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Physical Characterization of the *ilvHI* Operon of *Escherichia coli* K-12

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The *ilvHI* and *leu* genes of *Escherichia coli* K-12 are contained on a single 10.9-kilobase *Eco*RI fragment of deoxyribonucleic acid derived from the *leu* transducing phage λG4. Since the expression of all of these genes is controlled by leucine, we investigated whether they are part of single operon or whether they constitute separate but adjacent operons controlled from a common site. Both cloning and hybridization studies indicated that *ilvHI* and *leu* are distinct operons. They are transcribed in opposite directions and are separated by approximately 1,500 base pairs of deoxyribonucleic acid. Hybridization experiments showed that the expression of *ilvHI* is regulated chiefly at the level of transcription. The size of the *ilvHI* messenger ribonucleic acid is estimated to be 2,550 bases.

Escherichia coli K-12 possesses the genetic potential to express three acetohydroxy acid synthase (AHAS) isoenzymes (18, 19). Each of these isoenzymes (in mutants lacking the other two activities) can catalyze the first biosynthetic step common to isoleucine, valine, and leucine biosynthesis (Fig. 1). The properties of each isoenzyme and the mechanisms by which their synthesis is regulated are not well understood. Such information is crucial for an understanding of the roles that each isoenzyme plays in regulating the flow of carbon through the branched-chain amino acid pathways.

The three structural genes for these isoenzymes are *ilvB* (AHAS I), *ilvG* (AHAS II), and *ilvHI* (AHAS III). In vitro complementation studies (10) have suggested that AHAS III is composed of regulatory (*ilvH*) and catalytic (*ilvI*) subunits. The growth of *E. coli* strain K-12 is sensitive to valine, and both AHAS I and AHAS III are valine sensitive, whereas AHAS II is valine resistant (21). Also, a strain lacking both AHAS I and AHAS III requires isoleucine and valine for growth (15). These observations have suggested that *ilvG* (AHAS II) is a cryptic gene. Some valine resistant derivatives of *E. coli* K-12, formerly called *ilvO* mutants (28), express a functional AHAS II (16) and contain frameshift mutations within *ilvG* that allow translation of a complete AHAS II product (22) and increase the transcription of the downstream *ilvE, ilvD* and *ilvA* genes. Evidence has recently appeared, however, which suggests that the truncated *ilvG* product is functional, perhaps as part of an AHAS isoenzyme complex in wild-type *E. coli* K-12 (4).

The synthesis of AHAS III is affected by leucine, but not by isoleucine or valine (12, 21). Addition of leucine to the growth medium of a strain lacking AHAS I (*ilvB*) causes such a severe repression of AHAS III synthesis that the growth of the strain is inhibited. This observation is interesting in view of the fact that the *ilvHI* and *leu* operons are very closely linked (10, 11). We considered the possibility that *ilvHI* and *leu* are part of the same transcriptional unit or that they are transcribed bidirectionally from a common promoter region. By cloning and hybridization techniques, we studied the physical organization and regulation of *ilvHI* and its relationship to the *leu* operon. The results presented here indicate that *ilvHI* and *leu* are distinct operons, separated by about 1,500 base pairs, and that they are transcribed in opposite directions.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study were derivatives of *E. coli* K-12 and are listed in Table 1. The genetic symbols used are those of Bachmann and Low (2).

Growth of cells and enzyme assays. M9 (24) and SSA (6) were used as minimal media with the addition of 0.4% glucose as carbon source. Media were supplemented with amino acids (50 μg/ml except va-

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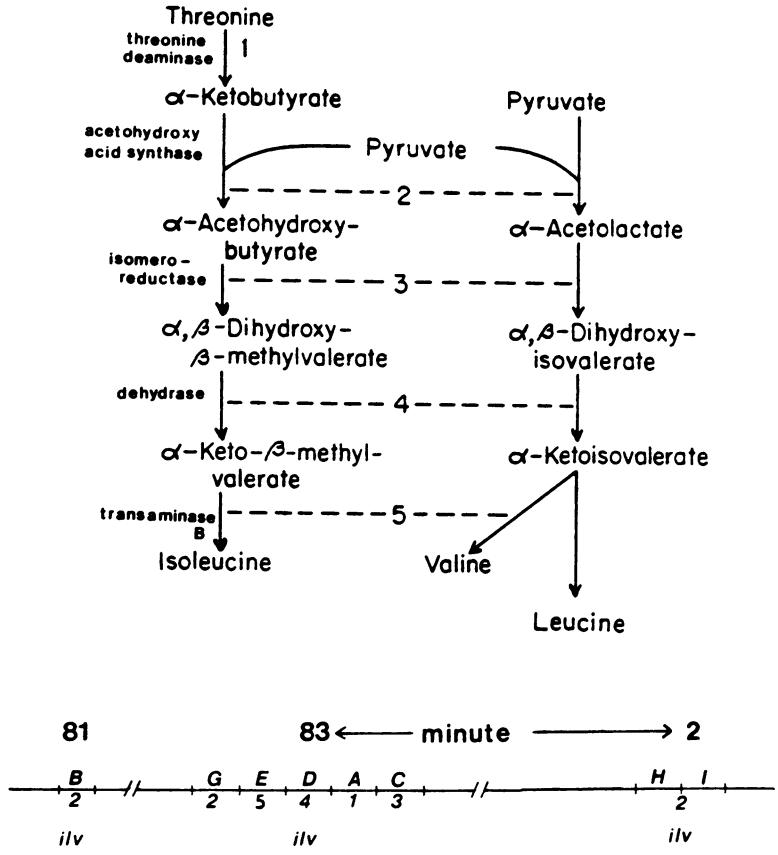

FIG. 1. *The biosynthetic pathways leading to leucine, valine, and isoleucine. The locations of several operons on the E. coli K-12 map (2) are given together with the relevant gene-enzyme relationships.*

TABLE 1. *Bacterial strains*[a]

Strain	Genotype	Origin
PS1035	*thi-1 glyA ilvB619*	DeFelice et al. (12)
MI316	*thi-1 glyA ilvB619 ilvH612 ilvI614 ara bgl*	DeFelice et al. (15)
LR16	*thi-1 glyA ilvB619 lrs-16*	M. V. Ursini et al., submitted for publication; a spontaneous leucine-resistant derivative of strain PS1035
CV655	MI316(pCV35)	This paper
CV669	MI316(pCV4)	This paper
CV670	MI316(pCV7)	This paper
CV671	MI316(λG4)	This paper
PS1199	*thi-1 trpR Δ(ara-leu-ilvHI)*	DeFelice et al. (12)

[a] The genetic nomenclature conforms to that of Bachmann and Low (2).

line [100 μg/ml]) and vitamins (1 μg/ml). Cells were harvested and extracted, and AHAS assays were done as previously described (13, 14). Protein concentrations in extracts were measured by the method of Groves et al. (17).

Nucleic acid procedures. Plasmid DNA preparation, transformations, preparative isolations of DNA fragments from agarose and acrylamide gels, isolation of RNA, and DNA-RNA hybridizations done in liquid were performed as described by Smith and Calvo (26, 27). λG4 DNA was separated into heavy and light strands by centrifugation with polyuridylate-polyguanylate as described by Hradecna and Szbalski (20). RNA-DNA hybridizations on filters were done as described by Bovre and Szbalski (5) and Cooper et al. (7). The hybridization of a DNA probe to RNA bound to diazobenzyloxymethyl-paper (see Fig. 5) was done by the procedure of Wahl et al. (29). RNA preparations were fractionated on a 1.5% agarose gel containing 1 mM methylmercury hydroxide (3). After transfer to diazobenzyloxymethyl-paper, hybridization was performed by using a nick-translated (25) *Hin*dIII/*Bam*HI (600-base pair [bp]) fragment of the *ilvHI* operon as probe.

Restriction and other DNA and RNA enzymes, largely purchased from New England Biolabs, were used under conditions recommended by the vendor.

Construction of plasmids. Plasmid pCV1 (see Fig. 3) has been previously described (9). Plasmid pCV35 contains the 10.9-kilobase (kb) *Eco*RI fragment of pCV1 inserted into pBR322. Plasmids pCV4 and pCV6 contain, respectively, the 4,000-bp *Hin*dIII and 3,300-bp *Hin*dIII/*Bam*HI fragments of pCV1 inserted

into pBR322 (see Fig. 3 for details). Plasmid pCV7, containing *ilvHI* but not *leu* DNA, was prepared as follows (see Fig. 3). Plasmid pCV4 (50 μg) was digested with 10 U of *Hin*dIII, precipitated with ethanol, and suspended in 50 μl of T4 DNA ligase buffer and 0.2 units of T4 DNA ligase. After incubation at 14°C for 16 h, the DNA was precipitated with ethanol, suspended in *Pvu*II buffer, and digested with 10 U of *Pvu*II for 2 h at 37°C. After phenol extraction and ethanol precipitation, the sample was resuspended in 20 μl of T4 DNA ligase buffer containing 1 U of T4 DNA ligase and incubated at 14°C for 24 h. This mixture was then ethanol precipitated and used to transform strain MI316 to ampicillin resistance.

RESULTS

Phage λG4 carries *ilvHI*. Strain MI316 (*ilvB619 ilvH612 ilvI614*) requires both isoleucine and valine for growth and contains little, if any, AHAS activity (15). When lysogenized with phage λG4 (8), it becomes Ilv$^+$ and contains AHAS activity. Six lines of evidence suggest that this activity is due to isoenzyme AHAS III. (i) The amount of AHAS activity in strain CV671 (MI316/λG4) is similar to that in a haploid strain expressing *ilvHI* (Table 2; compare lines 1 and 2). (ii) Flavine adenine dinucleotide (FAD), which stimulates the activity of AHAS I, does not stimulate AHAS activity in extracts of strain CV671 (data not shown). (iii) Valine inhibits growth and AHAS III activity in strains PS1035 and CV671 to the same extent (Fig. 2 and Table 2). It should be noted that AHAS II, when expressed, is resistant to valine inhibition (21). (iv) The growth of strain PS1035 (*ilvB619*) is inhibited by leucine (Table 2), presumably because excess leucine interferes with the synthesis of AHAS III (12). The growth of strain CV671 is also inhibited by leucine (Table 2). AHAS activity in extracts of PS1035 (15) and CV671 is not inhibited by leucine (data not shown). (v) The growth of strain PS1035 (*ilvB619*) is inhibited by α-ketobutyrate (Table 2), probably because excess α-ketobutyrate preempts AHAS III in favor of isoleucine synthesis, resulting in a valine limitation (15). Strain CV671 is similarly inhibited by α-ketobutyrate (Table 2). By comparison, the growth of strains producing only AHAS I is much less severely affected by leucine or α-ketobutyrate (13, 14). (vi) *ilvHI* (AHAS III)

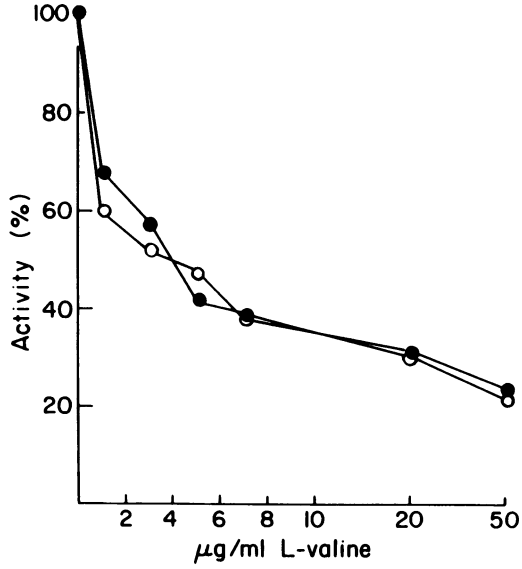

FIG. 2. *The effect of* L-*valine on AHAS activity in extracts of strains PS1035 (ilvB619)* (○) *and CV671 (ilvB619 ilvH612 ilvI614/λG4)* (●). *For strains PS1035 and CV671, 100% activity is 24.4 and 24.0 nmol/min per mg of protein, respectively.*

TABLE 2. *Relative AHAS activities and phenotypes in haploid and plasmid-containing strains of E. coli K-12*

Strain	Relative sp act of AHAS[a]	Inhibition caused by valine (%)[b]	Zone of inhibition[c] caused by:		
			Leucine	Valine	α-Ketobutyrate
Haploid					
PS1035	1.0	71	31	39	35
CV671 MI316(λG4)	1.18	78	27	41	33
Plasmid containing					
CV669 MI316(pCV4)	0.45	13	25	24	25
CV670 MI316(pCV7)	0.33	19	26	17	22
CV655 MI316(pCV35)	38.0	78	15	24	26

[a] The activities were measured at pH 9.0 without FAD added to the reaction mixtures. The addition of FAD did not increase the activity in any strain. The specific activity for the AHAS III from strain PS1035 was 16.4 nmol of acetolactate formed per min per mg of protein.

[b] Percent of total activity when reaction mixtures contained 0.5 mM valine.

[c] Supplements were applied (50 μg of each) to filter paper disks resting on plates containing the indicated strains in a minimal agar overlay. The numbers are the diameters (millimeters) of the zones of inhibition surrounding the disks.

is closely linked to *leu* (10, 11), and λG4 carries the entire leucine operon (8).

Subcloning of bacterial genes from λG4 onto plasmids. The approximate location of *ilvHI* relative to the *leu* genes was established by subcloning experiments (Fig. 3). Plasmid pCV1 contains a 10.9-kb *Eco*RI fragment from λG4 ligated into the *Eco*RI site of plasmid ColE1 (7). This 10.9-kb *Eco*RI fragment and a 4.0-kb *Hin*dIII fragment were separately transferred from plasmid pCV1 to plasmid pBR322, yielding plasmids pCV35 and pCV4, respectively. Removal of a *Bam*HI fragment from plasmid pCV4 generated plasmid pCV6. Treatment of plasmid pCV4 sequentially with endonuclease *Hin*dIII and DNA ligase yielded a mixture of two plasmids having the 4.0-kb *Hin*dIII fragment in opposite orientations relative to genes on pBR322. After treatment of this mixture sequentially with endonuclease *Pvu*II and T4 DNA ligase, plasmid pCV7 was recovered.

Plasmids pCV1, pCV35, pCV4, and pCV7 all confer prototrophy upon strain MI316 (*ilvB619 ilvH612 ilvI614*). These results support the gene order *azi ilv leu* and are shown in Fig. 4. This order has also been deduced from the results of genetic experiments employing P1 transductions (11). The results indicate further that *ilvHI* is located to the left of the *Pvu*II site shown in Fig. 4. On the other hand, plasmid pCV6, containing a 3.3-kb *Bam*HI/*Hin*dIII fragment subcloned on plasmid pBR322, did not confer prototrophy upon strain MI316, suggesting that there is a *Bam*HI site within *ilvI*, coding the catalytic subunit of AHAS III.

Expression of *ilvHI* in plasmid-contain-

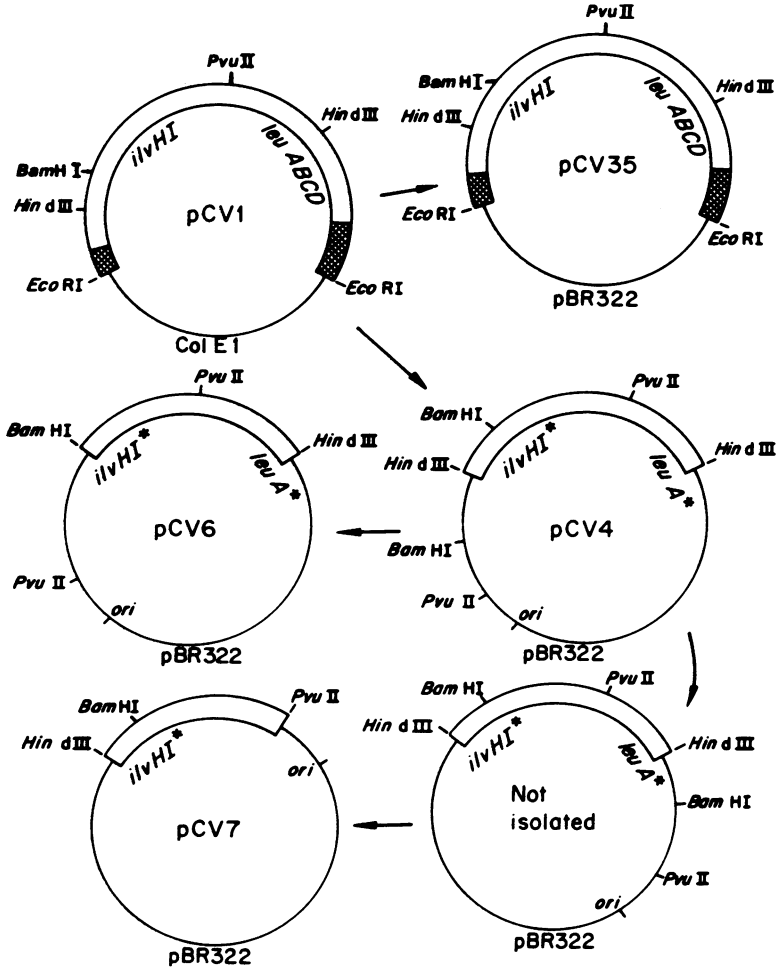

FIG. 3. *Construction of plasmids. Bacterial DNA is represented by double lines, plasmid DNA, by a single line, and λ DNA, by cross-hatching. An asterisk indicates that part of the operon is missing. The drawings are not to scale.*

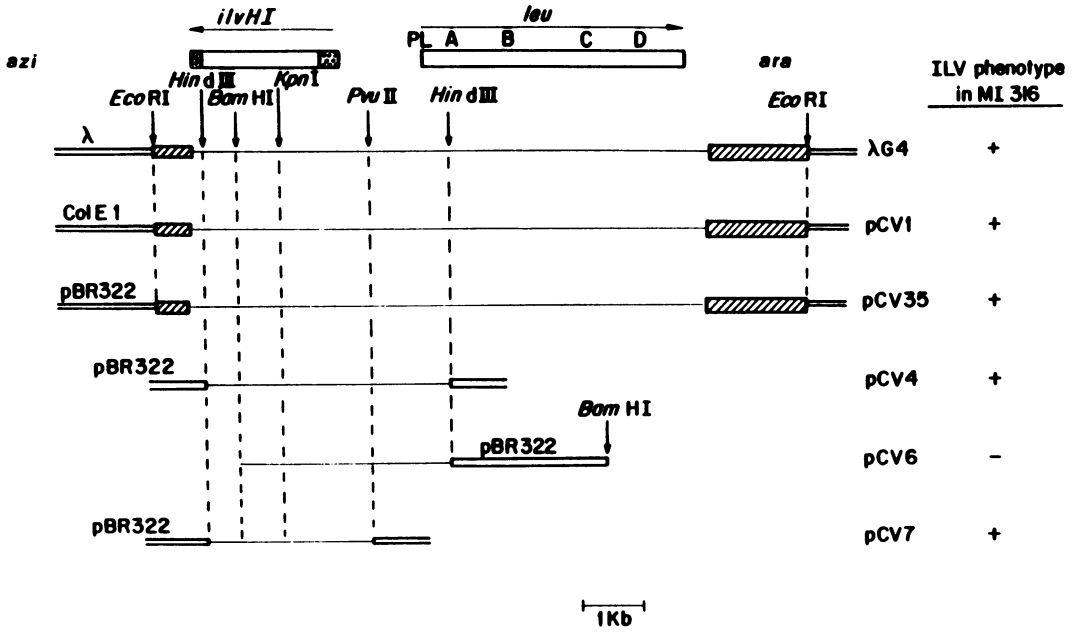

FIG. 4. *Coding potential of subclones derived from λG4. All of the DNA between the indicated EcoRI sites of λG4 is bacterial, with the exception of a small amount of λ DNA represented by cross-hatching (8). About 900 bp of λ DNA are at the ilvHI end, and 2,400 bp are at the leu end (8). The exact boundaries of ilvHI have not been determined. The ability (+) or inability (−) of strain MI316 (13) to grow in minimal medium lacking isoleucine and valine when carrying the indicated plasmid is shown. Horizontal arrows denote direction of transcription of the indicated operons.*

ing strains. The activity of AHAS was measured in extracts of *ilvHI* plasmid-containing strains CV669, CV670, and CV655 (Table 2). High AHAS activities were expected because these plasmids, derivatives of pBR322, should exist in 25 to 50 copies per cell. Although we did not measure plasmid copy number directly, the yield of plasmid from these strains was comparable to what we normally isolate from pBR322-containing strains. Surprisingly, strains carrying plasmids pCV4 and pCV7 had low AHAS activity, even less than that in the haploid wild-type strain (Table 2). Furthermore, the activity in extracts of strains CV669 and CV670 was not inhibited to a significant extent by L-valine (Table 2). By comparison, strain CV655 [MI316(pCV35)] had the expected high AHAS activity (38-fold elevated over the haploid level), and the latter was substantially inhibited by valine. The finding that strain CV655 has elevated AHAS activity is consistent with the fact that the growth of this strain is less susceptible to inhibition by valine and leucine than is the haploid strain PS1035 (Table 2). The reduced sensitivity of strains CV669 and CV670 to inhibition by valine (Table 2) is presumably due to the fact that, although these strains produce less AHAS activity than does a wild-type strain, that activity is not strongly inhibited by valine (Table 2). Since plasmids pCV4 and pCV7 differ from plasmid pCV35 in lacking a 1,000-base pair piece of DNA at the left end of *ilvHI* (Fig. 4), we conclude that at least part of this DNA belongs to the *ilvHI* operon and that it is required for the valine sensitivity and maximal activity of AHAS III. In this regard, these plasmids could be deleted either for a part of *ilvH* or that part of the *ilvI* sequence coding for *ilvH* product recognition (see below).

Leucine reduces the rate of synthesis of *ilvHI*-specific RNA. A culture of strain PS1035 (*ilvB619*) in the log phase of growth was divided in two and leucine was added to one half. After further incubation for 15 min, each culture was pulse-labeled for 3 min with [^{3}H]uridine, and RNA was isolated. Samples of RNA were hybridized to plasmid pCV7 DNA that had been denatured and bound to nitrocellulose filters. As demonstrated below, the bacterial DNA on plasmid pCV7 is almost entirely DNA from the *ilvHI* operon. Incubation with leucine resulted in an 8- to 10-fold decrease in the rate of synthesis of *ilvHI* mRNA (Table 3).

The *ilvHI* operon contains approximately 2,550 nucleotide bp. RNA was prepared from strain PS1035 (*ilvB619*) that had been grown in

TABLE 3. *Effect of leucine upon the rate of synthesis of ilvHI mRNA in strain PS1035*

Addition to culture medium[a]	Amt (μg) of:		cpm bound[b]	% of input bound
	pCV7 DNA	RNA		
None	0.7	10	497	0.053
None	0.7	40	1640	0.044
None	2.8	10	433	0.048
None	2.8	40	1654	0.044
Leucine	0.7	10	41	0.0061
Leucine	0.7	40	135	0.0051
Leucine	2.8	10	58	0.0087
Leucine	2.8	40	180	0.0067

[a] Strain PS1035 was grown in minimal medium to mid-log phase. At zero time, L-leucine (50 μg/ml) was added to one-half of the culture, and 15 min later, both cultures were labeled for 3 min with [^{3}H]uridine (20 μCi/ml).

[b] For each experiment, an equivalent amount of RNA was incubated with filters containing 2.8 μg of pBR322 DNA. These values, ranging from 10 to 60 cpm, have been subtracted.

minimal medium and separately in minimal medium after incubation for 15 min with L-leucine (50 μg/ml). RNA samples were fractionated by agarose gel electrophoresis in the presence of 15 mM methylmercury hydroxide (3), and after transfer to diazobenzyloxymethyl-paper (1, 29), they were hybridized to the *Hin*dIII/*Bam*HI (600-bp) fragment from plasmid pCV7 (Fig. 4). The latter had been labeled to high specific activity by nick translation (25). The probe hybridized to RNAs heterogeneous in size (Fig. 5A). a result consistent with the short half-life of almost all bacterial mRNA's. The track containing RNA derived from the leucine-treated culture of strain PS1035 hybridized only very weakly (Fig. 5B), confirming the specificity of the probe and the strong repression of *ilvHI* transcription by leucine reported above.

An estimate of the size of the operon was made by comparison of the largest hybridizable RNA that is observable in Fig. 5 with the mobilities of 23S, 16S, and 5S RNAs within the sample. The size of the *ilvHI* operon estimated by this method is 2,550 nucleotides. If the *ilvHI* operon ends within the small amount of bacterial DNA carried on phage λG4 that lies to the left of the *Hin*dIII site (Fig. 4), as suggested by the results shown in Fig. 4 and by recent S1 nuclease mapping experiments (C. Squires, unpublished data), then the 5′ end of the operon is estimated to be about 500 bp to the left of the *Pvu*II site (Fig. 4).

***ilvHI* and *leu* are transcribed in opposite directions.** The *Bam*HI/*Kpn*I (750-bp) fragment was chosen as a probe to measure *ilvHI* mRNA because it is entirely within the *ilvHI*

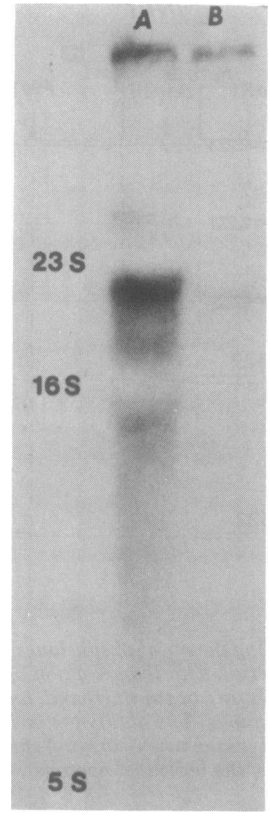

FIG. 5. *Analysis of ilvHI mRNA by the Northern blotting procedure. A 100-ml culture of strain PS1035 was grown in minimal medium (A) containing thiamine and glycine at 32°C to about 2 × 10^8 cells per ml. The culture was divided in two, and 50 μg of leucine per ml was added to one of the two parts (B). After 15 min at 32°C, RNA was prepared from both cultures. Samples (30 μg) were fractionated on a 1.5% agarose gel containing 1 mM methylmercury hydroxide (3), transferred to diazobenzyloxymethyl-paper (1, 24), and then hybridized to the HindIII/BamHI (600-bp) fragment (Fig. 4) containing about 8 × 10^6 cpm.*

operon (Fig. 4). This fragment was labeled to a high specific activity by nick translation (25); after denaturation the strands were separated by acrylamide gel electrophoresis (27). Each strand was then hybridized to a high C_0t value with each of the separated strands of λG4. *Bam*HI/*Kpn*I^s, the strand that migrated more slowly during electrophoresis, hybridized to $\lambda G4_H$ (Table 4, line 3). This same probe also hybridized specifically to the RNA derived from strain LR16 (Table 4, line 1). Strain LR16 was chosen for this experiment because it expresses the *ilvHI* operon constitutively (M. V. Ursini, P. Ascari, and M. DeFelice, submitted for publication). RNA derived from strain PS1199, deleted

TABLE 4. *Hybridization studies determining the direction of transcription of ilvHI*

Nucleic acid in excess[a]	Labeling probe[b]	Input (cpm)	cpm resistant to S1 nuclease
LR16 RNA	*Bam/Kpn*^{S}	1,570	760
LR16 RNA	*Bam/Kpn*^{F}	1,400	95
λG4$_H$ DNA	*Bam/Kpn*^{S}	1,570	760
λG4$_L$ DNA	*Bam/Kpn*^{S}	1,570	0
λG4$_H$ DNA	*Bam/Kpn*^{F}	1,400	120
λG4$_L$ DNA	*Bam/Kpn*^{F}	1,400	820

[a] A 75-μg amount of RNA was incubated with the probe for 24 h at 67°C in a total volume of 100 μl. Counts per minute resistant to S1 nuclease were measured as described by Smith and Calvo (21). A 20-μg amount of RNA protected the probes to the same extent as 75 μg (data not shown). DNA-DNA hybridizations were similar, except that 3 μg of λG4$_H$ or λG4$_L$ were employed.

[b] F and S identify the strands of *Bam*HI/*Kpn*I as moving faster or slower during electrophoresis. Approximate specific activity of each strand, 10^7 cpm/μg. These probes were obtained by nick translation (23).

for the *ilvHI* and *leu* operons, did not hybridize to either of the probes used in this experiment (data not shown). These results demonstrate that λG4$_H$ is the noncoding strand of *ilvHI*. Since λG4$_H$ is the coding strand of the *leu* operon (9), *ilvHI* and *leu* must be transcribed in opposite directions.

DISCUSSION

The *ilvHI* genes of *E. coli* K-12 are contained on an *Eco*RI fragment derived from the *leu*-transducing phage λG4. The following evidence indicates that the *ilvHI* genes are confined to a region of approximately 3,000 bp between the *Pvu*II site and a point about 100 bp to the left of *Hin*dIII site. Plasmid pCV35 apparently has an intact *ilvHI* operon because in strains carrying pCV35, AHAS III is synthesized in the expected amounts, the synthesis is repressed by leucine, and the activity is inhibited in a normal fashion by valine (Table 2). Strains carrying plasmids pCV4 or pCV7, on the other hand, produce small amounts of AHAS III activity, and that activity is relatively insensitive to inhibition by valine. These phenotypes do not relate to DNA between the *Pvu*II site and the *leu* operon because plasmid pCV4 contains this region, whereas plasmid pCV7 does not (Fig. 4). Rather, these two phenotypes are presumed to result from the fact that both plasmids pCV4 and pCV7 lack about 100 bp of bacterial DNA at their left end in comparison with plasmid pCV35. Recent DNA sequence analysis of the *ilvHI* operon (C. Squires, manuscript in preparation) supports the existence of both *ilvH* and *ilvI* genes and the gene order *ara leu ilvI ilvH azi* (Fig. 4). This means that plasmids pCV4 and pCV7 are deleted for a portion of the coding sequence for the regulatory subunit of AHAS III, *ilvH*. The valine resistant phenotypes of strains carrying these plasmids are consistent with this model. The small amount of AHAS III present in strains containing either pCV4 or pCV7 further suggests that the *ilvH* and *ilvI* gene products must interact to form an efficient AHAS III enzyme.

Since removal of DNA between the *Pvu*II site and the *leu* operon has no effect upon either the amount of AHAS activity produced or the inhibition of synthesis caused by leucine (Table 2), we conclude that the promoter and control region of the *ilvHI* operon lies to the left of the *Pvu*II site. On the basis of the hybridization experiments shown in Fig. 5, we tentatively assign the promoter to a region about 500 bp to the left of the *Pvu*II site. The results of recent subcloning experiments employing plasmids constructed by McKenney et al. (23) indicate that the relevant region indeed contains a functional promoter (C. Squires, unpublished data).

The above analysis, together with the results in Table 4, which demonstrate that the two gene clusters are transcribed in opposite directions, indicates that *leu* and *ilvHI* represent separate, nonadjacent units of transcription. Therefore, the fact that leucine represses both operons cannot be explained by some mechanism involving close physical proximity of the two.

The idea that *leu* and *ilvHI* are different transcriptional units is also supported by observations indicating that leucine affects them in different ways. When cells are grown in leucine-containing medium, the expression of *ilvHI* is reduced by a factor of 8 to 10 (Table 3), whereas the expression of the *leu* operon is reduced only by a factor of 2 to 3 over that found in minimal medium (9). Furthermore, the mutation in strain LR16 causes the synthesis of AHAS III to become insensitive to leucine but does not affect the expression of the leucine biosynthetic enzymes (Ursini et al., submitted for publication). Finally, the region between *leu* and *ilvHI* does not seem to be related to either of them. The 400-bp region of DNA immediately adjacent to and upstream from the *leu* promoter has been sequenced and cannot code for a protein, nor does this DNA hybridize to *E. coli* mRNA to a measurable extent (S. Wessler and J. Calvo, unpublished data).

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