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Roles of the GcvA and PurR Proteins in Negative Regulation of the *Escherichia coli* Glycine Cleavage Enzyme System

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When *Escherichia coli* was grown in medium containing both inosine and glycine, the PurR repressor protein was shown to be responsible for a twofold reduction from the fully induced glycine cleavage enzyme levels. This twofold repression was also seen by measuring β-galactosidase levels in cells carrying a λ*gcvT-lacZ* gene fusion. In this fusion, the synthesis of β-galactosidase is under the control of the *gcv* regulatory region. A DNA fragment carrying the *gcv* control region was shown by gel mobility shift assay and DNase I footprinting to bind purified PurR protein, suggesting a direct involvement of the repressor in *gcv* regulation. A separate mechanism of purine-mediated regulation of *gcv* was shown to be independent of the *purR* gene product and resulted in an approximately 10-fold reduction of β-galactosidase levels when cells were grown in medium containing inosine but lacking the inducer glycine. This additional repression was dependent upon a functional *gcvA* gene, a positive activator for the glycine cleavage enzyme system. A dual role for the GcvA protein as both an activator in the presence of glycine and a repressor in the presence of inosine is suggested.

The glycine cleavage (GCV) enzyme system in *Escherichia coli* is responsible for the oxidative cleavage of glycine into CO_2 and NH_3 and the transfer of a one-carbon (C_1) methylene unit to tetrahydrofolate (4). This C_1 donor, 5,10-methylenetetrahydrofolate, supplies the cell with carbon units necessary for the biosynthesis of purines, methionine, thymine, and other cellular components (12). Another source of C_1 units for the cell is the conversion of serine into glycine with the concomitant transfer of a C_1 methylene unit to tetrahydrofolate via the *glyA* gene product (12, 15) (Fig. 1). It has been estimated that 15% of all carbon atoms from glucose assimilated by *E. coli* enter the serine-glycine pathway, making this pathway of great importance to the organism (16).

Although regulation of the *glyA* gene has been studied extensively (for a review, see reference 27), very little is known about control of the glycine-inducible *gcv* system. The genes encoding the T, H, and P proteins of the GCV enzyme complex are known to form an operon (28) that maps at min 65.2 on the *E. coli* chromosome (17). At present, two regulatory proteins, Lrp (7) and GcvA (38), have been shown to be involved in regulation of *gcv*. Lrp, or leucine-responsive regulatory protein, is a global regulatory protein involved in the control of transcription of numerous genes relating to amino acid metabolism. A mutation in *lrp* results in very low levels of GCV enzymes (7). The *trans*-acting protein GcvA is responsible for glycine-induced activation of *gcv*, and mutations in *gcvA* prevent inducibility of *gcv* (38).

An earlier study suggested that purine nucleotides included in the growth medium of *E. coli* have an inhibitory effect on GCV enzyme levels (13). Since the PurR protein is known to repress transcription of purine biosynthetic genes (5, 20) and *glyA* (31) in the presence of exogenous purines, we tested the ability of PurR to regulate expression of *gcv*. During this study, a second PurR-independent mechanism for purine-mediated repression of *gcv* that requires a functional *gcvA* gene was identified.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Genotypes of strains and plasmids used in this study are listed in Table 1. Strain GS973 (*gcvA1*) was described previously (38). Strain GS852 (*purR6*::Tn*10*) was constructed by transducing wild-type strain GS162 to Tc^r with P1 phage grown on strain SØ5058, which contains a *purR6*::Tn*10* allele (5). Tc^r colonies were tested for resistance to 2 mM 6-mercaptopurine to verify the presence of the *purR6*::Tn*10* allele (5). Strain GS986 (*gcvA1 purR6*::Tn*10*) was constructed by transducing GS973 to Tc^r with P1 phage grown on strain Sφ5058 and testing Tc^r transductants for resistance to 2 mM 6-mercaptopurine.

Media. The minimal medium used was Vogel and Bonner minimal salts (35) supplemented with 0.4% glucose (GM) and appropriate amino acids and antibiotics when needed. The complex medium used was Luria broth (11). Amino acids and antibiotics were added at the following concentrations: phenylalanine, 50 μg/ml; inosine, 50 μg/ml; glycine, 300 μg/ml; vitamin B_1, 1 μg/ml; tetracycline, 10 μg/ml; spectinomycin, 50 μg/ml; and kanamycin, 20 μg/ml.

P1 transductions. P1 *cml clr-100* phage for transductions was used as described previously (11).

Growth of cells and preparation of extracts for GCV enzyme assays. Cultures for enzyme assays were grown overnight at 30 or 37°C in GM medium with appropriate supplements and then subcultured into GM medium at a density of about 15 Klett units. Growth was monitored by using a Klett-Summerson colorimeter with a blue no. 42 filter.

Cell extracts for GCV enzyme assays were prepared by growing 250-ml quantities of cells to a reading of about 100 Klett units (approximately 5×10^8 cells). Cells were pelleted by centrifugation at 5,500 × *g* and washed two times with 0.85% NaCl. The cells were stored as frozen pellets for no longer than 48 h. Cell pellets were suspended in 1-ml quantities of 50 mM potassium phosphate buffer (pH 7.4) and sonicated for 7 s on ice. Cell extracts were centrifuged for 5 min at 4°C in an Eppendorf centrifuge to remove debris. Extracts were used immediately for enzyme assays.

Enzyme assays. The GCV enzyme assay used was the assay developed by Sagers and Gunsalus (22) and modified by Meedel and Pizer (10). Protein concentrations were

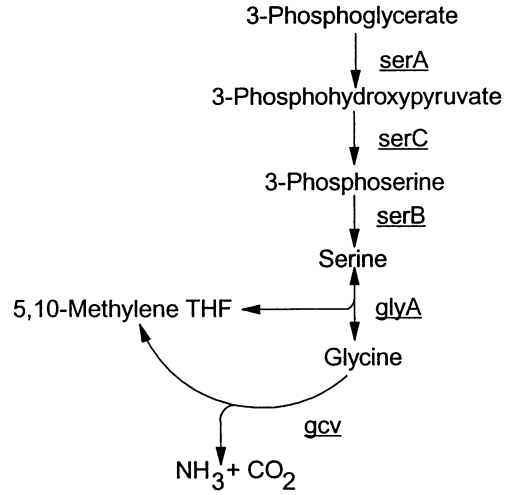

FIG. 1. Serine-glycine pathways of *E. coli*. The genes and the enzymes encoded are as follows: *serA*, 3-phosphoglycerate dehydrogenase; *serC*, 3-phosphoserine aminotransferase; *serB*, 3-phosphoserine phosphatase; *glyA*, serine hydroxymethyltransferase; and *gcv*, glycine cleavage enzyme system. THF, tetrahydrofolate.

determined by the method of Lowry et al. (8). The β-galactosidase assays were performed as described by Miller (11). All results are averages of two or more assays, with each sample done in triplicate.

Gel mobility shift assay. The gel mobility shift assay was based on the methods of Fried and Crothers (2) and Garner and Revzin (3). A 460-bp *Ssp*I-*Sma*I DNA fragment containing the *gcv* control region from plasmid pGS146 (30) was ^{32}P labeled at the 5′ terminus and used as the target DNA fragment. The assay was performed as described previously (34) with 52 to 104 ng of purified PurR protein in 20-μl reaction mixtures.

DNase I protection assay. The DNase I protection assay was a modified version of the method of Schmitz and Galas (25). The 5′ ^{32}P-labeled *Ssp*I-*Sma*I DNA fragment described above was incubated at 37°C for 5 min in 36 μl of 1× DNA binding buffer (10 mM Tris [pH 7.5], 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol) containing 125 μg of bovine serum albumin per ml. Four microliters (104 ng) of PurR protein was added, and the mixtures were incubated for 15 min at 37°C. DNase I was added (2 μl of a 1-mg/ml solution of DNase I in 20 mM sodium acetate [pH 7.0]–32 mM $CaCl_2$) and incubated at 37°C for 2 min. The reactions were stopped by the addition of 9 μl of a solution containing 3 M ammonium acetate, 0.25 M EDTA, and 15 μg of sonicated calf thymus DNA per ml. The samples were ethanol precipitated two times and suspended in sequencing dye mix. Maxam-and-Gilbert A+G and C+T sequencing reactions (9) were done on the same ^{32}P-labeled *Ssp*I-*Sma*I fragment and run next to the DNase I digestion reaction mixtures. After electrophoresis, the gel was dried and autoradiographed.

Site-directed mutagenesis and construction of lysogens. To alter the PurR binding site in the *gcv* control region, a 769-bp *Eco*RI-*Bam*HI fragment from the *gcvT-lacZ* fusion plasmid pGS239 (29) was cloned into phage M13mp19 (39). Oligonucleotide-directed mutagenesis was then performed by the method of Kunkel et al. (6) with the mutant oligonucleotide 5′-GGCAAAAGAGAACCAATGCGTTGGGGACC (altered bases are underlined). The mutations were confirmed by DNA sequencing (24). The 769-bp *Eco*RI-*Bam*HI fragment containing the mutated bases from the replicative form of phage DNA was cloned into the *Eco*RI-*Bam*HI fragment of the translational *lacZ* fusion plasmid pMC1403 (1), creating plasmid pGS264. pGS264 was digested with *Sal*I and filled in with T4 DNA polymerase, and *Eco*RI linkers were added with T4 DNA ligase. The ligated products were digested with *Eco*RI, and the 6,936-bp *Eco*RI-*Eco*RI DNA fragment carrying the *gcv* control region, the first 90 amino acids of *gcvT*, and the *lacZYA* genes was isolated and cloned into the *Eco*RI site of phage λgt2 (14), forming λ*gcvT-lacZ*+8C+10A. The wild-type phage λ*gcvT-lacZ* from plasmid pGS239 was constructed in a similar manner and is described elsewhere (29). Appropriate strains were lysogenized with the wild-type or mutant phage as described previously (33), except that the packaged phage was used to infect strain GS162. Lysogens were tested for a single copy of the λ phage by infection with λ *c*I90*c*17 (26).

DNA manipulations. Isolation of plasmid DNA, restriction enzyme digestions, and ligations were done as described previously (23).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strains[a]		
GS162	Wild type	This laboratory
GS852	*purR6*::Tn*10*	This laboratory
GS973	*gcvA1* λ*gcvT-lacZ*	This laboratory
GS986	*purR6*::Tn*10 gcvA1* λ*gcvT-lacZ*	This laboratory
Plasmids		
pRR1002	3.8-kb *Pst*I *purR*$^+$ fragment in pMS421; Sp^r	20
pGS146	7.12-kb *Sal*I-*Bam*HI *gcv* fragment in pACYC184; Cm^r	30
pGS239	769-bp *Eco*RI-*Bam*HI *gcv* fragment in pMC1403; Ap^r	29
pGS264	pGS239 with mutated PurR binding site; Ap^r	This laboratory
pGS266	1.5-kb *Eco*RI-*Eco*RI *gcvA* fragment in pGS225; Kn^r	This laboratory

[a] These strains also carry *thi pheA905* Δ*lacU169 araD129 rpsL150* mutations.

RESULTS

PurR-mediated repression of GCV enzyme activity. To test the effect of the *purR* gene product on GCV enzyme activity, *E. coli purR*$^+$ and *purR* strains with and without plasmid pPR1002, which contains a wild-type *purR* gene (20), were grown in GM medium containing no glycine or containing glycine or glycine and inosine and assayed for GCV enzyme activity. In the *purR*$^+$ strain, the addition of glycine and inosine to the growth medium resulted in a twofold decrease in GCV enzyme activity compared with the fully induced levels in medium containing only glycine (Table 2). The *purR* mutant, however, did not show this twofold repression when grown in the presence of glycine and inosine. The introduction of plasmid pPR1002, a multicopy plasmid carrying *purR*, restored inosine-dependent repression of GCV enzyme activity in the *purR* mutant (Table 2). We did not test the GCV enzyme activities of these strains grown in GM medium containing only inosine, since the uninduced levels of GCV

TABLE 2. GCV enzyme activities for *purR*$^+$ and *purR* strains

Strain	Sp act[a] of GCV enzyme from cells grown in GM medium		
	Alone	With glycine	With glycine + inosine
GS162 (*purR*$^+$)	0.04	1.27	0.59
GS852 (*purR*)	0.07	1.07	1.03
GS162 (*purR*$^+$) [pRR1002]	0.02	1.22	0.40
GS852 (*purR*) [pRR1002]	0.03	1.10	0.35

[a] Specific activities are expressed as nanomoles of HCHO generated per milligram of protein per minute.

activity (without glycine) were too low to detect with accuracy.

PurR protein binds to DNA containing the *gcv* control region. To determine whether the effect of the *purR* locus on GCV activity was due to a direct involvement of the PurR protein with the *gcv* regulatory region, a gel mobility shift assay was done. The addition of purified PurR protein to a ^{32}P-labeled DNA fragment containing the *gcv* control region resulted in a shift in mobility of the labeled DNA fragment to a more slowly migrating complex (Fig. 2). Increasing concentrations of PurR protein resulted in the shift of increasing amounts of DNA probe (Fig. 2, lanes 3 and 4). An additional band was detected above the shifted band; however, since the density of this band did not increase with increasing protein concentrations, it is most likely not due to a second binding site for PurR. A DNA fragment that is not specific for PurR did not show a shift in mobility at the concentrations of PurR used in this assay (data not shown). Higher concentrations of protein were not tested.

Localization of the PurR binding site on the *gcv* control region. To determine the exact sequence of *gcv* regulatory DNA to which PurR protein binds, a DNase I protection assay was performed on the same ^{32}P-labeled DNA fragment used in the mobility shift assay (see Materials and Methods).

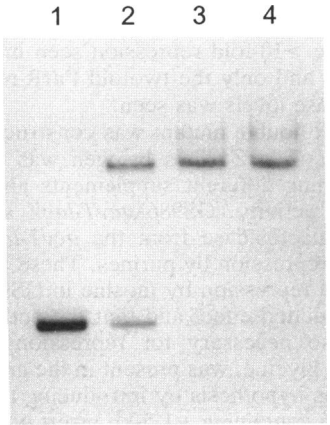

FIG. 2. Gel mobility shift assay for binding of PurR protein to the *gcv* control region DNA. A 460-bp ^{32}P-labeled DNA fragment (less than 10^{-9} M) containing the *gcv* control region was incubated with purified PurR protein, and specific DNA complexes were allowed to form. Reaction mixtures were then run on a nondenaturing 5% polyacrylamide gel, and the mobility patterns were visualized by autoradiography. Lane 1, DNA probe only; lane 2, 52 ng of PurR protein added; lane 3, 78 ng of PurR protein added; lane 4, 104 ng of PurR protein added.

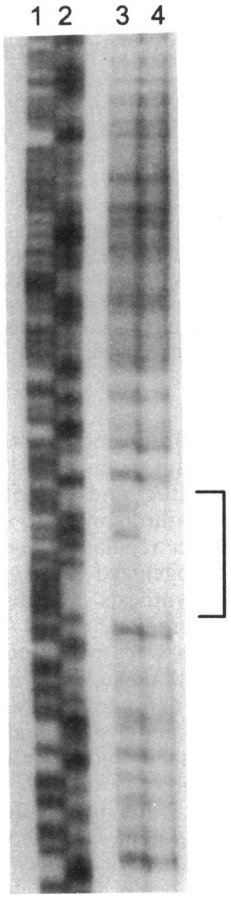

FIG. 3. Protection from DNase I digestion of the *gcv* control region by PurR. A ^{32}P-labeled DNA probe containing the *gcv* control region was incubated without or with purified PurR protein (see Materials and Methods). The mixtures were subjected to partial DNase I digestions, and the products were run on a denaturing polyacrylamide gel adjacent to the Maxam-and-Gilbert sequencing reaction mixtures of the labeled probe. Lane 1, A+G; lane 2, C+T; lane 3, no PurR protein added; lane 4, 4 μl of (104 ng) PurR protein added. The region of DNA protected from DNase I digestion is indicated by the brackets.

Because on numerous attempts this fragment showed a partial resistance to DNase I digestion in the absence of PurR protein, the precise boundaries of the PurR-protected region could not be determined. However, PurR is known to bind and protect a 16-bp region of DNA from DNase I digestion (21), and the region of *gcv* protected by PurR contains a 10- of 16-bp match to this PurR-binding consensus sequence (Fig. 3 and 4).

Mutations in the PurR binding site of *gcv* alter the ability of PurR to negatively regulate *gcv*. Using site-directed mutagenesis, we altered the PurR binding site identified in the DNase I footprint of the *gcv* regulatory region. A 769-bp DNA fragment containing the *gcv* regulatory region was cloned into M13mp19 phage and a 2-bp change in two highly conserved bases of the PurR consensus-binding sequence was made at positions +8 (G to C) and +10 (T to A) relative to the transcription initiation site of the *gcvT* gene, the first gene in the *gcv* operon (Fig. 4). A translational fusion of this mutated DNA fragment to the *lacZ* gene was made and cloned into phage λgt2. This phage, designated λ*gcvT*-

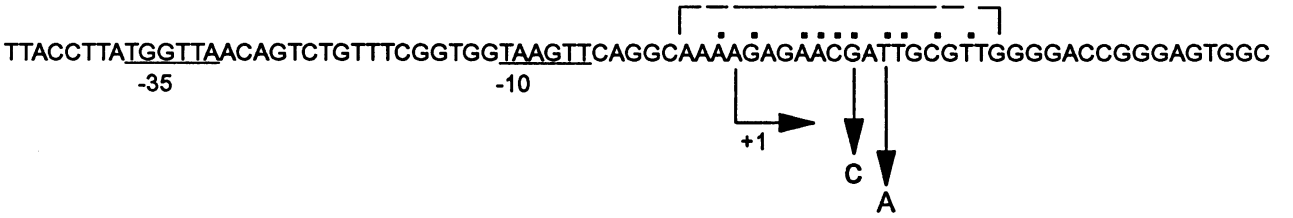

FIG. 4. Sequence of the *gcv* promoter region. The transcription start site for *gcvT* is indicated as +1 (29). *gcvT* is the first of three genes in the *gcv* operon (28). The −10 and −35 promoter sequence elements were determined previously (29) and are underlined. The region protected by PurR is indicated (⌜ and ⌝), and bases in that region that match the PurR consensus binding site 5′-aCGCAAACGTTTtCnT-3′ (lowercase letters indicate bases that are not 100% (conserved in all PurR binding sites, n is any base) (21) are indicated by small solid squares. Since the DNase I cleavage in this region was nonrandom in the unprotected lane (Fig. 3, lane 3), the endpoints of protection are not discernible and are represented by the broken line. The mutations introduced into the PurR binding site are indicated by arrows.

lacZ+8C+10A, was used to lysogenize the wild-type strain GS162 and the *purR* mutant strain GS852, generating strains GS162λ*gcvT-lacZ*+8C+10A and GS852λ*gcvT-lacZ*+8C+10A. β-Galactosidase synthesis in these strains is under the control of the mutated *gcv* regulatory region. Strains GS162 and GS852 were also lysogenized with λ*gcvT-lacZ* phage, in which β-galactosidase synthesis is under the control of a wild-type *gcv* regulatory region.

β-Galactosidase assays were performed on these strains grown in GM medium with no supplements, with glycine, or with inosine and glycine. In addition, since the uninduced levels of β-galactosidase activity are high enough to detect small variations, cultures were grown in inosine alone. This was not previously possible, since GCV enzyme activity is near the limits of detectability in uninduced cultures. The wild-type lysogen GS162λ*gcvT-lacZ* showed a fivefold increase in β-galactosidase activity when grown in the presence of glycine, the inducer of *gcv* (Table 3). The twofold repression seen in GCV enzyme levels when cells were grown in GM medium supplemented with glycine and inosine was also reflected in the β-galactosidase levels. This twofold repression was relieved in lysogen GS852λ*gcvT-lacZ*, indicating that repression of *gcvT-lacZ* is PurR dependent when both inosine and glycine are present in the growth medium. Lysogen GS162λ*gcvT-lacZ*+8C+10A also showed no repression of the glycine-induced levels when both inosine and glycine were present. Thus, either removal of the PurR protein or alteration of its binding site is sufficient to relieve this twofold repression. It should be noted that the altered bases in the mutant lysogens affected the promoter region of *gcvT-lacZ* and resulted in significantly reduced β-galactosidase levels compared with those of the wild-type lysogens under all four growth conditions.

TABLE 3. β-Galactosidase activities for lysogenized strains

Strain	β-Galactosidase activity[a] for cells grown in GM medium			
	No supplement	With glycine	With glycine + inosine	With inosine
GS162λ*gcvT-lacZ* (*purR*$^+$)	125	658	293	8
GS852λ*gcvT-lacZ* (*purR*)	100	616	650	7
GS162λ*gcvT-lacZ*+8C+10A (*purR*$^+$)	68	409	492	10
GS852λ*gcvT-lacZ*+8C+10A (*purR*)	69	339	339	9
GS973λ*gcvT-lacZ* (*gcvA*)	85	85	36	36
GS986λ*gcvT-lacZ* (*gcvA purR*)	94	100	88	88
GS973λ*gcvT-lacZ*[pGS266] (*gcvA*/*gcvA*$^+$)[b]	156	766	410	7

[a] In Miller units (11).

[b] This strain is a partial diploid: the chromosomal *gcvA* is inactive, and the plasmid copy is the wild-type gene.

To test whether removal of glycine results in greater repression of the uninduced *gcv* promoter by the PurR protein, we grew the lysogens in medium containing only inosine. In GS162λ*gcvT-lacZ*, β-galactosidase levels were decreased more than 10-fold compared with the levels in medium containing no supplements (Table 3). Surprisingly, however, the *purR* mutant GS852λ*gcvT-lacZ* and lysogens GS162λ*gcvT-lacZ*+8C+10A and GS852λ*gcvT-lacZ*+8C+10A still showed significant decreases in β-galactosidase levels, suggesting that another locus in addition to *purR* is involved in repression of *gcv* by purines.

GcvA-mediated purine repression. Since removal of glycine resulted in more severe purine-mediated repression of *gcv*, it was of interest to determine whether removing the activator for *gcv*, GcvA, would have the same effect. Strain GS973λ*gcvT-lacZ* (*gcvA1*) was grown in GM medium containing different supplements and assayed for β-galactosidase activity (Table 3). As previously reported, the *gcvA1* lysogen GS973λ*gcvT-lacZ* showed no induction of β-galactosidase in response to glycine (38). The twofold PurR-dependent repression of β-galactosidase levels was seen when the *gcvA1* strain was grown in the presence of glycine and inosine. However, when only inosine was included in the medium, the >10-fold repression seen in the wild-type strain was gone and only the twofold PurR-mediated effect on β-galactosidase levels was seen.

A *gcvA1 purR* double mutant was constructed and designated GS986λ*gcvT-lacZ*. This lysogen was grown in GM medium containing different supplements and assayed for β-galactosidase activity. GS986λ*gcvT-lacZ* showed no induction of β-galactosidase from the *gcvT-lacZ* fusion by glycine and no repression by purines. These results suggest that the twofold repression by inosine in GS973λ*gcvT-lacZ* was a PurR-mediated effect and that the activator for *gcv*, GcvA, was also necessary for repression of *gcv* when inosine, but not glycine, was present in the growth medium.

We tested this hypothesis by introducing the single-copy plasmid pGS266 containing a 1.5-kb insert which encodes a wild-type *gcvA* gene into lysogen GS973λ*gcvT-lacZ* (37). The wild-type *gcvA* gene should restore both activation by glycine and repression by purines of the λ*gcvT-lacZ* gene fusion. The transformed cells were grown in GM medium as described above and assayed for β-galactosidase activity. GS973λ*gcvT-lacZ*[pGS266] exhibited wild-type activation of *gcv* in the presence of glycine and wild-type repression of *gcv* in the presence of purines (Table 3).

DISCUSSION

It is becoming evident that the *E. coli gcv* operon is yet another example of a highly regulated bacterial gene system. Since it is believed that the GCV enzyme complex is important for balancing the cells' glycine and C_1 unit requirements, an integrated mechanism for sensing the levels of certain metabolic intermediates and end products would be expected for *gcv*.

The PurR repressor protein is involved in a twofold repression of *gcv* when cells are grown in medium containing inosine (Table 2). Since the C_1 unit from 5,10-methylenetetrahydrofolate is utilized in the formation of purine nucleotides (12), the need for additional C_1 units might be diminished when cells are supplied with excess purines. Both gel mobility shift assays and DNase I protection experiments indicate that repression of *gcv* by PurR is caused by direct interaction of the protein with the *gcv* regulatory region. This interaction occurs at approximately −2 to +15 of the *gcvT* promoter region, the first of three genes in the *gcv* operon (29). It appears that both the location of the PurR operator and its similarity to the PurR-binding consensus sequence are indicators of the level of repression exhibited by PurR on different operons (36). A consensus PurR-binding sequence at the −10 to −35 region of the *purF* gene has been shown to result in a 15-fold repression by PurR (19). The location of the PurR operator of *gcv* at −2 to +15 and the variation from the consensus sequence to only 10 of 16 bp probably results in the low level of PurR-mediated regulation of *gcv*.

Another locus necessary for purine-mediated regulation of *gcv* was identified in this study. It was found that a >10-fold repression of *gcv* that occurred in the presence of inosine but in the absence of glycine was caused by the GcvA protein, the *trans*-acting positive regulator for *gcv* (38). A mutation in *gcvA* prevents both activation of *gcv* by glycine and repression of *gcv* by inosine. Both phenotypes can be complemented in *trans* by a wild-type *gcvA* gene carried on the single-copy plasmid pGS266 (Table 3). Since the β-galactosidase levels from a λ*gcvT-lacZ* fusion in the *gcvA1 purR* double mutant GS986 are 10-fold higher than in the *purR* mutant GS852 when these mutants are grown in medium containing inosine alone, it appears that inosine does not simply prevent the ability of GcvA to induce *gcv*; rather, the GcvA protein plays an active role in repressing the *gcv* operon (Table 3). This inosine-mediated repression can be overcome by the addition of glycine.

Several other proteins have been shown to have the ability to both activate and repress gene expression. The *Saccharomyces cerevisiae* LEU3 protein (Leu3p) represses transcription of the leucine biosynthetic genes containing its binding site (UAS_{LEU}) in the absence of α-isopropylmalate. Upon addition of α-isopropylmalate, it is assumed that Leu3p undergoes a conformational change to an activator form and increases transcription from UAS_{LEU} (32). Many bacterial proteins are known to negatively autoregulate their own transcription as well as activate transcription from their target promoters. Other bacterial proteins, such as Lrp (7) and NR_1 (18), are known to activate at some promoters and repress at others. However, these examples differ from the dual role suggested for GcvA in that this single protein appears to respond differently to two different metabolites and presumably regulates gene expression from the *gcv* operator accordingly. It is not known whether GcvA acts alone in a direct role or whether another protein or cofactor interacts with GcvA in some manner. We are currently attempting to purify GcvA to elucidate the unusual mechanism by which GcvA accomplishes this apparent role as both an activator and repressor of *gcv*.

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