Genetic analysis of the GcvA binding site in the gcvA control region

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The GcvA protein both activates and represses the gcv operon and negatively regulates its own transcription. GcvA binds to three sites in the gcv control region and to one site in the gcvA control region; each of these binding sites contains the conserved 5 bp DNA sequence 5'-CTAAT-3'. This report describes the role this DNA sequence plays in autoregulation and expression of gcvA. Through single base-pair mutations, the importance of three of these five basepairs in the autoregulation of gcvA expression is shown. Two of the gcvA control region mutations described cause a gcvA::lacZ fusion to be overexpressed at 9–24 times the wild-type level. The increase in expression is due in part to a complete loss of autoregulation and in part to a GcvA-independent mechanism. One of the mutants was shown by Western blot analysis to increase the intracellular concentration of GcvA. This high level of gcvA expression subsequently causes the loss of purine-mediated repression of a gcvT::lacZ fusion. However, overexpression of gcvR re-established purine-mediated repression of the gcvT::lacZ fusion, supporting the model for gcv regulation that suggests the need for a relatively constant GcvA to GcvR ratio for appropriate regulation of gcv expression in response to glycine and purine availability.

Keywords: gcvA, GcvA, glycine cleavage, autoregulation, DNA binding site mutations

INTRODUCTION

The glycine cleavage (GCV) enzyme system in Escherichia coli catalyses the oxidative cleavage of glycine to CO₂ and NH₃, and the transfer a C-1 methylene unit to tetrahydrofolate (Kikuchi, 1973) (Fig. 1). The resulting 5,10-methylenetetrahydrofolate is used as a C-1 donor in the biosynthesis of purines, methionine, thymine and other cellular components (Mudd & Cantoni, 1964). In E. coli, three of the proteins of the GCV enzyme complex are encoded by the gcvTHP operon which maps at 62.5 min on the chromosome (Plamann et al., 1983; Stauffer et al., 1994). The fourth protein, encoded by lpd, is not a part of the gcv operon and maps at 2.5 min (Steiert et al., 1990).

Regulation of the gcv operon is complex and involves at least four proteins. PurR, a global regulator in E. coli involved in negative regulation of many purine and pyrimidine de novo biosynthesis genes (Kilstrup et al., 1989; Rolfes & Zalkin, 1988), also represses gcv expression twofold in the presence of exogenous purines. In vitro studies have shown that PurR binds the gcv control region at a site overlapping the transcriptional start site of gcvT, the first gene of the gcv operon, and presumably interferes with transcription initiation by RNA polymerase (Wilson et al., 1993a).

GcvR is also required for repression of gcv (Ghrist & Stauffer, 1995). Mutations in gcvR result in high constitutive expression of a gcvT::lacZ fusion and overexpression of gcvR results in superrepression of the fusion. Data suggest that repression is mediated through the GcvA protein (Ghrist & Stauffer, 1995). GcvR alone does not bind to the gcv promoter (A. Ghrist & G. Stauffer, unpublished data) and whether it is directly or indirectly involved in the repression mechanism has not been determined.

The leucine-responsive regulatory protein (Lrp) is another global regulator in E. coli and has been shown to activate or repress many genes involved in amino acid metabolism (Calvo & Matthews, 1994; Newman et al., 1992). An lrp mutant containing a gcvT::lacZ fusion shows low, uninducible β-galactosidase synthesis (Lin et
al., 1992; Stauffer & Stauffer, 1994). In vitro studies identified multiple Lrp binding sites in the gcv control region from base −229 to −92 relative to the transcription initiation site (Stauffer et al., 1995). Lrp appears to play primarily a structural role in gcv regulation, bending the DNA (Stauffer & Stauffer, 1999).

GcvA, a member of the LysR family of transcriptional regulators (Schell, 1993), plays a dual role in gcv regulation by mediating a fivefold, PurR-independent repression of gcv expression in the presence of purines and a six- to sevenfold activation in the presence of glycine (Wilson et al., 1993a, b). GcvA binds to three sites in the gcv control region from base −271 to −242 (site 3), from −242 to −214 (site 2) and from −69 to −34 (site 1) relative to the transcription initiation site (Wilson et al., 1995). All three sites are required for GcvA-mediated repression, but only the upstream sites 2 and 3 are required for GcvA-mediated activation (Wilson et al., 1995). GcvA also binds to a site in the gcvA control region from base −28 to +20 relative to the gcvA transcription initiation site, negatively regulating its own expression over an approximately threefold range (Wilson & Stauffer, 1994). A comparison of these four GcvA binding sites revealed a conserved 5 bp DNA sequence, 5′-CTAAT-3′, shown to be important for GcvA binding (Wilson et al., 1995) (Fig. 2a).

Overexpression of gcvA results in constitutive expression of a gcv T::lacZ fusion in the absence of exogenous glycine and in the presence of exogenous purines (Ghrist & Stauffer, 1995), and thus it appears critical that GcvA levels are maintained within a narrow range for the cell to respond appropriately to a glycine-inducing or purine-repressing signal. A model was previously proposed to explain how GcvA and GcvR might interact to regulate gcv expression, where GcvA homocomplexes function as activators and GcvA-GcvR heterocomplexes function as repressors (Ghrist & Stauffer, 1995; Wilson & Stauffer, 1994). This model proposes that the amount of each type of complex formed is likely to be due to the level of the possible coregulators (glycine and purines). High glycine levels would favour activator formation and high purine levels would favour repressor formation. This model also predicts that artificially raising the levels of either GcvA or GcvR would favour the formation of either activator or repressor complexes, respectively.

To define domains of the GcvA protein involved in activation and repression, a genetic selection was designed to screen for mutations in gcvA that produce proteins able to activate but unable to repress the gcv operon. One of the mutations isolated, however, was in the gcvA control region rather than in the structural gene and altered gcvA expression and subsequently gcv T::lacZ expression. The results are consistent with the model proposed above to explain how GcvA is involved in accomplishing activation and repression of the gcv operon based on GcvA being maintained at a relatively constant ratio with the GcvR protein.

**METHODS**

**Strains and plasmids.** The genotypes of strains and plasmids used in this study are listed in Table 1. All are laboratory strains or were constructed during this investigation.

**Media.** The minimal growth medium used was Vogel & Bonner (1956) minimal salts supplemented with 0.4% glucose (GM) or lactose (LM) and appropriate amino acids and antibiotics as needed. The complex medium used was Luria–Bertani broth (LB) (Miller, 1992). Supplements were added at the following concentrations: phenylalanine, 50 µg ml⁻¹; phenylethyl-D-thiogalactoside (TPEG), 2 mM; inosine, 50 µg ml⁻¹; glycine, 300 µg ml⁻¹; vitamin B₁, 1 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; ampicillin, 50 µg ml⁻¹ for single-copy plasmids and 100 µg ml⁻¹ for multicopy plasmids. Minimal medium was supplemented with phenylalanine and vitamin B₁ because all of the strains used in this study contain pheA905 and thi mutations.

**Enzyme assays.** β-Galactosidase assays were performed as described by Miller (1992). All results are the means of two or more assays, with each sample done in triplicate. Protein concentrations were determined by the method of Lowry.

**DNA manipulation.** Isolation of plasmid DNA, restriction digests, ligations, DNA sequencing and plasmid transformations were performed as described by Sambrook et al. (1989).

**PCR mutagenesis and site-directed mutagenesis.** Random mutagenesis of gcvA was performed using the PCR mutagenesis protocol (Zhou et al., 1991). The upstream primer was complementary to the DNA template upstream of the gcvA gene (which retains its own promoter) and a unique EcoRI site, and the downstream primer was complementary to the DNA template distal to the gcvA gene and a unique HindIII site. Several independently mutagenized PCR products were
Table 1. *E. coli* K-12 strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype*</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS162†</td>
<td>wt</td>
<td>This laboratory</td>
</tr>
<tr>
<td>GS973†</td>
<td><em>gcvA</em>::lacZ</td>
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</tr>
<tr>
<td>GS986†</td>
<td><em>gcvA</em> purR::Tn10 <em>gcvT</em>::lacZ</td>
<td>This laboratory</td>
</tr>
<tr>
<td>GS998†</td>
<td><em>gcvA</em></td>
<td>This laboratory</td>
</tr>
<tr>
<td>GS1039†</td>
<td><em>gcvA</em> serA25 purR::Tn10 <em>gcvT</em>::lacZ</td>
<td>This laboratory</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMC1403</td>
<td>lac fusion vector</td>
<td>Casadaban et al. (1980)</td>
</tr>
<tr>
<td>pGS272</td>
<td>pACYC177 derivative, <em>Smal</em> site converted to EcoRI site in <em>kan</em> gene, Kn Ap'</td>
<td>This laboratory</td>
</tr>
<tr>
<td>pGS311</td>
<td>Single-copy plasmid, Ap', derived from plasmid pDF41†</td>
<td>This laboratory</td>
</tr>
<tr>
<td>pGS334</td>
<td>1 kb EcoRI–HindIII <em>gcvR</em> fragment in pBR322, Ap' Tc'</td>
<td>This laboratory</td>
</tr>
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<td>pGS335</td>
<td>1.3 kb EcoRI–HindIII <em>gcvA</em> fragment in pGS272, Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pGS341</td>
<td>1.3 kb EcoRI–HindIII <em>gcvA</em> fragment from pGS335 in pGS311, Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pGS383</td>
<td><em>EcoRI</em> to <em>SacI</em> deletion in pGS334, Ap' Tc'</td>
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</tr>
<tr>
<td>pGS468</td>
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<td>This study</td>
</tr>
<tr>
<td>pGS469</td>
<td>1.3 kb EcoRI–HindIII <em>gcvA</em> + 13G fragment (A to G base change at +15) in pGS311, Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pGS470</td>
<td>1.3 kb EcoRI–HindIII <em>gcvA</em> + 16A fragment (T to A base change at +16) in pGS311, Ap'</td>
<td>This study</td>
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</table>

*Ap, ampicillin; Kn, kanamycin; Tc, tetracycline.† These strains also carry thi, pheA905, *lacU169*, *araD129* and *rpsL150* mutations.‡ Plasmid pDF41 (Kahn et al., 1979) was obtained from S. R. Kushner, University of Georgia, USA.

*GcvA* binding site for *gcvA*

Collected, digested with *EcoRI* and *HindIII*, purified from a low-melting-point agarose gel, ligated into the *EcoRI*–*HindIII* sites of the single-copy plasmid pGS311 and transformed into the *gcvA1* strain GS1039 lysogenized with an *gcvT*::*lacZ* translational fusion phage.

Site-directed mutagenesis of *gcvA* was performed using a PCR 'megaprimer' mutagenesis protocol (Sarkar & Sommer, 1990). Changes were introduced through downstream primers complementary to *gcvA* except at the position of the desired base change. PCR products were generated using a primer upstream of a unique *EcoRI* site and complementary to vector DNA outside of the *gcvA* insert. These PCR products were then used as the upstream megaprimers in another round of PCR synthesis against the same DNA template. The downstream primer was complementary to an internal segment of *gcvA* distal to the mutagenic primer and an internal *MluI* site. PCR products were cut with *EcoRI* and *MluI*, purified from a low-melting-point agarase gel and ligated into *EcoRI*–*MluI*-digested plasmid pGS341. The resulting plasmids (pGS468, pGS470 and pGS471) each contained specific base mutations that were verified by the complete DNA sequence analysis of each *EcoRI*–*MluI* fragment.

Constructions of lysogens. Plasmids pGS468, pGS469 and pGS470 were digested with *EcoRI* and *EcoRV* and the 519 bp fragment from each digest, containing the 303 bp region upstream of *gcvA* and the first 72 codons of *gcvA*, were isolated from a low-melting-point agarase gel and ligated into the *EcoRI*–*Smal* sites of plasmid pMC1403 (Casadaban et al., 1980), forming an in-frame translational fusion to *lacZ*. The resulting plasmids were sequenced across the fusion junction, confirming that the correct reading frame had been maintained. Each plasmid was cut at a unique *SalI* site at the end of the *lacZYA* segment, the ends filled in using T4 DNA polymerase and *EcoRI* linkers added using T4 DNA ligase. The plasmids were then digested with *EcoRI* and a 668 bp *EcoRI*–*EcoRI* fragment, containing each mutant *gcvA* control region, the first 72 codons of *gcvA* and *lacZYA*, was isolated and cloned into the *EcoRI* site of pGS272 (Panasenko et al., 1977). In the resulting phages β-galactosidase synthesis is under control of the *gcvA* control region. The phages were used to lysogenize appropriate strains as described previously (Urbanowski & Stauffer, 1986). The lysogens were tested for the presence of a single copy of λ phage by infection with λ2190e17 (Shimada et al., 1972). The effect of each of the mutations in the *gcvA* control region on *gcvA* expression was measured by β-galactosidase assay. As controls, a wild-type (wt) *gcvA*::*lacZ* fusion constructed previously in an identical manner (Wilson & Stauffer, 1994) was used to lysogenize appropriate strains and β-galactosidase activity was measured.

Gel mobility shift assays. Gel mobility shift (GMS) assays were performed based on the methods of Fried & Crothers (1981) and Garner & Revzin (1981). A 519 bp *EcoRI*–*BamHI* fragment from each of the *gcvA*::*lacZ* fusion plasmids, containing 303 bp upstream of the *gcvA* transcription start site and the first 72 codons of *gcvA*, was 32P-labelled at the unique *EcoRI* site using T4 polynucleotide kinase (Sambrook et al., 1989).
Western blot analysis. Cells were grown in 1 ml GM plus ampicillin (50 µg ml⁻¹) overnight at 30 °C, then diluted into 50 ml GM plus ampicillin and incubated at 30 °C until mid-exponential phase (OD₆₀₀ ~ 0.5). Cells were collected at 4 °C by centrifugation and the cell pellet was frozen overnight at −70 °C. The cell pellet was resuspended in 2 ml sodium phosphate buffer (50 mM sodium phosphate, 500 mM NaCl) and sonicated on ice until viscous and clear. Cytoplasmic and membrane fractions were separated by centrifugation and the supernatant was brought to 33% ammonium sulfate saturation. Precipitated protein was collected by centrifugation and resuspended in 120 µl TEG buffer (50 mM Tris/HCl, pH 7.9, 0.5 mM EDTA, 5% glycerol). Protein concentration was determined using the Bio-Rad Protein Assay Kit according to the manufacturer’s instructions, followed by reaction with secondary antibody [alkaline-phosphatase-conjugated polyclonal sheep anti-mouse IgG (H+L); ICN Biomedicals] diluted according to the manufacturer’s directions. Colorimetric detection was done according to the Qiangen protocol for Western blot immunodetection.

RESULTS AND DISCUSSION

Isolation of a gcvA mutant affecting purine repression of gcv

It was shown previously that GcvA functions to both activate and repress a gcvT::lacZ fusion (Wilson et al., 1993a, b). To define amino acids in GcvA important for its repressor function, we attempted to isolate gcvA mutants that encoded proteins that could no longer repress gcvT::lacZ expression in the presence of purines but could activate expression in the presence of glycine. The following selection strategy was used to obtain such gcvA mutants. Strain GS1039 is a serA gcvA double mutant that requires serine for growth. This double mutant cannot grow on a GM plate supplemented with only glycine because in the absence of GcvA there are insufficient C-1 units available from the GCV enzyme system for the conversion of glycine into serine via the serine hydroxymethyltransferase reaction (Fig. 1). If wt gcvA is supplied in trans, growth on a GM plate supplemented with glycine occurs because GcvA, in the presence of glycine, activates expression of the GCV enzyme system resulting in cleavage of glycine and the production of C-1 units. Although strain GS1039 carries a gcvT::lacZ translational gene fusion on an integrated λ phage, when wt gcvA is supplied in trans this lysogen cannot grow on a GM plate supplemented with serine, inosine and TPEG (a competitive inhibitor of β-galactosidase; Miller, 1992) because GcvA, in the presence of inosine, represses the fusion resulting in insufficient β-galactosidase expression to support growth on lactose. The presence of TPEG increases the stringency of the selective media. Lysogen GS1039 was transformed with a single-copy plasmid pool carrying PCR-induced random base changes in gcvA (including the promoter region) and plated on LB agar (appropriate antibiotics were added to all selection media). Colonies were patched to GM plates supplemented with serine, inosine and TPEG, and to GM plates supplemented with glycerol. Our selection assumed that cells containing mutations in gcvA that alter the repressor function but not the activator function of GcvA would grow on both of the scoring media. One of about 1000 transformants tested was capable of growth on both of the scoring media. The transformant was single-colony-purified and the plasmid carrying the putative gcvA repressor mutation was isolated.

To determine the effects of the gcvA repressor mutation on gcvT::lacZ expression, the plasmid carrying the putative gcvA mutation was used to transform the gcvA1 mutant strain GS986 which was lysogenized with a gcvT::lacZ fusion. Repression of the gcvT::lacZ fusion in strain GS986 is mediated through the plasmid-borne gcvA-encoded protein and not PurR. As a control, lysogen GS986 was also transformed with plasmid pGS341 which carries the wt gcvA gene. The transformants were grown in GM medium or GM medium supplemented with either glycine or inosine and assayed for β-galactosidase activity. GS986 transformed with the plasmid carrying the repressor mutation in gcvA displayed normal induction of gcvT::lacZ expression when grown in the presence of glycine, but in the presence of inosine there was an approximately twofold increase in gcvT::lacZ expression compared with that of the control strain GS986(pGS341) (data not shown). These results suggest that the plasmid carries the relevant gcvA repressor mutation allowing growth on the scoring plates.

The mutant gcvA gene from the plasmid was sequenced and a single base change from A to G at position +15 relative to the transcriptional start site was identified and designated +15G (Fig. 2b). This A to G transition occurs within the conserved 5 bp 5'-CTAAT-3' sequence common to all four known GcvA binding sites and is believed to be involved in binding of GcvA to DNA (Wilson et al., 1995) (Fig. 2). The plasmid carrying the +15G gcvA mutation was designated pGS469.

The +15G mutation in the GcvA binding site causes overexpression of gcv::lacZ

Overexpression of wt gcvA from the tac promoter results in a relief of purine-mediated repression of a gcvT::lacZ fusion (Ghrist & Stauffer, 1995). Because the +15G mutation also resulted in a relief of purine-mediated repression of a gcvT::lacZ fusion and lies in the putative autoregulatory GcvA consensus binding site, it was hypothesized that this mutant might over-
express GcvA. To examine this possibility, a lacZ translational fusion was constructed where β-galactosidase is under control of the +15G mutant control region (see Methods). The fusion was cloned into λgt2 and the phage designated λgcvA::lacZ+15G. Phage λgcvA::lacZ+15G was then used to lysogenize the wt strain GS162 and the gcvA1 mutant strain GS998. As controls, a wt λgcvA::lacZ phage was used to lysogenize the same strains. The lysogens were grown in GM medium and assayed for β-galactosidase activity. As reported previously (Wilson & Stauffer, 1994), expression of the wt λgcvA::lacZ fusion is autoregulated about threefold in the wt strain GS162 compared to the gcvA1 strain GS998 (Table 2). However, the λgcvA::lacZ+15G fusion did not show a significant difference in β-galactosidase levels in the wt strain compared to the gcvA1 strain (Table 2), suggesting that the +15G mutation prevents GcvA from autoregulating λgcvA::lacZ +15G. Furthermore, in the gcvA1 strain GS998, β-galactosidase synthesis from the λgcvA::lacZ +15G fusion increased about 3.9-fold compared to the wt fusion, indicating that the +15G mutation caused an additional GcvA-independent increase in gcvA::lacZ expression.

### Table 2. Effect of gcvA control region mutations on gcvA::lacZ expression

<table>
<thead>
<tr>
<th>gcvA::lacZ fusion*</th>
<th>β-Galactosidase activity (Miller units)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>GS162 (wt)</td>
</tr>
<tr>
<td>wt</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>+ 13A</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>+ 15G</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>+ 16A</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

* Denotes the λgcvA::lacZ wt and mutant phages used to lysogenize strains GS998 and GS162.

### Effects of additional base changes in the GcvA binding site on gcvA::lacZ expression

To determine if other bases in the 5′-CTAAT-3′ sequence of the GcvA binding site are important in autoregulation at the gcvA promoter, both bases +13 and +16 were each independently changed from T to A using the megaprimer PCR mutagenesis protocol (see Methods) (Fig. 2b). Each base change was translationally fused to the lacZYA reporter genes such that expression of these genes is under control of the gcvA promoter regions containing the altered GcvA binding sites. The fusions were cloned into λgt2 and the resulting phages, designated λgcvA::lacZ+13A and λgcvA::lacZ+16A, were used to lysogenize wt strain GS162 and the gcvA1 mutant strain GS998. The lysogens were grown in GM medium and assayed for β-galactosidase activity. The λgcvA::lacZ+13A and λgcvA::lacZ+16A fusions showed no significant difference in β-galactosidase levels in the wt strain GS162 compared to the gcvA1 strain GS998 (Table 2), suggesting the +13A base change prevented GcvA-mediated autoregulation, similar to the +15G mutation. In addition, in strain GS998 the +13A base change resulted in a 7.8-fold GcvA-independent increase in gcvA::lacZ expression. The +16A mutation in the gcvA control region also resulted in the loss of autoregulation of gcvA::lacZ expression similar to the +13A and +15G mutants (Table 2). However, unlike the +13A and +15G base changes, the +16A mutation showed a GcvA-independent decrease in gcvA::lacZ expression (Table 2). Why expression of gcvA::lacZ increased with the +13A and +15G base changes is unknown, although there are several possible explanations. These changes may have increased the strength of the gcvA promoter, thus increasing transcription initiation. Although changes in the initially transcribed...
region are known to affect promoter strength (Record et al., 1996), it should be noted that the changes reported here are a considerable distance from the +1 transcription initiation site. These changes may have generated a new promoter that is stronger than the wt promoter. However, the base changes do not create a recognizable promoter consensus sequence. Changing these bases may have altered the efficiency at which the mRNA is translated, or may have increased the stability of the mRNA. Finally, there could be an additional E. coli protein that regulates gcvA expression, or a protein that normally does not recognize the gcvA control region DNA but, when this DNA is mutated, now increases gcvA expression. We are developing in vitro assays and in vivo selections that should allow us to determine which of the above possible scenarios is most likely responsible for the altered gcvA expression in these mutants.

GcvA binding to the mutant gcvA control regions

GMS assays were performed to determine if the loss of autoregulation observed by changing bases in the GcvA binding site were due to an altered binding affinity of GcvA for DNA (Fig. 3). Purified GcvA protein used in all GMS assays is given in monomer concentrations (Jourdan & Stauffer, 1995). At the lower concentrations of protein used in the GMS assay the wt gcvA fragment was shifted to two bands of slower mobility (Fig. 3a, lanes 2–3; Fig. 3b, lanes 14–16; complexes A and B). At the higher concentrations of protein used the fragment was shifted to multiple bands of slower mobility (Fig. 3a, lanes 4–6; Fig. 3b, lanes 17–18). It should be noted that complex B is the predominant band at any GcvA concentration used in the assay. Previous results from a DNase I footprint analysis of gcvA identified a single target site of about 48 bp (Fig. 2) (Wilson et al., 1995). We believe that complex A and B indicate that the binding region is made up of two target sites and that GcvA binding to these two sites is cooperative. However, it is also possible that the two sites have essentially equal affinity for gcvA. The additional bands of slower mobility at higher concentrations of GcvA used in the assay are likely to be due to non-specific binding, as DNase I footprint assays did not show protection other than the GcvA binding region previously identified (Fig. 2) (data not shown). The +16A fragment had a GMS pattern essentially the same as the wt (Fig. 3a, compare lanes 7–12 with lanes 1–6). The amount of GcvA protein required to bind and shift the +13A and +15G gcvA

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**Fig. 3.** Gel mobility shift assay for the binding of GcvA to wt and mutant gcvA DNAs. The 519 bp fragments containing the wt and mutant gcvA control regions were incubated with GcvA protein and the samples were run on a 5% polyacrylamide/3% glycine gel (see Methods). Each reaction was performed in a 20 μl volume. (a) Lanes: 1–6, wt DNA; 7–12, gcvA +16A DNA; 1 and 7, no protein; 2–6 and 7–12, 5, 10, 20, 40 and 80 nM GcvA protein, respectively. (b) Lanes: 1–6, gcvA +13A DNA; 7–12, gcvA +15G DNA; 13–18, wt gcvA DNA; 1, 7 and 13, no protein; 2–6, 8–12 and 14–18, 5, 10, 20, 40 and 80 nM GcvA protein, respectively. The unbound DNA fragments are indicated. Complexes A and B are likely to represent GcvA bound to one half and the full 48 bp GcvA target sequence, respectively.
Table 3. Effects of varying gcvA expression on gcvT::lacZ expression

All plasmids were assayed in strain GS986. Cells were grown in GM medium with the indicated supplements and assayed for β-galactosidase activity.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>gcvA allele</th>
<th>β-Galactosidase activity (Miller units)</th>
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<td></td>
<td>None</td>
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<tr>
<td>pGS341</td>
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<tr>
<td>pGS468</td>
<td>gcvA + 13A</td>
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<td>pGS469</td>
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<tr>
<td>pGS470</td>
<td>gcvA + 16A</td>
<td>187 ± 11</td>
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</table>

Modifying the level of gcvA expression affects gcv regulation

Previous studies have shown that regulation of a gcvT::lacZ fusion is sensitive to the level of GcvA in the cell (Ghrist & Stauffer, 1995). Overexpression of gcvA leads to the loss of purine-mediated repression and constitutive expression of a gcvT::lacZ fusion in the absence of exogenously added glycine (Ghrist & Stauffer, 1995). Each of the mutants described above had either increased or decreased gcvA::lacZ expression. To determine the effects the altered levels of gcvA expression had on gcvT::lacZ expression, plasmids were constructed in which each mutant control region was translationally fused upstream of the gcvA structural gene (see Methods). The plasmids were then transformed into the gcvA1 purR::Tn10 mutant strain GS986 that was lysogenized with a gcvT::lacZ fusion. The transformants were grown in GM medium and GM medium supplemented with either glycine or inosine and assayed for β-galactosidase activity. The wt control transformant GS986(pGS341) showed a 4.7-fold glycine-mediated induction and a 6-fold purine-mediated repression of the gcvT::lacZ fusion (Table 3). Transformant GS986(pGS468), carrying the gcvA +15G mutation which caused an 8.7-fold overexpression of the gcvA::lacZ fusion (Table 2), resulted in a 2-fold loss of GcvA-mediated repression of the gcvT::lacZ fusion when grown in the presence of inosine (Table 3). The +15G base change had no significant effect on gcvT::lacZ expression when the lysogen was grown in GM medium or GM medium supplemented with glycine. Transformant GS986(pGS468), carrying the gcvA +13A mutation which caused a 22-fold overexpression of the gcvA::lacZ fusion (Table 2), resulted in constitutive expression of the gcvT::lacZ fusion under all growth conditions (Table 3). These results suggest that the ability of the gcvT::lacZ fusion to be regulated in response to normal physiological signals for activation and repression was altered by increased levels of GcvA. Transformant GS986(pGS470), carrying the gcvA +16A mutation which had no significant effect on levels of gcvA::lacZ expression (Table 2), showed essentially normal gcvT::lacZ expression compared to the wt gcvT::lacZ fusion (Table 3).

The +13A mutant control region increases intracellular levels of GcvA

We tested directly whether the +13A base change in the gcvA control region that caused gcvA::lacZ expression to increase 22-fold resulted in overproduction of the GcvA protein by Western blot analysis. The Tetra-His Antibody (Qiagen) reacts with four histidine residues present in a histidine-tagged protein. A sequence encoding six histidine residues (His₁₆) was added to the 3' end of the gcvA gene (gcvA::His₁₆) (Jourdan & Stauffer, 1998). Strain GS986 transformed with single-copy plasmids carrying the wt control region upstream of gcvA::His₁₆, the +13A control region upstream of gcvA::His₁₆, and the vector only were grown as described, protein prepared and used in Western blot analysis (see Methods). The vector only protein preparation did not react with the Tetra-His Antibody at any
of the protein concentrations used (Fig. 4, lanes 1–3). The GcvA–His$_a$ protein preparation under control of its native promoter gave a weak signal that increased as the protein concentration was increased (Fig. 4, lanes 7–9). The low level of GcvA–His$_a$ detected corresponds well with the low level of wt gcvA::lacZ expression in a GS162 wt strain (Table 2). The GcvA–His$_a$ protein preparation under control of the +13A control region displayed a strong reaction that increased as the amount of total protein increased (Fig. 4, lanes 4–6). The Western analysis agrees well with the gcvA::lacZ +13A expression data (Table 2). Since it was shown previously that overexpression of gcvA from the tac promoter causes a loss of gcvT::lacZ repression (Ghrist & Stauffer, 1995), the overproduction of the GcvA protein in the +13A mutant could account for the loss of gcvT::lacZ repression observed in cells containing plasmid pGS468 (Table 3).

**Overexpression of GcvR in mutant gcvA strains mediates the return of gcvT::lacZ regulation**

Overexpression of gcvR results in superrepression of a gcvT::lacZ fusion and this repression is dependent on a functional gcvA gene (Ghrist & Stauffer, 1995). We hypothesized that if the signal for activation and repression of a gcvT::lacZ fusion is dependent upon the ratio of GcvA to GcvR in the cell, then the altered regulation of the gcvT::lacZ fusion observed with the gcvA +13A and gcvA +15G mutations (Table 3) would be eliminated by overexpressing gcvR in the mutant gcvA backgrounds. To examine this hypothesis, strain GS973, a gcvA1 mutant carrying a $^{4}$gcvT::lacZ fusion, was transformed with plasmids pGS468 and pGS469 carrying the gcvA +13A and gcvA +15G mutations, respectively. These strains were then transformed with plasmid pGS383, a pBR322 derivative carrying wt gcvR, to produce two strains containing plasmids that overproduce both GcvA and GcvR. Two control strains were also constructed by transforming GS973 with plasmid pGS341, the single-copy plasmid carrying wt gcvA, and by transforming GS973 with both plasmids pGS341 and pGS383. The GS973(pGS341) transfectant contained one plasmid-borne copy of gcvA and a chromosomal copy of gcvR. The GS973(pGS341)-(pGS383) transformant contained one plasmid-borne copy of gcvA and multiple copies of gcvR. These strains were grown in GM medium and GM medium supplemented with either glycine or inosine and assayed for $^{4}$-galactosidase activity. The control strain GS973(pGS341) showed normal glycine-mediated activation and purine-mediated repression (Table 4). When both the single-copy gcvA plasmid pGS341 and the multicopy gcvR plasmid pGS383 were transformed into GS973, superrepression occurred under all growth conditions (Table 4). In the GS973(pGS469)(pGS383) double transformant, where gcvA was overexpressed about 87-fold and gcvR was overexpressed about 30-fold (based on the copy number of the vector (Bolivar et al., 1977) and the observation that gcvR is constitutively expressed (Ghrist & Stauffer, 1995)), superrepression of gcvT::lacZ occurred under all growth conditions (Table 4). In the GS973(pGS468)(pGS383) double transformant, where gcvA was overexpressed about 22-fold and gcvR was overexpressed about 30-fold, the level of gcvT::lacZ expression was still repressed under all growth conditions. However, there was a significant increase in $^{4}$-galactosidase activity in this transformant compared to the strain carrying a single copy of gcvA, or in the strain where gcvA was overexpressed only 87-fold. In addition, this transformant could mediate a 62-fold activation of gcvT::lacZ expression in the presence of glycine (Table 4), although the induced levels were 37-fold lower than the control strain with a single copy

**Table 4. Effects of overexpressing gcvR on gcvT::lacZ expression in cells overproducing gcvA**

All plasmids were assayed in strain GS973. Cells were grown in GM medium with the indicated supplements and assayed for $^{4}$-galactosidase activity.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relative levels of GcvA and GcvR</th>
<th>$^{4}$-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>pGS341</td>
<td>A', R'</td>
<td>163 ± 28</td>
</tr>
<tr>
<td>pGS341 + pGS383</td>
<td>A', R'</td>
<td>7 ± 0</td>
</tr>
<tr>
<td>pGS468 + pGS383</td>
<td>A', R'</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>pGS469 + pGS383</td>
<td>A', R'</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>
of gcvA and gcvR. These results indicate that the loss of gcvC:: LacZ repression caused by overexpression of gcvA can be overcome by proportionally overexpressing gcvR and suggest that the proper ratio of GcvA and GcvR, rather than their absolute levels, is critical for normal regulation of the gcv operon. We have designed a genetic screen to isolate mutations in the coding region of gcvA that would render the GcvA protein either unable to repress gcv in the presence of inosine or, alternatively, unable to activate gcv in the presence of glycine. One gcvA mutant isolated encodes a protein that no longer responds to GcvR for repression (A. D. Jourdan & G. V. Stauffer, unpublished data), supporting a model where GcvR and GcvA might interact.

Careful analysis of this and additional mutants will provide insight into the mechanisms by which GcvA and GcvR are involved in activating and repressing the gcv operon.

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REFERENCES


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