Positive and Negative Regulation of Sequences Upstream of the Form II *cbb* CO$_2$ Fixation Operon of *Rhodobacter sphaeroides*

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The unlinked form I and form II Calvin cycle CO$_2$ fixation (*cbb*) operons of the photosynthetic bacterium *Rhodobacter sphaeroides* are located on different genetic elements, yet both operons are positively regulated by the transcription activator protein CbbR, the product of the *cbbR* gene located immediately upstream of the form I operon. By employing deletion mutagenesis, and a newly constructed promoter probe vector, the form II operon promoter (*cbbF*$_{II}$p) and three other promoters (Up, Vp, and Wp) were localized within 2.1 kb upstream of the form II operon. Mutations in both *cbbR* and the first gene of the form I operon (*cbbF*$_I$) elicited both positive and negative responses when transcriptional fusions controlled by these four promoters were examined. With the exception of Wp, all these upstream promoters were repressed by oxygen. In addition, these promoters were associated with open reading frames of unknown function whose deduced amino acid sequences showed no significant relationship to proteins in current databases. The results of these experiments suggest that the promoter sequences and genes upstream of the form II *cbb* operon may be intimately involved with control of the *cbb* regulon of this photosynthetic organism.

The Calvin reductive pentose phosphate pathway (Calvin cycle) is the major pathway by which organisms fix carbon dioxide into organic carbon. This pathway is basically conserved in diverse organisms, from bacteria to algae to green plants. Two reactions are unique to this pathway: one is catalyzed by the bifunctional enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the most abundant protein on earth, and the other reaction is catalyzed by phosphoribulokinase (35). In certain photosynthetic bacteria, two distinct forms of RubisCO are found (form I and form II) (9, 35), which are the products of different genes in *Rhodobacter sphaeroides* (6, 10, 23, 28). Subsequent studies have shown that the genes encoding the two forms of RubisCO are clustered with other Calvin cycle structural genes at two unlinked loci (11), and these gene clusters were shown to constitute two separate operons (the form I and form II *cbb* operons) (7, 8, 11, 14, 15). Interestingly, the form I and form II genes were mapped to two distinct genetic elements in *R. sphaeroides*, the large and small chromosomes, respectively (33, 34).

The *R. sphaeroides* form I operon begins with the *cbbF*$_I$ gene, coding for fructose 1,6/sedoheptulose 1,7-bisphosphatase, followed by *cbbP*$_I$ (coding for phosphoribulokinase), *cbbA*$_I$ (coding for fructose-1,6/sedoheptulose-1,7-bisphosphate aldolase), and *cbbL*$_I$*cbbS*$_I$ (coding for the large and small subunits of form I RubisCO). The form II operon shows both similarities to and differences from the form I operon. The major difference is that the form II operon comprises two additional structural genes, *cbbT*$_{II}$ and *cbbG*$_{II}$, encoding transketolase and glyceraldehyde-3-phosphate dehydrogenase, respectively, and a distinct *cbbM*$_{II}$ gene (coding for form II RubisCO) is found in the form II operon in place of *cbbL*$_I$*cbbS*$_I$ (2). The *cbbT*$_{II}$ and *cbbG*$_{II}$ genes are located between *cbbP*$_{II}$ and *cbbA*$_{II}$ in the form II operon. The two RubisCO enzymes appear to have distinct enzymological and physiological properties (36), consistent with their low sequence homology, while the other

duplicated enzymes appear to exhibit strong sequence homology (6).

A variety of studies have shown that the regulation of the two *cbb* operons in *R. sphaeroides* is complex. For example, under dark aerobic (heterotrophic) growth conditions, low levels of *cbb* gene products are synthesized and the *cbb* regulon is transcriptionally quiescent compared with cells grown under anoxygenic photosynthetic growth conditions in the presence of organic carbon (photoheterotrophic growth). When cells are shifted to minimal medium lacking organic carbon (photoautotrophic growth conditions), where CO$_2$ is the sole carbon source in the light, the *cbb* operons are maximally derepressed, resulting in the synthesis of high levels of the Calvin cycle enzymes (8, 12, 17). Studies with various mutants (3, 39) and analysis of wild-type strains by quantitative immunological techniques (17) all indicate that the synthesis of the form I and form II gene products is independently controlled in *R. sphaeroides*. Thus, shifts from photoheterotrophic to photoautotrophic growth conditions result in an up to 17-fold increase in form I RubisCO synthesis, while form II RubisCO synthesis is increased by only 2- to 4-fold. On the other hand, since synthesis of both forms of RubisCO is maximally derepressed under photoautotrophic growth conditions, one might consider that the separate *cbb* operons are coordinately regulated. This hypothesis was strengthened when it was found that the synthesis of the form II RubisCO and the transcription of *cbbM*$_{II}$ were elevated in mutants lacking the ability to express the form I genes. The ability of *R. sphaeroides* to compensate for inactivated form I or form II *cbb* genes suggests cross-talk between the two operons (3, 8, 14, 15). In support of this hypothesis, the product of the divergently transcribed *cbbR* gene, related to the LysR family of autoregulatory transcriptional regulators (31), and located upstream of the form I operon, was shown to positively regulate transcription of both the form I and form II operons (12). Indeed, homologous *cbbR* genes appear to be important for regulating CO$_2$ fixation in a number of bacterial systems in addition to *R. sphaeroides* (4, 20, 37, 38, 40). Moreover, recombinant CbbR proteins from *Alcaligenes eutrophus* and *Xanthobacter flavus* specifically bind to the intergenic region between the *cbbR* gene and the *cbb*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relative characteristic(s)	Reference or source
R. sphaeroides strains		
HR	Wild type, Sm^r	39
1312	cbbR::Tp^r	12
1884	cbbF$_I$::Tp^r	8
Plasmids		
pK18	Derivative of pUC18, Km^r	27
pHP45Ω	Containing Ω cassette, Sm^r/Spc^r Ap^r	26
pRK2013	Km^r, self-conjugative	5
pHX200	Promoter probe vector, Tc^r, promoterless *xylE*	41
pHX300	Promoter probe vector; Tc^r Sm^r/Spc^r; promotorless *xylE*	This study
pJG3	pUC8 derivative containing part of form II operon and about 2.1 kb of upstream sequence	J. L. Gibson

operon it controls (37, 40), and CbbR of *Thiobacillus ferrooxidans* was shown to bind specifically to overlapping promoter elements of the RubisCO gene operon (*cbbLS*) and the divergently transcribed *cbbR* gene (20).

Since the separate form I and form II operons of the *cbb* regulon are found on distinct genetic elements in *R. sphaeroides*, our work has focused on further defining the mechanism of coordinated control, as well as determining factors which influence how each operon may be independently regulated. In the current study, we employed a newly constructed *xylE*-based promoter probe vector to identify promoter elements upstream from the form II operon of the *R. sphaeroides cbb* regulon. The results of our experiments clearly indicate that the region upstream of the first gene of the form II operon is important in CO_2 fixation control in the presence or absence of *cbbR* or form I operon expression. Moreover, diverse regulatory elements and unidentified open reading frames (ORFs) of this region all appear to play an important role in this control, with both positive and negative regulatory effects noted.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All the strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C. *R. sphaeroides* strains were grown heterotrophically in the dark at 30°C on peptone yeast extract medium (39) or in Ormerod's minimal medium (25) with 0.2% malate as the carbon and energy source; photoheterotrophic growth was in Ormerod's medium containing malate as the electron donor. In some instances, cells were grown photolithoautotrophically in organic-carbon-free Ormerod's medium under an atmosphere of 1.5% CO_2 in H_2. Antibiotics were supplied to the medium at final concentrations of 50 μg of ampicillin and kanamycin per ml for *E. coli* strains; 15 and 5 μg of tetracycline per ml and 40 and 50 μg of spectinomycin per ml for *E. coli* and *R. sphaeroides*, respectively; and 200 μg of trimethoprim per ml for *R. sphaeroides*.

Promoter probe vector construction. The promoter probe vector used in this study was constructed by inserting an Ω fragment (Sm^r/Spc^r) into the *Hpa*I site of pHX200 (41) (Fig. 1). Plasmid pHP45Ω (26) was digested with *Sma*I, and the digested products were separated by agarose gel electrophoresis. The DNA band corresponding to the 2.0-kb Ω fragment
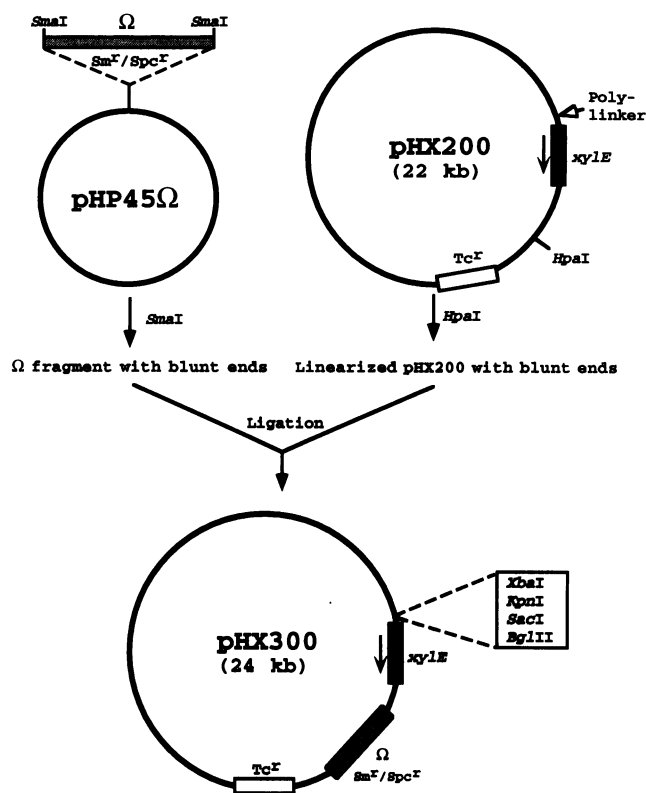

FIG. 1. Construction of a promoter probe vector suitable for studying promoter activity in *R. sphaeroides*. The *xylE* gene shown is promoterless; however, an *E. coli*-type promoter has been identified in the vector sequence upstream of the polylinker which helps to identify clones containing the inserted fragment inside the polylinker in an *E. coli* host (41). Abbreviations: Sm, streptomycin; Spc, spectinomycin; Tc, tetracycline.

was purified with a Geneclean kit and ligated with *Hpa*I-digested pHX200. Competent cells of *E. coli* JM109 were transformed with the ligation mixture, and several transformants with the Tc^r and Spc^r phenotypes were obtained; all were confirmed to contain the desired vector after restriction enzyme digestion analysis. This vector retained the broad host range and promoter probing capacity of its parent plasmid; however, the ability to express Spc^r/Sm^r markers makes it suitable for regulation studies with photosynthetic bacteria under conditions in which tetracycline is normally inactivated in the light. One property of vector pHX300 (as well as pHX200 [41]) worthy of mentioning is the presence of an *E. coli*-type promoter upstream of the polylinker which functions in *E. coli* but not in *R. sphaeroides*. This property enables efficient selection of recombinant plasmids after transformation of *E. coli* competent cells. Colonies containing the vector (pHX300) produce a yellow color in *E. coli* after being sprayed with catechol, while colonies containing recombinant plasmids, in which a DNA fragment is inserted within the polylinker, produce reduced or no yellow color after catechol addition.

Genetic manipulation. Plasmid isolation and purification, restriction endonuclease digestion, and ligation were performed as described by Sambrook et al. (30). *E. coli* JM109 cells were transformed with various plasmid constructs by electroporation with a Bio-Rad (Richmond, Calif.) Gene Pulser. A three-way patch mating was performed to mobilize
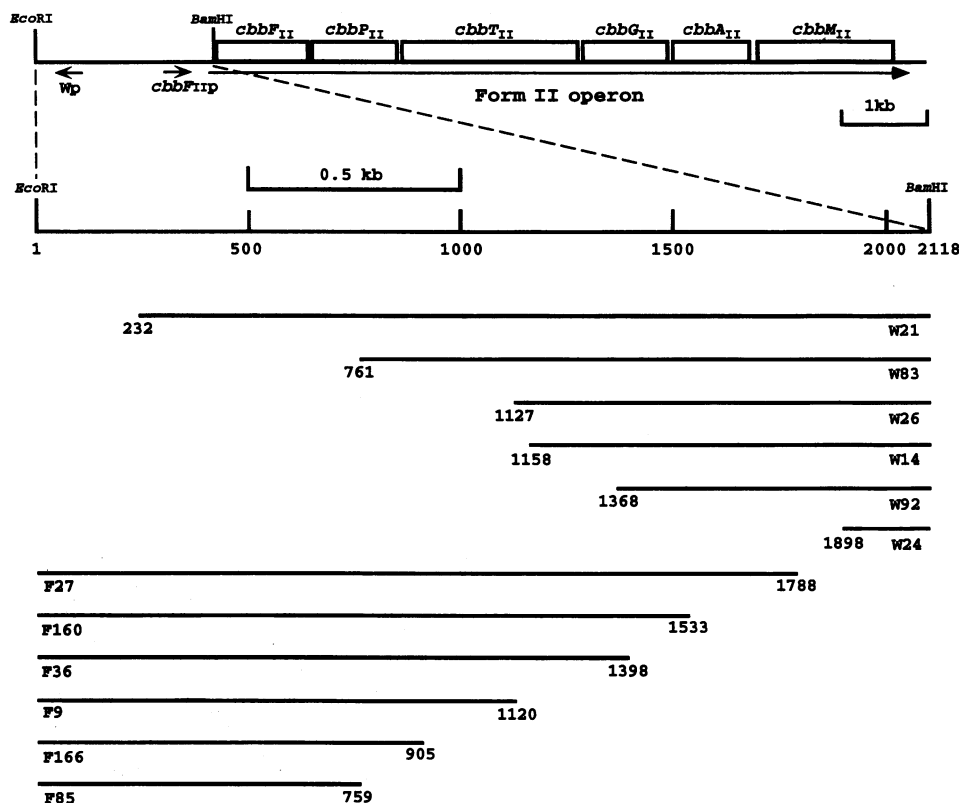
FIG. 2. Form II *cbb* operon and deletion mutagenesis of its 2.1-kb upstream fragment. Deletions starting from the distal end of the fragment (relative to the *cbb* operon) are designated the W series; deletion clones starting from the proximal end are part of the F series. The endpoints of each deletion construct were identified by DNA sequencing (see Fig. 5).

large numbers of plasmid constructs into *R. sphaeroides* strains, as previously described (1).

Construction of nested deletions of the 2.1-kb fragment. Plasmid pJG3 (kindly provided by J. L. Gibson of this laboratory) contains a 3.9-kb *Eco*RI insert, composed of the first two genes of the form II operon of *R. sphaeroides* and about 2.1 kb of upstream sequence (Fig. 2). The 2.1-kb *Eco*RI-*Bam*HI fragment was isolated, purified, and subsequently cloned into pK18 (27). Two derivatives (pHX13 and pHX31) were isolated, each with the insert in the opposite orientation. Two sets of nested deletions were made on this 2.1-kb fragment, representing both orientations, by using protocols described in the Promega (Madison, Wis.) Protocols and Applications Guide. These deletion clones not only were used in the structure-function analyses (Fig. 2 and 3) but also were employed for subsequent sequencing of the 2.1-kb fragment. The deletion fragments generated from the proximal end of the fragment (relative to the form II operon), where the *cbbF*$_{II}$ promoter is presumably located, were termed the F series of deletions. By contrast, deletions starting from the distal end of the fragment, where promoter W is located, resulted in the W series of deletion fragments (Fig. 2).

Cloning of deletion fragments into pHX300. Inserts from deletion derivatives of pHX13 and pHX31 were subcloned into vector pBluescript SK(+) (Stratagene, La Jolla, Calif.) since there is a lack of appropriate restriction sites on pK18 for direct cloning into the promoter probe vector. For the W series of deletion fragments, plasmids were first cut with *Hind*III and *Eco*RI and then ligated into pBluescript SK(+) after digestion of the vector with the same enzymes. After transformation, recombinant plasmids containing each deletion fragment were identified. These plasmids were then digested with *Kpn*I and ligated with *Kpn*I-digested pHX300. Following transformation of *E. coli* competent cells, colonies containing recombinant plasmids were identified on the basis of the lack or reduced levels of *xylE* reporter gene expression. Each fragment may be cloned into pHX300 in two possible orientations. In one case, the *cbbF*$_{II}$ promoter, or other promoters transcribed in the same direction, would direct transcription of the reporter gene; in the other orientation, promoter W, or additional internal promoters, could control the expression of the reporter gene (Fig. 3). The two types of insertions could be differentiated by restriction mapping. To clone the F series of deletion fragments, plasmids were first cut with *Kpn*I and *Hind*III and then cloned into *Kpn*I-*Hind*III-digested pBluescript SK(+). The resulting recombinant plasmids were then digested with *Bam*HI, and the deletion fragments were cloned into *Bgl*II-digested pHX300. Eventually, two series of recombinant plasmids, containing a wide range of fragment lengths in the two orientations, were prepared with the newly constructed promoter probe vector pHX300.

Preparation of cell extracts and enzyme assays. Cells were harvested in a 15-ml sterile plastic centrifuge tube after being spun at 3,430 × g for 10 min at 4°C. Cell pellets were washed once with cold TEM buffer (25 mM Tris-HCl [pH 8.0], 1 mM EDTA, 5 mM β-mercaptoethanol) and recentrifuged. Pellets were suspended in a 1/20 volume (compared with the original cell culture volume) of TEM buffer, and the cells were subsequently disrupted by sonication in an ice bath. Cell debris was removed after centrifugation at 4°C. Supernatants (cell
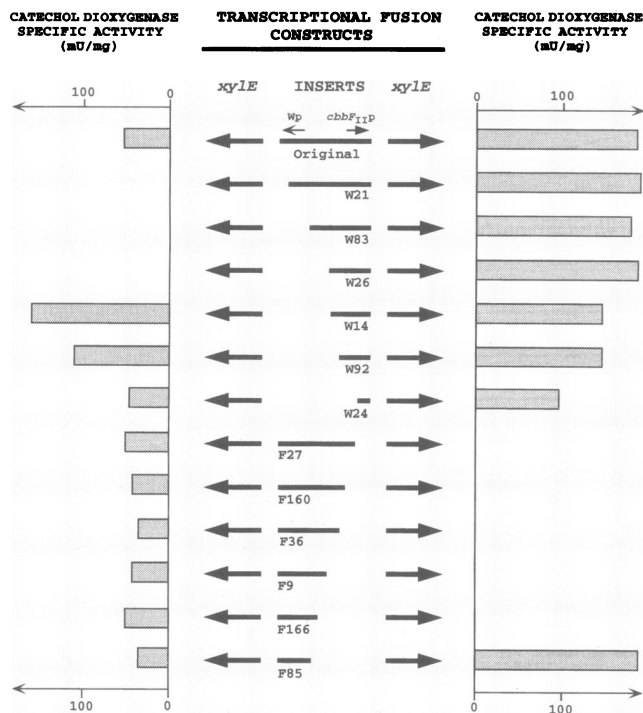
FIG. 3. Promoter activity of various deletion fragments in *R. sphaeroides* grown under photoheterotrophic conditions. Promoter activity was determined by reporter gene expression, manifested by the specific activity (nanomoles of product minute^{-1} milligram^{-1}) of catechol 2,3-dioxygenase (*xylE* gene product). Each DNA fragment was ligated to the reporter gene in two orientations relative to the *xylE* gene (central panel), producing two different transcriptional fusions whose expression is represented by the length of bars on the corresponding sides of the figure. Values are the result of triplicate assays.

extracts) were carefully collected for subsequent assay. Catechol 2,3-dioxygenase activity was measured by the methods of Zukowski et al. (42) and Kataeva and Golovleva (18), with slight modification (41).

Sequence analysis. The 2,118-bp *Eco*RI-*Bam*HI fragment of plasmid pJG3, which contains the upstream sequence of the form II *cbb* operon, was completely sequenced from both DNA strands by the dideoxynucleotide chain termination method. Sequenase version 2.0 (U.S. Biochemical, Cleveland, Ohio), in combination with deazanucleotide analogs, was used in all reactions. Plasmids containing the two sets of nested deletions were used directly for sequencing. Four oligonucleotides, synthesized by Bio-Synthesis, Inc. (Lewisville, Tex.), were also employed as internal primers to fill in sequencing gaps. Nucleotide sequence data were analyzed by sequence analysis programs of the University of Wisconsin Genetics Computing Group as well as by MacVector Sequence Analysis Software of International Biotechnologies, Inc. (New Haven, Conn.).

Primer extension analysis. Hybridization and primer extension reactions were performed as described previously (19). Total RNA was isolated from photolithoautotrophically grown *R. sphaeroides* as previously described (8). An oligonucleotide (5'-CATCACGTCCGCCACATC-3') corresponding to codons 13 to 18 of the first gene (*cbbF*$_{II}$) of the form II operon was synthesized and used as the primer for primer extension experiments. The same primer was used to synthesize a sequence ladder from template pJG3 in order to identify the transcriptional start site of the form II operon.

Nucleotide sequence accession number. The nucleotide sequences reported in this paper have been submitted to the GenBank-EMBL data bank under accession number U12430.

RESULTS

Promoter activity of the 2.1-kb fragment. A 2.1-kb *Eco*RI-*Bam*HI fragment, immediately upstream of the first gene (*cbbF*$_{II}$) of the form II operon, was cloned into pHX300. Recombinant plasmids were identified on the basis of the reduced level of expression of the *xylE* gene in *E. coli* cells, since there is an *E. coli*-type promoter upstream of the polylinker on vector pHX300 that enhances the expression of the reporter gene in *E. coli* cells but not in *R. sphaeroides*. Recombinant plasmids, with inserts in both orientations, were identified on the basis of differences in DNA band profiles after restriction enzyme digestion. Plasmid pHX300-A1 contains the fragment oriented such that the potential promoter of the form II operon directs the expression of the reporter gene. In contrast, in plasmid pHX300-A2, the fragment is in the opposite orientation. Photoheterotrophically grown *R. sphaeroides* strains (pHX300-A1 or pHX300-A2) each exhibited catechol 2,3-dioxygenase activity. Transconjugants containing pHX300-A1 exhibited high levels of *xylE* expression, suggesting that the promoter (termed *cbbF*$_{II}$p) of the form II operon is located on the 2.1-kb fragment (Fig. 3, first horizontal row). Surprisingly, analysis of *R. sphaeroides* (pHX300-A2) revealed significant *xylE* gene expression (Fig. 3). These results suggest that the 2.1-kb fragment might contain another promoter distal to the form II operon. For convenience, the second putative promoter is termed promoter W (or Wp).

Localization of the form II operon promoter, promoter W, and identification of internal promoters U and V. In order to further localize the *cbbF*$_{II}$ and W promoters on this fragment, 12 deletion derivatives were constructed from both ends of the *Eco*RI-*Bam*HI insert. The endpoints of these nested deletions on the 2.1-kb *Eco*RI-*Bam*HI fragment were precisely mapped by DNA sequencing (Fig. 2). Each of the deletion fragments was subsequently cloned individually into pHX300 in front of the *xylE* reporter gene, in both orientations (Fig. 3, central panel). *R. sphaeroides* cells harboring the various mobilized plasmids were then grown in malate medium under photoheterotrophic growth conditions; *xylE* gene expression was subsequently determined for each strain. The horizontal bars in Fig. 3 indicate the corresponding promoter activity of each transcriptional fusion (each fragment in both orientations relative to the *xylE* reporter gene) as determined by the specific activity (milliunits per milligram or nanomoles per minute per milligram) of the *xylE* gene product, catechol 2,3-dioxygenase. The first horizontal row presents results of the transcriptional fusions (pHX300-A1 and pHX300-A2) of the original 2.1-kb fragment. Obviously, this fragment exhibited promoter activity when the insert was cloned in either orientation relative to *xylE*. Deletions of various length (up to 1,897 bp) from the distal end (relative to the form II operon) of the fragment had little or no effect on the promoter activity of the form II operon promoter (*cbbF*$_{II}$p), indicating that the promoter of the form II operon is located within 221 bp of the proximal end of the fragment (Fig. 2 and 3, W series of deletions). Similarly, deletions of as much as 1,359 bp of sequence from the proximal end had little impact on the activity of promoter W (Fig. 2 and 3, F series of deletions). A deletion of 231 bp from the distal end (W21) apparently eliminated the promoter activity of promoter W. Since promoter W is divergently transcribed relative to *cbbF*$_{II}$p, promoter W must be situated near the distal end of the fragment (within 231 bp). Surprisingly, after

1,157 bp of sequence was removed from the distal end (fragment W14), the activity of a new promoter was revealed. Continued deletion in the same direction (see W92 and W24), even after the distal 1,897-bp sequence was removed, failed to completely eliminate this promoter activity. This putative promoter is transcribed in the same direction as Wp and is termed promoter U. Several deletion clones with distal deletions of 231, 760, and 1,126 bp (W21, W83, and W26, respectively), yielded no promoter activity, suggesting the existence of a transcriptional terminator for the putative gene(s) controlled by promoter U. From these results, the functional terminator structure should be located between nucleotides 1127 and 1157. Deletion of 330 bp of sequence from the proximal end (see fragment F27) led to the complete abolishment of $cbbF_{II}$p promoter activity. The deleted fragment that had lost the proximal 1,359-bp sequence (see fragment F85, Fig. 2 and 3) also exhibited promoter activity (designated promoter V, or Vp), with transcription apparently in the same orientation as that of the promoter of the form II operon. Further deletion of 330 nucleotides eliminated the activity of promoter V (data not shown), suggesting that promoter V is located between nucleotides 429 and 759.

Promoter activity in $cbbF_I$ and $cbbR$ mutants of *R. sphaeroides*. Recently, the $cbbR$ gene, which encodes a LysR-type positive regulatory protein (12), was identified in *R. sphaeroides*. This gene positively controls the expression of the form I operon and, to a lesser extent, the form II operon. Inactivation of the form I operon genes results in elevated transcription and increased synthesis of the form II enzymes, presumably to compensate for the lack of form I proteins such as form I RubisCO (encoded by $cbbL_IcbbS_I$) and fructose-1,6/sedoheptulose-1,7-bisphosphatase (encoded by $cbbF_I$) (8). Therefore, we analyzed whether the recently identified promoters were influenced by mutations in $cbbF_I$ and $cbbR$. Strain 1844 is a $cbbF_I$ mutant in which the first gene ($cbbF_I$) of the form I operon was disrupted by insertion of an antibiotic-resistant cassette (8). Similarly, $cbbR$ mutant strain 1312 was constructed by inserting the same cassette into the $cbbR$ gene (12). Four recombinant plasmids, representing four different transcriptional fusions, were mobilized into these two mutant strains, and transconjugants were grown in malate medium under photosynthetic conditions as before. Measurements of enzyme activity in cell extracts (Fig. 4) indicated that $cbbF_{II}$ promoter activity ($cbbF_{II}$p-$xylE$ fusion) was elevated significantly in the $cbbF_I$ mutant relative to the wild type. This is in accordance with observations of Gibson et al. (8) in which the form II operon genes were shown to be overexpressed, presumably to compensate for the absence of the form I enzymes. On the other hand, expression of the $cbbF_{II}$p-$xylE$ fusion was reduced in the absence of the functional positive regulator, CbbR, thus confirming the conclusion that the $cbbR$ gene positively regulates form I as well as form II operon expression. Surprisingly, the exact opposite scenario was observed with the Vp-$xylE$ fusion: decreased expression in the $cbbF_I$ mutant but increased activity in the $cbbR$ strain. The expression of the Wp-$xylE$ fusion appeared not to be affected by the mutations (Fig. 4), suggesting that either the Wp promoter is not regulated in the same way as the form I and form II operons or the fragment does not contain all the necessary regulatory elements. Interestingly, expression of the Up-$xylE$ fusion construct increased significantly in both mutants (Fig. 4). These results suggest that both the U and V promoters are negatively controlled by CbbR but that expression of the form I genes has both a positive and a negative effect on the activity of the V and U promoters, respectively.
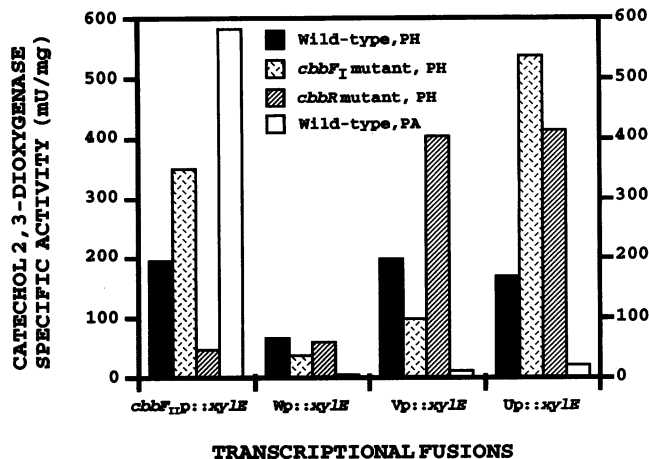

TRANSCRIPTIONAL FUSIONS

FIG. 4. Promoter activity of four transcriptional fusions in wild-type, $cbbF_I$, and $cbbR$ *R. sphaeroides* mutant strains grown under photoheterotrophic (PH) and photoautotrophic (PA) growth conditions. Transcriptional fusions $cbbF_{II}$p::$xylE$, Wp::$xylE$, Vp::$xylE$, and Up::$xylE$ contain, respectively, representative inserts W21, F27, F85, and W14 (Fig. 2) in front of the $xylE$ gene in the proper orientation. Values are the result of triplicate assays.

Promoter activity of representative transcriptional fusions when CO$_2$ is the sole carbon source for growth. Previous work had shown that the form I and form II structural genes of *R. sphaeroides* are maximally derepressed when the organism is grown in the light in minimal medium, with CO$_2$ as the sole source of carbon (photoautotrophic growth) (8, 12, 17). To test how each promoter responds to the lack of organic carbon when cells are grown under photosynthetic growth conditions, *R. sphaeroides* cells containing appropriate transcriptional fusions were first grown in malate-containing medium in the light and subsequently shifted to minimal medium under an atmosphere of 1.5% CO$_2$ in H$_2$. Measurements of $xylE$ expression in cell extracts indicated that form II operon promoter activity ($cbbF_{II}$p) was increased about threefold compared with extracts prepared from photoheterotrophically grown cells (Fig. 4), consistent with the levels of form II RubisCO synthesized under these growth conditions (8, 12, 17). By contrast, extracts from all the other transcriptional fusions, controlled by promoters Wp, Vp, and Up, showed drastically reduced $xylE$ expression under photoautotrophic growth conditions (Fig. 4), indicating that CO$_2$-dependent growth exerts a negative effect on these promoters.

Promoter activity in *R. sphaeroides* strains grown in malate medium anaerobically (under light) and aerobically (in the dark). Anaerobic growth on malate (photoheterotrophic growth) was described above. To culture *R. sphaeroides* strains, harboring various transcriptional fusions, aerobically in malate medium, purified colonies were inoculated into 50-ml flasks containing 20 ml of malate medium supplemented with spectinomycin and incubated in the dark at 30°C with shaking. Table 2 compares the promoter activity of various transcriptional fusions under both anaerobic and aerobic conditions. With the exception of fusion Wp-$xylE$, all the other transcriptional fusions were either completely or partially repressed by the presence of oxygen, suggesting that, like the $cbbF_{II}$ promoter, promoters U and V may be involved in anoxygenic metabolism. The fact that fusion Wp-$xylE$ was not influenced by oxygen confirms that promoter W functions independently of the cbb regulon.

TABLE 2. Comparison of promoter activity of *R. sphaeroides* under anaerobic and aerobic growth conditions in malate medium

Transcriptional fusion	Catechol 2,3-dioxygenase sp act (mU/mg)[a]	
	Anaerobic growth	Aerobic growth
*cbbF*IIp::*xylE*	160	0
Up::*xylE*	168	56
Wp::*xylE*	59	49
Vp::*xylE*	197	0

[a] Expressed in terms of the nanomoles of product formed minute^{-1} milligram^{-1} of protein in cell extracts prepared as described in Materials and Methods. Values are the result of triplicate assays.

Sequence analysis of the entire 2.1-kb upstream fragment of the form II *cbb* operon.

The sequence of both strands of the 2,118-bp DNA fragment was determined (Fig. 5), and this is shown along with 42 codons at the 5' end of *cbbF*II and 38 bp of upstream sequence previously reported (7). Several ORFs that correspond quite well with the recently discovered promoter activities were located. A partial ORF (ORFW) was found associated with promoter W at the distal end of the fragment (relative to the form II *cbb* operon); this ORF, which possesses a ribosome binding site typical of *R. sphaeroides* genes, begins at nucleotide 114 and is transcribed divergently with respect to the *cbb* operon. The deletion clone that eliminated the activity of promoter W was found to have lost the distal 231 nucleotides. Therefore, at least a part of the structure required for functional promoter W activity must be located between nucleotides 114 and 231. The deletion clone that illustrated the activity of promoter V was found to have lost the proximal 1,359 nucleotides (in relation to the form II operon). Further deletion of 330 nucleotides eliminated this activity (data not shown), indicating that promoter V resides between nucleotides 429 and 759. There are two potential ORFs downstream of promoter V, but only one of them has a canonical bacterial ribosome binding site. The ORF (ORFV), which has a recognizable ribosome binding site and is tran-
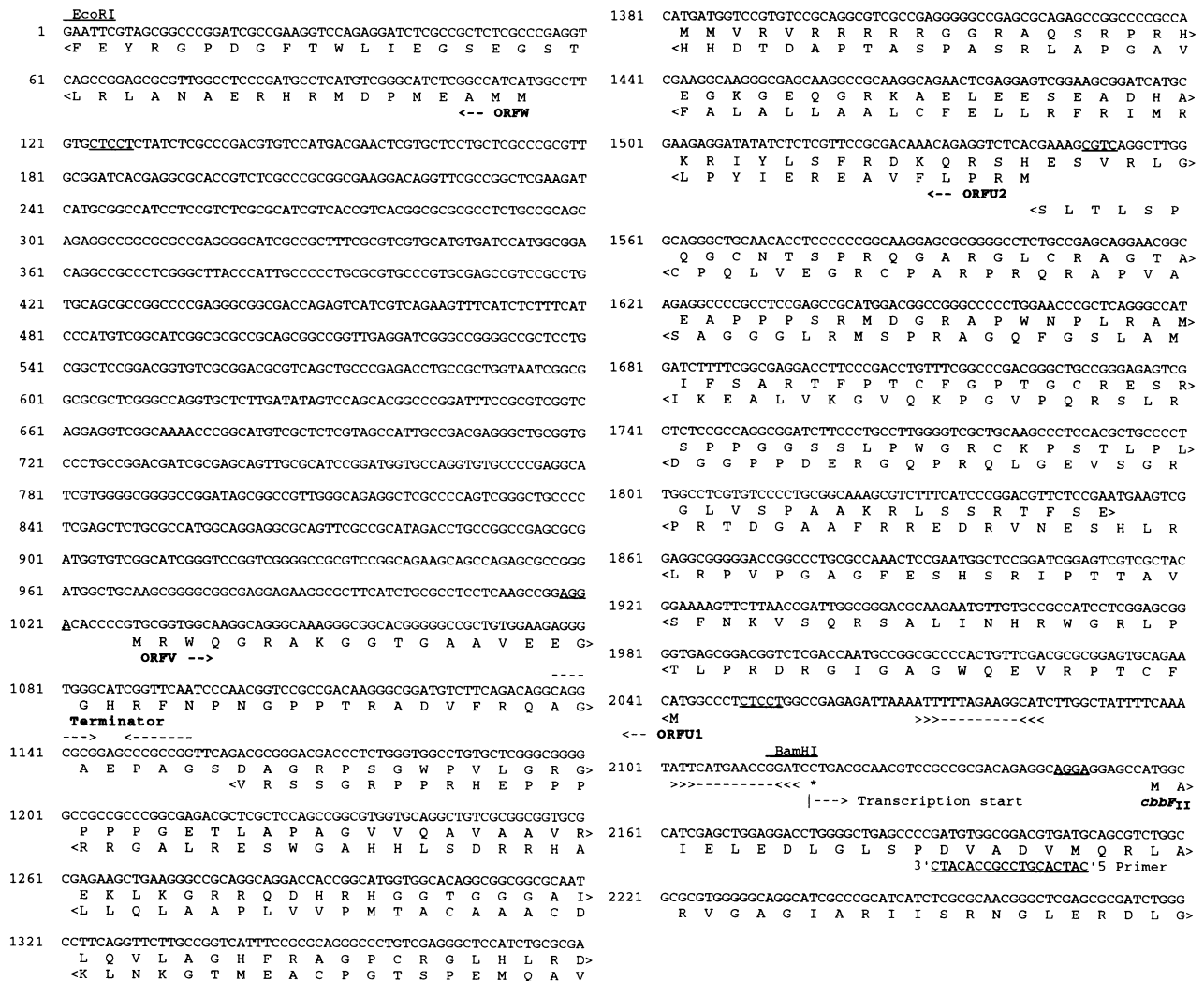
FIG. 5. Nucleotide sequence of the upstream region of the form II *cbb* operon of *R. sphaeroides*. The sequence of the first 42 codons of the first gene (*cbbF*II) previously published (7) is shown to demonstrate the primer used and the transcriptional start site of the operon. Potential ribosome binding sites are underlined. T-N$_{11}$-A symmetry sequences are indicated by >>>----<<<. Putative terminator structures are indicated by ----><----. The transcription start site is marked by an asterisk.

scribed in the same direction as the form II operon, begins at nucleotide 1028 and ends at nucleotide 1855. ORFV encodes a putative polypeptide of 275 amino acids, with an estimated molecular weight of 33,000. Between promoter U (located within nucleotides 1898 and 2118) and the terminator sequences previously identified and centered at nucleotide 1146 (Fig. 3), two tandemly located ORFs that are transcribed divergently relative to the form II operon were found. ORFU1 starts at nucleotide 2043 and ends at nucleotides 1540. It has an excellent ribosome binding site and encodes a putative polypeptide of 167 amino acids with a molecular weight of about 20,000. ORFU2 starts at nucleotide 1543 and ends at nucleotide 1157 but has a weak ribosome binding site. It encodes a putative polypeptide of 128 amino acids with an estimated molecular weight of 15,000. The starting codon for ORFU1 is 112 bp upstream from the first gene ($cbbF_{II}$) of the form II operon (7). In this intergenic region, two dyad symmetry sequences of the $T-N_{11}-A$ type (13) were identified; similar sequences are also found in the intergenic region of the $cbbF_I$ and $cbbR$ genes of the form I operon gene cluster of the same bacterium (12).

Identification of transcriptional start site of the form II operon. From deletion analyses, the form II operon promoter was localized within 258 bp (221 + 37) of the beginning of the first gene of the operon. To define the promoter region more precisely, a primer extension experiment was conducted to identify the transcriptional start site of the form II operon. These studies indicated that the form II operon transcript begins at 38 bp upstream of the first codon of the first gene (Fig. 6). No −35 or −10 hexamers were identified.

DISCUSSION

After the positive regulatory gene, $cbbR$, was identified upstream of the form I operon of *R. sphaeroides*, Southern hybridization experiments indicated that similar $cbbR$-type sequences were not present in the upstream region of the form II operon (12). Yet, there is considerable evidence to indicate that the form II cbb operon is independently (and interdependently) regulated with respect to the form I operon (6, 8, 14, 15). In the present investigation, sequence analysis of the 2,118-bp upstream region confirmed the absence of any $cbbR$-like sequences in the region upstream of $cbbF_{II}$ or, for that matter, any ORFs that resembled known families of prokaryotic regulatory proteins (16, 29, 31). However, a newly constructed *xylE*-based promoter probe vector (pHX300) proved extremely useful in identifying promoter sequences in the region upstream of the form II cbb operon of *R. sphaeroides*. Immediately upstream of the form II operon, a divergently transcribed promoter (promoter U) was found which appeared to control an operon composed of two closely linked genes (ORFU1 and ORFU2) of unknown function and whose deduced amino acid sequences failed to exhibit significant homology to any known proteins. From the expression studies (Fig. 3), evidence for the presence of a terminator at the end of the second ORF was obtained, in support of the conclusion that this region contains a discrete divergent operon controlled by promoter U. The fact that promoter U was regulated by oxygen (Table 2) and influenced by mutations in $cbbR$ and $cbbF_I$ (Fig. 4) lends credence to the idea that this putative operon is somehow related to carbon dioxide metabolism. The presumptive negative control of promoter U exerted by CbbR, exemplified by the significant enhancement of promoter U activity in a $cbbR$ background, is extremely interesting as is the fact that active transcription of the form I genes resulted in reduced promoter U activity. That CbbR is capable of posi-
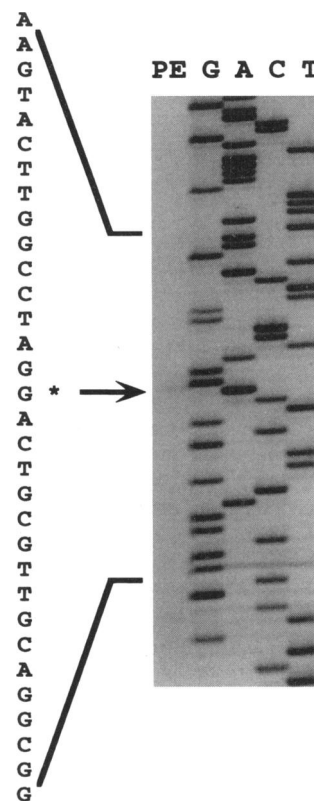

FIG. 6. Primer extension experiment. The 5' end of mRNA for the form II operon is indicated by an asterisk. PE, primer extension reaction mix. G, A, C, and T represent sequencing lanes containing reaction mixtures with appropriate dideoxy termination.

tively regulating the form II cbb operon, while also exerting an apparent reciprocal negative effect on an operon in close proximity, is not surprising since there are examples in which LysR-type transcriptional activator proteins exhibit both positive and negative effects on diverse target operons; these proteins also characteristically show either positive or negative autoregulation (31). However, to our knowledge, this is the first instance in which such a regulatory protein has been observed to influence both positive and negative control of distinct operons important for carbon metabolism. In this respect, $cbbR$ resembles the nitrogen assimilatory control or *nac* gene of *Klebsiella aerogenes*, whose product both activates and represses transcription of genes important for nitrogen assimilation (32). Unlike the *nac* system, and similar to most *lysR*-type systems, $cbbR$ is closely linked and divergently transcribed relative to one of the operons that its product regulates. Unique to the cbb regulator of *R. sphaeroides*, however, is the fact that the LysR homolog, CbbR, also regulates juxtaposed and divergently transcribed operons that are obviously unlinked to the transcriptional regulator gene, which itself is found on a completely distinct genetic element or separate chromosome of this bacterium. Certainly, further experiments are necessary to confirm whether the effects exerted by CbbR are direct; it will also be most important to identify the inducer molecule to facilitate initiation of the proper in vitro experiments.

Goethals et al. (13) proposed a conserved $T-N_{11}-A$ structure as the core of an inverted repeat required for specific binding to LysR-type regulatory proteins. Two copies of dyad symme-

```
        operon U                       >>>----------<<<          >>>----------<<<                                      cbbFII
RsII -CATGGCCCTCTCCTGGCCGAGAGATTAAAATTTTTAGAAGGCATCTTGGCTATTTTCAAATATTCATGAACCGGATCCTGACGCAACGTCCGCCGCGACAGAGGCAGGAGGAGCCATG-
     <M                                                                                                                     M>

        cbbR  >>>----------<<<          >>>----------<<<                                      cbbFI
RsI  -CATTTCCAAATTCCCGAACAGAACTTCTACCTTATTAAATTCCATAAAATCCGATCCGCGGCAAGCTGGGCACACGTCGCGGAGGAGATGCCAGTG-
     <M                                                                                      M>

        cbbR                           >>>----------<<<          >>>----------<<<                                      cbbL
Ae   -CATGGGCGGTTGGGGGCGGCTTTGGATGGTCCCGTGATGTGCAGCTTGGTCCGCACTTAAGGGATTGCTTATACAGGGGCTAAGAATATCTGAATTACCTTATGTGGGTGGGCTTATATC--49bp--ATG-
     <M                                                                                                                             M>

        cbbR             >>>----------<<<                                            >>>----------<<<                  cbbA
Cv   -CATGGTGGTGGAAGGCTCGTCAGACGATAGACAAACGCAATGGCGGATAATCGATTGAGGCATAGATCATAAGCTATGCGTTATATCTAGCATAAGCAATATTTAGTAGTAATTATGTGG--109bp--ATG-
     <M                                                                                                                             M>

        cbbR             >>>----------<<< >>>----------<<<                                                             cbbL
Tf   -CATGTCAATAGTAAACCATGGTAAATCATAATAGTAAATATATTTTACTTTTATAAATTAGAAGATCATCCACAATGCCAATCAAGCATTGGAACCCAATCAGTGGCTTCAATCGTCGTT--27bp--ATG-
     <M                              >>>----------<<<                                                                  M>

        cbbR >>>----------<<<          >>>----------<<<                              >>>----------<<<     cbbL
Xf   -CACTTCAGATTTCCTGAATGCCTACTTCATATCATTTAAATTTACCTGAAATCGGCGCGGGGGCAAGGTCACCTCAACAAACAGGAGGGCCGCCATCATG-
     <M                                                                                                  M>

        cbbR             >>>----------<<<                                            >>>----------<<<     cbbE
RrE  -CATTCCCCTGCCGATTGGCCTATAAGGTTTAGCTTATAGACTATGCCATAATAACTTTGTTGTGTTTATGTGTCCGTCCCGCCAGAATTTCCATGGTGGATTTAGGGGTTCACAAGGCCC--31bp--ATG-
     <M                                                                                                                             M>

        cbbR                                                     >>>----------<<<   >>>----------<<<     cbbM
RrM  -TGAGCGACGCCATCCGCACCCTGCCGCCGCGCGGCAACCGTCATGTCAGCGGCTGAAAAGCGCGGACAACGGAAAGTCGTATAATCTTTTACTTATGGGGAAGTCTAAAACAATAAATTA--60bp--ATG-
     >*                                                                                                                            M>
```
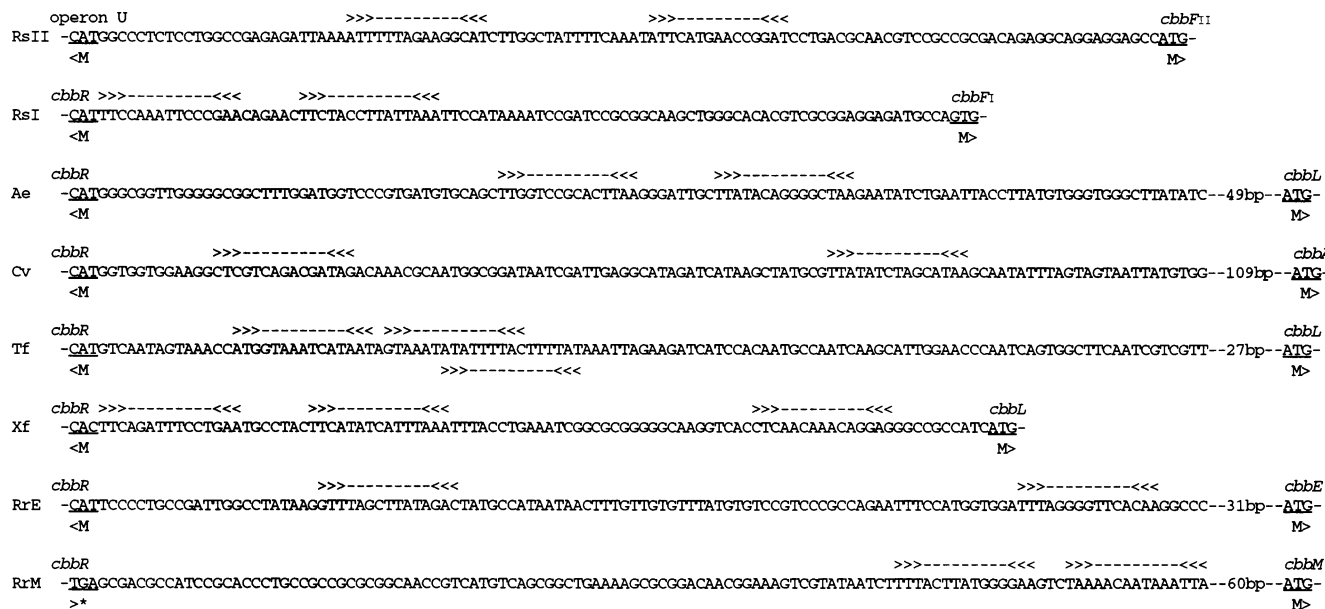
FIG. 7. Localization and comparison of T-N$_{11}$-A dyad symmetry sequences in the intergenic region between cbbR (or operon U in case of RsII) and cbb operons of R. sphaeroides and several species of phototrophic and autotrophic bacteria. The first codon of each ORF is shown. All cbbR (or operon U as in RsII) structural genes are shown on the left and begin with M with orientations shown by <, except in RrM, where the stop codon is shown; cbb operons shown to the right and the first structural gene begin with M>. Abbreviations: RsII, R. sphaeroides form II operon; RsI, R. sphaeroides form I operon (8); Ae, A. eutrophus (40); Cv, Chromatium vinosum (38); Tf, T. ferrooxidans; (20); Xf, X. flavus (37); RrE, Rhodospirillum rubrum intergenic region between cbbR and cbbE (4); RrM, R. rubrum intergenic region between cbbR and cbbM (4). An asterisk marks the stop codon, and T-N$_{11}$-A symmetry sequences are indicated by >>>----<<<.

try are located in the intergenic region between cbbR and the form I operon of this organism (12); on the basis of previous studies with similar systems (31), these sequences are presumably important for the binding of CbbR. Thus, it was not surprising to find that two copies of the T-N$_{11}$-A symmetry structure were present in the intergenic region of the form II operon and the putative U operon. In fact, at least two copies of the T-N$_{11}$-A symmetry structure have been located in the intergenic regions between cbb operons and cbbR genes in a number of phototrophic and autotrophic bacteria (4, 20, 37, 38, 40) (summarized in Fig. 7). Especially noteworthy is that mutations within the core of the region of dyad symmetry in the intergenic region between cbbR and the cbbL$_1$cbbS$_1$ genes of T. ferrooxidans lead to reduced capacity for CbbR binding in vitro (20). These observations strongly support the idea that the T-N$_{11}$-A dyad symmetry sequences within the intergenic regions between cbbR and the RubisCO structural genes in R. sphaeroides are important for gene expression.

Promoter V is followed by an ORF encoding a putative 275-amino-acid polypeptide. Data bank searches of ORFV also failed to identify any structurally similar proteins. This ORF overlaps with most of the proposed ORFU1-ORFU2 operon; however, promoter V and ORFV are divergently transcribed compared with promoter U and ORFU1 and ORFU2. Promoter V was regulated by oxygen in a manner similar to that for the form II operon promoter. In addition, since a mutation in cbbR resulted in a significant increase in promoter V activity, it will be important to establish whether CbbR exerts a direct negative influence on promoter V in the wild type. Much like promoter U, promoter V is also influenced by a mutation in cbbF$_I$ of the large chromosome; in this instance, however, promoter activity decreased in a cbbF$_I$ background. These results strongly suggest that ORFV, like

the genes of the U operon, is also somehow involved in CO$_2$ fixation via the Calvin cycle.

Promoter W appeared not to be regulated by oxygen or the cbbR and cbbF$_I$ genes. The putative gene (ORFW) under the control of promoter W therefore appears to be constitutively expressed, except under photoautotrophic conditions. Data bank searches of partial ORFW also did not help to identify this protein. Obviously, it will be interesting to continue characterizing the sequences and structures further upstream of the 2.1-kb fragment (downstream from ORFW), in order to better define and study the function of ORFW under more diverse growth conditions. Thus far, our results certainly suggest that ORFW is not involved with CO$_2$ metabolism; if this is verified, then we have presumably delimited the major portion of the form II CO$_2$ fixation gene region. It should also be noted that, although the unique role of the form II cbb operon in carbon dioxide fixation has been studied extensively (2, 3, 7, 11, 12, 17, 28), until the present study, direct evidence that cbbF$_{II}$ is the first gene of the operon had not been presented. Thus, the primer extension analysis clearly identified the transcription start site for the form II cbb operon as being 38 bp upstream of the first codon of the cbbF$_{II}$ gene. Most important, this work established the position of the form II operon relative to other upstream regulatory elements and ORFs. A model for the organization of the form II operon is presented (Fig. 8), in which known relationships of diverse regulatory elements and structural genes of the cbb regulon are depicted. Experiments are currently under way to further characterize ORFs U1, U2, V, and W; their promoters; and surrounding sequences. Earlier work had indicated that insertion of a mini-Tn10 cartridge about 2 kb upstream of the cbbF$_{II}$ gene prevented complementation by this fragment of cbbA$_I$-cbbA$_{II}$ or cbbP$_I$-cbbP$_{II}$ double mutants of R. sphaeroides under
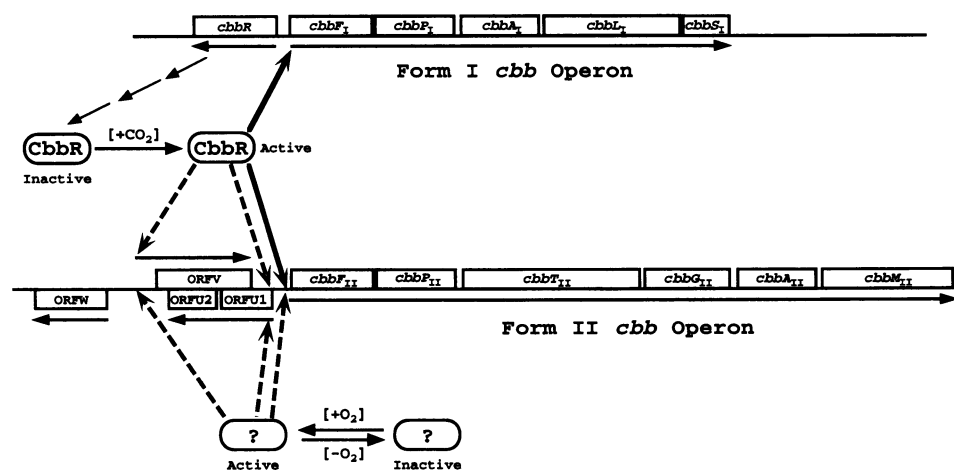
FIG. 8. Model for positive and negative Calvin cycle (*cbb* regulon) structural gene regulation in *R. sphaeroides*. CbbR exerts both a positive effect (solid diagonal arrows) and negative effect (broken diagonal arrows) on specific promoters. Some unknown gene product (?) is thought to negatively influence gene expression in the presence of oxygen at specific loci (broken diagonal arrows). In addition to the regulation caused by CbbR and molecular oxygen, the absence of each *cbb* operon of the regulon results in a compensatory level of expression of genes of the opposite *cbb* operon (8). The direction of transcription is indicated by the horizontal arrows.

photoheterotrophic growth conditions (14, 15). Since the exact location of the insertion is not known, it is possible that either ORFW, ORFV, or even the U operon (or noncoding region between these ORFs) was disrupted by the insertion. Nevertheless, this result accentuates the importance of sequences and genes upstream of the form II Calvin cycle operon in *R. sphaeroides*. It is apparent that the form I and form II operons and their promoters are positively regulated by CbbR and particularly up-regulated when CO_2 is used as the carbon source during photosynthetic growth. However, promoters V and U, and presumably the genes they control, behaved in exactly the opposite way; i.e., these promoters exhibited lower activity in the wild type compared with a *cbbR* mutant under photoheterotrophic growth conditions. Presumably, the activity levels in the wild type are due to some negative consequences of the presence of the *cbbR* product. Moreover, promoters U and V were down-regulated when CO_2 replaced malate as the source of carbon. These results suggest a complex regulatory circuit in which both positive and negative control by the CbbR regulator protein and changes in the carbon source directly influence gene expression (Fig. 8). Again, it must be stressed that considerable work remains in verifying aspects of this model; however, the results we have attained thus far are fully compatible with the indicated scenario. It should also be noted that the structural genes and promoters of both *cbb* operons as well as the U and V promoters, were repressed in the presence of oxygen. It is tempting to speculate that other regulatory factors, besides CbbR, might be involved in regulation of the *cbb* regulon in response to environmental factors such as oxygen tension and carbon source variations.

ACKNOWLEDGMENTS

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