GcvA interacts with both the α and σ subunits of RNA polymerase to activate the *Escherichia coli* gcvB gene and the gcvTHP operon

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Abstract

The glycine cleavage enzyme system in *Escherichia coli* provides one-carbon units for cellular methylation reactions. The gcvB gene encodes two small RNAs that in turn regulate other genes. The GcvA protein is required for expression of both the gcvTHP (P<sub>gcvT</sub>) and gcvB (P<sub>gcvB</sub>) promoters. However, the architectures of the two promoters are different, with the P<sub>gcvT</sub> promoter representing a class III activator-dependent promoter and the P<sub>gcvB</sub> promoter representing a class II activator-dependent promoter. The RNA polymerase holoenzyme was examined for its role in transcription activation of the gcvTHP operon and the gcvB gene by the GcvA protein. The results suggest that GcvA interacts with the RNA polymerase α subunit for activation of the gcvTHP operon and interacts with the RNA polymerase σ subunit for activation of the gcvB gene.

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Keywords: gcvTHP operon; gcvB gene; GcvA activator protein; RNA polymerase

1. Introduction

In *Escherichia coli* the glycine cleavage (GCV) enzyme system, encoded by the gcvTHP operon and lpd gene, catalyzes the cleavage of glycine into CO₂ + NH₃ and transfers a one-carbon unit (C1) to tetrahydrofolate, producing 5,10-methylenetetrahydrofolate [1]. The gcvB gene encodes two small RNAs that, in turn, regulate other pathways (for example, oppA and dppA, encoding the oligopeptide and dipeptide periplasmic binding proteins, respectively) [2]. Both gcvTHP and gcvB are activated by the GcvA protein in response to glycine and repressed by the GcvA + GcvR proteins in the absence of glycine; this repression is enhanced in the presence of purines [2,3]. GcvR’s ability to repress is dependent on GcvA [4] and genetic and biochemical evidence suggests that GcvR interacts directly with GcvA rather than binding to DNA to cause repression [5,6].

At many promoters, transcription activation occurs through the formation of specific contacts between a DNA-bound activator protein and RNA polymerase (RNAP) [7]. At class I promoters, activators bind upstream of the RNAP binding site [8]. At class II promoters, activators bind to sites that are adjacent to or overlap the RNAP binding sites [9,10]. At class III promoters, multiple activator molecules typically bind more than 90 bps upstream of the RNAP binding site [7,11].

The GcvA protein binds to 3 sites in the gcvTHP control region, from bp −271 to −242 (site 3), from bp −241 to −214 (site 2) and from bp −69 to −34 (site 1) relative to the transcription initiation site (Fig. 1(a)) [12]. All three binding sites are required for
GcvA · GcvR-mediated repression of the gcvTHP operon, whereas only sites 3 and 2 appear to be required for GcvA-mediated activation of the operon in response to glycine [12]. Lrp, a global regulator for many genes involved in amino acid metabolism [13], binds to multiple sites upstream of the P\textsubscript{gcvT} promoter, from bp –92 to –229 (Fig. 1(a)) and plays a structural role in gcvTHP regulation, bending the DNA to allow GcvA to function as either an activator or repressor [14]. The cAMP receptor protein (CRP) binds to a site from bp –299 to –326 relative to the transcription initiation site (Fig. 1(a)) and is required for a 4-fold activation of the gcvTHP operon in glucose minimal (GM) medium [15]. Thus, P\textsubscript{gcvT} is a class III promoter. The gcvB promoter is divergently transcribed from and overlaps the gcvA promoter [2,12]. GcvA binds to a single region in the gcvB control region from bp –29 to –76 relative to the transcription initiation site (Fig. 1(b)) [12], and binding of GcvA to this region is required for both GcvA-mediated activation in the presence of glycine and GcvA · GcvR-mediated repression in the absence of glycine [16]. Thus, P\textsubscript{gcvB} is a class II promoter.

Because of the different architectures of the P\textsubscript{gcvT} and P\textsubscript{gcvB} promoters, we tested if GcvA interacts with different subunits of RNAP at these two promoters. Our results are consistent with GcvA interacting with the RNAP α subunit to activate P\textsubscript{gcvT} and interacting with the RNAP σ subunit to activate P\textsubscript{gcvB}.

2. Materials and methods

2.1. Strains and plasmids

The E. coli strains and plasmids used in this study are listed in Table 1. Strain CAG20207 carries the \(\Omega(\text{Cm}\textsuperscript{R})\text{ptrp-rpoD}\) fusion and was provided by Dr. C. Gross. Plasmids encoding wild-type (WT) \(\sigma\textsuperscript{70}\) and alanine substitutions in the \(\sigma\textsuperscript{70}\text{CTD} in residues 590 through 613 [9] are variants of pGEX-2T in which the coding region of glutathionine S-transferase is fused to RpoD at amino acid 8 and expressed from the tac promoter and were provided by Dr. C. Gross.

2.2. Phage

The \(\lambda\text{gcvT::lacZ}\) translational fusion phage includes 466 bp upstream and 291 bp downstream of the P\textsubscript{gcvT} promoter.

### Table 1

| Strains/plasmids | Relevant genotypes | Source/references |
|------------------|--------------------|-------------------|
| Strains*         |                    |                   |
| CAG20207         | \(\Omega(\text{Cm}\textsuperscript{R})\text{ptrp-rpoD}\) | Dr. C. Gross [9]   |
| GS162            | WT                 | This laboratory    |
| GS986            | gcvA \textsuperscript{3}parR::Tn10 | This laboratory    |
| GS1053           | gcvR::Tn10         | This laboratory    |
| GS1142           | GS162 + \(\Omega(\text{Cm}\textsuperscript{R})\text{ptrp-rpoD}\) | This study         |
| Plasmids         |                    |                   |
| pGEX-2T\textsuperscript{3} WT (and derivatives) | \textsuperscript{R}rpoD WT (and derivatives with alanine substitutions at residues 590, 591, 593, 595–600, 602–605, 608, 612 and 613) | Dr. C. Gross [9]   |
| pREI\textsuperscript{H} | Multi-copy plasmid carrying WT rpoA controlled by the lpp-lacPUV5 promoter | [28] |
| pGS490           | rpoA::Δ239; amber stop at codon 239 in rpoA of pREI\textsuperscript{H} | [24] |
| pGS491           | rpoA::Δ245; amber stop at codon 245 in rpoA of pREI\textsuperscript{H} | [24] |
| pGS255           | Low-copy plasmid carrying WT gcvA | [29] |

* All strains except CAG20207 also carry \(\lambda(\text{argF-lac})\U169, \text{phe}A905, \text{thi}, \text{araD}129, \text{rps}L150, \text{rel}A1, \text{deo}C1, \text{fb}b301, \text{pts}F25 and \text{rps}R\) mutations.
transcription initiation site fused in phase with the lacZYA genes [17]. The λgcvB::lacZ transcriptional fusion phage includes 150 bp upstream of the P_{gcvB} transcription initiation site fused at bp +50 within gcvB to translationally competent lacZYA genes [2]. The λgcvA::lacZ translational fusion phage includes 231 bp upstream and 218 bp downstream of the gcvA transcription initiation site fused in phase with the lacZYA genes [18]. The λgcvR::lacZ translational fusion phage includes 348 bp upstream and 132 bp downstream of the gcvR transcription initiation site fused in phase with the lacZYA genes [19]. Each fusion carries all of the known regulatory sites for the respective promoters. These phage were used to lysogenize various host strains and each lysogen was tested to ensure that it carried a single-copy of the λ chromosome by infection with λc190c17 [20]. Lysogens were grown at 30 °C since all of the fusion phage carry the λc1857 mutation, resulting in a temperature sensitive repressor. Strains carrying the Ω(Cm)\textsuperscript{R}trp-rpoD allele were difficult to lysogenize directly with the λ fusion phage. Therefore, single-copy lysogens were first constructed in strain GS162 and the Ω(Cm)\textsuperscript{R}trp-rpoD allele introduced into the lysogens by P\textsubscript{1}C\textsubscript{hr} transduction as described [21] and are designated as GS1142 derivatives.

2.3. Media

The complex medium used was Luria–Bertani broth (LB) [21]. The GM medium used was the salts of Vogel and Bonner [22] supplemented with 0.4% glucose. Supplements were added at the following concentrations in μmol\textsuperscript{-1}: glycine, 300; phenylalanine, 50; inosine, 50; thiamine, 1. GM medium was always supplemented with phenylalanine and thiamine since all strains carry the pheA905 and thi mutations.

2.4. β-Galactosidase assays

Cells were grown in the media indicated in the text to an OD\textsubscript{600} of ~0.5, placed on ice for 20 min and β-galactosidase levels determined using the chloroform–SDS lysis procedure [21]. All assays were performed at least twice and the activity of each sample was determined in triplicate.

3. Results

3.1. Effects of deletions of the αCTD on P_{gcvT} and P_{gcvB} expression

The P_{gcvT} promoter shows characteristics of a class III promoter, whereas the P_{gcvB} promoter shows characteristics of a class II promoter (Fig. 1(a) and (b)). Despite the different architectures of the two promoters, a region of GcvA defined by the gcvA::F31L mutation in the helix-turn-helix (H–T–H) region is required for GcvA-mediated activation of both P_{gcvT} and P_{gcvB} [2,23]. Since GcvA contacts the αCTD to activate P_{gcvT} [24], we tested if the αCTD is also required for activation of P_{gcvB}. The lysogens GS1053, gcvT::lacZ and GS1053, gcvB::lacZ were transformed with plasmid pREI\textsubscript{λ}, encoding a WT RNAP α subunit, and plasmids pGS490 and pGS491, encoding deletions of the αCTD from amino acid residue 239 (RpoA\textsubscript{Δ239}) and 245 (RpoA\textsubscript{Δ245}), respectively. We used the gcvR mutant strain GS1053 for these studies to ensure that any decrease in gcvT::lacZ or gcvB::lacZ repression could not be attributed to the GcvA·GcvR repression system. The transformed cells were grown in LB + ampicillin (Ap) to mid-log phase of growth and assayed for β-galactosidase activity. As reported previously, GS1053, gcvT::lacZ transformed with plasmids pGS490 and pGS491 displayed about 2-fold lower β-galactosidase levels compared to the transformant encoding the WT α subunit (Fig. 2(a)). Although the effects of the rpoA mutations were not dramatic on GcvA activation of the gcvT::lacZ fusion, this is not unexpected as similar results have been reported in other studies for single amino acid changes and deletions of the αCTD [25]. GS1053, gcvB::lacZ transformed with plasmids pGS490 and pGS491 displayed β-galactosidase levels essentially the same as the transformant encoding the WT α subunit (Fig. 2(b)). These results suggest that the αCTD is required for activation of gcvT::lacZ, but is not required for activation of gcvB::lacZ.

![Fig. 2. Effects of deletions of the CTD of the α subunit of RNAP on gcvT::lacZ and gcvB::lacZ expression. The gcvB strain GS1053, lysogenized with either a λgcvT::lacZ or a λgcvB::lacZ fusion, was transformed with pREIλ, encoding WT α, or plasmids pGS490 and pGS491, encoding deletions of the αCTD. The transformants were grown in LB + Ap and assayed for β-galactosidase activity [21]. Activity is expressed in Miller units. (a) Results with the λgcvT::lacZ lysogen. (b) Results with the λgcvB::lacZ lysogen.](https://academic.oup.com/femsle/article-abstract/242/2/333/687664)
3.2. Effects of changes in the $\sigma^{70}$ CTD on $P_{gcvT}$ and $P_{gcvB}$ expression

Since the $\sigma$CTD is required for GcvA-mediated activation of $P_{gcvT}$, but not for GcvA-mediated activation of $P_{gcvB}$, it was of interest to examine whether the RNAP $\sigma^{70}$ subunit is required for activation of either $P_{gcvT}$ or $P_{gcvB}$. The $\Omega(Cm^{R})$ ptp–rpoD fusion was introduced into lysogens GS162$\Delta gcvT::lacZ$ or GS162$\Delta gcvB::lacZ$ by P1chr transduction, generating lysogens GS1142$\Delta gcvT::lacZ$ and GS1142$\Delta gcvB::lacZ$, respectively. In these lysogens, the chromosomal rpoD gene is under the control of the trp promoter to minimize expression of endogenous $\sigma^{70}$ [9]. These strains were transformed with pGEX-2TrpoD encoding the WT $\sigma^{70}$ and with pGEX-2T derivative plasmids encoding alanine substitutions in the $\sigma^{70}$CTD. Lysogen GS1142$\Delta gcvT::lacZ$ and GS1142$\Delta gcvB::lacZ$ transformed with pGEX-2T$\sigma^{70}$ WT and the alanine substitutions were grown in GM + glycine + Ap + Chloramphenicol (Cm) to mid-log phase of growth and assayed for $\beta$-galactosidase activity [21]. Lysogen GS1142$\Delta gcvB::lacZ$ transformed with pGEX-2T carrying the alanine substitutions E591A, L595A, H600A, S602A and R603A showed 1.5- to 2-fold reduced levels of $\beta$-galactosidase expression compared to the transformant carrying WT $\sigma^{70}$ (Fig. 3(b)). The remaining substitutions in rpoD had no significant effect on $gcvB::lacZ$ expression. The small effects of the rpoD mutations on GcvA-mediated activation of the $gcvB::lacZ$ fusion are not unexpected, as similar results have been reported in other studies for single amino acid changes in rpoD [9,26]. In vivo assays of the effect of alanine substitutions in $\sigma^{70}$CTD are difficult. The mutant forms of $\sigma^{70}$ must compete with the WT $\sigma^{70}$ for incorporation into the RNAP holoenzyme. Although the uninduced plasmid encoded pGEX-2T$\sigma^{70}$ WT levels and the levels of the alanine substitutions are equivalent to the expression levels of the chromosomal allele, it is likely that the mutant $\sigma^{70}$ proteins with alanine substitutions are unable to compete effectively with the WT $\sigma^{70}$ for incorporation into the RNAP holoenzyme [9,26]. Thus, although the rpoD mutations did not have dramatic effects on GcvA-mediated activation in vivo, the results suggest that several residues in the $\sigma^{70}$CTD are likely to be important for GcvA-mediated activation of $P_{gcvB}$. GS1142$\Delta gcvT::lacZ$ transformed with pGEX-2T carrying alanine substitutions in $\sigma^{70}$ displayed $\beta$-galactosidase levels essentially the same as the transformant encoding the WT $\sigma^{70}$ subunit (Fig. 3(a)). These results suggest that the $\sigma$CTD is not required for activation of $gcvT::lacZ$.

3.3. Reduced $gcvB::lacZ$ expression in the rpoD mutants is not due to increased GcvR levels or decreased GcvA levels

Activation of $gcvB$ requires a functional GcvA protein and repression requires a functional GcvR protein [2]. It is possible that the $\sigma^{70}$CTD mutants that reduce $gcvB::lacZ$ expression either decrease the levels of GcvA or increase the levels of GcvR. Either condition would be expected to decrease $gcvB::lacZ$ expression. Although one might expect such changes would also alter $gcvT::lacZ$ expression, it is possible a lower concentration of GcvA is required for activation of the $gcvT::lacZ$ fusion or that a higher concentration of GcvR is required for repression of the $gcvT::lacZ$ fusion. Thus, the $\Omega(Cm^{R})$ ptp–rpoD fusion was introduced into lysogens GS162$\Delta gcvB::lacZ$ and GS162$\Delta gcvA::lacZ$ by P1chr transduction, generating lysogens GS1142$\Delta gcvA::lacZ$ and GS1142$\Delta gcvR::lacZ$. The lysogens were transformed with pGEX-2T$\sigma^{70}$ WT and with pGEX-2T derivative plasmids encoding alanine substitutions in the $\sigma^{70}$CTD (E591A, L595A, H600A, S602A and R603A). The transformants were grown in GM + glycine + Cm + Ap to mid-log phase of growth.

**Fig. 3.** Effects of alanine substitutions in the $\sigma^{70}$CTD on chromosomal $gcvT::lacZ$ and $gcvB::lacZ$ expression. The $\Omega(Cm^{R})$ ptp–rpoD strain GS1142, lysogenized with either a $\lambda gcvT::lacZ$ fusion (a) or a $\lambda gcvB::lacZ$ fusion (b), was transformed with plasmid pGEX-2T$\sigma^{70}$ encoding WT $\sigma^{70}$ or alanine substitutions of the $\sigma^{70}$CTD, cells were grown in GM + glycine + Ap + Cm and assayed for $\beta$-galactosidase activity [21]. Gaps in the amino acid positions indicate that these positions are alanine in the native protein. The activities of the lysogens transformed with plasmids encoding WT $\sigma^{70}$ were set at 100%, and the activities of the mutants are given as percentages relative to the WT activity. Activity of GS1142$\Delta gcvT::lacZ$ transformed with a plasmid encoding WT $\sigma^{70}$ was 778 ± 48 Miller units and the activity of GS1142$\Delta gcvB::lacZ$ transformed with a plasmid encoding WT $\sigma^{70}$ was 202 ± 12 Miller units.
Discussion

P

is a class III activator-dependent promoter (Fig. 1(a)) and P

is a class II activator-dependent promoter (Fig. 1(b)). Activators for class III promoters generally make a protein–protein interaction with the σCTD of the RNAP [7,11], whereas activators for class II promoters generally make protein–protein interactions with the σ70 subunit of the RNAP and often with the α subunit as well [9,10]. Results presented here show that although the RpoAΔ239 and RpoAΔ245 deletions of the σCTD result in about 2-fold reduced levels of expression of a gcvT::lacZ fusion, the deletions have no significant affect on expression of a gcvB::lacZ fusion (Fig. 2(a) and (b)). In addition, a genetic screen of >15,000 PCR mutagenized ropA transformants failed to identify any amino acid in the σ subunit necessary for GcvA activation of gcvB::lacZ. Thus, the GcvA–σCTD interaction required for GcvA activation of the P

promoter is not required for GcvA activation of the P

promoter. The fact that Lrp and CRP are required for full activation of P

[14,15] complicates interpretation of the results. However, evidence suggest that Lrp’s role is structural, bending the P

DNA to allow a GcvA–RNAP interaction [14] and CRP’s role is to antagonize GcvA-mediated repression [15]. Thus, although it is possible that part of the effects of the mutations could be mediated through Lrp and CRP, the results are more consistent with a GcvA–σCTD interaction likely being required for GcvA-mediated activation at the P

promoter.

Five σ70CTD mutants, E591A, L595A, H600A, S602A and R603A, were found to be important for P

expression, resulting in 1.5- to 2-fold reduced levels of expression of a gcvB::lacZ fusion (Fig. 3(b)). However, none of the alanine substitutions in RpoD had a significant effect on gcvT::lacZ expression (Fig. 3(a)). These results suggest that a GcvA–σ70 interaction is likely required at the P

promoter, but is not required for GcvA activation of the P

promoter. Although decreased GcvA levels or increased GcvR levels could explain the decreased levels of gcvB::lacZ expression, none of the mutations that resulted in decreased gcvB::lacZ expression had a significant effect on either gcvR::lacZ or gcvA::lacZ expression (Fig. 4(a) and (b)). Thus, the decreases in gcvB::lacZ expression observed are likely due to direct effects of the alanine substitutions in the σ70 subunit preventing necessary GcvA–σ70CTD interactions required for activation rather than indirect effects of the mutations altering the levels of the GcvA and GcvR regulatory proteins.

Previous genetic results showed that a region of GcvA that is required for activation of P

defined by the gcvA-F31L positive control mutation, is also required for activation of the P

promoter [2,23]. Modeling studies suggest that the side chain of residue F31 of GcvA is surface exposed, does not interact directly with DNA, and is capable of interacting directly with the σCTD [27]. If the amino acid at position 31 defines a region of GcvA that interacts with RNAP, then the results from this study are consistent with a hypothesis where this region interacts with the RNAP σ subunit at a class III promoter (P

) and interacts with the RNAP σ subunit at a class II promoter (P

). We are constructing and testing...
additional mutations in the GcvA H–T–H region to determine if there are other amino acids in GcvA that are required for activation of both the P_{gcvT} and P_{gcvB} promoters, or are specific for only one of the promoters. Characterization of additional mutations and in vitro transcription assays showing the importance of the RNAP subunits in transcription from the respective promoters will provide results necessary to confirm the above model.

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