GcvA binding site 1 in the gcvTHP promoter of Escherichia coli is required for GcvA-mediated repression but not for GcvA-mediated activation

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GcvA binds to three sites in the gcvTHP control region, from base −34 to −69 (site 1), from base −214 to −241 (site 2) and from base −242 to −271 (site 3). Previous results suggested that sites 3 and 2 are required for both GcvA-dependent activation and repression of a gcvT::lacZ fusion. However, the results were less clear as to the role of site 1. To determine the role of site 1 in regulation, single and multiple base changes were made in site 1 and tested for their ability to alter GcvA-mediated activation and GcvA/GcvR-mediated repression. Several of the mutants were also tested for effects on GcvA binding to site 1 and the ability of GcvA to bend DNA at site 1. The results are consistent with site 1 playing primarily a role in negative regulation of the gcvTHP operon.

Keywords: gcvTHP, gcvT::lacZ, GcvA, GcvR, repression

INTRODUCTION

The Escherichia coli glycine cleavage (GCV) enzyme system catalyses the cleavage of glycine to CO$_2$, NH$_3$, and a one-carbon (C1) methylene unit that is transferred to tetrahydrofolate (Kikuchi, 1973). The resulting 5,10-methylenetetrahydrofolate is required as a donor of methyl groups in many biosynthetic reactions and for the methylation of DNA and proteins (Marinus, 1996; Mudd & Cantoni, 1964). Strains defective in the GCV reaction excrete glycine (Plamann et al., 1983), and strains that overexpress the GCV enzyme system are partial glycine auxotrophs (L. Stauffer, unpublished results), suggesting that the GCV reaction balances the levels of glycine and C1 units produced according to the needs of the cell. Four enzymes are required for the GCV reaction. Three are encoded by the gcvTHP operon located at 62–5 min on the E. coli map (Plamann et al., 1983; Stauffer et al., 1994) and the fourth enzyme is encoded by the lpd gene at 2–5 min (Steiert et al., 1990).

Regulation of the gcvTHP operon is complex, involving both global-acting and gcv-specific regulatory proteins (Fig. 1). Lrp, a global regulator involved in regulation of amino acid metabolic pathways (Calvo & Matthews, 1994), is required for expression of a gcvT::lacZ fusion (Lin et al., 1992; Stauffer & Stauffer, 1994). Lrp binds to and protects from DNase I digestion a large region of DNA upstream of the gcv promoter from about base −92 to −229 (Stauffer & Stauffer, 1994). In addition, Lrp binding to this region results in a sharp bend in the gcv DNA (Stauffer & Stauffer, 1998a). Its primary role may be structural, facilitating formation of stereospecific nucleoprotein complexes required to either activate or repress the gcv operon (Stauffer & Stauffer, 1999).

PurR, a repressor of several operons involved in nucleotide metabolism (Meng & Nygaard, 1990; Rolles & Zalkin, 1988), represses gcvT::lacZ expression two-fold when cells are grown in the presence of purines (Stauffer & Stauffer, 1994; Wilson et al., 1993a). PurR binds to a site from about base −3 to +17 relative to the transcription initiation site, and possibly interferes with RNA polymerase (RNAP) binding to the gcv promoter (Wilson et al., 1993a).

The cAMP receptor protein (CRP) has recently been shown to play a positive role in controlling gcvT::lacZ expression (Wonderling & Stauffer, 1999). CRP binds to a site from about base −324 to −303, and although its specific mechanism for regulating gcvT::lacZ ex-

Abbreviations: CRP, cyclic AMP receptor protein; GCV, glycine cleavage; RNAP, RNA polymerase; WT, wild-type.
expression has not yet been identified, CRP likely interferes with repression by the GcvA protein.

The GcvA protein is a member of the LysR family of activators that share amino acid homology and domain structure (Schell, 1993). GcvA binds to three sites in the gcv control region, from base −34 to −69 (site 1), from base −214 to −241 (site 2) and from base −242 to −271 (site 3) (Fig. 1) (Wilson et al., 1995). GcvA acts as an activator of gcv expression when glycine is present in the growth medium, causing a six- to sevenfold induction (Wilson et al., 1993b). GcvA also represses the gcv operon over a fivefold range when the purine inosine is added to the growth medium (Wilson et al., 1993a). In the presence of both glycine and purines, the activator function of GcvA is dominant (Stauffer & Stauffer, 1994). Activation and repression by GcvA requires Lrp, presumably to bend the DNA appropriately (Stauffer & Stauffer, 1998a, b, 1999). Mutations in GcvA have been isolated that affect either the activation or repression function without altering its DNA-binding capabilities (Jourdan & Stauffer, 1998), suggesting that these two activities lie in separate functional regions.

The GcvR protein is required for repression by GcvA (Ghrist & Stauffer, 1995). A Tn10 insertion in the gcvR gene causes high constitutive levels of gcvT::lacZ expression due to the loss of GcvA-mediated repression, and overexpression of gcvR leads to superrepression of the fusion that also requires the GcvA protein (Ghrist & Stauffer, 1995). Preliminary evidence suggests that GcvR interacts directly with GcvA rather than binding gcv DNA (A. Ghrist, unpublished results), but the mechanism by which GcvR causes repression is unknown.

Previous studies suggested that all three GcvA binding sites are required for repression by GcvA, but only sites 2 and 3 are necessary for activation (Wilson et al., 1995). However, interpretation of the role of site 1 was complicated because the single site 1 allele examined only reduced GcvA binding to site 1 about twofold, and also resulted in a twofold promoter-down phenotype. A prediction based on these early studies is that mutations in site 1 that prevent GcvA binding should affect GcvA-mediated repression without interfering with GcvA-mediated activation. Such mutations would be useful in understanding the mechanism of the GcvA/GcvR-mediated repression system. In this study, mutations were created in GcvA binding site 1 to determine which nucleotides are necessary for GcvA binding and the effects of these mutations on GcvA-mediated repression and activation. The results are consistent with the previous hypothesis that GcvA site 1 is primarily involved in GcvA-mediated repression. The results also suggest that GcvA has different requirements for binding at sites 2 and 3 compared to site 1.

METHODS

Strains and plasmids. The E. coli K-12 strains used all carry thr, pheA905, lacU169, araD129 and rpsL150 mutations. In addition, GS852 is purR::Tn10 (Kilstrup et al., 1989), GS986 is purR::Tn10 and gcvA1 (Wilson et al., 1993a), and GS1053 is gcvR::Tn10 (Ghrist & Stauffer, 1995). Plasmid pMC1403 is a lac fusion vector that has been previously described (Casadaban et al., 1980). Plasmid pGS239 is a derivative of pMC1403 that carries a wild-type (WT) gcvT::lacZ translational fusion (Stauffer et al., 1993). The single-copy plasmid pGS441 carries the gcvAF3IL positive control allele and was described previously (Jourdan & Stauffer, 1998) All other plasmids used are described in the text.

Media. The glucose minimal (GM) medium used was the minimal salts medium of Vogel & Bonner (1956) supplemented with 0.4% glucose. Supplements were added at the following concentrations in μg ml⁻¹: thiamin, 1; phenylalanine, 50; glycine, 300; inosine, 50; ampicillin, 100; tetra-
cycline. 10. GM medium was always supplemented with phenylalanine and thiamin since all strains used carry the pheA905 and thi mutations.

**Enzyme assays.** Cells for enzyme assays were harvested from mid-exponential phase cultures (OD600 ~ 0.6). β-Galactosidase assays were performed as described by Miller (1992), using the chloroform/SDS lysis procedure. All results are the means of two or more assays with each reaction performed in triplicate.

**Site-directed mutagenesis and construction of lysogens.** Using plasmid pGS239 as template, the PCR ‘megaprimer’ mutagenesis procedure (Sarkar & Sommer, 1990) was used to create nucleotide changes in GcvA binding site 1 in the gcv control region. The specific base changes were verified by DNA sequence analysis. The approximately 5400 bp EcoRI-MfeI fragments carrying mutant gcvT::lacZ fusions along with the lacY and lacA genes were isolated from each plasmid and ligated into the EcoRI site of phage λgt2 (Pansenko et al., 1977). The phages generated were purified as single plaques and designated lgcvT::lacZ(−55T), lgcvT::lacZ(−60G), lgcvT::lacZ(−61G), lgcvT::lacZ(−67A) and lgcvT::lacZ(−60G, −61G, −67A). The designations in parentheses after the fusions indicate the nucleotide changes and positions relative to the +1 transcription start site. The gcvT::lacZ(1-A) and gcvT:: lacZ(1-B) fusions have four and six base changes, respectively, in site 1 and are described in Fig. 4. Appropriate strains were lysogenized with the above phages and the lysogens verified by carrying a single copy of λ by infection with phage λct90c17 (Shimada et al., 1972).

**Gel mobility-shift assay.** The purified GcvA protein used in this study was described previously (Jourdan & Stauffer, 1998). It incorporates six histidine residues at the C-terminal end and shows normal regulation in vivo. The gel mobility-shift assay was carried out essentially as described (Fried & Crothers, 1981; Garner & Revzin, 1981). The 464 bp SspI-BamHI fragments from plasmid pGS239 and its derivatives were labelled with 32P at the BamHI ends using T4 polynucleotide kinase (Sambrook et al., 1989). The labelled DNA fragments (<65 pM) were included in 20 µl reactions containing DNA binding buffer (10 mM Tris/HCl, pH 7.0, 50 mM KCl, 0.5 mM EDTA, 5 (v/v)% glycerol, 1 mM DTT) and 125 µg bovine serum albumin ml⁻¹. Reaction mixtures were incubated for 5 min at 37 °C, and 2 µl purified GcvA diluted in DNA binding buffer was added to each mixture as indicated in Figs 2, 3 and 5. Incubation was continued at 37 °C for 15 min, 1 µl loading buffer (0.1% xylene cyanol and 50% (v/v) glycerol in H₂O) was added, and the samples were loaded onto a 5% polyacrylamide/3% glycine gel and electrophoresed at approximately 12 V cm⁻¹. The gels were transferred to Whatman 3MM paper, dried and autoradiographed. The autoradiographs were quantified by densitometry analysis at the University of Iowa Central Research Facility.

**DNA bending assay.** The DNA bending assay was essentially as described by Kim et al. (1989) using vector pBEND2. The GcvA binding site 1 from either pGS239 or mutant derivatives was amplified by a PCR reaction that added flanking XbaI restriction sites to each end, and the resulting 51 bp XbaI fragment containing nucleotide changes at both ends using T4 polynucleotide kinase (Sambrook et al., 1989). Gel mobility-shift assays were performed as described above and in Fig. 3.

**RESULTS**

**Point mutations in the conserved 5′-CTAAT-3′ sequence of GcvA binding site 1 alter GcvA-mediated repression**

There is little sequence conservation among the GcvA binding sites except for a short 5′-CTAAT-3′ sequence (Wilson et al., 1995). In a previous study, four of the five conserved bases in the CTAAT sequence were changed simultaneously in site 1 of the gcv::lacZ control region, causing a partial loss of GcvA-mediated repression, but having no significant effect on GcvA-mediated activation (Wilson et al., 1995). However, these changes also caused a GcvA-independent decrease in gcvT::lacZ expression, complicating interpretation of the results. In the present study, four individual point mutations were created by PCR ‘megaprimer’ mutagenesis (see Methods) in site 1 at positions −55, −60, −61 and −67 relative to the transcription start site (+1) with the expectation that at least one of these mutations would affect GcvA-mediated regulation without affecting promoter strength. Nucleotides −60 and −61 are located in the conserved sequence 5′-CTAAT-3′, and nucleotides −55 and −67 are located at positions that may serve as end points of the T-N1-A motif for binding of GcvA (Fig. 1). The DNA fragments carrying these nucleotide changes were cloned as gcvT::lacZ fusions into bacteriophage λgt2 and used to lysogenize strains GS852 and GS986. GS852 is a purR strain, and purine-mediated repression of gcv in this strain is due solely to the GcvA/GcvR regulatory system (Wilson et al., 1993a). Strain GS986 is a gcvA purR double mutant used as a control since no purine repression of gcvT::lacZ occurs in GS986, providing a basal level of β-galactosidase activity for comparison (Wilson et al., 1993a). The lysogens were grown in GM medium supplemented with the purine inosine and β-galactosidase levels were measured. In the GS852 WT lgcvT::lacZ lysogen, growth in GM + inosine resulted in 4-4 fold repression of lgcvT:: lacZ expression compared to the GS986 lysogen (Table 1, compare rows 1 and 6). The β-galactosidase level in the GS852 lgcvT::lacZ(−55T) lysogen was slightly higher than the WT lysogen. In the GS852 lgcvT::lacZ(−60G), (−61G) and (−67A) lysogens, lgcvT::lacZ expression was two- to threefold higher than in the WT lysogen. In the GS986 lgcvT::lacZ(−55T), (−60G), (−61G) and (−67A) lysogens, lgcvT::lacZ expression was not significantly different from the GS986 WT lysogen. The results suggest that the higher levels of lgcvT::lacZ expression of the mutant lysogens in GS852 are primarily due to the loss of GcvA-mediated repression.

Although the above results suggest that the mutations in site 1 interfere with the ability of GcvA to repress gcvT::lacZ, it is also possible that the mutations created a promoter that allows better GcvA-dependent acti-
were grown in GM medium and β-galactosidase levels were measured (Table 1). The −55T, −61G and −67A mutations at site 1 did not cause a significant increase in gcvT::lacZ expression in GS1053 compared to the WT fusion (Table 1). Although −60G caused a slight 1.3-fold increase in β-galactosidase activity compared to the WT fusion, the increase was small compared to the 2.7-fold increase seen in strain GS852. These results suggest that the base changes at positions −60, −61 and −67 relative to the transcription initiation site interfere with GcvA-mediated repression rather than activation. However, it is possible that a gcvR strain expresses gcvT::lacZ at a maximal level, and higher levels may be difficult to achieve.

### A three-base mutation in the conserved 5’-CTAAT-3’ sequence of site 1 causes a phenotype similar to the single-base changes

Despite the loss of GcvA-mediated repression caused by the point mutations when the GS852 lysogens were grown in GM+inosine, the levels of gcvT::lacZ expression are still significantly lower than the levels seen in the gcvR strain GS1053 (Table 1). If binding site 1 is required for repression by GcvA and GcvR, why are the levels of expression lower in the site 1 mutants compared to a gcvR mutant? One possibility is the inability of single-base changes to eliminate binding of GcvA to site 1. To test this possibility, a triple mutant was constructed, combining the changes at bases −60, −61 and −67 in a single gcvT::lacZ fusion. This fusion was cloned into the λgt2 vector and the resulting phage, designated gcvT::lacZ(−60G, −61G, −67A) was used to lysogenize strains GS852 and GS986. Cells were grown in GM, GM+glycine or GM+inosine and β-galactosidase levels were measured (Table 2). Expression of the gcvT::lacZ(−60G, −61G, −67A) fusion was two- to threefold higher in GS852 compared to the WT gcvT::lacZ fusion when cells were grown in either GM or GM+inosine. The mutations in site 1 had no significant effect on β-galactosidase expression when the lysogen was grown in GM+glycine. In the gcvA purR strain GS986, the site 1 triple mutant no longer caused a two- to threefold increase in gcvT::lacZ expression compared to WT, although there was a slight but reproducible increase (~1.4-fold). Since GS986 is a gcvA strain, these results indicate that the site 1 mutations cause a small GcvA-independent effect, probably an increase in promoter strength. Despite this small GcvA-independent effect, the majority of the increase seen in GS852 can be attributed to a loss of GcvA-mediated repression. However, the site 1 triple mutant did not completely relieve GcvA-mediated repression of the gcvT::lacZ fusion as seen in a gcvR strain, but exhibited a phenotype similar to the single-base mutants.

## Gel mobility-shift assay

Gel mobility-shift assays were performed to determine whether GcvA binds to the triple mutant site 1 with a reduced affinity compared to the WT site 1. The WT
The mutations in the conserved sequence of site 1 are involved in DNA conformation

Recent experiments demonstrated that GcvA bends DNA when it binds to any of the three binding sites in the gcv control region (G. Stauffer, unpublished results). Since the mutations in the conserved 5′-CTAAT-3′ sequence of site 1 alter repression to varying degrees but reduce GcvA’s binding affinity less than twofold, it is possible that some other function, such as DNA bending, is affected by mutations in the region that leads to altered regulation. To test this possibility, the triple site 1 mutant –60G, –61G, –67A, the four-base mutant site 1 described previously (Wilson et al., 1995) (Fig. 1) and the WT site 1 were cloned into the vector pBEND2. The plasmids were digested with restriction enzymes to generate equal length DNA fragments with site 1 located at varying distances from the centre of the fragments (Fig. 3). These fragments were then used as templates in gel mobility-shift assays. Since protein-induced DNA bending alters the end-to-end distance of a fragment, a determinant of mobility through a polyacrylamide gel, DNA bending was detected by a change in mobility when site 1 was located at different distances from the centre of the fragment. As expected, when the WT site 1 was located at the right end or the left end of the fragment, it showed a faster mobility than when site 1 was located in the centre of the fragment (Fig. 3, lanes 1, 4 and 7). The mobilities of the two mutant fragments were faster than the WT fragment when the target site was located at the right end of the fragment (Fig. 3, lanes 1–3), but slower when the target sites were located at the left end of the fragment (lanes 7–9). When the target sites were located near the middle of the DNA fragment, the three-base mutant fragment exhibited a similar mobility to the WT and the four-base mutant fragment exhibited a faster mobility than the WT fragment (lanes...
bases that have any of the four nucleotides incorporated at nucleotides, referred to as oligo site 1-A and oligo site 1-B, that were chosen to further characterize site 1. A partially random mutagenesis was performed that could potentially alter site 1 at six different nucleotides in the region surrounding the conserved 5’-CTAAAT-3’ sequence, or at seven positions promoter proximal to the conserved sequence (Fig. 4). This was accomplished by the use of two oligonucleotides, referred to as oligo site 1-A and oligo site 1-B, that have any of the four nucleotides incorporated at bases -58, -59, -64, -65, -66, -68 or at bases -50, -51, -52, -53, -54, -56 and -57 relative to the transcription initiation site at +1. Using a second WT primer, a pool of PCR products was generated, the fragments were cloned into pMC1403 to reconstruct gcvT::lacZ fusions and used to transform GS852. One transformant from each selection was sequenced to determine the nucleotide changes. The transformants isolated using oligo 1-A and oligo 1-B had four and five base changes, respectively (Fig. 4).

To test for their abilities to relieve repression by GcvA, the 1-A and 1-B fragments were cloned as gcvT::lacZ fusions and used to lysogenize strains GS852 and GS986. The resulting lysogens were grown in GM, GM + glycine and GM + inosine, and assayed for β-galactosidase activity. The 1-A mutations had little effect when compared to the WT gcvT::lacZ fusion in purR strain GS852 (Table 3, compare rows 1 and 2). However, in the gcvA purR strain GS986, the 1-A mutations caused a two- to threefold decrease in expression compared to the WT, indicating that one or more of the altered nucleotides in the 1-A mutant are important for general promoter strength (compare rows 4 and 5). In addition, these nucleotides might be important for normal gcvT::lacZ expression since any effect on GcvA-mediated regulation may be masked by the promoter-down effect. The 1-B fusion had about twofold higher levels of gcvT::lacZ expression than the WT fusion since any effect on repression by GcvA would be relieved.

**Table 3.** The effects of the 1-A and 1-B mutations on gcvT::lacZ expression in GS852 (purR) and GS986 (gcvA purR)

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Galactosidase activity of cells grown in GM with</th>
</tr>
</thead>
<tbody>
<tr>
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<td>No addition</td>
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<tr>
<td>GS852 gcvT::lacZ</td>
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<tr>
<td>GS852 gcvT::lacZ(1-A)</td>
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<tr>
<td>GS852 gcvT::lacZ(1-B)</td>
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<tr>
<td>GS986 gcvT::lacZ(1-A)</td>
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</tr>
<tr>
<td>GS986 gcvT::lacZ(1-B)</td>
<td>41</td>
</tr>
</tbody>
</table>

β-Galactosidase activity is expressed in Miller units (Miller, 1992). All standard deviations were within 19% of the mean.

**Isolation of a GcvA site 1 that relieves repression**

Since previous mutations (Wilson et al., 1995) and the mutations described above did not completely relieve GcvA/GcvR-mediated repression of gcvT::lacZ, the role of site 1 in regulation is difficult to ascertain. To further characterize site 1, a two-fold random mutagenesis was performed that could potentially alter site 1 at six different nucleotides in the region surrounding the conserved 5’-CTAAAT-3’ sequence, or at seven positions promoter proximal to the conserved sequence (Fig. 4). This was accomplished by the use of two oligonucleotides, referred to as oligo site 1-A and oligo site 1-B, that have any of the four nucleotides incorporated at bases -58, -59, -64, -65, -66, -68 or at bases -50, -51, -52, -53, -54, -56 and -57 relative to the transcription initiation site at +1. Using a second WT primer, a pool of PCR products was generated, the fragments were cloned into pMC1403 to reconstruct gcvT::lacZ fusions and used to transform GS852. One transformant from each selection was sequenced to determine the nucleotide changes. The transformants isolated using oligo 1-A and oligo 1-B had four and five base changes, respectively (Fig. 4).

4–6). The results of the DNA bending assays show that the mutations cause some disruption of the bending at site 1.
that the 1-B mutations cause an even larger relief of repression than reflected by the β-galactosidase levels in Table 4, that is concealed by the GcvA-independent repression when compared to the WT fusion due to the absence of gcvA this hypothesis using the rather than interfere with its ability to repress. We tested changes have enhanced the ability of GcvA to activate expression of the WT fusion is repressed 10-fold in the presence of GcvA-F31L, the 1-B fusion shows decreased expression when compared to the WT fusion due to the promoter-down effect (Table 4, compare rows 1 and 3). In the presence of GcvA-F31L, the gcvT::lacZ(1-B) fusion is repressed only 1.3-fold (compare rows 3 and 4).

An alternative interpretation of the results is that the 1-B changes have enhanced the ability of GcvA to activate rather than interfere with its ability to repress. We tested this hypothesis using the gcvAF31L allele. The gcvAF31L mutation is a positive control mutation (Jourdan & Stauffer, 1998). The altered amino acid at position 31 of GcvA presumably prevents an interaction with RNAP that is required for activation of gcvT::lacZ; however, GcvA-F31L is still able to repress gcvT::lacZ normally. We tested the effects of the gcvAF31L allele on the expression of gcvT::lacZ(1-B). GS986 i gcvT::lacZ and GS986 i gcvT::lacZ(1-B) were transformed with the single-copy plasmid carrying the gcvAF31L allele and β-galactosidase levels were measured (Table 4). Expression of the WT fusion is repressed 10-fold in the presence of the gcvAF31L allele. As noted above, in the absence of gcvA the 1-B fusion shows decreased expression when compared to the WT fusion due to the promoter-down effect (Table 4, compare rows 1 and 3). In the presence of GcvA-F31L, the gcvT::lacZ(1-B) fusion is repressed only 1.3-fold (compare rows 3 and 4).

**Table 4.** GcvA is unable to repress gcvT::lacZ when the 1-B mutations are present in site 1

<table>
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<th>Strain</th>
<th>Relevant genotype</th>
<th>β-Galactosidase activity</th>
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<td>GS986 i gcvT::lacZ</td>
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<td>GS986 i gcvT::lacZ(1-B) + pGS441</td>
<td>gcvA purR + gcvAF31L</td>
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</tbody>
</table>

* Value is the average of 9 and 11 units.

**Fig. 5.** Mobility-shift assay for binding of GcvA to the 464 bp BamHI–SspI WT, 1-A and 1-B fragments. GcvA was added at the following dimer concentrations: lanes 1, 5 and 9, 0 nM; lanes 2, 6 and 10, 16 nM; lanes 3, 7 and 11, 32 nM; lanes 4, 8 and 12, 64 nM.

**Binding of GcvA to the site 1-A and site 1-B mutants**

The 464 bp BamHI–SspI fragments, identical to those described in the mobility-shift assays above except for the base changes, were isolated from the 1-A and 1-B mutants, 32P-labelled at the BamHI ends and used in gel mobility-shift assays. The 1-A mutant site 1 fragment was able to bind GcvA with approximately a twofold decreased affinity compared to WT site 1, suggesting that the nucleotides at positions −57, −58, −64 and −65 have some role in GcvA binding (Fig. 5, compare lanes 1–4 with 5–8). The 1-B mutant fragment showed at least a fourfold decrease in affinity for GcvA, indicating that one or more of the nucleotides altered are important for binding of GcvA (Fig. 5, compare lanes 1–4 with 9–12). Although the 1-B mutations did not completely abolish binding by GcvA, the 1-B mutant is the first site 1 mutant to exhibit more than a twofold decrease in binding affinity for GcvA, suggesting that the central region of site 1 is important for GcvA binding, rather than the conserved 5′-CTAAT-3′ sequence important for recognition and binding of GcvA at sites 2 and 3 (Wilson et al., 1995).

To separate the GcvA-dependent and -independent effects of the 1-B mutations, site-directed mutagenesis was used to create single-base changes at positions −50, −52, −53, −56 and −57. We expected that one or more of these nucleotides would be required for GcvA binding and repression, and for general promoter strength independent of GcvA. The fragments containing the single-base changes were verified by DNA sequencing and tested for their effects on these various
functions. Surprisingly, none of the single-base changes had any significant effect on regulation by GcvA, general promoter strength or the ability to bind GcvA (data not shown).

Superrepression by multi-copy gcvc in the presence of the 1-B mutations in site 1

Our current model proposes that GcvA binding to site 1 is required for GcvA-mediated repression in the presence of inosine. However, we do not know if the repression mediated by the GcvR protein requires the GcvA/site 1 interaction or if GcvR represses through a separate mechanism. To determine if repression by GcvR is affected by the mutations at site 1, the multi-copy plasmid pGS334 that overexpresses gcvc and causes superrepression of a WT fusion was transformed into GS852::lacZ, GS852::lacZ(1-B), GS852::lacZ(1-B)+pGS334, GS986::lacZ, GS986::lacZ(1-B), GS986::lacZ(1-B)+pGS334, and GS986::lacZ(1-B)+pGS334. The transformants were grown in GM medium and assayed for β-galactosidase activity (expressed in Miller units) (Miller, 1992). All standard deviations were within 8% of the mean.

<table>
<thead>
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<th>Strain</th>
<th>Relevant genotype</th>
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</table>

DISCUSSION

GcvA-mediated repression of the gcvc operon has been suggested to require GcvA binding to site 1 (Wilson et al., 1995). This study demonstrates that the sequence requirement at site 1 is complex. Single and multiple base changes throughout the potential T-N₁₁-A sequence, which includes the conserved 5’-CTAAT-3’ sequence of site 1 (Fig. 1), relieve repression by GcvA about two- to threefold in the purR strain GS852 (Table 1), but show less than a twofold decrease in GcvA binding affinity compared to WT site 1 (Fig. 2). It is likely that the loss of repression is due in part to a loss of GcvA binding to site 1. In addition, the three and four base site 1 mutations examined show altered GcvA-induced DNA bending at site 1 (Fig. 3). Thus, an altered bend in the DNA may in some way also be responsible for part of the loss of repression. DNA bending by the phage φ29 p4 protein has been shown to be important in its ability to act as a repressor to hinder RNAP binding (Rojo & Salas, 1991). Of interest is that the three-base mutant isolated in this study runs more slowly than the four-base mutant isolated previously (Wilson et al., 1995) when site 1 is at the right end of the DNA fragment, but faster when located at the left end of the fragment (Fig. 3). One interpretation to explain the gel-shift pattern is that the base changes alter the centre of the bend angle as well as or instead of the degree of bending.

The most severe allele examined that alters both GcvA binding and repression is the 1-B mutant, with five base changes (Fig. 4). These changes result in a fourfold decrease in GcvA binding affinity in vitro when compared to WT site 1 (Fig. 5), and in vivo caused a sixfold loss of GcvA-mediated repression when cells were grown in GM + inosine (Table 3). The mutations also caused a two- to threefold decrease in promoter strength (Table 3). However, when the five bases were changed individually, none of the changes had a significant effect on gcvcT::lacZ expression or on the binding affinity of GcvA, again suggesting that several bases must be altered to interfere with binding and repression by GcvA.

The 1-A and 1-B mutants isolated in this study, as well as the four-base mutant isolated previously (Wilson et al., 1995), showed GcvA-independent promoter-down
effects (Table 3). It should be noted that the promoter-down effects are only observed in the absence of GcvA. Thus, it is possible that RNAP interacts with this region of gcv in the absence of GcvA to maintain basal promoter activity, and the mutations interfere with this interaction. It is known that the C-terminal domain of the α subunit of RNAP interacts with up-elements located upstream of the promoter —33 sequence element (Estrem et al., 1999). Although a sequence is present in gcv from base —44 to —54 that shows a 7 out of 11 base match with the proposed up-element consensus distal subsite (Fig. 1), this degree of homology is considered insignificant as a subsite (Estrem et al., 1999). Furthermore, the 1-A mutant and the four-base mutant (Wilson et al., 1995) lie upstream of this sequence. We have been unsuccessful at footprinting RNAP at the gcv promoter to verify an RNAP–DNA interaction in this region.

Previous studies showed that overexpressing gcvR favours repression of a gcvT::lacZ fusion even under activating conditions (glycine supplementation), and overexpressing gcvA favours activation even under repressing conditions (purine supplementation) (Ghrist & Stauffer, 1998). Since GcvA activates a gcvT::lacZ fusion in the absence of GcvR, and GcvR-mediated repression is dependent on a functional GcvA protein, a model was proposed that GcvA homo-oligomers activate the gcv operon, whereas GcvA/GcvR hetero-oligomers repress the gcv operon (Ghrist & Stauffer, 1995). The small co-regulators glycine and purines possibly determine whether the activator or the repressor form of GcvA is favoured in the cell. We believe our results are consistent with this model. Since GcvA/GcvR, in the presence of inosine, represses a gcvT::lacZ fusion four- to fivefold in a site 1-dependent manner, GcvA binding at site 1 in the presence of GcvR appears to be necessary to repress transcription. Furthermore, as none of the changes have a significant effect on activation, site 1 appears necessary only for repression. However, the role(s) of GcvR in the repression mechanism is still unclear. Also unclear are the different sequence requirements for site 1 compared to sites 2 and 3. It is possible that the difference is related to the mechanism GcvA employs to switch between activation and repression.

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