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Involvement of the Cyclic AMP Receptor Protein

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Previous studies with *Salmonella enterica* serovar Typhimurium LT2 demonstrated that transcriptional activation of the prpBCDE operon requires the function of transcription factor PrpR, sigma-54, and IHF. In this study, we found that transcription from the prpBCDE and prpR promoters was down-regulated by the addition of glucose or glycerol, indicating that these genes may be regulated by the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex. Targeted mutagenesis of a putative CRP-binding site in the promoter region between prpR and prpBCDE suggested that these genes are under the control of CRP. Furthermore, cells with defects in cya or crp exhibited reduced transcriptional activation of prpR and prpBCDE in *Escherichia coli*. These results demonstrate that propionate metabolism is subject to catabolite repression by the global transcriptional regulator CRP and that this regulation is effected through control of both the regulator gene prpR and the prpBCDE operon itself. The unique properties of the regulation of these two divergent promoters may have important implications for mechanisms of CRP-dependent catabolite repression acting in conjunction with a member of the sigma-54 family of transcriptional activators.

In *Escherichia coli*, glucose controls utilization of alternative carbon sources by regulating gene expression in response to glucose depletion (8, 19, 21). Transcriptional regulation is modulated by the level of cyclic AMP (cAMP) synthesized by the membrane-bound adenylate cyclase and cAMP receptor protein (CRP), a global transcriptional regulator. Phosphorylated EIIA$^{\text{cIC}}$, an intermediate in the phosphorylation cascade of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) for the uptake of glucose, is thought to stimulate adenylate cyclase. As a consequence, the active cAMP-CRP complex binds to specific DNA sites located at or upstream of CRP-dependent promoters. Binding of cAMP-CRP to these DNA sites modulates transcription initiation by RNA polymerase (RNAP). In addition, recent studies on catabolite repression caused by non-PTS sugars such as glucose-6-phosphate or glucosamine concluded that it is the amount of CRP, as well as cAMP, that is altered in response to non-PTS sugars (14, 16, 44). Interestingly, Eppler et al. (9) proposed that glycerol-3-phosphate or glycerol also causes catabolite repression by interference with the stimulation of adenylate cyclase by EIIA$^{\text{cIC}}$-P.

The cluster of genes required for the catabolism of propionate was first identified in and characterized for *Salmonella enterica* serovar Typhimurium (12, 15), and a closely related gene cluster was found in *E. coli* during the sequencing of this bacterium (1). These genes constitute a locus composed of two divergently transcribed units. One unit is the single gene prpR, which encodes a member of the sigma-54 ($\sigma^{54}$)-dependent activator family (32, 40). The second transcriptional unit contains the prpBCDE operon, which encodes the enzymes for propionate metabolism (also known as the 2-methylcitrate pathway), allowing growth on propionate as a sole carbon and energy source (15). Interestingly, the region between the two transcriptional units contains a putative $\sigma^{54}$-dependent promoter for prpR and a $\sigma^{54}$-dependent promoter 5′ to prpBCDE (Fig. 1). To date, four regulatory elements have been shown to participate in the transcriptional activation of the prpBCDE operon of *S. enterica* serovar Typhimurium LT2: PrpR, a co-activator of PrpR such as 2-methylcitrate or a derivative, $\sigma^{54}$, and integration host factor (IHF) (15, 32, 47). Very little is known about the regulation of prpR expression itself.

Expression of prpBCDE is dependent on the $\sigma^{54}$ RNAP holoenzyme (Esr$^{35}$) and PrpR, an NtrC-like protein in *S. enterica* (15, 32, 47). Promoter sequences recognized by Esr$^{35}$ are well conserved (TGGCAC-5 nucleotides [nt]-TTGCA/T, situated between −26 and −11 bp) and distinct from classical −35, −10 $\sigma^{70}$-type consensus promoters (Fig. 1A). Esr$^{54}$ binds to its cognate promoters as a transcriptionally inactive closed complex. Activator proteins in the NtrC family interact directly with Esr$^{35}$ to stimulate transcription from $\sigma^{54}$-dependent promoters (27), which are generally involved in nitrogen regulation in *E. coli*. Since nitrogen assimilation consumes energy and intermediates of central metabolism (35, 38), the direct action of the cAMP-CRP complex as a modulator of Esr$^{54}$ could provide a regulatory link between carbon and nitrogen metabolism by the dual regulatory role of PTS components and the CAMP-CRP complex (the activation of $\sigma^{70}$-dependent promoters involved in carbon metabolism and the downregulation of $\sigma^{54}$-dependent promoters involved in nitrogen assimilation) (5, 36, 37). Recently, it has been reported that CRP/cAMP can bind at $\sigma^{54}$-dependent promoters and inhibit the ability of Esr$^{35}$ to activate transcription (46, 48). This effect appears to be both CRP and $\sigma^{54}$ dependent.
In this work, the overlapping and opposing promoter elements for _E. coli_ prpR and _prpBCDE_ within the propionate catabolic gene cluster were investigated by using site-specific mutations and transcriptional fusion reporter constructs. We show that the catabolism of propionate in _E. coli_ and _S. enterica_ serovar Typhimurium is modulated by catabolite repression together with PrpR. Our results imply that the cAMP-CRP complex can act as a positive transcriptional regulator of both the σ70-dependent _prpR_ promoter and the σ54-dependent _prpBCDE_ promoter.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. Cultures were grown in Luria-Bertani (LB) broth at 37°C. Cell growth was monitored as the optical density at a wavelength of 600 nm (OD_{600}). Media were amended with 10 mM sodium propionate (pH 8.0), 25 μg of tetracycline (TC) per ml as an inducer, and 0.2 or 0.4% glucose or glycerol, as indicated.

**Plasmid and strain construction.** The low-copy-number pAP vector was created by using _pACYC184_ and _pPROBE_-gfp plasmids for promoter activity assay. First, the gene encoding the green fluorescent protein, gfp, was removed from pPROBE-gfp by self-ligation of HindIII-digested pPROBE-gfp vectorless. The resulting construct was digested with PvuI and BsaBI. The small fragment (2,057 bp) containing four tandem copies of the TC terminator from the _E. coli_ rnb1 operon, a multicloning site, a phage T7 promoter, and a single T1 terminator was added in with _T4_ DNA polymerase and ligated to the _pACYC184_ vector, which had been digested with ClaI and BsaBI and filled in with _T4_ DNA polymerase. The pAPLPR reporter plasmid, a bidirectional transcriptional fusion vector, was constructed by splice overlap extension-PCR. Initially, three separate PCRs were performed to amplify _lacZ_ (the gene encoding β-galactosidase [LacZ]), _prpBCDE_ promoter sequences, and _rfp_ (the gene encoding the red fluorescent protein [RFP]) with the _pTC40_ plasmid, _E. coli_ BL21 genomic DNA, and the p70GL plasmid as templates, respectively. The three products were then mixed, and a second PCR was performed with a pair of primers, one each for the 3' ends of _lacZ_ and _rfp_. The resultant splice overlap extension-PCR products were ligated into the HindIII-digested pAP vector. A series of _pAPR_ and _pAPF_ plasmids were constructed by PCR amplifying _P_{prpR-lacZ}_ and _P_{prpR-rfp}_ with pAPLPR as the template and by ligating the PCR products into the HindIII/KpnI-digested pAP vector. For serial deletions of _prpBCDE_ upstream regions, the _rfp_ gene and the upstream sequence were individually amplified from the pAPLPR plasmid by PCR with specific primers containing KpnI and HindIII restriction sites. The DNA fragments obtained from PCR were digested with KpnI and HindIII and ligated into the _KpnI_ and HindIII sites. The resulting plasmids were used as promoters of promoter activity. The sequences of the promoter region were confirmed by DNA Sanger dye deoxy terminator sequencing (Elim Biopharmaceuticals, Inc., Hayward, Calif.). pZB, an expression vector, was constructed by removing chaperone genes from pGKJ8 carrying an origin of replication derived from _pACYC_ a chloramphenicol resistance gene, and the necessary regulatory components (araC and _tetR_) for the arabinose-inducible araBAD promoter (P_{araBAD}) and the TC-inducible promoter (P_{Tet}). Two multicloning sites were created by PCR with primers containing sequences corresponding to restriction enzyme sites. Plasmids pZBR and pZBRR were constructed from pZB by cloning sequences corresponding to restriction enzyme sites. Plasmids pZBR and pZBRR were constructed from pZB by cloning _rnb_ under control of the _prpBCDE_ promoter and by cloning _prpR_ under control of the _P{prpR}_{lacZ} promoter and by cloning _prpR_ under control of the _prpBCDE_ promoter, respectively. A _lacZ_ mutant of _E. coli_ W3110 was constructed by allelic exchange with the integration plasmid pBRINT-T-Gm (2). Mutations in _crp_ and _cya_ in _E. coli_ W3110 and DH10B strains were constructed by a PCR-mediated gene disruption method (7).

**DNA manipulation and site-directed mutagenesis.** Bacterial genomic DNA and plasmid DNA were routinely prepared with QIAGEN miniprep kits (QIAGEN Inc., Chatsworth, Calif.). PCR was performed with _Pfu_ DNA polymerase under standard conditions. Site-directed mutagenesis of the cAMP-CRP binding site in probes used in the promoter assays was performed by PCR with primers containing the desired mutation.

**FIG. 1.** Nucleotide sequence of the _prpR-prpBCDE_ bidirectional promoter region in _E. coli_ and _S. enterica_ (A). On the basis of previous work (32), a σ70 promoter for _prpR_, a consensus σ54 binding region 5' to _prpBCDE_, and two ribosome-binding sites (RBS) are underlined and labeled in the promoter region between the two transcriptional units. The proposed ATG start sites for PrpR and PrpB are boxed. Putative CRP-binding sequences are identified, shaded, and labeled. An inverted repeat (GTTCAT-10 nt-ATGAAAC), which may be a PrpR-binding site for activation of the _prpBCDE_ promoter, is in italics. Nucleotides in the region between the two genes are numbered 5’ to 3’ on the basis of the _E. coli_ sequence. Putative binding sites for regulator proteins are shown (B). The inferred −10 and −35 region and −12 and −24 region of each promoter are indicated. Reporter plasmids were constructed by fusion of the _prpBCDE_ promoter to the gene encoding RFP and/or the _prpR_ promoter to the gene encoding LacZ.
RESULTS

Influence of glucose and glycerol on \(pprBCDE\) and \(prpR\) expression. Recently, we found that propionate catabolism may be affected by the presence of glycerol or glucose. To determine how these carbon sources might affect \(pprBCDE\) expression, we constructed a reporter plasmid containing a bidirectional transcriptional fusion of the \(pprBCDE\) promoter to the gene encoding RFP (\(P_{pprBCDE}\)-rfp) and the \(prpR\) promoter to the gene encoding LacZ (\(P_{prpR}\)-lacZ). The \(pprBCDE\) promoter activity, as measured by the \(P_{pprBCDE}\)-rfp reporter fusion, was high in the presence of propionate in \(E. coli\) JSW1 and \(S. enterica\) TR6583 (Fig. 2A and B). However, activity from this promoter decreased to background levels when glucose or glycerol was added to cells growing in the presence of propionate. The \(pprBCDE\) promoter in both strains displayed this catabolite repression in response to glucose or glycerol. As expected, no \(pprBCDE\) activity was detectable in the absence of propionate, regardless of glucose or glycerol addition (Fig. 2A and B), consistent with a previous report that PrpR is the central positive regulator of \(pprBCDE\) expression and requires a coactivator derived from propionate catabolism (47).

To determine if the carbon source could affect the transcription of the gene encoding the regulatory protein PrpR, we measured the \(pprP\) promoter activity in the presence and absence of glucose or glycerol with the sensitive \(P_{pppR}\)-lacZ reporter construct. The promoter responsible for \(pprP\) expression appears to be significantly weaker than the \(pprBCDE\) promoter, as there was no detectable fluorescence when the \(pprP\) promoter was fused to \(gfp\), which is a less sensitive reporter than \(lacZ\) (data not shown). In both \(E. coli\) JSW1 and \(S. enterica\) TR6583, \(P_{pppR}\)-lacZ expression decreased to background levels in the presence of glucose or glycerol (Fig. 2C and D). Interestingly, \(pprP\) promoter activity was unaffected by the addition of propionate in strains JSW1 and TR6583 (Fig. 2C and D) and in the cloning strain DH10B, which lacks the \(ppr\) operon (data not shown). These results indicate that, unlike \(pprBCDE\) expression, expression of \(pprP\) is not regulated by propionate or its catabolism but is regulated by glucose or glycerol availability, suggesting a classic catabolite repression mechanism mediated by cAMP-CRP. These results indicate that \(pprBCDE\) transcription can also be mediated by catabolite repression through control of \(pprP\) expression.

Catabolite repression affects transcription from \(P_{pprBCDE}\) independently of \(pprP\) expression. To determine if transcription from the \(pprBCDE\) promoter is directly subject to catabolite repression or if this effect is purely the result of catabolite...
repression of prpR expression, we used the P_{prpBCDE}−rfp reporter fusion in conjunction with a plasmid containing prpR under control of the TC-inducible promoter, Pzt1. This promoter is not regulated by glucose or glycerol (data not shown) (41), so that PrpR will be expressed at a consistent level regardless of catabolite repression. Nevertheless, P_{prpBCDE} activity consistently decreased to near background levels in the presence of glucose or glycerol in cells expressing PrpR from Pzt1 (Fig. 3A). Therefore, we conclude that the decrease in P_{prpBCDE} activity is not mediated solely by a decrease in PrpR in the cell. Interestingly, P_{prpBCDE} activity was threefold greater in cells harboring extra Pzt1-prpR on a multicopy plasmid (pZBRR) even in the absence of TC, compared with other strains in which prpR was present only on the chromosome (Fig. 3). This may be attributable to basal or “leaky” transcription from Pzt1, resulting in an increase in the levels of PrpR, indicating that prpBCDE expression might be tightly controlled by the levels of the secondary activator, PrpR (13). Unexpectedly, addition of TC showed some negative effect on P_{prpBCDE−rfp} activity. Previous studies have shown that transcription factors encoded on a plasmid under the control of a nonnative promoter are expressed at high levels relative to native expression levels (20). Recently, it has been reported that PrpR bound to two regions in S. enterica that have the consensus sequence 5'-CGTTTCATGAAACG-3' and span bases 55 to 68 and 77 to 90 from the start codon of the prpR gene, as shown in Fig. 1 (33). Gel shift assays suggested that two sites show different affinities for PrpR and PrpR may bind in different oligomerization states to the two binding sites. Regulation of prpBCDE expression might be affected by different concentrations of PrpR, which could explain how high concentrations of PrpR would lead to a decrease in promoter activity, but we do not know how this inhibition might occur.

**CRP modulates transcription from prpR and prpBCDE promoters.** It has been well documented that the cAMP-CRP

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**FIG. 2.** Regulation of the prpBCDE and prpR promoters by glucose (Glc) or glycerol (Gly) in *E. coli* JSW1 and *S. enterica* TR6583. Strains JSW1 (A, C) and TR6583 (B, D) harboring the dual P_{prpBCDE−rfp}/P_{prpR−lacZ} reporter plasmid pAPLPR were grown in LB medium plus the indicated carbon sources at 0.2 or 0.4% and in the absence of propionate (black bars) or in the presence of 10 mM propionate (hatched bars). RFP fluorescence per unit of OD_{600} (A and B; prpBCDE promoter) and β-galactosidase activity (C and D; prpR promoter) were measured.

**FIG. 3.** Regulation of the prpBCDE promoter by glucose or glycerol in cells expressing PrpR. *E. coli* JSW1 strains harboring the P_{prpBCDE−rfp} reporter with no extra copy of prpR (pZBR, black bars) or the P_{prpBCDE−rfp} reporter and prpR under the control of Pzt1 (pZBRR, hatched bars) were grown in LB medium plus 0.4% glucose (Glc) or glycerol (Gly) with 10 mM propionate (Prop) or 5 ng of TC per ml.
complex plays an important role in \( E. \) coli gene regulation and mediates catabolite repression. To investigate the role of CRP and adenylate cyclase in catabolite repression of \( prpR \) or \( prpBCDE \) by glucose and glycerol, we compared the activities of the \( prpBCDE \) and \( prpR \) promoters (with the \( P_{prpBCDE}^{rfp} \) or \( P_{prpR}^{lacZ} \) reporter fusion) in strains lacking CRP or adenylate cyclase activity (\( crp \) and \( cya \) mutants) to promoter activity in the parent strains. As expected, there was little to no expression from the \( prpBCDE \) promoter in wild-type or mutant strains when propionate was omitted from the medium (Fig. 4A). However, while expression of the \( prpBCDE \) promoter was high in wild-type cells grown with propionate, expression from this promoter was reduced to near background levels in \( crp \) or \( cya \) mutant strains JSW2 and JSW3 (Fig. 4A) grown in the presence of propionate.

Regardless of the presence of propionate, \( P_{prpR} \) activity was reduced 2.5-fold in strains lacking CRP or adenylate cyclase (JSW2 or JSW3, respectively) compared to that in the wild type (JSW1), indicating that the cAMP-CRP complex acts as a positive regulator of \( prpR \) transcription (Fig. 4B). Furthermore, the \( crp \) and \( cya \) mutations created in a strain lacking \( prpRBCDE \) (JSD1 or JSD2, respectively) reduced \( P_{prpR} \) activity approximately fourfold compared to that in the parent strain (DH10B) (Fig. 4C), supporting the notion that cAMP-CRP regulates \( prpR \) transcription regardless of \( PrpRBCDE \) expression or activation. To further confirm the involvement of the cAMP-CRP complex in the activation of the \( prpBCDE \) promoter, \( P_{prpBCDE}^{rfp} \) expression was measured in \( crp- \) and \( cya- \) deficient strains expressing PrpR in \( \text{trans} \). As illustrated in Fig. 5, the fluorescence due to \( P_{prpBCDE}^{rfp} \) expression in both mutants was lower than that in the parent strain in the presence of propionate. However, unlike the data shown in Fig. 4A, significant expression was detected in both mutants in the presence of propionate. This may be due to the overexpression of PrpR, similar to the effect shown in Fig. 3.

Taken together, these results demonstrate that the dramatic decrease in \( prpBCDE \) promoter activity is most likely the result of decreased PrpR production in the \( crp- \) and \( cya- \) mutant strains in combination with the loss of cAMP-CRP activation of \( P_{prpBCDE} \) itself. These results, taken together with previous data (32, 47), indicate that no fewer than four proteins (IHF, \( \sigma^+ \), PrpR, and CRP) are involved in the regulation of \( prpBCDE \) transcription.

**Mutations with a putative cAMP-CRP binding site.** A potential binding site for the cAMP-CRP complex within the promoter region of \( prpRBCDE \) was identified on the basis of similarity to the consensus binding site (11) (Fig. 1A). To demonstrate that the putative CRP-binding sequence plays a role in the regulation of the \( prpBCDE \) and \( prpR \) promoters, the consensus sequence was altered by site-directed mutagenesis. To examine the effect of the putative cAMP-CRP binding site on \( prpBCDE \) expression, six nucleotide substitutions were made at the consensus CRP-binding site (\( \text{AAACGTATAACT} \rightarrow \text{TTACCGGACC} \) [changed nucleotides are underlined]) in the promoter region of the \( P_{prpBCDE}^{rfp} \) fusion (Fig. 6C). These substitutions resulted in a significant decrease in promoter activity compared to that in the wild type (Fig. 6A), reflecting the effect of the \( crp \) or \( cya \) mutation in JSW2 and JSW3 (Fig. 4A).

Base pair substitutions at this site resulted in a twofold decrease in \( P_{prpR} \) activity in strain JSW1 (Fig. 6B), which is also consistent with the decrease in promoter activity observed in \( crp \) or \( cya \) mutant strains JSW2 and JSW3 (Fig. 4B). Taken together, these results strongly suggest that the putative cAMP-CRP binding site is involved directly in the positive regulation of \( prpBCDE \) and indirectly involved via \( prpR \) promoter regulation.
PrpR-binding region. To outline the PrpR-binding region, serial deletions were made in the prpBCDE promoter sequence and promoter strength was measured with an rfp reporter construct (Fig. 7). Interestingly, only the reporter plasmid carrying the entire intergenic sequence between the start codons of the divergent prpR and prpB genes (pAPF8) showed $P_{prpBCDE}$-rfp expression. The function of the divergent prpBCDE promoter was completely destroyed even by deleting prpR promoter sequences (with PrpR supplied in trans) (pAPF7), suggesting that the PrpR-binding site for activation of the prpBCDE promoter overlaps the prpR promoter itself (Fig. 1). Indeed, an inverted repeat sequence (GTTTCAT-10 nt-ATGAAAC) was found to encompass the $P_{prpR}$ region of the prpR promoter, and this sequence is conserved in both S. enterica and E. coli. Since the submission of this report, Palacios and Escalante-Semerena have reported footprinting experiments that confirm a PrpR-binding site encompassing this repeat sequence (33).

DISCUSSION

The enzymes involved in the catabolism of propionate by E. coli (1, 45) and S. enterica (15, 32, 47) are encoded by the prpBCDE operon. These genes are controlled by a specific regulator encoded by the divergent prpR gene. PrpR of S. enterica belongs to the NtrC family of transcriptional activators and has been known to regulate the expression of the prpBCDE operon in response to propionate catabolism (32). In this report, we show that transcriptional activation of the E. coli prpBCDE operon and the divergent prpR gene depends on the function of the global regulator CRP. The present results, together with previous data, enlarge our understanding of the regulation of propionate metabolism in E. coli and S. enterica and provide insight into this complex, divergent promoter structure requiring the coordinate function of multiple regulators (Fig. 1B).

The prpBCDE promoter is a $\sigma^{54}$-dependent promoter activated by CRP. CRP can act as a global regulator in E. coli by binding to specific DNA sites in or near target promoters and enhancing the ability of RNAP to bind and initiate transcription (3, 4). Commonly, cAMP-CRP is an activator of the $\sigma^{70}$-dependent transcription of genes coding for catabolism of alternative carbon sources (19). Work presented here supports the hypothesis that cAMP-CRP participates in the PrpR-mediated activation of a $\sigma^{54}$-dependent prpBCDE promoter that allows catabolism of the alternative carbon source propionate. This result adds another layer of complexity to a promoter already known to require two other proteins, IHF and PrpR, for transcriptional activation (Fig. 1B).

In this study, we have shown that the $\sigma^{54}$-dependent prpBCDE promoter is positively regulated by CRP together with PrpR, which implies direct contact between CRP and $\Sigma^{54}$ or activation via DNA conformational changes. CRP may function, at least in part, through (i) direct protein-protein interaction with a second activator that facilitates interactions...
between a second activator and DNA, (ii) CAP-induced DNA bending that facilitates interactions between a second activator and RNAP, and/or (iii) CRP-induced DNA bending that facilitates interactions between PrpR and its target site and stabilizing the DNA-PrpR interaction (10, 22, 26, 34, 39). The exact molecular mechanism will be the focus of future study and may elucidate new mechanisms of CRP-mediated transcription activation. In fact, cAMP-CRIP-mediated activation at $\sigma^{54}$-dependent promoters may occur by one or more mechanisms.

The prpR promoter behaves like a class I CRP-dependent promoter. In class I CRP-dependent promoters, CRP is known to activate transcription from a DNA site located upstream of the DNA-binding site for $\sigma^{70}$, with the site for CRP usually centered at position $-62.5$, $-72.5$, or $-92.5$ relative to the transcription start site. At these promoters, CRP interacts with the C-terminal domain of the RNAP $\alpha$ subunit ($\alpha$CTD), facilitating the binding of $\alpha$CTD to the DNA segment adjacent to the CRP-binding site (4). By sequence alignment and site-directed mutagenesis, we have identified a putative CRP-binding site required for activation of the prpR promoter centered near position $-62$ relative to $\sigma^{54}$, suggesting that this is a class I CRP-activated promoter. Although the data presented here make a strong argument for a class I CRP-binding site, we cannot rule out the possibility that other regulatory elements outside of this binding site (Fig. 1) can contribute to the transcriptional regulation of prpR. Indeed, when serial deletions in the prpR promoter region were analyzed for promoter function, a deletion that eliminated the 153-to-237 region (Fig. 1A) displayed two- to threefold higher prpR promoter activity in DH10B compared to the fusions containing whole promoter sequences (data not shown). As this region overlaps the $\sigma^{54}$-dependent prpBCDE core promoter, this finding suggests that the binding of $\sigma^{54}$ could inhibit transcription of the divergent prpR promoter or that another regulatory element acting on the 153-to-237 region may negatively regulate prpR gene expression. Because the effect is observed in DH10B, which does not carry prpR, the negative regulation might be due to a secondary DNA structure formed by binding of IHF to the region.

Implications of PrpR and CRP binding. Transcriptional factors that regulate the $\sigma^{54}$ class of sigma factors are specific for this class of sigma factor (25, 30). On the other hand, CRP is capable of regulating genes transcribed by a variety of sigma factors such as $\sigma^{32}$, $\sigma^{16}$, $\sigma^{24}$, and $\sigma^{54}$ (25). Recently, however, it has been shown that Escherichia coli $\sigma^{32}$ promoters can be responsive to CRP, since the $\sigma^{54}$-dependent promoters dctA and glnAP2 are down-regulated via an interaction between Escherichia coli $\sigma^{32}$ and the cAMP-CRIP complex (46, 48, 49). The authors proposed a regulatory role for the cAMP-CRIP complex as a switch balancing carbon metabolism and nitrogen assimilation in Escherichia coli by cAMP-dependent repression of a $\sigma^{54}$-dependent promoter via CRP. Also, Lu and Abdelal proposed that expression of the putative $\sigma^{54}$-dependent astC promoter in S. enterica serovar Typhimurium is subject to carbon catabolite repression and requires CRP, together with arginine and ArgR, for activation of the ast operon (23). On the other hand, despite the effect of catabolite repression on the expression of astC in Escherichia coli, Kiu-pakis and Reitzer (18) were unable to demonstrate CRP binding at the astC promoter. Interestingly, unlike the $\sigma^{54}$-dependent promoters described above, $\sigma^{54}$-dependent prpBCDE genes are not involved in nitrogen metabolism (38). Our finding that the cAMP-CRIP complex activates a $\sigma^{54}$-dependent promoter in concert with a member of the $\sigma^{54}$-dependent activator family suggests that the CRP family might interact with the $\sigma^{54}$-dependent expression system regardless of nitrogen metabolism.

Since the submission of our work, the inverted repeat sequence GTTTTCAT-10 nt-ATGAAAC was demonstrated to encompass the PrpR-binding site by in vitro footprinting experiments (33). This binding site overlaps $P_{pR}^d$’s own $\sigma^{70}$ promoter-binding site for prpR transcription. The implication that prpR expression might be autoregulated by the prpR gene product will be the subject of future study.

In conclusion, data presented here clearly show a role for the cAMP-CRIP complex in the activation of the two divergent promoters: $\sigma^{70}$-dependent $P_{pR}^d$ and $\sigma^{54}$-dependent $P_{pRBCDE}$. The tightly overlapping promoters between $\sigma^{70}$- and $\sigma^{54}$-dependent promoters are influenced by the expression levels of the opposing promoters, offering new perspectives for further studies of CRP-mediated gene expression and interactions between $\sigma^{70}$-dependent promoters and $\sigma^{54}$-dependent promoters. Future work will focus on the precise mechanisms by which the cAMP-CRIP complex interacts with IHF and PrpR to simultaneously control expression from these two promoters.

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