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In Vivo and In Vitro Characterization of the Light-Regulated *cpcB2A2* Promoter of *Fremyella diplosiphon*†

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When exposed to different spectral qualities of light, many cyanobacteria dramatically alter their phycobilisome rod composition in a process termed complementary chromatic adaptation. In the cyanobacterium *Fremyella diplosiphon*, this response is associated with differential expression of the *cpcB2A2*, *cpeBA*, and *cpeCDE* operons, which code for the phycobiliproteins phycocyanin and phycoerythrin and the phycoerythrin linker polypeptides, respectively. To define components of the signal transduction pathway involved in light-regulated expression of genes encoding phycobilisome polypeptides, we have used in vivo and in vitro techniques to identify *cis*-acting sequences and *trans*-acting factors necessary for the regulation of the red-light-inducible *cpcB2A2* operon. Deletion of the *cpcB2A2* upstream sequences to −76 bp with respect to the transcription start site had no effect on red-light induction of a *cpcB2A2*–β-glucuronidase (GUS) chimeric gene, while deletion to −37 bp abolished GUS expression. Furthermore, a fragment of the *cpcB2A2* gene from −76 to +25 bp linked to the untranslated leader of *cpcB1A1* (a constitutively expressed operon encoding phycocyanin) is sufficient to drive high-level GUS expression in red light. Therefore, the sequence between positions −76 and −37 is necessary for the expression of *cpcB2A2*, and the region extending from −76 to +25 is sufficient for red-light induction of the operon. Attempts were made to correlate the in vivo data with protein binding in the region upstream of the transcription start site of *cpcB2A2*. Using in vitro analysis, we detected two protein-binding sites in the *cpcB2A2* promoter which were localized to positions −162 to −122 and −37 to +25. Proteins from both red- and green-light-grown cells interacted with the former site, while only proteins present in extracts from red-light-grown cells interacted with the latter site. The data from both the in vivo and in vitro analyses suggest that while two regions upstream of the *cpcB2A2* transcription initiation site specifically bind proteins, only the binding site bordering the transcription start site is important for complementary chromatic adaptation.

To maximize the capture of light energy, some cyanobacteria acclimate to different light environments by altering the number and composition of the major light-harvesting antennae, the phycobilisomes (PBS). These multiprotein complexes are composed of chromophorylated phycobiliproteins and nonchromophorylated linker polypeptides which are organized into two structural domains, the core and the rods (3, 15–17, 40). The core is directly associated with thylakoid membranes and consists of two or three cylinders that are composed primarily of hexamers of the phycobiliprotein allophycocyanin (A_{max} = 650 nm). The rods radiate from the core and consist entirely of hexamers of phycocyanin (PC) (A_{max} = 620 nm) or of both PC and phycoerythrin (PE) (A_{max} = 566 nm). The allophycocyanin of the core and the PC and PE of the rods are each composed of two dissimilar subunits designated α and β. Specific nonchromophorylated linker polypeptides associate with each phycobiliprotein hexamer of the rod substructure. The organization of the phycobiliproteins in the PBS allows for the efficient vectoral transfer of energy through the rods and core to the chlorophylls of the photosynthetic reaction centers.

The filamentous cyanobacterium *Fremyella diplosiphon* (similar to *Calothrix* sp. strain PCC 7601) is one of a subset of cyanobacteria that sense light wavelength variations and respond by altering the levels of both PC and PE in the PBS rods. This process is termed complementary chromatic adaptation (CCA) (2, 36). The PBS rods of organisms that exhibit CCA contain high PE and low PC levels in green light (GL) and high PC and low PE levels in red light (RL).

Genes encoding PE, PC, and associated linker polypeptides from a number of different organisms have been characterized, and specific DNA probes have been used to determine the organization of these genes on the cyanobacterial genome and the effect of light quality on their transcription. These studies have revealed that phycobiliprotein and linker polypeptide genes are often clustered and that the control of PC and PE abundance in the PBS in response to different wavelengths of light is regulated primarily at the level of transcription (12, 27).

In *F. diplosiphon* there are three operons encoding PC. One of these operons, *cpcB3A3* (*B* and *A* designate genes for the β and α subunits, respectively), is expressed only under conditions of sulfur limitation (25). The other two, *cpcB1A1* and *cpcB2A2*, are relevant to CCA; transcription from the former is constitutive, while transcription from the latter occurs only in RL (10–12, 24). The PC encoded by *cpcB1A1* has been designated PC_c, while that encoded by *cpcB2A2* has been designated PC_i. Furthermore, genes encoding the linker polypeptides, *cpcH2I2D2*, which are required for the assembly of PC_i into PBS rods, are located downstream of and cotranscribed with *cpcB2A2* (22).

In contrast to the multiple genes encoding PC, there is only one operon encoding PE subunits, designated *cpeBA*, in *F. diplosiphon* (23). The PE-associated linker polypeptide operon, *cpeCDE*, is not contiguous to *cpeBA*; however, it is coordinately regulated with *cpeBA* with respect to light quality (13, 14).

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Our laboratory is interested in identifying the components of the signal transduction pathway controlling the process of CCA. One approach to characterizing this system is to define the *cis*-acting elements and *trans*-acting factors necessary for differential transcription of *cpcB2A2* and *cpeBA*. Recent studies have demonstrated that proteins bind to DNA sequences upstream of the transcription start site of *cpcB2A2* (37) and that at least one protein binds to sequences 45 to 67 bp upstream of the transcription start of *cpeBA* (33, 34). Phosphorylation may also play a role in the CCA signal transduction pathway. This is suggested by the findings that the affinity with which a protein binds to the *cpeBA* promoter is altered by its phosphorylation state (34) and that *rcaC*, a gene that complements a mutant that exhibits aberrant CCA, encodes a protein homologous to the response regulators of bacterial two-component regulatory systems (4, 31). The activation state of response regulators is controlled by the phosphorylation of specific aspartate residues (for a review, see reference 35).

In this paper we report the use of both in vivo reporter gene studies and in vitro binding studies in an initial characterization of the *cis* sequences and *trans*-acting factors that regulate the *cpcB2A2* operon. We have demonstrated that the sequence at −76 to +25 bp relative to the transcription start site of *cpcB2A2* is sufficient for RL-GL regulation. It was also demonstrated that a protein that interacts specifically with this region of the promoter can be detected only in extracts from RL-grown cells.

MATERIALS AND METHODS

Strains and growth conditions. Fd33, the short-filament strain of *F. diplosiphon*, was used in these studies (6). Cells were grown at 30°C on solid BG-11 medium buffered at pH 8.0 with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) or in liquid BG-11 medium aerated with 3% CO_2. Illumination was with RL or GL at 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ or with white light at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (27).

DNA methods. DNA restriction and -modifying enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and Pharmacia Biochemicals (Piscataway, N.J.). Amplitaq was purchased from Perkin-Elmer (Branchburg, N.J.). Sequencing and in vitro mutagenesis reactions were primed with the M13 universal and reverse primers, the pBluescript SK and KS primers (Stratagene, LaJolla, Calif.), or specific primers synthesized on a Biosearch 8600 oligonucleotide synthesizer. [α-^{32}P]dCTP and deoxyadenosine 5'-[α-[^{35}S]thio]triphosphate were from Dupont/NEN (Boston, Mass.).

DNA manipulations, including restriction enzyme analysis, agarose and polyacrylamide gel electrophoreses, ligations, transformations into *Escherichia coli*, production of single-stranded DNA templates, and nucleic acid hybridizations were performed as described by Sambrook et al. (32).

Generation of plasmids. All plasmids containing *cpcB2A2* sequences were derivatives of pFD126 (10). All *cpcB1A1* subclones were from a pUC8 clone containing a 4.1-kbp *Eco*RI fragment from λEMBL3 clone 4-10 (11, 12). A 713-bp fragment containing the *cpcB2A2* promoter was amplified from a 3.2-kbp *Hin*dIII-to-*Cla*I subclone by PCR in a Precision Scientific (Chicago, Ill.) thermal cycler. This PCR fragment extends from 298 bp upstream of the transcription start site to 51 bp downstream of the translation initiation site. The 3' oligonucleotide primer used in the amplification reaction contained a 2-bp substitution 36 and 40 bp downstream of the translation initiation site to create a *Bam*HI site at one end of the amplified fragment. This 703-bp *Hin*dIII-*Bam*HI fragment was cloned into pBluescript SK(+) (Stratagene) and sequenced with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio). This sequence was compared with that of the *Hin*dIII-*Cla*I subclone to ensure that no nucleotide changes occurred during the PCR; the resulting plasmid was designated $pPC_i(298)$. (The number in the parentheses indicates the number of nucleotides upstream of the *cpcB2A2* transcription start site in the promoter fragment.) A fragment containing the *cpcB1A1* promoter was also generated by PCR amplification. Nucleotides 36, 39, and 40 bp downstream of the translation initiation site were altered on one oligonucleotide to introduce a *Bam*HI site during the PCR. This 705-bp *cpcB1A1* promoter fragment, extending from 412 bp upstream of the transcription start site to the *Bam*HI site, was subcloned into pBluescript SK(+) as an *Eco*RI-*Bam*HI fragment and sequenced. This construct was designated $pPC_c(412)$.

To fuse these sequences to the β-glucuronidase (GUS) gene, the GUS gene plus a 5' polylinker region and a NOS terminator were excised from pB101.2 (Clonetech Laboratories, Inc., Palo Alto, Calif.). The ends of the fragment were made blunt with the Klenow fragment of DNA polymerase I and cloned into the *Pst*I site of pPL2.7 (5), in both orientations, creating p2.7GI (see Fig. 2A) and p2.7GII. The unique *Bam*HI site downstream of the *npt1* gene of pPL2.7 was eliminated prior to insertion of the GUS gene by digestion with *Bam*HI, creation of blunt ends, and religation. The 703- and 705-bp fragments containing the promoters of *cpcB2A2* and *cpcB1A1*, respectively, were fused to the 5' end of the GUS gene in p2.7GI and -II (see Fig. 2 for details) to generate translational fusions. The resulting plasmids in p2.7GI were designated $pPC_i(298)$G and $pPC_c(412)$G.

To create a series of 5' deletion constructs, $pPC_i(298)$G was linearized with *Pst*I and *Sal*I, and exonuclease III was used to digest the DNA from the *Sal*I restriction site in a processive manner (18). At various times after initiation of the exonuclease III reaction, the reaction was stopped and the truncated plasmids were religated. The plasmids, designated $pPC_i(182)$G, $pPC_i(159)$G, $pPC_i(76)$G, $pPC_i(37)$G, and $pPC_i(+73)$G, are depicted in Fig. 3. A + sign indicates a nucleotide position downstream of the transcription start site. To generate $pPC_i(123)$G, oligonucleotide-directed mutagenesis (1) was used to introduce a *Pst*I site in $pPC_i(298)$ at a position 122 bp upstream of the *cpcB2A2* transcription start site. The *cpcB2A2* fragment (*Sph*I-*Bam*HI) with this *Pst*I site was ligated into p2.7GI. A 176-bp fragment extending from the new *Pst*I site to an upstream *Pst*I site in the polylinker was excised, and the *Pst*I ends were ligated, leaving 123 bp of the original sequence upstream of the transcription start site.

To generate the internal deletion plasmid $pPC_i(\Delta 162\text{-}122)$G, a *Pst*I site and an *Eco*RV site were introduced into $pPC_i(298)$ by oligonucleotide-directed mutagenesis at positions 122 and 162 bp, respectively, upstream of the transcription start site. The 40-bp fragment from −162 to −122 bp was removed by digestion with *Pst*I and *Eco*RV. The *Pst*I overhang was made blunt with the Klenow fragment of DNA polymerase I, and the blunt ends were ligated. The altered promoter was transferred into the shuttle vector p2.7GI. The junctions of all of the constructs were sequenced.

Generation of chimeric PC_i/PC_c-GUS fusions. To make the chimeric constructs presented in Fig. 4, the *cpcB2A2* promoter region was linked to the *cpcB1A1* untranslated leader in pUC118. A *Hin*dIII (made blunt)-to-*Bam*HI fragment from $pPC_c(412)$ containing a 278-bp leader fragment extending from +10 to 41 bp downstream of the translation initiation site was cloned into the *Xba*I (made blunt) and *Bam*HI sites of pUC118, creating $pPC_c(+10)$, which we are abbreviating as pL

(for plasmid with the PC_c leader). *cpcB2A2* promoter fragments from $pPC_i(298)G$ and $pPC_i(76)G$ were excised with *Sph*I (a site just upstream of the *cpcB2A2* sequence) and *Pvu*II (a site 25 nucleotides downstream of the transcription start site). These fragments were cloned into the *Sph*I and *Pst*I (made blunt) sites of pL, resulting in the plasmids designated $pPC_i(298)L$ and $pPC_i(76)L$. To fuse these fragments to GUS, the *Sph*I-to-*Bam*HI fragments containing both the PC_i promoter and PC_c leader sequences were excised from the pPC_iL plasmids and cloned into the *Sph*I-*Bam*HI polylinker sites of p2.7GI, creating $pPC_i(298)LG$ and $pPC_i(76)LG$. As a control, the leader region of *cpcB1A1* was fused to the 5′ end of the GUS gene by cloning the PC_c leader from pL (*Sal*I-*Bam*HI fragment) into p2.7GI to create pLG.

Gene transfer, plasmid rescue, and copy number determination. Gene transfer by electroporation and plasmid rescue were performed as previously described (5). Rescued plasmids were mapped by restriction enzyme analysis. The plasmid copy number (relative to the genome copy number) was determined in the electroporants by the following method. Total genomic DNA (3 to 5 μg) isolated from cells grown in RL or GL was digested to completion with *Eco*RI, electrophoresed in a 0.8% agarose gel, and transferred to a charged nylon membrane (Schleicher and Schuell, Keene, N.H.) by the method of Reed and Mann (30). Nylon membranes with DNA from strains harboring plasmids with the PC_i promoter-GUS fusion constructs were hybridized to a gel-purified (Qiagen, Chatsworth, Calif.) 379-bp *Pvu*II-*Bam*HI fragment derived from $pPC_i(298)$ that contains 339 bp of the untranslated leader and 40 bp of the PC_i coding sequence. The hybridization probe was labeled by the random-primer method (14a). Two restriction fragments hybridize to this probe: a plasmid-containing fragment of 8 kbp and a genomic fragment of 5 kbp. The relative levels of hybridization were quantitated with a Molecular Dynamics PhosphorImager (400A). The same strategy was used to determine the relative plasmid copy number in strains harboring the PC_c promoter-GUS fusion constructs except that a 282-bp *Hin*dIII-*Bam*HI fragment from $pPC_c(412)$ that contains the untranslated leader region was used as a hybridization probe.

GUS assays. Axenic electroporants were grown in white light in 50 ml of BG-11 medium with 25 μg of kanamycin per ml [BG-11 (kan_{25}) medium] to a cell density corresponding to an A_{750} of 0.5 to 1.8. Approximately 1 ml of this culture was inoculated into 50 ml of BG-11 (kan_{25}) medium that was placed in RL or GL for 60 h, at which time the cells attained an A_{750} of 0.5 to 1.8. Duplicate samples of 1 ml were pelleted by centrifugation for 5 min at full speed in a microcentrifuge at room temperature. Cell pellets were rinsed with 1 ml of GUS assay buffer (50 mM sodium phosphate [pH 7.0], 1 mM EDTA) and either frozen in liquid nitrogen or resuspended in 1 ml of the same buffer. Cell lysis and quantitation of GUS activity were performed by using 10 to 100 μl of cell suspensions (39), and activity was quantitated as nanomoles of product per milligram of protein per minute. Protein concentrations were determined with the Pierce (Rockford, Ill.) BCA protein assay reagent kit. All reactions were done in duplicate, and A_{405} values were taken at two to three time points between 10 and 30 min. The GUS values were not affected if cell pellets were frozen at −80°C prior to the assays.

RNA isolation and Northern (RNA) blot analysis. Electroporants were grown in GL in liquid BG-11 (kan_{25}) medium to an A_{750} of 0.5 to 1.0, at which time 50 ml of the culture was transferred to RL. RNA was isolated by the method of Collier and Grossman (9) from cells continuously maintained in GL and from those transferred from GL to RL. The RNA was resolved by electrophoresis on formaldehyde-agarose gels, transferred to nitrocellulose membranes, and hybridized (8) to the ^{32}P-labeled GUS gene (*Hin*dIII-*Eco*RI fragment from pB101.2).

Protein extract preparation. Protein was isolated from 250-ml cultures of Fd33 cells grown in BG-11 medium in either RL or GL. The cell densities ranged from an A_{750} of 0.6 to 1.2. Cells were pelleted at 6,500 × *g*, rinsed with 25 ml of binding buffer (50 mM Tris [pH 7.0], 50 mM KCl, 10 mM $MgCl_2$, 15% glycerol, and 1 mM dithiothreitol), and resuspended in 3 to 6 ml of the same buffer. After the cells were lysed by passage through a French pressure cell at 18,000 lb/in^2, the cell debris and membranes were removed by two centrifugations; the first was at 10,000 × *g* for 10 min, and the second was at 100,000 × *g* for 90 min. The high-speed supernatant was fractionated by the addition of solid ammonium sulfate to 35 and 65% saturation. Ammonium sulfate-precipitated proteins were pelleted, resuspended in a minimal volume of binding buffer, and dialyzed twice against the same buffer at 1,000 times the volume of the samples. All steps of the isolation were done at 4°C. Crude protein extracts and ammonium sulfate-fractionated proteins were quantitated with the Pierce BCA protein assay reagent kit and stored at −80°C.

DNA mobility shift assays. Restriction fragments of the *cpcB2A2* promoter (4 μg) were end labeled by filling in 5′ overhang sequences with the Klenow fragment of DNA polymerase I and α-^{32}P-labeled deoxyribonucleosides (1). The radioactive fragments were purified by polyacrylamide gel electrophoresis and eluted overnight at room temperature in 0.5 ml of 0.5 M ammonium acetate–1 mM EDTA. The eluate was passed through a 0.2-μm sterile syringe filter and ethanol precipitated with 1 μg of pBluescript SK(+) DNA. The quantity of the probe was determined by measuring the counts per minute of the DNA fragment in the polyacrylamide gel slice and of the purified fragment. The percentage of the labeled fragment recovered is assumed to be the percentage of DNA recovered from the labeling reaction (i.e., percentage of 4 μg). The two radiolabeled *cpcB2A2* DNA fragments extended from −298 to +25 bp (from a *Hin*dIII to a *Pvu*II restriction site [see Fig. 1]) and from −76 to +25 bp [a *Sph*I-to-*Sal*I fragment from $pPC_i(76)LG$ (see Fig. 4)]. DNA binding reactions, competition experiments, and gel electrophoresis conditions were as described previously (33) with some modifications; the reactions were performed with 5 fmol of labeled DNA and 1 to 4 μg of polynucleotide dIdC (Pharmacia) as a nonspecific competitor, and the electrophoresis reservoir buffer was 90 mM Tris–90 mM borate–1 mM EDTA (1× TBE). The DNA fragments used in the competition assays were purified from agarose gels by using the Qiaex DNA gel extraction kit from Qiagen and quantitated on agarose gels. The fragments were derived from the plasmids as follows: (i) $PC_i(-298/+25)$ and $PC_i(-76/+25)$ as described above; (ii) $PC_i(-170/-124)$ was synthesized as two 46-mer oligonucleotides, cloned into the pBluescript SK(+) at an *Eco*RV site and excised from the vector by digestion with *Hin*dIII and *Eco*RI; (iii) $PC_i(-37/+25)$ is an *Sph*I-*Sal*I fragment from $pPC_i(37)G$; (iv) $PC_i(-76/-34)$ is a *Hin*dIII-*Eco*RI fragment from a *Dra*I-to-*Sph*I clone of $pPC_iG(76)$ in pUC118; (v) pPC (−412/+15) is a *Hin*dIII-*Eco*RI fragment from $pPC_c(412)$; and (vi) $PC_c(-61/+15)$ is a *Dde*I-*Hin*dIII fragment from $pPC_c(412)$.

RESULTS

Constructs and transformation by electroporation. The change in PC levels in response to RL and GL is controlled

primarily by the rate of *cpcB2A2* transcription in *F. diplosiphon* (27). To define the regions of the promoter necessary for light-regulated expression of the *cpcB2A2* operon, constructs were generated in which a PC promoter fragment was fused to GUS. The promoter and leader sequences used in these fusion constructs are presented in Fig. 1. The transcription start sites for *cpcB2A2* and *cpcB1A1* and a partial sequence of the upstream regulatory region of both operons were previously reported (11). Figure 1 provides additional sequence information extending the characterized regions to 298 bp upstream of the transcription start site of *cpcB2A2* and 412 bp upstream of the transcription start site of *cpcB1A1*. This sequence also corrects some errors in the previously published sequence. The transcription start sites for both *cpcB2A2* and *cpcB1A1* are marked by thin arrows and designated +1. Sequences upstream of the transcription initiation site are preceded by a minus sign, while those downstream are preceded by a plus sign. Both *cpcB2A2* and *cpcB1A1* contain direct repeats in the −35 region of the promoter. These repeats, 13 bp each in *cpcB2A2* and 6 bp each in *cpcB1A1*, are underlined with boldface arrows.

Promoter fragments extending from −298 bp (*cpcB2A2*) or −412 bp (*cpcB1A1*) to a *Bam*HI site introduced in the coding sequence of both operons were fused to the GUS gene in the vector p2.7GI (Fig. 2A). This resulted in translational fusions; the junctions are shown in Fig. 2B. For each, the fusion products contain 13 amino acids of the PC β subunit, 7 amino acids derived from the polylinker region of the vector (designated linker in Fig. 2B) and the complete GUS open reading frame. One orientation of the promoter-GUS fusion is shown in Fig. 2A, but the opposite orientation was also generated by cloning the promoter insert into p2.7GII. The shuttle vector, plasmids with both orientations of the promoterless-GUS gene construct, and the *cpcB2A2*-GUS and *cpcB1A1*-GUS plasmids were electroporated into Fd33. Electroporants (stably transformed with pPL2.7 constructs) were selected for kanamycin resistance.

GUS activity and deletion analysis. The GUS activities in electroporants grown in RL and GL were quantitated. Initial experiments demonstrated that strains harboring plasmids containing the promoterless GUS gene, in either orientation, had little or no GUS activity (data not shown). Electroporants with $pPC_i(298)G$ exhibited high levels of GUS activity in RL and low levels (equivalent to that of the promoterless GUS construct) in GL, while electroporants harboring $pPC_c(412)G$ exhibited high levels of GUS activity in both RL and GL (Fig. 3). The orientation of the chimeric gene within the vector appeared to have no effect on expression (data not shown).

These results demonstrate that the 703-bp *cpcB2A2* fragment consisting of 298 bp upstream of the transcription start site, the 364-bp untranslated leader, and 41 bp of coding sequence contains the necessary elements for photoregulation of the *cpcB2A2* operon. To localize *cis*-acting sequences required for the light-regulated expression of *cpcB2A2*, a 5′ deletion analysis was performed. The *cpcB2A2* promoter was truncated to positions −182, −159, −123, −76, −37, and +73 bp relative to the transcription initiation site. The 5′ endpoints of the promoter deletions are marked by boldface, underlined letters in the sequence in Fig. 1. These deletion constructs are represented diagrammatically in Fig. 3. All 5′ deletions extending to 76 bp upstream of the transcription start site had little effect on the RL-GL regulation of GUS. Strains containing $pPC_i(76)G$ express high levels of GUS in RL and low levels in GL. However, in strains that contain $pPC_i(37)G$, RL induction of GUS is abolished and there is a low basal level of GUS expression in both RL and GL. This indicates that the sequence located downstream of position −76, including at least part of the sequence between −76 and −37, is important for expression of *cpcB2A2* in RL.

Since all promoter-GUS constructs are maintained as self-replicating plasmids in *F. diplosiphon*, it is possible that the copy number of the plasmid in each strain may vary and affect the GUS activity level. To address this problem, at least two isolates of each strain were examined for both plasmid copy number and GUS activity after growth in RL and GL (Fig. 3). The average plasmid copy number of most strains grown in either RL or GL was five times the copy number of the genome. The strain harboring $pPC_i(76)G$ exhibits a slightly higher copy number and also a twofold-higher level of GUS activity in GL, although on the basis of statistical analysis this latter difference may not be significant ($P = 0.013$).

***cpcB2A2/cpcB1A1* chimeric GUS constructs.** The deletion analysis revealed that a fragment containing 76 bp upstream of the *cpcB2A2* transcription start site plus the complete 364-bp leader is sufficient for the RL induction of the *cpcB2A2* operon. To determine if the elements required for RL-GL regulation are located in the 5′ untranslated leader region of the *cpcB2A2* operon, we replaced these sequences with those from the constitutive PC operon (*cpcB1A1*) and tested whether transcription of these constructs was induced in RL. Specifically, a 101-bp *cpcB2A2* fragment extending from −76 bp to +25 bp was linked to the *cpcB1A1* leader (+10 to the *Bam*HI fusion site) and fused to the GUS coding sequence [$pPC_i(76)$LG in Fig. 4]. Two additional GUS constructs, one with a larger region of the *cpcB2A2* promoter fused to the *cpcB1A1* leader [$pPC_i(298)$LG] and a second in which no promoter is fused to the *cpcB1A1* leader (pLG), served as controls in these experiments. The individual constructs are diagrammed in Fig. 4B along with the in vivo GUS activities for strains harboring these constructs. In strains with the *cpcB1A1* leader linked to GUS (pLG) there is constitutive GUS activity that is approximately 40 times greater than the activity observed in strains containing p2.7GI, the promoterless GUS construct (data not shown), and between 5 and 10 times greater than that in strains containing the *cpcB2A2* leader linked to the GUS gene [$pPC_i(+73)G$]. In GL, the strains harboring $pPC_i(298)$LG exhibit GUS activities similar to those of strains harboring pLG, while strains containing $pPC_i(76)$LG express GUS at slightly higher levels. This latter difference may not be statistically significant ($P = 0.08$). In RL, the levels of GUS activity in strains harboring $pC_i(298)$LG and $PC_i(76)$LG increase dramatically; they are between 7- and 16-fold higher than the levels observed in GL. These data provide strong evidence that the 101-bp fragment from *cpcB2A2* that extends from −76 to +25 bp is sufficient for RL induction of the *cpcB2A2* operon.

The average plasmid copy numbers for strains with pLG, $pPC_i(298)$LG, and $pPC_i(76)$LG are two- to eightfold higher than those for the strains in the deletion analysis (Fig. 3). Both the plasmid copy numbers and GUS activities for the former strains were determined for cultures that were grown to an A_{750} of between 0.9 and 1.8, while these values for the latter strains were determined for cultures grown to an A_{750} of 0.5 to 1.5. Differences in cell densities make a considerable difference in plasmid copy number (unpublished data); cells grown to an A_{750} of below 0.7 can have a plasmid-to-genome ratio of less than half of that observed in cells grown to an A_{750} of above 1.0. In spite of the high plasmid copy number in the strains with $pPC_i(298)$LG and $pPC_i(76)$LG, the total GUS activity in RL is no more than twofold higher than the values measured in strains harboring $pPC_i(298)G$ and $pPC_i(76)G$ (Fig. 3). This suggests that factors other than the copy number of the

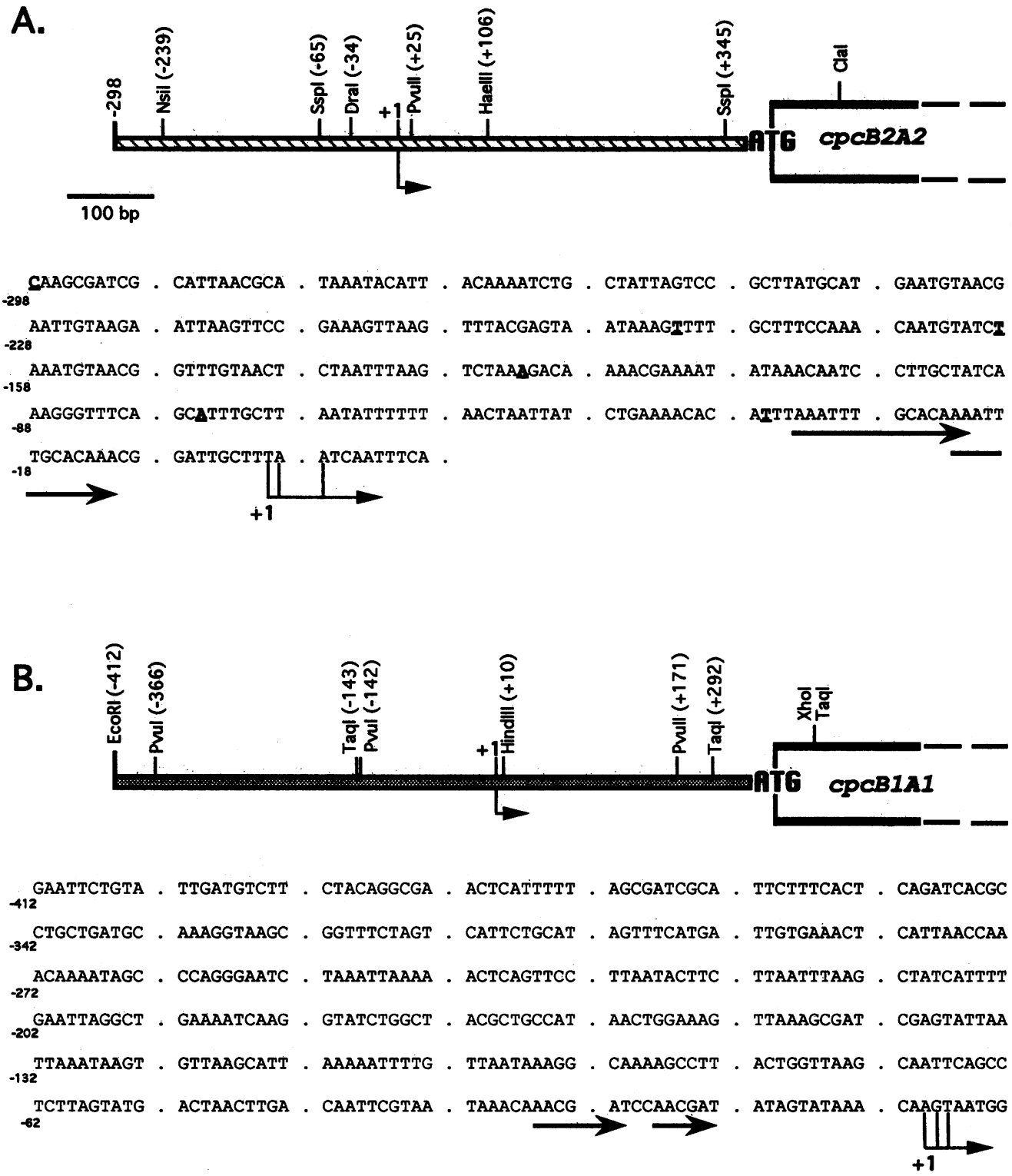
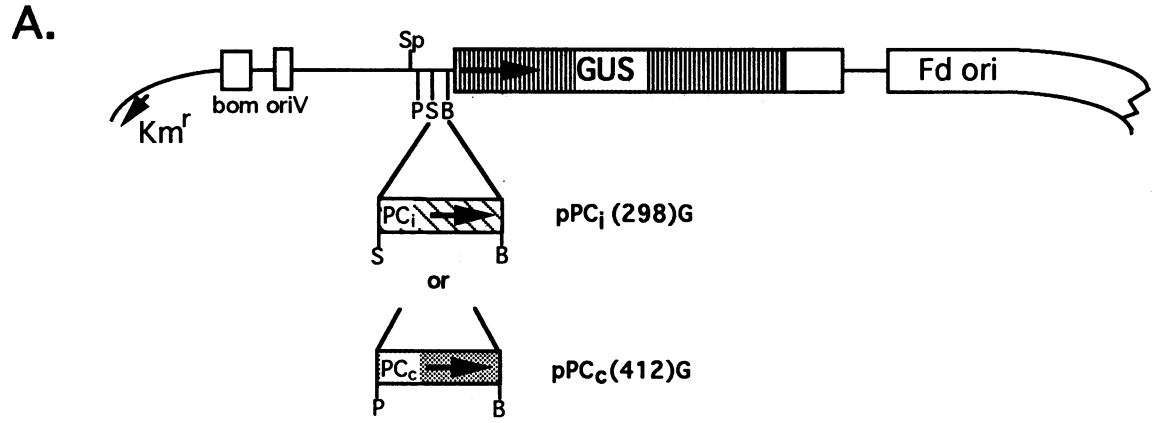


FIG. 1. Restriction map and nucleotide sequences of the upstream regulatory regions of the *cpcB2A2* (A) and *cpcB1A1* (B) operons. Hatched and stippled boxes represent the regions upstream of the translation start sites of the *cpcB2A2* and *cpcB1A1* operons, respectively, with thin arrows and a +1 marking the transcription start sites. The open boxes represent the coding regions. Relevant restriction enzyme sites are shown and numbered (in parentheses) with respect to the transcription start site. The sequences of the two operons from position −98 to the ATG start codon were previously reported (11). Changes from the published sequence of *cpcB2A2* are at nucleotides −8, −17, and −18, and a change from that of *cpcB1A1* is at position −92. The nucleotide position with respect to the transcription start site is marked at the left of each line of sequence. Boldface arrows mark direct repeats, and the nucleotides that are boldface and underlined are the 5′ ends of the deletion constructs depicted in Fig. 3.



B.

PC$_i$ Linker GUS

ATG CTT GAC GCT TTT ACT AAA GTA GTT TCC CAG GCG GAT CCC CGG GTA GGT CAG TCC CTT ATG TTA CGT
Met Leu Asp Ala Phe Thr Lys Val Val Ser Gln Ala Asp Pro Arg Val Gly Gln Ser Leu Met Leu Arg

PC$_c$ Linker GUS

ATG TTA GAC ACA TTT GCA AAA GTA GTC TCC CAA GCG GAT CCC CGG GTA GGT CAG TCC CTT ATG TTA CGT
Met Leu Asp Thr Phe Ala Lys Val Val Ser Gln Ala Asp Pro Arg Val Gly Gln Ser Leu Met Leu Arg

FIG. 2. PC-GUS translational fusions. (A) Physical maps of the *cpcB2A2* (PC_i) and *cpcB1A1* (PC_c) promoters fused to GUS in the p2.7GI shuttle vector. The shuttle vector includes the *F. diplosiphon* origin of replication (Fd ori), *bom* site, *oriV*, and kanamycin resistance gene (Km^r), as previously described (5), and the GUS gene, as described in Materials and Methods. The *cpcB2A2* and *cpcB1A1* upstream regions and the GUS open reading frame are represented by hatched, stippled, and striped boxes, respectively. The direction of transcription is indicated by a boldface arrow. The unmarked open box to the right of GUS represents the NOS terminator. The designations of the plasmids harboring each of these constructs are at the right of the PC promoter boxes, and restriction enzymes sites shown are abbreviated as follows: B, *Bam*HI; P, *Pst*I; S, *Sal*I; Sp, *Sph*I. (B) Nucleotide and amino acid sequences at the junctions of the PC-GUS fusion constructs. The 5′ ends of the coding regions of *cpcB2A2* and *cpcB1A1* were ligated to GUS at a *Bam*HI site 21 nucleotides (linker) upstream of the ATG translation initiation codon. The *Bam*HI site is underlined, and the nucleotides altered by site-directed mutagenesis to introduce this site into the PC coding sequence are in boldface.

plasmid in a given strain may limit the absolute level of expression from the *cpcB2A2* promoter.

Northern blot analysis. To ensure that GUS activity accurately represents GUS transcript levels, RNA isolated from electroporants either grown in GL or transferred from GL to RL were analyzed by Northern blot analysis. GUS transcripts were not detectable in GL-grown cells but accumulated to high levels 3 and 6 h after the cells had been transferred to RL (Fig. 5). The level of GUS activity in electroporants with pPC_i(298)G, pPC_i(76)G, and pPC_i(37)G was reflected by the level of GUS mRNA. The diffuse mRNA signal seen in Fig. 5 is indicative of the unstable nature of the GUS transcript. In strains harboring pPC_i(37)G, essentially no GUS transcript could be detected. To check the integrity of the RNA, to ensure that equivalent levels of RNA were loaded in each gel lane, and to demonstrate proper regulation of the *cpcB2A2* operon in the electroporants, the blots were stripped of the labeled GUS probe and rehybridized with a fragment containing the *cpcB2A2* or the *cpcB1A1* leader sequence. Transcripts from the endogenous *cpcB2A2* operon were 1.6 and 3.8 kb, as previously noted (10), and were detected only in RL-grown cells or GL-grown cells that had been transferred to RL and left for 3 and 6 h; they were never detected in GL-grown cells (data not shown). No significant degradation of the transcripts was observed, and the hybridization signals were approximately the same for RNA samples isolated from strains that were exposed to similar light conditions.

DNA binding activities in protein extracts from RL- and

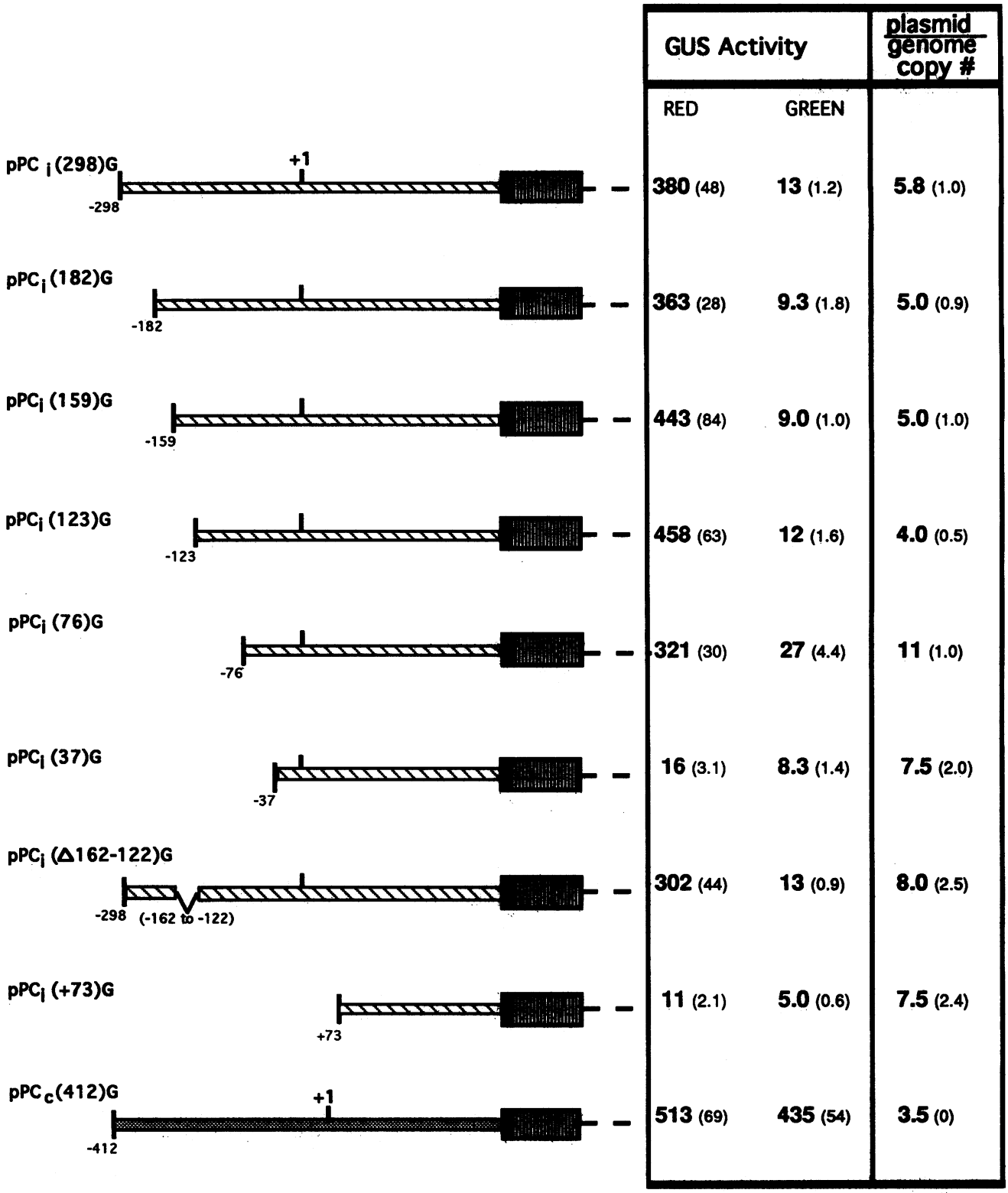
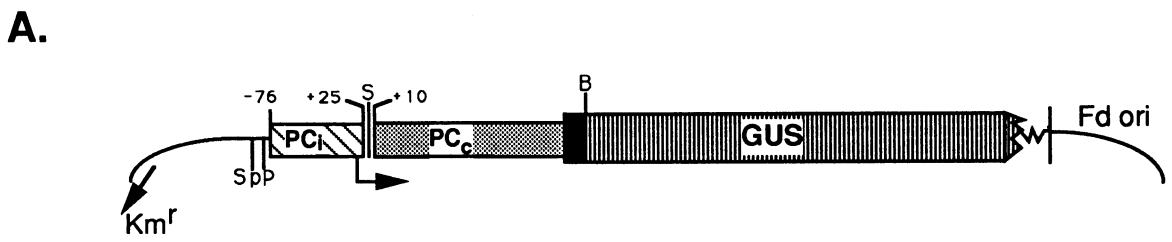


FIG. 3. Deletion analysis of the RL-inducible *cpcB2A2* promoter. The various *cpcB2A2* promoter-GUS fusion constructs are represented schematically. The designations of the plasmids containing these constructs and the positions of the 5′ ends with respect to the transcription start site are to the left. GUS activities and plasmid copy numbers are to the right. The hatched box represents the region of *cpcB2A2* upstream of the translation start site, with a +1 marking the transcription start site. A stippled box represents the region of *cpcB1A1* upstream of the translation start site (in the last construct presented). The GUS open reading frame is depicted by a striped box. The V in the schematic for pPC$_i$(Δ162-122)G represents an internal deletion. The GUS activity is given as the average nanomoles of product per milligram of protein per minute for two independent electroporants assayed in 4 to 10 separate experiments. The A_{750} of the cultures used in these experiments ranged from 0.5 to 1.5. The standard errors for both GUS activity and plasmid copy number are in parentheses.



B.

	GUS Activity		plasmid / genome copy #
	RED	GREEN	
pPC$_c$(412)G	513 (69)	435 (54)	3.5 (0)
pLG	72 (15)	59 (10)	24 (5.0)
pPC$_i$(76)LG	605 (78)	91 (11)	22 (3.0)
pPC$_i$(298)LG	644 (51)	38 (4.1)	34 (8.8)
pPC$_i$(+73)G	11 (2.1)	5.0 (0.6)	7.5 (2.4)

FIG. 4. PC$_i$ promoter/PC$_c$ leader-GUS fusion constructs. (A) Physical map of the PC$_i$(−76)/PC$_c$-GUS construct in the shuttle vector pPL2.7. The hatched, stippled, solid, and striped boxes represent *cpcB2A2* (PC$_i$) upstream sequences, *cpcB1A1* (PC$_c$) upstream sequences, part of the *cpcB1A1* coding sequence, and the GUS gene, respectively. Restriction enzyme sites used in cloning are designated as in Fig. 2. Numbers shown above the upstream regions represent the 5′ or 3′ end, in base pairs, relative to the transcription start site of the *cpcB2A2* (−76 to +25) and the *cpcB1A1* (+10 to ATG) operons. (B) Effect of replacing the *cpcB2A2* leader with the *cpcB1A1* leader on GUS activity in RL and GL. The designations of the strains containing the constructs and the positions of the 5′ ends (relative to the transcription start site) are to the left of the schematic representation. The average GUS activities and plasmid copy numbers with respect to that of the genome are on the right. The nucleotide position of the 5′ end with respect to the transcription start site (+1) is indicated. For pLG, pPC$_i$(298)LG, and pPC$_i$(76)LG, the average GUS activity and plasmid copy number were derived from four separate experiments with two independent isolates that were grown to an A_{750} of 0.9 to 1.8. For strains harboring pPC$_c$(412)G and PC$_i$(+73)G, averages were obtained from 5 to 10 experiments with two independent isolates grown to an A_{750} of 0.5 to 1.5. Standard errors are in parentheses.

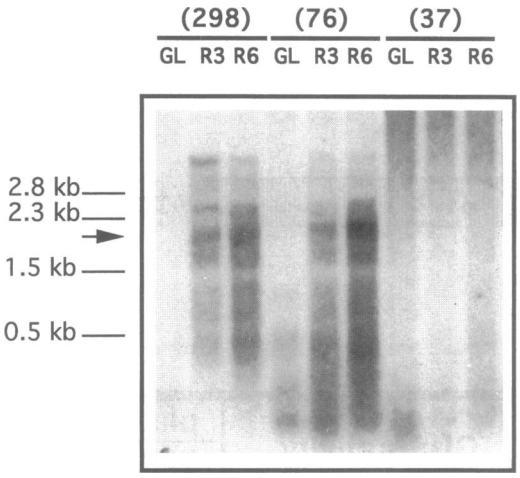


FIG. 5. Northern blot analysis of *cpcB2A2* promoter-GUS deletion constructs. An autoradiogram of total RNA isolated from strains harboring $pPC_i(298)G$, $pPC_i(76)G$, and $pPC_i(37)G$ (designated 298, 76, and 37, respectively) grown in GL or transferred from GL to RL and left for 3 (R3) or 6 (R6) h and then probed with the GUS gene is shown. The sizes marked on the left were derived from the positions of the rRNA (2.8 and 1.5 kb) and two rRNA degradation products (2.3 and 0.5 kb).

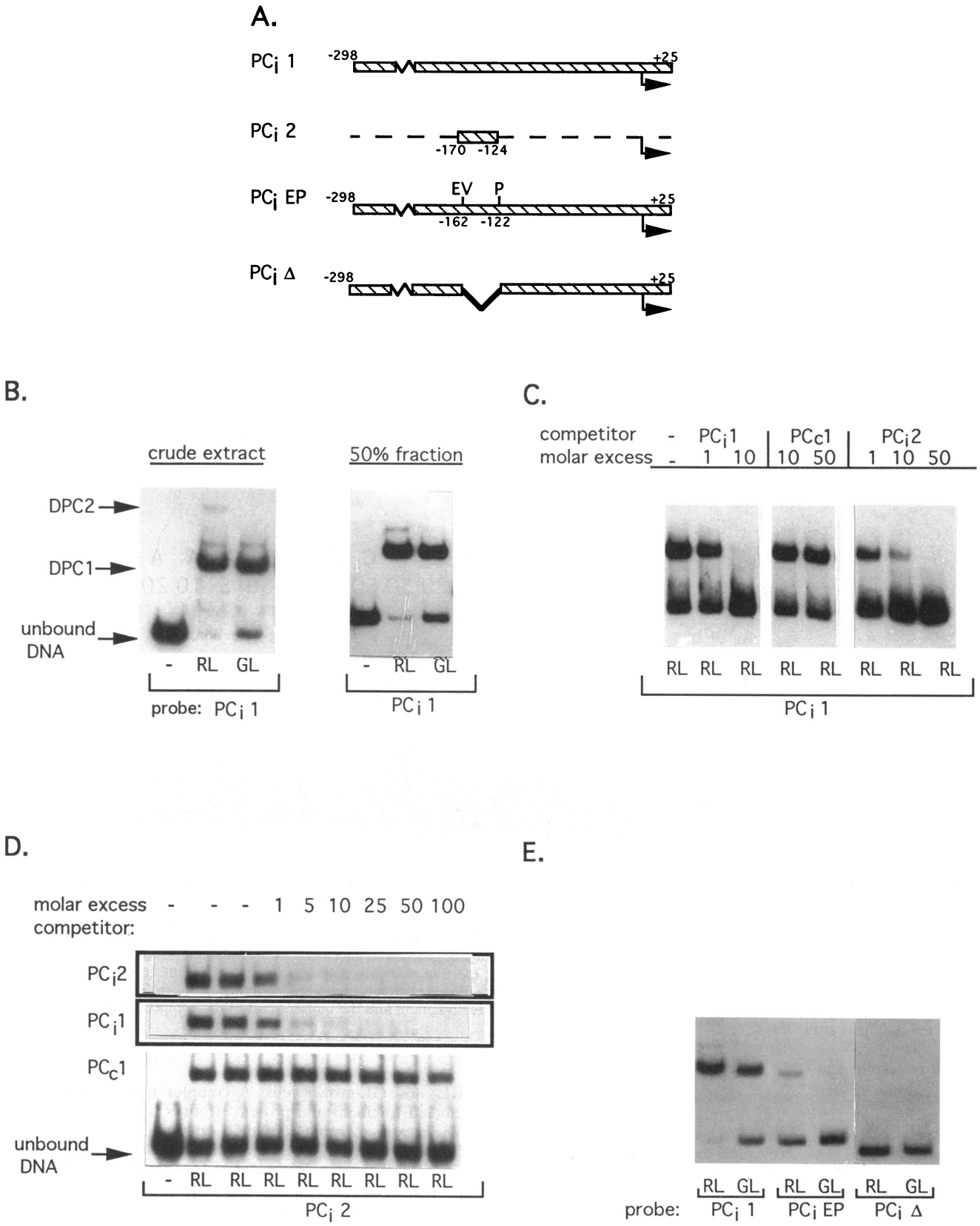
GL-grown cells. Gel mobility shift assays were used to characterize *trans*-acting factors that bind specifically to *cpcB2A2* upstream regulatory sequences. Both crude and fractionated extracts (30, 40, 50, 60, and 80% ammonium sulfate) were isolated from RL- and GL-grown cells and incubated with DNA from upstream of the *cpcB2A2* coding region. DNA complexes were detected as bands of reduced mobility on autoradiograms when the fragment designated PC_i1 (Fig. 6A), a *cpcB2A2* promoter fragment extending from −298 to +25 bp, was used. One abundant shifted band (labeled DPC1 for DNA-protein complex in Fig. 6B) was observed when PC_i1 was incubated with crude protein extracts or proteins precipitated by 50% saturation of the extract with ammonium sulfate (Fig. 6B). The band shift was observed with extracts from either RL- or GL-grown cells; however, the quantity of the labeled probe in the shifted band was slightly greater after incubation with the former. A second, less abundant band (marked DPC2) was detected when PC_i1 was incubated with crude RL protein extracts. This protein-DNA binding interaction was not seen in any of the ammonium sulfate fractions under these assay conditions, and the intensity of the band varied with different preparations of the crude protein extracts. A minor band was also sometimes seen immediately above DPC1; it was not consistently observed.

Localization by competition and deletion analysis. Competition studies were performed to determine the relative affinities of the RL and GL binding proteins for the *cpcB2A2* and *cpcB1A1* promoter sequences. Competition experiments demonstrated that a 5- to 10-fold excess of unlabeled PC_i1 eliminated the binding of labeled PC_i1 to the RL and GL proteins that formed DPC1 (Fig. 6C). However, a 50-fold excess of PC_c1 (*Hin*dIII-*Eco*RI fragment from the *cpcB1A1* promoter) had no effect on complex formation (Fig. 6C).

DNase I footprint analysis of DPC1 indicated that the DNA-protein binding site extended from −162 to −132 bp in the *cpcB2A2* promoter (data not shown). However, the complex was unstable and difficult to footprint, and in order to verify the location of DPC1, the 46-bp region extending from −170 to −124 bp was synthesized as two oligonucleotides, annealed, cloned into pBluescript, and used directly as an unlabeled competitor fragment (designated PC_i2 in Fig. 6A). A 10-fold excess of unlabeled PC_i2 greatly reduced and a 50-fold excess prevented complex formation with labeled PC_i1 (Fig. 6C). When labeled PC_i2 was incubated with RL and GL extracts, a band with reduced mobility was detected (Fig. 6D [GL data not shown]). A 10-fold excess of unlabeled PC_i1 and PC_i2 eliminated complex formation with labeled PC_i2, while a 100-fold excess of PC_c1 reduced the amount of complex formed by only about one-half (Fig. 6D). These competition experiments show that proteins in RL and GL extracts bind specifically to and have similar affinities for PC_i1 and PC_i2.

To confirm that DPC1 is formed by the association of a protein with the binding site centered at position −142, an internal deletion between positions −162 and −122 ($PC_i\Delta$) was made after an *Eco*RV site was introduced at −162 and a *Pst*I site was introduced at −122 (PC_iEP). PC_iEP and $PC_i\Delta$ (Fig. 6A) were labeled and used in gel mobility shift assays with RL and GL ammonium sulfate-fractionated proteins (Fig. 6E). DPC1 formation was significantly reduced when RL and GL proteins were incubated with the labeled PC_iEP fragment, and there was no detectable protein complex formation when labeled $PC_i\Delta$ was used in binding reactions. The *cpcB2A2* fragment (−298 to +25) with the 40-bp deletion was ligated to GUS, creating the plasmid designated $pPC_i(\Delta-162-122)G$, which was then electroporated into Fd33 (Fig. 3). The GUS activity in these electroporants was the same as that seen with those containing $pPC_i(298)G$; high levels of GUS activity were exhibited in RL, and very low levels were exhibited in GL. Taken together, the competition assays and the gel mobility shift assays with $PC_i\Delta$ demonstrate that proteins in RL and GL extracts bind specifically to a region centered at 142 bp

FIG. 6. DNA gel mobility shift assays. (A) Four fragments from the *cpcB2A2* operon. The horizontal lines represent the upstream region of the *cpcB2A2* operon extending from position −298 to +25, and the arrow marks the transcription initiation site. The *Eco*RV (EV) and *Pst*I (P) sites introduced by site-directed mutagenesis and the corresponding nucleotide positions are marked. The V represents a deletion between the EV and P sites, and the zigzag line indicates that the region extending from −298 to −162 bp is not to scale. (B to E) Crude or ammonium sulfate-purified proteins were isolated from *F. diplosiphon* cultures grown in RL or GL and incubated with one of four fragments from the *cpcB2A2* operon (PC_i1, PC_i2, PC_iEP, and $PC_i\Delta$) shown in panel A. Reactions were performed as described in Materials and Methods with 6 to 12 μg of protein and 0.001 to 0.005 pmol of DNA. (B) Crude and 50% ammonium sulfate-saturated protein extracts were isolated from RL- and GL-grown cells (as marked) and incubated with probe PC_i1. Unbound DNA and DNA-protein complexes 1 (DPC1) and 2 (DPC2) are marked. (C) Competition of PC_i1, PC_c1 (positions −412 to +10 of *cpcB1A1*), and PC_i2 for RL proteins (precipitated with 50% ammonium sulfate) that bind to the PC_i1 probe and form DPC1. The probe (PC_i1) and light conditions in which the cells used for protein extraction were grown (RL) are given at the bottom of the gel. The competitor DNA fragments and molar quantity of competitor relative to the probe are indicated at the top. (D) Competition of PC_i1, PC_i2, and PC_c1 for RL proteins (50% ammonium sulfate purified) that bind to the PC_i2 probe. The notations are as in panel C except that the competitor DNA is indicated to the left. (E) Gel mobility shift assays with RL and GL proteins (50% ammonium sulfate purified) and labeled PC_i1, PC_iEP, or $PC_i\Delta$ as indicated.


A.
PCi 1
-298
+25
PCi 2
-170 -124
PCi EP
-298
EV P
+25
-162 -122
PCi Δ
-298
+25
B.
crude extract
50% fraction
DPC2
DPC1
unbound DNA
- RL GL
probe: PCi 1
- RL GL
PCi 1
C.
competitor - PCi1 PCc1 PCi2
molar excess - 1 10 10 50 1 10 50
RL RL RL RL RL RL RL RL
PCi 1
D.
molar excess - - - 1 5 10 25 50 100
competitor:
PCi2
PCi1
PCc1
unbound DNA
- RL RL RL RL RL RL RL RL
PCi 2
E.
RL GL RL GL RL GL
probe: PCi 1 PCi EP PCi Δ

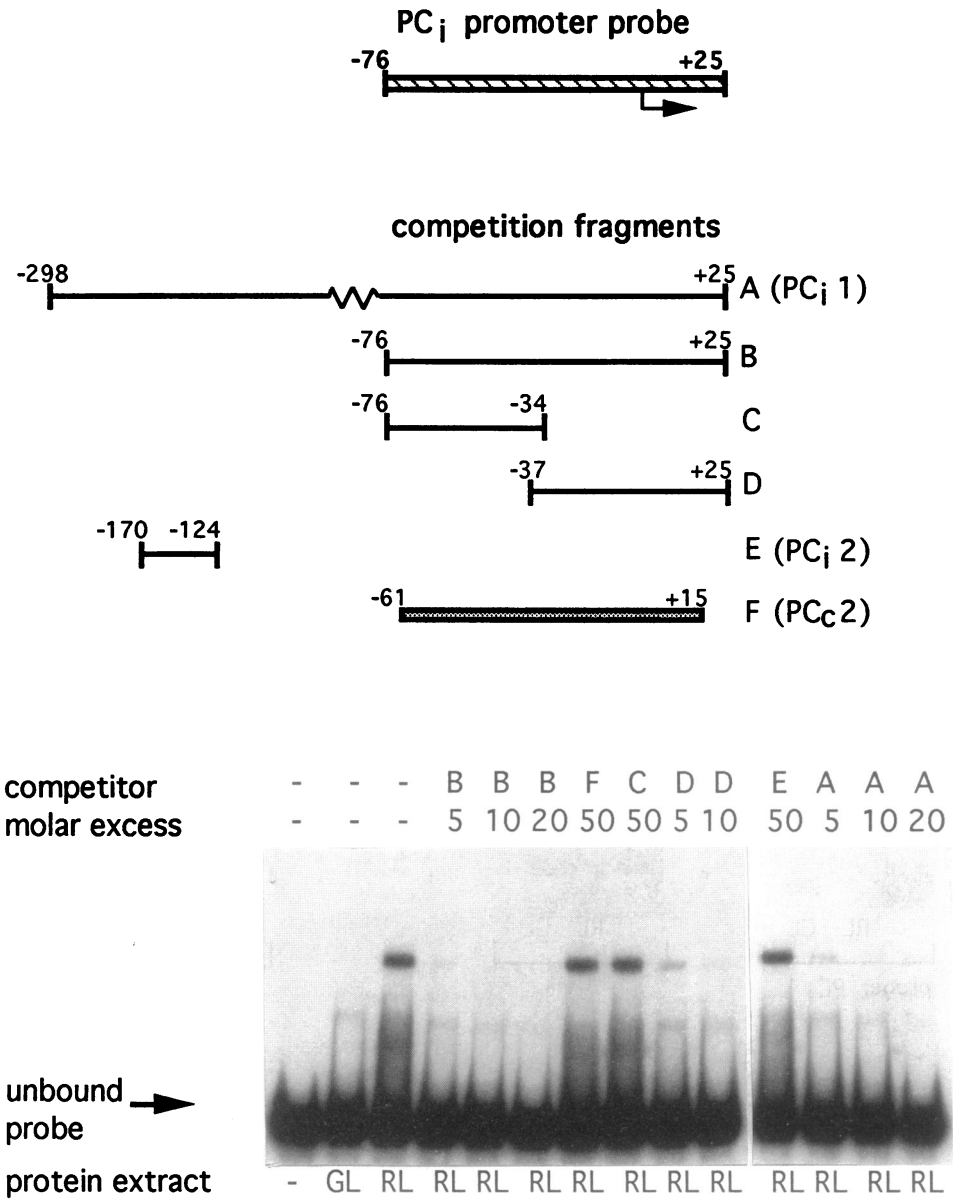


FIG. 7. Competition of *cpcB2A2* (PC_i) and *cpcB1A1* (PC_c) upstream regulatory sequences for RL proteins that bind to a 101-bp fragment bordering the transcription start site of *cpcB2A2*. Forty micrograms of crude protein extract from RL- and GL-grown cells was incubated with 0.001 pmol of a *cpcB2A2* labeled fragment extending from position −76 to +25 (hatched) relative to the transcription start site (top). RL proteins that bind to this fragment were competed for by five fragments (A through E) from the upstream regulatory region of *cpcB2A2* and one 76-bp fragment (F) bordering the *cpcB1A1* transcription start site. The *cpcB2A2* competition fragments are indicated by horizontal lines, and the *cpcB1A1* fragment is indicated by a stippled box. The nucleotide positions at the ends of the fragments are marked with respect to the transcription site, and a single-letter designation is given to the right of the competition fragments. Protein extracts used were from cells grown in GL or RL; the unbound probe is indicated at the bottom of the autoradiogram, and the competitor fragment and the molar excess of the competitor with respect to the probe are indicated at the top.

upstream of the transcription start site of *cpcB2A2*; however, this binding interaction does not play a major role in CCA.

Detection of an RL-specific DNA-protein interaction. Initial gel mobility shift assays revealed a second, unstable protein-DNA complex (DPC2; Fig. 6B) that formed by binding of a protein present only in RL extracts to PC_i1. To determine if this RL-specific binding activity was localized to the region of the *cpcB2A2* operon shown to be necessary for RL induction, a 101-bp fragment extending from −76 to +25 bp (Fig. 7) was radiolabeled and incubated with crude protein extracts from RL- and GL-grown cells. A complex was formed with the labeled fragment and proteins from RL-grown cells (Fig. 7). These extracts from RL- and GL-grown cells were also assayed for and shown to have DPC1 binding activity (data not shown). To delimit the *cis*-acting sequences to which the RL-specific protein bound and to assess the specificity of binding, DNA competition assays were performed. The complex was nearly eliminated when a fivefold excess of unlabeled fragment A, B, or D was used as a competitor. A 50-fold excess of fragment C, E, or F had no effect on complex formation. These data

demonstrate that a DNA binding activity, detected only in RL-grown cells, can specifically interact with the region of the *cpcB2A2* operon between −37 and +25 bp.

DISCUSSION

We have used in vivo expression studies and gel mobility shift assays to begin to characterize *cis*-acting elements and *trans*-acting factors necessary for light-regulated expression of the *cpcB2A2* operon. In vivo expression studies demonstrated that a fragment that includes 76 bp immediately upstream of the transcription start site and the entire untranslated leader of the *cpcB2A2* operon drives high-level expression of the GUS gene in RL but not in GL. When the promoter is truncated to position −37, there is a loss of this RL-stimulated induction. To determine if the 364-bp leader of the *cpcB2A2* operon is necessary for RL induction, a 101-bp *cpcB2A2* promoter fragment (deleted for the leader sequence downstream of +25) was linked to the leader of the constitutively expressed *cpcB1A1* operon and fused to GUS. From these in vivo studies, we conclude that the *cpcB2A2* leader sequence downstream of +25 is not involved in the RL-GL regulation, that the fragment from −76 to +25 bp is sufficient for the RL induction of the *cpcB2A2* operon, and that the region between −76 and −37 bp is necessary for the expression of this operon.

Gel mobility shift assays were used to identify *trans*-acting factors that might control expression of *cpcB2A2*. These studies have identified two DNA-binding sites in the upstream regulatory region of *cpcB2A2*. There is a stable association between a protein present in both RL and GL extracts of *F. diplosiphon* with a region of the promoter positioned at −162 to −122 bp relative to the transcription initiation site. There is a second interaction between proteins in extracts specifically from RL-grown cells and sequences bordering the transcription start site (between −37 and +25 bp).

Combining the results of the in vivo and in vitro analyses of the *cpcB2A2* promoter has given us considerable insight into the regulation of this operon. Even though there is strong binding to the sequence at −162 to −122 bp, 5′ deletions downstream of this region and a 40-bp internal deletion spanning this region did not dramatically alter RL activation of the reporter gene. Hence, the upstream binding site is not necessary for RL induction, although it may have another regulatory function. For example, it may be involved in fine tuning the level of *cpcB2A2* induction in RL. The GUS protein is very stable, making it difficult to detect small differences in expression under conditions in which samples are collected several hours after the transfer of cells to RL. Such differences may be detected if cells are assayed shortly after transfer to inductive conditions (prior to the buildup of the chimeric protein). The variable plasmid copy number among the different strains may also obscure subtle expression differences. Alternatively, it is possible that the sequence from −162 to −122 bp is involved in the regulation of *cpcB2A2* in response to nutrient limitation or light intensity, since such conditions alter both the number of PBS and the PBS rod length.

The second DNA-protein interaction is more likely to play a role in CCA since the complex forms between an RL-specific protein and the 101-bp fragment from −76 to +25 bp, the region found to be necessary for RL-dependent expression of chimeric genes. Competition assays indicated that proteins from the RL extracts do not bind to the region between −76 and −37 bp, which is necessary for expression of the *cpcB2A2* operon, but rather to sequences downstream of −37. In a simple model that accommodates both the in vivo and in vitro data, we suggest that sequences upstream of −37 contain promoter elements necessary for expression of the *cpcB2A2* operon and that the region from −37 to +25 contains an activator-binding site. This binding is proposed to be that of an activator since it is present only in RL-grown cell extracts. The synthesis of this activator may be promoted by RL, or it may be modified in RL in a way that favors a stable association with the DNA. In *E. coli*, activator-binding sites are centered around −40 bp, presumably to allow interaction with RNA polymerase in the −35 region of the promoter (7). The activators cII of phage λ (19, 20), PhoB in *E. coli* (for a review, see reference 38), and MerR in *Pseudomonas aeruginosa* (28) bind slightly farther downstream. cII activates P_{RE} and P_I (20) and P_{AQ} (19) by binding to the same region of the promoter as RNA polymerase (−35) but on the opposite face of the double helix.

For *cpcB2A2*, a potential DNA-binding site in the region downstream of position −37 is composed of two 13-bp direct repeats that extend from −36 to −11 bp of the *cpcB2A2* promoter. While activators most commonly bind to inverted repeat sequences, a number of proteins from the OmpR family of bacterial activators, such as PhoB (39), OmpR (29), and ToxR (26) bind to direct repeats. A 7-bp direct repeat located 42 bp upstream of the *cpeBA* promoter, which may play a role in the regulation of the *cpeBA* operon, has been shown to bind to proteins found in both RL and GL extracts (33). Recently we have identified a protein with a DNA-binding motif with some similarity to that of the OmpR family that is necessary for normal CCA (4, 21).

It is also possible that the RL-specific binding interaction with the fragment at −76 to +25 bp is due to RNA polymerase binding. RNA polymerase binding has been detected in gel shift assays using the *cpeBA* promoter from *Calothrix* sp. strain 7601 (34). The results of the studies described in this paper may be the consequence of the synthesis of a unique sigma factor in RL that allows RNA polymerase to bind and transcription to occur only in RL. DNA sequences involved in establishing an open transcription complex may be removed when the region upstream of position −37 is deleted from promoter-GUS constructs. The exact functions of the regions from −76 to −37 bp and −37 to +25 bp are still speculative.

We have demonstrated that a 101-bp region surrounding the transcription start site is required for the RL induction of the *cpcB2A2* operon and that at least one protein that is probably involved in this induction can be assayed through the use of conventional in vitro binding studies. A more detailed analysis of the promoter, including additional DNA footprint analysis, site-directed mutagenesis, and DNA binding studies with protein extracts from PBS regulatory mutants will help to elucidate the significance of the RL-specific protein-DNA interaction and the regulatory mechanisms involved in the light-regulated expression of the *cpcB2A2* operon.

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