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Amino Acid Residues of RegA Important for Interactions with the CbbR-DNA Complex of *Rhodobacter sphaeroides*

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CbbR and RegA (PrrA) are transcriptional regulators of the Calvin-Benson-Bassham (CBB) CO_2 fixation pathway (*cbb*$_I$ and *cbb*$_{II}$) operons of *Rhodobacter sphaeroides*. The CbbR and RegA proteins interact, but CbbR must be bound to the promoter DNA in order for RegA-CbbR protein-protein interactions to occur. RegA greatly enhances the ability of CbbR to bind the *cbb*$_I$ promoter or greatly enhances the stability of the CbbR/promoter complex. The N-terminal receiver domain and the DNA binding domain of RegA were shown to interact with CbbR. Residues in α-helix 7 and α-helix 8 of the DNA binding domain (helix-turn-helix) of RegA directly interacted with CbbR, with α-helix 7 positioned immediately above the DNA and α-helix 8 located in the major groove of the DNA. A CbbR protein containing only the DNA binding motif and the linker helix was capable of binding to RegA. In contrast, a truncated CbbR containing only the linker helix and recognition domains I and II (required for effector binding) was not able to interact with RegA. The accumulated results strongly suggest that the DNA binding domains of both proteins interact to facilitate optimal transcriptional control over the *cbb* operons. *In vivo* analysis, using constitutively active mutant CbbR proteins, further indicated that CbbR must interact with phosphorylated RegA in order to accomplish transcriptional activation.

CbbR and RegA (PrrA) are transcriptional regulators of the Calvin-Benson-Bassham (CBB) pathway operons *cbb*$_I$ and *cbb*$_{II}$, comprising genes that encode enzymes necessary for CO_2 assimilation in *Rhodobacter sphaeroides*. A complex multilayered system was found to activate gene expression (1–4). CbbR is a LysR-type transcriptional regulator (LTTR). In almost all cases, LTTRs employ a coinducer (effector metabolite) to influence gene expression, adding to the complexity of gene regulation (5, 6). For *R. sphaeroides* CbbR, ribulose 1,5-bisphosphate (RuBP) serves as a major coinducer (7–10). With regard to regulating *cbb* gene expression in nonsulfur purple bacteria, response regulator RegA also plays an important role. RegA is part of a global regulation system, also consisting of RegB and RegC, which establishes redox control over many energy-associated pathways, including CO_2 fixation and photosynthesis (11–15), in nonsulfur purple bacteria. When RegA is phosphorylated, catalyzed by the RegB membrane-bound kinase, phosphorylated RegA (RegA~P) then facilitates gene regulation by binding to appropriate gene promoters, providing additional complexity to the control of CO_2 fixation. Moreover, it was found that CbbR and RegA interact with each other on the *cbb*$_I$ promoter, adding another dimension to the regulation of *cbb* gene expression (16). Indeed, the interaction of RegA with CbbR greatly enhances the affinity of CbbR for the *cbb*$_I$ promoter-DNA complex, thus contributing to a finely tuned transcriptional control scenario. Finally, CbbR interacts with RegA only when CbbR is bound to DNA, providing specificity such that CbbR and RegA interact only when associated with *cbb* promoter sequences (16).

CbbR and RegA bind the *cbb* promoters via well-characterized DNA binding domains (DBDs) of the helix-turn-helix (HTH) motif family (17, 18). Ninety-five percent of all prokaryotic DNA binding proteins possess the HTH motif (6). Like prototypical LTTR proteins, CbbR binds DNA as a dimer of a dimer, referred to as a tetramer, placing a total of four HTH motifs along the DNA (19–22). RegA must bind DNA as a dimer to function as a global regulator; this involves two HTH motifs per site. The consensus DNA binding sequences for both RegA and LTTRs are well established (6, 23). The functional domains of CbbR consist of a DBD (residues 1 to 69), a linker helix (residues 70 to 95), recognition domain I (RDI; residues 96 to 170 and 275 to 310), and recognition domain II (RDII; residues 171 to 274). RDI and RDII are required for coinducer (effector metabolite) binding. The functional domains of RegA consist of a receiver domain (residues 1 to 130), a short linker region (residues 131 to 138), and a DBD (residues 139 to 184). Most DBDs are connected to their "effector" domains through rigid linker helices or small linkers that prevent interactions between domains (24). Most commonly, DBDs are localized either at the N terminus or C terminus of the protein. In the case of CbbR and RegA, the DBDs are located at the N terminus and at the C terminus, respectively. DBDs are typically separated from the rest of the protein complex when bound to DNA, creating an isolated region on the promoter. The structure of the HTH motif consists of two α-helices that are positioned on separate planes oriented at approximately 120° relative to each other (17, 18, 25). The first α-helix is positioned just above the DNA, and the second α-helix is located within the major groove of the DNA, often referred to as the recognition helix (17, 18, 25). A third α-helix runs along the DNA backbone and precedes the two aforementioned α-helices. This HTH model is known as a simple trihelical structure (18). CbbR (and the LysR family members) possesses a winged HTH which incorporates two roughly antiparallel β-strands after the HTH, called the wing, and a long α-helix after

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the wing, referred to as the linker helix (18). RegA has a simple trihelical arrangement (23). The HTH binds DNA in a manner that places specific regions of the HTH in locations that are readily available for interactions with residues from other proteins.

There are several examples of DBDs interacting with other proteins (or domains), illustrating an important secondary function for DBDs, in addition to binding DNA (26–32). It is therefore no surprise that DBDs have evolved the ability to bind other proteins or other DBDs to gain additional functions, since many promoters contain DNA binding sites that are within a few base pairs of each other or even overlap. Indeed, the cbb_I promoter contains one DNA binding site for CbbR and four DNA binding sites for RegA (3). The CbbR DNA binding site and RegA DNA binding site 1 overlap, placing their DBDs in close proximity. As previously shown (16), CbbR gains a greater affinity for the cbb_I promoter when it interacts with RegA; however, exactly how these two regulators interact was not clear. In the present study, interaction sites between the DBDs of the two transcriptional regulators, CbbR and RegA, were identified and shown to be important for *cbb* transcription and subsequent CO_2 fixation. In addition, this report illustrates the potential for additional functions for DBDs.

MATERIALS AND METHODS

Strains, plasmids, growth conditions, and triparental matings. Bacterial strains and plasmids are described in Table 1. *R. sphaeroides* strains were grown under aerobic chemoheterotrophic conditions in Ormerod's medium (42) supplemented with 0.4% malate with shaking at 30°C in the dark. For anaerobic photoheterotrophic growth, cultures were grown in Ormerod's medium supplemented with 0.4% malate in completely filled screw-cap glass tubes under incandescent light at 30°C (43). For photoautotrophic growth, cultures were grown in Ormerod's medium under an atmosphere of 1.5% CO_2 and 98.5% H_2 in the presence of incandescent light (44). Optical density measurements were taken at 660 nm. Antibiotics were used at the following concentrations (μg/ml): for *Escherichia coli*, ampicillin (100), chloramphenicol (12.5), kanamycin (50), spectinomycin (50), and tetracycline (12.5); and for *R. sphaeroides*, kanamycin (50), spectinomycin (50), tetracycline (5), and trimethoprim (50). Where appropriate, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 40 μg/ml. Mating of various broad-host-range plasmids (in strain JM109) into *R. sphaeroides* strains was accomplished using a triparental mating strategy and the pRK2013 helper plasmid (37).

Site-directed mutagenesis of the *regA* ORF and construction of CbbR truncation mutants. Specific nucleotide changes in the *regA* open reading frame (ORF) were generated using a kit (Agilent Technologies/QuikChange) of pJC407 and pJC414(wt) to produce specific single-amino-acid substitutions in the RegA protein. The pJC407 vector is an intein-chitin binding/RegA fusion construct (33) used for the purification of the RegA proteins in *E. coli* (New England BioLabs). A list of oligonucleotides used to introduce nucleotide substitutions in each *regA* mutant is provided (see Table S1 in the supplemental material). The reverse complement is not listed. For the CbbR truncation mutant consisting of the DBD plus linker helix, a NdeI/BamHI-digested PCR product encoding the first 96 amino acids of the CbbR protein was ligated into NdeI/BamHI-digested pET28a (Novagen) (N-terminal His_6-tagged/CbbR), designated pETCbbR/201, to synthesize the truncated CbbR-DBD-plus-linker protein for gel mobility shift assays. For the CbbR truncation mutant consisting of the linker helix plus RDI/RDII, a NdeI/BamHI-digested PCR product, encoding amino acids 66 to 310 of the CbbR protein, was ligated into NdeI/BamHI-digested pET28a, designated pETCbbR/204, to synthesize the truncated CbbR-linker-plus-RDI/RDII protein for gel mobility shift assays. The p12EH/442 plasmid, for *in vivo* studies using CbbR-DBD-plus-linker helix, was constructed by modification of pUC12EH with the introduction of KpnI sites at nucleotide positions 302 and 925 of the *cbbR* ORF. A stop codon was incorporated at position 298 of the ORF so that a truncated CbbR consisting of the first 98 residues would be translated. The KpnI-digested plasmid and subsequent religation removed DNA encoding residues 99 through 310. The resulting p12EH/442 plasmid was linearized at the EcoRI site and ligated into EcoRI-digested pVK101, creating plasmid pVK442, and mated into the *cbbR* deletion strain (strain 87). A list of the oligonucleotides used for the construction of pETCbbR/201, pETCbb/R204, and pVK442 is provided (see Table S2).

Synthesis and purification of CbbR and RegA. CbbR and RegA (encoded by wild-type [wt], point mutant, and truncation mutant genes) were overexpressed in *E. coli* and recombinant proteins purified as previously described (16).

Chemical mutagenesis for the generation of CbbR*. Constitutive mutants of CbbR, designated CbbR*, were generated by random chemical mutagenesis using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as previously described (10).

Gel mobility shift assays. Gel mobility shift assays were performed as previously described (10, 16), with the following modifications. The pKCl-5 plasmid contains the cbb_I promoter region of *R. sphaeroides* and was used to amplify the DNA used for ^{32}P-labeled probes for gel mobility shift assays. The probes had BamHI sites incorporated at their 5′ and 3′ ends. Probes were digested with BamHI before labeling with [^{32}P]CTP via an end-filling reaction using Klenow DNA polymerase. Each reaction mixture for binding between CbbR or RegA and DNA contained 0.1 nM labeled DNA. Probe-0 is 165 bp in length and contains only the CbbR DNA binding site, and probe-1234 is 551 bp in length and contains all four RegA DNA binding sites plus the CbbR binding site (16). Oligonucleotides used to generate the probes were as follows: for probe-0, 5′-GATTGGATCCACCATTTCCAAATTCCCGAACAG-3 and 5′-GATTGGATCCGGTCCATCACGTCCTGCAACTC-3′; and for probe-1234, 5′-GATTGGATCCGATTCGGATCTCGGGGCAGGCGA-3′ and 5′-GATTGGATCCGGTCCATCACGTCCTGCAACTC-3′.

RubisCO assays. For chemoheterotrophic, photoheterotrophic, and photoautotrophic cultures, cells were grown to an optical density of 1.0 (at 660 nm), subjected to centrifugation in a microcentrifuge at 4°C, resuspended in sonication buffer (25 mM Tris-Cl [pH 8.0], 1 mM EDTA, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM $NaHCO_3$), and sonicated for 2 min on ice. Lysates were subjected to centrifugation in a microcentrifuge at 4°C, and the resulting clear supernatant was used in RubisCO assays as previously described (45).

Extraction of protein/DNA complexes from polyacrylamide gels. CbbR/RegA/probe-0 and *RegA*/probe-1234 complexes were excised from native polyacrylamide gels with a scalpel. The excised polyacrylamide was placed in dialysis tubing (12,000-kDa cutoff), and electrophoresis was performed in a horizontal gel apparatus for 1 h using 50 mM Tris (pH 8.0), 380 mM glycine, and 2 mM EDTA as a running buffer to electroelute the protein/DNA complexes from the polyacrylamide. The supernatant containing the isolated CbbR and RegA proteins was subsequently used for immunoblot analysis.

Immunoblot analysis. CbbR and RegA preparations isolated from protein/DNA complexes were subjected to SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Penta-His monoclonal antibody (Qiagen, Valencia, CA) was used to detect the presence of His-tagged CbbR in immunoblot assays. Anti-RegA polyclonal antibodies were used to detect the presence of RegA in immunoblot assays (a kind gift from Tim Donohue, University of Wisconsin) (33). The immunoblots were developed as previously described (10).

RT-PCR analysis. RNA was isolated from *R. sphaeroides* strains using Tri reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. A 150-ng volume of RNA was used for determination of expression of *cbbR*(wt) and *cbbR*(trunc). Reverse transcription-PCR (RT-PCR) was performed using a Universal SYBR green One-Step kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Oligonu-

TABLE 1 Plasmids and strains

Plasmid or strain	Relevant characteristics[a]	Source or reference
Plasmids		
pJC407	Ap^r; contains the *regA* coding region from *R. sphaeroides* cloned into the intein/chitin-binding fusion vector, pTYB4; overexpression in *E. coli*	33
pJC417	Ap^r; contains the *regA* and *D63A* coding region from *R. sphaeroides* cloned into the intein/chitin-binding fusion vector, pTYB4; overexpression in *E. coli*	33
pETCbbR	Kn^r; contains the *cbbR* coding region from *R. sphaeroides* cloned into the NdeI/BamHI-digested His_6-tagged vector pET28a; overexpression in *E. coli*	16
pETCbbR/201	Kn^r; NdeI/BamHI-digested PCR product encoding the first 96 residues of the *cbbR* coding region cloned into pET28a; overexpression in *E. coli*	This study
pETCbbR/204	Kn^r; NdeI/BamHI-digested PCR product encoding residues 66–310 of the *cbbR* coding region cloned into pET28a; overexpression in *E. coli*	This study
pVK101	Kn^r; Tc^r; broad-host-range vector	34
pVK102	Kn^r; Tc^r; broad-host-range vector	34
pUC12EH	Ap^r; pUC9 containing a 1.8-kb EcoRI-HindIII fragment carrying *cbbR*	1
p12EH/442	Ap^r; pUC12EH modified by the introduction of two KpnI sites and subsequent removal of DNA between the KpnI sites to create a truncated *cbbR*, encoding the first 99 residues of CbbR	This study
pVK442	Ap^r; Kn^r; Tc^r; pVK101 carrying p12EH/442 inserted at the EcoRI site	This study
pVK12	Ap^r; Tc^r; pVK102 containing pUC12EH inserted at the HindIII site	1
pRK415	Tc^r; broad-host-range vector	35
pJC414	Tc^r; 1.2-kb insert containing *regA* and *D63A* cloned into XbaI/HindIII-digested pRK415	33
pJC414(wt)	Tc^r; pJC414 modified by site-directed mutagenesis to restore wild-type *regA*	This study
pBBR1MCS-2	Kn^r; broad-host-range vector	36
p12EH(P160L)	Ap^r; pUC12EH modified by site-directed mutagenesis to create the CbbR constitutive mutant carrying the P160L amino acid substitution	This study
p12EH(R158C)	Ap^r; pUC12EH modified by site-directed mutagenesis to create the CbbR constitutive mutant carrying the R158C amino acid substitution	This study
p12EH(R274W)	Ap^r; pUC12EH modified by site-directed mutagenesis to create the CbbR constitutive mutant carrying the R274W amino acid substitution	This study
pMCS/CbbR(wt)	Ap^r; Kn^r; pBBR1MCS-2 containing pUC12EH inserted at the EcoRI site	This study
pMCS/CbbR*(P160L)	Ap^r; Kn^r; pBBR1MCS-2 containing p12EH(P160L) inserted at the EcoRI site	This study
pMCS/CbbR*(R158C)	Ap^r; Kn^r; pBBR1MCS-2 containing p12EH(R158C) inserted at the EcoRI site	This study
pMCS/CbbR*(R274Q)	Ap^r; Kn^r; pBBR1MCS-2 containing p12EH(R274Q) inserted at the EcoRI site	This study
pKCl-5	Kn^r; pK18 containing the 719-kb EcoRI-AvaII fragment of pUC12EH; promoter region of cbb_I from *R. sphaeroides*	2
pRK2013	Kn^r; helper plasmid for conjugation; Mob$^+$ (RK2)	37
R. sphaeroides strains		
HR	Sm^r; wild type	38
1312	Tp^r; trimethoprim cassette inserted into *cbbR*; inactivation of CbbR	1
87	Sp^r; Tp^r; 1312 with the cbb_I promoter-*lacZYA* (Sp^r) fusion inserted at the cbb_I genomic region	10
PrrA2	2.4.1 *prrA*ΔBstBI-PstI::Ω; Sm^r; Sp^r; deletion of *regA*	39
E. coli strains		
JM109		40
BL21(DE3)	Overexpression strain carrying an IPTG-inducible T7 RNA polymerase gene	41
ER2566	Overexpression strain carrying an IPTG-inducible T7 RNA polymerase gene	New England BioLabs

[a] Ap, ampicillin; Kn, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; Tp, trimethoprim; IPTG, isopropyl-β-D-thiogalactopyranoside.

cleotides used in the RT-PCR analysis are listed in Table S3 in the supplemental material.

RESULTS AND DISCUSSION

The DBD of RegA interacts with CbbR. Previous studies indicated that RegA specifically interacts with CbbR but does so only when CbbR is bound to promoter DNA sequences; in addition, RegA influences the migration of the protein-DNA complex in gel mobility shift assays. Interactions of the two transcriptional regulator proteins were shown to be specific, and cross-linking studies indicated that a stoichiometric RegA-CbbR-DNA complex was formed (16). To identify the region(s) of the RegA protein (Fig. 1) that interacted with the CbbR-bound DNA complex, single-amino-acid substitutions were constructed in RegA throughout the protein, including 14 changes in the receiver domain (e.g., between residues 1 and 130), one mutation in the linker domain (between residues 131 and 140), and 30 mutations in the DBD (between residues 141 and 184). Initially, residue substitutions were chosen as nonconservative changes to efficiently identify regions of the RegA molecule that would affect interaction with

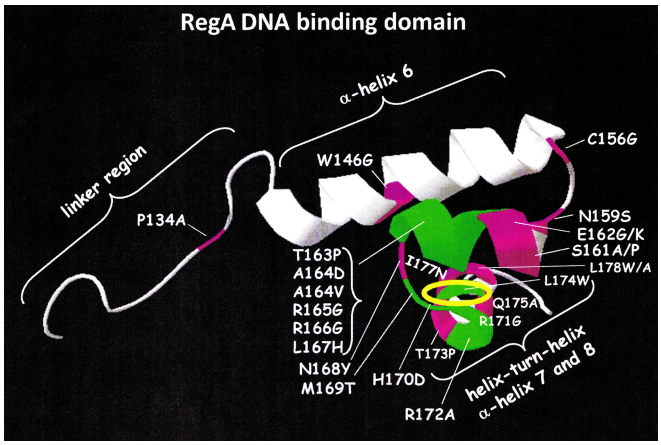

FIG 1 Structural model of the RegA DBD from *R. sphaeroides* (23). RegA mutant proteins from this study are highlighted. The mutated residues highlighted in green abolish interaction with CbbR. The mutated residues highlighted in magenta do not abolish interaction with CbbR. A yellow circle identifies the region where residues of the DBD of CbbR likely interact with residues of the DBD of RegA.

CbbR, such as charge alterations or size modifications. Subsequently, alanine substitutions were employed; finally, several residues in the DBD were targeted with multiple substitutions to facilitate interpretations about protein domain interactions and protein conformations. A 165-bp cbb_I promoter DNA fragment containing the CbbR binding site, but excluding any RegA binding site (probe-0), was used in gel mobility shifts (Fig. 2) to determine the potential effect on interactions between CbbR and the various RegA mutant proteins. Probe-0 was used in these studies since CbbR binds to probe-0 but RegA does not (16) and since previous studies had indicated that RegA is not required to bind DNA in order to interact with CbbR (16). Since RegA can bind to CbbR only when CbbR is in the CbbR/probe-0 complex, retardation of the mobility of the complex is clearly manifested. Such a supershift thus signifies interaction between CbbR and a particular RegA mutant protein, while the absence of a supershift signifies a loss of interaction between the two proteins. A second group of gel mobility shifts using a 551-bp cbb_I promoter fragment containing RegA sites 1, 2, 3, and 4 (probe-1234) (16) was used to determine the functionality and conformational integrity of the RegA mutant proteins, since binding to probe-1234 identifies those mutant proteins that were able to bind DNA and form dimers (Fig. 3). Furthermore, it has been observed that RegA oligomerizes when it binds to CbbR or its DNA binding sites on the cbb_I promoter region (16), where oligomerization is defined as the formation of incrementally larger multimeric complexes as the concentration of RegA is increased. Due to oligomerization of RegA as seen with binding to either CbbR or probe-1234, mutated RegA molecules produced different mobilities from those seen with wild-type

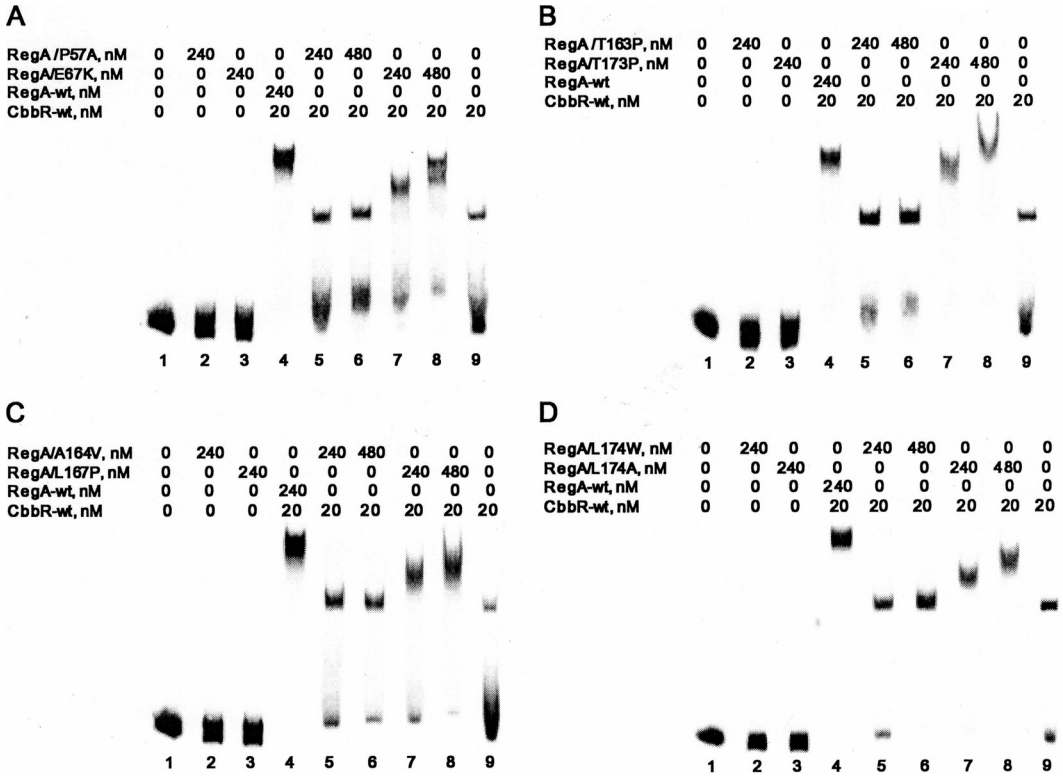

FIG 2 Phosphorimages of gel mobility shift assays, illustrating that certain RegA mutants will not interact with CbbR. CbbR was bound to ^{32}P-labeled probe-0. RegA mutant proteins that interact with CbbR create a supershifted complex consisting of CbbR, RegA, and probe-0. RegA mutants that abolish interaction with CbbR did not create a supershifted complex. Examples of RegA mutants that both abolished interaction with CbbR and retained interaction with CbbR are given. (A) RegA mutations located in the receiver domain show that RegA/P57A did not interact with CbbR and that RegAE67K did interact with CbbR. (B) RegA mutants located in the DBD show that RegAT163P did not interact with CbbR while RegA/T173P did interact with CbbR. (C) Additional RegA mutants located in the DBD. RegAA164V did not interact with CbbR, while RegAL167P did. (D) Additional RegA mutants from the DBD. RegAL174W did not interact with CbbR, while RegAL174A did. For the complete list of all RegA mutant proteins and their interactions with CbbR, see Tables 2 and 3. wt, wild type.

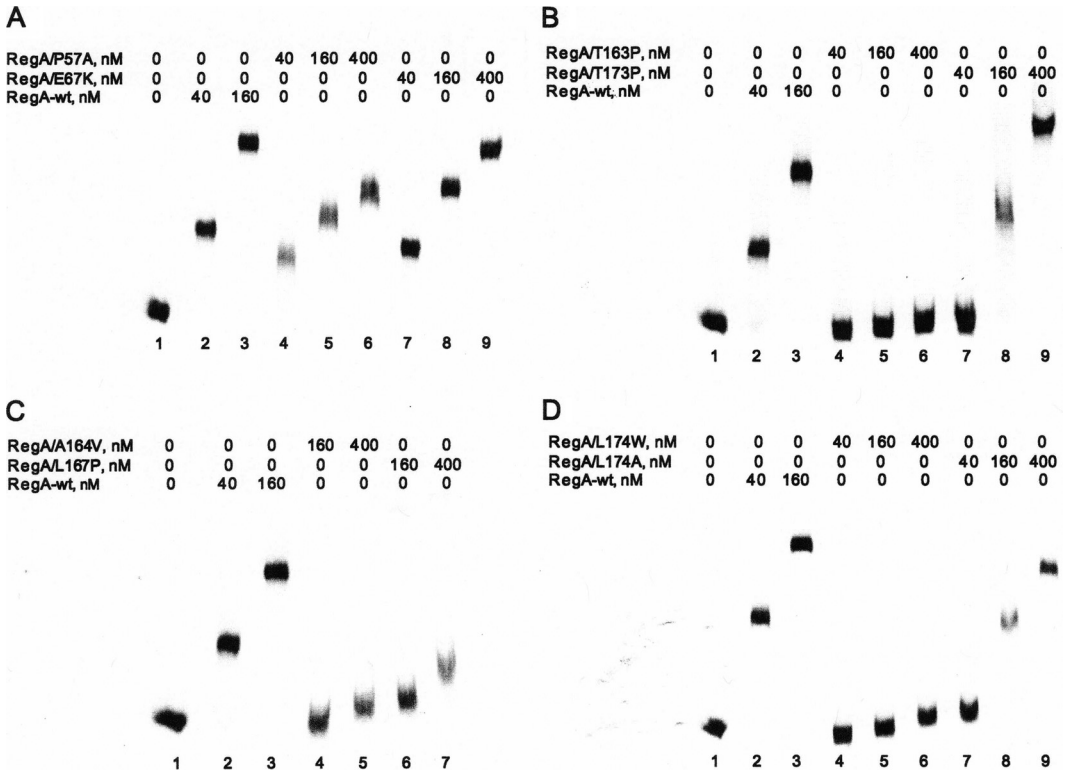

FIG 3 Phosphorimages of gel mobility shift assays illustrating the DNA binding function of the RegA mutant proteins analyzed as described in the Fig. 2 legend. ^{32}P-labeled probe-1234 was used to bind RegA. RegA mutant proteins were assessed relative to RegA-wt for DNA binding and oligomerization as a function of the RegA concentration. (A) RegAP57A and RegAE67K. (B) RegAT163P and RegAT173P. (C) RegAA164V and RegAL167P. (D) RegAL174W and L174A. For the complete list of all RegA mutants and their binding of probe-1234 DNA, see Tables 2 and 3.

RegA used at the same concentrations (Fig. 2 and 3). Most likely, these variable mobilities were caused by subtle stoichiometric changes between the interactions of mutant RegA with CbbR or probe-1234.

Several gel mobility shift experiments, using probe-0, were employed to determine interactions of each RegA mutant protein with CbbR-bound DNA (Fig. 2). Several RegA proteins with mutations in the receiver domain, including P57A and E67K (Fig. 2A) and D20A/D21A (conserved acidic pocket), M32K, P49E, and R79G, lost the ability to interact with CbbR (summarized in Table 2). Mutant proteins that show this propensity tend to contain alterations that cluster toward the N terminus of the receiver domain. All of these RegA mutant proteins, except for M32K, retain some or all of their DNA binding function when assessed for their ability to interact with probe-1234 (Fig. 3A and Table 2). Interestingly, loss of the ability of the RegA mutant proteins to interact with CbbR-bound DNA was usually accompanied by a reduction in DNA binding function (Table 2), suggesting a relationship between CbbR interaction and the ability of the RegA molecules to bind DNA. Possibly, this relationship is manifested because the residues of RegA important for binding DNA are the same residues that are required for CbbR interaction.

Mutational analysis of the DBD of RegA identified many single-amino-acid changes that abolished or severely reduced the interaction with CbbR (Fig. 2B to D and Table 3). These residues are located in the HTH region of RegA (Fig. 1) (e.g., α-helix 7, α-helix 8, and the small turn region between the two helices). These altered proteins and their residue changes are T163P, A164D, A164V, R165G, R166G, L167H, M169T, H170D, R171G, R172A, L174W, and L178W. Figure 3 depicts a representative sample of gel mobility shifts using probe-1234 to determine the inherent DNA binding capability of each RegA mutant protein. As found with mutations in the receiver domain of RegA, loss of the ability of RegA to interact with CbbR was accompanied by a reduction in cognate DNA binding to probe-1234 (Fig. 2B to D, Fig. 3B to D, and Tables 2 and 3). It is also no surprise that several RegA mutant proteins that retain their capacity to interact with CbbR have a reduction in inherent DNA binding function since these mutations are located in the DBD. These include E162G, A164G, R165H, L167P, L174A, Q175A, and L178A (Fig. 1 and Table 3). Several mutants have no or almost no DNA binding function at 40 nM and yet have good DNA binding at 160 nM or 400 nM. These include R165G, R166G, L167H, R171G, T173P (Fig. 3B), L174A (Fig. 3D), L178A, and L178W. Severely compromised DNA binding at low protein concentrations is a characteristic of many of the RegA mutants located in the DBD. In addition to reporting the lowest concentration of each RegA mutant protein that binds probe-1234, a relative scale comparing mutant RegA/promoter complex size to wild-type RegA/promoter complex size was used and is illustrated (Tables 2 and 3), where +++ denotes RegA/promoter complex sizes comparable to the wild-type RegA size, ++ denotes complex sizes that were reduced relative to the wild-type RegA size, + denotes a severe reduction of complex size relative to the wild-type RegA size, −/+ denotes complex mobility that was slightly more than that seen with the probe only, and − denotes no DNA binding relative to wild-type RegA. No

TABLE 2 Summary of RegA mutant proteins generated from the receiver domain and linker domain indicating RegA-CbbR (DNA) interactions (using probe-0) and DNA binding function manifested by the ability to bind probe-1234

RegA protein	CbbR interaction (probe-0)[a]	DNA binding	
		Probe-1234[b]	Lowest [RegA] (nM)[c]
wt	+	+++	40
Receiver domain			
D20A/D21A	−	+	160
R27L	+	++	40
M32K	−	−	No binding
R35L	+	++	40
K49E	−	++	40
P57A	−	++	40
D63A(-P)	+	+++	40
E67K	+	++	40
R79G	−	+	40
D84A	+	+++	40
T91A	+	+++	40
A97S	+	+++	40
D109A	+	+++	40
K113M	+	+++	40
Linker			
P134A	+	+++	40

[a] For CbbR interaction, + denotes interaction and − denotes no interaction.
[b] For DNA binding, +++ denotes RegA/promoter complex size comparable to wild-type RegA size, ++ denotes complex size reduced relative to wild-type RegA size, + denotes severe reduction of complex size relative to wild-type RegA size, and − denotes no DNA binding relative to wild-type RegA binding.
[c] Lowest concentration of RegA that bound probe-1234.

TABLE 3 Summary of RegA mutant proteins from the DBD indicating RegA-CbbR (DNA) interactions (probe-0) and DNA binding function (probe-1234)

RegA protein	CbbR interaction (probe-0)[a]	DNA binding	
		Probe-1234[b]	Lowest [RegA] (nM)[c]
wt	+	+++	40
α-6			
W146G	+	+++	40
C156G	+	+++	40
N159S	+	+++	40
α-7			
S161A	+	+++	40
S161P	+	+++	40
E162G	+	+++	40
E162K	+	+++	40
T163A	+	+++	40
T163P	−	−/+	400
A164D	−	+	400
A164V	−	+	400
A164G	+	++	160
A164S	+	+++	40
R165G	−	+	160
R165H	+	++	40
R166G	−	++	160
L167H	−	++	160
L167P	−/+	+	400
Turn			
N168Y	+	+++	40
M169T	−	++	40
H170D	−	++	160
α-8			
R171G	−	++	160
R172A	−/+	+	40
T173P	+	++	160
L174A	+	++	40
L174W	−	+	400
Q175A	+	++	40
I177N	+	+++	40
L178A	+	++	160
L178W	−/+	++	160

[a] For CbbR interaction, + denotes interaction, − denotes no interaction, and −/+ denotes barely discernible interaction.
[b] For DNA binding, +++ denotes RegA/promoter complex size comparable to wild-type RegA size, ++ denotes complex size reduced relative to wild-type RegA size, + denotes severe reduction of complex size relative to wild-type RegA size, and −/+ denotes complex mobility was slightly more than that seen with probe only.
[c] Lowest concentration of RegA that bound probe-1234.

RegA mutant proteins, whether interacting with CbbR or binding DNA, were associated with protein/DNA complexes that were larger (i.e., representing reduced mobility) than wild-type RegA complexes, at equal RegA concentrations. Therefore, it would appear that a reduction in protein/DNA complex size is a departure from optimum function.

Tables 2 and 3 summarize the entire gel mobility shift data for all RegA mutants used in this study. Many of the residues within the DBD of RegA that are important for DNA binding are illustrated in this report and confirm structural predictions from Laguri et al. (23).

Structural implications of RegA mutations. Fig. 1 is a three-dimensional (3-D) ribbon model of the linker region and DBD of RegA (residues 125 to 184). DNA interaction with this RegA fragment indicates that α-helix 6 runs along the backbone of the DNA, while α-helix 7 locates just above the major groove and α-helix 8 rests in the major groove (23). Residues highlighted (colored) in Fig. 1 denote the RegA mutations used in this study; the mutated residues highlighted in green abolish interaction with CbbR, and the mutated residues highlighted in magenta do not abolish interaction with CbbR. Residues in α-helix 7 (residues 163 to 167) and in α-helix 8 (residues 171, 172, 174, and 178) and the three residues between the two helices (residues 168 to 170) that form a pocket in the DBD of RegA may serve as a localized region of interaction with CbbR (Fig. 1). Point mutations that abolish or severely reduce interaction with CbbR define this pocket and reveal this to be a region critical for RegA/CbbR interaction. Mutagenesis of residue Ala-164 (within α-helix 7) indicates that mutant A164D or conservative mutant A164V was sufficient to abolish interaction between RegA and CbbR, but small-residue substitutions, A164G and A164S, still retained the ability to interact with CbbR (Fig. 2C and Table 3). Ala-164 is the amino acid closest to α-helix 8 (positioned directly above residues His-170 and Arg-171 of α-helix 8), so any residue larger than alanine appears to inhibit interaction with CbbR (Fig. 1). This inhibition of interaction may be due to steric hindrances or conformational

changes produced by larger negatively charged residues (e.g., aspartic acid) or larger hydrophobic residues (e.g., valine) at position 164 that interfere with the positively charged residues, His-170 and Arg-171, and reduce their ability to interact with CbbR.

Judging on the basis of the foregoing results, it is possible that changing the conformation of this localized pocket of RegA prevents certain residues within the CbbR protein from entering the pocket. Alternatively, α-helix 7 interacts directly with CbbR because of its position directly above the DNA helix and its accessibility. Substitution of alanine at position 164 with a larger residue or a more hydrophobic residue was sufficient to disrupt interaction with CbbR. Any nonconservative substitution within the second half of α-helix 7 (T163P, A164D, A164V, R165G, R166G, or L167H) destroyed CbbR interactions (Fig. 2B and C and Table 3). Changing the charge of any residue within this region (A164D, R165G, R166G, L167H, H170D, R171G, or R172A) also disrupted CbbR interaction (Table 3). Large hydrophobic substitutions of two residues in RegA positioned on the top of α-helix 8 oriented near the pocket, L174W and L178W, provided further evidence that steric hindrance may play a role in the disruption of CbbR interactions (Fig. 1 and Table 3). A small-amino-acid substitution, L174A or L178A, did not disrupt CbbR interaction, but the large-amino-acid substitutions, L174W and L178W, did disrupt CbbR interaction, suggesting that CbbR is required to fit into this region and is excluded by bulky residues (Fig. 2D and 3D).

Direct evidence for mutant RegA/CbbR interactions. Verification of the interaction between CbbR and the mutant RegA proteins was accomplished by isolation of the CbbR/RegA/DNA complex from nondenaturing polyacrylamide gels and subsequent identification of each protein from the complex. This experiment provided direct evidence for CbbR/RegA interactions (Fig. 4). A representative group of six RegA mutant proteins with amino acid substitutions in the DBD (see Fig. 2 and 3) were chosen to illustrate that supershifted complexes contained both CbbR and mutant RegA. Three of the RegA mutants chosen (RegAL167P, RegAT173P, and RegAL174A) interacted with CbbR (Fig. 4A to C). Three of the RegA mutants chosen (RegAT163P, RegAA164V, and RegAL174W) did not interact with CbbR (Fig. 4D to F), as manifested by the lack of recovery of RegA proteins from the gel. CbbR/RegA/probe-0 complexes were separated on gel mobility shifts, and the complexes containing unlabeled probe-0 were excised from the polyacrylamide gel and the proteins extracted as described in Materials and Methods. A positive control for each mutant RegA protein (Fig. 4) and subsequent immunoblotting confirmed that the RegA mutant proteins bound and formed complexes with probe-1234 and that the mutant RegA proteins were recognized by the antibody. As negative controls, CbbR and RegA mutant proteins were analyzed on polyacrylamide gels without probe DNA; gel slices were excised at the position where CbbR/RegA/probe complexes would be expected to migrate and were processed in the same manner as potential RegA/CbbR/DNA complexes. The isolated proteins were separated by SDS-PAGE (12% acrylamide) and subjected to immunoblot analysis using either specific anti-RegA- or anti-His-tagged antibodies (detecting His-tagged CbbR). From this analysis, it was apparent that the immunoblots detected the presence of the mutant RegA proteins and CbbR in the appropriate complexes and verified that the RegA mutants interact with the CbbR/probe-0 complex (Fig. 4). The negative controls verified that neither RegA protein nor CbbR protein migrated at the same position as protein/DNA complexes and that free proteins did not contaminate the assays.

A truncation mutant of CbbR containing only the DBD and linker helix region is sufficient to interact with the DBD of RegA *in vitro*. To determine if the DBD of CbbR interacts with RegA, three truncation mutants of CbbR were constructed and were used in gel mobility shift assays. The first truncation mutant protein contains only the DBD (residues 1 to 65), the second truncation mutant protein contains the DBD and the linker helix, designated CbbR-DBD-plus-linker (residues 1 to 96), and the third truncation mutant protein contains the linker helix and recognition domains I and II, designated CbbR-linker-plus-RDI/RDII (residues 66 to 310). A gel mobility shift using probe-0 illustrated that the CbbR-DBD-plus-linker protein interacted with RegA (Fig. 5A). Interestingly, the CbbR-DBD-plus-linker molecule did not bind probe-0 alone (Fig. 5A, lane 3) but was able to bind probe-0 when RegA was present to create a CbbR-DBD-plus-linker/RegA/probe-0 complex that shifted probe-0 (Fig. 5A, lane 6).

It is known that RegA enhances the binding affinity of CbbR for the cbb_I promoter DNA as much as 11-fold (16). This enhancement of DNA binding affinity allows the CbbR-DBD-plus-linker protein to bind to probe-0. As expected, CbbR-wt/RegA/probe-0 migrated as a complex that was larger than the CbbR-DBD-plus-linker/RegA/probe-0 complex, most likely because CbbR-wt is approximately 3-fold larger than the CbbR-DBD-plus-linker protein (Fig. 5A, lanes 6 and 7). The CbbR-linker-plus-RDI/RDII protein served as a negative control in these experiments since it will not bind probe-0 (Fig. 5A, lanes 3 and 5). The first CbbR truncation mutant protein that contained only the DBD cannot bind probe-0 in either the presence or the absence of RegA (data not shown). This suggests that the linker helix provides stability, possibly for dimerization. Dimerization is necessary for DNA binding of LTTR proteins. Long (greater than 20 residues) α-helices often provide coil-coil interaction for stability to facilitate dimerization, as is the case with several LTTR proteins (19–22, 46), as well as other transcription factors, including the leucine zipper family and basic leucine zipper (bzip) proteins (47, 48).

A second gel mobility shift assay using probe-1234 was performed to determine if the CbbR-DBD-plus-linker protein would bind to a larger cbb_I promoter probe and also to determine whether the CbbR-linker-plus-RDI/RDII protein would interact with RegA when RegA was bound to probe-1234. Surprisingly, the CbbR-DBD-plus-linker protein was able to bind probe-1234 in the absence of RegA (Fig. 5B, lane 4), in contrast to probe-0, with which the CbbR-DBD-plus-linker protein cannot bind in the absence of RegA. The CbbR-DBD-plus-linker and RegA proteins were able to bind probe-1234 simultaneously to generate a supershift (Fig. 5B, lane 6). The CbbR-linker-plus-RDI/RDII protein was not able to interact with RegA that is bound to probe-1234 (Fig. 5B, lane 5), indicating that the linker helix, RDI, or RDII was not sufficient to facilitate interaction with RegA. The data in Fig. 5 established that the DBD of CbbR interacted with RegA.

To summarize these results, a general model depicting the interaction of CbbR and phosphorylated RegA on the cbb_I promoter is provided (Fig. 6) where both proteins are shown to interact at their DBD regions. Thus, RegA-DBD mutants that effectively interact with CbbR, such as L167P, T173P, and L174A (Fig. 4; others are summarized in Tables 2 and 3), interact through their DBD regions, as depicted by the white shaded area of both proteins (Fig. 6). Clearly, RegA-DBD mutants such as T163P, A164V, and

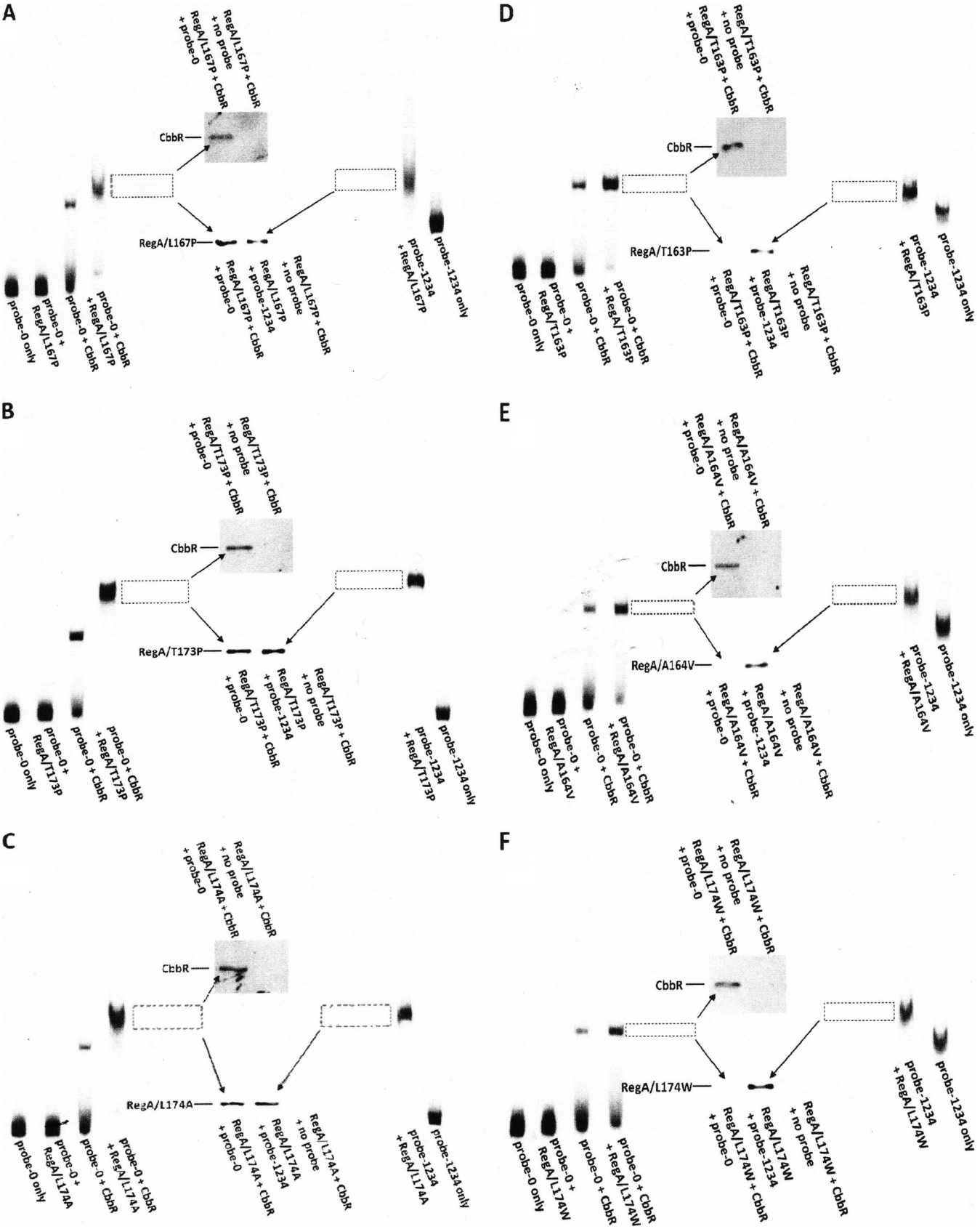

FIG 4 A combination of immunoblot and gel mobility shift analyses illustrates the presence of mutant RegA and CbbR proteins extracted from CbbR/RegA/*cbb* promoter complexes. Proteins from protein/DNA complexes contained in-gel fragments (represented by dashed boxes) excised from nondenaturing polyacryl-

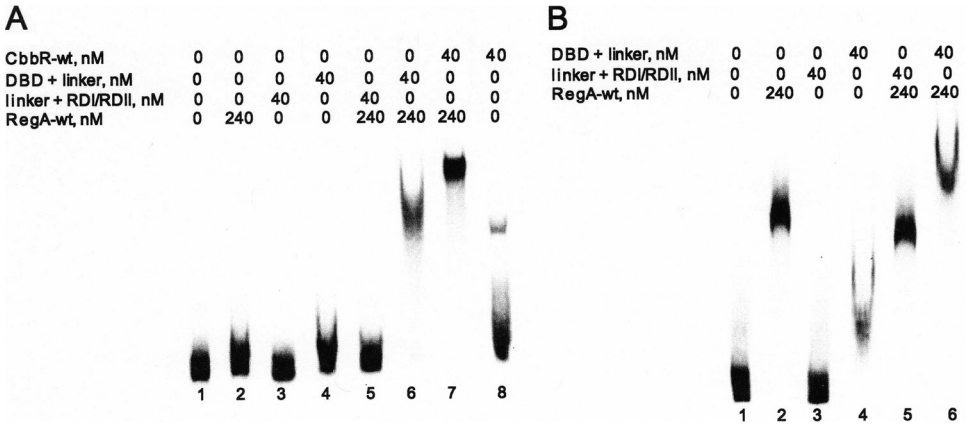

FIG 5 Phosphorimages of gel mobility shift assays illustrating that the DBD of CbbR interacts with RegA. (A) CbbR-DBD-plus-linker helix truncation mutant protein bound to ^{32}P-labeled probe-0 in the presence of RegA. (B) CbbR-DBD-plus-linker helix truncation mutant protein bound to ^{32}P-labeled probe-1234 in the absence or presence of RegA; CbbR-linker-plus-RDI/RDII truncation mutant protein did not bind to probe-1234 or RegA.

L174W (Fig. 4) do not interact with CbbR. Also illustrated by this model is the interaction of the positive coinducer metabolite RuBP, which is thought to influence the conformation of CbbR so that it is better able to bind to the promoter and/or subsequently influence transcription by RNA polymerase.

Protein-protein interactions of DBDs of other prokaryotic or eukaryotic proteins have been previously reported, but these appear to be an infrequent occurrence. Similarly to CbbR interacting with RegA only when bound to the *cbb* promoter, the DBDs of the *Drosophila* nuclear receptors, EcR and DHR38, interact only on the *hsp27pal* promoter (32). In *Vibrio cholerae*, the wing region of the HTH of ToxR interacts with a second transcriptional regulator, TcpP, to activate gene expression (28), and in the marine bacterium *Erythrobacter litoralis*, interaction between the HTH domain and the light-oxygen-voltage domain of the blue-light-activated photosensory protein (EL222) inhibits DNA binding (29). Other examples of DBD interactions have also been reported (26, 27, 30, 31).

Clearly, the interaction between the DBDs of CbbR and RegA enhances the affinity of CbbR for the cbb_I promoter (16), which also establishes a finer attenuation of gene expression through the communication of the HTH domains, thus illustrating the gain of two functions for the DBD of CbbR by the acquired interaction with RegA. In addition, the interaction between DBDs of the two regulators most likely alters the conformation of the DBD of CbbR, which facilitates a modified orientation along the cbb_I promoter to enhance or stabilize DNA binding.

The DBD/linker helix truncation mutant of CbbR cannot complement photoheterotrophic or photoautotrophic growth in a *cbbR* deletion strain. To determine whether the CbbR-DBD-plus-linker helix truncation (residues 1 to 99) protein can function *in vivo*, DNA encoding this truncation was placed on the pVK101 broad-host-range vector (expression driven by the *cbbR* promoter) and mated into *cbbR* deletion strain 87 (10). For this experiment, strains HR and 87 contained the empty pVK101 vector to serve as controls (Table 4). Photoheterotrophic growth studies indicated that no growth advantage was elicited for strain 87 containing the CbbR-DBD-plus-linker helix truncation, designated 87/CbbR(trunc) (Table 4) relative to strain 87 alone. In addition, the truncated protein supported slower growth relative to wild-type strain HR or strain 87 complemented with the wild-type *cbbR* gene, designated 87/CbbR(wt) (Table 4). In fact, a modest reduction in photoautotrophic (CO_2-dependent) growth was observed for strain 87/CbbR(trunc) relative to strain 87 (Table 4) and a substantial reduction in the growth rate relative to strain 87/CbbR(wt) was observed. This reduction in the growth rate may have been a consequence of the CbbR(trunc)/RegA complex not being able to properly contact RNA polymerase, thus causing a reduction in *cbb* transcription. RegA alone (without CbbR) can initiate photoheterotrophic and photoautotrophic growth in *R. sphaeroides*, but growth is considerably slower than that of wild-type strains since *cbb* transcription is active in the absence of CbbR only partially (1). The CbbR(trunc)/RegA complex may interfere with *cbb* transcription relative to RegA alone (strain 87), and RegA-only-dependent photoautotrophic growth was reduced relative to CbbR(wt)/RegA growth (Table 4). RubisCO-specific activities also reflected these growth data (Table 4). RubisCO-specific activities for strain 87 were 4- to 5-fold lower than for strain 87/CbbR(wt) or wild-type strain HR during photoheterotrophic or photoautotrophic growth, but the RubisCO activity of strain 87/CbbR(trunc) was almost half that of strain 87 during photoautotrophic growth, illustrating the possible interference of the CbbR truncation mutant protein with the initiation of *cbb* transcription relative to the strain without CbbR.

amide gels that were extracted, isolated, and subjected to immunoblotting as described in Materials and Methods. Gel mobility shifts are shown on the far left and far right of each individual panel (A to F), and immunoblots (detecting mutant RegA and CbbR) are located in the center. Polyclonal antibodies generated against RegA from *R. sphaeroides* were used to identify and detect the presence of the RegA mutant proteins; a monoclonal antibody generated against His-tagged proteins identified and detected the presence of His-tagged CbbR (see Materials and Methods). Every lane from the gel mobility shifts and immunoblots was identified with respect to the protein(s) and *cbb* probe DNA in each reaction. A control using probe-1234 was run for each mutant RegA protein (to the right in panels A to F) to verify that the antibodies would detect mutant RegA proteins, thus validating the results obtained with RegA/CbbR/cbb_I promoter complexes (to the left in panels A to F). The RegA mutants used in this study were as follows: RegAL167P (A); RegAT173P (B); RegAL174A (C); RegAT163P (D); RegAA164V (E); RegAL174W (F).

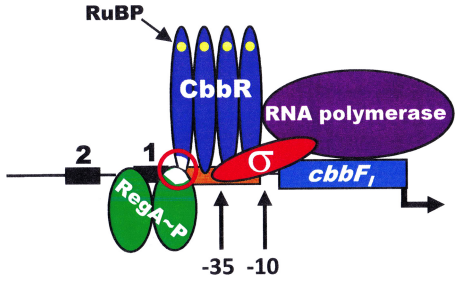

FIG 6 Model of CbbR-RegA interactions on the *cbb*$_I$ promoter illustrating the overlapping DNA binding sites of CbbR and RegA site 1. The CbbR DNA binding site also overlaps the −10 and −35 positions recognized by the *R. sphaeroides* sigma factor associated with RNA polymerase. RegA DNA binding site 2 is also pictured. A CbbR tetramer is shown to bind the positive-effector molecule, RuBP, which is thought to change the conformation of CbbR so that it is better able to recruit RNA polymerase and influence transcription. CbbR and the phosphorylated RegA (dimer) interact with each other via their DBD regions (depicted by white shadowing of each protein, encircled in red) near the DNA helix. Thus, both transcriptional regulators are poised to activate transcription of *cbbF*$_I$ (black arrow), the first gene in the *cbb*$_I$ operon.

Reverse transcription-PCR (RT-PCR) was employed to determine whether the CbbR(trunc) construct (pVK442) was transcriptionally active *in vivo*. As controls, RNAs were isolated from the pVK12 construct (wild-type CbbR) in strain 87, strain 87 itself, and strain HR under both photoheterotrophic and photoautotrophic growth conditions. Setting wild-type strain HR at 1.0 for the relative transcription of the *cbbR* gene, strain 87/CbbR(wt) yielded 1.9-fold relative transcriptional activity, strain 87/CbbR(trunc) yielded 1.4-fold relative transcriptional activity, and strain 87 yielded 0 relative transcriptional activity under photoautotrophic growth conditions. Similar transcriptional activities of the *cbbR* gene were obtained for these strains under photoheterotrophic growth conditions. The CbbR(trunc) protein can be synthesized and is stable in *E. coli* and has DNA binding activity *in vitro* (Fig. 5). Direct quantification of the CbbR protein (via immunoblotting or enzyme-linked immunosorbent assay [ELISA]) in *R. sphaeroides* is not reliable due to its low abundance.

Constitutively active CbbR mutant proteins require interaction with phosphorylated RegA (RegA~P) (not unphosphorylated RegA) to activate *cbb* transcription *in vivo* in *R. sphaeroides*. Chemoheterotrophic growth normally leads to strong repression of *cbb* expression since CbbR's ability to activate transcription is compromised by the binding of negative effectors that accumulate under these growth conditions (10, 49, 50). CbbR constitutive mutant proteins (CbbR*) are defined as CbbR molecules that can activate both *cbb*$_I$ and *cbb*$_{II}$ transcription under chemoheterotrophic growth conditions, which are conditions under which the wild-type CbbR protein cannot activate transcription (10). This unique characteristic of constitutively active CbbR proteins (CbbR*s) was exploited to determine if the phosphorylation site of RegA, whether phosphorylated (RegA~P) or unphosphorylated, is required for interaction with CbbR* to activate transcription/translation of *cbb* operon genes. Like many major regulators, CbbR synthesis itself is not regulated (51). The PrrA2 strain is a RegA deletion strain of *R. sphaeroides* (39), while the PrrA2(D63A) strain contains a point mutation in RegA (D63A) that abrogates the ability of the protein to be phosphorylated by RegB/PrrB catalysis (33, 52); the PrrA2(wt) strain is the RegA deletion strain complemented with wild-type RegA. Expression of both the wild-type *regA* gene and the *regA*(*D63A*) mutant gene was driven by the *regA* promoter, and both *regA* genes were placed on the pRK415 broad-host-range vector and mated into strain PrrA2. The *cbbR** constitutive mutants employed were previously described (10). Expression of the *cbbR** genes, *cbbR**(*P160L*), *cbbR**(*R158C*), and *cbbR**(*R274W*), was driven from the *cbbR* promoter, and the genes were placed on the pBBR1MCS-2 broad-host-range vector and mated into strains PrrA2, PrrA2(D63A), and PrrA2(wt). Activation of the *cbb* operons and subsequent accumulation of active RubisCO under chemoheterotrophic growth conditions in strains PrrA2(wt) and PrrA2(CbbR*) provided a sensitive indication of the role of the CbbR* proteins in RubisCO gene expression, as well as reflecting the stability of the CbbR* proteins *in vivo*. The expression and stability of the RegAD63A protein in strain PrrA2 and the wild-type RegA protein in strain 2.4.1 were previously demonstrated (33).

The sensitive radiometric RubisCO activity assays, where RubisCO was encoded by the *cbbLS* genes of the *cbb*$_I$ operon, detected whether *cbb* expression might occur under chemoheterotrophic growth conditions with malate as the carbon source. Only strains containing the constitutively active CbbR* proteins, in association with phosphorylated RegA (RegA~P), showed RubisCO activity (specific activities ranging 4 to 16 nmol CO_2 fixed/min/mg protein). No RubisCO activity whatsoever, and no *cbb* expression, was found in strains containing the RegA phosphorylation mutant, D63A, or in the RegA deletion mutant, PrrA2. Clearly, as manifested by the detection of RubisCO activity, these results illustrate that CbbR requires interaction with RegA~P to activate expression and subsequently translate key *cbb* operon genes *in vivo*.

There are other salient points that reflect the interaction of the two transcription regulator proteins. Thus, despite the requirement for RegA~P for productive expression of *cbb* genes, it is important that RegAD63A does interact with CbbR and can bind the *cbb*$_I$ promoter, as indicated in Table 2 and a previous study (16). Moreover, it is apparent that there is some basal level of RegA phosphorylation even under chemoheterotrophic growth conditions, which are conditions where RegA is assumed to be unphosphorylated or poorly phosphorylated (15). In addition, the RegA/D63A mutant has severely impaired functional capabilities (affected in structural integrity or activity or both) since the RegA/D63A protein was unable to stimulate *in vitro* transcription of the *cycA P2* gene. Interestingly, however, unphosphorylated wild-type

TABLE 4 RubisCO-specific activities and doubling times for *R. sphaeroides* strain 87 complemented with wild-type and truncated CbbR mutant proteins[a]

Strain	Photoheterotrophic activity		Photoautotrophic activity	
	RubisCO (nmol product/mg of protein)	Doubling time (h)	RubisCO (nmol product/mg of protein)	Doubling time (h)
HR	21	5.5	280	20.2
87/CbbR(wt)	23	7.1	245	18.5
87	5	10.5	52	27.7
87/CbbR(trunc)	4	11.9	27	34.5

[a] Enzyme activities are expressed in nmol product per minute per milligram of protein in lysates. Values are the averages of results of three independent determinations with standard deviations not exceeding 10%. Doubling times are the averages of results of three independent determinations with standard deviations not exceeding 12%.

RegA was also found to partially stimulate *in vitro* transcription (33). Clearly, with wild-type unphosphorylated RegA, it is difficult to demonstrate that there was not some low level of phosphorylation that had occurred *in vivo* with wild-type unphosphorylated RegA that might not be detected *in vitro*. Thus, it was apparent that RegAD63A most certainly was not phosphorylated to any significant extent under any circumstances. This interpretation is supported by the lack of detectable RubisCO activity in strains that contained this protein. Finally, the present study illustrated that the CbbR* proteins retain their constitutive properties even in the presence of wild-type CbbR since the PrrA2 strain has an active native *cbbR* gene on chromosome one. Most likely, CbbR and CbbR* form heterodimers that can still function constitutively and activate the *cbb* operons under chemoheterotrophic growth conditions. Alternatively, a subpopulation of the dimers formed are CbbR*/CbbR* homodimers and function constitutively, while CbbR/CbbR* heterodimers may not be constitutively capable of activating *cbb* expression.

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