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Promoter Characterization and Constitutive Expression of the *Escherichia coli gcvR* Gene

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The *Escherichia coli* glycine cleavage repressor protein (GcvR) negatively regulates expression of the glycine cleavage operon (*gcv*). In this study, the *gcvR* translational start site was determined by N-terminal amino acid sequence analysis of a GcvR-LacZ fusion protein. Primer extension analysis of the *gcvR* promoter region identified a primary transcription start site 27 bp upstream of the UUG translation start site and a minor transcription start site approximately 100 bp upstream of the translation start codon. The -10 and -35 promoter regions upstream of the primary transcription start site were defined by mutational analysis. Expression of a *gcvR-lacZ* fusion was unaltered in the presence of glycine or inosine, molecules known to induce or repress expression of *gcv*, respectively. In addition, it was shown that *gcvR-lacZ* expression is neither regulated by the glycine cleavage activator protein (GcvA) nor autogenously regulated by GcvR. From DNA sequence analysis, it was predicted that the translation start codon of the downstream *bcp* gene overlaps the *gcvR* stop codon, suggesting that these genes may form an operon. However, a down mutation in the -10 promoter region of *gcvR* had no effect on the expression of a downstream *bcp-lacZ* fusion, and primer extension analysis of the *bcp* promoter region demonstrated that *bcp* has its own promoter within the *gcvR* coding sequence. These results show that *gcvR* and *bcp* do not form an operon. Furthermore, the deletion of *bcp* from the chromosome had no effect on *gcv-lacZ* expression.

The *Escherichia coli* glycine cleavage enzyme system catalyzes the cleavage of glycine into carbon dioxide, ammonia, and 5,10-methylenetetrahydrofolate (9). Glycine is required for both protein and purine biosynthesis, while 5,10-methylenetetrahydrofolate serves as a one-carbon donor in the biosynthesis of purines, methionine, thymine, and numerous methylated products (15). Three components of the glycine cleavage enzyme system, the GcvT, GcvH, and GcvP proteins, are encoded by the *gcv* operon (17). Induced by glycine (13, 17, 28) and repressed by purines (8, 27), it appears that expression of the *gcv* operon is regulated in order to balance cellular requirements for glycine and one-carbon units.

Currently, four proteins, the leucine-responsive regulatory protein (Lrp), the purine repressor protein (PurR), the glycine cleavage activator protein (GcvA), and the glycine cleavage repressor protein (GcvR), have been shown to be involved in regulating expression of the *gcv* operon. Lrp is a global regulatory protein involved in the control of transcription of numerous genes involved in amino acid metabolism (6) and is required for normal induction of *gcv* (11, 21). Lrp binds to multiple sites upstream of the *gcv* promoter, suggesting a direct role for Lrp in *gcv* expression (21). Whether Lrp interacts with RNA polymerase, one of the other regulatory proteins, or plays a structural role by bending DNA is unknown.

PurR is a negative regulator of many genes involved in nucleotide metabolism (30), including *gcv* (27). PurR mediates a twofold decrease in *gcv* transcription in response to purine supplementation and has been shown to bind to the *gcv* control region, overlapping the *gcv* transcription start site (20, 27).

GcvA and GcvR work in concert to further regulate *gcv* expression. In glucose minimal medium, GcvR negatively regulates *gcv* expression, resulting in a low, basal level of *gcv* expression (8). In glycine-supplemented cultures, GcvR repression is relieved, and GcvA activates *gcv* expression (8, 28). In purine-supplemented cultures, both GcvA and GcvR are required to mediate a PurR-independent repression of *gcv* (8, 27). GcvA has been shown to bind to three sites upstream of the *gcv* promoter, and mutations have verified that all three sites are required for normal GcvA-mediated activation and repression of *gcv* (29).

It has been shown that *gcv* expression can be altered by changing the ratio of GcvA to GcvR (8). Overexpression of GcvA leads to constitutive activation of *gcv*, even in the absence of glycine, while overexpression of GcvR causes superrepression of *gcv*, even in the absence of purines. Therefore, it is important to understand how the expression of *gcvA* and *gcvR* is regulated. Expression of *gcvA* is negatively autoregulated and is unaffected by glycine or purine supplementation (26). It has also been shown that GcvR has no effect on *gcvA* expression (8). In this study, we characterized the promoter region of *gcvR* and investigated transcriptional regulation of *gcvR*. In addition, we show that *bcp*, a gene located immediately downstream of *gcvR*, is not in an operon with *gcvR* and plays no role in the regulation of *gcv* expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 strains and plasmids used in this study are described in Table 1.

Media and growth conditions. The complex medium used was Luria-Bertani (LB) broth (14). The glucose minimal (GM) medium used was minimal salts (25) supplemented with 0.4% glucose, phenylalanine (50 μg/ml), and thiamine (1 μg/ml). Additional supplements were added, where indicated, at the following concentrations: inosine, 50 μg/ml; glycine, 300 μg/ml; ampicillin, 150 μg/ml; and kanamycin, 30 μg/ml. λ lysogens carry the *c*I857 mutation resulting in a temperature-sensitive repressor and were grown at 30°C. All other strains were grown at 37°C.

Nucleotide sequencing. Dideoxynucleotide DNA sequencing was performed by using a Sequenase version 2.0 sequencing kit (United States Biochemical, Cleveland, Ohio).

Construction of *gcvR-lacZ* and *bcp-lacZ* translational fusions. The *gcvR-lacZ* fusion plasmid pGS343 was constructed by cloning the *Eco*RI-*Ssp*I fragment carrying the control region and initial coding sequence of *gcvR* from plasmid

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

Strain or plasmid	Genotype or description	Source or reference
Strains[a]		
GS162	Wild type	This lab
GS597	*metJ97*	This lab
GS1029	Δ(*gcvA*):Σ*aadA*	26
GS1053	*gcvR*::Tn*10*	8
GS1066	*recD*::Tn*10*	This lab
GS1111	Δ(*gcvR bcp*)Σ*neo*	This study
Plasmids		
pGS311	Single-copy cloning vector	8
pGS334	Multicopy $gcvR^+$ plasmid	8
pGS338	Single-copy $gcvR^+$ plasmid	8
pGS343	*gcvR-lacZ* fusion plasmid	This study
pGS393	pGS343 with -13A *gcvR* promoter mutation	This study
pGS394	pGS343 with -36A *gcvR* promoter mutation	This study
pGS429	pGS334 with -13A *gcvR* promoter mutation	This study
pGS430	*bcp-lacZ* fusion plasmid	This study
pGS431	pGS430 with -13A *gcvR* promoter mutation	This study
pGS433	pBR322 with *Ssp*I site replaced by *Sma*I site	This study
pGS434	Multicopy $gcvR^+$ bcp^+ plasmid	This study
pGS437	Δ(*gcvR bcp*)Σ*neo* in pGS434	This study
pGS451	pGS437 with extra 2 kb of homologous DNA upstream of the Δ(*gcvR bcp*)Σ*neo*	This study

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

pGS334 (Fig. 1) into the *Eco*RI-*Sma*I sites of the *lac* fusion plasmid pMC1403 (7), forming an in-frame fusion of *gcvR* to the *lacZYA* genes. Plasmids bearing mutations in the -10 (-13A) and -35 (-36A) regions of the *gcvR-lacZ* fusion promoter (pGS393 and pGS394, respectively) were created by the PCR megaprimer mutagenesis procedure (18), using primers GcvR7 (5′-GCATACATCA ATCAGAACGG-3′) and GcvR8 (5′-GCATGTTTTTTTTATGCATTCCTTAA G-3′) (mutations are underlined; see Fig. 2). Plasmid pGS343 was used as a template.

The *bcp-lacZ* fusion plasmid pGS430 was constructed by cloning the *Eco*RI-*Eco*RV fragment carrying *gcvR* and the initial coding sequence of *bcp* from pGS334 (Fig. 1) into the *Eco*RI-*Sma*I sites of pMC1403. Plasmid pGS431, carrying a *gcvR* promoter mutation upstream of a *bcp-lacZ* fusion, was constructed in two steps. First, the *Eco*RI-*Afl*II fragment carrying the wild-type *gcvR* promoter was deleted from plasmid pGS334 (Fig. 1) and replaced with the same fragment carrying the -13A mutation from pGS393. This plasmid was designated pGS429. Then, a *bcp-lacZ* fusion was constructed downstream of this *gcvR* promoter mutation by cloning the *Eco*RI-*Eco*RV fragment from pGS429 into the *Eco*RI-*Sma*I sites of pMC1403.

All fusions were cloned into bacteriophage λgt2 (16) as previously described (23), and the resultant phage were used to lysogenize the appropriate strains. Lysogens were tested for the presence of a single copy of the λ phage by infection with phage λ*c*I90*c*17 (19).

Purification and N-terminal amino acid sequencing of a GcvR-LacZ fusion protein. Wild-type strain GS162 carrying the *gcvR-lacZ* fusion plasmid pGS343 was grown in LB-ampicillin, and the GcvR-LacZ fusion protein was extracted and partially purified by affinity chromatography (22). To further purify the fusion protein, approximately 5 μg of fusion protein was electrophoresed on a sodium dodecyl sulfate–10% polyacrylamide (PA) gel and electroblotted onto a polyvinylidene difluoride membrane. The sequence of the first 14 amino acids was determined by automated N-terminal amino acid sequencing at the University of Iowa Protein Facility.

Primer extension analysis. To determine the location of the *gcvR* promoter, primer extension mapping was performed with a Promega (Madison, Wis.) primer extension kit. Wild-type strain GS162 carrying the $gcvR^+$ plasmid pGS334 was grown in LB-ampicillin, and total RNA was isolated as previously described (3). Primer extension reactions were carried out by the Promega protocol, using the ^{32}P-labeled primer GcvR4 (5′-GCAACTACTGACATGACGGGTG-3′), which hybridizes 71 bp downstream of the *gcvR* translation start site. Reaction products were electrophoresed on a 5% PA gel next to DNA sequencing reactions also generated with primer GcvR4.

To determine the location of the *bcp* promoter, primer extension mapping was performed as described below, using SuperScript II RNase H^- reverse transcriptase (RT) (Gibco BRL, Gaithersburg, Md.). Total RNA was isolated from strain GS597 as previously described (3). Approximately 5 μg of RNA and either 100 fmol (reaction 1) or 1 pmol (reaction 2) of ^{32}P-labeled primer Bcp2 (5′-CTCC GTCTTGATCCGGCAAGC-3′) were mixed in 1× RT buffer (50 mM Tris-HCl [pH 8.3], 75mM KCl, 3 mM $MgCl_2$), heated to 60°C for 10 min, and then allowed to anneal at 25°C for 10 min. Extension reactions were carried out in 1× RT buffer, 400 nM deoxynucleoside triphosphates 100 μg of bovine serum albumin per ml, and 1 mM dithiothreitol in a total volume of 10 μl at 42°C for 30 min, using 10 (reaction 1) or 100 (reaction 2) U of RT. Reactions were stopped by adding 8 μl of sequencing stop mix. The products of each reaction were electrophoresed on a 5% PA gel next to DNA sequencing reactions also generated with primer Bcp2.

β-Gal assays. β-Galactosidase (β-Gal) enzyme assays were performed as described by Miller (14). All results are the average of two or more assays, with each sample being determined in triplicate. All standard deviations are ≤12% of the mean.

Construction of a *gcvR-bcp* chromosomal deletion. An approximately 1.9-kb fragment carrying *gcvR* and *bcp* was cloned by PCR amplification of Kohara λ phage 424 (10), using primers GcvR1 (5′-GAATTCGCAATTACCGGAATGC GCC-3′) and Bcp1 (5′-GCGCATGGGCATTCACCGGACGGC-3′). This fragment was then digested with *Eco*RI and *Hin*dIII, and the resulting fragment was cloned into the *Eco*RI-*Hin*dIII sites of plasmid pGS433, which is a pBR322 (5) derivative in which the *Ssp*I site has been replaced with a *Sma*I site. This plasmid was designated pGS434 (Fig. 1). A deletion from codon 35 of *gcvR* through codon 49 of *bcp* (Fig. 1) was made by digesting pGS434 with *Ssp*I and *Stu*I. The deleted fragment was then replaced with a blunt-ended, *Sal*I-*Hin*dIII fragment carrying the kanamycin resistance gene (*neo*) from Tn*5* (4). This plasmid was designated pGS437.

Initial attempts to integrate this deletion into the chromosome by linear transformation were unsuccessful, most likely due to a limiting amount of homologous DNA flanking the *neo* gene. Therefore, an additional 2 kb of flanking DNA was introduced upstream of the *neo* gene by cloning an *Eco*RI-*Ppu*10I fragment from Kohara λ phage 424 carrying approximately 2.3 kb of DNA upstream of the *gcvR* coding sequence into the *Eco*RI-*Ppu*10I sites of pGS437, creating plasmid pGS451. This plasmid was digested with *Eco*RI and *Hin*dIII and used to perform a linear transformation of strain GS1066 (*recD*::Tn*10*). Transformants were selected on LB-kanamycin and checked for ampicillin sensitivity

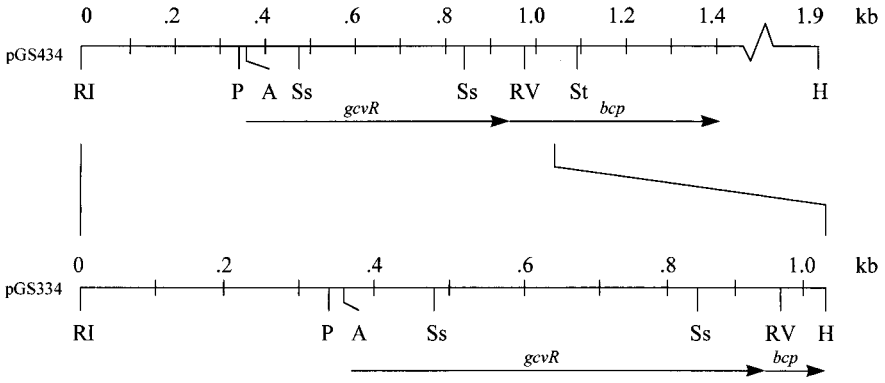

FIG. 1. Diagram of the *Eco*RI-*Hin*dIII fragments of plasmids pGS334 and pGS434. The locations and directions of transcription of *gcvR* and *bcp* are indicated by arrows. The *Eco*RI sites of both fragments as well as the *Hin*dIII site of the pGS334 fragment were PCR generated. A, *Afl*II; H, *Hin*dIII; RI, *Eco*RI; RV, *Eco*RV; P, *Ppu*10I; Ss, *Ssp*I; St, *Stu*I.

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CATGGGCCATCCTCTGTGCAAACAAGTGTCTCAATGGTACGTTTGGTATGGCATTAAAAG   60

                          -35                    -10
CAAGCAGACAGAACCGTTCTGATTGTTGTATGCATGTTTTTTTTATGCTTTCCTTAAGAA  120
                          ↓                      ↓
                         -36A                   -13A
+1            RBS
CAACTCACCCCTTAAAGGAATAACCAGTTTGACACTGTCATCGCAACATTATCTGGTGAT  180
                            M  T  L  S  S  Q  H  Y  L  V  I
                          gcvR→

CACTGCGTTGGGTGCCGATCGCCCTGGAATTGTGAACACCATCACCCGTCATGTCAGTAG  240
 T  A  L  G  A  D  R  P  G  I  V  N  T  I  T  R  H  V  S  S

TTGCGGCTGTAATATTGAAGACAGTCGCCTGGCGATGCTGGGAGAAGAGTTCACGTTTAT  300
 C  G  C  N  I  E  D  S  R  L  A  M  L  G  E  E  F  T  F  I

TATGCTGCTTTCCGGTTCATGGAATGCCATTACTCTGATTGAATCAACGTTACCGTTGAA  360
 M  L  L  S  G  S  W  N  A  I  T  L  I  E  S  T  L  P  L  K

AGGTGCCGAACTGGATCTTTTAATCGTGATGAAGCGCACGACGGCGCGTCCGCGTCCGCC  420
 G  A  E  L  D  L  L  I  V  M  K  R  T  T  A  R  P  R  P  P

AATGCCAGCATCTGTCTGGGTTCAGGTCGATGTGGCAGACTCCCCGCATTTAATTGAACG  480
 M  P  A  S  V  W  V  Q  V  D  V  A  D  S  P  H  L  I  E  R

CTTCACAGCACTTTTCGACGCGCATCATATGAACATTGCGGAGCTGGTGTCGCGCACGCA  540
 F  T  A  L  F  D  A  H  H  M  N  I  A  E  L  V  S  R  T  Q

ACCTGCTGAAAATGAACGGGCTGCGCAGTTGCATATTCAGATAACCGCCCACAGCCCCGC  600
 P  A  E  N  E  R  A  A  Q  L  H  I  Q  I  T  A  H  S  P  A

                                                  -35
ATCTGCGGACGCAGCAAATATTGAGCAAGCGTTCAAAGCCCTATGTACAGAACTCAATGC  660
 S  A  D  A  A  N  I  E  Q  A  F  K  A  L  C  T  E  L  N  A

       -10        +1                              RBS
ACAAGGCAGTATTAACGTCGTCAATTATTCCCAACATGATGAACAGGATGGAGTTAAGTA  720
 Q  G  S  I  N  V  V  N  Y  S  Q  H  D  E  Q  D  G  V  K  *

ATGAATCCACTGAAAGCCGGTGATATCGCACCGAAATTTAGCTTGCCGGATCAAGACGGA  780
M  N  P  L  K  A  G  D  I  A  P  K  F  S  L  P  D  Q  D  G
bcp→

GAACAAGTTAATTTGACCGACTTCCAGGGACAGCGT                          816
E  Q  V  N  L  T  D  F  Q  G  Q  R
```

FIG. 2. Complete nucleotide and deduced amino acid sequences of the *E. coli gcvR* gene and partial nucleotide and deduced amino acid sequences of the *E. coli bcp* gene. The *gcvR* and *bcp* transcription start sites (+1), -10 and -35 promoter regions, and ribosome binding sites (RBS) are underlined and in boldface. A potential secondary *gcvR* transcription start site and its promoter are underlined. Mutations in the *gcvR* promoter constructed in vitro are shown below the sequence. The *gcvR* amino acid sequence in boldface was verified by N-terminal amino acid sequencing.

by spotting on LB-ampicillin. One kanamycin-resistant, ampicillin-sensitive transformant was designated GS1111.

Nucleotide sequence accession number. The nucleotide sequence data reported here are in the GenBank database under accession no. AFO23337.

RESULTS AND DISCUSSION

Nucleotide sequence of *gcvR*. Plasmid pGS334 carries the *E. coli gcvR* gene and a portion of the downstream *bcp* gene on an approximately 1-kb *Eco*RI-*Hin*dIII fragment (Fig. 1). In this study, the nucleotide sequence of most of this PCR-generated fragment was determined (Fig. 2). This sequence differs from the original sequence of this region as determined by S. C. Andrews et al. (GenBank accession no. M37689) but is identical to the sequence of this region as determined by F. R. Blattner et al. and Y. Yamamoto et al. (GenBank accession no. ECAE000335 and D90877, respectively).

***gcvR* translation start site.** To determine where translation of *gcvR* begins, a *gcvR-lacZ* fusion plasmid was constructed, the GcvR-LacZ fusion protein was purified, and the sequence of the first 14 N-terminal amino acids was determined (Fig. 2; see Materials and Methods). This sequence indicates that translation initiates at a UUG codon located 9 bp downstream of a potential ribosome binding site (Fig. 2). The formylmethionine encoded by the UUG translation initiation codon is apparently removed from the nascent polypeptide. These data suggest

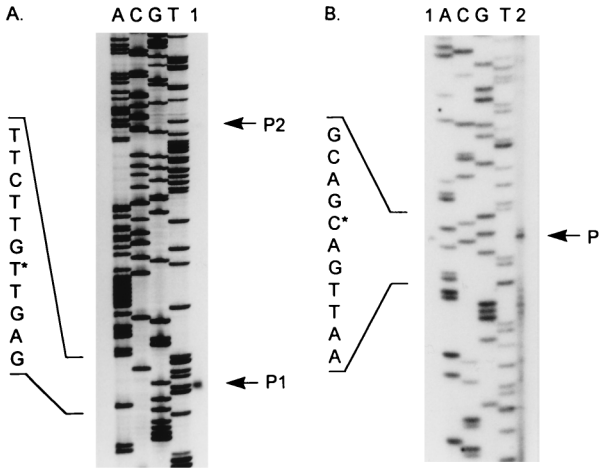

FIG. 3. Primer extension analysis of *gcvR* and *bcp* transcripts. Primer extension products were synthesized as described in Materials and Methods and are marked by arrows. DNA sequencing ladders were generated with the same primer as that used in the primer extension reactions. The nucleotide sequence of the complementary strand of each promoter region is indicated to the left. The nucleotide designated +1 for each promoter is indicated with an asterisk. (A) *gcvR* primer extension analysis. Lanes: A, C, G, and T, DNA sequencing ladders; 1, primer extension products (P1, primary transcription start site; P2, minor transcription start site). (B) *bcp* primer extension analysis. Lanes 1, primer extension reaction 1; A, C, G, and T, DNA sequencing ladders; 2, primer extension reaction 2.

that GcvR is a 189-amino-acid protein with a molecular mass of 20.6 kDa. A similarity search performed with the BLAST (1) program failed to identify any proteins with significant similarity to the deduced amino acid sequence of GcvR.

Only 1% of all sequenced *E. coli* genes use a UUG codon to initiate translation (91% use AUG; 8% use GUG) (12), and in such cases, translational efficiency is reduced three- to fivefold (24). Since overproduction of the GcvR protein results in repression of the *gcv* operon (8), the use of this codon as the *gcvR* translation start site may serve to reduce translation of *gcvR*, preventing superrepression of *gcv*.

***gcvR* promoter mapping.** To determine the location of the *gcvR* promoter, primer extension mapping was performed on RNA isolated from the wild-type strain GS162 bearing the multicopy *gcvR*$^+$ plasmid pGS334. As shown in Fig. 3A, two primer extension products were synthesized. The major primer extension product (P1) migrated in alignment with an A residue located 27 bp upstream of the *gcvR* translation start site (Fig. 2). This A residue was designated +1. The minor primer extension product (P2) indicates that a second transcription start site may also exist approximately 100 bp upstream of the *gcvR* translation start codon. A potential σ^{70} promoter sequence is present upstream of both start sites (Fig. 2).

To verify the -10 and -35 regions of the promoter sequence upstream of the primary start site, a mutation was introduced in each region, and the effect of each mutation on promoter function was determined. Wild-type strain GS162 was lysogenized with λ phage carrying either wild-type (λ*gcvR-lacZ*) or mutant (λ*gcvR-113A-lacZ* and λ*gcvR-36A-lacZ*) fusions. The resulting lysogens were grown in GM medium and GM medium supplemented with either glycine or inosine (molecules known to induce or repress *gcvT-lacZ* expression, respectively) and assayed for β-Gal activity (Table 2). Expression of the wild-type *gcvR-lacZ* fusion is not affected by the addition of glycine or inosine, suggesting that these molecules do not indirectly affect *gcv* operon expression by altering *gcvR* expres-

TABLE 2. Expression of a *gcvR-lacZ* fusion from wild-type and mutant *gcvR* promoters

Strain	β-Gal activity (Miller units)[a]		
	GM	Glycine	Inosine
GS162λ*gcvR-lacZ*	73	78	69
GS162λ*gcvR-13A-lacZ*	5	5	4
GS162λ*gcvR-36A-lacZ*	7	8	7

[a] Cells were grown in GM medium with supplements as indicated and assayed for β-Gal activity. Each value is the average of two separate determinations, each performed in triplicate. All standard deviations are ≤11% of the mean.

sion. The -13A mutation resulted in a 15-fold decrease in *gcvR-lacZ* expression compared to the wild-type level, and the -36A mutation resulted in a 9-fold decrease in *gcvR-lacZ* expression compared to the wild-type level, regardless of supplementation. Thus, it appears that the primary transcription start site determined by primer extension mapping is correct.

The importance of the minor promoter of *gcvR* is still unclear. If this promoter functions in vivo to control expression of *gcvR*, it appears to be relatively weak compared to the primary promoter, since the -13A and -36A mutations in the primary promoter eliminate more than 90% of *gcvR-lacZ* expression under all growth conditions (Table 2). These results are consistent with the primer extension results which show that nearly all of the *gcvR* mRNA transcript initiates from the primary promoter (Fig. 3A).

***gcvR* expression is not regulated by GcvA or GcvR.** Since it is known that GcvR-mediated regulation of *gcv* requires GcvA, it is possible that GcvA regulates *gcvR* expression. To test this hypothesis, lysogens GS162λ*gcvR-lacZ* and GS1029λ*gcvR-lacZ* (*gcvA*) were grown in GM medium and GM medium supplemented with either glycine or inosine and assayed for β-Gal activity. As shown in Table 3, GcvA has no effect on *gcvR-lacZ* expression. This was true for all growth conditions.

Since many regulatory proteins are known to regulate their own expression, we tested the ability of GcvR to regulate *gcvR-lacZ* expression. Lysogens GS162λ*gcvR-lacZ* and GS1053 λ*gcvR-lacZ* (*gcvR*) were grown in GM medium and GM medium supplemented with either glycine or inosine and assayed for β-Gal activity. As shown in Table 3, GcvR does not autoregulate its own expression. This is true for all growth conditions. Furthermore, when GS162λ*gcvR-lacZ* was grown in rich (LB) medium, or when GM medium-grown cells were harvested from stationary phase rather than exponential phase, there was no significant change in β-Gal activity (data not shown), suggesting that *gcvR* expression does not respond to any other component in LB medium or to growth phase. Thus, whatever the role is for GcvR in the negative regulation of the *gcv* operon, the mechanism appears to be independent of changes in GcvR levels in the cell.

TABLE 3. *gcvR-lacZ* expression is not regulated by GcvA or GcvR

Strain	Relevant genotype	β-Gal activity (Miller units)[a]		
		GM	Glycine	Inosine
GS162λ*gcvR-lacZ*	Wild type	91	91	88
GS1029λ*gcvR-lacZ*	*gcvA*	94	94	90
GS1053λ*gcvR-lacZ*	*gcvR*	101	94	97

[a] Cells were grown in GM medium with supplements as indicated and assayed for β-Gal activity. Each value is the average of two separate determinations, each performed in triplicate. All standard deviations are ≤10% of the mean.

The *bcp* gene is not in an operon with *gcvR*. The *bcp* gene, encoding the bacterioferritin comigratory protein, is located immediately downstream of *gcvR*. It is predicted that this open reading frame, first reported by Andrews et al. (2), encodes a 156-amino-acid protein of unknown function. Our sequence of *gcvR* suggests that *gcvR* and *bcp* may form an operon, as the UAA stop codon of *gcvR* overlaps the predicted AUG start codon of *bcp* (Fig. 2). To test this hypothesis, a translational *bcp-lacZ* fusion was constructed downstream of the wild-type *gcvR* gene as well as downstream of a mutant *gcvR* gene carrying the -13A promoter mutation described above. Both fusions were cloned into phage λgt2, and the phage was used to lysogenize the wild-type strain GS162. The resulting lysogens were grown in GM medium and assayed for β-Gal activity. The lysogen carrying the wild-type *gcvR* promoter had 325 U of activity, and the lysogen carrying the mutant *gcvR* promoter had 323 U of activity. Thus, a mutation in the -10 region of the primary *gcvR* promoter, which results in a 15-fold decrease in *gcvR-lacZ* expression, has no effect on the expression of a downstream *bcp-lacZ* fusion. These results suggest that *gcvR* and *bcp* do not form an operon.

To further demonstrate that *gcvR* and *bcp* do not form an operon, we tested whether *bcp* has its own promoter within the *gcvR* coding sequence. Primer extension mapping was performed on RNA isolated from strain GS597. As shown in Fig. 3B, transcription of *bcp* begins at a G nucleotide, 8 bp downstream of a potential σ^{70} promoter and 41 bp upstream of the putative *bcp* translation start site as predicted by Andrews et al. (2) (Fig. 2). The presence of multiple products in lane 2 compared to the presence of a single product in lane 1 is most likely due to nonspecific primer annealing and extension due to the 10-fold increase in the amount of primer and RNA polymerase used in this extension reaction.

The *bcp* gene has no effect on the regulation of *gcv* expression. Even though *gcvR* and *bcp* are transcribed separately, their proximity still suggested that *bcp* might play a role in the regulation of *gcv* expression. To test whether *bcp* plays a role in *gcv* expression, we constructed strain GS1111, which carries a chromosomal deletion of both the *gcvR* and *bcp* genes (see Materials and Methods). Lysogens GS162λ*gcvT-lacZ*, GS1053 λ*gcvT-lacZ* (*gcvR*), and GS1111λ*gcvT-lacZ* (Δ*gcvR bcp*), each transformed with the single-copy vector pGS311, and lysogen GS1111λ*gcvT-lacZ*, transformed with the single-copy $gcvR^+$ plasmid pGS338, were grown in GM medium and GM medium supplemented with either glycine or inosine and assayed for β-Gal activity (Table 4). GS1053λ*gcvT-lacZ*[pGS311] and GS1111λ*gcvT-lacZ*[pGS311] had similar levels of *gcvT-lacZ* expression under all conditions. In addition, transformation of GS1111λ*gcvT-lacZ* with pGS338 resulted in wild-type levels of *gcvT-lacZ* expression under all conditions, confirming that *bcp* is not involved in the regulation of *gcv* expression.

Possible roles for GcvA and GcvR in the regulation of *gcv*. Expression of the *gcv* operon is induced in the presence of glycine and repressed in the presence of purines (8, 13, 17, 27, 28). Since overproduction of GcvA leads to activation of *gcv*, while overproduction of GcvR results in repression of *gcv* (8), it is possible that glycine and inosine alter *gcv* expression indirectly by altering the ratio of GcvA to GcvR. However, previous results (8, 26) as well as those presented here demonstrate that glycine and inosine have no effect on the expression of *gcvA* and *gcvR*. Both genes also appear to be transcribed constitutively with respect to medium richness and growth phase.

Since both GcvA and GcvR are required for normal regulation of *gcv*, it is also possible that they regulate *gcv* indirectly by regulating the expression of one another. However, it has

TABLE 4. Bcp is not involved in the regulation of *gcvT-lacZ* expression

Strain	Relevant genotype	β-Gal activity (Miller units)[a]		
		GM	Glycine	Inosine
GS162λ*gcvT-lacZ*[pGS311]	Wild type	159	1020	15
GS1053λ*gcvT-lacZ*[pGS311]	*gcvR*	1,443	1,710	1,012
GS1111λ*gcvT-lacZ*[pGS311]	*gcvR bcp*	1,400	1,559	909
GS1111λ*gcvT-lacZ*[pGS338]	*gcvR bcp*[*gcvR*$^+$]	205	1,150	16

[a] Cells were grown in GM medium with supplements as indicated and assayed for β-Gal activity. Each value is the average of two separate determinations, each performed in triplicate. All standard deviations are ≤12% of the mean.

been shown that no reciprocal regulation occurs. Only a twofold autoregulation by GcvA occurs.

Several models to explain how GcvA and GcvR interact to regulate *gcv* expression have been proposed. In one model, GcvA homocomplexes function as activators, while GcvA-GcvR heterocomplexes function as repressors. In this model, the coregulators determine the type of complex formed; glycine leads to the formation of activation complexes, while inosine leads to the formation of repression complexes. Increasing the amount of either GcvA or GcvR would force the formation of activation or repression complexes, respectively. In a second model, GcvR may synthesize the corepressor required for the repressor function of GcvA. In a *gcvR* mutant, insufficient corepressor would lead to constitutive *gcv* expression. If GcvR is overproduced, too much corepressor causes superrepression of *gcv*. In another model, GcvR may negatively regulate *gcv* by modifying the structure of GcvA, changing it from an activator to a repressor in response to the coregulators. Purification of GcvR protein and identification of the actual coregulators of *gcv* expression should help us to determine which of these models, if any, is correct.

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