected separately upon buoyant-density centrifugation in the presence of ethidium bromide.

RESULTS

The plasmid vector pAQE17 replicates in *E. coli* and *Synechococcus* sp. PCC 7002 (6). It encodes a β-lactamase and an aminoglycoside phosphotransferase, thus conferring resistance in either organism to ampicillin and kanamycin. The plasmid pCpcPst7.8 (Fig. 1) contains a segment of the *C. paradoxa* cyanelle genome which includes the APα and APβ subunit apoprotein genes, *apcA* and *apcB* (2). To introduce these genes into *Synechococcus* sp. PCC 7002, we inserted two different restriction fragments of pCpcPst7.8 into pAQE17 (Fig. 1). To construct pAQE17CpAP1, the 5.2-kilobase (kb) *Sal*I-*Xho*I fragment was ligated into the *Sal*I site of pAQE17 in the orientation shown. The plasmid pAQE17CpAP2 was constructed by ligating the 1.4-kb *Bam*HI-*Bgl*II fragment of pCpcPst7.8 into the *Bgl*II site of pAQE17 in the orientation shown. As this *Bgl*II site lies between the promoter and structural gene for aminoglycoside phosphotransferase (18), the *C. paradoxa* AP genes might be expressed from this promoter.

Purified pAQE17CpAP1 or pAQE17CpAP2 DNA isolated from *E. coli* was added to growing cultures of *Synechococcus* sp. PCC 7002 to effect transformation. Transformants were selected with kanamycin for pAQE17CpAP1 and with ampicillin for pAQE17CpAP2. In each case, drug-resistant colonies arose at a frequency of 1×10^{-5} to 2×10^{-5}/CFU. An isolate from the pAQE17CpAP1 transformation was designated PR6006, and an isolate from the pAQE17CpAP2 transformation was designated PR6007.

Both PR6006 and PR6007 were similar to PR6000 (wild type) on the basis of color, growth rate, and colony morphology. Each transformant was also resistant to the antibiotic, either ampicillin or kanamycin, which was not used in the original selection.

The state of transformed DNA in PR6006 and PR6007 was examined to assess its integrity. DNA from PR6000 and the two transformant strains was separated into plasmid and chromosomal fractions and digested with *Sal*I or *Hin*dIII-*Eco*RI. Digested DNA was fractionated by agarose gel electrophoresis, blotted to a nitrocellulose membrane, and probed with a labeled restriction fragment of pCpcPst7.8 (1.4-kb *Bam*HI-*Bgl*II) containing the *C. paradoxa* APα and APβ genes. Autoradiograms of the resulting Southern blots are shown in Fig. 2.

A single *Sal*I site is present in pAQE17CpAP1 and pAQE17CpAP2 (Fig. 1). Thus *Sal*I digestion should have linearized these plasmids. Multiple *Sal*I fragments in plasmid fractions of PR6006 and PR6007 are homologous to *C. paradoxa apc* genes, but no homology is evident in plasmid DNA of PR6000 (Fig. 2A). The smallest fragment in each transformant corresponds in length to the size of the transforming plasmid, either pAQE17CpAP1 (12.5 kb) or pAQE17CpAP2 (8.7 kb). The two larger species in each case correspond in length to cointegrates with either one or two

copies of the 4.6-kb endogenous plasmid pAQ1, which has no *Sal*I site (5). These types of cointegrates are commonly observed in plasmid transformation of *Synechococcus* sp. PCC 7002 (5). Chromosomal DNAs of the two transformants showed the same *apc*-homologous *Sal*I fragments as in plasmid fractions. This was due to plasmid DNA contamination of the chromosomal fraction. In all chromosomal DNAs, a large (35-kb) fragment showed a weak signal (not visible in Fig. 3A) due to *Synechococcus* sp. PCC 7002 AP genes. The weak signal was caused by poor transfer from the gel.

*Eco*RI-*Hin*dIII double digests (Fig. 2B) of plasmid fractions of PR6006 and PR6007 show single *apc*-homologous fragments of the sizes (2.9 and 2.1 kb) expected from the structure of pAQE17CpAP1 and pAQE17CpAP2. Multiple fragments with *apc* homology do not appear because cointegrate formation did not affect the *apc*-containing *Eco*RI-*Hin*dIII fragment. As in Fig. 2A, plasmid DNA of PR6000 shows no homology to the probe.

All *Eco*RI-*Hin*dIII digests of chromosomal DNAs show a 1.0-kb *apc*-homologous fragment. This was due to a *Hin*dIII fragment containing the endogenous *apc* genes of *Synechococcus* sp. PCC 7002 (1). Chromosomal DNAs of PR6006 and PR6007 also show the same *Eco*RI-*Hin*dIII band typical

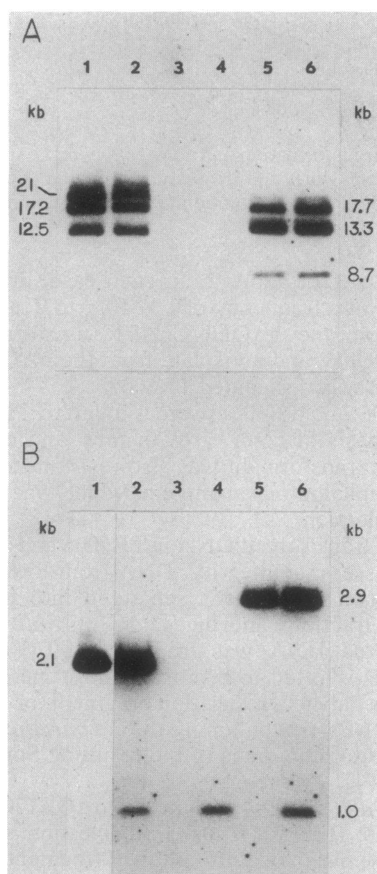

FIG. 2. Southern blot of plasmid and chromosomal DNAs. Data are from wild-type and transformant *Synechococcus* sp. PPC 7002 probed with the 1.4-kb *Bam*HI-*Bgl*II fragment of pCpcPst7.8, which carries the *C. paradoxa apc* genes. (A) *Sal*I digests. (B) *Eco*RI-*Hin*dIII double digests. Lanes (panels A and B): 1, PR6006 plasmid; 2, PR6006 chromosomal; 3, PR6000 plasmid; 4, PR6000 chromosomal; 5, PR6007 plasmid; 6, PR6007 chromosomal.

of their plasmid DNAs, because of contamination of chromosomal DNA by relaxed plasmid circles. These species were not detected in chromosomal DNA of PR6000. Stained gels of undigested chromosomal fractions also displayed plasmid species (not shown). Thus, these two fragments of 2.9 and 2.1 kb in chromosomal fractions of PR6006 and PR6007, respectively, did not result from integration of plasmid DNA into the chromosome at the endogenous *apc* locus. Further, had such an event occurred, the expected pattern of *apc*-homologous *Eco*RI-*Hin*dIII fragments would have been quite different from the observed locations of *Eco*RI and *Hin*dIII sites in the endogenous AP genes (1).

These results demonstrate that transformed *C. paradoxa* AP genes are intact and located only on plasmids. From staining intensities of intact plasmid species in agarose gels, we estimate that there are fewer than five copies of cyanelle *apc*-bearing plasmids per cell. In this organism, recombination between homologous sequences on plasmid and chromosomal DNAs can lead to integration into the chromosome (17). Failure to find such a rearrangement in the present case was likely due to insufficient homology between the two sets of *apc* loci. There is 72% nucleotide homology between the transformed and endogenous AP subunit genes (unpublished data), with the longest perfectly matched stretch being 14 base pairs. On the basis of results for *E. coli*, this length of matching seems insufficient to allow general recombination (21).

Phycobilisomes from wild-type and transformant *Synechococcus* sp. PCC 7002 were purified by a procedure which included sedimentation through a sucrose gradient. The appearance of the gradients after centrifugation was similar for PR6000, PR6006, and PR6007. There was a major blue zone in the middle of all tubes, with less than 5% of the phycobiliprotein (A_{620}) contained in a slowly sedimenting zone. The faster sedimenting zone was collected as purified phycobilisomes. The absorption spectroscopic properties and steady-state fluorescence emission and excitation properties of the phycobilisomes isolated from PR6006 and PR6007 were identical to those of the phycobilisomes of the wild-type PR6000. The slowly sedimenting zone consisted primarily of PC in all cases and was not enriched with AP in the transformants (data not shown).

Phycobilisome components were analyzed by IEF gel electrophoresis, IEF-SDS two-dimensional gel electrophoresis, and cross-reactivity with immune serum directed to purified AP. Unstained IEF gels of phycobilisome components are shown in Fig. 3A. Phycobiliproteins were detected by their intrinsic absorption at visible wavelengths. Stained IEF-SDS gels of PR6007 and PR6000 phycobilisomes are shown in Fig. 3B and C, respectively. A double immunodiffusion test for AP antigenicity in certain IEF gel fractions is depicted in Fig. 3D. As discussed below, these results demonstrate the presence of two phycobiliproteins in PR6006 and PR6007 which appear to be identical to *C. paradoxa* AP subunits.

The presence of a polypeptide in phycobilisomes of PR6006 and PR6007 with properties like those of the *C. paradoxa* APα subunit is established by the following observations. (i) Gel 1 of Fig. 3A is of AP subunits partially purified from *C. paradoxa*. The two major bands are identified as APα and APβ based on the finding that in all cases so far examined, the APα subunit has a lower isoelectric point than APβ has (7). In whole phycobilisomes of PR6007 (gel 2) and AP-enriched phycobiliproteins of PR6006 (gel 4), a blue polypeptide of the same isoelectric point of *C. paradoxa* APα is detectable. This band is not seen in whole phycobili-

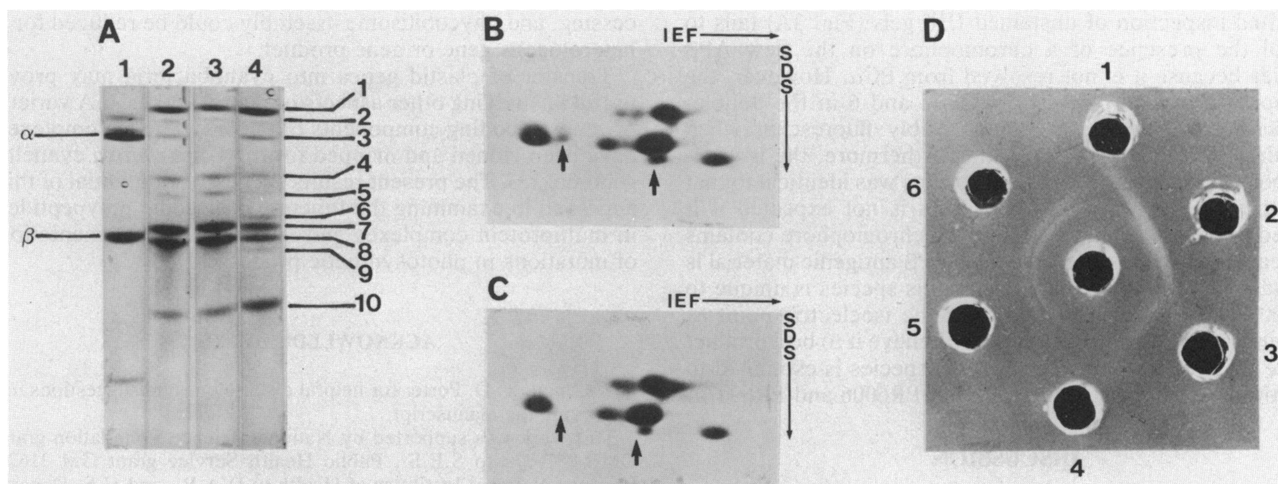

FIG. 3. Gel electrophoresis and double immunodiffusion of phycobiliproteins from wild-type and transformant phycobilisomes. (A) Unstained IEF gels: 1, *C. paradoxa* AP; 2, PR6007 phycobilisomes; 3, PR6000 phycobilisomes; 4, PR6006 AP-enriched phycobiliproteins. In gel 1 the α and β subunits of *C. paradoxa* are indicated. Bands: 1, *Synechococcus* sp. PCC 7002 APα; 2, *Synechococcus* sp. PCC 7002 $\beta^{18.3}$; 3, *C. paradoxa* APα; 4, *Synechococcus* sp. PCC 7002 PCα (variant); 5, *Synechococcus* sp. PCC 7002 PCβ (variant); 6, *Synechococcus* sp. PCC 7002 α^{APB}; 7, *Synechococcus* sp. PCC 7002 PCα (with underlying *C. paradoxa* APβ in gels 2 and 4); 8, *Synechococcus* sp. PCC 7002 PCβ; 9, *Synechococcus* sp. PCC 7002 α^{APB}; 10, *Synechococcus* sp. PCC 7002 APβ. (B and C) Stained IEF-SDS two-dimensional gels of PR6007 and PR6000 phycobilisomes, respectively. Left-hand arrows indicate presence (B) or absence (C) of putative *C. paradoxa* APα. Right-hand arrows indicate a reproducible elongation of the α^{APB} spot for PR6007 (B) which is not observed for PR6000 (C). (D) Double immunodiffusion of proteins contained in bands excised from the IEF gels shown in Fig. 3A. Center well holds antiserum to *Synechococcus* sp. PCC 7002 AP. Wells: 1, band 7 of gel 3 (PCα); 2, band 1 of gel 4 (*Synechococcus* sp. PCC 7002 APα); 3, band 3 of gel 4 (putative *C. paradoxa* APα); 4, band 8 of gel 3 (PCβ); 5, band 7 of gel 4 (PCα plus underlying putative *C. paradoxa* APβ); 6, band 10 of gel 4 (*Synechococcus* sp. PCC 7002 APβ). Cross-reactions are denoted for antigens contained in wells 2, 3, 5, and 6 but not for antigens contained in wells 1 and 4. A cross-reaction of partial identity, indicated by the spur extending toward well 3, establishes that the antigen in well 3 is an APα-type subunit. A cross-reaction of identity was noted between the antigens in wells 5 and 6; no cross-reaction was noted for the equivalent band excised from an IEF gel of wild-type phycobilisomes. This result establishes that the transformant produces an antigen which is not present in wild-type phycobilisomes, which is immunologically indistinguishable from the *Synechococcus* sp. PCC 7002 APβ subunit and which has an isoelectric point identical to that of authentic *C. paradoxa* APβ. For additional discussion, see Results.

somes of PR6000 (gel 3). (ii) The species corresponding to band 3 in gel 2 is also seen in a stained IEF-SDS two-dimensional gel of PR6007 phycobilisomes (Fig. 3B, left-hand arrow). This species has the apparent molecular mass expected of an AP subunit (approximately 18 kilodaltons) and is not detectable in a similar two-dimensional gel of PR6000 phycobilisomes (Fig. 3C, left-hand arrow). (iii) IEF gel 4 (PR6006) in Fig. 3A was sliced to separate various bands to test them for AP antigenicity. Band 1 is known to be the endogenous APα subunit of *Synechococcus* sp. PCC 7002 (unpublished results). This band was placed in well 2 of the double-diffusion plate shown in Fig. 3D. Band 3 (the putative *C. paradoxa* APα subunit) of gel 4 was placed into well 3. Both species were precipitated by the anti-AP serum, with a slight spur extending toward well 3. Individual AP subunits, α or β, are known to be antigenically conserved across taxonomic lines, with little or no cross-reactivity between α and β subunits (7). Thus, the immunodiffusion result establishes the protein species of band 3 in PR6006 phycobilisomes as an APα subunit which is not identical to *Synechococcus* sp. PCC 7002 APα. These lines of evidence demonstrate the presence of a protein species in PR6006 and PR6007 which is not distinguished from authentic *C. paradoxa* APα. The amount of this species relative to *Synechococcus* sp. PCC 7002 APα was estimated by scanning densitometry of acidified (5% trichloroacetic acid) IEF gels. In both PR6006 and PR6007, the *C. paradoxa* APα subunit constituted 9 to 13% of total APα absorbance.

The presence of *C. paradoxa* APβ in transformant *Synechococcus* sp. PCC 7002 cells is less evident because the authentic subunit has an isoelectric point very near that of the endogenous PC and α^{APB} subunits (Fig. 3A). Hence, the *C. paradoxa* APβ subunit is not resolved in IEF gels of phycobiliproteins from PR6006 or PR6007 (Fig. 3A, gels 2 and 4). Resolution is also not achieved in two-dimensional gels because the zone from this species overlaps with that of the *Synechococcus* sp. PCC 7002 α^{APB} subunit in the SDS dimension. The right-hand arrow in Fig. 3B (PR6007 phycobilisomes) indicates a reproducible elongation of the α^{APB} spot which is not observed with PR6000 phycobilisomes (Fig. 3C), consistent with the presence of an unresolved component. The presence of a new APβ species in transformant phycobilisomes is revealed by double immunodiffusion. In well 6 of the double-diffusion plate (Fig. 3D) was placed an IEF gel slice containing *Synechococcus* sp. PCC 7002 APβ from PR6006 (Fig. 3A, gel 4, band 10). Well 5 held a gel slice of PR6006 phycobilisomes which contained PCα and underlying species (Fig. 3A, gel 4, band 7). The corresponding PCα band of wild-type phycobilisomes (Fig. 3A, gel 3, band 7) was placed in well 1. This latter band shows no cross-reactivity with anti-AP serum. The equivalent IEF band from PR6006 gives a reaction of identity to *Synechococcus* sp. PCC 7002 APβ. Thus, an APβ species is found in PR6006 phycobilisomes which is not present in PR6000 and which has the isoelectric point of *C. paradoxa* APβ. APβ subunits from various taxa often show identity reactions in the double-immunodiffusion test (7); APβ subunits exhibit a higher degree of amino acid sequence conservation (approximately 85%) (2) than do APα subunits (approximately 80%) (2).

Visual inspection of unstained IEF gels (Fig. 3A) fails to reveal the presence of a chromophore on the new APβ species because it is not resolved from PCα. However, the precipitin bands formed with wells 5 and 6 in the double-diffusion experiment were both visibly fluorescent when illuminated with ultraviolet light. Furthermore, the isoelectric point of the unresolved APβ species was identical to that of authentic *C. paradoxa* APβ. This is not expected if it lacked phycocyanobilin, because the chromophore contains two carboxyl groups (9). Thus, the APβ antigenic material is bound to a chromophore. Because this species is unique to PR6006 and PR6007 and has the same isoelectric point as authentic *C. paradoxa* APβ has, we believe it to be a product of the *C. paradoxa apc*B gene. This species is estimated to constitute about 10% of total APβ in PR6006 and PR6007.

DISCUSSION

We have presented evidence that *apc* genes from the cyanelle genome of *C. paradoxa* are expressed as polypeptides in a cyanobacterium. These polypeptides carry a blue chromophore which is likely phycocyanobilin. Since this chromophore remains bound in the presence of 8 M urea, it is probably covalently linked. The cyanelle AP subunits were found in purified phycobilisomes from transformant *Synechococcus* sp. PCC 7002 cells and thus appear to be assembled into functional cores, since the spectroscopic properties of the phycobilisomes containing the cyanelle AP subunits were identical to those of the wild type. In vitro experiments have demonstrated that the intersubunit contact surfaces of APs are conserved, since hybrid APs can be assembled from purified α and β subunits from different cyanobacterial and red algal species (7). The present results suggest conservation of other structural features, such as those required for chromophore attachment and assembly into phycobilisomes.

The fact that cyanelle AP subunits are synthesized in the cyanobacterium suggests some functional homology in the mechanisms of gene expression in the cyanelle and cyanobacterium. We have previously shown that cyanelle AP apoprotein subunits are expressed in *E. coli*, probably from a cyanelle promoter (3). The 5′ termini of stable RNA species encoding cyanelle AP and PC genes have been mapped (15). Sequences upstream of these sites resemble both chloroplast and *E. coli* promoter sequences. Comparison of these cyanelle sequences and putative promoter sequences for *Synechococcus* sp. PCC 7002 *cpc* and *apc* genes shows similar sequence motifs (2, 8; unpublished data). *apc* genes in pAQE17CpAP2 might be expressed from the promoter for aminoglycoside phosphotransferase. However, the *apc* genes in pAQE17CpAP1 are not aligned with known vector-borne promoters and thus are probably transcribed from a cyanelle promoter.

Polypurine sequences of the type believed important to the initiation of protein synthesis are found in both cyanelle and *Synechococcus* sp. PCC 7002 PC and AP genes (2, 8, 15; unpublished data). Codon frequencies in *C. paradoxa apc* genes differ in some respects from those of *Synechococcus* sp. PCC 7002 *cpc* and *apc*. For example, the most common leucine codon of cyanelle *apc*, UUA, is not found in *Synechococcus* sp. PCC 7002 *cpc* and appears only once in *apc* (8; unpublished data). Codon usage differences such as this may account in part for the relatively low levels of cyanelle AP subunits found in transformant phycobilisomes. However, there are numerous other steps in which the efficiencies of transcription, translation, polypeptide processing, and phycobilisome assembly could be reduced for a heterologous gene or gene product.

Transfer of plastid genes into cyanobacteria may prove useful in studying other aspects of photosynthesis. A variety of genes encoding components of photosynthetic complexes have been cloned and mapped for the *C. paradoxa* cyanelle genome (13). The present results suggest the potential of this approach in examining the function of specific polypeptides in multiprotein complexes and in genetic complementation of mutations in photosynthetic processes.

ACKNOWLEDGMENTS

We thank R. D. Porter for helpful discussions and suggestions for improving the manuscript.

This work was supported by National Science Foundation grant DMB-8510770 to S.E.S., Public Health Service grant GM 31625 from the National Institutes of Health to D.A.B., and U.S. Department of Agriculture grants 82-CRCR-1-1080 to S.E.S. and 83-CRCR-1336 to D.A.B.

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