Regulation of the *gltBDF* Operon of *Escherichia coli*: How Is a Leucine-Insensitive Operon Regulated by the Leucine-Responsive Regulatory Protein?

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The regulon controlled by the leucine-responsive regulatory protein (Lrp) of *Escherichia coli* consists of over 40 genes and proteins whose expression is regulated, either positively or negatively, by Lrp. The *gltBDF* operon, encoding glutamate synthase, was originally identified as a member of the Lrp regulon through a two-dimensional electrophoretic analysis of polypeptides from isogenic strains containing or lacking a functional Lrp protein. We have now demonstrated that Lrp regulates the transcription of *gltBDF::lacZ* operon fusions. Relative to expression in glucose minimal 3-(*N*-morpholino)propanesulfonic acid (MOPS) medium, *gltBDF::lacZ* expression in an *lrp*⁺ strain is repressed 2.2-fold in the presence of 10 mM exogenous leucine and 16-fold in Luria broth. Repression of *gltBDF::lacZ* expression by leucine or Luria broth is not seen for an isogenic strain containing a Tn10 insertion in *lrp*, and expression of *gltBDF::lacZ* is 44-fold lower than in the *lrp*⁺ strain when both are grown in glucose minimal MOPS medium. Lrp binds specifically to DNA fragments containing the *gltBDF* promoter region. Saturating levels of leucine do not abolish binding of Lrp upstream of *gltBDF* but merely increase its apparent dissociation constant from 2.0 to 6.9 nM. Electrophoretic analysis of the Lrp regulon established that target proteins differ greatly in the degree to which the effect of Lrp on their expression is antagonized by leucine. On the basis of our present results, we present a model for positive regulation of target genes by Lrp. Insensitivity to leucine would be expected when the effective intracellular concentration of Lrp is high relative to the affinity of Lrp binding sites required for transcription of the target gene. At lower concentrations of Lrp, transcription of the target gene should be sensitive to leucine. This model suggests that regulation of the concentration of active Lrp is critical to control of the Lrp regulon.

The Lrp (leucine-responsive regulatory protein) regulon of K-12 strains of *Escherichia coli* consists of over 40 polypeptides whose expression is regulated, either positively or negatively, by Lrp (2, 6, 8, 11, 12, 17, 20, 21). The first genes and operons of the Lrp regulon to be recognized were regulated in response to the presence or absence of exogenous leucine. For these leucine-sensitive genes and operons, Lrp can be a positive or a negative regulator of expression, and the effect of Lrp is antagonized in the presence of exogenous leucine. The best studied of these operons is the *ilvIH* operon, encoding aceto-hydroxy acid synthase form III (5). Lrp is a transcriptional activator of *ilvIH* expression (29), and the presence of leucine in the medium reduces this activation 5- to 10-fold (9).

Two-dimensional gel electrophoretic analysis of net protein synthesis in strains containing or lacking a functional *lrp* gene and grown in the presence and absence of leucine detected a number of known and unknown proteins regulated by Lrp in a leucine-sensitive manner. That study also revealed an even larger number of proteins regulated by Lrp but insensitive to exogenous leucine (6). Among these leucine-insensitive polypeptides are the small subunit of glutamate synthase (encoded by the *gltBDF* operon) and glutamine synthetase. We have demonstrated that the regulation of glutamine synthetase expression by Lrp is indirect and apparently is due to the effect of Lrp on the expression of glutamate synthase (6).

An important question arising from this work concerned the nature of leucine-insensitive regulation by Lrp. How is the

expression of these proteins regulated by Lrp if not in response to leucine? We suggested (6) that the regulation of leucine-insensitive genes and operons by Lrp might be modulated in response to altered physiological conditions. This modulation could be brought about either by altering the expression of Lrp itself or by altering the activity of existing Lrp molecules. Lin and coworkers have investigated the regulation of Lrp expression using *lacZ* fusions to the *lrp* gene (11). Their work showed that the expression of Lrp is more than 10-fold lower when cells are grown in Luria broth (LB) than when they are grown in a minimal medium. Regulation of the concentration of Lrp in response to changing growth conditions might provide a way to regulate Lrp-dependent genes that appear to be insensitive to leucine. Low concentrations of Lrp resulting from continued growth in LB might lead to levels of Lrp insufficient to regulate some genes and operons. Furthermore, we wished to test the possibility that when concentrations of Lrp are low, target genes may be more sensitive to the presence of leucine. In other words, genes and operons that seem to be leucine insensitive in a minimal medium might be regulated in response to leucine present in a rich medium. To probe the regulation of leucine-insensitive genes by Lrp, we investigated the regulation of glutamate synthase by Lrp.

gltBDF mutant strains grow at the same rate as their isogenic parent strains on media containing high levels of ammonia but grow poorly on low levels of ammonia or on poor nitrogen sources. Enzyme assays have shown that *lrp::Tn10* strains lack detectable glutamate synthase activity (6) and that these strains cannot grow on media in which nitrogen is supplied by arginine or ornithine or by concentrations of ammonia lower than 0.1

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TABLE 1. *E. coli* strains used in this work

Strain	Description	Source or reference
BE1	W3110 <i>lrp-201::Tn10</i>	6
BE40	XL1 Blue/pBE10	This work
BE52	XL1 Blue/pBE15	This work
BE53	XL1 Blue/pBE16	This work
BE54	PS2209 <i>ilvIH::Mu</i> d1-1734	This work
BE55	PS2209 <i>ilvIH::Mu</i> d1-1734 <i>lrp-35::Tn10</i>	This work
BE3471	PS2209 <i>gltB (psiQ39)::lacZ</i> (Mu d1-1734)	This work
BE3479	PS2209 <i>gltB (psiQ32)::lacZ</i> (Mu d1-1734)	This work
BE3480	PS2209 <i>gltB (psiQ35)::lacZ</i> (Mu d1-1734)	This work
BE3771	PS2209 <i>gltB (psiQ39)::lacZ</i> (Mu d1-1734) <i>lrp-35::Tn10</i>	This work
BE3779	PS2209 <i>gltB (psiQ32)::lacZ</i> (Mu d1-1734) <i>lrp-35::Tn10</i>	This work
BE3780	PS2209 <i>gltB (psiQ35)::lacZ</i> (Mu d1-1734) <i>lrp-35::Tn10</i>	This work
BW12671	Δ <i>lac-169 gltB (psiQ39)::lacZ</i> (Mu d1-1734) <i>creB510 rps-267 crp-72 aroB thi</i>	B. Wanner (13)
BW12679	Δ <i>lac-169 gltB (psiQ32)::lacZ</i> (Mu d1-1734) <i>creB510 rps-267 crp-72 aroB thi</i>	B. Wanner (13)
BW12680	Δ <i>lac-169 gltB (psiQ35)::lacZ</i> (Mu d1-1734) <i>creB510 rps-267 crp-72 aroB thi</i>	B. Wanner (13)
CV975	F ⁻ <i>ara thi</i> Δ (<i>lac-pro</i>) <i>ilvIH::Mu</i> d1-1734	J. M. Calvo (20)
CV1008	F ⁻ <i>ara thi</i> Δ (<i>lac-pro</i>) <i>ilvIH::Mu</i> d1-1734 <i>lrp-35::Tn10</i>	J. M. Calvo (20)
CV1014	F ⁻ <i>ara thi</i> Δ (<i>lac-pro</i>) <i>ilvIH::Mu</i> d1-1734 (pCV168) (<i>lrp</i> ⁺)	J. M. Calvo (20)
JM105	<i>supE endA sbcB15 hsdR4 rpsL thi</i> Δ (<i>lac-proAB</i>)	Promega
JWD2	JM105/pJWD2	This work
JWD3	BE1/pJWD2	This work
PS2209	W3110 Δ <i>lac-169</i>	F. C. Neidhardt
PS3110	F ⁻ prototroph	F. C. Neidhardt
XL1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1</i> Δ <i>lac-pro</i> [F' <i>proAB lacI</i> ^q Z Δ M15 Tn10]	Stratagene

mM. Assays of glutamate synthase activity have also shown that *lrp*⁺ cells grown in the presence of 10 mM leucine have approximately 50% of the glutamate synthase activity seen in cells grown in the absence of leucine. The regulation of *gltBDF* expression by Lrp is not absolutely independent of leucine but is better described as insensitive to leucine, relative to the regulation of *ilvIH* transcription.

The following work demonstrates that expression of the *gltBDF* operon is regulated by Lrp at the level of transcription and that Lrp plays a role in the repression of glutamate synthase expression in a rich medium in which nitrogen is present in the form of amino acids and nucleic acid bases as well as ammonia. We show that Lrp binds specifically to a site or sites upstream of the *gltBDF* operon and compare this binding to the binding of Lrp to the *ilvIH* promoter region. On the basis of a quantitative analysis of the in vitro binding affinities of Lrp for these two promoters in the presence and absence of leucine, we propose a model to explain the differing effects of leucine on the in vivo expression of *ilvIH* and *gltBDF*. This model is consistent with the observed effect of exponential growth in a rich medium on the expression of *gltBDF::lacZ* fusions, which is predicted to arise in part from the presence of leucine and in part from decreased levels of Lrp.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used in this work are described in Table 1.

Media and growth conditions. All cultures were grown aerobically in rotary-action shakers at various temperatures. The growth of cells was monitored spectrophotometrically at 420 or 550 nm. Glucose minimal MOPS medium was 3-(*N*-morpholino)propanesulfonic acid (MOPS) minimal medium (15) supplemented with 10 mM thiamine, 0.4% glucose as a carbon source, and amino acids as indicated. The concentrations of amino acids were those used in defined rich medium, including 0.4 mM isoleucine and 0.6 mM valine (30), unless noted otherwise. LB (24), glucose rich MOPS medium (30),

and STG medium (LB containing 0.2% glycerol and 50 mM potassium phosphate buffer [pH 7.4]) were used as rich media. Media sometimes contained ampicillin (100 μ g/ml), kanamycin (50 μ g/ml), or tetracycline (20 μ g/ml). Cultures were maintained on LB agar plates supplemented with appropriate antibiotics.

Construction of strains. Generalized transduction mediated by P1 *vir* was carried out as described by Miller (14). Strains BE3471, BE3479, and BE3480 were derived from strain PS2209 and were isolated by selection for kanamycin resistance following transduction with P1 *vir* lysates of strains BW12671, BW12679, and BW12680 (13), respectively. Strains BW12671, BW12679, and BW12680 carry *gltBDF::lacZ* fusions and were isolated as phosphate starvation-inducible fusion strains. Strains BE3771, BE3779, and BE3780 were derived from strains BE3471, BE3479, and BE3480, respectively, and were isolated by selection for tetracycline resistance following transduction with a P1 *vir* lysate of strain CV1008 (20). Strain CV1008 carries a Tn10 insertion in the *lrp* gene. In a similar fashion, *ilvIH::lacZ* operon fusion strains BE54 and BE55 were constructed by P1 *vir* transductions. Strains BE40, BE52, and BE53 were constructed by transformation of strain XL1 Blue (Stratagene) with plasmids pBE10, pBE15, and pBE16, respectively, followed by selection for ampicillin resistance. Strain JWD3 was constructed by transformation of strain BE1 with plasmid pJWD2 followed by selection for ampicillin resistance. Cells were made competent for transformation by the method of Chung et al. (4). Cells were grown in 100 ml of LB at 37°C until an *A*₅₅₀ of 0.3 to 0.6 was reached, harvested by centrifugation, and resuspended in a 10 ml of LB (pH 6.1) containing 10% polyethylene glycol 3500, 5% dimethyl sulfoxide, 10 mM MgCl₂, and 10 mM MgSO₄. After a 15-min incubation on ice, the cells were competent for transformation.

DNA methods. Double-stranded plasmid DNA was sequenced by the dideoxy chain termination method (25) (Sequenase kit from U.S. Biochemicals). Synthetic 17-mers were used as sequencing primers. Plasmid DNA was purified from overnight cultures grown in the presence of ampicillin. Cells

were lysed in the presence of RNase A, and plasmid DNA was isolated by use of a plasmid midiprep kit (Qiagen). DNA fragments were separated on agarose gels with $1 \times$ TAE (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) as a running buffer. Fragments cut from agarose gels were purified with a GeneClean II kit (Bio 101). Restriction endonucleases, including *TfiI*, were purchased from New England Biolabs.

Overexpression and purification of Lrp protein. The coding sequence for the *lrp* gene was amplified from plasmid pCV168 (20) by the polymerase chain reaction (PCR) (23) with a GeneAmp kit and a DNA thermal cycler (Perkin-Elmer). Thirty five cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 1 min were performed. The primers used are shown below, with sequences complementary to the *lrp* gene (32) in italics. Each of these primers contains 19 or 20 nucleotides (nt) complementary to the *lrp* gene. The upstream primer includes nt 1 to 19 of the *lrp* coding sequence, including the ATG start codon, while the downstream primer is complementary to nt 476 to 495 of the *lrp* coding sequence, including the TAA stop codon.

upstream primer: 5' CCGGATCCATGGTAGATAGCAAGAAGC 3'
downstream primer: 5' CGGAATTCATAGATTAGCGCTTAATAACCA 3'

The upstream primer contained *Bam*HI and *Nco*I sites, while the downstream primer contained *Xba*I and *Eco*RI sites. The presence of these sites allowed the amplified product to be cut with *Bam*HI and *Xba*I and cloned directly into the corresponding sites of plasmid pTrc99A, generating plasmid pJWD1. This plasmid was transformed into strain XL1 Blue, isolated, and cut with *Nco*I to remove DNA between the *Nco*I sites in pTrc99A and in the upstream portion of the insert. The DNA fragments resulting from this digest were separated on an agarose gel, and the fragment containing the *lrp* gene was purified and religated. This ligation brought the *lrp* gene under the control of the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible hybrid promoter in pTrc99A. The resulting plasmid (pJWD2) was transformed into strain JM105 and purified, and the insert containing the *lrp* gene was sequenced. We determined that a single base mutation had occurred during the amplification and cloning process, a T-to-C substitution 444 nt from the beginning of the *lrp* coding sequence, resulting in the change of a GTC codon to a GTT codon. Both of these codons specify valine, and the codon usage frequencies were unchanged by this mutation. Plasmid pJWD2 was transformed into *lrp*::Tn10 strain BE1. The resulting strain, JWD3, was used as the source of Lrp protein for purification.

Strain JWD3 was grown in STG medium at 37°C until the A_{550} was between 1 and 1.5. At this time, IPTG was added to a final concentration of 0.5 mM, and the cells were incubated at 37°C for a further 2 h. After the induction, cells were harvested by centrifugation, resuspended in TG₁₀ED (10 mM Tris-Cl [pH 8.0], 10% [vol/vol] glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol), and sonicated in the presence of the protease inhibitors phenylmethylsulfonyl fluoride (20 μ M) and tosyl-L-lysine chloromethylketone (2 μ M). The sonicate was centrifuged at $33,000 \times g$ for 1 h. Three liters of induced culture yielded 34.5 g of wet cells, with Lrp present at approximately 2.5% of the total soluble protein in the supernatant. The supernatant was loaded onto a carboxymethyl acrylate (Bio-Rad) column with a bed volume of 30 ml. The column resin had been previously equilibrated with TG₁₀ED–0.2 M NaCl (pH 8.0). The column was washed with TG₁₀ED–0.2 M NaCl until the A_{280} of the eluate dropped to 0.05, and a linear 200-ml NaCl gradient from 0.2 to 1 M NaCl in TG₁₀ED was used for elution. Three- to 4-ml fractions were collected,

and the conductivity of each fraction was monitored. Denaturing polyacrylamide gel electrophoresis was used to monitor the proteins in each fraction. The Lrp protein was eluted from the column at a NaCl concentration of 0.4 M. Fractions containing Lrp were pooled and concentrated to 2.5 mg of protein per ml with an Amicon YM10 concentrator. The concentrate was loaded onto a Superose-12 MR 10/30 fast protein liquid chromatography gel filtration column (Pharmacia) that had been equilibrated with TG₁₀ED–0.2 M NaCl and then was isocratically eluted with the same buffer. Fractions containing electrophoretically homogeneous Lrp were pooled, and glycerol was added to a final concentration of 50%. The Lrp protein was concentrated to 2.1 mg/ml and stored at –20°C. The final yield of homogenous Lrp from 3 liters of induced culture was 38.5 mg.

Mapping, cloning, and sequencing of the *glbBDF* promoter region. The *glbBDF* operon has been reported to map near 69 min on the *E. coli* chromosome (3). Using the method of Versalovic et al. (28), we isolated the Kohara phages (27) in the 69-min region (Kohara miniset phages 520 to 528) and subjected the lysates to PCR. The primers used to detect the presence of *glbBDF* sequences were 5'-GTTAACACCTTATGAC-3' and 5'-ACGCGCGCCTCCGATTTCG-3'. PCR assays with these primers were performed with 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. The products from each PCR were separated on agarose gels. Phage lysates from Kohara phages 523 and 524 yielded the expected 300-bp PCR product. Larger (50-ml) lysates of these two phages were prepared, and phage DNA was isolated. The DNA was cut with *Kpn*I or with *Kpn*I and *Hind*III. There are two *Hind*III sites in the 300-bp region between the primers, so any *Hind*III digest should not yield an amplified product. The resulting fragments were separated on a 1.5% agarose–TAE gel. Each of the fragments was cut from the gel and purified. These fragments were subjected to PCR with the same primers. One *Kpn*I fragment of approximately 2,100 bp and common to both PCR-positive phages yielded the 300-bp PCR product, while no fragment resulting from the *Hind*III digestions yielded an amplified PCR product. The PCR-positive 2,100-bp *Kpn*I fragment was ligated into the *Kpn*I site of plasmid pBSII (KS)+ (Stratagene) to construct plasmid pBE10 (Fig. 1). The sequence of the upstream portion of the insert extending from –573 bp to +145 bp relative to the start of transcription was determined (GenBank accession number L20253). When our sequencing overlapped that previously reported by Oliver and coworkers (19), the two sequences were identical.

*Tfi*I digests of pBE10 did not produce the expected number of fragments. Several of the restriction digests used in the binding studies included the restriction endonuclease *Tfi*I. Whenever *Tfi*I was included in a restriction digest, the numbers and sizes of fragments were consistent with a lack of *Tfi*I cleavage at the predicted *Tfi*I site located at +110 bp relative to the transcription start. We sequenced this region on both strands, confirming the presence of the predicted *Tfi*I site. This site was not included in the 680 bp of *glbBDF* DNA that was subcloned for further binding studies.

Plasmid pBE15 (Fig. 1) carries a subclone of the *glbBDF* promoter region from pBE10 inserted into the *Bam*HI and *Sal*I sites of plasmid pKK232-8 (Pharmacia). Plasmid pBE10 was subjected to PCR with five cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 1 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The following primers were used for the PCR.

FIG. 1. Plasmids pBE10 and pBE15 containing the *gluBDF* upstream sequences. The positions of restriction sites used in the manipulation of these plasmids are indicated. Heavy lines indicate DNA derived from the *gluBDF* structural genes and upstream region. The *Tfi*I site in parentheses is predicted by sequence analysis but is not cut with *Tfi*I under the conditions tested (see Materials and Methods). T_1 in pBE15 is the first transcription terminator sequence downstream of *rrnB*.

primer 3: 5' CGAGGGATCCGGTACCGCGGTCTAGATACCGTCACGGTTAGGGCAG 3'
 primer 4: 5' CGCCGTCGACTCGCCCCCTTGTGTCTTT 3'

Sequences in italics are complementary to the *gluBDF* promoter sequences. The upstream primer (primer 3), complementary to the *gluBDF* sequence beginning at 572 bp upstream of the transcription start, contains an added *Bam*HI site. The downstream primer, complementary to the *gluBDF* sequence ending 109 bp downstream of the transcription start, contains a *Sal*I site. The 716-bp PCR product was cleaved with *Bam*HI and *Sal*I and ligated into the *Bam*HI and *Sal*I sites of plasmid pKK232-8 so that the *gluBDF* promoter was inserted upstream of the chloramphenicol acetyltransferase reporter gene. *Bam*HI-*Sal*I digestion fragments of pBE15 were end labeled and used for gel mobility shift assays.

Gel mobility shift assay. The gel retardation assay for the binding of Lrp protein to the 406-bp fragment of the *ilvIH* promoter region from plasmid pCV112 was carried out essentially as described by Ricca et al. (22). Plasmid pCV112 DNA was cut with *Bgl*I, *Eco*RI, and *Hind*III and diluted to 0.01 μ g/ μ l in labeling reaction buffer (50 mM Tris-Cl [pH 8], 50 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol) in the presence of 0.5 μ l of [α -³²P]dATP (10 μ Ci/ μ l; 25 Ci/mmol; ICN 33002) and 2 U of avian myeloblastosis virus reverse transcriptase. The labeling reaction mixture was incubated at 37°C for 30 min. Unincorporated nucleotides were removed by gel filtration with a Bio-Rad Bio-Spin-6 column, and labeled DNA was stored at -20°C until its use. Binding reaction buffer contained 20 mM Tris-acetate (pH 8), 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 mM NaCl, 4 mM magnesium acetate, 12.5% glycerol, and 0.15 μ g of sonicated calf thymus DNA per μ l as a nonspecific competitor. Various amounts of Lrp protein and leucine were added to the binding reaction mixture, and the mixture was incubated at room temperature for 10 min. Following incubation, the binding reaction mixture was separated on a 1.25% agarose gel with 1 $\times$ TAE buffer. After the gel had run for 1.5 to 2 h at a 200-V constant voltage, it was dried at 60°C and exposed overnight on a storage phosphor screen (Molecular Dynamics) or on Kodak XAR film. Gels exposed on storage phosphor screens were visualized on a Molecular Dynamics Phosphorimager. Image Quant software supplied by Molecular Dynamics was used to quantitate the intensity of each resolved band. For DNA fragments that bound Lrp, the intensities of

the shifted and unshifted bands were added, and each band was represented as a percentage of the total fragment intensity. There was no density above the background between the shifted and unshifted bands. Gel retardation assays for Lrp binding to various fragments of the *gluBDF* promoter from plasmids pBE10 and pBE15 were similar, except that plasmid pBE10 was cut with *Tfi*I and either *Kpn*I or *Nsi*I and diluted to 0.017 μ g/ μ l and plasmid pBE15 was cut with *Bam*HI and *Sal*I and diluted to 0.019 μ g/ μ l. These dilutions ensured that DNAs from all three plasmids were present at equal molar concentrations (240 pM) in the binding reaction mixture. Fragments derived from pBE15 were labeled with [α -³²P]dGTP (10 μ Ci/ μ l; 25 Ci/mmol; ICN 33005) as described above.

β -Galactosidase assay. β -Galactosidase activity and culture density were measured at intervals during exponential growth. The assay used was that described by Miller (14), as modified by Platko et al. (20). At each time point, two samples were removed from the culture. One sample was diluted 1:5 in fixer (minimal MOPS medium containing 0.9% formaldehyde) and used to determine A_{420} of the culture. The cells in the second sample were permeabilized by mixing with an equal volume of an aqueous solution of cetyl trimethylammonium bromide (200 μ g/ml)-sodium deoxycholate (100 μ g/ml) and incubating at 4°C overnight. Permeabilized cells (0.5 ml) were mixed with 0.5 ml of assay buffer (0.1 M sodium phosphate buffer [pH 7.0], 1 mM magnesium sulfate, 2 mM manganese sulfate, 50 mM β -mercaptoethanol) and incubated at 28°C in the presence of 0.15 ml of 4 mg of *o*-nitrophenyl- β -D-galactopyranoside (ONPG) per ml. The assay was stopped by the addition of 0.325 ml of 1 M sodium carbonate. Cells were removed by centrifugation, and the A_{420} of the supernatant was measured. One unit of activity was defined as 1 nmol of ONPG hydrolyzed per min, assuming a molar extinction coefficient of 5,000 at 420 nm for *o*-nitrophenol (20).

RESULTS

The expression of *gluBDF::lacZ* operon fusions is regulated by Lrp. Prior studies had indicated that glutamate synthase expression and activity are significantly lower in strains that do not produce a functional Lrp protein than in isogenic wild-type strains (6). We used transcriptional fusions of the *lacZ* reporter

FIG. 2. Effects of Lrp and leucine on *gltBDF* and *ilvIH* operon fusions. Cells were grown in glucose minimal MOPS medium containing either isoleucine and valine (closed symbols) or isoleucine, valine, and 10 mM leucine (open symbols). The slopes of these plots, units of β -galactosidase activity per A_{420} unit, represent Miller units of β -galactosidase activity. (A) In the absence of leucine, strain BE3479 (*gltBDF::lacZ lrp*⁺; diamonds) showed a slope of 441 Miller units. In the presence of leucine, this strain expressed β -galactosidase at a level of 196 Miller units. Strain BE3779 (*gltBDF::lacZ lrp::Tn10*; circles) expressed β -galactosidase at 9.6 Miller units in the presence and absence of leucine. (B) Strain BE54 (*ilvIH::lacZ lrp*⁺ diamonds) showed slopes of 54.0 Miller units in the absence of leucine and 9.9 Miller units in the presence of 10 mM leucine. Strain BE55 (*ilvIH::lacZ lrp::Tn10*; circles) showed a slope of 1.75 Miller units in the absence of leucine.

gene to the *gltBDF* operon to confirm the effects of Lrp and leucine on the expression of this operon and to determine whether the effect of Lrp was exerted at the level of transcription. We investigated three transcriptional fusions of *lacZ* to the *gltBDF* operon, *psiQ32*, *psiQ35*, and *psiQ39* (Table 1) (13). The *psiQ32* and *psiQ35* fusion points are located in the *gltB* gene at nt 1613 and 1389, respectively, in the *gltBD* sequence published by Oliver et al. (19). The *psiQ39* fusion point is located in the *gltD* gene at nt 5877. These three fusions behaved identically to one another in both *lrp*⁺ and *lrp::Tn10* backgrounds. The results of assays with the *psiQ32* fusion are shown in Fig. 2A. In an *lrp*⁺ background, 10 mM leucine repressed *gltBDF::lacZ* expression 2.2-fold, consistent with the effect of leucine on glutamate synthase activity reported pre-

TABLE 2. Expression of a *gltBDF::lacZ* operon fusion^a in various media

Medium	β -Galactosidase activity (Miller units) in strain:	
	BE3479 (<i>lrp</i> ⁺)	BE3779 (<i>lrp::Tn10</i>)
Glucose minimal MOPS	440	11.5
LB	26.0	7.8
Glucose rich MOPS	31.0	ND ^b

^a Strains BE3479 and BE3779 contain the *psiQ32* fusion.

^b ND, not determined.

viously (6). In an *lrp::Tn10* background, the expression of *gltBDF::lacZ* was unaffected by leucine and was 44-fold lower than expression in an *lrp*⁺ strain grown in the absence of leucine.

The expression of *gltBDF::lacZ* operon fusions is regulated by medium composition. As shown in Table 2, strain BE3479, containing the *psiQ32* fusion in an *lrp*⁺ background, expresses β -galactosidase at a level 16-fold lower when grown in LB than when grown in glucose minimal MOPS medium. Strain BE3479 shows a similar reduction in the level of *gltBDF::lacZ* expression when grown in the defined rich medium glucose rich MOPS. The same fusion in an *lrp::Tn10* background shows less than a twofold difference in β -galactosidase activity when expression in LB and expression in glucose minimal MOPS medium are compared. The reduced levels of *gltBDF* expression seen in rich media and in *lrp* strains may be causally connected if growth in rich media leads to reduced concentrations of Lrp; evidence on this point will be presented in the Discussion section.

Mapping and cloning of the *gltBDF* promoter region. We next wished to study in vitro interactions between Lrp and the *gltBDF* promoter region. The *gltBDF* operon was cloned and sequenced by Oliver et al. (19), but this published sequence extended only 178 bp upstream of the reported start site of transcription. It is possible that this region would not contain all of the determinants for Lrp regulation of the operon. To construct a clone carrying more of the *gltBDF* upstream region, we used PCR to establish that Kohara phages 523 and 524 (27) contain the *gltBDF* operon and isolated a 2,100-bp *KpnI* fragment containing the *gltBDF* promoter region as described in Materials and Methods. Plasmid pBE10, containing this fragment, and plasmid pBE15, containing the 5' region of this fragment, were used for gel mobility shift assays.

Lrp binds specifically to DNA containing the *gltBDF* promoter. Restriction digests of plasmid pBE10 yielded a mixture of fragments containing either vector DNA, *gltBDF* DNA, or a combination of vector and *gltBDF* DNAs. We carried out gel mobility shift assays with purified Lrp and various restriction digests of the plasmid, which generated a number of radiolabeled fragments. Fragments binding Lrp showed altered mobility, while fragments that did not bind Lrp at the concentrations tested showed normal mobility, acting as internal controls for the specificity of each binding reaction. As an additional control, we carried out mobility shift assays of Lrp binding to the 406-bp fragment of the *ilvIH* promoter (20). The results of one of these experiments are shown in Fig. 3. Lanes 1 through 8 show the 1,370- and 406-bp fragments of the *ilvIH* promoter. As the Lrp dimer concentration is increased from 0 to 25 nM, the 406-bp fragment, which contains the *ilvIH* promoter, shifts first into a high-affinity complex containing Lrp dimers bound at two sites located 200 to 250 bp upstream of the start of transcription and then into a lower-affinity complex containing

FIG. 3. Lrp binds specifically to the *glbBDF* promoter region. Gel mobility shift assays were performed with an agarose gel as described in Materials and Methods. Lanes 1 to 8 each contain an end-labeled *EcoRI*-*BglI*-*HindIII* digest of plasmid pCV112, carrying the *ilvIH* promoter, with Lrp concentrations as indicated. Lanes 9 to 16 each contain an end-labeled *KpnI*-*TfiI* digest of plasmid pBE10, carrying the *glbBDF* promoter, with the same concentrations of Lrp as in lanes 1 to 8. Lrp binds only to the 406-bp fragment of the *ilvIH* promoter and to the 916-bp fragment of the *glbBDF* promoter. For all lanes, labeled DNA was present in the binding reaction mixture at 240 pM.

Lrp dimers bound at four additional sites located 50 to 150 bp upstream of the transcription start (29). It is this second complex, containing Lrp dimers bound at six sites, that is required for transcriptional activation (29). Lanes 9 through 16 show the same range of Lrp concentrations incubated with a *KpnI*-*TfiI* digest of plasmid pBE10, containing the *glbBDF* promoter region. The 916-bp fragment resulting from this digestion extends from the *KpnI* site at -572 bp to the *TfiI* site at +344 bp and contains the *glbBDF* promoter. This fragment binds Lrp in a single complex at an affinity comparable to that of the high-affinity Lrp-*ilvIH* complex.

Since the 916-bp fragment from pBE10, containing the *glbBDF* promoter region, is rather large, we wished to perform mobility shift assays with a smaller fragment to determine whether intermediate complexes were formed during an Lrp titration. A 666-bp *NsiI*-*TfiI* fragment extending from -322 to +344 of the *glbBDF* promoter region was also shown to bind Lrp with the same affinity as the 916-bp *KpnI*-*TfiI* fragment (data not shown), and only one bound complex with Lrp was seen on both acrylamide and agarose gels.

Lrp binds to the *glbBDF* and *ilvIH* promoters in the presence and absence of leucine. As described above, *glbBDF::lacZ* fusion experiments and enzyme assays showed that the presence of 10 mM leucine in the growth medium leads to a decrease of approximately twofold in both the level of glutamate synthase activity in crude extracts and the level of β -galactosidase activity expressed from a *glbBDF* operon fusion. *ilvIH* expression, as measured from similar operon fusions, is decreased 5.5-fold when leucine is present in the growth medium at 10 mM (Fig. 2B). The relative insensitivity of glutamate synthase expression to the presence or absence of leucine in the growth medium could be explained by leucine-insensitive binding of Lrp to the *glbBDF* promoter. To compare Lrp binding to these promoters, we needed to choose a concentration of leucine that would be saturating for both *ilvIH* and *glbBDF*.

Figure 4 shows the results of gel mobility shift assays of Lrp binding to the 692-bp fragment of plasmid pBE15, containing the *glbBDF* promoter, at various concentrations of leucine. Increasing concentrations of leucine, at a fixed Lrp concentration, decrease binding, but this effect is saturable. The de-

creased binding effected by leucine is not seen when isoleucine, valine, or glutamate is substituted for leucine (data not shown). At concentrations of leucine higher than 20 mM, no further decrease in Lrp binding is seen. Similar results were seen in experiments with Lrp binding to the *ilvIH* promoter: at a fixed concentration of Lrp, increasing the leucine concentration beyond 20 mM does not lead to further decreases in Lrp binding (data not shown).

The saturability of this system allowed us to investigate Lrp binding to the *ilvIH* and *glbBDF* promoters in the absence of leucine and under conditions in which the leucine effect is maximal. Figure 5 shows Lrp binding to the *glbBDF* promoter

FIG. 4. The leucine effect on Lrp binding to the *glbBDF* promoter is saturable. Plasmid pBE15 was cut with *Bam*HI and *Sal*I and end labeled. The labeled DNA, present at 240 pM in the binding reaction mixture, was electrophoresed in the presence of Lrp with or without leucine. Data from gel mobility shift assays were quantified by Phosphorimager scanning and plotted as percent DNA bound at various concentrations of leucine in the binding reaction mixture. Lrp was present at the concentrations indicated.

FIG. 5. Lrp binds to the *gltBDF* promoter in the presence and absence of leucine. An end-labeled *Bam*HI-*Sal*I digest of plasmid pBE15 was electrophoresed in the presence or absence of 30 mM leucine. Only the 692-bp fragment containing the *gltBDF* promoter is shown here. Lanes contained the following Lrp concentrations: 1 and 12, no Lrp; 2, 0.0625 nM; 3, 0.125 nM; 4, 0.25 nM; 5, 0.5 nM; 6, 1 nM; 7, 2 nM; 8, 3 nM; 9, 4 nM; 10, 5 nM; 11, 10 nM Lrp. Labeled DNA was present at 240 pM in the binding reaction mixture.

in the presence and absence of 30 mM leucine. The Lrp binding data from this experiment were quantified by Phosphorimager scanning and plotted in Fig. 6A. The data in Fig. 6 were fitted to equation 1, the Hill equation (10), assuming n Lrp binding sites:

$$y = ([Lrp]^{\alpha}/K^{\alpha}) / (1 + [Lrp]^{\alpha}/K^{\alpha}) \quad (1)$$

In this equation, K is the apparent dissociation constant for the Lrp-DNA complex, y is the fractional saturation of a site or sites with ligand, and $1 \leq \alpha \leq n$ is a measure of the apparent degree of cooperativity of binding. For n thermodynamically identical sites, $\alpha = 1$ indicates no cooperativity while $\alpha = n$ indicates perfect cooperativity of the binding event. Hill binding curves fitted to the plotted data showed that, in the presence of leucine, the affinity of Lrp for *gltBDF* promoter DNA was reduced 3.3-fold and that the apparent cooperativity of binding was increased from $\alpha = 1.6$ to $\alpha = 2.6$. Similar results were seen when the binding assays were carried out with the *ilvIH* promoter. In these experiments, Lrp initially formed a complex involving cooperative binding to two high-affinity sites upstream of the *ilvIH* promoter (resulting in the smaller mobility shift) and then bound cooperatively to lower-affinity sites located closer to the start site for transcription (resulting in the larger mobility shift). Since transcriptional activation is associated with the occupancy of the lower-affinity sites (29), we have plotted the percent DNA bound in the second complex, in which Lrp is bound to both low- and high-affinity sites. The apparent affinity of Lrp for occupancy of the lower-affinity sites was decreased twofold in the presence of leucine, while the apparent cooperativity was increased from $\alpha = 1.1$ to $\alpha = 2.6$. The results are plotted in Fig. 6B. Although the assumption of n thermodynamically identical binding sites for Lrp is clearly incorrect, the data are quite well fitted by the Hill equation.

From the data in Fig. 6, we constructed curves describing the effect of leucine on the binding of Lrp to these two promoters (Fig. 7). The leucine sensitivity was determined for each system by dividing each point on the fitted binding curve measured in the absence of leucine by the corresponding point on the fitted binding curve measured in the presence of 30 mM leucine. At low concentrations of Lrp, the leucine sensitivity is infinite. At high concentrations of Lrp, at which the DNA is fully bound in the presence and absence of leucine, leucine has no effect, and

FIG. 6. Effect of leucine on Lrp binding to the *gltBDF* and *ilvIH* promoters. Data from gel mobility shift assays carried out in the absence or presence of 30 mM leucine were quantified by Phosphorimager scanning and plotted as percent DNA bound at various concentrations of Lrp. Theoretical Hill binding curves (10) were fitted to the data. Lrp binds to the *gltBDF* promoter (A) with an apparent K value of 2.0 nM and a value for α of 1.6 in the absence of leucine and an apparent K value of 6.6 nM and a value for α of 2.6 in the presence of 30 mM leucine. Apparent K and α values for Lrp binding to the low-affinity complex (the more retarded band), required for activation of transcription of the *ilvIH* promoter (B), are $K = 6.9$ nM and $\alpha = 1.1$ in the absence of leucine and $K = 14.1$ nM and $\alpha = 2.6$ in the presence of 30 mM leucine. The vertical line is drawn at the concentration of Lrp at which leucine has a 2.2-fold effect on Lrp binding to the *gltBDF* promoter.

the value on the y axis is 1. From the *in vivo* *gltBDF::lacZ* fusion experiments (Fig. 2A), we estimated a 2.2-fold effect of leucine on the expression of glutamate synthase. This degree of sensitivity to leucine defines a unique effective intracellular Lrp concentration of 5.5 nM under these growth conditions, assuming that our mobility shift assays reflect the effect of Lrp on the transcription of *gltBDF::lacZ* fusions. The vertical lines in Fig. 6 and 7 are drawn at this concentration of Lrp. At this concentration of Lrp, leucine decreases the binding of Lrp to the *ilvIH* promoter by a factor of approximately 5.5. These data from the *in vitro* binding studies match the 5.5-fold effect of leucine on the expression of the *ilvIH* operon fusions *in vivo* (Fig. 2B). Thus, independent data from two DNA binding studies predict an identical effective intracellular concentration of Lrp during exponential growth in glucose minimal MOPS medium.

DISCUSSION

The identification of genes and proteins whose expression is regulated by Lrp, but not apparently in response to the presence or absence of leucine in the growth medium, was the

FIG. 7. The in vitro effect of leucine on Lrp binding to the *gltBDF* and *ilvIH* promoters explains the leucine sensitivity of these operons in vivo. The curves shown were constructed by dividing the percent DNA bound at each Lrp concentration in the absence of leucine by the percent DNA bound at the corresponding Lrp concentration in the presence of 30 mM leucine, using the data for *gltBDF* and *ilvIH* shown in Fig. 6. The vertical line is drawn at the concentration of Lrp at which leucine has a 2.2-fold effect on Lrp binding to the *gltBDF* promoter. At this concentration of Lrp, leucine has a 5.5-fold effect on the binding of Lrp to the *ilvIH* promoter.

most surprising result to emerge from an analysis of the Lrp regulon (6). The *gltBDF* operon, encoding glutamate synthase, was among the group of genes and proteins positively regulated by Lrp but insensitive to leucine. In this work, we have provided evidence consistent with the direct regulation of *gltBDF* expression by Lrp and demonstrating that 10 mM exogenous leucine decreases the expression of this operon approximately twofold. Lrp binds specifically to DNA fragments containing the *gltBDF* promoter, and this binding occurs even in the presence of 30 mM leucine in the binding reaction mixture, although with a reduced affinity. The discovery of a high-affinity specific site or sites upstream of the *gltBDF* operon allowed us to compare the Lrp regulation of the *gltBDF* operon and the more leucine-responsive *ilvIH* operon.

Lrp has been shown to bind to the *ilvIH* promoter in two steps (29). At low concentrations of Lrp, Lrp dimers bind to two sites more than 200 bp upstream of the transcription start in a single highly cooperative step. As Lrp concentrations are increased, a second complex containing Lrp dimers bound to four additional sites near the transcription start is formed in a second highly cooperative step. It is this second complex, containing Lrp dimers bound at six sites, that is required for the transcriptional activation of *ilvIH* transcription (29). The *K* for the Lrp-*ilvIH* activation complex is 6.9 nM, while the *K* for the Lrp-*gltBDF* complex is 2.0 nM. The *K* for the Lrp-*ilvIH* promoter reported by Wang and Calvo (29) was somewhat higher (22 nM) and may reflect an Lrp preparation of lower activity.

Leucine sensitivity reflects the affinity of Lrp for target promoters. It is possible that the differing responses to leucine of these two operons are a function of the differing affinities of the two promoters for Lrp. The observation that the *gltBDF* promoter, which is relatively insensitive to leucine, has a higher affinity for Lrp than does the leucine-sensitive *ilvIH* promoter suggests a model: positively regulated genes and operons that bind Lrp with a high affinity are intrinsically less sensitive to the presence or absence of exogenous leucine than are positively

regulated genes and operons that bind Lrp with a lower affinity. This model predicts that at sufficiently high concentrations of Lrp, promoters will form complexes with an activator regardless of the presence or absence of leucine and that all positively regulated genes and operons will be insensitive to leucine. Conversely, at sufficiently low concentrations of Lrp, all positively regulated genes and operons will be sensitive to leucine.

The binding of Lrp to the *gltBDF* and *ilvIH* promoters in the presence and absence of leucine is in accord with this model. In the presence of 30 mM leucine, the affinity of Lrp for DNA is reduced, but as Lrp concentrations are increased the DNA can nonetheless be driven into the bound complex. We estimate that at an effective Lrp concentration of 5.5 nM, binding to the *gltBDF* promoter would be reduced 2.2-fold in the presence of leucine, while binding to the *ilvIH* promoter would be reduced 5.5-fold. These in vitro leucine sensitivities are in excellent agreement with the in vivo leucine sensitivities measured during exponential growth in glucose minimal MOPS medium with operon fusions. According to this model, the binding of leucine by Lrp would affect only the affinity of the DNA-Lrp interaction; Lrp would retain its ability to activate transcription regardless of the presence or absence of bound leucine.

The effective intracellular concentration of Lrp during exponential growth in glucose minimal MOPS medium is 5.5 nM. The fact that a unique Lrp concentration yields the proper leucine sensitivities for both *gltBDF* and *ilvIH* suggests that this is the effective intracellular concentration of Lrp during steady-state growth in glucose minimal MOPS medium. Willins and coworkers have estimated that there are approximately 3,000 copies of Lrp dimer per cell during growth in glucose minimal medium (32). Given a cellular volume of 6.7×10^{-13} ml (16), this value predicts that the Lrp concentration in the cell is on the order of 7.5 μ M, over 1,000-fold higher than the effective concentration of Lrp that accounts for the leucine sensitivities of the *gltBDF* and *ilvIH* operons. This difference may be explained, at least in part, if most of the Lrp molecules in the cell are bound nonspecifically or specifically to chromosomal DNA and are therefore not readily available for interaction with any given site. It is important to note that the conditions in the cell are quite different from the conditions in our binding experiments. In the cell, Lrp may interact with a complex mixture of solutes and proteins, some of which may be bound to DNA near Lrp binding sites. Our binding reaction mixture consisted of a simple mixture of solutes and buffers, without proteins other than Lrp. In the cell, the chromosome is in a negatively supercoiled state, while fragments used in the binding reaction were linear. Nevertheless, the model based on the results of the in vitro binding experiments provides a reasonable explanation of the difference between leucine-sensitive and leucine-insensitive operons.

The effects of changing the Lrp concentration in vivo predict two-stage regulation of *gltBDF*. The model described above predicts that altering the level of Lrp in the cell provides a method of modulating the expression of Lrp-regulated genes. During growth in LB, the expression of a chromosomal *lrp::lacZ* fusion in an *lrp* background is repressed more than 10-fold, in comparison with that during growth in glucose minimal medium (11). Transformation of this strain with a plasmid-encoded *lrp* gene results in only twofold repression in LB. Experiments in our own laboratory with chromosomal *lrp::lacZ* operon fusions, inserted at the *attB* locus in a mero-diploid *lrp*⁺ background, have shown a 3.9-fold repression of *lrp::lacZ* expression in LB (4a).

We predict that a shift from growth in a minimal medium to growth in a rich medium containing leucine would have an immediate effect, as the level of Lrp binding to the *gltBDF*

promoter is reduced twofold by leucine. Continued growth in this medium would result in slowly falling concentrations of Lrp, as preexisting Lrp molecules are diluted through degradation, doubling, and growth, leading to progressively less binding and activation by Lrp at the *gluBDF* promoter. Furthermore, as the concentration of Lrp drops, Lrp-activated promoters should become more sensitive to leucine. The 16-fold effect of a rich medium on the expression of *gluBDF* supports this hypothesis. The *gluBDF* binding data plotted in Fig. 6 predict that the effective concentration of Lrp will have decreased approximately 3.5-fold under these conditions. This is the reduction in the Lrp concentration required to reduce binding from 84% in the absence of leucine to 5.3% in the presence of leucine. This proposed two-stage regulation of *gluBDF* expression in response to a rich medium would allow the cell to regulate *gluBDF* expression over a wide range in response to changes in the form of supplied nitrogen. In a rich medium, in which nitrogen is present in the form of amino acids, oligopeptides, and nucleic acids, in addition to ammonia, the demand for glutamate and glutamine synthesis is greatly reduced, in comparison with the situation during growth in a minimal medium, in which nitrogen is supplied solely in the form of ammonia.

Does leucine affect Lrp cooperativity? The data plotted in Fig. 6 have been fitted to the Hill equation. The parameters specified for these curve fits are the apparent K value for the Lrp-DNA complex and the apparent α value. Higher α values denote increased cooperativity for a given number of identical sites (n , where $\alpha < n$). In these experiments, we have neither shown that the sites to which Lrp is binding are thermodynamically identical nor distinguished between cooperativity arising from interactions between Lrp dimers bound at adjacent sites on the DNA and cooperativity due to assembly of Lrp monomers to form dimers during DNA binding. The increased cooperativity in the presence of leucine suggests that much of the cooperativity may be due to interactions between Lrp monomers associated with binding events. If at nanomolar Lrp concentrations, leucine shifts the monomer-dimer equilibrium toward the monomeric form, a larger fraction of Lrp dimers will be dissociated in the presence of leucine. Under these conditions, putative cooperative interactions between monomers bound to DNA would become more important than in the absence of leucine, and increased cooperativity of the binding event might be seen. Lrp binding to an isolated site in the presence and absence of leucine will be studied to gain further insight into the nature of the observed cooperativity.

The basis of the differing affinities of Lrp for the DNA of the *gluBDF* and *ilvIH* promoters remains a major question. Wang and Calvo (29) have shown that the highest-affinity site in the upstream region of the *ilvIH* promoter contains the palindromic sequence AGAATtttATTCT. This exact palindrome does not occur in the *gluBDF* promoter region, even though this promoter region binds Lrp with an affinity comparable to that of Lrp for the high-affinity sites upstream of the *ilvIH* operon. The definition of the DNA sequences important for the Lrp regulation of *gluBDF* expression will be useful in the continuing effort to define DNA sequences recognized by Lrp in specific binding events and is currently under investigation in our laboratory.

Binding of other regulatory proteins to regulated promoters. The binding of regulatory proteins to DNA has been studied with many systems. Typically, such regulatory proteins alter the level of transcription of a target gene, and the regulation is modulated either by binding of a ligand to or by covalent modification of the regulatory protein. The Lrp-DNA interactions studied in this work suggest that the primary effect

of leucine is to modulate the affinity of Lrp for DNA over a relatively narrow range. In contrast, the affinity of the Lac repressor with a bound inducer for the *lac* operator is 2×10^4 -fold weaker than the affinity of the unligated Lac repressor (7). The catabolite activator protein does not bind specifically to DNA in the absence of cyclic AMP (1). Upon binding cyclic AMP, the catabolite activator protein undergoes a conformational change that allows it to bind at its regulatory sites and activate transcription. The Lac repressor and the catabolite activator protein represent a class of regulators which, like a switch, may exist in either an "off" or an "on" state, with only the on state able to bind DNA and bring about the regulation of target genes.

Ligation or modification of other transcriptional regulators is known to alter their affinity for DNA, without abolishing specific binding. OxyR, the regulatory protein of the oxidative stress regulon, is bound specifically to the DNA of its target genes even in the absence of the covalent modification required for the activation of transcription (26). Upon oxidation, in a process that alters the structure of the complex, the already-bound OxyR becomes competent to activate transcription. Similarly, the MerR regulatory protein binds to the DNA of the *merT* promoter in the presence and absence of the inducer Hg(II). This regulator, though, acts as a repressor in the absence of Hg(II) and as an activator in the presence of Hg(II) (18). Both of these regulatory functions involve MerR bound at a single site, with the activator (ligated) form binding with an approximately threefold-lower affinity than the repressor (unligated) form.

The binding of the phosphorylated and nonphosphorylated forms of regulatory protein NR₁ to the positively regulated σ^{54} -dependent *gluAp₂* promoter has been characterized by Weiss et al. (31). In this system, a 20-fold increase in the affinity of phosphorylated NR₁ over the nonphosphorylated form is brought about by a large increase in the cooperativity of binding of the phosphorylated form. Phosphorylation of the NR₁ protein therefore leads to increased binding and more efficient transcriptional activation of the *gluAp₂* promoter.

Our model for Lrp binding to the DNA of the *ilvIH* and *gluBDF* operons is distinct from systems in which the action of the regulator is either off or on. We have demonstrated that the binding of Lrp to specific DNA sequences occurs in the presence and absence of leucine and that the activation of transcription occurs under both conditions. In contrast to the on or off binding of the switch-like Lac repressor and catabolite activator protein, leucine has a relatively subtle effect on the binding of Lrp to target genes. Major effects on the expression of genes regulated by Lrp may occur through a two-stage process involving immediate effects of leucine following a shift to a rich medium and then slowly decreasing Lrp concentrations in response to continued growth in a rich medium.

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