JOURNAL OF BACTERIOLOGY, Sept. 1984, p. 951–957
0021-9193/84/090951-07$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 159, No. 3

Regulation of *Salmonella typhimurium ilvYC* Genes

D. L. BLAZEY†* AND R. O. BURNS‡
Department of Microbiology and Immunology, Duke University School of Medicine, Durham, North Carolina 27710

Received 15 March 1984/Accepted 5 June 1984

The *Salmonella typhimurium* LT2 *ilvYC* genes were studied by fusion of each gene to the *Escherichia coli* K-12 *galK* gene. The expression of *ilvY* and *ilvC* could then be determined by measurement of the *galK*-encoded galactokinase enzyme. The promoter for *ilvC*, p_C, was located by this technique to a 0.42-kilobase *BglII*-*EcoRI* fragment of the *S. typhimurium ilvGEDAYC* gene cluster. This sequence was completely sufficient for α-acetohydroxyacid-inducible *galK* expression. The *ilvY* gene was located within a 1.0-kilobase *XhoI*-*SalI* fragment. *ilvY* gene expression was constitutive with respect to *ilv*-specific control signals. The *ilvY* gene was transcribed in the same direction as the other two transcriptional units in the *ilvGEDAYC* gene cluster, *ilvGEDA* and *ilvC*. Transcription of the *ilvC* gene was completely dependent upon the activity of its own promoter, p_C, and independent from transcription of the *ilvY* gene. The role of the intervening region between *ilvY* and *ilvC* in regulation of *ilvC* expression was explored.

The *Salmonella typhimurium* LT2 *ilvGEDAYC* gene cluster specifies the proteins necessary for the biosynthesis of isoleucine and valine (6, 21). Four of the biosynthetic enzymes are encoded by the *ilvGEDA* genes, which constitute a single operon (4). Expression of *ilvGEDA* is negatively regulated by isoleucine, valine, and leucine (8). The control mechanism involves translational control of transcription termination, i.e., attenuation (20). The *ilvC* gene encodes the remaining enzyme of the isoleucine-valine biosynthetic pathway, α-acetohydroxy acid isomeroreductase (2, 11). The *ilvC* gene constitutes a separate transcriptional unit from *ilvGEDA* (4), the expression of which is induced by the presence of the substrates of the isomeroreductase, α-acetolactate and α-acetohydroxybutyrate (1, 2). Action of these inducers, as in *Escherichia coli* K-12 (22), requires the participation of a regulatory protein encoded by the *ilvY* gene. The *S. typhimurium ilvY* gene was mapped between *ilvA* and *ilvC* by analysis of cloned fragments of the gene cluster (6). The *ilvY* gene product has an essential role in expression of *ilvC* as demonstrated by the isolation of an *ilvY* mutant that displays a *trans*-recessive $IlvC^-$ phenotype (5). The *ilvYC* genes constitute a distinctive regulatory system that employs positive control, which is uncommon for biosynthetic genes (21) and sharply contrasts with the control of the *ilvGEDA* operon. In addition, the apparently contiguous relationship between the regulatory gene *ilvY* and its target gene *ilvC* is unusual, and this propinquity may allow for more complex interactions between *ilvY* and *ilvC*. This work presents further characterization of the *S. typhimurium ilvYC* genetic system.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are shown in Tables 1 and 2, respectively. The primary *S. typhimurium* ilv^+ recombinant plasmid was pDU1 (6); the vector was a derivative of RSF2124, and the pertinent genotype of the chromosomal insert was *ilvGEDA*Y^+C'. pDU5 is a derivative of pDU1 that contains a 6.0-kilobase (kb) *SalI* *ilvEDA*Y^+ fragment inserted in pBR322 (6). The pKO *galK* vectors pKO5 and pKO6 are derivatives of pKO1 (19) and contain different arrays of cloning sites proximal to *galK*. The *galK* gene is expressed only when a properly oriented promoter is inserted within one of these sites.

Media. The liquid medium contained Davis-Mingioli salts (7) modified by the omission of citrate and supplemented with 0.5% glucose. Repressing medium contained 100 mg of L-valine, 100 mg of L-leucine, and 50 mg of L-isoleucine per liter. Limitation for isoleucine or limitation for leucine was achieved by decreasing the concentration of that amino acid to 5 mg/liter. Limitation for valine was effected by substituting 25 mg of glycyl-L-valine for valine. Additional amino acid requirements were met with 50 mg of the appropriate amino acid per liter except for tryptophan, which was used at 10 mg/liter. The thiamine concentration was 10 mg/liter. All plasmid-containing organisms were grown in media containing 40 mg of ampicillin per liter. Transformants were selected on nutrient agar (Difco Laboratories) or MacConkey agar (Difco) containing 0.5% D-galactose; both media contained 40 mg of ampicillin per liter.

Enzymes. All restriction endonucleases were purchased from either New England Biolabs or Bethesda Research Laboratories. T4 DNA ligase was from Bethesda Research Laboratories. BAL-31 nuclease was prepared from *Alteromonas espejiani* BAL-31 as described previously (9).

DNA isolation. Plasmid DNAs were prepared from chloramphenicol-amplified cultures as described previously (6). The rapid screening of transformants was performed as described by Birnboim and Doly (3).

Construction and characterization of recombinant plasmids. All restriction endonuclease digestions were performed under the conditions suggested by Maniatis et al. (17). Subcloning experiments were performed with a vector/fragment ratio of 1:1. T4 DNA ligation reactions were performed as described by Weiss et al. (23) at a DNA concentration of 25 μg/ml. The ligase concentration was 1 to 2 U/ml for staggered-end DNA and 100 U/ml for blunt-end DNA. BAL-31 nuclease digestions were generally performed under the low-salt conditions described by Legerski (15). Digestion of λ DNA was performed to quantify the BAL-31 nuclease activity and to determine appropriate digestion rates as suggested previously (9). After BAL-31 nuclease digestion, the DNAs were extracted once with

* Corresponding author.
† Present address: Department of Medicine, University Hospital, University of Washington, Seattle, WA 98105.
‡ Deceased.

TABLE 1. Strains

Strain	Genotype	Source (reference)
E. coli K-12		
DU650	F$^-$ Δ(*ilvEDAYC*) *leuB5 hsdR hsdM*$^+$ Δ*trpE5*	Laboratory collection
C600	F$^-$ *galK pro leu*	McKenney et al. (19)
N100	F$^-$ *galK recA pro*	McKenney et al. (19)
S. typhimurium LT2		
DU2	F$^-$ *hsdR hsdM ilvD18 ilvA228 leuA409*	Laboratory collection (5)
DU703	F$^-$ *hsdR hsdM ilvA ilvY702 leuA409 recA1*	Laboratory collection (5, 6)
AA111	F$^-$ *ilv*$^+$ *rho-111 metE338 hisC2124 trpE49*	Housley et al. (12)

phenol-chloroform (1:1 [vol/vol]) and twice with water-saturated diethyl ether. Residual ether was boiled away at 50°C, and the DNAs were ethanol precipitated, collected by centrifugation, and suspended in an appropriate volume of T4 ligase buffer. Transformation was performed as described by Lederberg and Cohen (14).

Agarose gel electrophoresis. Electrophoresis was generally performed in a horizontal apparatus with 1.0% agarose gels and with the E buffer described by Loening (16). Electrophoresis of small fragments for molecular weight determinations was performed in either 1.4% agarose gels or 5% polyacrylamide gels with a Tris-borate buffer (10). Molecular weight standards were either λ phage or pBR322 restriction fragments.

Preparation of cells for assay. All cells were grown at 37°C in the defined media described above. When isomeroreductase activity was measured, the cells were grown as 125-ml batch cultures, and most of the culture was used to prepare a cell extract (4). A 2-ml sample of each culture was processed for galactokinase assay (19). In all cases, the cells were first grown in repressing medium and then inoculated into various media to effect limitation for each branched-chain amino acid. The cells were generally harvested after about 8 h of growth. Limitation for the specific amino acid was manifest when the cells reached a certain maximal optical density of less than that seen with fully supplemented medium and was confirmed by measurement of derepressed levels of *ilv* enzymes (4).

Enzyme assays. Galactokinase was measured by the method of McKenny et al. (19), and the units were expressed as nanomoles of galactose phosphorylated per minute per unit of absorbance at 650 nm. The α-acetohydroxyacid isomeroreductase was measured by the method of Arfin et al. (1) with α-acetohydroxybutyrate as the substrate. The isomeroreductase units were nanomoles of NADPH reduced per minute per milligram of protein.

Chemicals. α-Acetolactate and α-acetohydroxybutyrate were prepared by saponification of the corresponding methyl esters, which were the gift of Frank Armstrong. D-[^{14}C]galactose was purchased from Amersham Corp. (product no. CFA435).

RESULTS

***ilvC*-directed *galK* expression.** We initially cloned an *Eco*RI fragment from the *S. typhimurium* LT2 chromosome that was *ilvGEDAY*$^+$ but lacked an intact gene *ilvC* as shown by complementation analysis and enzyme assay (6). An *S. typhimurium Bam*HI-*Eco*RI *ilvDAY*$^+$*C'* subclone is shown in Fig. 1. Approximately 0.9 kb of DNA contained in this fragment is distal to the *ilvY* gene, and, because of the contiguous location of *ilvC* to *ilvY*, we concluded that some of the *ilvC* gene must be present between the *Sal*I and *Eco*RI sites. If the *S. typhimurium ilvC* gene is transcribed in the same direction as that of *ilvGEDA* (that is, from *ilvG* to *ilvA*), then the *ilvC* promoter probably resides within the *Sal*I-

TABLE 2. Plasmids

Plasmid	Pertinent genotype	Source (reference)
pDU1	*ilvGEDAY*$^+$*C' rrnC'*	Original *S. typhimurium ilv* clone (6)
pDU5	*ilvEDAY*$^+$	*Sal*I fragment of pDU1 inserted into pBR322 (6)
pKO6	(*Bam*HI-*Eco*RI) *galK*	pKO vector (19)
pDU60	*ilvDAY*$^+$*C' galK*$^+$	*Bam*HI-*Eco*RI fragment of pDU1 inserted into pKO6
pDU61	*ilvDA*$^+$ Δ*ilvY ilvC' galK*$^+$	Deletion of internal *Xho*I-*Sal*I fragment from pDU60
pDU62	*ilvD*$^+$ Δ*ilvAY ilvC'-galK*$^+$	Deletion of internal *Bgl*II fragment from pDU60
pDU63	Δ*ilvDAY ilvC'-galK*$^+$	Deletion of *Bam*HI-*Bgl*II and *Bgl*II fragments from pDU60
pDU60.43	*ilvDA*$^+$ Δ*ilvY ilvC'-galK*$^+$	BAL-31 nuclease-mediated deletion of ca. 0.4 kb of the N-terminal region of *ilvY*
pDU64	*ilvDAY*$^+$ *ilvC-galK*$^+$	Inversion of the internal *Xho*I-*Sal*I fragment of pDU60
pKO5	(*Bam*HI) *galK*	pKO vector (19)
pDU71	*ilvA' ilvY*$^+$ *galK*	*Bgl*II fragment from pDU60 inserted into *Bam*HI site of pKO5
pDU72	*ilvY*$^+$ *ilvA' galK*	*Bgl*II fragment from pDU60 inserted into *Bam*HI site of pKO5 but in opposite orientation from pDU71
pDU71.1	*ilvA' ilvY'-galK*$^+$	BAL-31 nuclease-mediated fusion of *ilvY* to *galK*

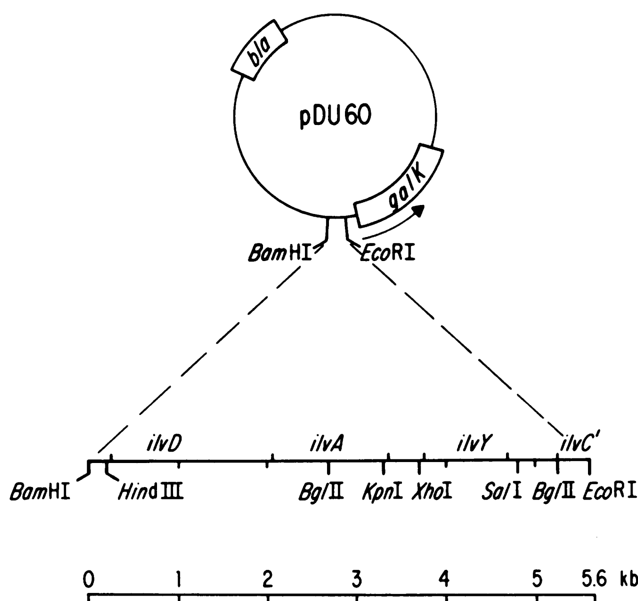

FIG. 1. pDU60 *ilvDAY*$^+$ *ilvC'-galK*. The vector is pKO6.

*Eco*RI region. This was demonstrated by insertion of the *Bam*HI-*Eco*RI fragment into the *galK* vector pKO6 (Fig. 1). Expression of *galK* from the resulting plasmid, pDU60, was studied under conditions known to modulate transcription of the *ilvC* gene (Table 3). In strain C600(pDU60), *galK* was induced by the acetohydroxyacids α-acetohydroxybutyrate and α-acetolactate. The less-efficient induction of *ilvC* by α-acetolactate has been noted previously for an *E. coli* K-12 in vitro transcription system (24). Since the products of the acetohydroxyacid synthases are inducers of *ilvC* expression,

TABLE 3. Expression of *galK* from the *S. typhimurium ilvC* promoter

Strain	Medium[a]	Galactokinase sp act (*galK*)[b]
C600 *ilv*$^+$(pKO6 *galK*)[c]	Repressing	10
	Repressing + AL	10
	Repressing + AHB	8
C600 *ilv*$^+$(pDU60 *ilvDAY*$^+$ *ilvC-galK*$^+$)	Repressing	80
	Repressing + AL	400
	Repressing + AHB	710
DU650 Δ(*ilvEDAYC*)(pDU60)[d]	Repressing	43
	Limiting valine	660

[a] The modified Davis-Mingioli medium described in the text was used. Repressing medium was supplemented with either 50 mg of L-proline per liter (for strain C600) or 10 mg of L-tryptophan per liter (for strain DU650). The concentration of α-acetohydroxybutyrate (AHB) or α-acetolactate (AL) was 2 mM.

[b] Nanomoles of galactose phosphorylated per minute per unit of absorbance at 650 nm. The *galK* assay was as described by McKenny et al. (19).

[c] Low-level *galK* expression from pKO6 results from plasmid promoters.

[d] Expression of the chromosomal *galK* gene in strain DU650 cannot be detected under these conditions.

TABLE 4. Expression of *galK* from deletion derivatives of pDU60

Strain	Medium[a]	Galactokinase sp act (*galK*)[b]
C600 *ilv*$^+$(pDU61 *ilvDA*$^+$ Δ*ilvY ilvC'-galK*$^+$)	Repressing	250
	Repressing + AHB	630
DU650 Δ(*ilvEDAYC*)(pDU61)	Repressing	200
	Repressing + AHB	220
C600 *ilv*$^+$(pDU62 *ilvD*$^+$ Δ[*ilvAY*] *ilvC'-galK*$^+$)	Repressing	220
	Repressing + AHB	580
DU650 Δ(*ilvEDAYC*)(pDU62)	Repressing	250
	Repressing + AHB	240
C600 *ilv*$^+$(pDU63 Δ[*ilvDAY*] *ilvC'-galK*$^+$)	Repressing	300
	Repressing + AHB	600
DU650 Δ(*ilvEDAYC*)(pDU63)	Repressing	380
	Repressing + AHB	280

[a] Modified Davis-Mingioli medium described in the text was used. Repressing medium was supplemented with either 50 mg of L-proline per liter (for strain C600) or 10 mg of L-tryptophan per liter (for strain DU650). The concentration of α-acetohydroxybutyrate (AHB) was 2 mM.

[b] See Table 3, footnote *b*.

derepression of the synthases by valine limitation (*ilvB*) or leucine limitation (*ilvB* and *ilvI*) is correlated with an endogenous induction of *ilvC*. Accordingly, pDU60 *galK* expression was also induced under these conditions as shown by assay of strains DU650 Δ(ilvEDAYC)(pDU60) and C600 *ilv*$^+$(pDU60). These results demonstrate that the pDU60 *galK* gene is expressed from the *ilvC* promoter (p_C) and that the transcription of *ilvC* is in the same direction as that of the *ilvGEDA* operon. Furthermore, the induction of *galK* in the DU650 Δ(ilvEDAYC) background confirms that the *Bam*HI-*Eco*RI fragment contains the intact *ilvY* gene.

A more precise location for p_C and its relationship to the contiguous *ilvY* gene was determined by deleting specific restriction endonuclease fragments from pDU60. These deletion derivatives were compared with pDU60 by agarose gel electrophoresis to confirm their compositions. Expression of *galK* from each plasmid was measured in strains C600 and DU650, and the responsiveness of *galK* to induction by α-acetohydroxybutyrate was determined (Table 4).

Previous analysis demonstrated that the *ilvY* gene is located between *Xho*I and *Sal*I (6). When the *Xho*I-*Sal*I fragment was deleted from pDU60, the resulting plasmid pDU61 conferred inducible *galK* expression in strain C600 *ilv*$^+$ and constitutive expression in strain DU650 Δ(*ilvEDAYC*), in which *ilvY* was absent from both the plasmid and chromosome. This result confirms the location of *ilvY* within the *Xho*I-*Sal*I fragment and indicates that p_C is entirely contained within the 0.88-kb *Sal*I-*Eco*I region. Deletion of the internal *Bgl*II fragment or both the *Bam*HI-*Bgl*II and *Bgl*II fragments from pDU60 (pDU62 and pDU63, respectively) also resulted in derivatives which expressed inducible *galK* activity in strain C600 *ilv*$^+$ and constitutive activity in strain DU650 Δ(*ilvEDAYC*). These results demonstrate that the p_C sequence must be contained within the 0.42 kb *Bgl*II-*Eco*RI region of the *ilv* insert. Furthermore,

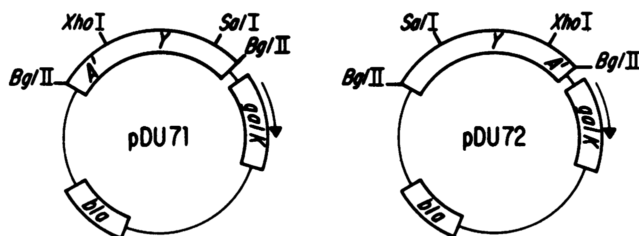

FIG. 2. *ilvY*-containing subclones pDU71 and pDU72. The 2.4-kb *Bgl*II fragment of pDU60 was inserted in either direction into the *Bam*HI site of pKO5. The orientations were confirmed by digesting the plasmids with *Ava*I endonuclease, which generates two distinct restriction profiles.

this region includes the *cis*-active regulatory information through which the acetohydroxyacids and the *ilvY* gene product effect induction of the *ilvC* gene.

ilvY transcriptional unit. The intact *ilvY* gene is contained within the 1.0-kb *Xho*I-*Sal*I segment of the *S. typhimurium ilv* cluster (Fig. 1). This fragment is only slightly larger than the predicted *ilvY* structural gene sequence (0.95 kb) based upon the 35,000-dalton *E. coli* K-12 *ilvY* gene product (22). Therefore, limited digestion with exonuclease BAL-31 at either end of the *Xho*I-*Sal*I segment should result in deletions extending into the *ilvY* transcriptional unit. If this digestion were done so as to fuse the BAL-31 nuclease-digested *ilvY* gene to *galK*, then *ilvY*-directed *galK* expression should occur with fusions at either the *Xho*I or *Sal*I termini of *ilvY*, depending upon the direction of transcription of *ilvY*. The BAL-31 nuclease-mediated fusion of *galK* to the *ilvY* transcriptional unit also affords the opportunity to study the regulation of *ilvY* expression by measuring *ilvY*-directed *galK* activity under various conditions.

The 2.4-kb $ilvY^+$ fragment generated by *Bgl*II endonuclease digestion of pDU60 (Fig. 1) was inserted in both orientations into the *Bam*HI site of the *galK* vector pKO5 (Fig. 2). The resulting plasmids, pDU71 and pDU72, showed no *galK* expression. The plasmids were cleaved with either *Sal*I (pDU71) or *Xho*I (pDU72) endonucleases and then subjected to limited digestion with BAL-31 nuclease. The nuclease-treated plasmids were restored to closed circular form by blunt-end ligation and used to transform strain N100 to Amp^r on MacConkey-galactose agar. Amp^r transformants that were red on the indicator medium, indicating expression of *galK*, were obtained only with the pDU71 DNA preparation (25 red Amp^r transformants per 120 Amp^r transformants). Analysis of the partially purified plasmids from the red transformants demonstrated that each plasmid contained a deletion extending into *ilvY* but not as far as the *Xho*I site. Enzyme assay confirmed that the deletion plasmids expressed the *galK* gene; the galactokinase activity of these plasmids ranged from 150 to 400 U. On the other hand, all 100 Amp^r transformants obtained with the pDU72 DNA preparation were white. Analysis of 20 of these transformants indicated that many contained plasmids with deletions extending into *ilvY* without affecting the distal *Sal*I site; these deletions also did not extend into the plasmid-encoded *galK* gene. Enzyme assay of these transformants showed no *galK* expression. The ability to promote *galK* expression by fusion of *galK* to *ilvY*, then, can occur by BAL-31 digestion only at the *Sal*I terminus of the *ilvY* gene. The *ilvY* promoter must be located near the *Xho*I site, so that transcription of *ilvY* proceeds in the direction from *Xho*I to *Sal*I. Therefore, the *ilvY* gene is transcribed in the same direction as the other

TABLE 5. *ilvY*-directed *galK* expression from pDU71.1

Strain	Medium[a]	Galactokinase sp act (*galK*)[b]
DU650 Δ(*ilvEDAYC*)(pDU71.1 *ilvA' Y' galK*$^+$)	Repressing	360
	Limiting isoleucine	280
	Limiting valine	320
	Limiting leucine	270
	Repressing + AL	300
	Repressing + AHB	320
C600 ilv^+(pDU71.1)	Repressing	310
	Repressing + AL	270
	Repressing + AHB	280

[a] Media are described in the text. The media were supplemented with either 10 mg of L-tryptophan per liter (for strain DU650) or 50 mg of L-proline per liter (for strain C600). The concentration of α-acetolactate (AL) was 5 mM, and that of α-acetohydroxybutyrate (AHB) was 2 mM.

[b] See Table 3, footnote *b*.

two transcriptional units contained in the *S. typhimurium ilv* cluster, namely, *ilvGEDA* and *ilvC*.

The relationship between the *ilvY* promoter and the *Xho*I site was more closely examined by cleaving two distinct pDU71-derived *ilvY-galK* fusion plasmids with *Xho*I endonuclease and then introducing small deletions about the *Xho*I site with BAL-31 nuclease. BAL-31-degenerated deletions as small as 50 base pairs destroyed *ilvY*-mediated *galK* expression; galactokinase activity encoded by these plasmids was less than 10 U, which is the background level observed with the pKO5 vector. Therefore, the maximum distance between the *Xho*I site and the *ilvY* promoter is 50 base pairs. We have recently exploited the proximity of the *Xho*I site to the *ilvY* promoter and N-terminal coding sequence by fusing the λ bacteriophage leftward promoter (p_L) to *ilvY* at an *Sst*I site that overlaps with *Xho*I. The *ilvY* gene product is then under regulation by the temperature-sensitive *c*I857 lambda repressor. Heat induction of the p_L-*ilvY* plasmid results in overproduction of the gene product *ilvY* to ca. 5% of the total cellular protein. We have used the p_L-*ilvY* system to determine that the subunit molecular weight of the *S. typhimurium ilvY* gene product is 34,000 (unpublished data).

One representative *ilvY-galK* fusion plasmid, pDU71.1, was selected for preliminary studies on the regulation of *ilvY* expression. This fusion lacks about 0.5 kb of the *ilvY* gene as determined by agarose gel electrophoresis (data not shown). Strain DU650(pDU71.1) was assayed under conditions of excess branched-chain amino acids or under limitation for isoleucine, valine, or leucine; the *ilvY*-directed *galK* expression was not multivalently regulated by the branched-chain amino acids (Table 5). In addition, the pDU71.1-determined *galK* expression was not affected by the presence of α-acetohydroxybutyrate or acetolactate in either the DU650 Δ(*ilvEDAYC*) or C600 ilv^+ backgrounds, thus providing no evidence for autogenous regulation via the *ilvY* gene product and the acetohydroxyacids. The *ilvY* promoter, therefore, is constitutive with regard to *ilv*-specific regulation. The *ilvY* promoter appears to be of low to intermediate strength when compared with the *galK*-determined expression of other procaryotic promoters in the pKO system (19).

Is the *ilvC* gene transcribed from the *ilvY* promoter? Recent work indicates that the *E. coli* K-12 *ilvY* gene is transcribed in the same direction as *ilvC* (J. Falk and H. E. Umbarger,

TABLE 6. *ilvC*-directed *galK* expression in the absence of transcription of a contiguous *ilvY* gene

Strain	Medium[a]	Galactokinase sp act (*galK*)[b]
DU650 Δ(*ilvEDAYC*)(pDU60.43 *ilvDA*$^{+}$Δ*ilvY ilvC'-galK*)	Repressing	80
	Limiting valine	120
C600 *ilv*$^{+}$(pDU60.43)	Repressing	70
	Repressing + AL	320
	Limiting leucine	260
DU650 Δ(*ilvEDAYC*)(pDU64 *ilvDAY*$^{+}$ *ilvC-galK*)[c]	Repressing	200
	Repressing + AHB	800

[a] Modified Davis-Mingioli medium was used, and the conditions were as described previously (4). The concentration of α-acetolactate (AL) or α-acetohydroxybutyrate (AHB) was 2 mM.
[b] See Table 3, footnote *b*.
[c] The *ilvY* gene of pDU64 is opposite in orientation from the native *ilvY* gene.

Fed. Am. Abstr. 1983, no. 1651, p. 2039). The insertion of transposon Tn*10* or phage Mu into the chromosomal *ilvY* gene prevented *trans* complementation by a plasmid-borne, intact *ilvY* gene. It was concluded that the *ilvC* gene is dependent upon transcription from the contiguous *ilvY* promoter and that the *ilvY* gene acts as an antiterminator at a site between *ilvY* and *ilvC* to allow transcription to proceed into the *ilvC* gene. The *cis*-dominant effect of *ilvY*::Tn*10* or *ilvY*::Mu insertion mutations on *ilvC* expression was explained by the ability of these elements to effect termination of transcription initiated at the *ilvY* promoter.

Two *S. typhimurium ilvY* mutants have been described that arose by imprecise excision of Tn*10* from *ilvA*::Tn*10* insertion mutations (5, 6). Chromosomal alterations associated with imprecise excision of Tn*10* are either inversions or deletions (13). Since the pertinent gene order of the *S. typhimurium* chromosome is *ilvA*-p_Y-*ilvY*-*ilvC*, then *ilvA*::Tn*10*-generated mutations affecting *ilvY* expression would necessarily disrupt the association between p_Y and *ilvC* by inverting p_Y or deleting p_Y. However, both *S. typhimurium ilvY* mutants could be complemented in *trans* by appropriate F′ *ilv* or recombinant plasmids (5, 6). In view of the apparent difference between the *S. typhimurium* and *E. coli* K-12 *ilvY-ilvC* systems, we used the *S. typhimurium ilvYC-galK* plasmids to define further the relationship between *ilvY* and *ilvC*. Deletion of the entire *ilvY* gene from pDU60 did not affect *ilvC-galK* expression other than to impose a *trans*-recessive *ilvY* requirement for acetohydroxyacid induction (Table 4). This is particularly striking with pDU63, in which the 0.42-kb *Bgl*II-*Eco*RI fragment directs acetohydroxyacid-responsive *galK* expression, localizing p_C within this short sequence.

If *ilvC* is transcribed independently from *ilvY*, then small deletions removing the *ilvY* promoter should not affect p_C function. This was demonstrated by BAL-31 nuclease digestion of pDU60 at the *Sst*I site, which is in close proximity to p_Y. A total of 50 derivatives were obtained which lacked *ilvY* function but retained *ilvC*-directed *galK* expression. One representative plasmid, pDU60.43, contains a 0.7-kb deletion, of which 0.4 extends into *ilvY*. In strain DU650 Δ(*ilvEDAYC*), pDU60.43 *galK* expression was constitutive, reflecting the absence of *ilvY* function (Table 6). Enzyme assay of strain C600 *ilv*$^{+}$(pDU60.43), however, showed that *galK* expression was responsive to induction by added acetohydroxyacids or by endogenous induction, varying from 70 U under repressing conditions to 320 U with α-acetolactate or 260 U in response to leucine limitation. Deletion of the *ilvY* promoter and N-terminal coding region, therefore, has no effect on *ilvC*-directed *galK* activity.

Dissociation of the p_C sequence from the *ilvY* transcriptional unit can also be effected by inverting the *ilvY* gene with respect to *ilvC*. The *ilvY* structural gene and promoter are contained within the *Xho*I-*Sal*I fragment. Both *Xho*I and *Sal*I restriction endonucleases generate staggered ends with the sequence 5′-TCGA, but *Xho*I-*Sal*I hybrid sites are resistant to cleavage with either enzyme (17). Inversion of *ilvY* was accomplished by digesting pDU60 with both *Xho*I and *Sal*I endonucleases and then ligating the fragments. The derivative of pDU60 containing *ilvY* in the opposite orientation, designated pDU64, was recognized by its identical size and its resistance to digestion by either *Xho*I or *Sal*I. Enzyme assay of strain DU650 Δ(*ilvEDAYC*)(pDU64) showed an α-acetohydroxybutyrate-inducible *galK* activity, indicating that the activity of p_C persisted despite the inverted orientation of *ilvY*. In fact, inversion of the *Xho*I-*Sal*I fragment was associated with an enhancement of basal p_C activity. Whether this results from a *cis*-active modulator of p_C activity which is present near the *Sal*I site is considered below.

The *E. coli* K-12 *ilvY* gene product is proposed to act as an antiterminator that permits readthrough transcription from *ilvY* to *ilvC*. The possibility of readthrough transcription from the *S. typhimurium ilvY* gene was examined with pDU71, which contains the intact *ilvY* gene and an additional 0.45 kb of DNA sequence beyond the *Sal*I site. Because of the propinquity of the C-terminal end of *ilvY* to *Sal*I and the p_C sequence to the *Bgl*II terminus of the pDU71 insert, it is likely that the *ilvY* transcriptional terminator resides within this region. If antitermination occurs, readthrough transcription of the adjacent *galK* gene should be detected as increased *galK* expression. The pDU71-determined *galK* activity was increased to 15 U when measured in a *S. typhimurium rho* mutant, AA111 (12). This level of activity was much lower than that seen with *ilvY-galK* fusion plasmids such as pDU71.1, suggesting that most transcription from p_Y was still properly terminated. Expression of *galK* was not enhanced by the presence of α-acetohydroxyacids in the strain AA111 *rho* background, measuring 10 U with α-acetolactate and 15 U with α-acetohydroxybutyrate. Therefore, readthrough transcription from the *S. typhimurium ilvY* gene can occur at low levels in a *rho* mutant, but this activity is not enhanced by *ilvC*-specific regulatory signals.

The results with *ilvC-galK* fusion plasmids demonstrate that the *S. typhimurium* promoter p_C is distinct and is active independently of the *ilvY* transcriptional unit. The α-acetohydroxyacids and *ilvY* gene product modulate the level of transcription initiated at p_C. Readthrough transcription of *ilvC* from the *ilvY* transcription unit probably can occur but this does not contribute significantly to the apparent activity of the *ilvC* promoter.

***trans* complementation of an *S. typhimurium ilvY* mutant.** The *ilvY702* mutation was generated by imprecise excision of Tn*10* from strain TT69 *ilvA*::Tn*10*. The phenotype of strain DU702 *ilvA ilvY702* is an isoleucine-valine bradytrophy which is explained by the low-level, noninducible *ilvC* expression permitted by the *ilvY702* mutation (see Table 7). The *ilvY702* mutation was transduced into a readily transformable *S. typhimurium* strain DU2 resulting in strain DU703, and various *ilv* recombinant plasmids were introduced for complementation studies. The original pDU1

TABLE 7. Induction of *ilvC* in an *S. typhimurium ilvY* mutant by certain *S. typhimurium ilvY*$^+$ recombinant plasmids

Strain	Medium[a]	Isomero-reductase sp act (*ilvC*)[b]
DU703 *ilvY702 leuA409*	Repressing	50
	Limiting leucine	45
DU703(pDU1 *ilvGEDAY*$^+$*C'*)	Repressing	60
	Minimal	420
DU703(pDU60 *ilvDAY*$^+$*C'*-*galK*$^+$	Repressing	40
	Limiting leucine	200
DU703(pDU63 Δ[*ilvDAY*] *ilvC'*-*galK*$^+$	Repressing	40
	Limiting leucine	42
DU703(pDU5 *ilvDAY*$^+$)	Repressing	50
	Minimal	190
DU703(pDU71 *ilvA'Y*$^+$)	Repressing	50
	Limiting leucine	50

[a] Modified Davis-Mingioli medium was supplemented with 10 mg of L-tryptophan per liter. Minimal medium additionallly contained 50 mg of L-leucine per liter.

[b] Isomeroreductase activity is expressed as nanomoles of NADPH oxidized per minute per milligram of protein.

ilvGEDAY$^+$ plasmid conferred inducibility to *ilvC* expression, indicating *trans* complementation by the plasmid *ilvY* gene (Table 7). The same result occurred with pDU60 *ilvDAY*$^+$-p_C-*galK*, but *ilvC* expression was not inducible with pDU63 p_C-*galK* since both plasmid and chromosome lack functional *ilvY* genes. These results confirm the genetic assignment of the *ilvY702* mutation and further demonstrate the *trans*-recessive nature of *S. typhimurium ilvY* mutations.

The effects of plasmids containing intact *ilvY* but lacking the p_C sequence were also examined. One plasmid, pDU5, contains a *SalI ilvEDAY*$^+$ insert that ends at the *SalI* site immediately distal to *ilvY*. Enzyme assay of strain DU703 *ilvY702*(pDU5) showed the expected inducible expression of *ilvC*. The pDU71 plasmid contains the intact *ilvY* gene as well as the distal 0.45-kb region between *SalI* and *BglII*. No induction of *ilvC* occurred in strain DU703(pDU71), suggesting that the presence of the additional sequence between *SalI* and *BglII* prevents *trans*-active complementation by *ilvY*. This effect is apparently obviated by the presence of the contiguous p_C (contained within the *BglII*-*EcoRI* region) since *trans*-complementation does occur with pDU1 and pDU60. The lack of *trans* complementation is also seen with pDU72, ruling out some orientation-specific effect peculiar to the pKO system (data not shown). The *SalI*-*BglII* region probably does not act as a negative regulator of *ilvY* expression because normal levels of the *ilvY* gene product encoded by pDU71 or pDU72 are detected in maxicell preparations (unpublished data). The possible role of the *SalI*-*BglII* region as a *cis*-active regulatory element is considered below.

DISCUSSION

The results presented herein demonstrate that the *S. typhimurium ilvC* gene is transcribed from a promoter located within the 0.42-kb *BglII*-*EcoRI* region of the cloned *ilv* fragment. This sequence is completely sufficient for acetohydroxyacid-inducible *galK* expression in the *ilvC*-*galK* fusion plasmids. The specific activities of the purified *E. coli* K-12 galactokinase and *S. typhimurium* LT2 α-acetohydroxyacid isomeroreductase enzymes have been reported previously (11, 25). If it is assumed that the copy number of pDU60 (a pBR322 derivative) is 30 per cell, then, based upon the activities of the purified enzymes, the strain C600(pDU60)-encoded *galK* expression (Table 3) corresponds to a haploid, noninduced *ilvC* activity of 3 U (nanomoles of NADPH oxidized per minute per milligram of protein) and an induced level of 25 U. Typical isomeroreductase levels for *S. typhimurium ilvG* mutants (which are most similar to *E. coli* K-12 because of the cryptic nature of *ilvG* in that organism) range from 10 to 40 U for basal expression and 100 to 500 U upon induction (D. Primerano and R. O. Burns, unpublished data). The induction ratio of *ilvC*-directed *galK* expression, therefore, is similar to that seen with the native *ilvC* gene, although the normalized *galK* activity is about 10- to 20-fold lower than the haploid *ilvC* levels. Since the *galK* gene sequence contains its own ribosome-binding site and should be translated with invariant efficiency regardless of the contiguous promoter (19), one explanation for this discrepancy is that the *galK* mRNA sequence is translated with less efficiency than the *ilvC* sequence, thus producing fewer protein products from each *galK* mRNA molecule than from *ilvC* mRNA.

Although the 0.42-kb *BglII*-*EcoRI* fragment contains the intact p_C element, the adjacent sequence also appears to modulate p_C activity. For instance, each deletion derivative of pDU60, such as pDU61 [Δ(*Xho*-*SalI*)], pDU62 (Δ*BglII*), or pDU63 [Δ(*BamHI*-*BglII*)], demonstrates a higher basal activity of p_C than does pDU60. If the region immediately 5' to p_C is altered by inversion (pDU64), an increase in p_C also occurs, although the promoter remains responsive to acetohydroxyacid induction. It appears, therefore, that the region defined by the *SalI*-*BglII* termini may exert a negative modulation of p_C activity, reducing the basal p_C level. Negative modulation is circumvented by the putative induction complex composed of the *ilvY* gene product and acetohydroxyacids, and a large component of the *ilvC* gene induction ratio can be ascribed to action at this negative regulatory region. Some facilitation of p_C activity must also occur at a site closer to p_C since all of the pDU60 derivatives (both deletion and inversion) retain some capacity for induction by acetohydroxyacids. This characteristic of pDU60 derivatives is reflected by the phenotype of the *ilvY702* mutation which was generated by an *ilvA*::Tn*10*-mediated chromosomal alteration (probably a deletion mutation). This mutation results in a fivefold elevation in the basal expression of *ilvC* (Table 6), and when *ilvY* is provided in *trans*, normal induction of *ilvC* can occur. We infer that the *ilvY702* deletion extends into the negative regulation region. Negative modulation of p_C does not require intact *ilvY* gene product since small deletions of the N-terminal region of *ilvY* do not alter p_C basal activity (Table 6, pDU60.43). Similarly, we have isolated one *ilvA*::Tn*10*-generated *ilvY* mutation which shows low basal p_C activity (5).

An additional effect of the interface region between *ilvY* and *ilvC* is suggested by the inability of certain *ilvY*$^+$ plasmids containing the *SalI*-*BglII* fragment to act in *trans* (Table 7, pDU71 and pDU72). If the *SalI*-*BglII* region is altered by deletion or if it is coupled with the contiguous p_C sequence, then the *ilvY* gene can act in *trans* to effect *ilvC* induction (Table 7, pDU5 or pDU6 and pDU60, respectively). Although these observations are as yet preliminary, we are intrigued by the finding that this region also appears to exert negative modulation of p_C and may be a site at which the *ilvY* gene product acts to increase p_C activity. One

possibility is that the *ilvY* gene product avidly binds to this sequence in the absence of acetohydroxyacids but does not interact with p_C. The binding of the coinducers to the *ilvY* gene product could then permit the induction complex to interact with the contiguous p_C sequence and increase transcription of *ilvC*. The activated induction complex may then be *trans* active. In the absence of the contiguous p_C sequence (as in pDU71 and pDU72), the necessary transcriptional interaction to form and then release activated complex could not occur. The nature of the *ilvY-ilvC* interface is currently under investigation.

Analysis of the *E. coli* K-12 *ilvY-ilvC* system employing *ilvY* insertion mutations has led to the conclusion that *ilvC* expression is dependent upon transcription initiated at the *ilvY* promoter. This is in clear distinction to the *S. typhimurium* system, in which it is apparent that *ilvY* and *ilvC* constitute separate transcriptional units. It is certainly possible that these two species utilize disparate control mechanisms to govern *ilvC* expression. Another interpretation is that the Tn*10* and Mu insertion mutations in the *E. coli ilvY* gene are located within the interface region between *ilvY* and *ilvC*. These mutations could be positioned so as to permit binding of the *ilvY* gene product but prevent its interaction with the contiguous *ilvC* promoter. This would negate *cis*-active induction of *ilvC* as well as *trans* induction since the putative release of activated induction complex after interaction with p_C could not occur. Further analysis of both the *E. coli* K-12 and *S. typhimurium ilvY-ilvC* systems is needed to clarify these possibilities.

ACKNOWLEDGMENTS

As always, we thank Dorothy Thompson and Dayle Wilkins for their considerable assistance.

This work was supported in part by Public Health Service grant GM12551 from the National Institutes of General Medical Sciences. D.L.B. was a trainee in the Medical Scientist Training Program and was funded by Public Health Service grant GM07171 from the National Institutes of Health.

LITERATURE CITED

1. **Arfin, S. M., B. Ratzkin, and H. E. Umbarger.** 1969. The metabolism of valine and isoleucine in *Escherichia coli*. XVII. The role of induction in the derepression of acetohydroxy acid isomeroreductase. Biochem. Biophys. Res. Commun. **37:** 902–908.
2. **Arfin, S. M., and H. E. Umbarger.** 1969. Purification and properties of acetohydroxy acid isomeroreductase of *Salmonella typhimurium*. J. Biol. Chem. **244:**1118–1127.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
4. **Blazey, D. L., and R. O. Burns.** 1979. Genetic organization of the *Salmonella typhimurium ilv* gene cluster. Mol. Gen. Genet. **177:**1–11.
5. **Blazey, D. L., and R. O. Burns.** 1980. Gene *ilvY* of *Salmonella typhimurium*. J. Bacteriol. **142:**1015–1018.
6. **Blazey, D. L., R. Kim, and R. O. Burns.** 1981. Molecular cloning and expression of the *ilvGEDAY* genes from *Salmonella typhimurium*. J. Bacteriol. **147:**452–462.
7. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring either methionine or vitamin B12. J. Bacteriol. **60:**17–28.
8. **Freundlich, M., R. O. Burns, and H. E. Umbarger.** 1962. Control of isoleucine, valine and leucine biosynthesis. I. Multivalent repression. Proc. Natl. Acad. Sci. U.S.A. **48:**1804–1808.
9. **Gray, H. B., D. A. Ostrander, J. L. Hodnett, R. J. Legerski, and D. L. Robberson.** 1975. Extracellular nucleases of *Pseudomonas* BAL-31. I. Characterization of single-strand specific deoxyribonuclease and double-strand deoxyribonuclease activities. Nucleic Acids Res. **2:**1459–1492.
10. **Greene, P. J., M. C. Betlach, H. W. Boyer, and H. M. Goodman.** 1974. The *Eco*RI restriction endonuclease. Methods Mol. Biol. **7:**87–105.
11. **Hofler, J. G., C. J. Decedue, G. H. Lunginbuhl, J. A. Reynolds, and R. O. Burns.** 1975. The subunit structure of α-acetohydroxyacid isomeroreductase from *Salmonella typhimurium*. J. Biol. Chem. **250:**877–882.
12. **Housley, P. R., A. D. Leavitt, and H. J. Whitfield.** 1981. Genetic analysis of a temperature-sensitive *Salmonella typhimurium rho* mutant with an altered *rho*-associated polycytidylate-dependent adenosine triphosphatase activity. J. Bacteriol. **147:**13–24.
13. **Kleckner, N., K. Reichardt, and D. Botstein.** 1979. Inversions and deletions of the *Salmonella* chromosome generated by the translocatable tetracycline resistance element Tn*10*. J. Mol. Biol. **127:**89–115.
14. **Lederberg, E. M., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119:**1072–1074.
15. **Legerski, R. J., J. L. Hodnett, and H. B. Gray.** 1978. Extracellular nucleases of *Pseudomonas* BAL-31. III. Use of the double-strand deoxyribonuclease activity as the basis of a convenient method for mapping of fragments of DNA produced by cleavage with restriction enzymes. Nucleic Acids Res. **5:**1445–1464.
16. **Loening, U. E.** 1969. The determination of the molecular weight of ribonucleic acid by polyacrylamide gel electrophoresis. The effects of changes in conformation. Biochem. J. **113:**131–138.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **McCorkle, G. M., T. D. Leathers, and H. E. Umbarger.** 1978. Physical organization of the *ilvEDAC* genes of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U.S.A. **75:**89–93.
19. **McKenney, K., H. Shimatake, D. Court, U. Schmeissner, C. Brady, and M. Rosenberg.** 1981. *In* J. Chirikian and T. Papas (ed.), Gene amplification and analysis, vol. 2, p. 383–415. Elsevier, Amsterdam.
20. **Taillon, M. P., D. Amanda-Gotto, and R. P. Lawther.** 1981. The DNA sequence of the promoter-attenuation of the *ilvGEDA* operon of *Salmonella typhimurium*. Nucleic Acids Res. **9:**3419–3432.
21. **Umbarger, H. E.** 1978. Amino acid biosynthesis and its regulation. Annu. Rev. Biochem. **47:**533–608.
22. **Watson, M. D., J. Wild, and H. E. Umbarger.** 1979. Positive control of *ilvC* expression in *Escherichia coli* K-12: identification and mapping of regulatory gene *ilvY*. J. Bacteriol. **139:**1014–1020.
23. **Weiss, B., A. Jacquemin-Sablon, T. R. Live, G. C. Fareed, and C. C. Richardson.** 1968. Enzymatic breakage and joining of deoxyribonucleic acid. VI. Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4. J. Biol. Chem. **243:**4543–4555.
24. **Wild, J., J. M. Smith, and H. E. Umbarger.** 1977. *In vitro* synthesis of β-galactosidase with *ilv-lac* fusion deoxyribonucleic acid as template. J. Bacteriol. **132:**876–883.
25. **Wilson, D. B., and D. S. Hogness.** 1969. The enzymes of the galactose operon in *Escherichia coli*. The frequencies of translation of the terminal cistrons of the operon. J. Biol. Chem. **244:**2143–2148.