Activation from a Distance: Roles of Lrp and Integration Host Factor in Transcriptional Activation of gltBDF

LIGI PAUL,1 ROBERT M. BLUMENTHAL,2 AND ROWENA G. MATTHEWS1,3*

Biophysics Research Division1 and Department of Biological Chemistry,3 University of Michigan, Ann Arbor, Michigan 48109, and Department of Microbiology and Immunology, Medical College of Ohio, Toledo, Ohio 436142

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The leucine-responsive regulatory protein (Lrp) binds to three sites centered 252, 216, and 152 bp upstream of the transcription start site of the Escherichia coli glutamate synthase operon (gltBDF) and activates transcription.

Escherichia coli synthesizes glutamate under ammonia-limiting conditions using the enzymes glutamate synthase, specified by the gltBDF operon, and glutamine synthetase, specified by glnA. The genes gltB and gltD specify the large and small subunits of glutamate synthase, respectively (7), and gltF specifies a protein of unknown function (8, 20). The gltBDF operon is positively regulated by the leucine-responsive regulatory protein (Lrp) (12, 13) and is transcribed at very low levels in the absence of Lrp (12). The coregulator for Lrp is leucine, and exogenous leucine reduces the expression of the glutamate synthase operon (70), allowing transcriptional activity to be monitored via β-galactosidase assays. Even replacing all gltBDF sequences downstream of and including the −35 hexamer did not eliminate Lrp-dependent activation of transcription. When a 91-bp region between the −35 hexamer and the proximal Lrp binding site (−48 to −128) was replaced with heterologous DNA of the same length, transcription was reduced nearly 40-fold. Based on the presence of a consensus binding sequence, this region seemed likely to be a binding site for integration host factor (IHF). Experiments to study the effects of a himD mutant on expression of a gltB::lacZ transcriptional fusion, gel mobility shift analyses, and DNA footprinting assays were used to confirm the direct participation of IHF in gltBDF promoter regulation. Based on these results, we suggest that IHF plays a crucial architectural role, bringing the distant Lrp complex in close proximity to the promoter-bound RNA polymerase.

MATERIALS AND METHODS

Escherichia coli synthesizes glutamate under ammonia-limiting conditions using the enzymes glutamate synthase, specified by the gltBDF operon, and glutamine synthetase, specified by glnA. The genes gltB and gltD specify the large and small subunits of glutamate synthase, respectively (7), and gltF specifies a protein of unknown function (8, 20). The gltBDF operon is positively regulated by the leucine-responsive regulatory protein (Lrp) (12, 13) and is transcribed at very low levels in the absence of Lrp (12). The coregulator for Lrp is leucine, and exogenous leucine reduces the expression of the glutamate synthase operon (70), allowing transcriptional activity to be monitored via β-galactosidase assays. Even replacing all gltBDF sequences downstream of and including the −35 hexamer did not eliminate Lrp-dependent activation of transcription. When a 91-bp region between the −35 hexamer and the proximal Lrp binding site (−48 to −128) was replaced with heterologous DNA of the same length, transcription was reduced nearly 40-fold. Based on the presence of a consensus binding sequence, this region seemed likely to be a binding site for integration host factor (IHF). Experiments to study the effects of a himD mutant on expression of a gltB::lacZ transcriptional fusion, gel mobility shift analyses, and DNA footprinting assays were used to confirm the direct participation of IHF in gltBDF promoter regulation. Based on these results, we suggest that IHF plays a crucial architectural role, bringing the distant Lrp complex in close proximity to the promoter-bound RNA polymerase.

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The leucine-responsive regulatory protein (Lrp) binds to three sites centered 252, 216, and 152 bp upstream of the transcription start site of the Escherichia coli glutamate synthase operon (gltBDF) and activates transcription. Activators of σ70-dependent promoters usually bind closer to the −35 hexamer of the core promoter sequence. To study the mechanism by which Lrp-dependent activation occurs over this relatively large distance, the gltBDF upstream region was sequentially replaced with corresponding portions from the well-characterized σ70-dependent promoter lacZAp. The glt-lac promoter hybrids were placed upstream of lacZ, allowing transcriptional activity to be monitored via β-galactosidase assays. Even replacing all gltBDF sequences downstream of and including the −35 hexamer did not eliminate Lrp-dependent activation of transcription. When a 91-bp region between the −35 hexamer and the proximal Lrp binding site (−48 to −128) was replaced with heterologous DNA of the same length, transcription was reduced nearly 40-fold. Based on the presence of a consensus binding sequence, this region seemed likely to be a binding site for integration host factor (IHF). Experiments to study the effects of a himD mutant on expression of a gltB::lacZ transcriptional fusion, gel mobility shift analyses, and DNA footprinting assays were used to confirm the direct participation of IHF in gltBDF promoter regulation. Based on these results, we suggest that IHF plays a crucial architectural role, bringing the distant Lrp complex in close proximity to the promoter-bound RNA polymerase.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. Cultures were grown in LB medium (30) or glucose minimal MOPS (morpholinopropanesulfonic acid) medium (25) at 37°C. The antibiotics ampicillin (80 to 100 μg/ml), tetracycline (20 μg/ml), and chloramphenicol (25 μg/ml), were added to the medium as indicated below.

Replacement of the gltBDF upstream region with the lac operon sequences and construction of Lrp regulon. Fusions of the lac and glt operon regions (Fig. 1) were constructed from fragments of lac and glt operons obtained by PCR amplification using the primers listed in Table 1. The glt regions were amplified from plasmid pBE10 (13), and the lac regions were amplified from E. coli strain W3110 genomic DNA purchased from Sigma Aldrich (St. Louis, Mo.).

The PCR mixtures contained 2 ng of template plasmid DNA or 200 ng of E. coli genomic DNA, 1 U of Vent polymerase (New England Biolabs, Beverly, Mass.), 200 mM concentrations of each primer, and 200 μM concentrations of

*Corresponding author. Mailing address: Biophysics Research Division, 4028 Chemistry, 930 N. University Ave, University of Michigan, Ann Arbor, MI 48109-1055, Phone: (734) 764-5257. Fax: (734) 764-3323. E-mail rmatthew@umich.edu.
TABLE 1. Strains of E. coli and primers used in this work

<table>
<thead>
<tr>
<th>Strains, lysogen, or primer</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td>Strains</td>
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<tr>
<td>BE1</td>
<td>W3110 lac-201; Tn10</td>
<td>12 Life Technologies</td>
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<tr>
<td>DH5α</td>
<td>supE44 lacZYA-argF)U169 (680&lt;laczΔM15) deoR hsdR17 (trk⁺ m₅k⁺) recA1 endA1 gvy96 thi-1 relA1 λ⁻ + F'</td>
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<tr>
<td>JWD3</td>
<td>BE1/pJWD2</td>
<td>13 F. C. Neidhardt</td>
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<td>PS2209</td>
<td>W3110 lac-169</td>
<td>F. C. Neidhardt</td>
</tr>
<tr>
<td>RJ1413</td>
<td>MC1000 himD::cat</td>
<td>R. Osuna (originally from R. Ely)</td>
</tr>
<tr>
<td>W3110 glucosylase prototroph</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supF44 relAI lacF' proAB lacPlZΔM15 Tn10 (TerI')</td>
<td>Stratagene</td>
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Recombinant λ lysogens of strain PS2209

<table>
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<tr>
<th>Primers</th>
<th>Description</th>
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<tr>
<td>LP1008</td>
<td>glt-lacZ transcriptional fusion with −406 to +8 of glt fused to +1 of the lac operon</td>
<td>This study</td>
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<tr>
<td>LP1023</td>
<td>glt-lacZ transcriptional fusion with −406 to −23 of glt fused to −24 of the lac operon</td>
<td>This study</td>
</tr>
<tr>
<td>LP1035</td>
<td>glt-lacZ transcriptional fusion with −406 to −35 of glt fusion to −36 of the lac operon</td>
<td>This study</td>
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<tr>
<td>LP1048</td>
<td>LP1007 with the region from −48 to −128 replaced with a fragment of equal length from the cat gene</td>
<td>This study</td>
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<tr>
<td>LP1000</td>
<td>gltB-lacZ transcriptional fusion with −406 to +246 of the gltBDF operon</td>
<td>This study</td>
</tr>
<tr>
<td>LP2000</td>
<td>LP1000 with the himD::cat allele from strain RJ1413</td>
<td>This study</td>
</tr>
</tbody>
</table>

Each deoxynucleoside triphosphate (Life Technologies, Rockville, Md.) was used in thermodenatured reaction buffer [10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO₄, and 0.1% Triton X-100] in a total volume of 100 µL. The reaction was carried out for 20 cycles of 90°C for 30 s, 60°C for 1 min and 72°C for 1.5 min, followed by 10 cycles of 90°C for 30 s, 50°C for 1 min, and 72°C for 1.5 min. The PCR products were purified using a Qiaquick PCR purification kit (Qiagen Inc., Valencia, Calif.). A single deoxyadenosine was ligated to the amplified fragments, and the PCR products were purified using the Qiaquick PCR purification kit (Qiagen Inc., Valencia, Calif.).

The restriction enzyme sites present in the primes are underlined, and sequences different from the template are given in lowercase letters. GLT primers correspond to the glt operon sequences. "GLTF P GTCGTCAGTTCAAGGCAGGATAAGG (−54 to −28)".

The chimera in strain LP1003 was obtained using primers glt1 and glt2 (−406 to −35) and lac1 and lac2 (−24 to +1166). Primers glt1 and lac2 each contain a PstI site, which was used to ligate the two fragments together.

The chimera in strain LP1048 was constructed by replacing the glt operon with a fragment of the chloramphenicol acetyltransferase gene (cat). The cat fragment was amplified from the plasmid pKK232-8 (Pharmacia) using the primers cat1 and cat2. The flank regions were amplified from plP1008, which contains the glt-lac chimera introduced into strain LP1008, with primers glt1 and glt2 for the upstream flank region and glt3 and lac1 for the downstream flank region. Primer glt3 contains an NruI site, and primer lac1 contains complementary sequence, allowing ligation of the upstream fragment to the cat insert. Primer glt1 contains a PstI site, as does primer cat2, allowing ligation of the downstream fragment to the cat insert.

The glt operon in strain LP1000 (Table 1) was generated using primers glt1 and glt2 (−406 to +246), resulting in a fragment with an EcoRI site at the 5’ end and a BamHI site at the 3’ end which was ligated into plasmid pRS415 (31) cut with EcoRI and BamHI.

The glt-lac fusions from the pRS vectors were transferred into the chromosome of E. coli strain PS2209 (W3110 lac) at the attB site using ARZ5 (2) according to the procedure described in the work of Weise et al. (35). Single-copy lysogens were identified using the PCR method described by Powell et al. (27).
Transferring Lrp and himD mutations into $\lambda$ lysogens. An $\text{lp-201::Tn10}$ allele from the $E.\ coli$ strain BE1 (13) and a $\text{himD-2cat}$ allele from the $E.\ coli$ strain RJ1413 (from Robert Osuna, University at Albany, Albany, N.Y.) were transferred into the $\lambda$ lysogens carrying $glt$-$\text{lac}$ fusions by P1 vir transduction (24). The transductants were selected on LB medium-ampicillin plates containing tetracycline or chloramphenicol.

**Gel mobility shift assays.** The $\lambda$ lysogens were grown in glucose minimal MOPS medium supplemented with 0.4 mM isoleucine and 0.6 mM valine (13). IPTG or chloramphenicol.

**Beta-galactosidase activities from the chromosomal lacZ gene in $E.\ coli$ strain W3110 ($\text{lp}^+$) and isogenic strain BE1 ($\text{lp}^+\text{::Tn10}$) are also shown for comparison. NA, not applicable.

**Gel mobility shift assays.** The DNA fragments used for gel mobility shift assays were amplified by PCR from plasmid $\text{pBE10}$ (13) using primers $\text{glt1 and glt2}$ and $\text{gltBDF}$ (5). The $\text{gltBDF}$ sequences are shown with a black line, and the $\text{lac}$ sequences are shown by a gray line. The base pairs that were changed to introduce restriction sites in the chimeras are indicated in lowercase letters in the sequences. The region from the hatched line to the underlined A indicate the start sites of transcription determined for the native $\text{lac}$ promoters. The arrows and the underlined A indicate the start sites of transcription determined for the native $\text{gltBDF}$ (26) or $\text{lacZYA}$ (23) promoters. The $\beta$-galactosidase activity from each chimeric strain in the presence or absence of Lrp is presented on the left side of the construct diagrams. The $\beta$-galactosidase activities from the chromosomal $\text{lacZ}$ gene in $E.\ coli$ strain W3110 ($\text{lp}^+$) and isogenic strain BE1 ($\text{lp}^+\text{::Tn10}$) are also shown for comparison. NA, not applicable.

**DNase I footprinting.** Footprinting assays were carried out as described earlier (5). The $\text{gltBDF}$ DNA fragment used for footprinting of the template strand was amplified by PCR from plasmid $\text{pBE10}$ (13) using primers $\text{glit1}$ and $\text{glit2}$ and digested with the restriction enzyme $\text{HincII}$, resulting in a fragment from $-176$ to $+8$. For footprinting of the nontemplate strand, DNA was amplified with primers $\text{glit5}$ and $\text{glit3}$ and digested with $\text{Bsp1286I}$, resulting in a fragment from $-203$ to $+161$. The DNA fragments were labeled at the $\text{glit2}$ and $\text{glit5}$ ends for the template and nontemplate strands, respectively, using T4 polynucleotide kinase and $[\gamma-32\text{P}]\text{ATP}$ as described above for gel mobility shift assays. Various concentrations of $\text{IHF}$ were incubated with the labeled DNA in a buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM $\text{MgCl}_2$, 1 mM $\text{CaCl}_2$, 2 mM diethiothreitol, 50 mM $\text{NaCl}$, 4 mM $\text{Mg}$ acetate, 12.5% glycerol (vol/vol), and 200 ng of poly(dil-dc) · poly(dl-dc) (Amersham Pharmacia Biotech, Piscataway, N.J.) in a total volume of 20 $\mu\text{L}$. The mixtures were left at room temperature for 5 min before being incubated at 28°C for 15 min. The samples were loaded directly onto a 6% polyacrylamide gel (8.3 cm wide by 7.3 cm long by 1 mm) in 0.5X TBE and 5 mM $\text{MgCl}_2$. The gel was fixed in a solution of 10% acetic acid and 10% methanol for 5 min before being incubated at 28°C for 15 min. The samples were loaded directly onto a 6% polyacrylamide gel (8.3 cm wide by 7.3 cm long by 1 mm) in 0.5X TBE and 5 mM $\text{MgCl}_2$. The gel was fixed in a solution of 10% acetic acid and 10% methanol for 5 min and dried at 80°C. BioMax MS film (Kodak, Rochester, N.Y.) was used for autoradiography. The gels were scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.), and the intensities of the bands were determined using ImageQuant version 1.2 software. The data were fitted to the Michaelis-Menten equation using Kaleidagraph (Synergy Software, Reading, Pa.).
ments were resolved on a 6% acrylamide gel containing 8 M urea (30). A dideoxy sequencing reaction of the $glbDF$ upstream region with the primer corresponding to the labeled end, carried out using a T7 Sequenase version 2.0 deaza-dGTP kit (Amersham Pharmacia Biotech), was used to generate the ladder.

### RESULTS

**Effect of replacing the $glbDF$ promoter regions with those of the $lac$ promoter on Lrp-dependent activation.** To study the role of the DNA sequences downstream of the proximal Lrp binding site centered at $-152$ in the regulation of the glutamate synthase ($glbDF$) operon of *E. coli*, these downstream sequences were systematically replaced with the corresponding regions of the $lacZYA$ operon. Single copies of the chimeric constructs were introduced into *E. coli* strain PS2209 (W3110 Δ$lac$) at the att$\lambda$ site by lambda integration. Transcription from these hybrid constructs was monitored using lacZ as the reporter gene. Figure 1 illustrates the sequence replacements and the β-galactosidase activities of each construct. As described below, replacing the $glbDF$ region downstream of and including the −35 hexamer with the corresponding regions of the $lac$ operon did not abolish Lrp-dependent regulation. Thus, Lrp is clearly able to regulate the well-characterized $σ^{70}$-dependent promoter $lacZAp$ from its binding sites more than 110 bp upstream of the −35 hexamer.

In strain LP1008, the region downstream of +8 of the $glbDF$ operon was replaced by the $lac$ operon sequence. Bases at +4 and +7 were changed (C to T and T to A, respectively) to introduce a $PstI$ site, which was used for ligating the $glb$ and $lac$ fragments. In strain LP1008, lacZ was still under the control of Lrp and showed a nearly 30-fold reduction in β-galactosidase levels in an Lrp$^{-}$ background relative to the wild-type strain.

Strain LP1023 has a chimeric promoter, with the −35 hexamer being from $glbDF$ and the −10 hexamer being from the $lac$ operon. The sequence in the spacer region derived from $glb$ was changed in two positions (T to G at −23 and T to C at −25) to generate a $PstI$ site for preparing the construct, and the spacer sequence downstream of −23 was from the $lac$ operon. The length of the spacer sequence is 17 bp as in $glb$; the wild-type $lac$ spacer region is 18 bp. This construct showed an extremely high level of β-galactosidase activity: 200-fold higher at both the basal and activated levels compared to levels in strain LP1008. Nonetheless transcription from this chimeric promoter was activated over 20-fold by Lrp.

In strain LP1035 the −35 hexamer and the entire region downstream of it have been replaced with the corresponding sequences from the $lac$ operon. The distance between the proximal Lrp binding site and the −35 hexamer was 1 bp shorter than in the other constructs (111 versus 110 bp). The β-galactosidase level from this construct was reduced sevenfold in an Lrp$^{-}$ background, but the basal level of transcription was higher than in strain LP1008.

In the $glbDF$ operon, the proximal Lrp binding site centered at −152 and the RNA polymerase recognition sequence at −34 are farther apart (more than 110 bp) than is usually seen in other $σ^{70}$-dependent promoters. To see if the intervening region is involved in transcriptional regulation of the $glb$ operon, the region from −48 to −128 was replaced with a portion of the chloramphenicol acetyltransferase (cat) gene of the same length (LP1048). This replacement had no effect on basal transcription but completely abolished Lrp-mediated activation.

**IHF is required for positive regulation of transcription from the $glbDF$ promoter.** In preliminary experiments we used surface-enhanced laser desorption and ionization (SELDI) ProteinChip technology (Ciphergen Biosystems, Fremont, Calif.) to identify proteins that might bind to the upstream region of the $glbDF$ operon. In this approach, biotin-labeled DNA fragments of the $glt$ region (from −406 to +132) were attached to streptavidin-coated chips and incubated with cell extracts. The masses of proteins that bound to the DNA fragments were determined using mass spectroscopy. This binding experiment was carried out under nonstringent conditions, and there were multiple peaks corresponding to various proteins that bound the DNA nonspecifically. Even though the results of these experiments were not conclusive, they suggested that two polypeptides with molecular masses of 10.6 and 11.2 kDa bound to the $glbDF$ upstream region. The molecular masses of these polypeptides matched those of the two subunits of IHF. These preliminary results led us to focus our study on the possible role of IHF in $glbDF$ transcription.

Upstream of the $glbDF$ promoter, within the region replaced by the cat fragment in strain LP10048, there are two regions resembling the consensus IHF binding sequence $WATCAANNNTTR$, where $W$ is A or T and $R$ is A or G (10, 17). The region from −95 to −83 (TTTCAGTCATTTA) has two mismatches and the overlapping region from −91 to −79 (AGTCAATTAA) has three mismatches to the consensus sequence. An insertion in the him$D$ gene encoding the β subunit of IHF (him$D$::cat) was transferred by P1 vir transduction into strain LP1000, which contains a $glbB$::$lacZ$ transcriptional fusion. The IHF$^{-}$ strain had a β-galactosidase level over 30-fold lower than that of the IHF$^{+}$ strain (Fig. 2).
and IHF binding to \( \text{gltBDF} \) DNA. The data were fitted using the Michaelis-Menten equation. The quantitated using a phosphorImager and used to calculate the bound IHF. (B) The free DNA in the above gel mobility shift assay was IHF. The reaction mixtures contained 1.15 nM DNA and 0 to 100 nM 1

To see if IHF affects promoter. The assays used a DNA fragment containing the region from 406 to 246 was digested with the restriction enzyme NsiI to obtain the above-described fragment. The reaction mixtures contained 1.6 to 100 nM Lrp and 6.3 to 100 nM IHF as noted above the lanes. In the presence of both Lrp and IHF in the reaction mixture, we observed an additional band corresponding to the ternary complex, which was seen between those corresponding to IHF or Lrp alone (Fig. 3C). In the presence of saturating amounts of Lrp and IHF, most of the DNA was bound to both IHF and Lrp.

The binding site of IHF on the \( \text{gltBDF} \) upstream DNA was determined using DNase I footprinting assays of both strands of the DNA (Fig. 4). IHF protected a 41-bp region from -115 to -75 on the non-template strand and a 38-bp region from -113 to -76 on the template strand (Fig. 5). The bases -78 on the non-template strand and -82 on the template strand were made hypersensitive to DNase I digestion by IHF binding. Upstream of the IHF binding consensus site there are two short tracts of adenine (-106 to -109 and -113 to -115), both of which were protected in the DNase I footprinting assays. High-affinity IHF binding sites are often preceded by a poly(A) tract (21), whose narrow minor groove helps optimize IHF-DNA contacts (29). In Fig. 6, the sequences upstream of the \( \text{gltBDF} \) operon of \( \text{E. coli} \) are compared with those in other genera of Enterobacteriaceae: \( \text{Salmonella enterica} \) serovar Typhimurium LT2, \( \text{Klebsiella pneumoniae} \), and \( \text{Yersinia pestis} \). The approximate distance between the proximal Lrp binding site and the IHF site is conserved among the different genera, but the sequence of the intervening region is not highly conserved except for the poly(A) region. Though the sequence of the IHF binding site is not conserved among the four genera, the cytosine that is present in all known natural IHF binding sites (16) is conserved. An adenine following the cytosine is also generally conserved (17), but this residue is a T rather than an A in \( \text{K. pneumoniae} \).

Purified IHF binds to the upstream region of the \( \text{gltBDF} \) promoter. To see if IHF affects \( \text{gltBDF} \) transcription directly, gel mobility shift assays were carried out using purified IHF and the region upstream of the \( \text{gltB} \) translational initiation codon. Assays with both the region from -406 to +246 that included the first 30 bp of the coding region of \( \text{gltB} \) and a shorter fragment (-406 to +8) showed similar band shift patterns. A gel mobility shift assay with the shorter fragment is shown in Fig. 3A. Migration of the DNA fragment was retarded in the presence of IHF. IHF binds to the \( \text{gltBDF} \) upstream region with an apparent \( K_d \) of 5.7 ± 0.3 nM (Fig. 3B).

A DNA fragment from -406 to +246 but with the region from -48 to -128 replaced with an equivalent length of the \( \text{cat} \) gene sequence did not bind IHF under identical binding conditions (not shown).

When both Lrp and IHF were present in the gel mobility shift assays, we observed an additional band that was not present in assays with either IHF or Lrp alone. The position of the shifted DNA fragment corresponding to the ternary complex depended on the distance of the IHF and Lrp binding sites from the ends of the DNA fragment used for the assay (37). When the \( \text{glt} \) DNA fragment from -324 to +246 was used, the band corresponding to the ternary complex was seen between those corresponding to IHF or Lrp alone (Fig. 3C). In the presence of saturating amounts of Lrp and IHF, most of the DNA was bound to both IHF and Lrp.

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In Fig. 6, the sequences upstream of the \( \text{gltBDF} \) operon of \( \text{E. coli} \) are compared with those in other genera of Enterobacteriaceae: \( \text{Salmonella enterica} \) serovar Typhimurium LT2, \( \text{Klebsiella pneumoniae} \), and \( \text{Yersinia pestis} \). The approximate distance between the proximal Lrp binding site and the IHF site is conserved among the different genera, but the sequence of the intervening region is not highly conserved except for the poly(A) region. Though the sequence of the IHF binding site is not conserved among the four genera, the cytosine that is present in all known natural IHF binding sites (16) is conserved. An adenine following the cytosine is also generally conserved (17), but this residue is a T rather than an A in \( \text{K. pneumoniae} \).

**DISCUSSION**

**\( \text{glt:} \text{lac} \)** hybrid promoters are activated by Lrp. To study the minimum DNA sequence required for Lrp-dependent regulation, the \( \text{gltBDF} \) operon sequence downstream of and including the -35 hexamer was sequentially replaced with the corresponding regions of the \( \text{lacZYA} \) operon. The effects of these substitutions on transcription were studied by monitoring the activity of the reporter gene \( \text{lacZ} \) (Fig. 1).

The \( \text{gltBDF} \) sequences downstream of -23, including the -10 hexamer, are not required for Lrp-dependent activation of transcription. Strain LP1023 has the -35 hexamer of \( \text{glt-} \)
BDF, the -10 hexamer of the lac operon, and a hybrid spacer region, yet Lrp-dependent activation (23.4-fold) was similar to that of strain LP1008 (27.5-fold), in which the gltBDF core promoter regions were intact. β-Galactosidase expression in strain LP1023 was >200-fold higher than in strain LP1008 and over 10-fold higher than the IPTG-induced level from the native chromosomal lacZ gene in W3110. Apparently, the combination of the -35 and the -10 hexamers or the hybrid

FIG. 4. DNase I footprinting analysis of IHF binding to the glt DNA. DNA fragments extending from -176 to +8 of the template strand (the strand complementary to the mRNA sequence) (A) or from -203 to +161 of the nontemplate strand (B) of the gltBDF operon were footprinted in the presence of 0 to 100 nM IHF. Dideoxy sequencing reactions of the gltBDF promoter region with the primer glt2 (for the template strand) and gltFP (for the nontemplate strand) were used to generate the ladders. IHF protected the region (shown in brackets) from -113 to -76 on the template strand and the region from -115 to -75 on the nontemplate strand against DNase I digestion. The positions of the sequences are given with respect to the transcription start site.

FIG. 5. Diagramatic representation of the region upstream of the gltBDF promoter that was protected against DNase I digestion by IHF binding. Open circles indicate protection against cleavage in the presence of IHF, and the filled circles indicate unchanged cleavage. Where no circles are shown, the DNA was not cleaved by DNase I in the absence of IHF. The two gray circles indicate hypersensitivity to DNase I digestion due to IHF binding. The underlined sequences indicate the region protected from DNase I digestion.
spacer region affected the strength of the promoter. A study using random sequences in the spacer region of *Lactococcus lactis* promoters in *L. lactis* and *E. coli* backgrounds has shown that the sequence of the spacer region can affect promoter strength up to 400-fold (22).

Lrp was able to activate transcription even when the *gltBDF* core promoter sequence (the −35 hexamer and downstream sequence) was completely replaced with that of the σ70-dependent *lac* promoter in strain LP1035. The *lac* promoter normally requires catabolite activator protein for activation. Dove et al. (11) have shown that any DNA binding protein that contacts RNA polymerase can activate transcription by strengthening the interaction of RNA polymerase with the promoter region. The extent of Lrp-dependent activation was lower in LP1035 than in strain LP1023 with the chimeric promoter or in strain LP1035 mutant (Fig. 2) confirmed that IHF is required for Lrp-dependent activation of the *gltBDF* upstream 110-bp distance between the proximal Lrp binding site and the −35 hexamer or −10 hexamers or 95 to 83, and the −35 and −10 hexamers. The non-*E. coli* sequences are prepublication communications from the Genome Sequencing Center at Washington University and The Sanger Centre.

IHF binding is required for Lrp-dependent activation of the *gltBDF* operon. The mechanism by which the leucine-responsive regulatory protein (Lrp) regulates the genes under its control is not completely understood. In some operons, e.g., *gev*, Lrp exerts its effect in tandem with other regulatory proteins (32); in others, such as *ih*, Lrp is sufficient for regulation (36). Lrp has been reported to play an architectural role in the transcription of the *gev* operon (32). Although the *gltBDF* operon of *E. coli* is positively regulated by Lrp (13), in vitro transcription with purified Lrp produced minimal activation of transcription from the *gltBDF* promoter (B. R. Ernsting, unpublished results). This observation suggested that additional factors might be involved in the transcriptional activation of the *gltBDF* operon of *E. coli*.

The 110-bp distance between the proximal Lrp binding site (centered at −152) and the −35 hexamer of the *gltBDF* operon is unusual for a σ70-dependent promoter, since the *lac* operon is controlled by what is probably the best-categorized such promoter.

### FIG. 6. Comparison of sequences upstream of the *gltBDF* operon from *E. coli* (4) with corresponding sequences from related genera: *S. enterica* serovar *Typhimurium* LT2 (WUGSC 99287/stml2-contig1496), *K. pneumoniae* (WUGSC 573/kpneumo B KPN.contig272), and *Y. pestis* (Sanger 632/Y pestis Contig850). The sequences of the related genera were obtained from unfinished genomes using a BLAST search (1), and the alignment was generated using CLUSTAL W (33). Bases conserved in all four genera are shown in bold, and the shaded regions indicate the best-categorized such promoter.

**IHF binding site**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>TTAGAC-CT---TTAAGAATTTACCCTATTTAACTTCGACATTAACTACATTTTAAT-AAAATATT</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>TTAGACGAC-CTTTTATATTTATTATAATTTTGTTAGCTTTTACTTTAATTTTACTTTTACTTTAAT</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>CTTGACTATAGACATTTTGTATTAATAGATTTGACTTATATAATTTATTTATTTATTTATTTATTTAT</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>GACATATATATCCATTTAAAGATATATACATTTATTTATTTATTTATTTATTTATTTATTTATTTAT</td>
</tr>
</tbody>
</table>

**Poly A IHF binding site**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>TGCGCTAAAGCAACTTTTGAGGACCATAGCTTCCATTACCCTGCTATAGCTT</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>CTGTCTACAGACATTTTGTAGCTTTGTTAGCTTTAGCTTTAGCTTTAGCTTTAGCTTTAGCTTTAGCTT</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>CTTGACTATAGACATTTTGTATTAATAGATTTGACTTATATAATTTATTTATTTATTTATTTATTTAT</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>GACATATATATCCATTTAAAGATATATACATTTATTTATTTATTTATTTATTTATTTATTTATTTAT</td>
</tr>
</tbody>
</table>

**Lrp binding site**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>AAACACCTTTGACATGAGATTCTCCTGT</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>CGCCAGCAGCTTTTATATTTATTATAATTTTGTTAGCTTTTACTTTAATTTTACTTTAATTTAAT</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>CTTGACTATAGACATTTTGTATTAATAGATTTGACTTATATAATTTATTTATTTATTTATTTATTTAT</td>
</tr>
<tr>
<td><em>Y. pestis</em></td>
<td>TTGCGCTAAAGCAACTTTTGAGGACCATAGCTTCCATTACCCTGCTATAGCTT</td>
</tr>
</tbody>
</table>
This effect was not as drastic as the approximately 30-fold effect seen in our assays with the himD mutant or with a strain where the IHF binding region was replaced by heterologous DNA (strain LP1048). It is possible that functional himD homodimers were formed in the himA mutant used for the microarray assay, resulting in activation of the gltBDF operon transcription to a certain extent (3, 38).

We confirmed that IHF binds to the gltBDF upstream region using gel mobility shift and footprinting assays (Fig. 3 to 5). The two overlapping matches to the consensus IHF binding sequence in the glt upstream region (−95 to −83 and −91 to −79) are protected in footprinting assays. Site-specific mutational studies have to be conducted to identify the site actually recognized by IHF. The activation of the gltBDF operon by Lrp is totally dependent on the presence of IHF, since introduction of an Lrp mutation in strain LP1048 did not lead to a further decrease in β-galactosidase level.

IHF has been shown to be involved in transcriptional regulation of many operons on its own or in concert with additional activator proteins (14, 18). The crystal structure of IHF bound to DNA shows that this protein bends DNA at an 160° angle (29). This facilitates looping of DNA, allowing an upstream element or upstream-bound activator protein to come in close contact with RNA polymerase. Transcriptional activation of the gltBDF operon depends completely on the presence of both proteins, suggesting that a DNA looping mechanism may be involved in gltBDF regulation.

The proposed IHF-induced bend in the gltBDF upstream region would bring the proximal Lrp binding site close to the region just upstream of the −35 hexamer, a position where activators of σ70-dependent promoters usually bind (19). So far, activation of transcription by direct contacts between Lrp and RNA polymerase has not been documented for any promoter. The effect of IHF on transcription from the gltBDF promoter is phase dependent: transcription from the gltBDF promoter was reduced fivefold when the IHF binding site was taken out of phase with respect to the −35 hexamer and the Lrp binding sites by 5-bp insertions at −40 and −120 (D. E. Weise II, unpublished results).

It is not known at present whether IHF acts as a direct regulator of gltBDF transcription or whether it plays a structural role in bringing the transcriptional machinery together. When IHF plays an architectural role, it can be replaced by an intrinsically bent region of DNA (15). Such an experiment remains to be done for the gltBDF operon. Since the IHF binding site and the Lrp binding sites upstream of the glt promoter are conserved in S. enterica, K. pneumoniae and Y. pestis, the glutamate synthase operons of enteric bacteria appear to be regulated by a common mechanism.

Our results suggest an exception to the general rule that transcriptional activators of σ70-dependent promoters bind close to the −35 hexamer (19). When a confirmed binding site for IHF is present, activators may bind farther upstream and retain function. Since the proximal Lrp binding site in gltBDF operon is positioned 110 bp upstream of the −35 hexamer, an intermediate IHF binding site appears to be essential to Lrp-mediated activation. Future experiments will probe for evidence of direct contact between Lrp and/or IHF and RNA polymerase.

ROLES OF IHF AND Lrp IN gltBDF REGULATION

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REFERENCES


