Roles for GcvA-binding sites 3 and 2 and the Lrp-binding region in *gcvT::lacZ* expression in *Escherichia coli*

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GcvA and Lrp are both necessary for activation of the *gcv* operon. The upstream GcvA-binding sites 3 and 2 were separated from the Lrp-binding region and the rest of the *gcv* control region. Moving these sites by 1 or 2 helical turns of DNA further from the *gcv* promoter reduces, but does not eliminate, either GcvA-mediated activation or repression of a *gcvT::lacZ* gene fusion. However, moving these sites by 1.5 or 2.5 helical turns of DNA results in a GcvA-mediated super-repression of the operon. This repression is dependent on Lrp and is partially dependent on GcvR. Lrp bound to the *gcv* control region induces a bend in the DNA. Based on these results, a model for *gcv* regulation is presented in which Lrp plays a primarily structural role, by bending the DNA and GcvA functions as the activator protein.

Keywords: glycine cleavage, *gcvT::lacZ*, GcvA, Lrp, DNA bending

INTRODUCTION

In *Escherichia coli*, the biosynthesis of glycine and C₁ units via the serine hydroxymethyltransferase reaction (*glyA* gene product) plays a central role in cell physiology. Glycine is used for both protein and purine biosynthesis, and C₁ units are used in the biosynthesis of purines, methionine, thymine and other cellular components (Mudd & Cantoni, 1964). The oxidative cleavage of glycine by the glycine cleavage (GCV) enzyme system provides a second pathway for the production of C₁ units (Kikuchi, 1973). Strains that are defective in the GCV enzyme system excrete glycine (Plamann *et al.*, 1983; Ghrist & Stauffer, 1995b), and it has been proposed that the physiological role for the GCV enzyme complex is to balance the requirements of the cell for glycine used in protein and purine biosynthesis and the C₁ units used in other biosynthetic reactions.

The *gcv* regulatory region is complex, with both global-acting and *gcv*-specific regulatory proteins. Lrp, a global regulator of numerous genes relating to amino acid metabolism (Calvo & Matthews, 1994), is required for induction of the *gcv* operon (Lin *et al.*, 1992; Stauffer & Stauffer, 1994), but is insensitive to the co-regulator leucine (Ghrist & Stauffer, 1995b). Lrp binds to multiple

sites upstream of the *gcv* promoter, from about base –92 to –229 (Fig. 1a) (Stauffer & Stauffer, 1994). However, the mechanism of Lrp-mediated activation of the *gcv* operon is unknown.

The GcvA protein functions as both an activator for *gcv* operon expression in glycine-supplemented medium and a repressor for the operon when cells are grown in the presence of inosine, but without glycine (Wilson *et al.*, 1993a, b). GcvA binds to three sites in the *gcv* control region, from bases –271 to –242, –242 to –214 and –69 to –34, relative to the transcription initiation site (Fig. 1a) (Wilson *et al.*, 1995). The upstream GcvA-binding sites 3 and 2 are required for GcvA-mediated activation of the operon, whereas all three sites are required for GcvA-mediated repression of the operon (Wilson *et al.*, 1995).

The GcvR protein also negatively regulates the *gcv* operon, although its ability to repress is dependent on a functional GcvA protein (Ghrist & Stauffer, 1995a). Whether the GcvR protein is directly or indirectly involved in the mechanism for repression is not known.

PurR, a negative regulatory protein for genes involved in nucleotide metabolism (Kilstrup *et al.*, 1989; Rolfes & Zalkin, 1988), represses the *gcv* operon twofold in response to purine supplementation (Stauffer *et al.*, 1994; Wilson *et al.*, 1993a). PurR binds to a region from about base –3 to +17 relative to the transcription initiation site (Fig. 1a). PurR-mediated repression is

Abbreviations: GCV, glycine cleavage; GM, glucose minimal; RNAP, RNA polymerase.

Fig. 1. (a) Diagram of the *gcv* operon control region showing the locations of the GcvA upstream (sites 3 and 2) and downstream (site 1) binding sites (Wilson *et al.*, 1995), the Lrp-binding region (Stauffer & Stauffer, 1994) and the PurR-binding site (Wilson *et al.*, 1993a), relative to the *gcv* promoter -35, -10 and +1 sequences (Stauffer *et al.*, 1993). (b) Nucleotide sequence of the *gcv* control region from base -209 to -232, relative to the *gcv* transcription initiation site. The 5' end of the Lrp-binding region (overlined) (Stauffer & Stauffer, 1994) and the 3' end of GcvA-binding site 2 (underlined) (Wilson *et al.*, 1995) are indicated, as well as the position of the insertions. The +11, +15, +21 and +25 bp insertions between bases -213 and -214 are indicated in lower case. The XbaI sites used to generate the +15 and +25 bp insertions are indicated above the sequences; the bases (ctag) added by fill-in reactions of the XbaI sites are underlined.

independent of the GcvA and GcvR proteins (Wilson *et al.*, 1993a; Ghrist & Stauffer, 1995a).

Previous studies have demonstrated that although the spacing of the GcvA-binding sites 3 and 2 and the Lrp-binding region relative to the *gcv* promoter is somewhat flexible, a strict stereospecific alignment of these sites relative to the *gcv* promoter is required for both glycine-mediated activation and inosine-mediated repression of a *gcvT::lacZ* gene fusion (Stauffer & Stauffer, 1998). These results are consistent with a protein interaction of either GcvA or Lrp with RNA polymerase (RNAP) at the *gcv* promoter for activation, or possibly with GcvA bound at site 1 for repression of the operon. Since the GcvA-binding sites 3 and 2 and the Lrp-binding region are a considerable distance upstream of the *gcv* promoter (Fig. 1a), it is reasonable to assume that if such a protein-protein interaction occurs, a DNA conformational change is involved as part of the regulatory mechanism. In this study, we separated, by integral and half integral turns of DNA, the upstream GcvA-binding sites 3 and 2 required for activation and repression of a *gcvT::lacZ* fusion from the rest of the *gcv* control region and tested the effects of these insertions on *gcvT::lacZ* expression. Based on our results, a model is discussed for GcvA and Lrp involvement in the *gcv* regulatory mechanism.

METHODS

Bacterial strains. The *E. coli* K-12 strains used are listed in Table 1.

Media. The glucose-minimal (GM) medium used was the minimal salts of Vogel & Bonner (1956) supplemented with 0.4% glucose. GM medium was always supplemented with

phenylalanine and thiamin, since all strains carry the *pheA905* and *thi* mutations. Supplements were added at the following concentrations ($\mu\text{g ml}^{-1}$): glycine, 300; phenylalanine, 50; inosine, 50; thiamin, 1.

DNA manipulation. Plasmid DNA isolation, restriction digestion, PCR reactions, etc. were as described by Sambrook *et al.* (1989). DNA sequencing was by the method of Sanger *et al.* (1977) using the Sequenase version 2.0 kit (USB).

Plasmid construction. Plasmid pGS318 carries an in-frame *gcvT::lacZ* gene fusion on a 769 bp *EcoRI*-*Bam*HI fragment (Stauffer & Stauffer, 1998) and was used as the starting template. pGS416 was constructed using the megaprimer mutagenesis method (Sarkar & Sommer, 1990) and carries an 11 bp insertion with a unique *Xba*I site between bases -213 and -214 relative to the transcription initiation site (Fig. 1b). pGS420 was constructed by digesting pGS416 with *Xba*I, filling in the ends with the large fragment of DNA polymerase I and ligating, resulting in a total of 15 bp inserted between wild-type positions -213 and -214. pGS427 was constructed using the megaprimer mutagenesis method (Sarkar & Sommer, 1990) and pGS420 as template. Six additional bases, carrying a unique *Xba*I site, were introduced into pGS420, resulting in a 21 bp insertion (Fig. 1b). pGS428 was constructed by digesting pGS427 with *Xba*I, filling in the ends with the large fragment of DNA polymerase I and ligating, resulting in a total of 25 bp inserted between wild-type positions -213 and -214. The insertions were verified by DNA sequencing. pGS281 carries the Lrp-binding region from base -69 to -236 on a 168 bp *Xba*I DNA fragment (Fig. 1a) cloned into the *Xba*I site of pBend2 (Kim *et al.*, 1989). The *Xba*I sites were generated by PCR. The orientation of the insert was verified by restriction enzyme analysis.

Phage and lysogen construction. An approximately 6 kb *EcoRI*-*Mfe*I DNA fragment carrying the *gcvT::lacZ* fusion along with the *lacYA* genes was isolated from each plasmid and ligated into the *EcoRI* site of λ gt2 (Panasencko *et al.*, 1977)

Table 1. *E. coli* strains used in this study

GS162 is a derivative of MC4100 (Casadaban, 1976). All other strains were constructed in this laboratory using GS162 as the parent strain. The *purR6::Tn10* allele is from strain S ϕ 5068 (Kilstrup *et al.*, 1989). The *lrp::Tn10* allele is from strain CV1008 (Platko *et al.*, 1990). The *gcvA1* (Wilson *et al.*, 1993b) and *gcvR::Tn10* (Ghrist & Stauffer, 1995a) mutations were isolated in this laboratory.

Strain	Relevant genotype	Other markers
GS162	Wild-type	<i>thi pheA905 ΔlacU169 araD129 rpsL150</i>
GS852	<i>purR6::Tn10</i>	As GS162
GS997	<i>lrp::Tn10</i>	As GS162
GS998	<i>gcvA1</i>	As GS162
GS1053	<i>gcvR::Tn10</i>	As GS162

as previously described (Urbanowski & Stauffer, 1986). The phages generated were single-plaque-purified and designated λ *gcvT::lacZ* + 11₋₂₁₃, λ *gcvT::lacZ* + 15₋₂₁₃, λ *gcvT::lacZ* + 21₋₂₁₃ and λ *gcvT::lacZ* + 25₋₂₁₃. The numbers after *lacZ* indicate the number of bases inserted; the subscript indicates that the insertions were immediately upstream of base -213, relative to the transcription initiation site. Bacterial strains were lysogenized with the various λ *gcvT::lacZ* fusion phages as previously described (Urbanowski & Stauffer, 1986). All the λ lysogens carry the *cI857* mutation, resulting in a temperature-sensitive repressor, and were grown at 30 °C. After single-colony purification, all the lysogens were verified to carry a single copy of the λ phage by testing for their ability to support lytic infection by λ *cI90c17* (Shimada *et al.*, 1972).

β -Galactosidase assays. β -Galactosidase activities were determined from exponential-phase cultures harvested at an OD₆₀₀ of approximately 0.5 as described by Miller (1992), using the chloroform/SDS lysis procedure. The results are from two separate assays in which the activity of each sample was determined in triplicate.

Gel mobility-shift assay. Double-stranded 299 bp *Mlu*I, *Xho*I and *Bam*HI DNA fragments from pGS281 carrying the Lrp-binding region at different positions were ³²P-labelled at the 5' ends using T4 polynucleotide kinase and [γ -³²P]ATP (Sambrook *et al.*, 1989). Less than 0.2 ng labelled fragment was added to 20 μ l reaction mixtures containing 1 $\times$ DNA-binding buffer (10 mM Tris/HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 1 mM DTT) and 125 μ g BSA ml⁻¹. The reaction mixtures were pre-incubated for 5 min at 37 °C and then 2 μ l purified Lrp protein was added to the reaction mixtures to give final concentrations of approximately 2.2 nM of Lrp dimer. Incubation was continued for 15 min, then 1 μ l of loading dye (0.1% xylene cyanol and 50% glycerol) was added to each reaction. The samples were loaded onto a 5% polyacrylamide, 3% glycine gel and run at 12 V cm⁻¹. Gels were transferred to 3MM Whatman paper, dried and autoradiographed.

RESULTS AND DISCUSSION

Effects on *gcvT::lacZ* expression of separating GcvA-binding sites 3 and 2 from the *gcv* control region by integral turns of DNA

Both Lrp and GcvA are required for expression of the *gcv* operon (Stauffer & Stauffer, 1994; Wilson *et al.*, 1993b; Lin *et al.*, 1992). One possible model for

regulation of the operon is that one of these two proteins interacts with RNAP and the other is required either for co-operative binding of the two proteins to the DNA, or to induce an altered conformation of the DNA to allow for an appropriate DNA-protein interaction to occur. We separated the upstream GcvA-binding sites 3 and 2 from the rest of the *gcv* control region to determine the distance and orientation requirements of GcvA bound at these sites for *gcvT::lacZ* expression, and whether GcvA and Lrp bind co-operatively. We assumed that separating the Lrp- and GcvA-binding regions by one or two helical turns of DNA would disrupt possible Lrp-GcvA interactions required for co-operative binding. We inserted DNA between bases -213 and -214 of the *gcvT::lacZ* control region, immediately downstream of GcvA-binding site 2 (Fig. 1b). Because GcvA-binding site 2 overlaps the upstream Lrp-protected region, insertion of DNA at this position not only separates the GcvA-binding sites 3 and 2 from the Lrp-binding region, but separates the upstream 16 bp of the Lrp-protected region from the rest of the Lrp-binding region (Fig. 1b). Therefore, we initially determined whether insertions of integral turns of DNA between bases -213 and -214 would inactivate the *gcv* promoter or would still show glycine-mediated activation and inosine-mediated repression of a *gcvT::lacZ* gene fusion. λ *gcvT::lacZ* phages carrying the +11₋₂₁₃ and +21₋₂₁₃ insertions were used to lysogenize the *purR* strain GS852, in which inosine repression is due solely to the GcvA/GcvR system. The lysogens were grown in GM media with the appropriate supplements, and β -galactosidase levels were measured. The GS852 + 11₋₂₁₃ lysogen showed about fourfold lower levels of expression in GM medium than the 852 λ *gcvT::lacZ* control lysogen (Table 2). Although the β -galactosidase levels were induced about 9-fold in the presence of glycine in the GS852 + 11₋₂₁₃ lysogen, the induced levels were still about 2.5-fold lower than in the control lysogen. The GS852 + 11₋₂₁₃ lysogen showed about a 4-fold repression when grown in GM medium + inosine compared to GM medium alone and the repressed levels were 2.6-fold lower than the GS852 control lysogen. The GS852 + 21₋₂₁₃ lysogen showed about a 2-fold higher basal level of expression in GM medium than the +11₋₂₁₃ lysogen (Table 2). The β -galactosidase levels

Table 2. Effects of insertion of integral or half-integral turns of DNA between base -213 and -214 on *gcvT::lacZ* expression

Lysogens were grown in GM medium with the indicated supplements. All standard deviations were within 12% of the mean, except for values marked *, for which it was within 25% of the mean (mean of 3 and 5 units of activity).

Lysogen	β -Galactosidase activity (Miller units)		
	None	Glycine	Inosine
852 λ <i>gcvT::lacZ</i>	126	704	21
852 λ <i>gcvT::lacZ</i> + 11 ₋₂₁₃	31	286	8
852 λ <i>gcvT::lacZ</i> + 15 ₋₂₁₃	2	6	2
852 λ <i>gcvT::lacZ</i> + 21 ₋₂₁₃	68	327	24
852 λ <i>gcvT::lacZ</i> + 25 ₋₂₁₃	2	8	1
997 λ <i>gcvT::lacZ</i>	24	28	10
997 λ <i>gcvT::lacZ</i> + 11 ₋₂₁₃	19	30	5
997 λ <i>gcvT::lacZ</i> + 15 ₋₂₁₃	20	25	7
997 λ <i>gcvT::lacZ</i> + 21 ₋₂₁₃	8	12	4*
997 λ <i>gcvT::lacZ</i> + 25 ₋₂₁₃	19	22	9
998 λ <i>gcvT::lacZ</i>	62	72	27
998 λ <i>gcvT::lacZ</i> + 11 ₋₂₁₃	115	131	65
998 λ <i>gcvT::lacZ</i> + 15 ₋₂₁₃	33	31	16
998 λ <i>gcvT::lacZ</i> + 21 ₋₂₁₃	112	103	56
998 λ <i>gcvT::lacZ</i> + 25 ₋₂₁₃	46	44	24
1053 λ <i>gcvT::lacZ</i>	1242	1236	705
1053 λ <i>gcvT::lacZ</i> + 11 ₋₂₁₃	784	825	476
1053 λ <i>gcvT::lacZ</i> + 15 ₋₂₁₃	6	12	3
1053 λ <i>gcvT::lacZ</i> + 21 ₋₂₁₃	326	543	281
1053 λ <i>gcvT::lacZ</i> + 25 ₋₂₁₃	19	24	10

were induced about 4.8-fold in the presence of glycine, and were repressed about 2.8-fold in the presence of inosine. Since GcvA-binding sites 3 and 2 were separated from the Lrp-binding region and still allow good activation of the *gcvT::lacZ* fusion, these proteins might not interact cooperatively in binding to the DNA as part of the activation mechanism. It should be noted that the final 16 bp of the upstream Lrp-binding region is still present in the insertion mutants, and it is possible that Lrp bound to this site alone is sufficient for cooperative binding with GcvA. However, we determined by the gel mobility shift assay that an Lrp dimer concentration of about 24 nM is required to bind and shift about 50% of a DNA fragment carrying the final 22 bp of the Lrp-binding region (not shown). This is about 10-fold higher than the affinity determined previously for Lrp binding to the wild-type *gcv* control region (50% binding at an Lrp dimer concentration of about 2.2 nM) (Stauffer & Stauffer, 1994) and about fourfold higher than the estimated physiologically relevant level of Lrp in the cell (5.5 nM) (Borst *et al.*, 1996). Thus, the upstream 16 bp of the Lrp-binding region that is separated from the rest of the Lrp-protected region in the insertion mutants is probably not sufficient to support Lrp's role in *gcvT::lacZ* expression.

Activation and repression in the +11₋₂₁₃ and +21₋₂₁₃ lysogens are dependent on GcvA, Lrp and GcvR

To demonstrate whether the glycine-mediated activation was GcvA and Lrp dependent and the inosine-mediated repression was GcvA and GcvR dependent, we lysogenized the *gcvA* strain GS998, the *lrp* strain GS997 and the *gcvR* strain GS1053 with the +11₋₂₁₃ and +21₋₂₁₃ insertion phase. The lysogens were grown in GM media with the appropriate supplements and β -galactosidase levels were measured. The 997 λ *gcvT::lacZ* control lysogen showed β -galactosidase levels that were reduced about fivefold in GM medium compared to the 852 λ *gcvT::lacZ* control lysogen (Table 2). These levels were not induced by glycine, or repressed by inosine, except for an expected approximately twofold PurR-mediated repression. The GS997 +11₋₂₁₃ lysogen showed levels of β -galactosidase that were essentially the same as the GS997 control lysogen. The GS997 +21₋₂₁₃ lysogen showed a similar pattern of regulation, except that the levels were about two- to threefold lower than in the GS997 control lysogen. Since the β -galactosidase levels observed in the GS852 +11₋₂₁₃ and +21₋₂₁₃ lysogens were reduced in the *lrp* mutant, the results suggest that Lrp is still required for *gcvT::lacZ* expression when GcvA-binding sites 3 and 2 are shifted by helical turns of DNA.

The GS998 +11₋₂₁₃ and +21₋₂₁₃ lysogens showed levels of β -galactosidase activity in GM, GM + glycine and GM + inosine media that were elevated compared to the 998 λ *gcvT::lacZ* control lysogen (Table 2). These levels were not induced by glycine, or repressed by inosine, except for the approximately twofold PurR-mediated repression. Thus, GcvA is still required for both glycine-mediated activation and inosine-mediated repression of the +11₋₂₁₃ and +21₋₂₁₃ fusions. Furthermore, the increase in basal levels of expression in the GS998 +11₋₂₁₃ lysogen in both GM and GM + inosine media to the same level observed in the GS998 +21₋₂₁₃ lysogen suggests that the low β -galactosidase levels found in the GS852 +11₋₂₁₃ lysogen are possibly due to increased GcvA-mediated repression. The reason for the increase in basal levels of expression observed in the GS998 +11₋₂₁₃ and +21₋₂₁₃ lysogens compared to the GS998 control lysogen is unknown.

The maximum level of expression of a *gcvT::lacZ* fusion is observed when the negative regulator GcvR is inactivated (Ghrist & Stauffer, 1995a). The GS1053 +11₋₂₁₃ lysogen showed less than twofold lower levels of expression under all growth conditions compared to the 1053 λ *gcvT::lacZ* control lysogen (Table 2). Both the GS1053 control lysogen and the GS1053 +11₋₂₁₃ lysogen showed the expected PurR-mediated repression in the presence of inosine. The effects of the +21 bp insertion were more severe in the *gcvR* background. The GS1053 +21₋₂₁₃ lysogen showed about a 3.8-fold decrease in β -galactosidase levels when grown in GM medium compared to the control lysogen, and a 2.3-fold decrease when grown in GM + glycine medium. Since the GS1053 +21₋₂₁₃ lysogen showed a significant

decrease in activity compared to the +11₋₂₁₃ lysogen, it is likely that although the absolute distance of GcvA sites 3 and 2 relative to the *gcv* promoter can be lengthened, the increase is limited to a few helical turns without loss of activation and repression. A similar distance-dependent loss of regulation was observed previously when both the GcvA sites 3 and 2 and the Lrp-binding region were moved together by helical turns of DNA (Stauffer & Stauffer, 1998). In both the +11₋₂₁₃ and +21₋₂₁₃ GS1053 lysogens, the β -galactosidase levels were significantly higher than in the GS852 lysogens, indicating that GcvR was still operating in the regulation of the *gcvT::lacZ* fusion. In addition, since in GS1053 the β -galactosidase levels for the mutant lysogens are lower than for the wild-type fusion, moving the GcvA sites 3 and 2 further upstream must disrupt some interaction necessary for *gcvT::lacZ* expression.

Effects on *gcvT::lacZ* expression of separating GcvA-binding sites 3 and 2 from the *gcv* control region by half integral turns of DNA

We also tested the effects of inserting half-integral turns of DNA at position -213 on *gcvT::lacZ* expression. When the GS852 +15₋₂₁₃ and +25₋₂₁₃ lysogens were grown in GM medium there was a more than 60-fold reduction in β -galactosidase levels compared to the 852 λ *gcvT::lacZ* control lysogen (Table 2). When these mutant lysogens were grown in GM+glycine medium, there was a slight increase in *gcvT::lacZ* expression. However, the induced levels for the GS852 +15₋₂₁₃ and +25₋₂₁₃ lysogens were still about 88- and 117-fold lower than in the GS852 control lysogen. When the mutant lysogens were grown in GM + inosine medium, the β -galactosidase levels were not significantly different from the levels found in GM medium alone (Table 2). Furthermore, since the β -galactosidase levels observed in the GS852 +15₋₂₁₃ and +25₋₂₁₃ lysogens were significantly lower than the levels observed even when both known positive regulators (GcvA and Lrp) were inactivated (about 14, 22 and 7 units of activity in GM, GM+glycine and GM+inosine media, respectively; Stauffer & Stauffer, 1994), it is clear that there is a strict stereospecific requirement for GcvA to bind on the appropriate side of the DNA helix for activation of the *gcv* promoter. It appears that putting GcvA on the wrong side of the DNA helix relative to RNAP not only eliminates activation, but results in super-repression of the operon.

We showed previously that moving the entire Lrp-binding region and GcvA-binding sites 3 and 2 by half integral turns of DNA did not result in super-repression, but rather resulted in levels of *gcvT::lacZ* expression similar to the levels found in an *lrp* mutant (Stauffer & Stauffer, 1998). Thus, the super-repression observed in the GS852 +15₋₂₁₃ and +25₋₂₁₃ lysogens is possibly due to moving only GcvA bound at sites 3 and 2. We tested this possibility by lysogenizing the *gcvA* strain GS998 with the control λ *gcvT::lacZ* phage and the +15₋₂₁₃ and +25₋₂₁₃ insertion phage. The lysogens were grown in GM medium with the appropriate supplements and

the β -galactosidase levels were measured. The levels were significantly elevated in the GS998 background compared to the GS852 background under all three growth conditions, suggesting that the decreased levels of expression in GS852 are possibly due to enhanced GcvA-mediated repression (Table 2). As expected in a *gcvA* background, these levels were not induced by glycine or repressed by inosine, except for the expected twofold PurR-mediated repression.

To confirm that the repressed levels of β -galactosidase observed in the GS852 +15₋₂₁₃ and +25₋₂₁₃ lysogens were mediated through the GcvA protein, the control 998 λ *gcvT::lacZ* lysogen and the mutant GS998 +15₋₂₁₃ and +25₋₂₁₃ lysogens were each transformed with the single-copy *gcvA*⁺ plasmid pGS341 (Wilson *et al.*, 1993b), the transformants were grown in GM or GM+glycine medium and the β -galactosidase levels were measured. The presence of pGS341 restored GcvA-mediated activation in the 998 λ *gcvT::lacZ* control lysogen (Table 3, compare rows 1 and 2). However, when the GS998 +15₋₂₁₃[pGS341] and +25₋₂₁₃[pGS341] lysogens were grown in GM medium, there was about an 11-fold and a sixfold repression of β -galactosidase levels, compared to the non-transformed lysogens (Table 3). When grown in GM+glycine medium, there was an increase in the β -galactosidase levels, but the induced levels were still about 2.5-fold lower than in the non-transformed lysogens.

Effects of *gcvR* and *lrp* mutations on the +15₋₂₁₃ and +25₋₂₁₃ insertions

As mentioned above, the maximum level of expression of a *gcvT::lacZ* fusion is observed when the negative regulator GcvR is inactivated (Ghrist & Stauffer, 1995a). Although the mechanism of GcvR-mediated repression is unknown, it is dependent on the GcvA protein (Ghrist & Stauffer, 1995a). Therefore, we lysogenized the *gcvR* strain GS1053 with the +15₋₂₁₃ and +25₋₂₁₃ insertions. The lysogens were grown in GM media with the appropriate supplements and β -galactosidase levels were measured. The insertions essentially inactivated *gcvT::lacZ* expression, compared to the 1053 λ *gcvT::lacZ* control lysogen (Table 2), confirming a strict face-of-the-helix dependency of GcvA bound at sites 3 and 2 for activation of *gcvT::lacZ*. Although the GS1053 +15₋₂₁₃ and +25₋₂₁₃ insertions showed slightly elevated levels of β -galactosidase compared to the GS852 +15₋₂₁₃ and +25₋₂₁₃ lysogens (Table 2), these levels were still significantly lower than the levels found in the *gcvA* GS998 +15₋₂₁₃ and +25₋₂₁₃ lysogens (Table 2). Thus, only part of the GcvA-dependent repression observed in the GS852 +15₋₂₁₃ and +25₋₂₁₃ lysogens is dependent on the GcvR protein.

The GS997 +15₋₂₁₃ and +25₋₂₁₃ lysogens also showed an increase in β -galactosidase levels compared to the levels observed in the GS852 +15₋₂₁₃ and +25₋₂₁₃ lysogens (Table 2). In addition, these levels were not significantly different from the levels observed in the 997 λ *gcvT::lacZ* control lysogen (Table 2). As shown

Table 3. GcvA represses the GS998 +15₋₂₁₃ and +25₋₂₁₃ lysogens *in trans*

Lysogens were grown in GM medium with the indicated supplements. All standard deviations were within 15% of the mean.

Lysogen	β -Galactosidase activity (Miller units)	
	None	Glycine
998 λ <i>gcvT::lacZ</i>	66	72
998 λ <i>gcvT::lacZ</i> [pGS341]	108	602
998 λ <i>gcvT::lacZ</i> + 15 ₋₂₁₃	45	41
998 λ <i>gcvT::lacZ</i> + 15 ₋₂₁₃ [pGS341]	4	17
998 λ <i>gcvT::lacZ</i> + 25 ₋₂₁₃	46	45
998 λ <i>gcvT::lacZ</i> + 25 ₋₂₁₃ [pGS341]	8	18

(a) Plasmid pGS281 **(b)**

Fig. 2. (a) Part of plasmid pGS281 showing the location of the *XbaI* fragment carrying the *gcv* Lrp-binding region cloned into the *XbaI* site of pBend2. (b) Location of the Lrp-binding region in the 299 bp *MluI*, *XhoI* and *BamHI* fragments from pGS281. The centre of each fragment is indicated by an arrow. Only relevant restriction enzyme sites are shown.

above, Lrp is required for expression of the +11₋₂₁₃ and +21₋₂₁₃ insertions, suggesting that the role of Lrp has not been significantly altered by insertions at the -213 position. Thus, the GcvA super-repression in the GS852 +15₋₂₁₃ and +25₋₂₁₃ lysogens appears to require not only that sites 3 and 2 are on the opposite side of the DNA helix relative to the wild-type *gcvT::lacZ* fusion, but also that a functional Lrp protein be present.

Bending of the *gcv* control region by Lrp

Lrp is known to be a DNA-bending protein; it has been suggested that Lrp might be an architectural element, facilitating the assembly of a nucleoprotein complex regulating transcription (Wang & Calvo, 1993). We considered the possibility that Lrp might function as an architectural DNA bending factor in *gcv* regulation. Using the gel-shift bending assay of Kim *et al.* (1989), we tested whether Lrp binding induces bending in a DNA fragment carrying the Lrp-binding region for *gcv*. A 168 bp DNA fragment carrying the Lrp-binding region was cloned into the *XbaI* site of plasmid pBend2 (see Methods). Three different restriction enzymes were used to generate fragments of identical length (299 bp), but with different locations of the Lrp-binding region (Fig. 2b). Gel electrophoresis of these fragments alone showed

no significant intrinsic DNA bending (Fig. 3). The addition of Lrp caused a significant change in the mobility of each different Lrp-DNA complex, with the magnitude of change being dependent on the position of the binding site relative to the fragment end-points (Fig. 3). The apparent bending angle for the *gcv* control region was calculated using the formula $\mu M/\mu E = \cos \alpha/2$, where μM is the mobility of the DNA with the protein bound in the centre of the fragment and μE is the mobility of the DNA fragment with the binding site located near one end of the DNA fragment (Kim *et al.*, 1989). When an Lrp dimer is bound to a single site, bending angles of about 30° and 26° were calculated for the *XhoI* and *BamHI* fragments relative to the *MluI* fragment, with the bending centre located near the centre of the *XhoI* fragment. However, when Lrp dimers are bound to two sites, bending angles of about 81° and 89° were calculated for the *XhoI* and *BamHI* fragments relative to the *MluI* fragment. These results suggest that when two sites are bound, the bending centre is located closer to the centre of the *BamHI* fragment than the *MluI* or *XhoI* fragments, near what corresponds to the promoter-proximal end of the Lrp-binding region (Fig. 2b). Lrp binds DNA in a highly cooperative manner (Wang & Calvo, 1993). Higher concentrations of Lrp in the gel-shift assay resulted in multiple bands of altered

Fig. 3. Lrp binding to the *gcv* control region induces DNA bending. Three ^{32}P -labelled DNA fragments from pG5281 of identical length were prepared with the Lrp-binding region at various locations. Lanes: 1, *Mlu*I fragment; 2, *Xho*I fragment; 3, *Bam*HI fragment. Lrp was added at a concentration of approximately 2.2 nM, resulting in binding of Lrp dimers to essentially two sites in the DNA. Higher concentrations resulted in multiple bands of slower mobility, making interpretation difficult. The samples were run on a 5% polyacrylamide, 3% glycine gel. The positions of the unbound (U) and two Lrp-retarded (A and B) bands are indicated.

Fig. 4. Hypothetical model for activation and repression of the *gcv* operon. For simplicity, regulatory proteins are represented as circles. An Lrp-induced DNA bend facilitates GcvA bound at sites 3 and 2 to interact with RNAP and activate transcription. GcvA binding to sites 1, 2 and 3 plus the GcvR protein are required for repression. Whether GcvR interacts directly with DNA or with GcvA to mediate repression is unknown.

mobility and it was difficult to predict with certainty which bands resulted from various multimers of the Lrp protein. Thus, we did not attempt to analyse the degree of bending in these higher-order complexes. However, it is clear that Lrp bends the DNA in the *gcv* control region and could play a structural role in *gcv* operon control.

Hypothesis for Lrp and GcvA involvement in *gcv* expression

We propose that the role of Lrp in *gcv* expression is probably one of bending the DNA such that GcvA bound at the upstream sites 3 and 2 can interact either with RNAP to activate *gcv* or possibly with GcvA bound at site 1 to repress *gcv* (Fig. 4). In this model, Lrp plays a structural role and GcvA is the activator protein. DNA bending has been shown to play a role in transcriptional regulation in prokaryotic promoters (Perez-Martin & de Lorenzo, 1997; Werner & Burley, 1997). GcvR is required for repression of the operon (Ghrist & Stauffer, 1995a). Although it is unknown whether its role is direct or indirect, GcvR could influence the degree of bending, or possibly the protein-protein interactions that occur. Bending of the DNA by Lrp might also be part of the mechanism for the artificially induced super-repression observed when GcvA sites 3 and 2 are shifted by half integral turns of DNA. It is conceivable that an Lrp-induced bend might allow GcvA bound on the wrong side of the helix to make an inappropriate contact with RNAP, destabilizing the transcription complex and decreasing promoter activity, or possibly allow GcvA to bind directly to binding site 1, occluding the *gcv* promoter from RNAP binding. We have isolated an *rpoA* mutation, encoding the α subunit of RNAP, that results in a GcvA-dependent decrease in *gcvT::lacZ* expression (A. Jourdan & G. V. Stauffer, unpublished). The isolation of an allele-specific suppressor in *gcvA* that restores activation would support a direct interaction between GcvA and RNAP. In addition, we are replacing the *gcv* Lrp-binding region with the integration host factor binding site I1A from λ phage. Integration host factor binding to this site results in a DNA bend of about 140° (Xin & Feiss, 1993). An integration-host-factor-dependent activation of *gcvT::lacZ* by GcvA in such a construct would support an architectural role for Lrp in bending *gcv* DNA.

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