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Use of an In Vivo Titration Method To Study a Global Regulator: Effect of Varying Lrp Levels on Expression of *gltBDF* in *Escherichia coli*

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Most studies of global regulatory proteins are performed in vitro or involve phenotypic comparisons between wild-type and mutant strains. We report the use of strains in which the gene for the leucine-responsive regulatory protein (*lrp*) is transcribed from isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoters for the purpose of continuously varying the in vivo concentration of Lrp. To obtain a broad range of Lrp concentrations, strains were employed that contained the *lrp* fusion either in the chromosome (I. C. Blomfield, P. J. Calie, K. J. Eberhardt, M. S. McClain, and B. I. Eisenstein, J. Bacteriol. 175:27–36, 1993) or on a multicopy plasmid. Western blot (immunoblot) analysis with polyclonal antiserum to Lrp confirmed that Lrp levels could be varied more than 70-fold by growing the strains in glucose minimal 3-(*N*-morpholino)propanesulfonic acid (MOPS) medium containing different amounts of IPTG. Expression of an Lrp-regulated *gltB*::*lacZ* operon fusion was measured over this range of Lrp concentrations. β-Galactosidase activity rose with increasing Lrp levels up to the level of Lrp found in wild-type strains, at which point expression is maximal. The presence of leucine in the medium increased the level of Lrp necessary to achieve half-maximal expression of the *gltB*::*lacZ* fusion, as predicted by earlier in vitro studies (B. R. Ernsting, J. W. Denninger, R. M. Blumenthal, and R. G. Matthews, J. Bacteriol. 175:7160–7169, 1993). Interestingly, levels of Lrp greater than those in wild-type cells interfered with activation of *gltB*::*lacZ* expression. The growth rate of cultures correlated with the intracellular Lrp concentration: levels of Lrp either lower or higher than wild-type levels resulted in significantly slower growth rates. Thus, the level of Lrp in the cell appears to be optimal for rapid growth in minimal medium, and the *gltBDF* control region is designed to give maximal expression at this Lrp level.

The leucine-responsive regulatory protein (Lrp) is a global transcriptional regulator in *Escherichia coli*. Lrp affects the expression of several dozen genes and operons, many of which are involved in amino acid metabolism and transport or pilin synthesis (6, 23, 28). It is thought that Lrp enables the cell to respond to its external environment, primarily sensing the availability of amino acids and nitrogen-containing bases, in order to regulate many metabolic pathways (6, 28). Genes involved in amino acid biosynthesis are positively regulated by Lrp, whereas genes involved in catabolic reactions are negatively regulated. Consistent with this role of Lrp, the expression of the *lrp* gene is high in minimal media and lower in rich media (6, 22a). One of the most interesting aspects of regulation by Lrp is the number of different modes of regulation which have been observed and the varied responses to the coregulator, leucine. Lrp can either activate or repress expression from target genes, and leucine may antagonize, potentiate, or have little effect on the regulation by Lrp.

We are studying the glutamate synthase operon (*gltBDF*) because it is a positively regulated operon that is highly sensitive to Lrp and relatively insensitive to leucine. Previous studies have shown that the *gltBDF* operon is regulated by Lrp at the level of transcription and that Lrp binds specifically upstream of *gltBDF* (12). These studies used in vitro methods such as gel mobility shift assays to measure the binding of Lrp to the promoter DNA of target genes and led to the proposal that sensitivity of Lrp-regulated genes to leucine depends on the affinity of Lrp for the promoter DNA of the target genes and the effective intracellular Lrp levels. According to this proposal, target promoters with a high affinity for binding Lrp (such as the promoter for *gltBDF*) are intrinsically less sensitive to leucine at normal in vivo concentrations of Lrp than are low-affinity promoters. That in vitro work enabled us to determine a unique in vitro Lrp concentration of 5.5 nM, which, under the growth conditions used, could explain the observed effect of leucine and Lrp on the target gene, *gltBDF*.

There are inherent difficulties in basing a physiological model on in vitro data. DNA-binding proteins such as Lrp have a certain proportion of their intracellular concentration sequestered by binding to nonspecific DNA, and this concentration may vary as the total DNA concentration varies in response to changes in growth rate (3). Therefore, the concentration of free versus nonspecifically bound Lrp in the cell cannot be predicted from the in vitro binding curves, and the intracellular concentration of Lrp responsible for the observed patterns of regulatory expression remains unknown. Other proteins in the cell, not present in the purified in vitro system, may also affect the expression of target operons and the concentration of Lrp required for their maximal expression. In the case of regulation by Lrp, it is likely that other players are involved, and this may help explain the number of modes of regulation observed (6). There are a number of cases in which regulation by Lrp is affected by other proteins. Many fimbrial operons are regulated both by Lrp and by a local fimbrial regulator (23): for the *pap* operon producing P pili in *E. coli*, it

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has been shown that the regulatory protein, PapI, affects the DNA binding pattern of *pap* DNA by Lrp (20, 29). Similarly, both Lrp and the fimbrial regulatory protein, FaeA, control expression from the fimbrial K88 (*fae*) operon (18). For the glycine cleavage (GCV) enzyme system, *gcv* expression is regulated by Lrp as well as by the regulatory proteins GcvA and PurR (37). The histone-like protein, H-NS, has also been found to bind specifically to a promoter segment of *lrp* and to play a role in the autogenous regulation of Lrp (22, 31). The *osmY* gene is regulated by at least three global regulators: Lrp, cyclic AMP receptor protein complex, and integration host factor (21). Thus, from the in vitro data alone, it is impossible to know whether the interactions between Lrp and *gltBDF* are sufficient to explain in vivo physiology.

To determine if the model based on in vitro data is physiologically relevant, we performed an in vivo titration of Lrp and studied the effects of changes in Lrp concentration on expression from a *gltB*::*lacZ* fusion. An in vivo titration of a regulatory protein involves changing the intracellular level of the protein over a wide range of concentrations in response to some means of induction. This method allows one to observe how target operons respond to changes in the concentration of the regulatory protein and can lead to a determination of its effective intracellular concentration. Several methods have been reported for the titration of regulatory proteins or enzymes. Haggerty et al. were able to titrate the level of the regulator of the arabinose operon, AraC, in *E. coli* by using an amber mutation in *araC* that was present in a strain with temperature-sensitive amber suppressors (15). By manipulating the temperature, they were able to vary the level of AraC and observed a linear relationship between the level of AraC and the inducibility of the arabinose operon. Interestingly, they found that the expression of the arabinose operon was maximal at the wild-type level of AraC. Other researchers have used extrachromosomal elements to adjust the in vivo level of enzymes or protein factors in the cell (30, 35, 41). A method for modulating the expression of enzymes in *E. coli* by expressing the desired gene from a *lacUV5* or *tacI* promoter in which the gene of interest remains on the chromosome has been described (19). With this method, the concentration of the H^+-ATPase *c* subunit varied between very low levels and up to five times the wild-type level with a dependence on the concentration of inducer, isopropyl-β-D-thiogalactopyranoside (IPTG).

In this study, we have developed a method to allow us to examine the effect of varying the Lrp concentration in the cell on the expression of a *gltB*::*lacZ* fusion. We were able to obtain a wide range of intracellular Lrp concentrations by using two different constructs. In one construct yielding low concentrations of Lrp, the *lrp* gene is chromosomally encoded and under the transcriptional control of the *lacUV5* promoter; in the second construct, higher concentrations of Lrp are achieved by placing *lrp* on a multicopy plasmid transcribed from the strong *trc* promoter. The data obtained by this method allow us to compare in vitro binding data for the interaction of Lrp with *gltBDF* with in vivo expression data. This study has enabled us to estimate the concentration of Lrp present in a wild-type strain during growth on glucose minimal MOPS [3-(*N*-morpholino)propanesulfonic acid] medium, and by comparison to the in vitro work, we are able to calculate the fraction of Lrp within cells that is nonspecifically bound to DNA (or sequestered in some other way). This method has also allowed us to examine the effect of varying intracellular Lrp levels on the doubling time of cells grown in this medium. The goal of these studies was to better understand how regulation of target genes by Lrp and by its coregulator, leucine, is affected by changes in the intracellular concentration of Lrp.

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

Strain	Description	Source or reference
AAEC546	W3110 Δ*lacZYA* p*lacUV5*::*lrp lrp-35*::Tn*10*	I. C. Blomfield (2)
BE2	W3110 *lrp-35*::Tn*10*	12
BE3479	PS2209 *gltB*(*psiQ*32)::*lacZ* (Mu d1–1734)	12
BW12679	Δ*lac-169 gltB*(*psiQ*32)::*lacZ* (Mu d1–1734) *creB510 rps267 crp-72 aroB thi*	B. Wanner (24)
DB3	AAEC546 *gltB* (*psiQ*32)::*lacZ* (Mu d1–1734)	This work
DB7	DB3/pDWB2 (p*trc99*::*lrp lacI*^{q})	This work
JM105	*supE endA sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*)	Promega
JWD2	JM105/pJWD2 (p*trc99*::*lrp*)	12
PS2209	W3110 Δ*lac*-169	F. C. Neidhardt
W3110	F$^-$ prototroph	F. C. Neidhardt
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ*lac-pro* [F′ *proAB lacI*^{q}Z*ΔM15* Tn*10*]	Stratagene

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 at 37°C in a rotary shaker (180 rpm) with an 8:1 flask volume-culture volume proportion. Cell growth was monitored spectroscopically at A_{420}. Cells were grown in MOPS minimal medium (27) supplemented with 10 μM thiamine, 0.4% glucose as the 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 (43). When indicated, leucine was present at a concentration of 10 mM on the basis of previous results in which this concentration of leucine resulted in a maximal effect in an in vitro gel shift assay (12). The intracellular leucine concentration in a wild-type strain is 1.7 mM when grown in glucose minimal medium; upon addition of 0.4 mM exogenous leucine, the intracellular leucine concentration rises transiently to 11.7 mM and then is maintained at 5.3 mM during steady-state growth (34). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; or tetracycline, 20 μg/ml. Cultures were maintained on Luria-Bertani agar plates supplemented with the appropriate antibiotics. IPTG (Boehringer Mannheim) was prepared as a 0.83 M stock in sterile water and was stored at −20°C in small aliquots; it was added to the growth medium prior to inoculation with an overnight bacterial culture.

Strain construction. Generalized transduction mediated by P1 *vir* was carried out as described by Miller (25). Strain DB3 was constructed by transduction of the transcriptional fusion *gltB*::*lacZ* from strain BW12679 into AAEC546, with selection for Km^r. pDWB2 was constructed by subcloning a 2-kb *Sph*I fragment containing *lacI*^{q} and *lrp* under the transcriptional control of the IPTG-inducible promoter p*trc99* (p*trc99*::*lrp*) from pJWD2 into the *Sph*I site of pBR322. Strain XL1 Blue was transformed with the ligation mixture with a selection for Ap^r. Strain DB7 was obtained after transformation of strain DB3 with pDWB2 and selection for Ap^r.

β-Galactosidase assay. β-Galactosidase activity and culture density were measured at six intervals during exponential growth (from an A_{420} of approximately 0.1 to 1.0). The assay used was described by Miller (25), as modified by Platko et al. (33). At each sampling time, two portions were removed from the culture. One was diluted in fixer (MOPS minimal medium containing 0.9% [vol/vol] formaldehyde) and used within 1 h to determine the A_{420}. The cells in the second portion were permeabilized by being mixed with an equal volume of an aqueous solution of cetyl trimethylammonium bromide (200 μg/ml) and sodium deoxycholate (100 μg/ml) and then being incubated overnight at 4°C. 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 managnesium sulfate, and 50 mM β-mercaptoethanol) and incubated at 28°C in the presence of 0.15 ml of 4-mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG). When the reaction tubes were visibly light yellow, 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; this value (ΔA_{420}) is associated with hydrolysis of ONPG by β-galactosidase. The units for β-galactosidase activity were calculated by dividing ΔA_{420} by the time allowed for the reaction and by the volume of permeabilized cells used for the reaction. The units of β-galactosidase activity are arbitrary units: $1{,}000 \times \Delta A_{420}\ \text{min}^{-1}\ \text{ml}^{-1}$. The specific activity reported was obtained by determining the slope of a plot of β-galactosidase activity versus the A_{420} of the culture, and the units are $(1{,}000 \times \Delta A_{420}\ \text{min}^{-1}\ \text{ml}^{-1})/A_{420}$ of the culture (Fig. 1). A series of similar plots (not shown) of the β-galactosidase activity versus the optical density of the culture for all strains and growth conditions were constructed, and the slopes of the lines are used as the reported values for β-galactosidase specific activity in Table 3. The slopes from these plots were found to be linear up to an A_{420} of the culture of at least 1.2 and indicate that all measurements were made during balanced growth.

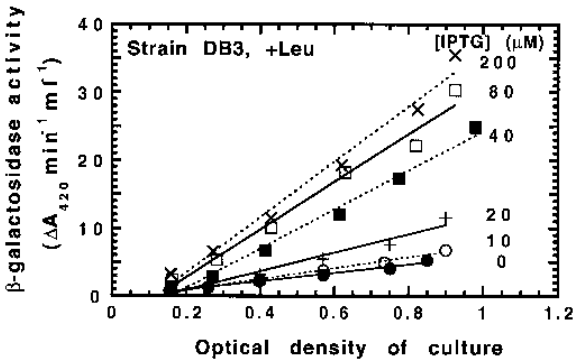

FIG. 1. Effect of IPTG titration on β-galactosidase activity from a *gltB*::*lacZ* operon fusion. Cultures of strain DB3 (low range of Lrp concentration) were grown in glucose minimal MOPS medium containing isoleucine, valine, and 10 mM leucine. IPTG was used to induce Lrp expression; the micromolar concentration of IPTG used is indicated to the right of the lines. The growth of the cultures was spectroscopically monitored at A_{420}. The slopes of the lines (β-galactosidase activity/A_{420} of the culture) determined by linear regression are reported as β-galactosidase specific activity in Table 3. Similar experiments were done in the absence of leucine for strain DB3 and in the presence and absence of leucine for strain DB7.

Western blot (immunoblot) analysis. Western blot analysis was used to quantify Lrp levels in cell extracts from 1-ml samples harvested at an A_{420} of approximately 1.0. Extracts were obtained by treating the cell pellets with 46 μl of a sodium dodecyl sulfate (SDS)–β-mercaptoethanol solution (0.3% SDS–5% β-mercaptoethanol in 58 mM Tris-Cl [pH 8]), boiling them for 2 min, and then incubating them on ice for 10 min with 4 μl of a nuclease solution (1 mg of DNase I and 0.3 mg of RNase A per ml [Worthington Biochemical Corp.]). When necessary, the extracts were diluted with extract prepared in the same way from strain BE2, which contains *lrp-35*::Tn*10*. Small aliquots of the extracts were mixed with an equal volume of 2× sample buffer (0.1 M Tris-HCl [pH 6.8], 10% β-mercaptoethanol, 20% glycerol, 2% SDS, trace amount of bromophenol blue) and boiled for 5 min, and then 4 μl of each sample was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% polyacrylamide gels. After fractionation in electrophoresis buffer (0.025 M Tris [pH ~8.3], 0.192 M glycine, 0.1% [wt/vol] SDS), proteins were electrophoretically transferred to Immobilon-P Transfer Membrane (Millipore) with a Mini Trans-blot cell (Bio-Rad) at 100 V for 1 h in transfer buffer (0.025 M Tris [pH ~8.3], 0.192 M glycine, 20% methanol [vol/vol], chilled to 4°C).

The blots were incubated for 0.5 h in a Tris-buffered saline solution with 0.05% Tween 20 (TBST) and 1% bovine serum albumin as a blocking agent. Blots were then incubated for 0.5 h with 15 ml of TBST containing polyclonal rabbit anti-Lrp serum (kindly supplied by J. M. Calvo), washed three times with TBST, and then incubated with TBST containing a 1:7,500 dilution of anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Promega). After three more washes, the antibody complexes were visualized by incubating the blots with the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Proto-Blot II AP System; Promega).

For quantitation, dilutions of purified Lrp were included on every blot. The Lrp was diluted with cell extract from strain BE2 (*lrp*::Tn*10*); dilution into an Lrp^- cell extract was found to be essential for optimal sensitivity, as previously reported (1, 45). By this method, we were able to detect Lrp with a sensitivity of approximately 50 pg per lane, and we were able to quantify Lrp levels over the range from 50 pg to 4 ng by using an Lrp standard dilution-response curve constructed for each blot. When necessary, sample extracts were also diluted with cell extract from strain BE2 in order to have an Lrp concentration for all samples in the range between 50 pg and 4 ng. To normalize for slight differences in sensitivity between blots, identical samples of extract from strain BE3479 (lrp^+) were included on each blot.

The intensities of Lrp signals on each blot were quantified with an XRS scanner with BioImage version 4.6Q software. A standard curve of signal intensity versus Lrp concentration was constructed for the dilutions of purified Lrp. The concentration of the experimental samples was determined by comparison to this standard curve. The concentration of the undiluted sample was determined by the Protein and Carbohydrate Structure Facility at the University of Michigan by amino acid analysis by the phenylthiocarbamyl method (16, 39).

RESULTS

Development of an in vivo system in which the intracellular concentration of Lrp can be varied continuously over a wide range. Our goal was to develop a method by which the intracellular concentration of Lrp could be varied over a continuous range from near zero to greater than the wild-type level and to study the effects of these changes on the expression of the Lrp-regulated gene, *gltB*. Lrp expression is affected by growth conditions: expression is maximal in glucose minimal medium and repressed in rich media such as Luria-Bertani medium (6, 22a). All of our measurements were made from cultures grown in glucose minimal MOPS medium, and all samples for quantitation of Lrp levels by Western blot analysis were obtained at an A_{420} of approximately 1.0. Since Lrp-dependent regulation of many genes is affected by leucine (6), experiments were performed both in the presence and in the absence of exogenous leucine.

The wild-type concentration of Lrp for cells grown in glucose minimal MOPS medium was determined for strain BE3479, which contains a wild-type chromosomal *lrp* gene and a transcriptional *gltB*::*lacZ* fusion (Table 2). *lrp* is known to be autogenously regulated (22a, 42), but studies measuring β-galactosidase expression from *lrp*::*lacZ* transcriptional fusions showed little effect of leucine on expression (22a, 32). We found that for strain BE3479, the presence of leucine resulted in slightly greater intracellular Lrp levels than its absence. This observation is consistent with autogenous repression of Lrp that is relieved by leucine. The abundance of Lrp in an lrp^+ strain (CSH26) has been previously reported to be about 3,000 dimers per cell, corresponding to an intracellular concentration of about 5 μM (45). Although we used a different strain, our values are in close agreement (Table 2). We generally report Lrp concentration in terms of nanograms per microliter of cell extract: conversions into units of dimers per cell or molarity introduce increased error since they require knowledge of the number of viable cells and the cellular volume, which are strain and growth rate dependent. These calculations have been made for strain BE3479, and the values are listed in Table 2; however, it must be stressed that these conversion factors should not be applied to other strains or growth conditions.

We used strain AAEC546, constructed by I. C. Blomfield, as the basis for development of an Lrp-titratable strain. In strain AAEC546, *lrp* expression is controlled by the IPTG-inducible *lacUV5* promoter (2). Strain DB3 was constructed by transduction of a *gltB*::*lacZ* transcriptional fusion into AAEC546. Initial experiments showed that Lrp levels in strain DB3 were lower than those of the wild type, even with maximal induction by IPTG. To increase the range of in vivo Lrp levels, a second strain was constructed that was isogenic to strain DB3, except

TABLE 2. Intracellular Lrp concentration in wild-type strain BE3479

Presence of leucine[a]	Intracellular Lrp concn		
	ng/μl of cell extract[b]	Dimers/cell[c]	μM[d]
−	1.21 ± 0.3	2,485	4.95
+	1.57 ± 0.2	3,224	6.42

[a] Strain BE3479 was grown in glucose minimal MOPS medium containing isoleucine and valine with or without 10 mM leucine.

[b] Lrp concentrations were determined by Western immunoblot analysis of cell extracts with antiserum raised against Lrp and dilutions of pure Lrp as standards. Lrp concentrations are represented as nanograms of Lrp per microliter of cell extract ± standard deviation, and the values were adjusted for slight differences in culture density by dividing them by the A_{420}.

[c] Values for Lrp dimers per cell were calculated with the experimentally determined value of 3.9×10^8 cells per ml at an A_{420} of 1.0 for strain BE3479 and a molecular mass of 37,600 g/mol for a dimer of Lrp (45).

[d] The intracellular concentration of Lrp was calculated with a value of 0.833 fl for the volume of *E. coli* B/r cells with a doubling time of approximately 1 h (10).

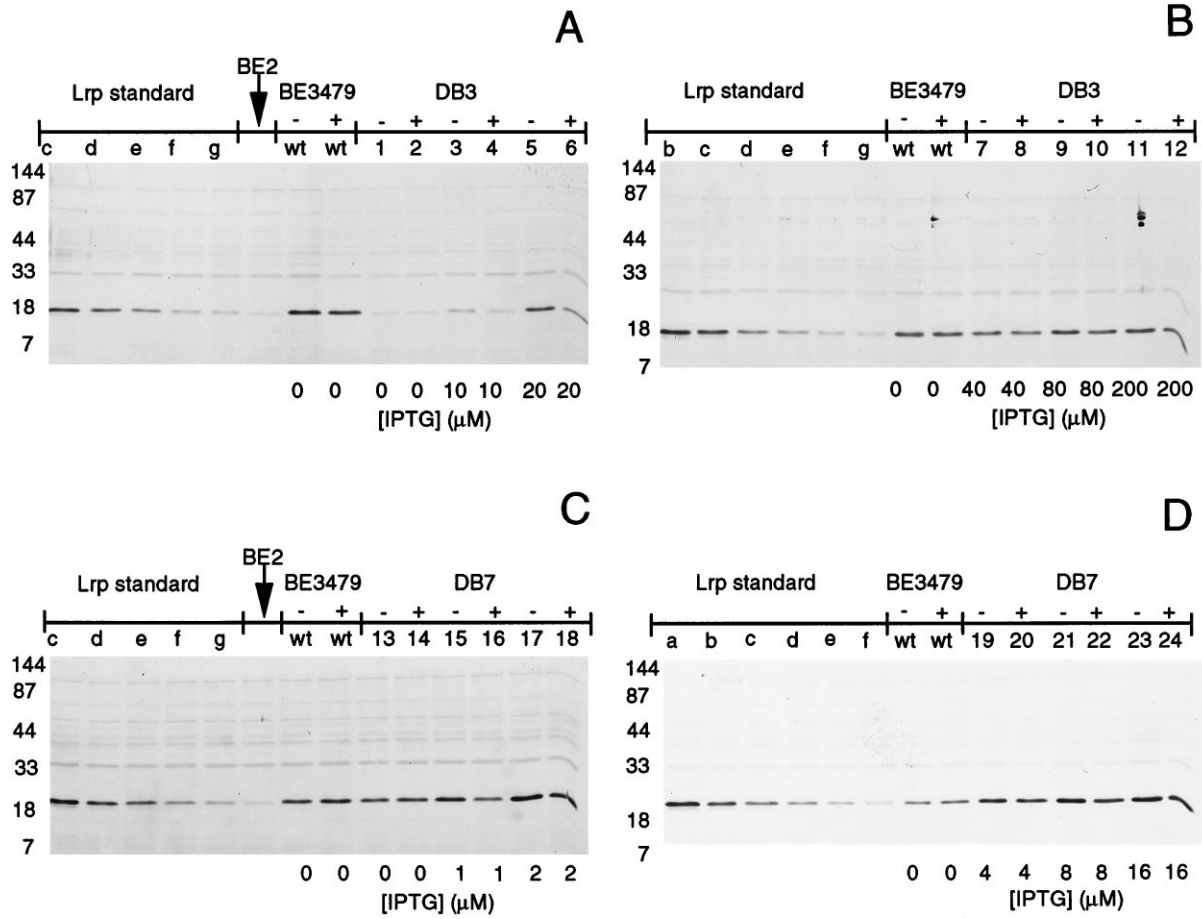

FIG. 2. Western blot analysis with polyclonal antiserum to Lrp of cell extracts from strains BE3479 (*lrp*$^+$), DB3 (p*lacUV5*::*lrp lrp-35*::Tn*10*), and DB7 (p*lacUV5*::*lrp lrp-35*::Tn*10*/pDWB2 p*trc99*::*lrp lacI*^{q}). Cells were grown in glucose minimal MOPS medium containing either isoleucine and valine or isoleucine, valine, and 10 mM leucine. Cultures were induced with IPTG at the concentrations indicated below the samples. Cell extracts from 1 ml of culture were obtained at an A_{420} of approximately 1.0. Prestained markers (Bio-Rad; not shown) were included on each blot, and the approximate molecular weights are given to the left of each blot. Equal volumes of cell extract plus sample buffer (4 μl) were loaded into each lane. Strain designations and the absence (−) or presence (+) of leucine are indicated above each sample. Lrp standards are amounts of purified Lrp diluted into an Lrp$^-$ cell extract (strain BE2 [*lrp-35*::Tn*10*]) and added to the lanes in the following amounts: a, 4.0 ng; b, 2.0 ng; c, 1.0 ng; d, 0.5 ng; e, 0.25 ng; f, 0.125 ng; g, 0.0625 ng. The lane indicated by the arrow is an equivalent volume of extract from strain BE2. Lanes labeled "wt" are samples of cell extract from strain BE3479 (PS2209 *lrp*$^+$ *gltB*::*lacZ*), which were diluted 1:4 with extract from strain BE2. (A) Strain DB3 samples (lanes 1 to 6) from cultures induced with IPTG at concentrations of 0, 10, and 20 μM. (B) Strain DB3 samples (lanes 7 to 12) from cultures induced with IPTG at concentrations of 40, 80, and 200 μM. (C) Strain DB7 samples (lanes 13 to 18) from cultures induced with IPTG at concentrations of 0, 1, and 2 μM. Strain DB7 samples were diluted 1:4 (samples 13 and 14) or 1:20 (samples 15 to 18) with cell extract from strain BE2. (D) Strain DB7 samples (lanes 19 to 24) from cultures induced with IPTG at concentrations of 4, 8, and 16 μM; these samples were diluted 1:20 with cell extract from strain BE2.

for the addition of a pBR322-derived plasmid containing *lrp* expressed from the IPTG-inducible *trc99* promoter. In this strain, DB7, transcription from the *trc99* promoter is very leaky, and thus Lrp levels are quite high even when no IPTG is present.

Quantification of Lrp levels involved Western blot analysis followed by image scanning and signal quantitation. Strains DB3 and DB7 were grown in glucose minimal MOPS medium in the presence or absence of leucine and at many different concentrations of IPTG. Samples for Western blot analysis were obtained at an A_{420} of approximately 1.0 for all cultures. Figure 2 shows representative Western blots for cell extracts obtained from the cultures. After the scanning and quantifying of each Western blot, a standard curve of Lrp concentration versus signal intensity was constructed with purified Lrp standards diluted into a cell extract from an *lrp*::Tn*10* strain, BE2. The Lrp concentrations in cell extracts from cultures of strains DB3 and DB7 were quantified by interpolation of their values for signal intensity to the Lrp standard curve. DB3 produces Lrp levels ranging from near zero, with no IPTG induction, to approximately two-thirds of the wild-type level with 0.2 mM IPTG induction; strain DB7 produces levels of Lrp similar to those of the wild type with no induction and much higher than the wild-type level with IPTG induction.

Since transcription of Lrp is controlled by IPTG-inducible promoters in these strains, we were able to continuously vary the intracellular concentration of Lrp as a function of IPTG concentration. As shown in Fig. 3, even very low levels of IPTG resulted in substantial induction. For strain DB3, Lrp levels became maximal at 40 μM IPTG both in the presence and in the absence of leucine. Even lower levels of IPTG resulted in a huge induction of Lrp in strain DB7. In the presence of leucine, 4 μM IPTG resulted in maximal Lrp concentrations, whereas in the absence of leucine, further induction with IPTG led to increased Lrp levels. At an IPTG concentration of 16 μM, the intracellular Lrp level in strain DB7 (in the absence of

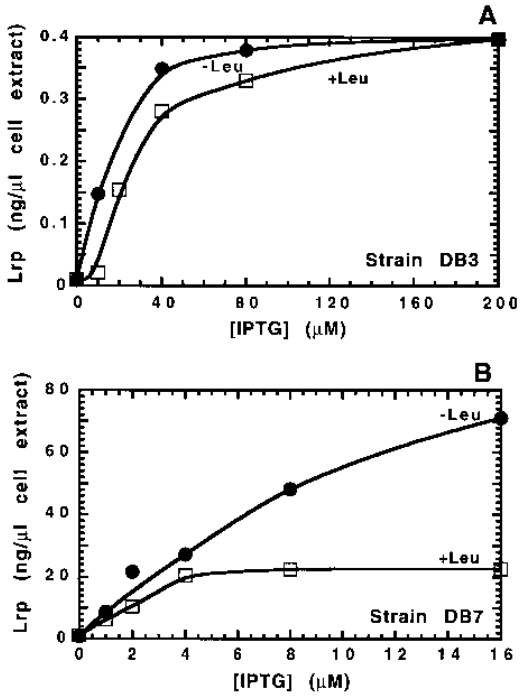

FIG. 3. In vivo titration of Lrp. Cells were grown in glucose minimal MOPS medium containing either isoleucine and valine (solid circles) or isoleucine, valine, and 10 mM leucine (open squares). Lrp concentrations were determined by Western immunoblot analysis of cell extracts obtained at an A_{420} of approximately 1.0 with antiserum raised against Lrp and dilutions of purified Lrp into an Lrp$^-$ extract as standards. Lrp concentrations are represented as nanograms of Lrp per microliter of cell extract, and the values were adjusted for slight differences in A_{420} at the time of cell harvest by dividing by A_{420}. (A) Strain DB3 (p*lacUV5*::*lrp lrp-35*::Tn*10*) was induced with 0, 10, 20, 40, 80, and 200 μM IPTG. (B) Strain DB7 (p*lacUV5*::*lrp lrp-35*::Tn*10*/pDWB2 p*trc99*::*lrp lacI*^{q}) was induced with 0, 1, 2, 4, 8, and 16 μM IPTG.

leucine) was approximately 70 times the wild-type level. In contrast to our results with wild-type *lrp* in strain BE3479 (Table 2), levels of Lrp were lower in the presence of leucine than in its absence for both strains DB3 and DB7. We expected no effect of leucine on Lrp levels, since *lrp* is no longer under the control of its own promoter in these strains. Moderate effects of Lrp and leucine on expression of β-galactosidase from the *lac* promoter have been reported previously (40); Lrp activates expression, and leucine antagonizes the effect of Lrp. Our finding of reduced Lrp expression from p*lacUV5* in the presence of leucine is consistent with these observations.

The level of expression from a *gltB*::*lacZ* operon fusion depends upon the level of induction with IPTG. Previous studies with a *gltB*::*lacZ* operon fusion in strain BE3479 (*lrp*$^+$) showed that expression of the fusion is positively regulated by Lrp and decreases in the presence of 10 mM leucine (11, 12). We examined how expression of the same *gltB*::*lacZ* fusion responded to changes in the intracellular Lrp levels obtained by IPTG titration of strains DB3 and DB7. Cultures of strains DB3 or DB7 were grown in the presence or absence of leucine with a ramp of IPTG concentrations. Figure 1 shows the series of plots obtained for β-galactosidase activity versus A_{420} of the culture for strain DB3 grown in the presence of leucine and various levels of IPTG. The same cultures were used to quantitate Lrp levels and to obtain samples for β-galactosidase expression from the *gltB*::*lacZ* fusion. As the concentration of IPTG (and therefore Lrp [Fig. 3]) increased, the specific activity of the *gltB*::*lacZ* fusion, as measured by the slope of the line, also increased. Data for a complete set of experiments for strains DB3 and DB7 are presented in Table 3. For strain DB3, wild-type levels of β-galactosidase activity were never reached; this is consistent with the level of Lrp (~70% of wild type) present in this strain at even the highest levels of IPTG. In strain DB7, the highest level of β-galactosidase activity occurred with no induction by IPTG. Induction of strain DB7 with IPTG resulted in very high levels of Lrp (Fig. 3) but somewhat surprisingly led to a decrease in β-galactosidase activity from the *gltB*::*lacZ* fusion. Addition of exogenous leucine resulted in an approximate threefold decrease in β-galactosidase activity for strain BE3479 (which contains *lrp* under the control of its own promoter) compared with the activity from cells grown in the absence of leucine.

Maximal expression from a *gltB*::*lacZ* fusion occurs at the wild-type intracellular Lrp concentration. Combining our measurements of in vivo Lrp levels (Fig. 2) with our data on *gltB*::*lacZ* expression from the same set of experiments (Table 3) allowed us to construct a plot showing the effect of intracellular Lrp concentration on the β-galactosidase specific activity of the fusion (Fig. 4). The vertical line in Fig. 4 was drawn at the average Lrp concentration found in wild-type cells grown in glucose MOPS minimal medium supplemented with Ile and Val with or without Leu (Table 2). At Lrp concentrations lower than the wild-type level, expression from the *gltB*::*lacZ* fusion increased with an increase in Lrp concentration. Maximal expression of the fusion was obtained at the wild-type Lrp concentration. At Lrp concentrations greater than those of the wild type, interference with activation of fusion expression was observed.

The data in Fig. 4 show a pattern reminiscent of theoretical Hill binding curves (17) and were fitted to equation 1, the Hill equation, with the addition of a factor allowing for the interference with activation at high Lrp concentrations:

$$y = \{Y_{\max} \cdot [\mathrm{Lrp}]^{1.5}/(K_{d1} + [\mathrm{Lrp}]^{1.5})\} - \{\Delta Y \cdot [\mathrm{Lrp}]/(K_{d2} + [\mathrm{Lrp}])\} \quad (1)$$

The first bracketed term of this equation describes the activation of expression observed in the left half of Fig. 4. K_{d1} is the

TABLE 3. β-Galactosidase specific activity from a *gltB*::*lacZ* fusion in strains BE3479, DB3, and DB7 in response to IPTG titration

Strain	IPTG concn (μM)	β-Galactosidase sp act[a]	
		− Leu	+ Leu
BE3479 (*lrp*$^+$)	0	308	102
DB3 (p*lacUV5*::*lrp lrp-35*::Tn*10*)	0	7	6
	10	36	8
	20	138	14
	40	181	29
	80	192	35
	200	184	41
DB7 (p*lacUV5*::*lrp lrp-35*::Tn*10*/ pDWB2 p*trc99*::*lrp*)	0	289	75
	1	191	69
	2	155	64
	4	185	54
	8	179	48
	16	192	43

[a] β-Galactosidase specific activity is reported in arbitrary units of ΔA_{420} min^{-1} ml^{-1}/A_{420} of the culture, and was calculated by linear regression of the slopes of the lines of plots (such as in Fig. 1) of β-galactosidase activity (ΔA_{420} min^{-1} ml^{-1}) versus A_{420} of the culture.

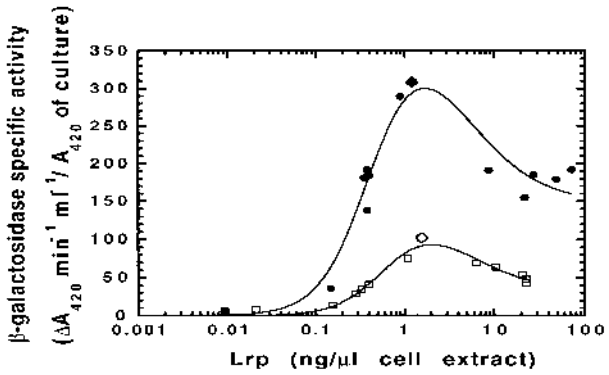

FIG. 4. Expression from a *gltB*::*lacZ* operon fusion as a function of intracellular Lrp concentration. Cultures of strains DB3 and DB7 were grown in glucose minimal MOPS medium containing either isoleucine and valine (closed circles) or isoleucine, valine, and 10 mM leucine (open squares). IPTG was used to induce Lrp expression. The diamonds represent the wild-type intracellular concentration of Lrp for strain BE3479 (*lrp*$^+$) in the absence (solid diamonds) and presence (open diamonds) of leucine. The vertical line is drawn at the average wild-type intracellular Lrp concentration. Lrp concentrations were determined by Western immunoblot analysis of cell extracts obtained at an A_{420} of approximately 1.0 with antiserum raised against Lrp and dilutions of pure Lrp as standards (data taken from Fig. 2). Lrp concentrations are represented as nanograms of Lrp per microliter of cell extract, and the values were adjusted for slight differences in culture density at the time of cell harvest by dividing by A_{420}. The β-galactosidase activity represents the values reported in Table 3, calculated from the slopes of plots of β-galactosidase expression versus optical density of the culture at various levels of IPTG induction (Fig. 1). Theoretical Hill binding curves (17) were fitted to the data with the addition of a term allowing for interference with activation at high Lrp concentrations.

apparent dissociation constant for the Lrp-DNA complex that activates expression of *gltB*::*lacZ*, and Y_{max} is the maximal β-galactosidase specific activity. To obtain the best fit to the data, Y_{max} in the absence of leucine was empirically set at 400 U, and the degree of cooperativity of binding was set at 1.5. Given these constraints, the curve-fitting program in KaleidaGraph (Synergy Software, Reading, Pa.) was used to find an optimal value for K_{d1}, resulting in a K_{d1} in the absence of leucine of 0.23 ng/μl of cell extract and a K_{d1} in the presence of leucine of 0.51 ng/μl of cell extract. The second bracketed term of the equation allows for the interference with activation at high Lrp concentrations, as observed in the right half of Fig. 4, and assumes that interference with activation is due to simple, noncooperative binding of more Lrp. An Lrp-binding motif has been identified within the *gltBDF* gene at nucleotide +36 relative to the start site of transcription (13); since this site is located within *gltBDF*, it might be expected to have a negative effect on transcription if bound by Lrp. ΔY is the difference between Y_{max} and y at infinite [Lrp], and K_{d2} is the apparent dissociation constant associated with this interference. In the absence of leucine, if Y_{max} is set at 400, then K_{d2} is predicted to be 3.46 ng/μl of cell extract. Our assumption in fitting the data was that leucine has no effect on the observed interference with activation. It is apparent from the data in Fig. 4, that the addition of leucine to the medium results in a shift of the binding curve to the right. Although the precise values of the apparent dissociation constants are dependent on the assumptions made for the degree of cooperativity and Y_{max}, the K_d of activation in the presence of leucine remains approximately twofold greater than that in the absence of leucine. This indicates that the level of Lrp necessary to produce half-maximal expression of the *gltB*::*lacZ* fusion is greater in the presence of leucine than in its absence. If these activation curves directly reflect binding, their shape and position would indicate a shift in the apparent dissociation constant of Lrp for *gltBDF* DNA with the presence of leucine resulting in a decrease in affinity.

The optimal growth rate in glucose minimal MOPS medium occurs at the wild-type intracellular Lrp concentration. In the course of growing strains DB3 and DB7 under various levels of IPTG induction, it became obvious that there were significant differences in the doubling times. Figure 5 shows a plot of the doubling times for strains DB3 and DB7 as a function of intracellular Lrp concentration. For strain BE3479 containing the wild-type *lrp* gene, the doubling time was 57 min (triangle, lower dashed line). The most rapid doubling times for strains DB3 and DB7 were approximately 65 min and occurred at Lrp concentrations near the wild-type level. At Lrp concentrations either lower or higher than wild-type levels, the doubling times increased significantly to approximately 84 min, a value very similar to the doubling time of 85 min for strain BE2 containing *lrp-35*::Tn*10* (upper dashed line).

DISCUSSION

Lrp-controlled genes show a surprising range of regulatory patterns. In beginning this study, our goal was to explore the relationship between in vitro behavior and in vivo effects of Lrp. This exploration has led us to five main conclusions.

Leucine sensitivity of target operons is a function of intracellular Lrp levels. Comparing the effect of in vivo titration of Lrp levels on the expression of an Lrp-regulated target operon, *gltBDF*, to the in vitro results from gel mobility shift assays of Lrp binding to the control region DNA of *gltBDF* (12) has enabled us to test a model for Lrp regulation of target genes. In this model, proposed by Ernsting et al. (12), the sensitivity of Lrp-regulated target operons to leucine is dependent on the affinity of the promoter DNA for Lrp. Lrp has a high affinity for the *gltBDF* promoter, and expression from *gltBDF* is relatively insensitive to leucine (the dissociation constant for the

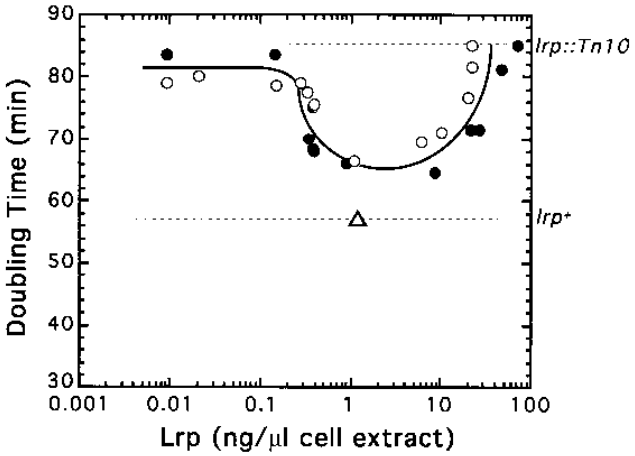

FIG. 5. Effect of intracellular Lrp levels on growth rate. Cultures of strains DB3 and DB7 were grown in glucose minimal MOPS medium containing either isoleucine and valine (solid circles) or isoleucine, valine, and 10 mM leucine (open circles). IPTG was used to induce Lrp expression. Growth of the cultures was monitored by spectrophotometric measurement at A_{420}, and the doubling times were calculated from the exponential portion of the growth curves. Lrp concentrations were determined by Western immunoblot analysis of cell extracts obtained at an A_{420} of approximately 1.0 with antiserum raised against Lrp and dilutions of purified Lrp as standards. Lrp concentrations are represented as nanograms of Lrp per microliter of cell extract, and the values are adjusted for slight differences in A_{420} at the time of cell harvest by dividing by A_{420}. The dashed lines are drawn to illustrate the doubling times of 85 min for strain BE2 (*lrp-35*::Tn*10*) and 57 min for strain BE3479 (*lrp*$^+$). The triangle indicates the growth rate and Lrp content of the wild-type strain (BE3479).

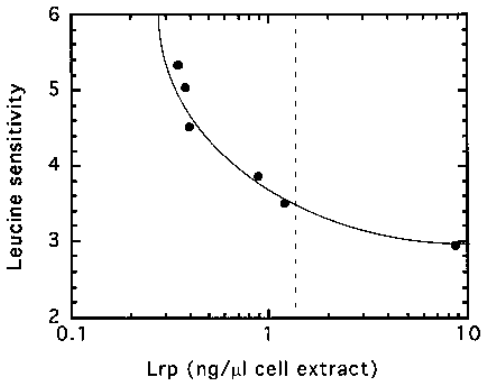

FIG. 6. Leucine sensitivity as a function of intracellular Lrp concentration. Lrp concentrations are represented as nanograms of Lrp per microliter of cell extract and represent the values determined by Western immunoblot analysis of cell extracts obtained from cultures grown in glucose minimal MOPS medium containing isoleucine and valine. Leucine sensitivity was calculated as the ratio of β-galactosidase specific activity in the absence of leucine to that in the presence of leucine for a given concentration of Lrp. The values used for the β-galactosidase specific activity for cultures grown in the absence of leucine were the actual values shown in Table 3. Since there are no corresponding values for β-galactosidase specific activity of cultures grown in the presence of leucine at these exact Lrp concentrations, the β-galactosidase values for these cultures were obtained by interpolation of the lower curve shown in Fig. 4. Measurements of both β-galactosidase activities and Lrp concentrations are less accurate at very low levels of Lrp (below 0.3 ng of Lrp per μl of cell extract); therefore, those data have been excluded from this analysis. The region of 0.3 to 10 ng of Lrp per μl of cell extract, however, does encompass the physiologically relevant range of Lrp concentrations. The curve represents an empirical fit to the data. The vertical dashed line is drawn at the wild-type level of Lrp.

Lrp-*gltBDF* complex is 2.0 nM in the absence of leucine and 6.6 nM in the presence of 30 mM leucine). In contrast, the operon coding for acetohydroxy acid synthase form III (*ilvIH*) is more sensitive to leucine, and the Lrp-*ilvIH* complex has a lower affinity (the dissociation constant is 6.9 nM in the absence of leucine and 14.1 nM in the presence of leucine).

The model predicts that the leucine sensitivity of high-affinity operons will increase as intracellular Lrp levels decrease. Figure 6 shows the combined data from strains BE3479, DB3, and DB7 for leucine sensitivity as a function of intracellular Lrp levels obtained by the in vivo titration of Lrp reported in this study. The range of Lrp concentrations shown in Fig. 6 encompasses the physiologically relevant range of concentrations for regulation of *gltBDF* by Lrp. The effect of changing the level of intracellular Lrp on expression from *gltB*::*lacZ* is just what is predicted by the model. At the wild-type Lrp concentration indicated by the vertical line, there is a ~3.5-fold effect on β-galactosidase specific activity from *gltB*::*lacZ* (calculated as the ratio of β-galactosidase specific activity in the absence of leucine to that in the presence of leucine). Leucine sensitivity progressively increases as the concentration of Lrp decreases. Leucine sensitivity remains constant at about a threefold effect for Lrp concentrations up to at least 10-fold higher than the wild-type level of Lrp.

Activation of *gltBDF* is a function of Lrp binding, but the maximal level of activation is also decreased in the presence of leucine. Figure 7 shows the result of overlaying the activity curves obtained from the in vivo titration experiments reported in this paper (Fig. 4, solid symbols) onto the binding curves from the earlier in vitro gel shift assays (open symbols) (12). The two sets of curves were overlaid such that a 100-fold difference in Lrp concentration used in the in vitro work corresponded to a 100-fold difference in Lrp concentration obtained by the in vivo titration, and the curves were shifted with respect to each other in order to maximize agreement of the two data sets obtained in the absence of leucine. There is a remarkably good fit between the two sets of data, up to concentrations of Lrp near the wild-type level in glucose minimal MOPS medium. This indicates that Lrp binding can explain all or most of the activation seen.

A surprising result of the in vivo titration was the observation that very high levels of Lrp actually lead to decreased expression of the *gltB*::*lacZ* fusion (Fig. 4). We interpret this result as interference with activation from the *gltBDF* promoter region and propose that it may be due to additional binding of Lrp within the operon that interferes with transcription. An Lrp binding site starting at nucleotide +36 in *gltB* was predicted by a computer analysis identifying an Lrp motif based on 23 gene sequences and a consensus sequence of experimentally defined binding sites (6–8, 13). The in vitro model proposed that at very high concentrations of Lrp, the final level of activation would be the same in the presence and absence of leucine (see Fig. 6 of reference 12); however, because of the competing effects of activation by Lrp and interference with activation at high concentrations of Lrp, we were unable to fit our data to the in vitro model at these higher levels of Lrp.

We also observed an effect of leucine on the maximal activation of expression of *gltB*::*lacZ* by bound Lrp. For the curves shown in Fig. 4, we assumed a threefold-lower maximal level of activation in the presence of leucine (Y_{max} = 133) than in its absence (Y_{max} = 400; empirically determined for equation 1) based on the in vivo observation that maximal β-galactosidase specific activity from a *gltB*::*lacZ* fusion is approximately threefold lower in the presence of leucine. This assumption implies that leucine decreases both the affinity of binding for Lrp-DNA and the efficiency of transcription of *gltB*::*lacZ*; however, it is a formal possibility that transcriptional efficiency could be unaltered or even increased in the presence of leucine but that this effect is masked by the competing effect of interference with activation at high Lrp concentrations. In vitro studies with mutant DNAs or mutant Lrp could be useful in separating the effects of binding and activation.

Most Lrp in the cell is sequestered. The vertical line in Fig. 7 represents the Lrp concentration determined by analysis of

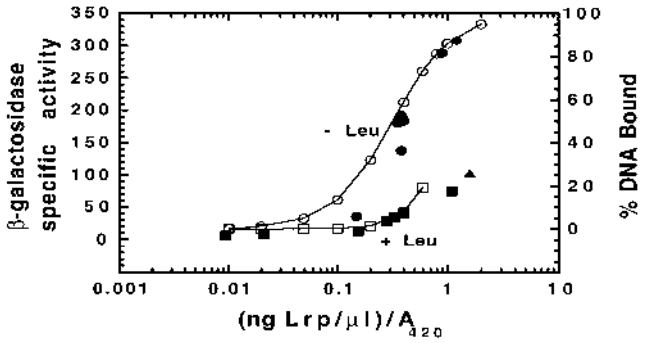

FIG. 7. Comparison of in vivo titration results with an in vitro model of leucine sensitivity for the *gltBDF* promoter. The effect of leucine on β-galactosidase activity from a *gltB*::*lacZ* transcriptional fusion is shown as a function of intracellular Lrp concentration (− leucine, solid circles; + leucine, solid squares). The intracellular concentration of Lrp in strain BE3479 (*lrp*$^+$) is indicated as a solid diamond (− Leu) and a solid triangle (+ Leu). The open symbols represent the in vitro data from gel mobility shift assays of Lrp binding to the *gltBDF* promoter (Fig. 6 of reference 12): open circles, − Leu; solid circles, + Leu. Theoretical Hill binding curves (17) were fitted to these in vitro data. The vertical line is drawn at the concentration of Lrp predicted by the in vitro model to represent the effective intracellular concentration of Lrp during exponential growth in glucose minimal MOPS medium.

the gel shift assays to result in the observed in vivo effects of leucine and Lrp on *gltBDF* and therefore represents a theoretical effective concentration of Lrp in the cell. This theoretical concentration is 5.5 nM. The solid diamond and triangle symbols represent the in vivo concentration of Lrp determined in this study for strain BE3479 (which contains a wild-type *lrp* gene) grown in glucose minimal MOPS in the absence or presence of leucine, respectively. This in vivo concentration is approximately 5 μM (Table 2), which is the same value determined by Willins et al. for strain CSH26 (45). This concentration corresponds to a value of 3,000 dimers of Lrp per cell, or approximately 0.1% of the total protein molecules in the cell (calculated with the value of 2,350,000 molecules of protein per cell [26]). This concentration of Lrp makes it a moderately abundant protein, but it is significantly less abundant than other global regulators such as Fis and HU, which have concentrations near 30,000 dimers per cell (1, 36), and integration host factor, which has a concentration of approximately 10,000 dimers per cell during exponential growth (9, 44).

By overlaying the in vitro gel shift data onto the in vivo expression data as shown in Fig. 7, we may estimate the effective concentration of free Lrp in the cell by calculating the ratio of the amount of Lrp added in vitro, which results in the observed leucine sensitivity, to the total intracellular Lrp, which also results in the observed effect of leucine. This ratio is ~1:1,000, and indicates that only 1 in 1,000 dimers is "available" at any moment. Thus, ~99.9% of the Lrp in the cell is associated with other specific and nonspecific sites.

Wild-type levels of Lrp are optimal for activation of expression from *gltBDF*. From Fig. 4, we can see that the in vivo wild-type Lrp level is associated with the peaks of the activation curves and is poised for the greatest responsiveness to changes in Lrp concentration due to physiological changes. Thus, as with the local regulator, AraC, for which the normal intracellular level is just high enough for optimal activation (15), the global regulator, Lrp, also has a concentration which is optimal for maximal expression.

Changes in the intracellular concentration of Lrp are known to occur. The expression of Lrp changes in response to growth conditions. Experiments with *lacZ* fusions to *lrp* have shown that β-galactosidase expression from the fusions is 4- to 10-fold lower when cells are grown in Luria broth or in a defined rich medium than when they are grown in a minimal medium (6, 22a). Our studies with a strain with a wild-type *lrp* gene have likewise shown a significant decrease in intracellular Lrp levels in cells grown in a rich medium (4). These changes in Lrp levels are consistent with its role in the positive regulation of anabolic pathways and negative regulation of catabolic pathways (6). Consistent with lower levels of Lrp in cells grown on a rich medium, such cells also show decreased glutamate synthase expression (5, 12). Preliminary data from our laboratory indicate that Lrp levels may also vary in response to the growth phase of the culture (4).

Lrp levels affect the growth rate of cultures. As shown in Fig. 5, it is apparent that the level of Lrp in the cell is a determinant of the growth rate of the culture. Significantly slower growth rates, similar to the growth rate for a strain containing *lrp-35*::Tn*10*, were observed for cultures in which the Lrp level was either much lower than or much higher than the wild-type level. The fastest growth rate occurred at an Lrp concentration similar to wild-type levels, indicating that normal Lrp levels are physiologically optimized for cell growth in glucose minimal medium. Decreases in the growth rate of strains containing a null mutation in *lrp* have been observed previously and are most evident in minimal medium (6). Strains containing a mutant *lrp* gene show a partial isoleucine and valine auxotrophy (33, 38), but this does not explain the decreased growth rate in this study, since isoleucine and valine were always present in the medium. Leucine is toxic to wild-type strains of *E. coli* (14, 34), and it has been proposed that an *lrp* mutation results in a partial leucine auxotrophy (22b), which would therefore be expected to result in a faster growth rate at low Lrp concentrations. Our results in Fig. 5 are consistent with both leucine auxotrophy at low Lrp concentrations and leucine toxicity at high Lrp concentrations: we observe a slight decrease in the doubling time in the presence of leucine at low Lrp concentrations, and this effect is reversed at high Lrp concentrations. We believe that the changes in growth rate at high concentrations of Lrp are indeed due to Lrp and not to the large fusion product from *gltB*::*lacZ*. Similar results have been seen for the universal stress protein (UspA): a decrease in growth rate occurs when UspA is overproduced 10-fold in *E. coli* in minimal medium, but a 10-fold over expression of LacZ has only a minor effect on growth rate (30).

Summary. The in vivo titration of Lrp has provided a means of examining the physiological significance of Lrp within growing cells under conditions known to result in regulation of the target operon, *gltBDF*. With this method, we were able to test the validity of a model describing the effect of the coregulator, leucine, on expression of a *gltB*::*lacZ* fusion. Our studies indicate that the model, which was based on in vitro gel mobility shift assays, is an accurate description of regulation by Lrp and leucine on *gltBDF*, at least at concentrations of Lrp lower than or equal to the wild-type level. Our current studies also revealed the unexpected finding that very high levels of Lrp result in interference with activation from the *gltBDF* promoter. The in vivo titration, in combination with the earlier studies, enabled us to determine the actual effective concentration of Lrp in the cell and to compare it to the total concentration of Lrp, thus estimating the amount of Lrp which is sequestered and unavailable for regulation. The observed effects of Lrp concentration on the growth rate of cultures are yet another indication of the overall physiological significance of Lrp.

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