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The Cyclic AMP Receptor Protein Is Dependent on GcvA for Regulation of the *gcv* Operon

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The *Escherichia coli gcv* operon is transcriptionally regulated by the GcvA, GcvR, Lrp, and PurR proteins. In this study, the cyclic AMP (cAMP) receptor protein (CRP) is shown to be involved in positive regulation of the *gcv* operon. A *crp* deletion reduced expression of a *gcvT-lacZ* fusion almost fourfold in glucose minimal (GM) medium. The phenotype was complemented by both the wild-type *crp* gene and four *crp* alleles that encode proteins with amino acid substitutions in known activating regions of CRP. A *cyaA* deletion also resulted in a fourfold decrease in *gcvT-lacZ* expression, and wild-type expression was restored by the addition of cAMP to the growth medium. A *cyaA crp* double deletion resulted in levels of *gcvT-lacZ* expression identical to those observed with either single mutation, showing that CRP and cAMP regulate through the same mechanism. Growth in GM medium plus cAMP or glycerol minimal medium did not result in a significant increase in *gcvT-lacZ* expression. Thus, the level of cAMP present in GM medium appears to be sufficient for regulation by CRP. DNase I footprint analysis showed that CRP binds and protects two sites centered at bp −313 (site 1) and bp −140 (site 2) relative to the transcription initiation site, but a mutational analysis demonstrated that only site 1 is required for CRP-mediated regulation of *gcvT-lacZ* expression. Expression of the *gcvT-lacZ* fusion in a *crp gcvA* double mutant suggested that CRP's role is dependent on the GcvA protein.

There are two pathways for the production of one-carbon (C_1) units in *Escherichia coli*. Serine hydroxymethyltransferase, the *glyA* gene product, catalyzes the cleavage of serine to glycine and the transfer of a C_1 unit to tetrahydrofolate to form 5,10-methylenetetrahydrofolate and is the primary source of C_1 units (24, 26). The glycine cleavage (GCV) enzyme system catalyzes the oxidative cleavage of glycine to form CO_2, NH_3, and 5,10-methylenetetrahydrofolate, providing a secondary pathway for C_1 units (19). The C_1 units produced by these pathways are used in cellular biosyntheses of methylated products such as methionine, thymine, and purines (26). It has been proposed that the physiological role of the GCV system may be to balance a cell's need for glycine and C_1 units.

The GCV enzyme system is composed of the GcvT, GcvH, and GcvP proteins, encoded by the *gcv* operon, and lipoamide dehydrogenase, encoded by the unlinked *lpd* gene. The regulation of the *gcv* operon is not fully understood, but there are four proteins known to affect *gcv* expression. The leucine-responsive protein, Lrp, is a global regulator of genes involved in amino acid metabolism (5) and is required for activation of the *gcv* operon (22, 41). The PurR protein is a negative regulator of nucleotide metabolic genes (14, 20, 31) and mediates a twofold repression of a *gcvT-lacZ* fusion when cells are grown in the presence of the purine nucleoside inosine (44). The GcvA protein is responsible for controlling *gcv* operon expression in two distinct ways. GcvA activates *gcv* expression when cells are grown in the presence of glycine and mediates a PurR-independent repression of *gcv* when cells are grown in the presence of inosine but without glycine (44, 45). A fourth protein, GcvR, is a GcvA-dependent negative regulator of *gcv* expression (12). However, GcvR has not been shown to bind to DNA, and its mechanism of regulation is unknown. Here we report a fifth protein that is involved in controlling *gcv* expression. The cyclic AMP (cAMP) receptor protein (CRP) mediates a fourfold positive effect on *gcv* expression as measured from a *gcvT-lacZ* fusion. In vitro binding experiments and a mutational analysis suggest that CRP binds to a site centered at bp −313 relative to the transcriptional start site for *gcv*. In addition, the CRP effect is dependent on a functional *gcvA* gene and its role may be to antagonize GcvA's repression of the *gcv* operon.

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MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. All strains used are listed in Table 1 and were constructed by P1 *clr* transduction (25). The λ*gcvA-lacZ* (46) and λ*gcvR-lacZ* (13) fusion phages were described previously. The λ*gcvA-lacZ* +15G phage carries a base pair change at position +15 relative to the transcription start site that results in a loss of GcvA-mediated autoregulation and about a sevenfold increase in *gcvA-lacZ* expression (15a). The λ*gcvT-lacZ* phage (39) used in earlier studies includes 466 bp upstream of the transcriptional start site. A derivative, λ$gcvT\text{-}lacZ_{\Delta-341}$, was constructed in this study and extends upstream only to bp −341; this 125-bp deletion does not alter regulation. Strains were lysogenized with lambda phages as previously described (42). Other λ*gcvT-lacZ* phages carrying mutations in the *gcv* control region were constructed during this investigation and are described below. Plasmids used are listed in Table 1 or were constructed during this investigation. Plasmids pYZcrp, p19A, p52N, p158A, and p162C were gifts from R. Ebright.

Media. Glucose minimal (GM) medium or glycerol minimal medium was Vogel and Bonner minimal salts (43) supplemented with 0.4% glucose or 0.4% glycerol, respectively. Supplements were added at the following concentrations: phenylalanine, 50 μg/ml; inosine, 50 μg/ml; thiamine, 1 μg/ml; glycine, 300 μg/ml; ampicillin, 30 μg/ml for single-copy plasmids and 100 μg/ml for all other Ap^r plasmids; chloramphenicol, 40 μg/ml; and kanamycin, 20 μg/ml. GM and glycerol minimal media were always supplemented with phenylalanine and thiamine since all strains used carry the *pheA905* and *thi* mutations.

DNA manipulations. Isolation of plasmid DNA, restriction enzyme digestions, ligations, and plasmid transformations were performed as described previously (32).

Enzyme assays. β-Galactosidase assays were performed by the method of Miller (25), by using the chloroform-sodium dodecyl sulfate lysis procedure. All results are the averages of results from two or more assays, with each reaction being performed in triplicate.

Site-directed mutagenesis and construction of lysogens. Starting with plasmid pGS239 as the template, bp −139 and −152 relative to the +1 transcription initiation site were changed to an A and a T, respectively (Fig. 1), by the PCR megaprimer mutagenesis method (33). The new plasmid was designated pGS484. Starting with plasmid pGS362 as the template, bp −306, −307, and −308 relative to the transcription initiation site were changed to a T, G, and T, respectively

TABLE 1. *E. coli* K-12 strains and plasmids used in this study

Strain[a] or plasmid	Relevant genotype or description	Source or reference
Strains		
GS162	Wild type	This lab
GS1068	Δ*crp*::Cm^r	This study
GS1079	Δ(*cyaA1400*)::Kn^r	This study
GS1080	GS1068, Δ(*cyaA1400*)::Kn^r	This study
GS1029	Δ*gcvA*::Sp^r	47
GS1081	GS1029, Δ*crp*::Cm^r	This study
GS986	*gcvA1 purR*::Tn*10*	44
GS1121	GS986, *gcvR*::Kn^r	This lab
GS1124	GS986, Δ*crp*::Cm^r	This study
GS1125	GS1121, Δ*crp*::Cm^r	This study
Plasmids		
pGS239	759-bp *Eco*RI-*Bam*HI *gcv* fragment in pMC1403; Ap^r	39
pGS258	606-bp *Eco*RI-*Bam*HI *gcv* fragment in pMC1403; a 153-bp 5′ deletion of the 759-bp fragment in pGS239; Ap^r	29
pGS294	Derivative of pDF41 (18), a single-copy plasmid; Ap^r	This lab
pGS362	634-bp *Eco*RI-*Bam*HI *gcv* fragment in pMC1403; a 125-bp 5′ deletion of the 759-bp fragment in pGS239; Ap^r	This study
pGS396	*Hpa*I fragment carrying *crp*$^+$ from pYZcrp cloned into pGS294	This study
pGS469	Derivative of pDF41 (18), a single-copy plasmid carrying the *gcvA* autoregulatory mutation +15G	A. Jourdan
pMC1403	*lac* fusion vector	6
pYZcrp	pBR322 derivative that carries the wild-type *crp* gene; Ap^r	49
p19A	pYZcrp carrying the AR2 *crp* allele H19A; Ap^r	27
p52N	pYZcrp carrying the AR3 *crp* allele K52N; Ap^r	1
p158A	pYZcrp carrying the AR1 *crp* allele with T158A; Ap^r	49
p162C	pYZcrp carrying the AR1 *crp* allele with G162C; Ap^r	49
pF31A	Derivative of pDF41 (18), a single-copy plasmid carrying a positive control *gcvA* allele with F31A; Ap^r	16

[a] All strains also carry *thi*, *pheA905*, Δ*lacU169*, *araD129*, and *rpsL150* mutations.

(Fig. 1). The new plasmid was designated pGS485. The specific base pair changes were verified by DNA sequence analysis. The approximately 5,400-bp *Eco*RI-*Mfe*I fragment carrying each mutant *gcvT-lacZ* fusion along with the *lacY* and *lacA* genes was isolated from each plasmid and ligated into the *Eco*RI site of phage λgt2 (28). The phages generated were single plaque purified and designated λ*gcvT-lacZ*−139A−152T and λ*gcvT-lacZ*$_{\Delta-341}$−306T−307G−308T. The extensions after each fusion indicate the nucleotide changes and positions relative to the +1 transcription initiation site. Appropriate strains were lysogenized with the above-described phages, and the lysogens were verified to carry a single copy of λ by infection with phage λc*I*90*c*17 (38).

CRP. The purified CRP used in the DNA mobility shift and DNase I footprinting assays was a gift from E. P. Greenberg.

Gel mobility shift assay. The gel mobility shift assay used was based on the methods described by Fried and Crothers (9) and Garner and Revzin (10). A 759-bp *Eco*RI-*Bam*HI fragment from pGS239 and a 606-bp *Eco*RI-*Bam*HI fragment from pGS258 were ^{32}P labeled at the *Eco*RI ends with T4 polynucleotide kinase (32). Samples of less than 22 ng of the labeled DNA fragments were included in 20-μl reaction mixtures containing DNA binding buffer (10 mM Tris HCl [pH 7.5], 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 1 mM dithiothreitol), 125 μg of bovine serum albumin per ml, and cAMP as indicated in the figures. Reaction mixtures were incubated for 5 min at 37°C, and 2 μl of purified CRP diluted in DNA binding buffer was added to the mixtures as indicated in Fig. 2 and 3. Incubation was continued for 15 min at 37°C, the reactions were stopped by the addition of 1 μl of loading buffer (0.1% xylene cyanol and 50% glycerol in H_2O), and the samples were loaded on a 5% polyacrylamide gel and run at approximately 12 V/cm. The gels were transferred to Whatman 3MM paper, dried, and autoradiographed.

DNase I protection assay. The DNase I protection assay was a modified version of the method of Schmitz and Galas (34) as previously described (47). The 759-bp ^{32}P-labeled fragment used in the gel mobility shift assay was used in the DNase I footprint assay. Less than 44 ng of labeled DNA was added to 18-μl reaction mixtures containing DNA binding buffer, 125 μg of bovine serum albumin, and 2 mM cAMP. The reaction mixtures were incubated for 5 min at 37°C, 2-μl samples of serial dilutions of CRP were added to the mixtures, and incubation continued at 37°C for 15 min. A 2-μl sample of a DNase I solution (0.1 U of DNase I per μl in 20 mM ammonium acetate–32 mM $CaCl_2$) was added for 30 s, reactions were stopped with the addition of 5 μl of stop solution (3 M ammonium acetate, 0.17 M EDTA, 33 μg of sheared calf thymus DNA per ml), and the samples were precipitated with ethanol. The DNA pellets were resuspended in DNA sequence loading buffer (0.1 M NaOH, 5 M urea, 1 mM EDTA, 0.05% xylene cyanol–bromophenol blue) and loaded onto a 5% polyacrylamide–7 M urea sequencing gel alongside the Maxam and Gilbert (23) A+G and C+T sequencing reaction mixtures loaded on the same labeled fragment.

RESULTS

CRP involvement in *gcvT-lacZ* expression. Previous results showed that a deletion that ends at bp −313 upstream of the *gcvT-lacZ* transcription initiation site results in reduced levels of expression of the fusion (41). An analysis of the DNA sequence in this region identified a possible CRP binding site, with 14 of 22 bp matching the consensus binding sequence (11) (Fig. 1). Therefore, we tested if the CRP protein plays a role in regulation of the *gcv* operon. The wild-type strain and the *crp* deletion strain were lysogenized with the λ*gcvT-lacZ*$_{\Delta-341}$ phage, and the lysogens were grown in GM medium with the appropriate supplements and assayed for β-galactosidase activity. The *crp* deletion caused more than a fourfold decrease in β-galactosidase levels compared to the level in the control strain when cells were grown in GM medium (Table 2). In contrast, the *crp* deletion had only a 1.5-fold effect on *gcvT-lacZ* expression when the cells were grown in GM medium supplemented with glycine and no significant effect when the GM medium was supplemented with inosine.

To confirm that the decrease in β-galactosidase levels in GM medium was due to the absence of the CRP protein, the *crp* deletion lysogen was transformed with a single-copy plasmid or with the single-copy plasmid carrying the *crp*$^+$ gene. The transformants were grown in GM medium, and β-galactosidase levels were measured. The single-copy vector had no significant effect on *gcvT-lacZ* expression (Table 2). However, the single-copy *crp*$^+$ plasmid complemented the *crp* deletion and restored expression of the *gcvT-lacZ* fusion to the wild-type level (Table 2).

In most well-studied CRP-regulated systems, CRP binding sites occur at three, five, or six helical turns upstream of the −10 region of the promoter (21). Two models have been proposed for CRP involvement in regulation when binding occurs within this region. First, certain amino acids of CRP have been shown to be part of three activation domains (AR1, AR2, and AR3) that interact with RNA polymerase (RNAP) to facilitate transcription initiation (3, 4, 7, 30). In addition, CRP binding has been shown to bend DNA 90 to 130°, and the bending may be involved in the activation of gene expression (2, 21). The putative CRP binding site on the *gcv* control region is centered around bp −313 relative to the transcription initiation site. Since this site is far upstream of RNAP's binding site, it seems unlikely that CRP is contacting RNAP to activate transcription of *gcv* unless DNA looping occurs at the *gcv* promoter, allowing contact between one of CRP's activating regions and RNAP. To determine if one or more of CRP's three activating regions may be involved in the regulation of *gcv*, four CRP mutants were tested for their ability to complement the *crp* deletion and restore expression of *gcvT-lacZ*. Each of these *crp* alleles encodes a mutant protein with an amino acid change in one of CRP's activating regions, but all of these mutant CRPs can

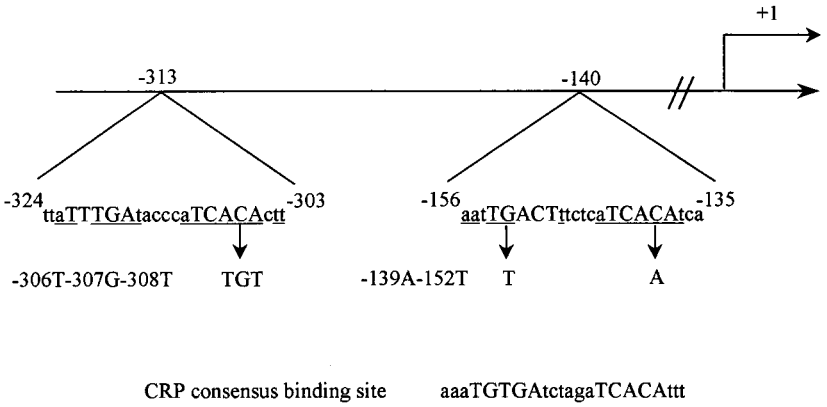

FIG. 1. CRP binding sites in the *gcv* control region. The transcription start site for *gcvT* is indicated as +1. The nucleotide sequences of the CRP binding sites centered at bp −313 and −140 relative to the transcription initiation site are shown. The inverted repeat sequence known to be important for CRP binding is in capital letters. Nucleotides conserved with respect to the CRP consensus site are underlined. The arrows indicate the nucleotide changes in the mutants *gcvT-lacZ*$_{\Delta-341}$−306T−307G−308T and *gcvT-lacZ*−139A−152T. The consensus CRP binding site is indicated for comparison.

bind DNA with an affinity similar to that of the wild-type protein (1, 7, 27). Since at least one CRP-regulated operon (*araBAD*) does not appear to require all of the amino acids defined by AR1 (48), two AR1 mutants were tested for the ability to restore *gcvT-lacZ* expression in the *crp* deletion lysogen. The transformants were grown in GM medium, and the β-galactosidase levels were determined. The T158A and G162C AR1 mutants and the K52N AR3 mutant complemented the *crp* deletion and restored expression of the *gcvT-lacZ*$_{\Delta-341}$ fusion to near the wild-type level (Table 2). The H19A AR2 mutant caused a twofold increase in *gcvT-lacZ* expression, suggesting that the wild-type amino acid histidine at position 19 is not essential for regulation of *gcv* and that an alanine at position 19 may allow CRP to regulate better at the *gcv* promoter. The results of these complementation experiments suggest that AR1, AR2, and AR3 are probably not involved in CRP's role in the regulation of the *gcv* operon. However, it is possible that other amino acids in AR1, AR2, or AR3 not tested or that amino acids that have not been defined as part of these activating regions may contact RNAP at the *gcv* promoter.

CRP requires cAMP to regulate *gcv* expression. Since CRP does not bind specifically to DNA in the absence of cAMP (21), we tested whether CRP's regulation of *gcvT-lacZ* requires cAMP. Strain GS1079 carries the Δ(*cyaA1400*)::Kn^r allele and is defective in the production of cAMP (36). This strain was lysogenized with λ*gcvT-lacZ*$_{\Delta-341}$ phage, the lysogen was grown in GM medium, and β-galactosidase activity was measured. The β-galactosidase level was not significantly different from the level measured in the *crp* deletion strain (Table 2). Since both the *crp* deletion and the *cyaA* deletion caused about a fourfold decrease in *gcvT-lacZ* expression compared to the level of expression in the wild-type strain when it was grown in GM medium, we wanted to confirm that the cAMP effect was mediated through CRP. Thus, we constructed a Δ*crp* Δ*cyaA* double mutant. This strain was lysogenized with the λ*gcvT-lacZ*$_{\Delta-341}$ phage, the lysogen was grown in GM medium, and β-galactosidase activity was measured. The β-galactosidase levels were not significantly different from the levels measured in either the *crp* deletion lysogen or the *cyaA* deletion lysogen (Table 2).

CRP and cAMP maximally regulate CRP-dependent genes when the level of cAMP is elevated due to growth on a poor carbon source (for reviews, see references 2 and 21). Since CRP and cAMP regulate *gcvT-lacZ* over a fourfold range in GM medium, a preferred carbon source where the cAMP level is low, we tested whether CRP would regulate *gcvT-lacZ* over a larger range if the level of cAMP was elevated. The wild-type strain lysogenized with λ*gcvT-lacZ*$_{\Delta-341}$ was grown in GM medium, GM medium plus cAMP, and glycerol minimal medium. The β-galactosidase levels were not significantly different when the lysogen was grown in any of the three media (Table 3). Thus, the level of cAMP in the wild-type strain grown in GM medium appears sufficient for CRP-mediated regulation of the *gcvT-lacZ* fusion. As controls, we also tested the effects of cAMP on the Δ*crp* and Δ*cyaA* lysogens. The addition of cAMP increased the β-galactosidase level over threefold in the Δ*cyaA* lysogen, up to the level observed in the control strain, confirming that the decreased expression in the Δ*cyaA* lysogen is due to the low concentration of cAMP. Since the cAMP added exogenously is sufficient to overcome the deletion of the *cyaA* gene, the results indicate that the cAMP level was probably sufficient in the wild-type lysogen to allow the maximum range of regulation by CRP. As expected, the addition of cAMP had no effect on *gcvT-lacZ* expression in the *crp* deletion strain (Table 3).

CRP binds to the *gcv* control region. To test whether the putative CRP site centered at bp −313 can be bound by CRP in vitro, gel mobility shift assays were performed with purified CRP and two different DNA templates. One template was the

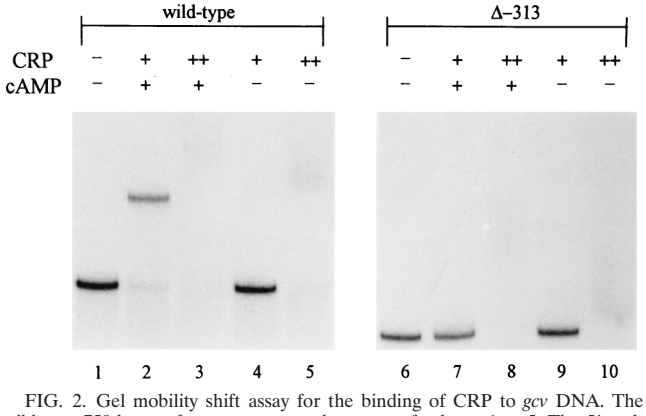

FIG. 2. Gel mobility shift assay for the binding of CRP to *gcv* DNA. The wild-type 759-bp *gcv* fragment was used as target for lanes 1 to 5. The 5′-end-truncated 606-bp fragment was used as the target for lanes 6 to 10. Where indicated, 20 mM cAMP was included. The CRP dimer was added at a concentration of either 10 nM (+) or 100 nM (++).

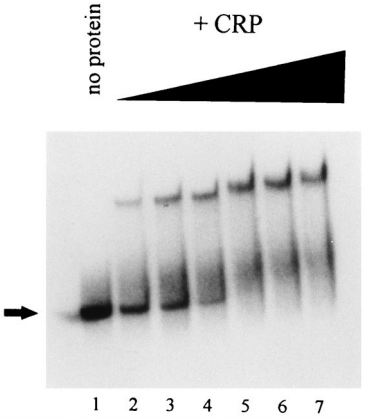

FIG. 3. Gel mobility shift assay for the binding of CRP to *gcv* DNA. The wild-type 759-bp *gcv* fragment was used as the target. The CRP dimer was added at the following concentrations: 0, 2.5, 5.0, 10, 25, 50, and 100 nM (lanes 1 to 7, respectively). cAMP was included in all reaction mixtures at a final concentration of 2 mM. The arrow denotes the unbound fragment.

759-bp *Eco*RI-*Bam*HI fragment carrying wild-type *gcv* DNA extending from bp −466 to +293 relative to the *gcv* transcriptional start site. The second template was a 606-bp *Eco*RI-*Bam*HI fragment carrying *gcv* DNA extending from bp −313 to +293. This 606-bp fragment lacks half of the potential CRP binding site centered near bp −313 (Fig. 1). CRP dimer at a concentration of 10 nM and cAMP at a concentration of 20 mM resulted in a shift of the 759-bp wild-type fragment to a single band of slower mobility (Fig. 2, compare lanes 1 and 2). Binding of CRP to DNA at this concentration was dependent on the presence of cAMP (Fig. 2, compare lanes 2 and 4). At 100 nM CRP dimer, all of the wild-type DNA fragment was shifted in the presence and absence of cAMP, probably the result of nonspecific binding by CRP. The truncated DNA fragment did not show a specific band shift at 10 nM CRP in the presence or absence of cAMP (Fig. 2, lanes 7 and 9). However, at 100 nM CRP dimer all of the truncated template shifted in the presence and absence of cAMP (Fig. 2, lanes 8 and 10). These results suggest that CRP, in the presence of cAMP, binds specifically to the wild-type DNA template but not when the DNA fragment lacks half of the putative CRP binding site.

TABLE 2. CRP is involved in *gcvT-lacZ* expression

Strain[a] (plasmid)	Relevant genotype[b]	β-Galactosidase activity[c] of cells grown in GM medium with:		
		No addition	Glycine	Inosine
GS162	Wild type	134	854	12
GS1068	Δ*crp*::Cm^r	32	564	12
GS1068 (pGS294)	Δ*crp*::Cm^r (vector)	38	—	—
GS1068 (pGS396)	Δ*crp*::Cm^r (*crp*$^+$)	145	—	—
GS1068 (pYZcrp)	Δ*crp*::Cm^r (*crp*$^{+++}$)	118	—	—
GS1068 (p158A)	Δ*crp*::Cm^r (*crp*$^{AR1+++}$)	143	—	—
GS1068 (p162C)	Δ*crp*::Cm^r (*crp*$^{AR1+++}$)	135	—	—
GS1068 (p19A)	Δ*crp*::Cm^r (*crp*$^{AR2+++}$)	236	—	—
GS1068 (p52N)	Δ*crp*::Cm^r (*crp*$^{AR3+++}$)	103	—	—
GS1079	Δ(*cyaA1400*)::Kn^r	34	—	—
GS1080	Δ(*cyaA1400*)::Kn^r Δ*crp*::Cm^r	33	—	—

[a] All strains carry the λ*gcvT-lacZ*$_{\Delta-341}$ fusion.
[b] *crp*^{AR1}, *crp*^{AR2}, and *crp*^{AR3}, *crp* with mutant activation domains AR1, AR2, and AR3, respectively. Superscript $^+$ and $^{+++}$ indicate single and multiple copies of the *crp* allele, respectively.
[c] β-Galactosidase activity is in Miller units (25). All standard deviations were within 15% of the means. —, not determined.

TABLE 3. Effects of high levels of cAMP on regulation of *gcvT-lacZ* by CRP

Strain[a]	Relevant genotype	β-Galactosidase activity[b] of cells grown in:		
		GM medium	GM medium plus cAMP	Glycerol minimal medium
GS162	Wild type	129	154	105
GS1068	Δ*crp*::Cm^r	40	39	—
GS1079	Δ(*cyaA1400*)::Kn^r	45	148	—

[a] All strains carry the λ*gcvT-lacZ*$_{\Delta-341}$ fusion.
[b] β-Galactosidase activity is in Miller units (25). All standard deviations were within 8% of the means, except with the result for GS1079 cells grown in GM medium, for which the standard deviation was within 20% of the mean. —, not determined.

A second gel mobility shift assay was performed to determine the lowest concentration at which CRP could bind and shift *gcv* DNA in the presence of cAMP. CRP dimer bound the wild-type fragment at a concentration as low as 2.5 nM, with more than half of the fragment being bound at a dimer concentration of about 5.0 nM (Fig. 3, lanes 2 and 3). At a concentration of 25 nM all of the DNA fragment was shifted (Fig. 3, lane 5).

Since in vivo regulation by CRP is observed in GM medium, where the cAMP concentration is low, a gel mobility shift assay was performed with 5 μM cAMP. It has been reported that micromolar rather than millimolar concentrations of cAMP often favor a higher affinity for DNA binding by CRP, and that millimolar concentrations of cAMP can even inhibit binding of CRP-cAMP to DNA (2, 21). At 5 μM cAMP, CRP dimer binds and shifts *gcv* DNA at protein concentrations similar to those seen in Fig. 3 (data not shown). This result suggests that CRP can bind to *gcv* DNA at similar concentrations of protein in the presence of high or low levels of cAMP, supporting the in vivo data demonstrating that CRP can regulate *gcv* optimally in GM medium.

Location(s) of the CRP binding site(s) in the *gcv* control region. DNase I footprinting assays were performed to determine where CRP binds in the *gcv* control region (see Materials and Methods). As the CRP concentration was increased from 5 to 100 nM, two regions were protected from DNase I cleavage; one site centered near bp −313 as expected and the other site centered at bp −140 (Fig. 4). The protected region centered near bp −313 extends over about 27 bp, from bp −299 to −326 relative to the transcription initiation site, and was designated site 1. This protected region contains 14 bp that match base pairs in the 22-bp CRP consensus binding site (Fig. 1) (11). The second CRP-protected site, designated site 2, extends from about bp −131 to −158 and contains 12 bp that match base pairs in the 22-bp consensus CRP binding site (Fig. 1). Site 1 has a two- to fourfold higher affinity for CRP than site 2 (Fig. 4), likely due to the higher degree of sequence conservation in site 1.

Genetic analysis of the CRP binding sites. Although the DNase I footprint analysis identified two binding sites for CRP, the results from the gel mobility shift assay suggested that CRP binds to a single site in the *gcv* control region (Fig. 2 and 3). To determine whether one or both sites were required for CRP-mediated activation of the *gcvT-lacZ* fusion, we carried out a genetic analysis of the two binding sites. A triple mutation (−306T−307G−308T) was created in CRP binding site 1, in the downstream half of the inverted repeat known to be important for CRP binding (11) (Fig. 1). A double mutation

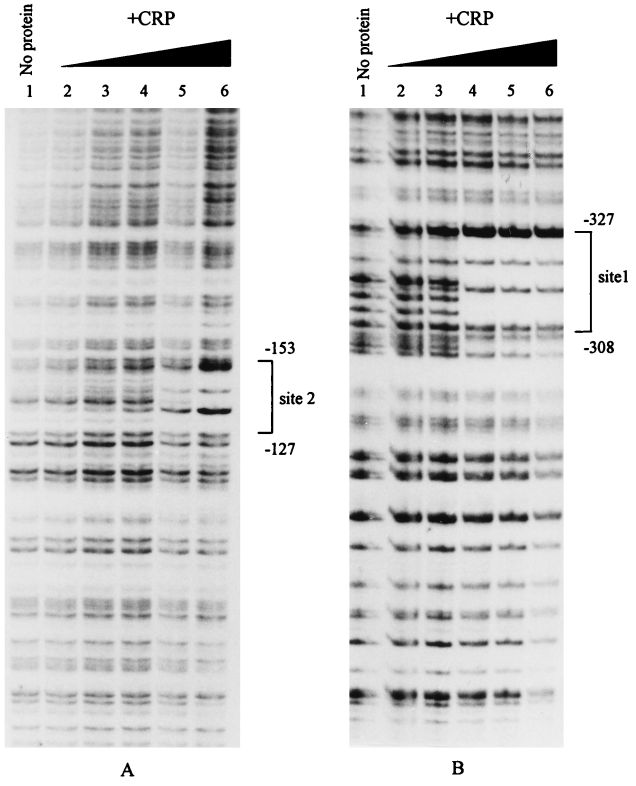

FIG. 4. Protection from DNase I digestion of *gcv* DNA by CRP plus cAMP. The ^{32}P-labeled wild-type 759-bp *gcv* fragment was incubated with dilutions of CRP and digested with DNase I (see Materials and Methods). cAMP (2 mM) was included in all reaction mixtures. The digestion products were electrophoresed on a denaturing 5% polyacrylamide gel adjacent to the Maxam-Gilbert sequencing reaction mixtures of the labeled DNA probe (not shown). (A and B) Long and short runs, respectively, of the digestion products. Lane 1, no protein; lanes 2 to 6, 5, 10, 25, 50, and 100 nM CRP dimer, respectively. The brackets indicate the two sites protected from digestion by DNase I.

(−139A−152T) was created in CRP binding site 2, with one change being in each half of the inverted repeat (Fig. 1). CRP was unable to bind and protect these two mutated binding sites from DNase I digestion (data not shown). λ*gcvT-lacZ* phage carrying the −306T−307G−308T and the −139A−152T mutations were used to lysogenize the wild-type strain and the *crp* deletion strain. The lysogens were grown in GM medium, and β-galactosidase levels were determined. The −306T−307G −308T triple mutation in site 1 caused about a twofold decrease of *gcvT-lacZ* expression in the wild-type strain (Table 4).

TABLE 4. Effects of mutations in CRP binding sites 1 and 2 on *gcvT-lacZ* expression

Strain	Relevant genotype	β-Galactosidase activity[a]
GS162 λ*gcvT-lacZ*	Wild type	144
GS162 λ*gcvT-lacZ*$_{\Delta-341}$	Wild type	141
GS162 λ*gcvT-lacZ*$_{\Delta-341}$−306T−307G−308T	Wild type	68
GS162 λ*gcvT-lacZ*−139A−152T	Wild type	97
GS1068 λ*gcvT-lacZ*	Δ*crp*::Cm^r	39
GS1068 λ*gcvT-lacZ*$_{\Delta-341}$	Δ*crp*::Cm^r	44
GS1068 λ*gcvT-lacZ*$_{\Delta-341}$−306T−307G−308T	Δ*crp*::Cm^r	56
GS1068 λ*gcvT-lacZ*−139A−152T	Δ*crp*::Cm^r	37

[a] Cells were grown in GM medium. β-Galactosidase activity is in Miller units (25). All standard deviations were within 17% of the means.

TABLE 5. CRP is dependent on GcvA for regulation of *gcvT-lacZ*

Strain	Relevant genotype	β-Galactosidase activity[a]
GS162 λ*gcvT-lacZ*$_{\Delta-341}$	Wild type	154
GS1068 λ*gcvT-lacZ*$_{\Delta-341}$	Δ*crp*::Cm^r	49
GS1029 λ*gcvT-lacZ*$_{\Delta-341}$	Δ*gcvA*::Sp^r	89
GS1081 λ*gcvT-lacZ*$_{\Delta-341}$	Δ*crp*::Cm^r Δ*gcvA*::Sp^r	105
GS162 λ*gcvA-lacZ*	Wild type	4.5
GS1068 λ*gcvA-lacZ*	Δ*crp*::Cm^r	2.0
GS162 λ*gcvR-lacZ*	Wild type	94
GS1068 λ*gcvR-lacZ*	Δ*crp*::Cm^r	97

[a] Cells were grown in GM medium. β-Galactosidase activity is in Miller units (25). All standard deviations were within 6% of the means.

In the *crp* deletion strain these changes did not cause a further decrease in *gcvT-lacZ* expression compared to that of the wild-type strain. These results suggest that the mutations in binding site 1 eliminated CRP's regulatory role in controlling *gcvT-lacZ* expression. The −139A−152T double mutation in binding site 2 decreased *gcvT-lacZ* expression 1.6-fold in the wild-type strain (Table 4). However, the *crp* deletion caused a further 2.6-fold decrease in expression (Table 4), suggesting that binding site 2 has no significant role in controlling *gcvT-lacZ* expression in vivo. The small decrease in β-galactosidase levels observed with the −139A−152T double mutation is possibly due to an alteration in Lrp-mediated regulation of *gcvT-lacZ*, as the changes are within the Lrp binding region (41). CRP binding to site 2 observed in vitro is likely due to the sequence similarity between the region and the consensus CRP binding site.

CRP's role is dependent on the GcvA protein. Of the many CRP-regulated promoters, the binding sites for CRP vary in their distances from the transcriptional start site and can often be correlated to CRP's mode of regulation (21). A distant upstream binding site, such as CRP binding site 1 for *gcv*, often indicates that CRP regulates in conjunction with another regulatory protein. To determine whether regulation is dependent on GcvA, a Δ*gcvA* Δ*crp* double mutant was constructed. This strain was lysogenized with the *gcvT-lacZ*$_{\Delta-341}$ fusion, the lysogen was grown in GM medium, and the β-galactosidase level was measured. The *crp* deletion caused approximately a threefold decrease in expression when the wild-type *gcvA* gene was present (Table 5). However, in the *gcvA* mutant background, a deletion of the *crp* gene had no effect on *gcvT-lacZ* expression. These results suggest that CRP is dependent on GcvA for its regulatory role and that the lower level of expression in the Δ*crp* strain than that in the Δ*crp* Δ*gcvA* strain was due to GcvA.

CRP regulates expression of *gcvA* but does not regulate *gcvR*. When CRP-mediated regulation is dependent on a second protein, CRP often regulates expression of the gene encoding the second regulatory protein (21). A possible explanation for CRP's dependence on GcvA is that CRP regulates expression of the *gcvA* gene and indirectly affects expression of *gcvT-lacZ*. In addition, GcvA is known to require the *gcvR* gene product for its role as a repressor (12), raising the possibilities that CRP also regulates expression of *gcvR* and indirectly alters *gcvT-lacZ* expression. These possibilities were tested by lysogenizing the wild-type strain and the *crp* deletion strain with the *gcvA-lacZ* (46) and *gcvR-lacZ* (13) fusions. The lysogens were grown in GM medium, and the cells were assayed for β-galactosidase activity. The deletion of the *crp* gene had no effect on *gcvR-lacZ* expression but caused a twofold decrease in expression of the *gcvA-lacZ* fusion (Table 5).

To determine if the twofold reduction in GcvA levels in a *crp*

TABLE 6. CRP does not indirectly regulate *gcvT* by controlling *gcvA* expression

Strain (plasmid)	Relevant genotype	β-Galactosidase activity[a]
GS162 λ*gcvT-lacZ*$_{\Delta-341}$	Wild type	152
GS162 λ*gcvT-lacZ*$_{\Delta-341}$ (pGS341)	Wild type (*gcvA*$^+$)	183
GS1068 λ*gcvT-lacZ*$_{\Delta-341}$	Δ*crp*::Cm^r	64
GS1068 λ*gcvT-lacZ*$_{\Delta-341}$ (pGS341)	Δ*crp*::Cm^r (*gcvA*$^+$)	51
GS1081 λ*gcvT-lacZ*$_{\Delta-341}$	Δ*crp*::Cm^r Δ*gcvA*::Sp^r	97
GS1081 λ*gcvT-lacZ*$_{\Delta-341}$ (pGS341)	Δ*crp*::Cm^r Δ*gcvA*::Sp^r (*gcvA*$^+$)	54
GS1081 λ*gcvT-lacZ*$_{\Delta-341}$ (pGS469)	Δ*crp*::Cm^r Δ*gcvA*::Sp^r (autoregulatory mutant *gcvA*$^+$ +15G)	57
GS162 λ*gcvA-lacZ* +15G	Wild type	29
GS1068 λ*gcvA-lacZ* +15G	Δ*crp*::Cm^r	49

[a] Cells were grown in GM medium. β-Galactosidase activity is in Miller units (25). All standard deviations were within 17% of the means.

deletion strain were responsible for part of the decrease in *gcvT-lacZ* expression in GM medium, the single-copy plasmid carrying the wild-type *gcvA* gene was transformed into the wild-type strain, the Δ*crp* strain, and the Δ*gcvA* Δ*crp* double mutant. This plasmid has been shown to complement a *gcvA* mutation on the chromosome and produce levels of GcvA comparable to those produced by the chromosomal *gcvA* gene (16). Since the *crp* deletion decreased the levels of GcvA to about half the levels in a wild-type strain, we assumed that two copies of *gcvA* in a Δ*crp* strain would restore GcvA levels. Thus, the transformant should allow us to determine if the decrease in *gcvT-lacZ* expression in the Δ*crp* strain is due to a decrease in GcvA production. The single-copy *gcvA*$^+$ plasmid had little effect on *gcvT-lacZ* expression in a wild-type strain (Table 6), indicating that two copies of *gcvA* are not sufficient to cause induction of the operon. In addition, the plasmid had no effect on *gcvT-lacZ* expression in the Δ*crp* strain, suggesting that the decrease in GcvA protein in the untransformed lysogen caused by the *crp* mutation was probably not responsible for the decrease in *gcvT-lacZ* expression. An important control for this experiment was the demonstration that the plasmid was able to complement *gcvA* on the chromosome in the Δ*gcvA* Δ*crp* strain, resulting in about a twofold decrease in the level of β-galactosidase (Table 6).

Since GcvA negatively autoregulates its own expression, a plasmid and a chromosomal copy of *gcvA* present in the same cell may allow more autoregulation and cause lower levels of *gcvA* than expected. To eliminate this possibility, we used a *gcvA* promoter mutant (*gcvA* +15G) that prevents autoregulation and that results in about sevenfold higher levels of *gcvA* expression and GcvA protein than those seen with a wild-type *gcvA* gene (15a). In addition, the mutation also results in elevated *gcvA-lacZ* expression in the presence and absence of CRP (Table 6) and presumably of the *gcvA* gene itself. In a *crp* strain, the *gcvA* autoregulatory mutant caused a twofold decrease in *gcvT-lacZ* expression, similar to what occurred with wild-type *gcvA* (Table 6). Since *gcvA* expression was much higher when the autoregulatory mutant was present, the lowered *gcvT-lacZ* expression was unlikely to have been due to less GcvA in the *crp* strain.

CRP requires the repressor function of GcvA to regulate *gcvT-lacZ* expression. GcvA activates and represses expression of *gcvT-lacZ*, and it is likely that both functions of GcvA are responsible for the basal levels of *gcvT-lacZ* expression in GM medium. Due to the dual action of GcvA, it is possible that CRP interferes with repression by GcvA or facilitates activation by GcvA, since both scenarios can explain the phenotypes of the Δ*crp* and the Δ*crp* Δ*gcvA* strains. To distinguish between these two possible roles for CRP, the positive-control (PC) *gcvA* mutant *gcvA*F31A (16) was used to separate the activation and repression functions of GcvA. In a *purR gcvA* strain, a single-copy plasmid carrying the *gcvA* PC allele allows binding to the *gcv* control region and repression by GcvAF31A but not activation, and in a *purR gcvA gcvR* strain, GcvAF31A has virtually no activity since it cannot activate efficiently due to the amino acid change and cannot repress in the absence of GcvR (16). If CRP-mediated regulation is dependent on the activator function of GcvA, a *crp* deletion would be expected to have no effect if the *gcvA*F31A allele is the only *gcvA* present in the cell. Conversely, if CRP's role is to interfere with repression by GcvA, then CRP would be expected to regulate normally when the repressor function is intact but would have no role when GcvAF31A cannot repress (in a *gcvR* strain). A *crp* deletion resulted in a fourfold decrease in *gcvT-lacZ* expression in a *purR gcvA* strain carrying the *gcvA*F31A allele (little activation) (Table 7). However, in the *purR gcvA gcvR* strain carrying the *gcvA*F31A allele (little activation and no repression), the *crp* deletion had no effect (Table 7), suggesting that CRP's role in the regulation of *gcvT-lacZ* is dependent on GcvA's ability to repress.

DISCUSSION

In this study CRP was shown to be a positive regulator of the *gcv* operon. A deletion of the *crp* gene resulted in a three- to fourfold decrease in β-galactosidase expression from a λ*gcvT-lacZ* fusion that was relieved by the introduction of a single-copy plasmid bearing the wild-type *crp* gene. A deletion of the *cyaA* gene also resulted in a fourfold decrease in β-galactosidase levels (Table 2), indicating that CRP requires cAMP for regulation of the *gcvT-lacZ* fusion. Although the addition of cAMP to GM medium restored CRP-mediated regulation in the *cyaA* mutant (Table 3), its addition resulted in no further increase in *gcvT-lacZ* expression in a wild-type strain, indicating that the cAMP level in glucose-grown cultures is sufficient for CRP-mediated regulation of *gcv*. In an early study of CRP-cAMP binding to DNA, a fragment with the CRP consensus site was shown to bind CRP with an increased affinity compared to that of naturally occurring CRP binding sites, even in the absence of high levels of cAMP (11). However, the CRP binding site on *gcv* diverges from consensus at many positions (Fig. 1) and it is not clear how CRP can achieve its maximum range of regulation of the *gcv* operon at the cAMP levels found in GM medium-grown cultures.

CRP binding to the *gcv* control region protected two sites

TABLE 7. CRP regulation requires the repressing function of GcvA

Strain (plasmid)	Relevant genotype	β-Galactosidase activity[a]
GS986 λ*gcvT-lacZ* (pF31A)	*gcvA1 purR*::Tn*10* + *gcvA*^{PC}	75
GS1124 λ*gcvT-lacZ* (pF31A)	*gcvA1 purR*::Tn*10* Δ*crp*::Cm^r + *gcvA*^{PC}	19
GS1121 λ*gcvT-lacZ* (pF31A)	*gcvA1 purR*::Tn*10 gcvR*::Kn^r + *gcvA*^{PC}	180
GS1125 λ*gcvT-lacZ* (pF31A)	*gcvA1 purR*::Tn*10 gcvR*::Kn^r Δ*crp*::Cm^r + *gcvA*^{PC}	169

[a] Cells were grown in GM medium with ampicillin. β-Galactosidase activity is in Miller units (25). All standard deviations were within 11% of the means.

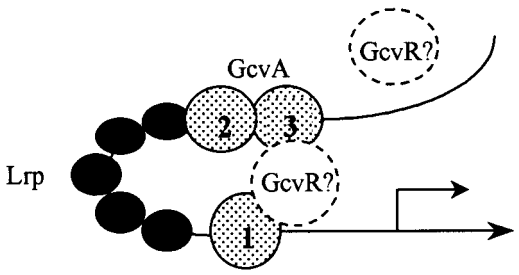

FIG. 5. Hypothetical model for repression of *gcv*. There is evidence that GcvA must bind to its three target sites for repression (47). GcvR is also required for repression (12, 13), but it is unknown if GcvR-GcvA contacts are required or if GcvR performs some other function. Lrp binds *gcv* DNA in the region depicted and bends DNA (29), possibly to allow the formation of a nucleoprotein complex.

from DNase I digestion, one centered near bp −313 (site 1) and the other centered at bp −140 (site 2) relative to the transcriptional start site (Fig. 4). A mutational analysis of the two binding sites demonstrated that CRP binding is required only at site 1 to effect regulation of *gcvT-lacZ* (Table 4), consistent with the results of the gel mobility shift assay that showed only a single shifted species that required an intact site 1 (Fig. 2 and 3). We cannot explain why CRP binds to site 2 in the footprinting assay and not in the gel mobility shift assay. It should be noted that site 2 is totally within the region shown previously to be protected from DNase I digestion by the Lrp protein (41), and we believe this site is probably not accessible to CRP in vivo due to Lrp binding to this sequence.

CRP-mediated regulation from site 1 centered at bp −313 is interesting; in other CRP-regulated genes, the binding sites are located from bp −40 to −200 relative to the transcription start sites (21). CRP binding at −313 does not appear to activate any upstream promoters since S1 nuclease mapping experiments and a genetic analysis of the *gcv* control region did not reveal any additional promoters (40). Although our results indicate that CRP's role is to inhibit repression by GcvA at the *gcv* promoter, it is still possible that direct contact occurs between CRP and RNAP via DNA bending at *gcv*. This possibility may be unlikely since four *crp* mutants, each with an amino acid change in a known activating region, were able to complement the *crp* deletion and restore *gcvT-lacZ* expression to near the wild-type level.

It was demonstrated in several other systems such as the *ara* and *mal* regulons that CRP regulates specific promoters in conjunction with another regulator and, in addition, regulates the expression of the coregulatory protein itself (21). CRP-mediated regulation of *gcvT-lacZ* is dependent on the GcvA protein, and CRP stimulates expression of a *gcvA-lacZ* fusion about twofold. However, the reduced level of GcvA in a *crp* deletion strain does not appear to be responsible for the reduced expression of *gcvT-lacZ*. The results from this study are consistent with a model where CRP's role in the regulation of *gcv* is to interfere with repression by GcvA, rather than to activate transcription via interactions with RNAP.

There is no clear definition of antirepression, although several modes of regulation have been described as antirepressive (15, 17, 35, 37). In our system, antirepression is characterized by the antirepressor (CRP) having no function in the absence of the repressor (GcvA). There are two similar examples of antirepression that have been described for *E. coli*. In the first example, the global regulator integration host factor binds to the *aceBAK* operon and appears to inhibit repression by IclR, but no mechanism for this antirepression has yet been characterized (29). The second example of antirepression occurs at the *pap* genes in *E. coli*. A single CRP binding site is located −115.5 and −215.5 bp upstream of the divergent transcription start sites for the *papI* and *papB* genes, respectively, and CRP positively regulates expression of these genes by inhibiting binding of the repressing H-NS protein (8). It is difficult to visualize CRP utilizing this mechanism at the *gcv* operon since CRP's binding site does not overlap the GcvA binding sites. How then can CRP binding at site 1 antagonize GcvA-mediated repression? Evidence indicates a requirement not only for GcvA and its three binding sites but also for both GcvR and Lrp (12, 13, 41, 44, 47), suggesting that a nucleoprotein complex may form with these regulatory components to cause repression (Fig. 5). The role of CRP, then, may be to antagonize the formation or function of the complex. Since repression by GcvA, GcvR, and Lrp is poorly understood, further elucidation of the roles of these proteins in the regulatory mechanism is necessary for further investigation into CRP's role in controlling the *gcv* operon.

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