The leucine-responsive regulatory protein (Lrp) regulates the expression of many operons in Escherichia coli including several involved in the metabolism of the branched-chain amino acids, L-isoleucine, L-valine, and L-leucine. The ilvGMEDA operon contains the genes for four of the five enzymes of the common pathway for the biosynthesis of these amino acids. A high affinity, consensus-like Lrp-DNA binding site has been identified at an unusual position in the leader region of this operon. 226 base pairs downstream of the transcriptional initiation site between the attenuator and the ilvG gene. Binding to this site facilitates the cooperative binding of a second Lrp protomer to an adjacent, upstream, secondary site. At higher Lrp concentrations, binding to a third site is observed. Chemical, enzymatic, and alkylation protection and interference footprinting experiments demonstrate that the Lrp homodimer contacts the DNA helix at symmetrical half-sites present in adjacent major grooves and that the primary and secondary binding sites are separated by one helical turn and aligned along the same face of the DNA helix. In vivo, Lrp represses transcription through the leader-attenuator region of the ilvGMEDA operon. Lrp-dependent production of attenuated RNA transcripts is also observed in vitro. No transcriptional effects are observed, in vivo or in vitro, in the absence of an intact Lrp primary binding site. A possible physiological role for Lrp in the regulation of ilvGMEDA operon expression is discussed.

The leucine-responsive regulatory protein, Lrp, is a global regulatory protein of Escherichia coli that affects the expression of many operons (reviewed in Refs. 1 and 2). The expression of some target operons is activated by Lrp, while the expression of others is repressed. In addition, the free amino acid, L-leucine, acts as an effector ligand of Lrp; and, at some DNA target sites, L-leucine is required for Lrp binding. At other sites, L-leucine can antagonize or have no effect on Lrp-DNA interactions. In most cases, the physiological role of Lrp is to activate biosynthetic operons and to repress degradative ones. Therefore, it has been suggested that Lrp and L-leucine might function to coordinate metabolic shifts between nutritional feast and famine conditions. However, the metabolic coherence of the operons regulated by Lrp remains unclear.

The structural gene for Lrp was initially identified as a mutation in a regulatory gene, leuR, for the branched-chain amino acid (L-leucine, L-isoleucine, and L-valine) transport system (3). Subsequently, additional mutations in this gene that affected the biosynthesis of L-leucine were identified (4). Further studies showed that the protein product of this gene (now referred to as lrp) activates transcription of the ilvIH operon, which encodes one of the three acetohydroxy acid synthase (AHAS) isozymes required for the first step in the biosynthesis of the three branched-chain amino acids (4). Interestingly, many of the other operons regulated by Lrp are involved in either the production or the dissipation of the carbon substrates, pyruvate and α-ketobutyrate, of the AHAS isozymes required for branched-chain amino acid biosynthesis (Fig. 1). For example, Lrp regulates the expression of the leucine biosynthetic operon, leuABCD (7), and the expression of the branched-chain amino acid transport genes livJ and livKHM (8). Thus, it appears that Lrp plays an important role in the maintenance of appropriate in vivo levels of the branched-chain amino acids. Such a role for Lrp in branched-chain amino acid metabolism is further underscored by the partial auxotrophy for the branched-chain amino acids observed in a lrp− strain of E. coli K12 (1).

In E. coli, the biosynthesis of the branched-chain amino acids occurs via a parallel pathway catalyzed by several bifunctional enzymes (Fig. 1) (9). The genes encoding these enzymes are specified by four separate operons (ilvBN, ilvGMEDA, ilvYC, and ilvIH). The ilvBN and ilvIH operons encode the genes for the subunits of the AHAS I and AHAS III isozymes, respectively (10). The ilvGMEDA operon encodes four of the five enzymes required for the biosynthesis of L-isoleucine and L-valine (11). The ilvGM genes encode the subunits of AHAS I, while the remaining genes encode two other enzymes of the common pathway, dihydroxy-acid dehydratase (ilvD) and transaminase B (ilvE), and one enzyme specific to the biosynthesis of L-isoleucine, L-threonine deaminase (ilvA). The α-keto acid precursor for L-valine, α-ketoisovalerate, is a branch point intermediate for the biosynthesis of L-leucine by the enzymes encoded in the leuABCD leucine-biosynthetic operon. Thus, the enzymes encoded by the ilvGMEDA operon are used for the production of all three branched-chain amino acids. Therefore, since Lrp is involved in the regulation of other operons involved in branched-chain amino acid metabolism, it might be expected that it would modulate the expression of this central operon.

In this report, we show that Lrp does, in fact, bind to a high affinity, consensus-like site located between the attenuator and the first structural gene of the ilvGMEDA operon. We further
**Lrp-DNA Interactions in the ilvGMEDA Operon**

**MATERIALS AND METHODS**

**Chemicals and Reagents—**All chemical reagents were purchased from Sigma. Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England Biolabs. *E. coli* RNA polymerase, pancreatic RNAse, and DNAse I were purchased from Boehringer Mannheim Biochemicals. Shrimp alkaline phosphatase was purchased from U. S. Biochemical Corp. Radiolabeled nucleotides were purchased from DuPont NEN. DNA oligonucleotides were synthesized by Operon Technologies. Site-directed mutagenesis was performed using the oligonucleotide-directed in vitro mutagenesis kit, version 2.0, from Amersham Corp. DNA sequencing was performed using the Sequenase kit from U. S. Biochemical Corp. The Lrp used in the experiments reported here is a 6-His-tagged derivative of Lrp containing 12 restriction fragment derived from the vector pIP16 (15) into the the EcoRI and 3′ pBS2 was created by cloning a polymerase chain reaction-amplified DNA fragment (containing ilvGMEDA sequence from base pair positions 110–278, flanked by HindIII and BamHI restriction endonuclease sites, respectively) into the HindIII and BamHI sites of pUC19. Plasmid pDHWT, used for the in vitro transcription reactions, was constructed by ligating an end-filled 515-bp EcoRI-BamHI DNA fragment containing a 494-bp ilvGMEDA-derived HindIII fragment (base pair position –245 to +249) into the EcoRI and BamHI sites of the lacZ-truncated transcriptional fusion plasmid pRS551A (17) (yielding the reporter plasmid pRS2490) and integrating this reporter plasmid construct into the bacterial chromosome of some of the polA-deficient strain, NO3454 (18), as described previously (19). Strain IH-G2492 was created by ligating a 515-bp EcoRI-BamHI DNA fragment containing a 494-bp ilvGMEDA-derived HindIII fragment (base pair position –245 to +249) into the EcoRI and BamHI sites of the lacZ-truncated transcriptional fusion plasmid pRS551A (17) (yielding the reporter plasmid pRS2490) and integrating this reporter plasmid construct into the bacterial chromosome of the polA-deficient strain, NO3443, in single copy as described previously (19). An isogenic lrp derivative of strain IH-G2492 was created by generalized P1 transduction of the lrp-35::Tn10 allele (4) into this strain according to the methods of Miller (20) to yield strain IH-G2491.

**Gel Mobility Shift Assays and Determination of Macroscopic Affinity Constants—**Gel retardation assays were performed as described by Wang and Calvo (21). Radiolabeled DNA fragments used in these experiments were present at a final concentration of 1 × 10⁻¹¹ m to ensure that the final free concentration of Lrp present in the binding reaction was essentially equivalent to the total Lrp concentration. Free and bound DNA fragments were visualized by autoradiography following the exposure of the dried gels to Kodak XAR-5 film overnight at 25°C. Quantitation of band intensity was performed utilizing the public domain NIH IMAGE gel quantitation software. Macroscopic equilibrium dissociation constants (KD) were determined as described by Senear and Brenowitz (22). According to this method, the binding curve is described by the Langmuir isotherm, \( Y = \frac{kP + kL}{1 + kP} \). The equilibrium binding data were analyzed by nonlinear least squares Levenberg-Marquardt method. The algorithm for this analysis (23) uses a variation of the Gauss-Newton procedure (24) to determine the best fit, model-dependent, parameter values corresponding to a minimum in the variance of each data point. The confidence levels for the curve fits reported correspond to ~1 S.D. (65% confidence). In fitting the data to the equations, the substitution, \( \Delta G = RT \ln K \), was made so that the \( \Delta G \) values for each experiment were the actual curve fit parameters.

**DNase I Footprinting Experiments—**DNase I footprinting reactions were performed as described by Wang and Calvo (25) using uniquely 5'-P-end-labeled DNA fragments (<1 × 10⁻¹⁰ M) containing ilvGMEDA base pair positions 6–278 and 110–278, derived from plasmids pUCR1TQ and pIS2, respectively. Binding reactions were performed under equilibrium binding conditions, and DNase I treatment was restricted to 1 min at 25°C to assure single hit kinetics. Conditions of single hit kinetics were verified by measuring the radioactivity of the remaining uncleaved DNA fragments by Cerenkov counting and demonstrating that this population of DNA fragments represented >70% of the total radioactivity included in the reaction (26). Samples were resolved by electrophoresis on an 8% denaturing polyacrylamide gel (7.6% acrylamide, 0.4% N,N'-methylenebisacrylamide) containing 8 M urea in TBE (14) and visualized by autoradiography following the exposure of the gels to Kodak XAR-5 film at ~70°C in the presence of a Cronex Quanta III intensifying screen (Dupont).

**Hydrolus Radical Footprinting Experiments—**Hydrolus radical footprinting was performed as described by Tullius and Dobrowsky (27) using the same DNA fragments (<1 × 10⁻¹⁰ M) as in the DNase I footprinting reactions. Binding reactions were performed under equilibrium binding conditions as described above with the exception that glycerol was omitted from the reaction mixture. Samples were treated with hydrolus radical for 2 min at 25°C and quenched with the addition of 20 μl of 0.2 M thionine to ensure conditions of single hit kinetics as
activities were calculated according to the method of Miller (20). Rates of acti-

mM dithiothreitol, 0.1 mM EDTA, 200  

acted with DMS at a final concentration of 26.5 mM for 5 min at 25°C  

amount of transcription between reactions, at topologically independent  

ing polyacrylamide gel (7.6% acrylamide, 0.4%  

region, gel mobility shift assays were performed under equilib-

The autoradiogram in Fig. 3 shows that Lrp binds with high affinity to a DNA fragment (ilvG base pair positions 6–278) containing this site. This gel mobility shift experiment also showed that Lrp binding to this DNA fragment results in the formation of three complexes. At an Lrp concentration of \( 1 \times 10^{-10} \) M, Lrp forms two complexes with the DNA fragment (complexes 1 and 2); while at an Lrp concentration of \( 1 \times 10^{-8} \) M, a third complex (complex 3) is observed. As the Lrp concentration was increased, a greater proportion of complexes 2 and 3 were formed. The overall macroscopic equilibrium dissociation constant \( (K_d) \) for these Lrp-DNA interactions, measured by the disappearance of the unbound (free) DNA fragment according to the methods of Senea et al. (22), is 1.35 ± 0.14 \times 10^{-9} \) M. The individual macroscopic \( K_d \) values for complexes 1, 2, and 3 are 2.40 ± 0.18 \times 10^{-9} \) M, 2.14 ± 0.23 \times 10^{-7} \) M, and 1.24 ± 0.27 \times 10^{-7} \) M, respectively (Fig. 4).

At Lrp concentrations as high as \( 1 \times 10^{-7} \) M, no detectable Lrp-DNA complexes were observed with a DNA fragment (ilv base pair positions 6–188) that does not contain the consensus-
Lrp-DNA Interactions in the ilvGMEDA Operon

Fig. 3. Gel mobility shift assay for Lrp binding in the ilvGMEDA leader-regulatory region. Autoradiogram of a gel mobility shift experiment performed with a 272-bp 32P-end-labeled DNA fragment (nt 9-6–278) as described under “Materials and Methods.” Lane 1, DNA probe only; lanes 2–12: 2.0 × 10−3 nM; 6.5 × 10−3 nM; 1.6 × 10−2 nM; 8.0 × 10−2 nM; 1.6 × 10−4 nM; 8.0 × 10−4 nM; 1.6 × 10−5 nM; 3.2 nM; 8.0 nM; 16 nM; 32 nM Lrp, respectively.

Fig. 4. Binding isotherm analysis of Lrp binding to the ilvGMEDA leader-regulatory region. These data were obtained from four experiments of the type illustrated in Fig. 3 performed as described under “Materials and Methods.” The percentage of DNA fragments bound by each of the Lrp-DNA complexes or the percentage of remaining uncomplexed (Free) DNA fragment is indicated on the ordinate. The curves are the best-fit isotherms for the gel shift analysis of each of the three Lrp-DNA complexes or the remaining uncomplexed DNA fragments. The overall macroscopic equilibrium dissociation constant (K_D ± S.D.) for these Lrp DNA interactions (measured by the disappearance of uncomplexed DNA fragment) is 1.35 ± 0.14 × 10−9 M. The individual macroscopic K_D values for complexes 1, 2, and 3 are 2.40 ± 0.18 × 10−9, 2.14 ± 0.23 × 10−9, and 1.24 ± 0.27 × 10−9 M (22). □, free DNA fragment; ■, complex 1; △, complex 2; ●, complex 3.

Lrp complexes observed in Fig. 3 might, alternatively, be explained by protein aggregation at high Lrp concentrations. If this were the case, then multiple complexes would be expected when high concentrations of Lrp are bound to only a single site contained on a small, double-stranded, DNA oligonucleotide. Cui et al. (12) have shown that double-stranded DNA oligonucleotides containing five base pairs flanking a consensus Lrp binding site are sufficient for site-specific, high affinity, Lrp-DNA interactions. Therefore, a 33-bp, double-stranded, DNA oligonucleotide containing the 15-bp, consensus-like, Lrp binding site in the ilvGMEDA leader region flanked on each side by an additional five ilv specific base pairs was synthesized (Fig. 5 legend). The autoradiogram in Fig. 5 shows that Lrp forms a single complex with this DNA oligonucleotide. However, over the same Lrp concentration range, multiple Lrp-DNA complexes are observed with a larger ilv DNA fragment (ilv base pair positions 6–278; Fig. 3). Thus, the appearance of the multiple complexes observed in Fig. 3 is not the consequence of protein aggregation at high Lrp concentrations. Instead, these experiments suggest that secondary Lrp-binding sites not clearly identifiable by sequence analysis are present in the ilvGMEDA leader region which give rise to complexes 2 and 3. The apparent affinity of Lrp for the single binding site in the double-stranded DNA oligonucleotide is greater than 1 order of magnitude less than the affinity of Lrp for the larger DNA fragment also containing the secondary binding sites. This result suggests that high affinity binding of Lrp to the consensus-like site in the ilvGMEDA operon requires additional 5’ and/or 3’ ilv-specific flanking DNA sequences beyond those previously defined by Cui et al. (33).

Chemical and Enzymatic Footprinting of Lrp-Binding Sites in the ilvGMEDA Leader Region—DNase I footprinting experiments were performed using DNA fragments uniquely 5’-32P-end-labeled on the transcribed (ilv base pair positions 6–278) or nontranscribed (ilv base pair positions 278–110) strand. The autora-
diagram in Fig. 6A shows that in the presence of increasing concentrations of Lrp (5.0 × 10⁻¹⁰ to 3.25 × 10⁻⁸ M), three regions of DNase I protection and two intervening regions of enhancement on the nontranscribed DNA strand are observed. Site 1 (approximate base pair position, 220–250), which contains the 15-bp Lrp consensus-like binding sequence located between base pair positions 226 and 240, appears at the lowest Lrp concentration followed by the appearance of site 2 (approximate base pair position 185–215) and then site 3 (approximate base pair position 155–180). The regions of DNase I protection and enhancement induced by Lrp on the transcribed DNA strand are identified in the autoradiogram shown in Fig. 6B. Again, site 1 appears before site 2 as the Lrp concentration is increased. On this strand, however, no DNase I protection in the region identified as site 3 on the transcribed strand was observed.

Since the DNase I-protected sites 1, 2, and 3 and the Lrp-DNA complexes 1, 2, and 3 observed with the gel mobility shift assays (Fig. 3) appear at nearly the same Lrp concentrations, these results suggest that the gel shift complexes 1, 2, and 3 are Lrp-DNA complexes containing 1, 2, and 3 Lrp protomers per DNA fragment, respectively. The regions of increased DNase I sensitivity suggest Lrp-induced changes in the structure of the DNA helix.

To more accurately determine the base pair-specific interactions between Lrp and the DNA helix, hydroxyl radical footprinting experiments were performed using DNA fragments uniquely 5'⁻³²P-end-labeled on either the transcribed or nontranscribed DNA strands described above. At a minimally saturating concentration of Lrp (2.6 × 10⁻⁸ M), protection from hydroxyl radical cleavage was observed on the transcribed DNA strand in all three of the regions identified as Lrp-binding sites by DNase I protection (Fig. 7B). Protection was observed in site 1 at base pair positions 220–225, 230–234, and 238–243. Protection was observed on the opposite (nontranscribed) DNA strand between base pair positions 223–227 and 233–238 (Fig. 7A). Thus, on one DNA strand or another, nearly every base pair between positions 220 and 243 was protected from hydroxyl radical cleavage by Lrp. Two other regions in site 2 on the transcribed strand (base pair positions 194–201 and 207–214) were also protected from hydroxyl radical cleavage by Lrp (Fig. 7B). No clear hydroxyl radical protection data were obtained for site 3. All of the protected regions lie within Lrp-binding sites identified by the DNase I footprinting experiments (Fig. 6).

To identify base-specific contacts between Lrp and its binding sites in the ilvGMEDA leader region, DMS protection assays were performed on DNA fragments uniquely 5'⁻³²P-end-labeled on either the transcribed or nontranscribed DNA strands described above. The autoradiogram in Fig. 8B shows that in the presence of a minimally saturating concentration of Lrp (2.6 × 10⁻⁸ M), 2 guanine residues separated by 9 base pairs (nearly one complete turn of the DNA helix) at base pair positions 229 and 238 on the transcribed DNA strand were not alkylated by DMS. Since DMS alkylates the N-7 position of guanine residues exposed in the major groove of the DNA helix, this result shows that the homodimeric Lrp molecule binds to adjacent major grooves positioned on the same face of the DNA helix. In addition, alkylation of the N-7 position of a guanine residue at position 228 on the nontranscribed strand was inhibited (Fig. 8A). Interestingly, this guanine is related by dyad symmetry to the protected guanine at position 238. Thus, binding of Lrp to the consensus-like site appears to involve symmetrical half-site contacts and interactions with adjacent ma-
major grooves of the DNA helix. A third guanine residue at base pair position 231 in site 1 on the transcribed strand was also protected from DMS alkylation (Fig. 8B). Because there is no sequence symmetry in the secondary Lrp-binding sites, no other DMS protections separated by an integral turn of the DNA helix were observed. However, a single DMS protection site at a guanine residue located at base pair position 199 in site 2 on the transcribed DNA strand was detected (Fig. 8B). In addition, a single, distal site of enhanced DMS reactivity was observed at base pair position 176 on the nontranscribed DNA strand (Fig. 8A).

Alkylation interference experiments were performed to identify additional nucleotide positions important for the binding of Lrp to its target site(s) within the ilvGMEDA leader-regulatory region and to complement the results of the alkylation protection experiments described above. To perform these experiments, DNA fragments uniquely 5'-32P-end-labeled on either the nontranscribed or transcribed strand (described above) were alkylated with DMS such that, on average, each DNA molecule contained only 1 modified nucleotide (30). These modified DNA fragments were incubated with a minimally saturating amount of Lrp (2.6 × 10^-8 M) and resolved by electrophoresis on a nondenaturating polyacrylamide gel to separate Lrp-bound and unbound fragments. In principle, DNA fragments containing modifications that interfere with binding of Lrp to its target site(s) should preferentially, if not exclusively, be contained in the unbound fraction. An autoradiogram of the results of the alkylation interference experiment is shown in Fig. 9. At the minimally saturating Lrp concentration used in this experiment, only the Lrp-DNA complex containing two Lrp protomers contained enough radioactivity to be suitable for further analysis. On the nontranscribed strand, two bands were identified in the unbound fraction that were missing in the bound fraction (Fig. 9). These bands are located at nucleotide positions 228 and 235 in site 1. Since the alkylation protection experiment identified the guanine at position 228 as a protected residue, it is likely that direct contacts between Lrp and this guanine residue are important for high affinity binding of Lrp to site 1. The interference observed with the modification of the adenine residue at nucleotide position 235 can be interpreted either as a site of direct contact with Lrp in the minor groove of the DNA helix or as a residue the modification of which alters the local conformation of the DNA helix. Analysis of Lrp-DNA complexes containing two protomers bound to an ilvGMEDA leader-containing DNA fragment labeled on the transcribed strand showed no detectable differences in the populations of modified DNA fragments in the bound versus unbound fraction. Also, no DMS interference sites were detected in site 2 on either DNA strand.

In summary, the results of the DNase I and hydroxyl radical footprinting experiments demonstrate that Lrp binds sequentially to three adjacent sites in the leader region of the ilvGMEDA operon (Fig. 10). The results of the DMS protection experiments show that the Lrp protomer binds to adjacent...
FIG. 10. Schematic representation of Lrp DNA interactions in the ilvGMEDA leader-regulatory region. ●, OH radical protection; ■, DMS protection; □, DMS interference. The DNA helix is oriented to the display the open face of the major grooves of the Lrp binding sites.

major grooves on the same face of the DNA helix at its primary binding site (site 1). The position of the DMS protection in site 2 at base pair position 199, three and four integral turns of the DNA helix away from the DMS protection sites in site 1 at base pair positions 229 and 238, suggests that Lrp molecules bound to sites 1 and 2 are separated by one helical turn and are aligned along the same face of the DNA helix. The hydroxyl radical and DNase I protection data suggest that site 3 is separated from site 2 by another turn of the DNA helix and are consistent with the possibility that site 3 is also aligned along the same face of the DNA helix as sites 1 and 2. The regions of Lrp-induced DNase I sensitivity suggest that Lrp alters the structure of the DNA helix in and around its primary and secondary binding sites (Fig. 6).

Preliminary in Vivo Effects of Lrp—To determine the in vivo consequence of Lrp-DNA interactions in the ilvGMEDA leader region, the expression of a reporter gene (lacZ) transcriptionally fused to the ilvP_G promoter-regulatory region at base pair position 249 and integrated in single copy into the bacterial chromosome of Lrp+ and Lrp− E. coli strains was examined. The results reported in Table I show that in the absence of a functional Lrp gene product, the expression of the reporter gene was increased nearly 3-fold (compare strains IH-G2490 and IH-G2491). To determine whether this increased transcription of the reporter gene was the consequence of a direct effect on the expression of the ilvGMEDA operon or an indirect pleiotropic effect of the Lrp− phenotype, the expression of the lacZ gene in a similar transcriptional fusion construct containing a mutationally altered primary Lrp-binding site (Fig. 2) was examined. In vitro, no detectable binding of Lrp to a DNA fragment containing this mutated site is observed at a Lrp concentration as high as 1 × 10−7 M. The results of this experiment are also shown in Table I. Again, the expression of the reporter gene was increased 3-fold (compare strains IH-G2490 and IH-G2492). These results demonstrate that Lrp represses the expression of the ilv::lacZ transcriptional fusion construct described above and that this repression is dependent on a site-specific interaction between Lrp and its consensus-like site within the ilvGMEDA leader region.

In Vitro Transcriptions in the Absence and Presence of Lrp—A purified in vitro transcription system was employed to examine the effects of Lrp on the production of attenuated RNA transcripts from the promoter-attenuator region of the ilvGMEDA operon on a negatively supercoiled DNA template. The autoradiogram in Fig. 11 shows that the production of the attenuated RNA transcripts which originate from the ilvP_G and ilvP_O2 promoters (36) is repressed by Lrp. Additionally, the concentration of Lrp required for half-maximal repression is comparable to the concentration of Lrp required for half-maximal saturation of the Lrp binding sites.

Leucine Inhibits but Does Not Abolish Lrp-DNA Interactions—To evaluate the effect of L-leucine on Lrp-DNA interactions in the ilvGMEDA leader region, gel mobility shift assays were performed under equilibrium binding conditions in the presence of increasing amounts of L-leucine. The results of these experiments showed that L-leucine coordinately destabilizes all of the Lrp-DNA complexes. The apparent macroscopic inhibition constant (K_i) for this L-leucine-mediated inhibition of Lrp-DNA binding activity, measured by the appearance of the uncomplexed (free) DNA fragment, is approximately 2 mM. However, even at a saturating concentration of L-leucine (20 mM) (37) inhibition is incomplete and about 20% of the DNA fragments remain complexed with Lrp. Thus, although L-leucine significantly destabilizes binding of Lrp in the ilvGMEDA leader region, it does not abolish it. While L-isoleucine or L-valine also inhibit Lrp-DNA interactions, significantly higher concentrations of each are required and different levels of maximal inhibition are observed for each complex.

DISCUSSION

Lrp Binds to a Consensus-like Primary Site in the Leader Region of the ilvGMEDA Operon—The results of the experiments reported here clearly identify a high affinity Lrp binding site located within the leader region of the ilvGMEDA operon. This site, located between the transcriptional termination site of the ilvGMEDA attenuator and the beginning of the ilvG structural gene exhibits a 13- of 15-bp match to a PCR-derived consensus sequence (33). Cui et al. (33) have estimated the relative binding affinities of sites with sequence similarities to the consensus site by assigning arbitrary values that were based on theoretical binding energies to each of the 15 base pair positions. This results in a predicted relative binding energy value for each consensus-like Lrp binding site. The consensus-like site identified within the ilvGMEDA leader region yields a predicted value of 4.81 which is similar to the predicted value (5.01) of a known high affinity Lrp binding site, site 2, in the ilvIH promoter region (33). A systematic search of the DNA sequence of the ilvGMEDA operon from base pair position −460 to +278 did not reveal any other Lrp consensus-like DNA binding sites with predicted values greater than 3.0. Cui et al. (33) suggest that sequences with predicted values less than 3.0 are not likely to be specific Lrp binding sites.

The Lrp binding site in the ilvGMEDA operon is highly conserved in sequence, position, and alignment orientation in the four closely related enterobacteria, S. typhimurium, E. tarda, S. marscescens, and K. aerogenes. The significance of these conserved features is underscored by the observation that little sequence conservation is observed in other noncoding regions of the ilvGMEDA operon (11).

Evidence for site-specific binding of Lrp to this consensus-like site comes from the observations that Lrp binds with high affinity to an ilv DNA fragment containing this site but binds with a much weaker affinity (~2 orders of magnitude) to an ilv DNA fragment lacking this site or containing a Lrp binding site altered by site-directed mutagenesis. The specific Lrp-DNA interactions at this site are defined by the results of the DNase I, hydroxyl radical, and DMS protection and interference foot-

K. Y. Rhee and G. W. Hatfield, unpublished results.
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**Fig. 11. In vitro transcription reactions of the ilvP2 promoter in the absence and presence of increasing amounts of purified Lrp.** Autoradiogram of in vitro transcription reactions, performed with a DNA template concentration of 8 nM, either in the absence of presence of increasing amounts of purified Lrp as described under “Materials and Methods.” Lane 1, no Lrp; lanes 2–5: 1.6 nM; 8.0 nM; 16 nM; 80 nM Lrp, respectively. Amp is a topologically independent runoff transcript from β-lactamase in a pUC13 DNA template linearized with ScaI. ilvP1 and ilvP2 identify the 258- and 186-nucleotide RNA transcripts that originate from these promoters, respectively. The ilvP1 transcript originates from an AT-rich region in the upstream DNA sequence (42); The 108-nucleotide ori transcript originates from the RNA-I promoter.

| Strain   | Relevant genotype                                    | β-Galactosidase specific activity ONP (nmol ONP/min/mg protein) |
|----------|------------------------------------------------------|---------------------------------------------------------------|
| IH-RS551 | ΔilvP1::lacZ                                         | ND*                                                           |
| IH-G2490 | ilvP1::att::lacZ                                     | 2973 ± 80                                                    |
| IH-G2491 | ilvP1::att::lacZ, lrp                                 | 8161 ± 642                                                   |
| IH-G2492 | ilvP1::att::lacZ, lbs (Lrp-binding site 1 mutation)  | 8516 ± 950                                                   |

* ONP, o-nitrophenol; ND, not detectable.
* Mean ± S.D. obtained from the results of at least three separate experiments.

**TABLE I**

**In vivo effect of Lrp on transcription through the ilvGMEDA leader-attenuator region**

| Strain   | Relevant genotype                                    | β-Galactosidase specific activity ONP (nmol ONP/min/mg protein) |
|----------|------------------------------------------------------|---------------------------------------------------------------|
| IH-RS551 | ΔilvP1::lacZ                                         | ND*                                                           |
| IH-G2490 | ilvP1::att::lacZ                                     | 2973 ± 80                                                    |
| IH-G2491 | ilvP1::att::lacZ, lrp                                 | 8161 ± 642                                                   |
| IH-G2492 | ilvP1::att::lacZ, lbs (Lrp-binding site 1 mutation)  | 8516 ± 950                                                   |

* ONP, o-nitrophenol; ND, not detectable.
* Mean ± S.D. obtained from the results of at least three separate experiments.

**consensus-like Secondary Sites**—In spite of the fact that only one, consensus-like, Lrp DNA binding site was identified in the nearly 750 base pairs around the promoter of the ilvGMEDA operon, the results of gel mobility shift experiments (Fig. 3) showed that lrp forms three complexes with an ilv DNA fragment containing this site. The results of DNase I footprinting experiments (Fig. 6) showed that these complexes are formed by the binding of Lrp to its primary, consensus-like, site followed by the sequential and directional binding to two adjacent secondary sites that possess no sequence similarity to the Lrp consensus site. The further observations that Lrp does not bind with high affinity to an ilv DNA fragment containing a mutationally altered or deleted primary binding site suggest that the binding of Lrp to these secondary sites requires cooperative interactions associated with the binding of an initial Lrp protomer to the primary consensus-like sequence. The DNase I footprinting results also suggest Lrp-induced conformational changes in the DNA helix in the regions flanking each of the Lrp binding sites (25). Since there are no identifiable consensus-like sequence elements at these secondary sites, it is possible that these Lrp-induced alterations of the DNA helix might be an important parameter of these cooperative interactions. It is unlikely that this cooperativity is solely attributable to specific protein interactions between adjacent Lrp protomers since Williams et al. (39) have demonstrated that, in solution, Lrp exists exclusively in a homodimeric form at concentrations up to and exceeding $1 \times 10^{-6}$ M.

**The Primary and Secondary Lrp-binding Sites Are Separated by One Helical Turn and Are Aligned along One Face of the DNA Helix**—The results of the hydroxyl radical footprinting experiments (Fig. 7) show that the primary consensus-like binding site (site 1) and the adjacent secondary binding site (site 2) are separated from one another by one integral turn of the DNA helix (Fig. 10). The results of the DMS protection experiments further demonstrate that these two sites (sites 1 and 2) are aligned along the same face of the DNA helix.

Since Lrp binding to site 3 did not reach saturation (Fig. 4), sufficient data was not obtained to accurately define the position of this third Lrp binding site. However, the results of *in vitro* DNase I and hydroxyl radical footprinting experiments in this region suggest that this site is most likely also separated from site 2 by one integral turn of the DNA helix and aligned along the same face of the DNA helix as sites 1 and 2. However, it is likely that Lrp does not bind to this site *in vivo*. This suggestion is based on the following observations: (i) Lrp concentrations in excess of $1 \times 10^{-8}$ M are required to form detectable Lrp DNA complexes at this site *in vitro* (Fig. 4); (ii) the estimated total intracellular concentration of Lrp is about $1 \times 10^{-6}$ M (39); and (iii) Lrp is thought to bind nonspecifically to over 1000 sites on the bacterial chromosome (6). Thus, the free intracellular concentration of Lrp might be too low to form Lrp-DNA complexes at this site *in vivo*.

**Lrp Represses Transcription through the ilvGMEDA Leader-Attenuator Region**—The results of both *in vitro* transcription printing data. In the presence of Lrp, a 30-bp region of DNase I protection from base pair position 220–250 covering the Lrp, consensus-like DNA binding site (base pair position 226–240), was observed. Lrp also protected a contiguous region of the DNA in this region (base pair positions 220–243) from cleavage by hydroxyl radical. These results suggest that the center of the Lrp binding site is coincident with the center of the dyad symmetry of the consensus-like site (Fig. 10). The observation that symmetrically spaced guanine residues on opposite DNA strands in the consensus-like site are protected from alklylation by DMS demonstrates that the homodimeric Lrp molecule forms symmetrical half-site contacts in this palindromic, consensus-like site. Since DMS alkylates the N7 position of guanines in the major groove of the DNA helix the DMS protection patterns also demonstrate that, like other homodimeric helix-turn-helix DNA binding proteins, the Lrp protomer binds in adjacent major grooves on the same face of the DNA helix. Together, these results support the sequence-derived inference that Lrp is a helix-turn-helix DNA binding protein (38).

**Lrp Binds directionally and Cooperatively to Adjacent Non-
assays with purified Lrp protein and in vivo assays of a reporter gene transcriptionally fused to the ilvGMEDA promoter-attenuator region demonstrate that Lrp represses transcription through the leader-attenuator region of the ilvGMEDA operon. However, given the unusual position of the primary Lrp binding site 226 bp downstream from the transcriptional initiation site and 40 bp downstream of the transcriptional termination site of the attenuator at base pair position 186, it is unclear how Lrp affects transcription from the upstream promoter.

i-Leucine Inhibits Lrp-DNA Interactions in the ilvGMEDA Leader Region—Ricca et al. (37) have shown that L-leucine decreases the affinity of Lrp for its consensus-like DNA binding site (site 2) in the ilvIH operon. However, even at high concentrations, i-leucine did not abolish the ability of Lrp to bind to this site. i-Leucine also inhibits but does not abolish Lrp binding to its primary and secondary sites in the leader region of the ilvGMEDA operon (Fig. 1). Given this observation and the results of the in vitro transcription and in vivo reporter construct assays (Fig. 11 and Table I), it is expected that high concentrations of i-leucine would relieve Lrp-mediated repression of ilvGMEDA operon expression.

A Possible Role for Lrp in the Regulation of the ilvGMEDA Operon—During i-leucine starvation, the synthesis of all three of the AHAS isozymes is increased (9). However, the activities of the two isozymes with substrate preferences for l-leucine (and L-valine) biosynthesis (40), AHAS I and AHAS III, are feedback inhibited by l-valine, while the activity of AHAS II, with a substrate preference for l-isoleucine biosynthesis, remains unchecked (41). It is possible that this situation might compromise the abilities of AHAS I and III to compete with AHAS II for pyruvate required for l-isoleucine biosynthesis. If this were the case, then an increase in the rate of pyruvate biosynthesis and a lowering of the expression of the ilvGM genes for AHAS II during i-leucine starvation might be advantageous under these conditions, the expression of the distal genes of the ilvGMEDA operon might be sufficiently sustained by the activity of the internal ilvPG promoter (16)]. Thus, Lrp might function to repress the production of AHAS II during conditions of l-leucine starvation. The results reported here are consistent with such a role for Lrp in the regulation of branched chain amino acid metabolism.

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REFERENCES
1. Calvo, J. M., and Matthews, R. G. (1994) Microbiol. Rev. 58, 466–490
2. Newman, E. B., and Lin, R. (1995) Annu. Rev. Microbiol. 49, 747–775
3. Anderson, J. J., Quay, S. C., and Oxender, D. L. (1976) J. Bacteriol. 126, 80–90
4. Platko, J. V., Willins, D. A., and Calvo, J. M. (1990) J. Bacteriol. 172, 4563–4570
5. Lin, R. T., Ari, R., and Newman, E. B. (1990) J. Bacteriol. 172, 4529–4535
6. Rex, J. H., Aronson, B. D., and Somerville, R. L. (1991) J. Bacteriol. 173, 5944–5952
7. Lin, Y., Ari, R., and Newman, E. B. (1992) J. Bacteriol. 174, 1948–1955
8. Haney, S. A., Platko, J. V., Oxender, D. L., and Calvo, J. M. (1992) J. Bacteriol. 174, 108–115
9. Umbarger, H. E. (1987) in E. coli and Salmonella typhimurium: Cellular and Molecular Biology, pp. 352–357, American Society for Microbiology, Washington, D. C.
10. Wek, R. C., Hauser, C. A., and Hatfield, G. W. (1985) Nucleic Acids Res. 13, 3995–4010
11. Lawther, R. P., Wek, R. C., Lopes, J. M., Pereira, R., Taillon, B. E., and Hatfield, G. W. (1987) Nucleic Acids Res. 15, 2132–2155
12. Cui, Y., Midkiff, M. A., Wang, Q., and Calvo, J. M. (1996) J. Biol. Chem. 271, 6611–6617
13. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989
15. Pagel, J. M., and Hatfield, G. W. (1991) J. Biol. Chem. 266, 1865–1966
16. Wek, R. C., and Hatfield, G. W. (1986) Nucleic Acids Res. 14, 2769–2777
17. Simons, R. W., Houman, F., and Kleckner, N. (1987) Gene (Amst.) 55, 85–96
18. Cole, J. R., and Nomura, M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4129–4133
19. Wek, R. C., and Hatfield, G. W. (1988) J. Mol. Biol. 203, 643–663
20. Miller, J. H. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972
21. Wang, Q., and Calvo, J. M. (1993) J. Mol. Biol. 229, 306–318
22. Senear, D. F., and Brenowitz, M. (1991) J. Biol. Chem. 266, 13661–13671
23. Johnson, M. L., and Frasier, S. G. (1985) Methods Enzymol. 117, 301–342
24. Hildebrand, F. B. Introduction to Numerical Analysis. McGraw-Hill Book Co., New York, 1956
25. Wang, Q., and Calvo, J. M. (1993) EMBO J. 12, 2495–2501
26. Brenowitz, M., Senear, D. F., Shea, M. A., and Ackers, G. K. (1986) Methods Enzymol. 130, 132–181
27. Tallius, T. D., and Dombroski, B. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5469–5474
28. Siedler, U., and Gilbert, W. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 122–127
29. Winkelman, J. W., and Hatfield, G. W. (1990) J. Biol. Chem. 265, 10055–10060
30. Wissmann, A., and Hillen, W. (1981) Methods Enzymol. 69, 365–379
31. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
32. Hauser, C. A., Sharp, J. A., Hatfield, L. K., and Hatfield, G. W. (1985) J. Biol. Chem. 260, 1765–1770
33. Cui, Y., Wang, Q., Stormo, G. D., and Calvo, J. M. (1995) J. Bacteriol. 177, 4872–4880
34. Friedberg, D., Platko, J. V., Tyler, B., and Calvo, J. M. (1995) J. Bacteriol. 177, 1624–1626
35. Fried, M. G., and Crothers, D. M. (1981) Nucleic Acids Res. 9, 6505–6525
36. Lawther, R. P., and Hatfield, G. W. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1862–1866
37. Ricca, E., Aker, D. A., and Calvo, J. M. (1989) J. Bacteriol. 171, 1658–1664
38. Platko, J. V., and Calvo, J. M. (1993) J. Bacteriol. 175, 1110–1117
39. Willins, D. A., Ryan, C. W., Platko, J. V., and Calvo, J. M. (1991) J. Biol. Chem. 266, 10768–10774
40. Barak, Z., Chipman, D. M., and Gollop, N. (1987) J. Bacteriol. 169, 3750–3756
41. Lawther, R. P., Calhoun, D. H., Adams, C. W., Hauser, C. A., Gray, J., and Hatfield, G. W. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 922–925
42. Parekh, B. S., and Hatfield, G. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1173–1177
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Kyu Young Rhee, Bhavin S. Parekh and G. Wesley Hatfield

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