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The *ilvIH* Operon of *Escherichia coli* Is Positively Regulated

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The *ilvIH* operon of *Escherichia coli* (located near min 2) encodes acetohydroxyacid synthase III, an isozyme involved in branched-chain amino acid biosynthesis. A strain with *lacZ* fused to the *ilvIH* promoter was constructed. Transposon Tn*10* was introduced into this strain, and tetracycline-resistant derivatives were screened for those in which *ilvIH* promoter expression was markedly reduced. In one such derivative, strain CV1008, β-galactosidase expression was reduced more than 30-fold. The transposon giving rise to this phenotype inserted near min 20 on the *E. coli* chromosome. Extract from a wild-type strain contains a protein, the IHB protein, that binds to two sites upstream of the *ilvIH* promoter (E. Ricca, D. A. Aker, and J. M. Calvo, J. Bacteriol. 171:1658–1664, 1989). Extract from strain CV1008 lacks IHB-binding activity. These results indicate that the IHB protein is a positive regulator of *ilvIH* operon expression. The gene that encodes the IHB protein, *ihb*, was cloned by complementing the transposon-induced mutation. Definitive evidence that the cloned DNA encodes the IHB protein was provided by determining the sequence of more than 17 amino acids at the N terminus of the IHB protein and comparing it with the nucleotide sequence. A mutation that prevents repression of the *ilvIH* operon by leucine in vivo and that alters the DNA-binding characteristics of the IHB protein in vitro was shown to be an allele of the *ihb* gene. The *ihb* gene is identical to *oppI*, a gene that regulates the *oppABCDF* operon (E. A. Austin, J. C. Andrews, and S. A. Short, Abstr. Mol. Genet. Bacteria Phages, p. 153, 1989). Thus, *oppI/ihb* encodes a protein that regulates both *ilvIH*, an operon that is repressed by leucine, and *oppABCDF*, an operon involved in peptide transport that is induced by leucine. We propose that the designation *lrp* be used in the future instead of *oppI* or *ihb* and that Lrp (leucine-responsive regulatory protein) be used in place of IHB.

The first step common to the biosynthesis of isoleucine (Ile), valine (Val), and leucine (Leu) is catalyzed by acetohydroxyacid synthases (AHAS). In *Escherichia coli*, genes in at least three operons code for distinct AHASs: *ilvBN* (AHAS I), *ilvGM* (AHAS II), and *ilvIH* (AHAS III) (for reviews, see references 11 and 36). The *ilvBN* and *ilvGM* operons are repressed in cells grown in media containing excess branched-chain amino acids and derepressed when Leu and Val (*ilvBN*) or Leu, Val, and Ile (*ilvGM*) are in short supply. This regulation in response to the supply of branched-chain amino acids is mediated by transcription attenuation (1, 14, 19, 26).

Expression of the *ilvIH* operon is repressed in cells growing in a medium containing Leu but apparently not by transcription attenuation (30). Rather, Leu appears to modulate expression of this operon by interfering with the action of a positively acting regulatory protein, the IHB protein (30). The IHB protein is a 43-kilodalton protein composed of two subunits of identical sizes. In vitro, the IHB protein binds to two regions upstream of the *ilvIH* promoter, one located between −260 and −190 and the other between −80 and −60 (30). Two pieces of evidence suggest that Leu-mediated repression acts through the IHB protein. (i) Binding of the IHB protein to the promoter/regulatory region in vitro is reduced by Leu but not by Ile, Val, or threonine (30). (ii) The *lrs-1* mutation, which prevents Leu-mediated repression of the *ilvIH* operon (37), affects the characteristics of IHB binding to DNA and eliminates the in vitro effect of Leu upon binding (30). The simplest model consistent with these data is that the IHB protein stimulates transcription from the *ilvIH* promoter and that Leu represses expression by interfering with the action of the IHB protein.

The work reported here supports one critical feature of the above-mentioned model, namely, that the IHB protein is an activator of *ilvIH* operon expression. A strain of *E. coli* containing a transposon in *ihb*, the gene that encodes the IHB protein, was isolated. Expression of the *ilvIH* operon in this strain was only 1/30th of that in the parent strain. In addition, the *ihb* gene was cloned through its ability to complement the *ihb*::Tn*10* mutation. The *ihb* gene is identical to *oppI*, a gene that negatively regulates the *oppABCDF* operon (E. A. Austin, J. C. Andrews, and S. A. Short, Abstr. Mol. Genet. Bacteria Phages, p. 153, 1989). The latter operon, which is involved with uptake of oligopeptides in *E. coli* and *Salmonella typhimurium* (4, 20, 23), is induced by Leu in *E. coli* (3, 5). Thus, the IHB protein apparently activates *ilvIH* expression and represses *oppABCDF* expression.

MATERIALS AND METHODS

Strains. The characteristics of the *E. coli* K-12 strains used are described in Table 1.

Media and growth conditions. Minimal medium contained SSA salts (18) supplemented with 5 μg of thiamine per ml 50 μg of proline per ml, 50 μg of Val per ml, 25 μg of Ile per ml, micronutrients (31), and 0.2% glucose. Media sometimes contained kanamycin (50 μg/ml), ampicillin (50 μg/ml), spectinomycin (50 μg/ml), tetracycline (20 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/ml; X-Gal), and Leu (100 μg/ml unless otherwise specified). In some experiments, minimal medium contained 0.2% lactose instead of glucose. LB was used as a rich medium (25). Strains were grown at 37°C, with the exception of strain LR16 and Mu *cts*-containing strains, which were grown at 30°C.

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TABLE 1. *E. coli* K-12 strains used in this study

Strain	Genotype	Source
W3102	F⁻ *galK2*	
LR16	HfrH *thi-1 glyA bglR20 ilvB619 lrs-1*	M. DeFelice
CSH26	F⁻ *ara thi* Δ(*lac-pro*)	J. H. Miller
VJS697	Mu dI1734 *recA*::*cam*	V. J. Stewart
VJS803	*recB21 recC22 sbcB15 sbcC201 argE3 his4 leuB6 proA2 thr-1 ara-14 galK2 mtl-1 xyl-5 thi-1 rpsL31 supE44 tsx-33* Δ(*argF-lac*)*U169* Δ(*trpEA*)*2*	V. J. Stewart
CV975	F⁻ *ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734	This study
CV976	F⁻*ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734 *lrs-1*	This study
CV1008	F⁻ *ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734 *ihb*::Tn*10*	This study
CV1009	F⁻ *ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734 *ihb*::Tn*10*(pCV168, *ihb*⁺)	This study
CV1010	F⁻ *ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734 *zca*::Tn*10*	This study
CV1011	F⁻ *ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734 *lrs-1 zca*::Tn*10*	This study
CV1012	F⁻ *ara thi* Δ(*lac-pro*) Mu *cts*(pCV170)	This study
CV1013	F⁻*ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734 *ihb*::Tn*10* Mu *cts*	This study
CV1014	F⁻*ara thi* Δ(*lac-pro*) *ilvIH*::Mu dI1734 (pCV168, *ihb*⁺)	This study

Genetic and recombinant DNA methods. P1-mediated transductions were performed as described by Miller (25). Transformations were carried out as indicated by Chung et al. (10), except that cells were grown to an A_{550} of 0.3 to 0.4 and a heat shock step (2 min at 45°C, followed by 2 min at 4°C) was included before addition of LB medium. Plasmid DNA isolations and ligations were done as described by Maniatis et al. (24). Nick translation was performed with a kit from Bethesda Research Laboratories as recommended by the manufacturer.

Members of the Kohara library (22) were screened by hybridization by spotting samples on a Hybond-N nylon membrane (Amersham Corp.) and incubating the membrane with nick-translated plasmid pCV168. Hybridization and washing conditions were those recommended by Amersham. Kohara library members 213, 214, 215, and 216 were grown on host CSH26, DNA was isolated from each (6), and samples cut with *Pvu*II were subjected to Southern blotting analysis (24) with nick-translated plasmid pCV168 as the probe.

The polymerase chain reaction (PCR) (32) was carried out with Taq DNA polymerase from New England BioLabs and the buffer they recommended, by using 0.5 μM primers, 0.3 μg of bacterial DNA (isolated as described in reference 7), and 50 μM deoxynucleoside triphosphates in a total volume of 100 μl. Twenty-four cycles of amplification were performed, a cycle being 1 min at 92°C, 2 min at 42°C, and 1.5 min at 70°C. The primers used were 5′ GGAAGGAATTCA GAAGGACAATA 3′ and 5′ TTGCACCGGATCCGTGT TAGC 3′.

DNA sequencing. Single-stranded DNA corresponding to plasmid pCV171 was isolated from strain NM522(pCV171) after infection with helper bacteriophage VCSM13 as described by Stratagene. This DNA was used as the template for dideoxy sequencing analysis (33) (Sequenase kit from U.S. Biochemicals) with a synthetic mixture of 15-mer oligonucleotides as primers.

Construction of strains and plasmids. Strain CV975, containing the *ilvIH* promoter fused to *lacZ*, was constructed as described by Groisman and Casadaban (15). Plasmid pCV7 (contains promoter, *ilvI*, and part of *ilvH*) (35) was introduced into strain VJS697 (Mu dI1734) by transformation. Phage derived from this strain following heat induction were used to transduce strain CSH26 [Δ(*lac-pro*)] with selection for growth on LB plates containing ampicillin, kanamycin, and X-Gal. Plasmids isolated from blue colonies were subjected to restriction analysis, and one which contained the Mu insert within the first 300 nucleotides of the *ilvI* structural gene and in the correct orientation for a transcriptional fusion was chosen. Φ(*ilvI-lacZ*) was transferred into the chromosome of strain VJS803 (*recBC sbc*) by recombination as described by Winans et al. (38) (selection for Kan^r) and then into the chromosome of strain CSH26 by P1 transduction. Transductional analysis of the resulting strain, CV975, verified that Φ(*ilvI-lacZ*) was located in the expected position in the chromosome, near *leu*.

Strains CV1008, CV1010, and CV1011 were isolated as follows. P1 phage grown on derivatives of strain VJS961 containing transposon Tn*10* inserted at random positions in the chromosome (a gift of V. Stewart) were mixed with recipient CV976 [Φ(*ilvI-lacZ*) *lrs-1*] and plated on minimal agar plates containing tetracycline, kanamycin, Leu, and X-Gal. Of some 600 transductants analyzed, about 99% were dark blue, about 1% were light blue (one such colony was named CV1010), and one was white (named CV1008). P1 phage grown on CV1010 were mixed with recipient CV976 and plated on minimal agar plates containing tetracycline, X-Gal, and Leu. One dark blue transductant was named CV1011.

Plasmid pCV170 was constructed by replacing the *kan* gene on plasmid pEG5005 (bounded by *Eco*RI sites) (15) with the spectinomycin resistance-encoding gene from plasmid pVJS983 (also bounded by *Eco*RI sites). Strain CV1012 (Mu *cts* pCV170) was heat induced (15), the resulting phage were mixed with cells of strain CV1013 [Φ(*ilvI-lacZ*) *ihb*::Tn*10* Mu *cts*], and transductants were selected on minimal agar plates containing spectinomycin and tetracycline with lactose as the sole carbon source. Plasmid pCV169, carrying 15 kilobases (kb) of bacterial DNA, was isolated from one of these transductants. Plasmid pCV168 (5.5 kb) was constructed by isolating an *Eco*RI-*Hin*dIII fragment from pCV169 and cloning it into the *Eco*RI-*Hin*dIII site of plasmid pBluescript II SK (−) (Stratagene). Plasmid pCV171 (4.1 kb) was prepared from pCV168 by cutting the latter with *Sac*I and *Eco*RI, treating it sequentially with exonuclease III and mung bean exonuclease, and performing blunt-end ligation as described by Stratagene.

Assays. Assays for β-galactosidase activity were performed as follows. Cells were made permeable by mixing them with an equal volume of buffer containing cetyl trimethylammonium bromide (200 μg/ml) and sodium deoxycholate (100 μg/ml) and placing them in a refrigerator overnight. The buffer was that described by Miller (25), except that it lacked KCl and contained 0.2 mM $MnSO_4$. Permeable cells (0.5 ml) were incubated with 0.15 ml of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml) at 28°C, and A_{420} and A_{550} were measured after addition of 0.325 ml of 1 M Na_2CO_3. Absorbance due to *o*-nitrophenol was calculated as $A_{420} - (1.65 \times A_{550})$. One unit of activity was defined as 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min, assuming a molar extinction coefficient of *o*-nitrophenol of 5,000. Cell density was estimated on the basis of a standard curve relating the A_{550}s of different dilutions of a culture to

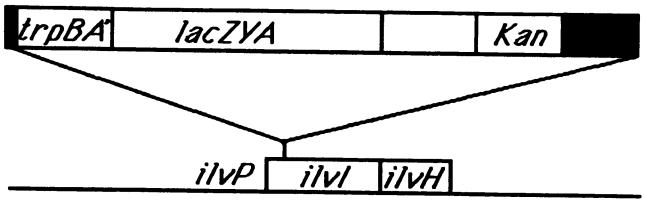

FIG. 1. Schematic representation of the *ilvI-lacZ* transcriptional fusion. Solid regions indicate Mu sequences. *Kan*, Kanamycin.

the dilution factor (Zeiss PM6 spectrophotometer). For data plotted as units of β-galactosidase per milliliter versus cell density, a cell density of 1 is approximately 2×10^9/ml.

Gel retardation assays for IHB protein were performed as described by Ricca et al. (30). For preparation of small amounts of crude extract, the cells in 1.5-ml cultures grown in minimal medium were harvested by centrifugation and the pellets were frozen at −70°C. After 10 min, samples were placed on ice and suspended in 50 μl of a solution containing 50 mM Tris chloride (pH 8), 100 mM EDTA, and 165 μg of lysozyme. Samples were incubated on ice for 5 min, at 37°C for 5 min, and then on ice for 5 min. After addition of 50 μl of a solution containing 50 mM Tris chloride (pH 8), 60 mM EDTA, and 1% Triton X-100, samples were mixed and incubated on ice for 10 min. The samples were microcentrifuged for 15 min, and the supernatant fluid was used in gel retardation assays.

Purification of IHB protein. IHB protein was purified from strain BL15 (kindly supplied by W. Studier) as described previously (30), by starting from extracts prepared with a French press. The Sephadex chromatography step was omitted, and three extra chromatographic steps were added, i.e., chromatography on herparin-agarose, on DNA-cellulose, and on octyl-Sepharose. The final product was at least 98% pure as judged by silver staining of a sodium dodecyl sulfate-polyacrylamide gel. N-terminal amino acid analysis by the Edman degradation procedure was performed on a purified sample of IHB protein by B. J. Krieger and J. Schloss.

RESULTS

A major aim of the work described here, to isolate and characterize a strain with a null mutation in the *ihb* gene, was facilitated by constructing a strain with a *lacZ* gene fused to the *ilvIH* promoter. We expected that a strain with an inactive *ihb* gene would have reduced expression of the *ilvIH* operon (30). In a wild-type strain of *E. coli* K-12 containing both *ilvBN* (codes for AHASI) and *ilvIH* (codes for AHAS III), reduction in the expression of *ilvIH* was not expected to have an easily measured phenotype because *ilvBN* by itself provides sufficient AHAS activity to support growth of cells (16). We could have started with a strain lacking a functional *ilvB* gene, but such strains are mucoid and are poor donors and recipients in genetic crosses (37). An *ilvI-lacZ* fusion provided a way of measuring expression from the *ilvIH* promoter in a wild-type strain and also a way of selecting for *ilvIH* promoter expression (by ability to utilize lactose as a carbon source).

Construction of an *ilvI-lacZ* fusion. Strain CV975, containing *lacZ* fused to a part of the *ilvIH* operon, was constructed by the Mu d technology of Groisman and Casadaban (15). Fusion to *lacZ* occurred within the first 300 nucleotides of the *ilvI* coding region (Fig. 1).

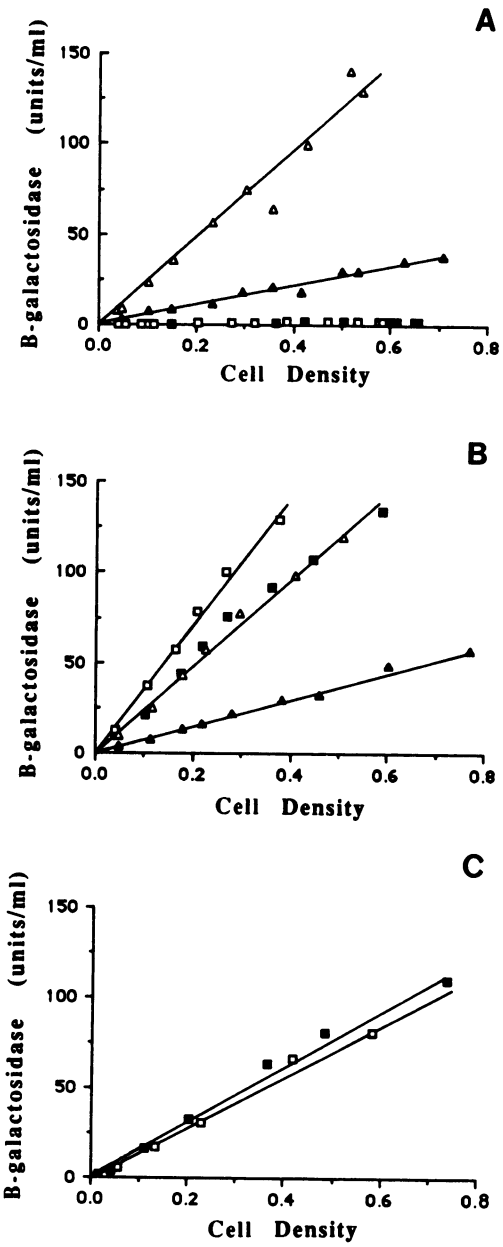

FIG. 2. Differential rates of β-galactosidase synthesis in some strains containing an *ilvI-lacZ* fusion. Cells were grown in minimal medium either lacking (open symbols) or containing (filled symbols) 100 μg of Leu per ml. (A) Triangles, CV975 (*ihb*$^+$); squares, CV1008 (*ihb*::Tn*10*). (B) Triangles, CV975; squares, CV1009 [*ihb*::Tn*10* (pCV168) *ihb*$^+$]. (C) CV976 (*lrs-1*).

The *ilvIH* operon is repressed 5- to 10-fold when wild-type cells are grown in a medium containing Leu (12, 35). As measured by β-galactosidase assays, expression from the *ilvIH* promoter in strain CV975 is similarly repressed (Fig. 2A). This Leu effect can be scored on minimal agar plates containing X-Gal; colonies of strain CV975 are light blue on plates containing Leu and dark blue on plates lacking Leu. Furthermore, strain CV975 grows on minimal agar plates containing lactose as the sole carbon source but not on the same medium containing Leu. These phenotypes are summarized in Table 2.

TABLE 2. Phenotypes of selected *E. coli* K-12 strains used in this study

Strain (relevant genotype)	Color on X-Gal		Growth on lactose	
	Without Leu	With Leu	Without Leu	With Leu
CV975 (wild type)	Dark blue	Light blue	Yes	No
CV976 (*lrs-1*)	Dark blue	Dark blue	Yes	Yes
CV1008 (*ihb*::Tn*10*)	White	White	No	No
CV1009 (*ihb*::Tn*10* pCV168, *ihb*$^+$)	Dark blue	Dark blue	Yes	Yes

Insertional inactivation of the *ihb* gene. A strain containing a transposon inserted in the *ihb* gene was isolated as follows. P1 phage grown on derivatives of strain VJS961 containing transposon Tn*10* inserted at random positions in the chromosome (a gift of V. Stewart) were mixed with strain CV976 [Φ(*ilvI-lacZ*) *lrs-1*], and transductants resistant to tetracycline (a property of Tn*10*) and kanamycin (a property of the Mu d phage that created the *ilvI-lacZ* fusion) were selected. The selective plates also contained X-Gal to assess expression from the *ilvIH* promoter. Because of the *lrs-1* mutation in the recipient (described in detail later), most transductants were dark blue. One transductant, however, was white. An extract prepared from this exceptional transductant (strain CV1008) did not retard electrophoretic migration of a DNA fragment carrying the *ilvIH* promoter/regulatory region (Fig. 3A). Transduction studies established that the *tet* gene associated with the transposon was tightly linked to the white colony phenotype (300 Tet^r transductants derived from recipient CV976 and phage grown on CV1008 were all white on X-Gal).

To determine quantitatively the effect of the insertion mutation upon *ilvIH* operon expression, we measured β-galactosidase activity in cells of the parent and mutant made permeable by treatment with a detergent. Such activity in the mutant was only 1/30th of that in the parent (Fig. 2A). The residual β-galactosidase activity in extracts of strain CV1008 was not reduced by growing the strain in medium containing 100 μg of Leu per ml. Primer extension experiments indicated that *ilvIH* mRNA levels in strain CV1008 were less than 1/20th of those in the wild type (data not shown).

The phenotypes of strain CV1008 are those expected of a strain with a Tn*10* insertion within a gene whose expression is needed for *ilvIH* operon expression. We show later that the transposon insertion in strain CV1008 is indeed within the *ihb* gene rather than in some other gene whose expression is required for *ihb* gene expression.

Cloning of the *ihb* gene. The *ihb* gene was cloned by complementing the Lac$^-$ phenotype of strain CV1013 [*ihb*::Tn*10* Φ(*ilvI-lacZ*) Mu *c*ts]. We used mini-Mu derivatives and the in vivo cloning strategy of Groisman and Casadaban (15) to clone the gene. In brief, a mini-Mu-containing plasmid was transferred to a Mu *c*ts lysogen carrying the wild-type *ihb* gene. Some of the phage formed after induction of Mu growth contained the *ihb* gene flanked by mini-Mu elements. Phage were mixed with strain CV1013 [Φ(*ilvI-lacZ*) *ihb*::Tn*10* Mu *c*ts], and transductants were selected for growth with lactose as the sole carbon source and resistance to spectinomycin (carried by the mini-Mu) and tetracycline (carried by Tn*10*). In this cloning procedure, the cloned DNA (in this case, the DNA that complements the Lac$^-$ phenotype of the recipient) ends up as part of a plasmid created by recombination between two mini-Mu elements (15). Each of seven colonies arising in this selection had plasmid DNA containing an insert of bacterial DNA ranging in size from 10 to 15 kb. Furthermore, in contrast to an extract from parent strain CV1013, which showed no IHB-binding activity, extracts from these isolates had IHB-binding activity. The complementing activity of one of these plasmids, pCV169, was localized first to a 2.6-kb *Hin*dIII-*Eco*RI fragment by subcloning (yielding plasmid pCV168) and then to a 1.2-kb fragment by exonuclease-promoted deletion (Fig. 4).

The following results suggest that the cloned DNA contains the *ihb* gene. Strains CV1009 [Φ(*ilvI-lacZ*) *ihb*::Tn*10*(pCV168)] and CV1014 [Φ(*ilvI-lacZ*)(pCV168)] contain the plasmid with the 2.6-kb insert. An analysis of IHB-binding activity in extracts of these strains and of parent strains lacking the plasmid is shown in Fig. 3. The presence of the plasmid in a strain containing *ihb*::Tn*10* restored IHB activity to extracts (Fig. 3B). Furthermore, the amount of IHB activity was increased in plasmid-containing strains at least 50-fold above the level in a haploid strain (Fig. 3C). In addition, the plasmid-promoted activity was reduced by including Leu in the binding buffer, as was the activity encoded by the single chromosomal gene (Fig. 3D).

To determine the effect of elevated IHB levels on *ilvIH* operon expression, β-galactosidase levels were measured in strain CV1009 [*ihb*::Tn*10* Φ(*ilvI-lacZ*)(pCV168)]. A more-than-50-fold increase in IHB protein level led to only an approximately 30% increase in *ilvIH* promoter expression (Fig. 2B). These results indicate that the amount of IHB protein in a wild-type strain is sufficient to give nearly maximal expression from the *ilvIH* promoter. Addition of Leu to the growth medium of the plasmid-containing strain reduced expression from the *ilvIH* promoter about 50% (Fig. 2B). Note that in a similar experiment with a strain containing a single copy of the *ihb* gene, expression was reduced more than fourfold (Fig. 2B). Thus, repression by Leu can be at least partially overcome by an increased concentration of IHB protein.

Definitive evidence that the cloned DNA contains the *ihb* gene was provided by the following experiment. The IHB

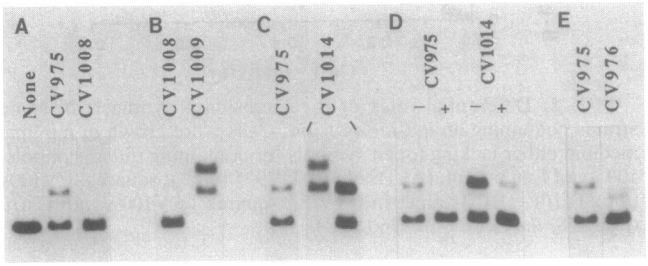

FIG. 3. Gel retardation assays for IHB protein. Crude extract (1 μl) from each of the indicated strains was mixed with a 406-base-pair ^{32}P-labeled fragment of DNA containing the *ilvIH* promoter/regulatory region, and the sample was fractionated by electrophoresis through agarose. When migration of the labeled DNA fragment was retarded because of bound wild-type protein, it migrated with an apparent size of 490 base pairs (protein bound to the upstream binding site) and/or 540 base pairs (protein bound to both upstream and downstream binding sites) (30) (see panel B for an example). In panel C, one sample contained undiluted extract from strain CV1014 whereas the other contained a 50-fold dilution of the same extract. In panel D, 50-fold-diluted extract from CV1014 was used for both samples; minus and plus refer to the absence or presence, respectively, of 50 mM Leu in the binding buffer.

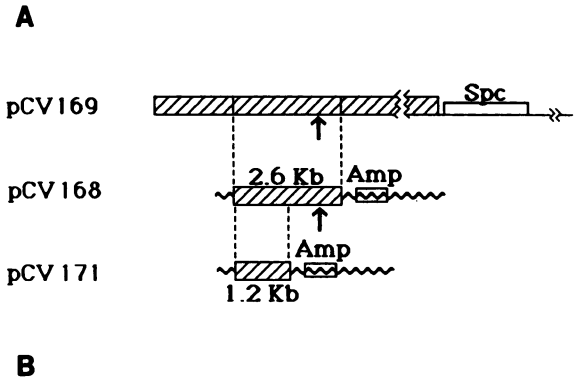

FIG. 4. (A) Schematic representation of some *ihb*-containing plasmids used in this study. Hatched regions represent cloned bacterial DNA, and wavy lines indicate vector sequences. The arrow represents a *Pvu*II site that is similarly emphasized in Fig. 5B. (B) Sequence of amino acids at the N terminus of the IHB protein determined by Edman degradation analysis. The N-terminal amino acid was Val. A mixture of 15-mer oligonucleotides that could code for the five amino acids underlined was synthesized. Use of these oligonucleotides as primers during dideoxy sequencing gave a nucleotide sequence which would code for the amino acids overlined. Spc, Spectinomycin; Amp, ampicillin.

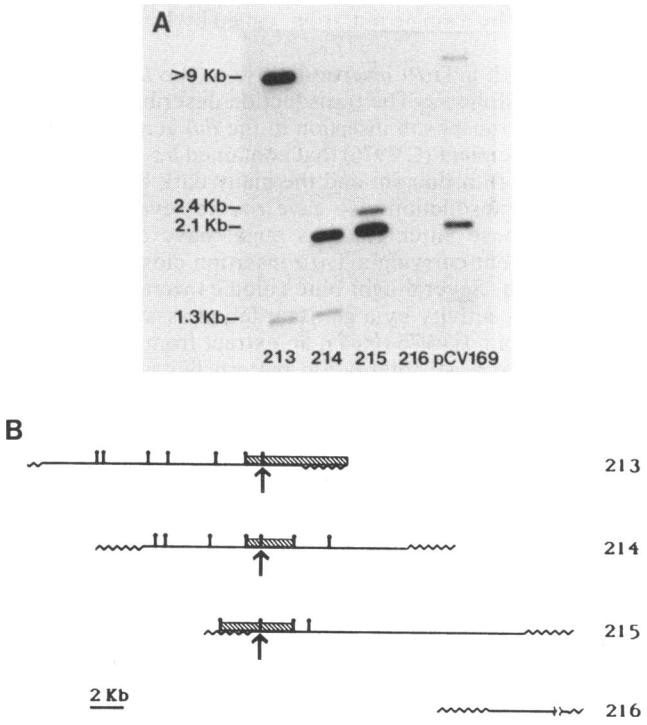

FIG. 5. (A) Southern blot analysis of Kohara phage library members 213, 214, 215, and 216 cut with *Pvu*II. Nick-translated plasmid pCV168 (*ihb*$^+$) was the probe. (B) Schematic representation of phages analyzed by Southern blotting. Vertical lines, *Pvu*II sites (22); hatched regions, *Pvu*II-generated fragments that hybridized with probe pCV168; horizontal lines, bacterial DNA; wavy lines, phage lambda DNA (not drawn to scale). The vertical arrows identify the *Pvu*II site present in the bacterial DNA carried on plasmid pCV168 (see Fig. 4A).

protein was purified to near homogeneity, and the sequence of 38 amino acids at the N-terminal end was determined. A mixture of 256 oligonucleotides that represent all of the oligonucleotides that could code for the five amino acids underlined in Fig. 4B was synthesized. These oligonucleotides were used as primers in dideoxy sequencing with DNA from single-stranded pCV171 as the template. A nucleotide sequence that corresponded exactly to the 17 amino acids identified in Fig. 4B by the wavy overline was obtained.

Map location of the *ihb* gene. While this work was in progress, we learned that the *oppI* gene, which lies near min 20 (Austin et al., Abstr. Mol. Genet. Bacteria Phages), is very likely identical to the *ihb* gene. The following two experiments confirmed that the *ihb* genes lies near min 20. The 2.6-kb fragment from plasmid pCV168 containing the *ihb* gene was labeled by nick translation and used as a probe to screen the library of *E. coli* fragments described by Kohara et al. (22). Three lambda phage clones hybridized to the probe: isolates 213, 214, and 215 (Fig. 5). These three phage clones share a region of bacterial DNA of about 2.6 kb (Fig. 5). The bacterial DNA in these clones is located near min 20 (22).

A transduction cross was carried out between recipient CGSC1321 (*aroA*, min 20) and phage P1 grown on strain CV1008 (*ihb*::Tn*10*). Among 35 transductants selected for Aro$^+$, 15 were Tet^r. This latter result confirms that the Tn*10* that inactivates expression of the *ihb* gene is closely linked to *aroA*.

A mutation that affects repression of *ilvIH* is an allele of the *ihb* gene. Ursini et al. isolated a mutant from a Leu^s strain of *E. coli* that grew in a medium supplemented with Leu. In this mutant strain, LR16, the *ilvIH* operon was not repressed by Leu, whereas it was in the parent strain (37). We found that IHB activity in an extract from strain LR16 behaved differently from that of a wild-type strain in the following ways. As judged by a gel retardation assay, the IHB protein in extracts of strain LR16 bound to the *ilvIH* promoter/regulatory region but the band pattern was different from that obtained with protein from the parent strain (30). Also, with protein from the mutant, binding was not reduced by addition of Leu to the binding buffer (30).

It was not clear whether the phenotypes of strain LR16 (*ilvIH* operon not repressed by Leu; altered binding characteristics of IHB protein) were caused by a single mutation. To investigate this question, P1 phage grown on strain LR16 were mixed with strain CV975 and transductants were selected for growth on minimal agar plates containing lactose as the sole carbon source and Leu. All 30 transductants analyzed had the same characteristics, those of the donor strain. On plates containing X-Gal, the transductants were blue, both for media lacking Leu and for those containing Leu. The results of a more quantitative analysis of this phenomenon are shown in Fig. 2C for one of the transductants, strain CV976. The *ilvIH* operon in this transductant was not repressed by Leu. Furthermore, the characteristics of the IHB protein in these transductants, as measured by a gel retardation experiment, were those of donor strain LR16 (Fig. 3E; results shown for CV976). Note that the mobility of the protein-DNA complex formed by using an extract from CV976 was higher than that formed by using an extract from CV975. Furthermore, Leu did not reduce the extent of binding when a CV976 extract was analyzed (data not shown). Thus, the phenotypes associated with the LR16 strain are caused by a single mutation or by several very closely linked mutations. We assume that the phenotypes

were caused by a single mutation, called by Ursini et al. *lrs-1* (37).

Strains with a Tn*10* insertion close to the *lrs-1* site were identified as follows. The transduction described above that yielded the transposon insertion in the *ihb* gene was carried out with a recipient (CV976) that contained *lrs-1*. Besides the single white transductant and the many dark blue transductants, this transduction also gave rise to several light blue colonies. These latter colonies might have arisen from a donor fragment carrying a Tn*10* insertion close to the wild-type *lrs* gene. Several light blue colonies were analyzed for IHB binding activity by a gel retardation assay. In contrast to parent strain CV976 (*lrs-1*), an extract from each of these colonies gave a gel retardation pattern that was similar to that shown by wild-type IHB protein. To confirm the linkage between Tn*10* and *lrs*, one of these isolates (CV1010) was used as a P1 phage donor with recipient CV976 (*lrs-1*). Among 544 Tet^r transductants, 84% had the donor Lrs^+ phenotype (light blue colonies on plates containing X-Gal and Leu). One *lrs-1*-containing transductant from this cross was called CV1011.

To test whether *lrs-1* is located in the min 20 region, recipient CGSC1321 (*aroA*) was crossed with phage grown on strain CV1011 (*zca*::Tn*10* *lrs-1*). Among 38 colonies selected as Aro^+, 20 were Tet^r. These results establish that *lrs-1* lies in the min 20 region rather than in the min 2 region as previously reported (37).

The *lrs-1* mutation could be an allele of the *ihb* gene, or it could be a mutation in a closely linked gene that in some way causes the IHB protein to be modified. To distinguish between these two possibilities, we performed the following experiment. Fragments of DNA corresponding to the ends of the *ihb* gene were synthesized by the PCR (32), with DNA from a wild-type strain as the template and, separately, DNA from an *lrs-1*-carrying strain. Primers were selected on the basis of the nucleotide sequence of the *oppI* gene (kindly provided by E. Austin and S. Short). Gel-purified products from the PCR were cloned into vector pBluescript SK (−) and transformed into strain CV1008 (*ihb*::Tn*10*). Eight transformants derived from the lrs^+ PCR product showed a band shift pattern characteristic of lrs^+-carrying strains, whereas eight transformants derived from the *lrs-1* PCR product showed a band shift pattern characteristic of *lrs-1*-carrying strains (data very similar to those shown in Fig. 3E). These results prove that the *lrs-1* mutation is located within the *ihb* gene between the two primers used for PCR amplification.

PCR was also used to investigate the location of the Tn*10* insertion that eliminated *ihb* expression in strain CV1008. With the primers described above, a PCR product of the correct size was not produced when genomic DNA from strain CV1008 (*ihb*::Tn*10*) was used as the template (data not shown). An internal control involving primers homologous to another region of the chromosome demonstrated that the template DNA was active and that amplification of DNA had occurred in the reaction tube. Southern blotting analysis of DNAs from strains CV975 (ihb^+) and CV1008 (*ihb*::Tn*10*) independently confirmed that the Tn*10* insertion that eliminated IHB binding activity is located within the *ihb* gene rather than within some closely linked gene (data not shown).

DISCUSSION

Expression of the *ilvIH* operon of *E. coli* is repressed by Leu. Our earlier studies, summarized below, suggested that this repression is mediated by a 43-kilodalton protein termed the IHB protein. We demonstrated that the IHB protein binds to two sites upstream of the *ilvIH* promoter and that this binding is reduced by Leu (30). A similar analysis of an *lrs-1*-containing strain (*ilvIH* operon not inhibited by Leu) provided a connection between the IHB protein and repression. The IHB protein in an *lrs-1*-containing strain is altered in two ways: it gives a different pattern of bands in a gel retardation assay and its binding to the *ilvIH* promoter/regulatory region is not reduced by Leu (30). Furthermore, in vitro mutagenesis experiments indicated that at least one of the IHB binding sites is necessary for transcription from the *ilvIH* promoter (Q. Wang and J. M. Calvo, unpublished data). On the basis of these results, we postulated a simple model for regulation of this system, namely, that the IHB protein is a positively acting regulator of the *ilvIH* promoter and that Leu represses expression by interfering with the action of the IHB protein (30). The results of this study, summarized below, support key features of this model.

Strains CV1008 lacks significant *ilvIH* operon expression because of a Tn*10* insertion located near min 20 on the *E. coli* chromosome. Extracts of strain CV1008 lacked IHB protein binding activity, and such activity was restored when the cloned *ihb* gene was introduced into the strain. These results indicate that the Tn*10* transposon inserted into the *ihb* gene or into a closely linked gene that affected *ihb* expression or IHB function. We demonstrated directly by the PCR and by Southern blotting that the insertion was located within the coding region of the *ihb* gene. Taken together, these results strongly support the idea that the IHB protein positively regulates *ilvIH* operon expression.

Another feature of our model is that repression of *ilvIH* operon expression caused by Leu is mediated by the IHB protein (30). Here we present two lines of evidence supporting this aspect of the model. (i) We showed that the residual *ilvIH* promoter activity in a strain containing an *ihb*::Tn*10* mutation is not affected by Leu. (ii) We demonstrated that *lrs-1* is an allele of the *ihb* gene. Thus, a mutation that alters the amino acid sequence of the IHB protein prevents repression by Leu. The *lrs-1* mutation could conceivably affect the affinity of the IHB protein for Leu or it could affect the affinity of an IHB-Leu complex for one or several DNA binding sites. Whether either of these two possibilities is correct remains to be established.

The *ihb* gene was cloned by a strategy that involved complementing the phenotype caused by the *ihb*::Tn*10* mutation. Proof that the cloned DNA corresponds to the *ihb* gene was provided by demonstrating that it encodes more than 17 amino acids at the N terminus of the IHB protein. This same sequence of amino acids is encoded by *oppI*, a regulatory gene that lies near min 20 on the *E. coli* map (Austin et al., Abstr. Mol. Genet. Bacteria Phages). Our nucleotide sequencing studies confirmed that *ihb* and *oppI* are the same gene (data not shown). The *oppI* gene in *E. coli* negatively regulates the expression of *oppABCDF*, one of several operons in *E. coli* that are required for transport of peptides across the inner membrane (4; Austin et al., Abstr. Mol. Genet. Bacteria Phages). The *oppABCDF*-encoded transport system, which transports primarily tripeptides containing a wide variety of amino acids (4), is induced by growing cells in the presence of Leu or Ala or by growing cells anaerobically (3, 5). One possibility consistent with all of these results is that the IHB protein is a positive regulator of *ilvIH* operon expression and a negative regulator of *oppABCDF* expression. By this interpretation, Leu represses *ilvIH* expression by preventing the IHB protein from activating the *ilvIH* promoter and activates *oppABCDF*

expression by preventing the IHB protein from repressing the latter operon.

The most recent genetic map of *E. coli* lists several loci near min 20 that are conceivably related to *ihb/oppI*. Mutations in *leuK*, originally determined to be near min 19.5 (8, 9), lead to elevated levels of enzymes involved in branched-chain amino acid biosynthesis. However, more recent data indicate that *leuK* mutations are really alleles of *hisT*, a locus lying near min 50 (34). Two loci that affect the regulation of systems involved in branched-chain amino acid transport are located near min 20 and are closely linked to *aroA*: *livR* (affects the LIV system involved in transport of Leu, Ile, and Val), and *lstR* (affects the Leu-specific transport system) (2). The relationship among *livR*, *lstR*, and *ihb/oppI* is being investigated.

Since it is clear that *ihb/oppI* is involved in the regulation of a number of operons with diverse functions, it seems appropriate to choose a locus designation that more accurately reflects this fact. We suggest that the designation *lrp* be used in the future in place of *ihb* or *oppI*, that the designation Lrp (leucine-responsive regulatory protein) be used in place of IHB, and that *lrp-1* be used as an allele designation in place of *lrs-1*. It may be noted that the designation *lrp* was chosen with some heuristic intent. There are a number of examples in the literature in which Leu plays a regulatory role. In some of these cases, there is a clear functional relationship to Leu, for example, the fact that Leu represses expression of genes involved in Leu transport (29). In some other cases, Leu has an effect on the regulation of genes, but for reasons that are not immediately apparent. For example, L-serine deaminase (21, 28), L-threonine dehydrogenase (27), and a transport system for L-serine (17) are induced in *E. coli* by Leu. It has even been suggested that Leu acts as a general regulatory molecule, serving to alert cells that it and, probably, other amino acids are available in the medium (13, 29). It will be of interest to determine whether Leu-related regulation of these other systems operates through the *lrp* gene.

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ADDENDUM IN PROOF

M. Levinthal and R. Somerville, E. Newman, and D. Oxender and their co-workers have isolated mutants that have properties similar to those of the *lrp* mutants described here. By mutual agreement, the locus mapping near *aroA* that affects expression of the *sdaA*, *serA*, *tdh*, and *ilvIH* operons is assigned the designation *lrp* (leucine-responsive regulatory protein).

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