Interaction at a Distance between Multiple Operators Controls the Adjacent, Divergently Transcribed \textit{glpTQ-glpACB} Operons of \textit{Escherichia coli} K-12*

(Received for publication, May 2, 1991)

Timothy J. Larson†, John S. Cantwell, and Ali T. van Loo-Bhattacharya

From the Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0308

---

The \textit{glp} regulon of \textit{Escherichia coli} encodes the proteins required for utilization of \textit{sn}-glycerol 3-phosphate and its precursors. Transcription of the divergently transcribed \textit{glpTQ} and \textit{glpACB} operons is initiated at sites separated by 132 base pairs (bp) of DNA. These operons are controlled negatively by \textit{glp} repressor and positively by the cAMP-cAMP receptor protein (CRP) complex. The locations of the binding sites for the \textit{glp} repressor and for cAMP-CRP in the control regions of these operons were determined by DNase I footprinting. Binding of the \textit{glp} repressor protected the region -32 to -51 (O_1) in the \textit{glpTQ} promoter, which was also the binding site for cAMP-CRP. Four repressor binding sites (-41 to -60 (O_2), -9 to -28 (O_3), +12 to +8 (O_4), and +52 to +35 (O_5)) and two cAMP-CRP binding sites (-11 to -11 and -30 to -51) were found in the \textit{glpACB} promoter region. Comparison of the sequences of the repressor binding sites found in the \textit{glpTQ-glpACB} control region with those operators previously described in the \textit{glpD} operon allowed formulation of a consensus operator sequence which was the palindrome 5'-WATGTTCCGWTAWC-GAACATW-3' (W is A or T). The role of each operator was assessed by measuring repression in constructs where individual operators were altered by site-directed mutagenesis. Alteration of O_1 did not significantly decrease repression of either operon. Each of the \textit{glpACB} operators contributed to repression of both operons. These results suggest involvement of \textit{glpACB} operator(s) in control of \textit{glpTQ} expression perhaps via formation of a repression loop. Evidence supporting this hypothesis was obtained by measuring the degree of repression of the \textit{glpTQ} promoter in constructs containing 6- or 10-bp insertions between the \textit{glpTQ} and \textit{glpACB} operators. A 6-bp insertion located within O_2 or between O_2 and O_1 eliminated repression of the \textit{glpTQ} promoter, whereas significant repression was maintained in the case of a 10-bp insertion within O_2.

---

The genes of the \textit{glp} regulon of \textit{Escherichia coli} encode the proteins needed for the utilization of glycerol, \textit{sn}-glycerol 3-phosphate (glycerol-P),¹ and glycerophosphodiesters (Fig. 1; Lin, 1976, 1987). These genes are arranged in five different operons positioned at three different locations on the chromosome of \textit{E. coli}. The \textit{glpTQ} operon encodes glycerol-P permease (Eigtmeier et al., 1987) and glycerophosphodiesterase (Tommassen et al., 1991). This operon is located near minute 49 on the linkage map and is transcribed in the counterclockwise direction. The \textit{glpACB} operon encodes the subunits of the anaerobic glycerol-P dehydrogenase (Cole et al., 1988). This operon is directly adjacent to and is transcribed divergently from the \textit{glpTQ} operon. The \textit{glpFK} operon encodes glycerol diffusion facilitator and glycerol kinase (Lupski et al., 1990; Sweet et al., 1990). The operon is located near minute 88 and is transcribed in the counterclockwise direction. The \textit{glpD} gene encodes aerobic glycerol-P dehydrogenase (Austin and Larson, 1991). This gene is located near minute 75 and is transcribed in the clockwise direction, divergently from \textit{glpEGR}. The \textit{glpR} gene encodes the \textit{glp} repressor, and \textit{glpE} and \textit{glpG} encode protein of unknown function (Schweizer et al., 1986; Schweizer and Larson, 1987).

The \textit{glp} operons are controlled transcriptionally in response to three types of environmental stimuli (Lin, 1987). Specific negative regulation is mediated by the \textit{glp} repressor. Its affinity for operator sites on DNA is decreased in the presence of glycerol-P, the inducer for the regulon. Results of studies employing a strain harboring a thermolabile repressor indicated that the order of sensitivity of the \textit{glp} operons to control by the \textit{glp} repressor is \textit{glpD > glpT > glpK} (Freedberg and Lin, 1973). The \textit{glp} operons are also subject to catabolite repression during growth in the presence of glucose, presumably because transcription is positively influenced by the cAMP-cAMP receptor protein (CRP) complex. The order of sensitivity of the operons to catabolite repression is opposite that observed for control by \textit{glp} repressor (Lin, 1987).

Expression of the operons encoding the glycerol-P dehydrogenases is sensitive to the respiratory state of the cell. Anaerobic glycerol-P dehydrogenase activity is maximal during anaerobic growth with fumarate present as electron acceptor. Under these conditions, the FNR protein has a positive influence on the transcription of genes involved in anaerobic respiration, including the \textit{glpACB} operon (Kuritzkes et al., 1984; Iuchi et al., 1990). Aerobic glycerol-P dehydrogenase activity is maximal under well oxygenated growth conditions. Anaerobiosis results in repression of the \textit{glpD} operon by the \textit{arcA/arcB}-encoded two-component regulatory system (Iuchi et al., 1990).

---

¹The abbreviations used are: glycerol-P, \textit{sn}-glycerol 3-phosphate; kb, kilobase pair(s); bp, base pair(s); CRP, cyclic AMP receptor protein; GlpR, \textit{glp} repressor protein; FNR, anaerobic activator protein.
The gfp represor has been overproduced and purified to near homogeneity in an active form which binds both inducer and operator DNA (Larson et al., 1987). The represor is tetrameric under native conditions and contains four identical 30-kDa subunits. The represor binding sites in the control region for the gfpD operon have been identified by DNase I footprinting (Ye and Larson, 1988). The represor binds to tandemly repeated operators of hyphenated dyad symmetry located just downstream from the start site of transcription. The present work was undertaken in order to elucidate molecular details of represor-mediated regulation of the divergently transcribed gfpTQ-gfpACB operons.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and DNA ligase were obtained from Boehringer Mannheim GmbH or New England Biolabs. Sequenase, Klenow fragment of DNA polymerase I, and the DNA sequencing kit were obtained from U.S. Biochemicals. Du Pont-New England Nuclear supplied [α-32P]dATP and [α-35S]dGTP. Cyclic AMP, o-nitrophenyl β-D-galactopyranoside, isopropyl-1-thio-β-D-galactopyranoside, p-nitrophenyl phosphate, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), 5-bromo-4-chloro-3-indolyl phosphate (X-P), and α-naphthyl phosphate were from Sigma. Casamino acids and maltose were from Difco Laboratories. Oligonucleotides were synthesized on an Applied Biosystems Model 381A synthesizer and purified by OPC (Applied Biosystems) as recommended by the manufacturer. A phosphorylated BglII linker (5’-pCATGATCTG-3’) was obtained from New England Biolabs. The gfp represor was purified to homogeneity as described previously (Larson et al., 1987). Cyclic AMP receptor protein (CRP) was purified to near homogeneity from strain N4830 (Harman et al., 1986). The conditions for induction of CRP synthesis were those described earlier for overproduction of the gfp represor (Larson et al., 1987). After incubation at 42°C for 5 h, a crude extract was prepared, and polyethyleneimine and ammonium sulfate fractionation were performed as described by Blazy and Ullmann (1986), followed by phosphocellulose chromatography (Larson et al., 1987). Fractions from the phosphocellulose column which contained the bulk of CRP activity were concentrated by ammonium sulfate precipitation. Chromatography on a Sephadex G-200 column (1.5 × 50 cm) developed with 0.05 M potassium phosphate (pH 7.5), 0.2 M KCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol resulted in a preparation that was nearly homogeneous as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The specific activity of the final product was 20 pmol of cAMP bound per mg of protein. Protein concentrations were estimated using the method of Bradford (1976) with bovine serum albumin as standard.

**Bacterial Strains and Growth Media**—LB medium (Miller, 1972) with 0.2% glucose and 100 µg/ml ampicillin was used for growth of bacteria prior to isolation of plasmid DNA. Strain JM110 [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 (lac-proAB) [F’ traD36 proB’ lacI258 lacZM15] (Yanisch-Perron et al., 1985) was used as host during construction of recombinant plasmids. Selection of the formants was on plates of LB medium supplemented with 100 µg/ml ampicillin, 40 µg/ml X-Gal, and 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Strain DH5αF’ [F’ 809lacZM15 recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 (lac-proAB)] (Liss, 1987) was used for propagation of recombinant M13 phase. Strain N4830 (F’ his strr lacI48 recA38::320cI857 ΔBAD ΔHflI) (Adhya and Gottesman, 1982) harboring pCRP-1 (Harmar et al., 1986) was used as the source of CRP. Strain TL73 (MC4100 gfpR2 recA1 (Larson et al., 1982)) harboring either pHS58 (gfpR+) or pACYC184 (the vector for cloning of gfpR+ in pSH58) was used to assess the affect of the gfp represor on various gfpT-phoA or gfpA-lac2 transcriptional fusions. Cells growing logarithmically on AB minimal medium (Clark and Maaske, 1967) supplemented with 0.2% maltose, 0.1% casamino acids, 30 µg/ml chloramphenicol, and 100 µg/ml ampicillin were used for these experiments. For aerobic growth, 5-ml cultures were incubated in tubes (18 × 150 mm) on a roller drum. For anaerobic growth, cells were grown in sealed tubes filled to the top starting with a 1 to 2% inoculum of a culture growing aerobically.

**Isolation of DNA**—For quick screening, plasmid DNA was isolated from 1.5-ml cultures using a rapid technique (Rodriguez and Tait, 1983). DNA was also isolated from 100-ml cultures using a protocol outlined by Promega. This DNA was used for sequencing reactions and for preparation of radiolabeled DNA. M13 single-stranded DNA was prepared by a procedure described in the Sequenase manual (U.S. Biochemicals).

**Sequencing of DNA**—Double-stranded plasmid or single-stranded M13 DNA was used as template for sequencing reactions carried out by the dideoxy chain termination technique (Sanger et al., 1977) using the Sequenase kit (U.S. Biochemicals).
A plasmid (pSH58) carrying the wild-type glpR gene was constructed by cloning of a 2.7-kb BglII fragment from pSH21 (Schweizer et al., 1985) into the BamHI site of pACYC184 (Chang and Cohen, 1978).

Details Footprinting—DNase I footprinting was performed using the conditions previously described (Ye and Larson, 1988), with details provided in the legends to the figures. Two sets of experiments were done, in which the DNA fragment was radiolabeled at either end. End-labeled DNA was prepared by digestion of pATHN with either BamHI or EcoRI followed by incubation with Sequenase in the presence of [α-32P]dATP and [α-32P]dGTP (for BamHI-cleaved DNA) or [α-32P]dATP (for EcoRI-cleaved DNA). A second digestion with the other enzyme followed by purification on an agarose gel resulted in 288-base pair fragments where the label was incorporated 11 and 12 bases away from the NruI site at the beginning of glpA, or, in the other case, 5 and 6 base pairs away from the HaeIII site near the beginning of glpT.

Oligonucleotide-directed Mutagenesis—The technique of Kunkel et al. (1987) was used for making specific alterations in the nucleotide sequence of the control region of the glpTQ-glpACB operons. Single-stranded M13 templates containing uracil were generated by three cycles of propagation in strain RZ1032 (dat1 ung1). The mutagenic oligonucleotides used are described in Figs. 2 and 3.

Construction of Insertion and Operator Mutations—Insertion mutations in the glpTQ-glpACB control region of pATHN were constructed as shown in Fig. 2. A 2-bp insertion at the unique BstBI site was generated by sequential treatment of pATHN with BstBI, SceI, and T4 DNA ligase, BglII, and T4 DNA ligase. The resulting plasmid (pATHN + 2) contained a unique NruI site in place of the original BstBI site. A 10-bp insertion at this position was generated by sequential treatment of pATHN + 2 with NruI, 8-bp BglII linkers, and T4 DNA ligase. The resulting plasmid (pATHN + 10) contained a unique BglII site. A 4-bp deletion at the BglII site of pATHN + 10 was created using oligonucleotide-directed mutagenesis. The HindIII-BamHI fragment of pATHN + 10 was cloned into M13mp19 for this purpose. The desired clone contained a unique Spel site and a 6-bp insertion relative to the wild type. The HindIII-BamHI fragment was recloned to pBluescript (pATHN + 6).

Mutations altering each of the operators were generated using oligonucleotide-directed mutagenesis (Fig. 3). Where convenient, operators were altered with simultaneous creation of restriction sites to facilitate identification of the desired clones. A construct containing a 6-bp insertion between O2 and O1 was generated using the same technique (AT + 6; Fig. 3). M13mp19 containing the HindIII-BamHI fragment of pATHN served as the uracil-containing template in the mutagenesis reactions. The nucleotide sequence of the control region of the glpTQ-glpACB operons was determined in each case to verify the presence of the desired changes.

To assess the effects of the above mutations on expression of the glpTQ-glpACB operons, the HindIII-BamHI fragments from the wild type and 10 mutant derivatives were cloned into the promoter-probe vector pCB267 (Schneider and Beck, 1987). This vector contains promoterless, divergently arranged phoA and lacZ genes with an intervening cloning site. The resulting plasmids were named pATCB, pATCB01M, pATCB01M, pATCB02M, pATCB04M, and pATCB04P. pATCB + 6, pATCB02 + 2, pATCB02, + 6, and pATCB02 + 10. In each case, β-galactosidase activity was controlled by a glpA-lacZ transcriptional fusion, and alkaline phosphatase expression was controlled by a glpT phoA transcriptional fusion. The structure of each plasmid was verified by restriction analysis, and the presence of the desired insert in each was confirmed by nucleotide sequence analysis of the entire HindIII-BamHI insert using a primer complementary to the 5′ end of the lacZ gene.

Enzyme Assays—β-Galactosidase activity was determined on cells permeabilized using chloroform and sodium dodecyl sulfate as described by Miller (1972). The absorbance at 420 nm was determined after removal of cells by centrifugation. Specific activities are expressed in Miller units and were determined by duplicate or triplicate assays on each batch of cells.

RESULTS

Identification of the Binding Sites for the glp Repressor in the glpTQ-glpACB Control Region—The control region for the divergently transcribed glpTQ-glpACB operons has been localized to a 272-bp region separating the two initiation codons (Eigleimer et al., 1987; Cole et al., 1988). Most of this region is contained on a 268-bp NruI-HaeIII restriction fragment. This fragment was employed in DNase I footprinting studies in order to locate the binding sites for the glp repressor and CAMP-CRP. Separate experiments were done where the fragment was radiolabeled on either end so the distance between

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>Wild-type Sequence</th>
<th>Control operator</th>
<th>wild type</th>
<th>Mutant operator</th>
<th>Mutant control</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-mgl oligo (27-mer)</td>
<td>AATCCAGGGTCATTGCGCCCAATY</td>
<td>AATCCAGGGTCATTGCGCCCAATY</td>
<td>野生</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5-mgl oligo (29-mer)</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>野生</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-mgl oligo (36-mer)</td>
<td>AATCCAGGGTCATTGCGCCCAATY</td>
<td>AATCCAGGGTCATTGCGCCCAATY</td>
<td>野生</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-mgl oligo (44-mer)</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>野生</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-mgl oligo (46-mer)</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>野生</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5-mgl oligo (36-mer)</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>野生</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5-mgl oligo (44-mer)</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>野生</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5-mgl oligo (46-mer)</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>TTCGAAAGATATCAATGCATGGATTA</td>
<td>野生</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Construction of insertion mutations in the glpACB-glpTQ control region. The region surrounding the unique BstBI site of pATHN is shown.

Fig. 3. Oligonucleotide-directed mutagenesis of the glpACB operators. The mutagenic oligonucleotides, the wild type sequences, and the operator consensus sequence are shown for each construct.
the labeled end and the binding sites could be minimized, and the regions protected on either strand could be analyzed.

The results of DNase I footprinting analysis involving purified glp repressor and end-labeled DNA are shown in Fig. 4. Binding of repressor protected several regions from digestion by DNase I. The areas protected are indicated on the nucleotide sequence shown in Fig. 5. It is apparent that there are multiple binding sites for the repressor in the control region for the glpACB operon. The largest area of protection (OA2 and OA3) included the start point of transcription and the −10 region. Protection of a relatively small area in the glpTQ promoter region was observed (Figs. 4 and 5). This region coincided with the binding site for cAMP-CRP. Binding of the glp repressor to all of these areas was greatly decreased when the inducer (glycerol-P) was included in the footprinting reactions (Fig. 4A).

The footprinting data were used to estimate the relative binding affinity of the repressor for the various operator sites. Repressor had the highest apparent affinity for OA1, OA2, and the tandem OA2-OA3 pair, and lower affinity for OA4. Protection of the high affinity operators was complete at a repressor concentration of 0.4 or 0.5 nM. Significant protection of OA4 did not occur until 5 nM or 20 nM repressor was used in the footprinting analysis (Fig. 4).

Cleavage by DNase I of a region of DNA between OA1 and OA2 was enhanced by binding of repressor (Fig. 4A, arrow). These results, along with results to be presented below, indicate that the DNA in this region may be bent or looped when bound to repressor.

Identification of the Binding Sites for the cAMP-CRP Complex—Expression of the glpTQ operon is dependent upon the presence of the cAMP-CRP complex. A sequence similar to the consensus sequence for interaction with cAMP-CRP was previously identified (Eiglmeier et al., 1987). CRP binding to this region was demonstrated directly by performing DNase I footprinting experiments. The results, shown in Fig. 4 and summarized in Fig. 5, show that CRP does indeed bind to the proposed site. Binding was dependent upon the presence of cAMP. The CRP site was centered 41–42 bp upstream from the start point of transcription of the glpTQ operon.

Two additional binding sites for CRP were discovered in the promoter region for the glpACB operon using this analysis (Figs. 4 and 5). One was centered 40–41 bp upstream from the first start point of transcription of the glpACB (Eiglmeier et al., 1989). This site coincides with the region predicted to interact with FNR (Eiglmeier et al., 1989). It is not surprising that FNR and CRP bind to the same site, because these two related transcription factors recognize very similar DNA sequences (Zhang and Ebright, 1990). The other CRP site identified by footprinting is centered 2–3 bp downstream from the start point of transcription documented by Eiglmeier et al. (1989; Fig. 5). Binding of cAMP-CRP to both of these areas was abolished when cAMP was omitted from the footprinting reactions.

Binding of CRP to each of its sites resulted in enhanced sensitivity of the DNA to cleavage by DNase I within the CRP binding sites. This is especially true for the sites labeled CT and CA1 (Fig. 4). The enhanced sensitivity is likely due to CRP-induced bending of the DNA (Kim et al., 1989; Schultz et al., 1991).

Comparison of the glp Operator Sites—Inspection of the sequences present in the regions protected by the glp repressor revealed that these sequences could be aligned with the previously identified tandem operators of the glpD operon (Ye and Lerson, 1988). By comparison of all 14 half-sites, the consensus half-site 5′-WATGTTCGWT-3′ (W = A or T) for repressor binding was derived (Fig. 6). The various operators matched the consensus operator sequence to varying degrees. It is interesting to note that the operators encoding the dehydrogenases are controlled by tandem operators and that the operator that matches the consensus most closely overlaps the start point of transcription in both cases. A single operator was found in the glpTQ promoter region overlapping directly the CRP site. The glpTQ operator did not match the consensus operator as well as most of the other operators.
Fig. 5. Positions of the operator and CRP sites in the glpACB-glpTQ control region. Regions protected by the glp repressor or CRP are indicated by dashes or double dashes, respectively. The -10 sequence and start point of transcription (+1) for each operon are underlined. The sequences of the insertions (+2, +6, and +10) into the BstBI site (TT|CGAA) are indicated for the top strand. The sequence changes present in the operator mutations (O2M, AT +6, O1M, O2M, O3M, O4M, and O4P) are indicated for the bottom strand.

<table>
<thead>
<tr>
<th>Operator</th>
<th>Sequence</th>
<th>Match with Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1M</td>
<td>TATGTTCCGAT ACGGAA CATT</td>
<td>100</td>
</tr>
<tr>
<td>O2M</td>
<td>TATGACCTTT ACGGAA AGTG</td>
<td>70</td>
</tr>
<tr>
<td>O3M</td>
<td>AATGTTCAAA ATGACGCA ATG</td>
<td>65</td>
</tr>
<tr>
<td>O4M</td>
<td>ACTTTTCGAAAT TATGACGCAA</td>
<td>50</td>
</tr>
<tr>
<td>O5M</td>
<td>TATGCGCGAA ATCAAACAAT</td>
<td>75</td>
</tr>
<tr>
<td>O6M</td>
<td>AATGTCGAAAA ACGGAACCTTC</td>
<td>70</td>
</tr>
<tr>
<td>OT</td>
<td>G T G T G G G C A A T T C A C A T T</td>
<td>65</td>
</tr>
</tbody>
</table>

Frequency of " Match with Consensus
Occurrence:

<table>
<thead>
<tr>
<th>Frequency of &quot;</th>
<th>10</th>
</tr>
</thead>
</table>

Half-site:

Consensus: 5'-WATGTTCCGWM-3'  

Fig. 6. Comparison of the glpD and glpACB-glpTQ operator sites. The operator sequences are written in the direction of transcription of the indicated gene. The gap in the center corresponds to the center of operator symmetry.

Effect of Operator Mutations on Repressor Control of glpTQ-glpACB Expression—To determine which of the operators identified by footprinting are important for regulation of the glpTQ-glpACB operons, constructs were generated where the five operators were individually altered. Mutations (O2M, O1M, O2M, O3M, and O4M) were designed so that the sequences resembled less closely the operator consensus, especially at positions located 4 and 7 bases from the center of operator symmetry.2 The specific alterations left the other promoter elements intact (+10 and CRP/TNR site). In a sixth construct, O4 was altered so that it matched the consensus operator sequence perfectly (O4P). DNA fragments harboring the wild type or mutant control regions were cloned into pCB267 (Schneider and Beck, 1987) such that glpTQ promoter activity could be monitored by measuring alkaline phosphatase activity, and glpACB promoter activity could be monitored by measuring β-galactosidase activity. The resulting plasmids were introduced into two strains, one lacking glp repressor (TL73(pACYC184)) and the other (TL73(pSH58)) producing glp repressor from a multicopy plasmid. The efficacy of the glp repressor was assessed by comparing alkaline phosphatase or β-galactosidase activities produced in the presence or absence of the repressor. The activities were measured after growth under aerobic or anaerobic conditions. Anaerobic growth conditions stimulated the glpACB and the glpTQ promoters by more than 10-fold and 5-fold, respectively.

The effects of the operator mutations on repressor-mediated regulation of the glpTQ and glpACB promoters are shown in the top portion of Table I. Alteration of O1 (O1M) did not result in a significant decrease in repression of the glpTQ operon during aerobic growth. Repression of glpTQ was decreased 2-fold during anaerobic growth (Table I). O2M had little influence on repression of the glpACB operon under anaerobic conditions, but increased repression aerobically.

Alterations in the glpACB operators had a very significant effect on regulation of both promoters. The greatest decrease in repression of the glpTQ promoter was observed in the case of the O4M mutation, where repression was almost eliminated. O3 and O4 were also important for regulation of glpTQ, however, as repression decreased relative to the wild type in the strains harboring the mutant constructs. Repression of glpTQ increased when O4 was changed to match the consensus operator perfectly (O4P, Table I). Mutation of O2 decreased repression of glpTQ only slightly. The relative importance of the operators for regulation of glpTQ was 0.1 > O3 > O4 > O2 > O1.

Results for regulation of the glpACB promoter involving the various constructs described above showed that each of the glpACB operators contributed to regulation by glp repressor. O3 was clearly the most critical for regulation of the


### Control of glpTQ-glpaCB Gene Expression in E. coli

#### TABLE I

Repressor control of divergent glpaCB-glpaTQ promoters in wild type and mutant constructs

<table>
<thead>
<tr>
<th>Construct^c</th>
<th>glpaTQ promoter^a</th>
<th>glpaCB promoter^a</th>
<th>glpaTQ promoter^a</th>
<th>glpaACE promoter^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic growth</td>
<td>Anaerobic growth</td>
<td>Aerobic growth</td>
<td>Anaerobic growth</td>
</tr>
<tr>
<td></td>
<td>glpR^+</td>
<td>glpR^*</td>
<td>Repression^a</td>
<td>glpR^+</td>
</tr>
<tr>
<td>Wild type</td>
<td>3.50</td>
<td>0.11</td>
<td>31 (11)</td>
<td>32.3</td>
</tr>
<tr>
<td>O2-M</td>
<td>10.10</td>
<td>0.37</td>
<td>27 (5)</td>
<td>80.4</td>
</tr>
<tr>
<td>O2+1M</td>
<td>2.48</td>
<td>1.06</td>
<td>2 (2)</td>
<td>21.9</td>
</tr>
<tr>
<td>O2+2M</td>
<td>3.27</td>
<td>0.22</td>
<td>15 (5)</td>
<td>11.0</td>
</tr>
<tr>
<td>O2+3M</td>
<td>2.16</td>
<td>0.39</td>
<td>6 (5)</td>
<td>14.0</td>
</tr>
<tr>
<td>O2+4M</td>
<td>3.15</td>
<td>0.31</td>
<td>10 (4)</td>
<td>8.7</td>
</tr>
<tr>
<td>O2+P</td>
<td>3.21</td>
<td>0.07</td>
<td>46 (4)</td>
<td>17.4</td>
</tr>
<tr>
<td>O2+2-6</td>
<td>3.36</td>
<td>0.71</td>
<td>5 (6)</td>
<td>13.6</td>
</tr>
<tr>
<td>O2+2+6</td>
<td>2.60</td>
<td>0.54</td>
<td>1 (3)</td>
<td>5.0</td>
</tr>
<tr>
<td>O2+2+10</td>
<td>4.30</td>
<td>0.63</td>
<td>7 (5)</td>
<td>6.7</td>
</tr>
<tr>
<td>AT+6</td>
<td>3.28</td>
<td>0.46</td>
<td>1 (4)</td>
<td>8.6</td>
</tr>
</tbody>
</table>

^a Promoter activity was monitored by measuring the specific activity of alkaline phosphatase.

^b Promoter activity was monitored by measuring the specific activity of β-galactosidase.

^c pCB267 containing the indicated control region was introduced into strain TL73(pAC71C84) [glpR] or TL73 (pSH58) [glpR+].

^d Repression is defined as the specific activity in the glpR strain divided by the specific activity in the glpR+ strain. Numbers in parentheses indicate the number of independent determinations performed in order to determine mean repression ratios.

glpaCB operon particularly under anaerobic conditions. During anaerobiosis, there was a striking increase in repression mediated by glp repressor. The relative importance of the operators for regulation of glpaCB under anaerobic conditions was O3 > O4 > O2 > O1. The same trend was observed under aerobic conditions, with O1 being least critical for repression (Table I).

**Interaction between the Operators**—The above results indicating that the glpTQ promoter is controlled by operators O1, O3, and O4 is unexpected in light of the relatively long distance between these operator sites and the glpTQ promoter. O1 is located -72 to -91 relative to the start point of transcription for glpTQ. This is farther upstream than usual for a negative control element influencing a σ-70 promoter (Collado-Vides et al., 1991; Gralla, 1991). However, a higher order DNA structure, such as a repressor-mediated DNA loop, possibly between O1 and O3 or O4, may influence expression of glpTQ over a larger distance. The following phasing experiments were designed to test this possibility.

First, the effects on repression of insertions of 2, 6, or 10 base pairs made at the unique BstBI site located in O2 were tested. If repressor-mediated DNA looping between operators is important for repression, then insertion of 6 base pairs (one-half of a helical turn) between the operators involved should decrease repression, whereas insertion of 10 base pairs (one helical turn) should have little influence on repression. Dissipation of O2 should not influence repression of glpTQ to a large extent, as this operator is least critical for control of glpTQ (see results for O2+2M, above).

Insertion of 6 base pairs at the BstBI site totally eliminated repression of the glpTQ promoter by glp repressor (O2+6; Table I). In fact, a small but reproducible stimulation by repressor of the O2+6 glpTQ promoter was observed during aerobic growth. Significant repression of the glpTQ promoter was retained in the case of the 2- or 10-base pair insertions (O2+2 + O2+10; Table I). The insertion mutations had similar effects on repression of the glpaCB promoter (Table I), except in this case repression was never totally eliminated, perhaps due to the presence of multiple operators. The results are consistent with a model for regulation in which repressor binds simultaneously to operator sites flanking the site of the insertions, with the intervening DNA forming a loop. Because O1 is apparently not critical for repressor control of the glpTQ promoter, a repression loop involving O1 and O3 or O4 seems the most plausible. Operators O3 and O4 are separated from O1 by 4.95 and 8.76 helical turns, assuming 10.5 base pairs per helical turn. The distance and phasing are appropriate for loop formation involving OA1 and either of the two most distant glpaCB operators (Hochschild and Ptaehne, 1986).

To provide further evidence for involvement of remote operators in the regulation of glpTQ, the effect on repression of a 6-base pair insertion between O1 and O2 was tested. It was found that this phasing mutation completely eliminated repression of glpTQ (AT +6; Table I). This alteration also decreased repression of glpaCB 3- to 4-fold. Although this insertion was made in the region between the promoter elements of the divergent operons, the insertion drastically reduced the strength of the glpTQ promoter and, to a lesser extent, the glpaCB promoter (Table I). These results are suggestive of interaction between transcription factors bound at the divergent promoters. One possibility would be interaction between the two CRP sites. The fact that O2-M increases glpaCB promoter activity supports this idea. O2+1M changes the CRP site for glpTQ to match the CRP binding site consensus more closely and might be expected to cause an increase in apparent promoter strength.

**DISCUSSION**

With the completion of this work and that described in the accompanying paper (Weissenborn and Larson, 1992), the control regions of each of the glp operons have been characterized with regard to the binding sites for the glp repressor and CAMP CRP. The common theme for the arrangement of the repressor binding sites is tandem repetition of the operator. This is especially noteworthy for the control of the operons encoding the aerobic and anaerobic glycerol-P dehydrogenases. Cooperative binding of repressor to adjacent sites, which is reminiscent of the binding of λ repressor to its operators (Gussin et al., 1983), is likely to play an important role in the relatively tight control of these operons by the glp repressor. As has been pointed out by Lin (1976, 1987), rapid shut down of the operons encoding the dehydrogenases would be desirable when glycerol-P in the medium is depleted.
Decreasing the levels of these enzymes would allow utilization of the remaining glycerol-P for the biosynthesis of membrane phospholipids.

Besides interaction of gpl repressor at adjacent operators, it is clear that interactions between operators separated by greater distances are important for complete repression of the gplTQ and gplACB operons. The degree of repression of the gplTQ promoter was decreased, to differing extents, by mutations in each of the gplACB operators. Somewhat unexpected was the finding that changes in O\textsubscript{2} had little effect on repression of gplTQ, while changes in O\textsubscript{1} completely eliminated repression. Mutation of O\textsubscript{3} reduced repression of gplTQ 5- to 6-fold. Elimination of O\textsubscript{2}, which is directly adjacent to O\textsubscript{3}, reduced repression only 2-fold. O\textsubscript{2} could exert a small effect on gplTQ due to its influence on binding of repressor to O\textsubscript{3}. O\textsubscript{4}, the operator most remote from gplTQ, also was required for full repression of the gplTQ promoter, especially during anaerobic growth.

One model which explains regulation of gplTQ from these remote operators invokes the existence of a repression loop. Evidence in support of such a model was obtained through analysis of the effects of phasing mutations and individual operator-constitutive mutations on repression of the divergent operators. Evidence for repressor-mediated DNA looping in the gplFK operon has also been obtained (Weissenborn and Larson, 1992). It is possible that tetrameric gpl repressor (Larson et al., 1987) is able to bind two appropriately oriented operators simultaneously, with the intervening DNA forming a loop. If this is the case, regulation of the gpl operons by gpl repressor would resemble control of other catabolic operons such as lac, gal, deo, and ara by the respective repressors. In each of these instances, repressor binding to widely separated operators has been invoked to explain repression (Adhya, 1989; Gralla, 1989).

If O\textsubscript{2} is not critical for repression of gplTQ, what is the nature of the repression complex responsible for regulation? We believe the data are most consistent with a model in which a primary repression loop involving O\textsubscript{1} and O\textsubscript{3} is formed. These operators are the most critical for regulation of gplTQ and are separated by five helical turns of DNA (assuming 10.5 base pairs per helical turn). A stable repression loop involving these two operators also would be responsible for the tight control of gplACB because the loop encompasses the entire promoter. Such a loop might also place O\textsubscript{4} and O\textsubscript{2} in juxtaposition such that repression of gplTQ results. O\textsubscript{4} and O\textsubscript{2} are positioned with precise symmetry relative to O\textsubscript{3} and O\textsubscript{1}, with operator centers of symmetry 40 base pairs removed from centers of the adjacent operators. An explanation must then be provided for the minimal influence of O\textsubscript{2} on repression. One possibility is that the three base pair change converting O\textsubscript{2} to O\textsubscript{3} was not severe enough to eliminate repression, especially if the primary repression loop involving O\textsubscript{1} and O\textsubscript{3} is very stable and places O\textsubscript{4} and O\textsubscript{2} in proper juxtaposition for simultaneous interaction with repressor. Another explanation would be that repressor bound at O\textsubscript{4} alone interferes with binding of CRP or polymerase at the gplTQ promoter due to the spatial proximity of the binding sites in the looped DNA. Thirdly, it is possible that repressor bound at one of the gplACB operators contacts CRP bound at O\textsubscript{2} or disrupts a positive interaction occurring between two CRP sites in the divergent promoters. The first possibility is favored, because the mutation AT + 6 decreased repression of gplACB, which indicates that an element on the gplTQ side of AT + 6 (most likely O\textsubscript{2}) is important for repression of gplACB. The observations that mutations affecting O\textsubscript{4} decreased repression of gplTQ and that gpl repressor footprints O\textsubscript{2} in vitro also support the first possibility.

Characterization of the influence of operator-constitutive mutations on expression of the gplACB promoter supports the conclusion that each of the four operators is important for regulation of the gplACB operon. The effects of the mutations on expression of gplACB are most readily apparent during anaerobic growth, where much higher repression ratios are observed. The differences in repression observed during anaerobiosis may be due to the increase in negative supercoiling of the DNA under these conditions (Dorman et al., 1988), which may favor formation of a repression complex. It is clear that the order of importance of the operators for regulation of gplACB is O\textsubscript{3} > O\textsubscript{4} > O\textsubscript{2} > O\textsubscript{1}. Thus, O\textsubscript{3}, which directly overlaps the −10 region of the gplACB promoter, is most critical for regulation of gplACB.

The spacing between FNR-binding site and the start of transcription of FNR-dependent promoters seems to be well conserved (Eiglmeier et al., 1989). The same is true for CRP-binding sites (Gaston et al., 1990; Usihide and Alba, 1990). Therefore, it was somewhat surprising to find that the insertion mutations, which were generated at the unique BstBI site located between the −10 promoter element of gplACB and the CRP/FNR site, did not eliminate gplACB promoter activity. Walker and DeMoss (1991) and Bell et al. (1990) found that a variety of insertions (including 4, 6, 8, or more nucleotides) between the FNR site and the start of transcription abolished FNR-dependent anaerobic induction. The presence of gplACB promoter activity both anaerobically and aerobically in the +2, +6, and +10 mutant constructs suggests that the second CRP site, which overlaps the start point for transcription (as indicated in Fig. 5), might promote initiation of transcription from a downstream promoter. An appropriately spaced −10 element for this putative promoter may be the sequence TAAATG which is located in O\textsubscript{4}. The corresponding −10 sequence in the O\textsubscript{4}M situation would be TAGCTA. The sequence changes in O\textsubscript{4}M would be expected to decrease promoter activity, which was observed (Table I). Although in vivo evidence for the function of the putative promoter sites (not been reported), it should be pointed out that the sequence of the downstream CRP/FNR site matches the consensus sequence for CRP and FNR binding more closely than that of the upstream CRP/FNR site.

**Acknowledgments**—The graduate students in the course Biochemistry 5104 (Summer 1991) are gratefully acknowledged for the isolation and sequencing of three of the oligonucleotide-directed mutations (O\textsubscript{1}M, O\textsubscript{3}M, and AT + 6). We thank James G. Harman for the gift of pCRP-1, Christopher Beck for providing pCB287, Heidi J. Hoffmann for construction of pHH11, Herbert Schweizer for construction of pH585, Deborah L. Weissenborn for preparation of Fig. 1, and Karin Eiglmeier and Stewart T. Cole for providing information regarding the start point of transcription of gplACB prior to publication.

**REFERENCES**


Control of glpTQ-glpACB Gene Expression in E. coli


