Mutations Affecting the Ability of *Escherichia coli* Lrp To Bind DNA, Activate Transcription, or Respond to Leucine

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Lrp is a regulatory protein in *Escherichia coli* that increases expression of some operons and decreases expression of others. Mutations in Lrp were isolated on the basis of their effects on *ilvIH*, one of the operons regulated positively by Lrp. The *ilvIH* operon encodes an enzyme involved in the biosynthesis of leucine, valine, and isoleucine, and expression of this operon is repressed when cells are grown in the presence of leucine. Three groups of mutants were isolated. Mutant strains that were resistant to the repressive effects of leucine were termed leucine response mutants. These mutants had changes in the Lrp amino acid sequence between amino acid residues 108 and 149. Mutant strains having low expression of *ilvIH* in vivo were identified as colonies having reduced expression of a reporter gene. For some of these mutants, called DNA-binding mutants, binding to *ilvIH* DNA in vitro was markedly reduced. The mutations in these strains caused changes in Lrp between amino acids 16 and 70. Six of ten of these mutations were within a region having a putative helix-turn-helix motif. A third group of mutants had low *ilvIH* expression in vivo but apparently normal DNA binding in vitro. These mutants were called activation mutants since they affected the ability of Lrp to activate expression. Lrp from these strains had changes in amino acids between residues 76 and 125. This study suggests that Lrp has separate domains responsible for binding DNA, activating transcription, and responding to leucine.

Lrp (leucine-responsive regulatory protein) has recently attracted attention as an *Escherichia coli* master regulatory protein that influences the expression of more than a dozen different operons (8, 13, 18). Some of these operons were detected by chance, others were detected by a two-dimensional electrophoretic analysis, and yet others were detected by analysis of mutants created by *placMu* transposition. One of the most striking aspects of the Lrp regulon is the number of different patterns of regulation that are caused by the interaction of Lrp and leucine. Like some other important global regulators, for example, cyclic AMP response protein (15), Lrp activates expression of some operons and represses expression of others. Among the operons activated by Lrp, in some cases that activation is abolished by leucine (as is the case for the *ilvIH* operon), in some cases the activation requires leucine, and in yet other cases the activation is independent of leucine (8, 13, 18). Similarly, for those operons whose expression is repressed by Lrp, the same three subcategories have been recognized; leucine abolishes the effect, leucine is required for the effect, and leucine has no effect. Thus, in total, six different regulatory patterns involving Lrp and leucine have been observed.

The *lrp* gene was cloned (2, 14, 20, 30) and sequenced (2, 30). It is evolutionarily related to *asnC*, a gene that positively regulates the *asnA* operon (6, 12, 30). Both Lrp and AsnC were predicted to have a helix-turn-helix (HTH) motif at their amino-terminal ends (7, 30). Lrp has been purified to near homogeneity. It exists in solution as a dimer composed of two identical subunits of predicted size 18.8 kDa (30).

Among the operons regulated by Lrp, the most detailed studies to date were done with *ilvIH*, an operon that encodes one of several acetoacetyl acid synthase isoenzymes catalyzing the biosynthesis of branched-chain amino acids (5). Expression of the operon is repressed 5- to 10-fold by

addition of leucine to the growth medium (11, 26). In a mutant lacking Lrp because of a Tn10 insertion in *lrp*, expression from the *ilvIH* promoter was reduced more than 30-fold, and leucine no longer affected the residual promoter activity (20). In vitro, Lrp binds specifically and with relatively high affinity to six sites upstream of the *ilvIH* promoter, and mutations in five of these sites reduce expression of the operon in vivo (28). A simple model that accounts for these results is that Lrp activates transcription from the *ilvIH* promoter and leucine reduces expression by interfering with the action of Lrp (20).

Lrp must have at least three functions with respect to the regulation of the *ilvIH* operon: it must bind DNA, activate transcription, and respond to leucine. This report describes the isolation and characterization of Lrp mutants that were altered in each of these functions.

MATERIALS AND METHODS

Construction of strains and plasmids. *E. coli* K-12 strains CV975 (*ilvI⁻-lacZ lrp⁺*) and CV1008 (*ilvI⁻-lacZ lrp-35::Tn10*) have been described by Platko et al. (20). Transformation of plasmids pCV180 and pCV179 into strain CV1008 yielded strains CV1061 and CV1075, respectively. Transformations were performed as described previously (20) or by electroporation with a Bio-Rad Gene Pulser (voltage, 12.5 kV/cm; capacitance, 25 μ F; pulse controller, 200 ohms) and procedures provided by Bio-Rad.

Plasmid pCV180 was constructed as follows. *lrp* from strain CV975 was amplified in a polymerase chain reaction using primers N1 and C2 (30) and cloned into the *Eco*RI and *Bam*HI restriction sites of pBluescript II SK⁻ (Stratagene), yielding plasmid pCV179. Strain CV1075 (*lrp-35::Tn10*) (pCV179) does not grow on a medium containing lactose as the sole carbon source because the *lrp* gene on plasmid pCV179 does not have a functional promoter. Strain CV1075 was mutagenized with nitrosoguanidine (17), and colonies

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TABLE 1. Characteristics of strains used in this study

Strain ^a	Plasmid	lrp genotype		Promoter for lrp on plasmid	Growth on lactose (color on X-Gal)	
		Chromosome	Plasmid		-Leu	+Leu
CV975		lrp ⁺	None		Yes (blue)	No (white)
CV1009	pCV168	lrp::Tn10	lrp ⁺	lrp promoter	Yes (blue)	Yes (blue)
CV1075	pCV179	lrp::Tn10	lrp ⁺	Promoterless	No (white)	No (white)
CV1061	pCV180	lrp::Tn10	lrp ⁺	Unknown	Yes (blue)	No (white)

^a All strains contain an *ilvI'-lacZ* transcriptional fusion on the chromosome.

that grew on lactose were selected. In one colony that was analyzed, the Lac⁺ phenotype was caused by a mutation on the plasmid. To determine whether the mutation was in the *lrp* gene or elsewhere, plasmid DNA was isolated and the *Bss*HII fragment containing the *lrp* gene was replaced by the analogous fragment from pCV179. The resulting plasmid, pCV180, conferred a Lac⁺ phenotype on strain CV1008. The increased expression of Lrp in strains containing pCV180 is therefore due to a mutation that creates a promoter on the plasmid at an unknown position. Sequence analysis confirmed that the *lrp* gene on plasmid pCV180 had no amino acid changes and that the fragment was in the correct orientation.

lrp in plasmid pCV180 contains a T-to-C change at nucleotide 444 compared with the sequence of pCV168 (30). This change did not alter the amino acid sequence of Lrp. Furthermore, the GTT and GTC codons at the site of the mutation occur in about equal frequencies in *E. coli* (in proteins expressed at an intermediate level) (25); therefore, the effect of the mutation is expected to be neutral. This nucleotide change, which was not assigned an allele designation, is found in the following *lrp*-containing plasmids: pCV179, pCV180, pCV193-pCV200, pCV202-pCV215, and pCV217-pCV220.

Mutagenesis and isolation of mutants. The *lrp* gene was mutagenized by treatment separately with hydrazine, formic acid, and nitrous acid, after which the three samples were mixed together (3). Single-stranded DNA used as the template was obtained from plasmid pCV180, using the methods recommended by Stratagene. Double-stranded DNA, formed from the mutagenized template by extension from primer BS (defined below), was digested with *Eco*RI and *Bam*HI, and the *lrp*-containing fragment was introduced into pCV180 at these same restriction sites, replacing the *lrp*⁺ allele from this plasmid with the chemically mutagenized gene. These plasmids were then introduced into strain CV975 or CV1008 by transformation.

lrp-2 and *lrp-3* were isolated by mutagenizing strain CV975 (*lrp*⁺ *ilvI'-lacZ*) with nitrosoguanidine and plating the cells on agar containing lactose as the sole carbon source and 100 µg of leucine per ml. The *lrp* genes from two strains isolated in this selection were amplified in a polymerase chain reaction (10), cloned into plasmid pBluescript II SK⁻, and then recloned by attaching them to the large *Eco*RI-*Bam*HI fragment from plasmid pCV180.

Sequencing of *lrp* alleles. Cells were lysed by an alkaline lysis procedure (16), and nucleic acids were precipitated with ethanol. RNA was removed by treatment with RNase A (20 µg/ml), and then plasmid DNA was purified by using Plasmid Quik columns from Stratagene. Sequencing of double-stranded DNA was performed as previously described (30), using reagents and protocols from U.S. Biochemical Corp. The primers used were as follows: N1, 5'-GGAAG GAATTCAGAAGGACAATA-3'; BS, 5'-CGCGGTGGCG

GACGCTCTAGAACTAGT-3'; C3, 5'-CATCCGGTGGCG CACGATTCAGAGTAA-3'; and N4, 5'-GCTTAACCCCCA TTATCTGGATGCAT-3'.

Growth conditions and assays. Strains were grown at 37°C in a minimal medium containing SSA salts (11) supplemented with thiamine (5 µg/ml), proline (50 µg/ml), valine (50 µg/ml), isoleucine (25 µg/ml), micronutrients (23), and 0.2% glucose. Ampicillin (50 µg/ml) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; 40 µg/ml) were used where indicated. β-Galactosidase assays were performed as described by Platko et al. (20). Differential rates of synthesis were measured for each strain, using four to five samples taken from cultures in logarithmic phase having A₅₅₀ from 0.1 to 0.9. Specific activity was calculated from the slope of the line resulting from a plot of activity per milliliter against corrected A₅₅₀ (20). Gel mobility shift assays (20) and Western immunoblot analysis (30) were performed as previously described.

Computer analysis of polypeptide sequences. Potential secondary structures (9) within Lrp were identified by using Peptidestructure and Plotstructure programs within version 6 of the University of Wisconsin Genetics Computer Group software package. The amino acid sequence of Lrp was also examined for 338 different patterns by using Prosite; no motifs were uncovered by this analysis other than the HTH motif described later.

RESULTS

Rationale for isolation of Lrp mutants. The goal of this study was to isolate mutants altered in specific functions of Lrp. The strategy that we used was to mutagenize the *lrp* gene in vitro, attach it to a plasmid vector, and introduce the resulting chimeric plasmids into a host strain having an *ilvI'-lacZ* transcriptional fusion. For the isolation of mutants with reduced promoter expression (DNA-binding and activation mutants), transformants were selected for resistance to ampicillin and reporter gene expression was monitored on X-Gal plates. Transformants containing the parent plasmid, pCV180 (*lrp*⁺), were blue (Table 1), and the desired mutants were light blue or white. For the isolation of mutants in which *ilvIH* promoter expression was not reduced by leucine (leucine response mutants), transformants were selected for the ability to grow on plates containing lactose as the sole carbon source and leucine. Transformants containing the parent plasmid, pCV180, grew in the absence but not the presence of leucine (Table 1).

It may be noted that the *lrp* gene on plasmid pCV180 does not have its normal promoter (Table 1). Transcription from *lrp* in plasmid pCV180 and its derivatives is initiated at a promoter of unknown location in the pBluescript vector, resulting in levels of Lrp that are about eight times higher than for Lrp in a haploid strain, as measured by Western blotting experiments (data not shown). pBluescript deriva-

FIG. 1. Differential rates of synthesis of β -galactosidase in strains CV1061 (*lrp*⁺ in multiple copies on plasmid pCV180; squares) and CV975 (*lrp*⁺ in single copy; triangles) grown in the absence (open symbols) or presence (closed symbols) of 100 μ g of leucine per ml. β -Galactosidase activity was measured in units per milliliter. Cell density is A_{550} normalized so that it is proportional to cells per milliliter.

tives containing either an *lrp* gene with its normal promoter (pCV168) or without a promoter (pCV179) produced levels of Lrp that were too high or too low to be useful in our mutant selection scheme (Table 1).

To determine quantitatively the effect of eightfold-elevated levels of Lrp on *ilvIH* expression, β -galactosidase was measured in *ilvI'*-*lacZ* strains containing either a single chromosomal copy of *lrp*⁺ (CV975) or multiple copies of plasmid pCV180 (*lrp*⁺) (CV1061). As shown in Fig. 1, eightfold-elevated Lrp levels led to only a relatively slight increase in expression from the *ilvIH* promoter. In addition, strain CV1061 appeared mucoid on minimal agar plates, a phenotype presumably resulting from elevated Lrp production.

DNA-binding and activation mutants. Plasmid pCV180 containing the *lrp* gene was treated with hydrazine, formic acid, and nitrous acid and introduced into strain CV975 (*ilvI'*-*lacZ*, single-copy *lrp*⁺) by transformation. The majority of colonies selected on minimal agar plates containing ampicillin and X-Gal were dark blue and mucoid. Individual colonies that either were nonmucoid or appeared light blue, phenotypes indicating a decrease in Lrp activity, were chosen. Thus, the mutations studied here are dominant negative mutations in the sense that their phenotype was observed in the presence of a functioning wild-type *lrp* gene. Plasmid DNA was isolated from each of these colonies and introduced into strain CV1008 (*ilvI'*-*lacZ* *lrp*::Tn10) so that the properties of the mutants could be studied in the absence of the wild-type *lrp* gene. A gel mobility shift experiment was performed, using crude extracts from the mutant strains and from parent strains containing either a single (CV975) or multiple (CV1061) copies of *lrp*⁺. In extracts from one class of mutants, called DNA-binding mutants, binding activity to *ilvIH* DNA was abolished or severely reduced (Fig. 2A). There were 10 of these mutants in total, and a Western blot analysis indicated that for all but two (*lrp-9* and *lrp-11*), Lrp was present in them in normal or near-normal amounts (see Fig. 5). Therefore, the absence of binding activity in vitro was not simply due to an absence of Lrp protein. In vivo expression from the *ilvIH* promoter, as measured by β -galactosidase specific activity, was also very low in these strains (less than 5% of that of the parent) (Table 2). Taken together, these results suggest that the strains in this group

FIG. 2. Gel mobility shift assays of DNA-binding mutants. (A) Crude extracts from strains CV975 (*lrp*⁺), CV1076 (*lrp-7*), CV1077 (*lrp-8*), CV1078 (*lrp-9*), CV1079 (*lrp-10*), CV1080 (*lrp-11*), CV1081 (*lrp-12,40*), CV1082 (*lrp-13*), CV1085 (*lrp-16,36*), CV1086 (*lrp-17*), and CV1087 (*lrp-18*) (lane numbers indicate allele numbers) were incubated with a 406-bp ³²P-labeled DNA fragment containing the *ilvIH* promoter/regulatory region and fractionated by electrophoresis through agarose (20). Note that the *lrp* gene in CV975 is in single-copy, whereas in the other strains it is in multiple copies on a plasmid related to pCV180 (Table 1). (B) As in panel A except that extracts were prepared from strains CV975 (*lrp*⁺) containing the same plasmids.

do not have normal *ilvIH* expression because they produce variant Lrp molecules that do not bind to *ilvIH* DNA.

As mentioned, the DNA-binding mutants are dominant negative in the sense that their phenotype, low reporter gene activity, was observed in the presence of a functioning wild-type *lrp* gene. Gel mobility shift experiments were performed to determine whether strains containing both a chromosomal *lrp*⁺ gene and a plasmid-borne mutant *lrp* gene had reduced binding activity. The results in Fig. 2B show that this was indeed the case for each of the mutants. Thus, in terms of binding to DNA in vitro, the mutant phenotype is also dominant to the wild-type phenotype.

Extracts from a second class of strains showed substantial binding of Lrp to *ilvIH* DNA in vitro (Fig. 3A). These strains were identified on plates on the basis of reduced *ilvI'*-*lacZ* expression, and quantitative β -galactosidase measurements confirmed that this was the case (Table 2). Since Lrp was able to bind to *ilvIH* DNA in vitro but in vivo expression was reduced, these strains were presumed to have mutations that affect the ability of Lrp to activate transcription. This conclusion must be qualified for one of the strains analyzed, that containing allele *lrp-21,37*, because Lrp binding activity in that strain was reduced and the low expression from the *ilvIH* promoter in this strain might be due in part to this factor.

The complexes formed by the mutant Lrp proteins seemed similar to those formed by the parent, at least as judged by their mobilities (Fig. 3A, -Leu lanes). The position of uncomplexed *ilvIH* DNA is indicated by the letter F. Note that for parent strain CV1061, there was sufficient Lrp in crude extracts to drive the DNA into three complexes, two of which are visible in Fig. 3A. Complexes 1A and 1B contain Lrp bound to DNA at one and two upstream sites, respectively, whereas complex 1C contains Lrp bound to those sites as well as to four sites further downstream (29). A schematic representation of these complexes is shown in Fig. 3B. Complex 1A, the complex that is not visible, is faint

TABLE 2. Characteristics of mutants isolated in this study

Strain ^a	<i>lrp</i> allele	Nucleotide change ^b	Amino acid change ^b	β-Galactosidase activity ^c	
				-Leu	+Leu
CV1061	<i>lrp</i> ⁺			100	20
DNA-binding mutants					
CV1076	<i>lrp-7</i>	G-37→T	Asp-13→Tyr	1.4	1.4
CV1077	<i>lrp-8</i>	T-101→C	Leu-34→Pro	1.9	1.9
CV1078	<i>lrp-9</i>	C-118→T	Leu-40→Phe	1.4	1.4
CV1079	<i>lrp-10</i>	T-121→C	Ser-41→Pro	0.7	0.7
CV1080	<i>lrp-11</i>	C-130→T	Pro-44→Thr	1.7	1.7
CV1081	<i>lrp-12,40</i>	T-137→C, C-154→T	Leu-46→Pro	1.4	1.4
CV1082	<i>lrp-13</i>	C-142→T	Arg-48→Cys	5.2	1.1
CV1085	<i>lrp-16,36</i>	T-181→C, T-480→C	Tyr-61→His	1.0	1.0
CV1086	<i>lrp-17</i>	T-194→C	Leu-65→Pro	1.0	1.0
CV1087	<i>lrp-18</i>	T-209→C	Leu-70→Pro	3.3	3.3
Activation mutants					
CV1088	<i>lrp-19</i>	T-227→C	Val-76→Ala	18 ± 5	13 ± 4
CV1089	<i>lrp-20</i>	T-268→C	Phe-90→Leu	23 ± 4	11 ± 3
CV1090	<i>lrp-21,37</i>	T-337→C, T-195→C	Phe-113→Leu	4 ± 3	6 ± 1
CV1091	<i>lrp-22</i>	C-356→T	Thr-119→Ile	31 ± 5	20 ± 3
CV1092	<i>lrp-23,38</i>	T-373→C, T-150→C	Ser-125→Pro	29 ± 9	16 ± 9
Leucine response mutants					
CV1093	<i>lrp-24</i>	A-324→T	Leu-108→Phe	132 ± 34	102 ± 40
CV1200	<i>lrp-1,34</i>	C-342→G	Asp-114→Glu	139 ± 39	111 ± 16
CV1099	<i>lrp-2</i>	T-371→G	Met-124→Arg	114 ± 8	103 ± 8
CV1095	<i>lrp-26</i>	T-407→G	Leu-136→Arg	88 ± 7	73 ± 7
CV1096	<i>lrp-27</i>	T-439→C	Tyr-147→His	70 ± 16	71 ± 19
CV1098	<i>lrp-3</i>	G-442→T	Val-148→Phe	65 ± 22	59 ± 15
CV1097	<i>lrp-28</i>	T-446→C	Val-149→Ala	110 ± 14	81 ± 25
Unclassified mutants					
CV1032	<i>lrp-5,6</i>	G-46→A T-371→A	Asp-16→Asn Met-124→Lys	110	80
CV1094	<i>lrp-25</i>	T-323→C	Leu-108→Ser	80	55
CV1202	<i>lrp-30</i>	C-433→T	Arg-145→Trp	30	10
CV1203	<i>lrp-30,31</i>	C-433→T C-490→T	Arg-145→Trp Arg-164→Cys	20	6.0
CV1204	<i>lrp-32,33</i>	C-250→T T-328→C	Arg-84→Cys Ser-110→Pro	90	60

^a All are derived from strain CV1008 (*lrp::Tn10 ilvI'-lacZ*) and have plasmid pCV180 or a plasmid related to pCV180 containing the *lrp* allele noted.

^b From reference 30. Lrp has 164 amino acids.

^c Specific activities obtained from differential rate of synthesis plots were normalized to that of CV1061 grown in the absence of leucine. Strains were grown in minimal medium in the absence or presence of 100 μg of leucine per ml. Values with standard deviations are averages from four experiments. Other values represent averages of two experiments with different cells; the results did not differ more than 20% from the average.

because there is a high degree of cooperativity in the binding of Lrp to the two upstream sites (28).

To determine the effect of leucine upon binding in vitro, reaction mixtures either lacked or contained 50 mM leucine. For the parent strain CV1061 (wild-type *lrp* gene on plasmid pCV180), leucine caused an increase in the amount of free DNA, a decrease in the amount of the faster-moving complex 1B, and a relative increase in the amount of the slower-moving complex 1C (Fig. 3A). Thus, leucine seems to decrease the overall affinity with which Lrp binds, but it seems to favor the formation of particular complexes over others. All of the activation mutants behaved similarly to the wild type with respect to the in vitro effect of leucine. This result will be discussed below.

Leucine response mutants. Mutations in *lrp* that resulted in a protein that no longer showed leucine-mediated repression of the *ilvIH* operon were termed leucine response mutations. Such mutations were isolated by workers in other laboratories and by us in several different experiments. *lrp-5,6*, the first *lrp* allele isolated (originally called *livR1*), was identified as a mutation leading to high, constitutive expression of the operons involved in the transport of branched-chain amino acids (1, 10). *lrp-1*, the second allele of *lrp* described (origi-

nally called *lrs-1*), was isolated as a mutation that allowed a strain lacking acetohydroxy acid synthase I to grow in the presence of leucine (20, 27). We also isolated alleles that conferred resistance to leucine. With the exception of two that are described in Materials and Methods, these alleles were isolated as follows. Mutagenized plasmid pCV180 was transformed into strain CV1008 (*ilvI'-lacZ lrp-35::Tn10*), and colonies were selected for growth on a solid medium containing lactose and 100 μg of leucine per ml. The *lacZ* gene in this strain is transcribed from the *ilvIH* promoter, and leucine reduces expression from this promoter to an extent that the strain does not grow with lactose as a sole carbon source. Six new mutations conferring resistance to leucine were isolated in this way; their effects on in vivo expression from the *ilvIH* promoter are shown in Table 2. In general, these mutations led to mutant Lrps that were competent as activators of *ilvIH* transcription but that did not respond to leucine.

Figure 4A shows the results of gel mobility shift experiments performed with extracts from the leucine response mutant strains. In comparison with the wild type, two patterns of binding were observed for the mutants. For the first pattern, characteristic of alleles *lrp-1,34*, *lrp-24*, and

FIG. 3. Gel mobility shift assays of activation mutants. (A) As in Fig. 2A except that extracts were prepared from strains CV1061 (*lrp*⁺), CV1088 (*lrp-19*), CV1089 (*lrp-20*), CV1090 (*lrp-21,37*), CV1091 (*lrp-22*), and CV1092 (*lrp-23,38*). The assays were done in the presence (+) or absence (-) of 50 mM leucine. F, free DNA. 1A, 1B, and 1C are Lrp-DNA complexes (1A is not visible in this photograph). (B) Schematic representation of complexes 1A, 1B, and 1C. P, *ilvIH* promoter. Open and closed circles represent sites unoccupied and occupied by Lrp, respectively.

lrp-27, we observed a single Lrp-DNA complex that corresponded in mobility to the wild-type complex having Lrp bound at a single upstream site. Purified Lrp from a strain containing *lrp-1,34*, when used at higher concentrations, formed complexes with mobilities identical to those of 1B and 1C (data not shown). Thus, it seems likely that the *lrp-1,34* mutation markedly reduces the cooperativity of binding to the two upstream sites but that it does not prevent binding to the four downstream sites.

The second pattern of binding was shown by alleles *lrp-2*, *lrp-3*, *lrp-26*, and *lrp-28*. Lrp encoded by these alleles gave rise to complexes having the same mobility as wild-type complexes 1B and 1C (Fig. 4A). For *lrp-2*, the pattern of bands was analyzed after Lrp was purified more than 20-fold by chromatography on phosphocellulose (30). As shown in Fig. 4B, the relative amounts of free DNA and complexes 1B and 1C were different for the mutant protein in comparison with the wild type. For Lrp-2, at concentrations of protein that left sizeable fractions of DNA free, there was clear evidence of complex 1C formation. This was not the case for wild-type Lrp. These results suggest that the binding constants for one or more sites have been altered by the Lrp-2 mutation.

In summary, Lrps from the leucine response mutants form the same complexes with *ilvIH* DNA as does wild-type Lrp, but at least in some cases, one or more binding constants are altered.

As mentioned, leucine affects the pattern of bands observed in gel mobility shift experiments with wild-type Lrp. In experiments with the leucine response mutants, leucine did not increase either the amount of free DNA or the amount of complex 1C relative to 1B (Fig. 4A). Similarly, in

FIG. 4. Gel mobility shift assays of leucine response mutants. (A) As in Fig. 2B except that extracts were prepared from strains CV1061 (*lrp*⁺), CV1200 (*lrp-1,34*), CV1099 (*lrp-2*), CV1098 (*lrp-3*), CV1093 (*lrp-24*), CV1095 (*lrp-26*), CV1096 (*lrp-27*), and CV1097 (*lrp-28*). (B) Gel mobility shift experiments performed with purified Lrp⁺ or Lrp-2 purified partially by phosphocellulose chromatography (30). The numbers represent nanograms of protein analyzed.

vivo, *ilvIH* expression in these mutant strains was not affected by leucine (Table 2).

A number of other mutations that did not fit into the three categories described above were characterized. This unclassified category includes alleles *lrp-25*, *lrp-30*, *lrp-30,31*, and *lrp-32,33*, in addition to *lrp-5,6*, which was described previously (10). The locations of these mutations, and the effects that they have on in vivo expression are given in Table 2. The Lrps in this group supported at least some *ilvIH* expression, but in vitro, they bound poorly or not at all to *ilvIH* DNA (data not shown). As shown by Western blotting experiments, the poor binding was not due to the absence of Lrp (Fig. 5). It is possible that these Lrps are stable and active in vivo but that they lose binding activity in vitro.

Identities of the mutations within the *lrp* gene. Plasmid DNA was isolated from each of the strains, and the nucleotide sequence of the *lrp* gene was determined. Table 2 shows the positions and identities of the nucleotide and amino acid changes. Note that a number of these mutants have second-site mutations within the *lrp* gene that do not change the amino acid sequence. An example is *lrp-1,34*, which has a silent A-to-G change at nucleotide 228, in addition to the C-to-G change at nucleotide 342 that leads to an Asp-to-Glu change.

DISCUSSION

One group of mutations defined by this work interfered with the ability of Lrp to bind to sites upstream of the *ilvIH* promoter. These mutations were isolated in a strain contain-

FIG. 5. Western blot analysis of Lrp mutant strains. Samples of crude extract (1 μ l) from strains containing mutant *lrp* alleles were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a filter, which was probed with anti-Lrp antibody (20). Positions of Lrp are indicated by arrows. Numbers refer to *lrp* alleles on plasmids that reside in host strain CV1008 (*lrp*-35::Tn10). The corresponding strain numbers are given in Table 2. *lrp*⁺ refers to extract from strain CV1061.

ing a wild-type *lrp* gene and were recognized on the basis of reduced *ilvI'*-*lacZ* reporter gene expression. The results of gel mobility shift experiment established that the mutant phenotype was also dominant to the wild type in terms of DNA binding. The molecular basis for this finding is likely the following. Lrp exists in solution as a dimer (30). A strain containing a single wild-type copy of *lrp* on the chromosome and multiple plasmid-borne copies of mutant *lrp* (*Lrp*⁺:*Lrp*^{*} about 1:8) would contain wild-type homodimers, heterodimers, and mutant homodimers in the ratio 1:16:64 if heterodimers had the same chance of formation as did homodimers. The mutant homodimers were shown not to bind DNA (Fig. 2A), and the results in Fig. 2B suggest that heterodimers also do not bind DNA. The implications of this analysis are that the mutant Lrps are competent to form dimers but that a heterodimer cannot bind to *ilvIH* DNA. It seems likely that the species active in binding is a dimer and that heterodimers are inactive because they have only a single wild-type Lrp subunit.

Prokaryotic DNA-binding proteins often contain a structural motif known as an HTH region consisting of a short α helix, a turn that almost always includes a glycine, and a second short helix that may form specific base contacts with the DNA binding site. The amino acid sequence of Lrp was searched for an HTH motif. With use of the algorithm of Brennan and Matthews (4), amino acids 29 to 50 had a calculated score of 0.84, which was higher than scores for a group of proteins with known HTH motifs (scores of less than 0.80) but lower than that for sequences of amino acids generated at random, 0.95 ± 0.03 (30). The similarity between the residues of the first putative helix and turn of Lrp (residues 29 to 41) and the comparison data set was relatively

FIG. 6. Schematic representation of the positions of amino acid changes in mutant Lrps. Only mutants that were the result of a single amino acid change are represented.

good (score of 0.76). However, the score for the putative recognition helix was poor (0.97) and contributed to the overall high score. Similar results were obtained when the amino acid sequence of Lrp was analyzed by the method of Dodd and Egan (7). Lrp had a standard deviation score of 0.54, which may be compared with 2.5 for proteins with a known HTH and zero for random sequences. Again, the relatively poor score was due to the low homology of the recognition helix region.

The structure of the HTH motif places constraints upon the residues allowed at particular positions within the motif (19, 24). Ohlendorf et al. (19) outlined the following guidelines: position 9 should be a glycine, positions 4, 8, 10, and 15 should be hydrophobic, positions 3 to 8 and 15 to 20 should not contain proline, and position 5 should not be a branched-chain amino acid. Lrp does not violate any of these constraints except that residue 15 is a cysteine, which is polar rather than hydrophobic.

DNA-binding mutants of Lrp were identified as strains that showed markedly reduced expression of the *ilvIH* operon in vivo and little or no binding to *ilvIH* DNA in vitro. The amino acid changes in these mutants were located between positions 13 and 70 (164 amino acids in total) (Fig. 6). Alleles *lrp*-8, *lrp*-9, and *lrp*-10 had changes at residues 34, 40, and 41, respectively, within the putative HTH motif. Each of these mutations decreased the standard deviation score of the putative HTH motif when analyzed by the algorithm of Dodd and Egan (7). Another group of mutations (*lrp*-11, *lrp*-12,40, and *lrp*-13) were also within the putative HTH region, but two of them had little effect on the standard deviation score and one of them actually increased it significantly (*lrp*-11; from 0.54 to 1.72). All of these mutations were within the recognition helix portion of the putative HTH, suggesting that the specific binding of Lrp to *ilvIH* DNA may require particular amino acids which are not normally present at those positions within the HTH regions of other DNA-binding proteins. A third group of mutations (*lrp*-7, *lrp*-16,36, *lrp*-17, and *lrp*-18) were close to but outside of the putative HTH motif. These mutations could be in regions that are important for stabilizing the putative HTH region, or they could define residues that are important to a structure, other than an HTH domain, that is involved in the binding of Lrp to *ilvIH* DNA.

In summary, a computer analysis indicated that one region of Lrp shows similarity, albeit weak, with regions of proteins known to contain HTH motifs. The fact the mutations that markedly reduce DNA binding are within or near the putative HTH region is at least consistent with the idea that Lrp has a functional HTH motif. Additional circumstantial support for this contention comes from the fact that Lrp shows distinct evolutionary relatedness to AsnC, an *E. coli* regulatory protein (12) that is strongly suspected of having an HTH motif (7).

FIG. 7. Helical wheel representations of Lrp residues 88 to 103. Circles represent hydrophobic residues. The charge of a residue is represented by plus or minus sign.

Mutations in *lrp* that reduced *ilvIH* expression in vivo 3- to 12-fold, but that did not markedly affect the pattern of Lrp binding to DNA in vitro, were termed activation mutants. Sequence analysis of these mutations revealed changes affecting amino acids between positions 76 and 125 (Fig. 6). This region was examined for potential amphipathic α helices having numerous negatively charged residues along one face because studies of both prokaryotic and eukaryotic transcription factors have implicated such amphipathic helices in the mechanism of activation (21). The algorithm developed by Garnier et al. (9) predicted that residues 87 to 106 of Lrp form an α helix. Representation of residues 88 to 103 on a helical wheel revealed the presence of a potential amphipathic α helix (Fig. 7). Four of the five mutations that affected activation were outside of the region that contained the hypothetical amphipathic α helix. One of the mutations, *lrp-20*, was within the putative helix region, but it did not affect any of the negatively charged residues, nor did it affect the computer-predicted structure of the region. In summary, the activation mutations isolated in this study were located within a limited region of the *lrp* gene but were not correlated with any known structural motif.

In general, Lrps in the leucine response mutants were competent as activators of *ilvIH* transcription in vivo and, as expected, were insensitive in vivo to excess leucine in the growth medium. In vitro, the binding of these Lrps to DNA was not affected by leucine, and for some of them, the affinities for one or more binding sites were altered. The mutations giving rise to these phenotypes were located in the part of the gene giving rise to the C-terminal third of Lrp (Fig. 6).

For most of the activation mutants, leucine had little or no effect on the residual in vivo expression of the *ilvIH* operon (Table 2). Therefore, these mutants might better be classified as both leucine response and activation mutants. On the other hand, in vitro leucine affected the binding of mutant Lrps in much the same way as it did the wild-type protein; i.e., it caused an increase in free DNA and changed the relative proportions of two Lrp-DNA complexes. It is difficult to assess the significance of this latter result because the mechanism by which leucine affects Lrp action has not been investigated in detail. The fact that in vitro, leucine reduces binding of wild-type Lrp, but not of Lrp-1 (22) or Lrps from other leucine response mutants, to *ilvIH* DNA is consistent with the idea that leucine acts by reducing the extent of binding of Lrp. However, the physiologically significant effect of leucine may be not on the extent of binding but

rather on the nature of the binding. This possibility remains to be explored.

In summary, the work reported here suggests that Lrp has domains responsible for binding DNA, activating transcription, and responding to leucine. Further study of these Lrp mutants should prove useful in understanding the regulation of the *ilvIH* operon and other operons controlled by Lrp.

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