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Analysis of Regulation of the *ilvGMEDA* Operon by Using Leader-Attenuator-*galK* Gene Fusions

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Five of the genes for the biosynthesis of isoleucine and valine form the *ilvGMEDA* operon of *Escherichia coli* K-12. Expression of the operon responds to changes in the availability of isoleucine, leucine, and valine (ILV). Addition of an excess of all three amino acids results in reduced expression of the operon, whereas limitation for one of the three amino acids causes an increase in expression. The operon is preceded by a leader-attenuator which clearly regulates the increased expression that occurs due to reduced aminoacylation of tRNA. To assess the factors that result in the reduced expression of this operon upon the addition of ILV, a series of plasmids were constructed in which the *ilv* regulatory region was fused to *galK*. In response to addition of the amino acids, expression of the *galK* gene fused to the leader-attenuator decreased five- to sevenfold, instead of the twofold observed for the chromosomal operon. A deletion analysis with these plasmids indicated that the ILV-specific decrease in expression required an intact leader-attenuator but not *ilvGp2* or the DNA that precedes this promoter. This conclusion was supported by both S1 nuclease analysis of transcription initiation and determination of *galK* mRNA levels by RNA-RNA hybridization.

Wild-type *Escherichia coli* K-12 can synthesize all its molecular components when grown on a minimal salts medium containing suitable carbon and nitrogen sources. During steady-state or exponential growth, the cells presumably synthesize the appropriate quantity of each metabolite so as to achieve optimal growth. This is facilitated by the ability of the cell to increase or decrease the synthesis of enzymes or alter the catalytic activity of specific enzymes in response to changes in the environment in which the cell is growing. For several amino acid-biosynthetic systems, the cell responds to an exogenous source of an amino acid (i.e., an excess) by decreasing (i.e., repressing) the expression of the relevant genes. Alternatively, if the amount of an amino acid becomes growth limiting (i.e., starvation), the bacteria will increase expression of the genes required for the biosynthesis of that amino acid.

The *trp* operon is probably the most thoroughly studied set of genes for the biosynthesis of an amino acid. For the *trp* operon, the molecular mechanisms (34, 50) that result in the change in expression of this operon in response to either an excess or limitation for tryptophan are distinct. The *trp* repressor protein regulates the operon by mediating the initiation of transcription by RNA polymerase (RNAP) in response to an excess of free tryptophan within the cell. When growth is limited by tryptophan, increased expression of the operon occurs as a result of reduced aminoacylation of *trp* tRNA. This change in *trp* operon expression is mediated by the leader-attenuator. As described by Yanofsky et al. (50), the combination of the repressor-operator and the leader-attenuator results in a 500- to 600-fold range in expression of the *trp* operon.

The *ilvGMEDA* operon encodes four of the enzymes for the biosynthesis of the branched-chain amino acids isoleucine, leucine, and valine (ILV) (26). These genes are multivalently regulated by all three amino acids, i.e., repression of the operon requires the presence of all three amino acids, while growth limitation for any one of the three results in an increase in expression of the operon (11). No regulatory protein has been identified that modulates expression of the operon in response to changes in the availability of the three amino acids. A leader-attenuator precedes the structural genes, and the proposed leader peptide includes residues of all three amino acids, consistent with the multivalent regulation of the operon (2, 25, 26, 35). The data accumulated for the response of this operon to amino acid limitation are consistent with its being mediated via the leader-attenuator (26). Using an *ilvD-lacZ* fusion, Bennett and Umbarger (4) isolated a pair of deletions that extended into the leader-attenuator. Their results indicate that the leader-attenuator sequences participate in changes in operon expression in response to either a limitation for one or an excess of all three amino acids. Clearly, limitation for an amino acid results in a decrease in aminoacylation of the cognate tRNAs. Both theoretical (30, 46, 47) and experimental (reviewed in reference 20) analyses indicate that reduced transcription termination at an attenuator requires a relatively large change in the level of aminoacylation. This contrasts with the response of an *E. coli* wild type grown on minimal medium to which the three branched-chain amino acids are added. In minimal medium, the level of aminoacylation is very high (approaching 100% in some studies); therefore, it seems unlikely that the addition of ILV will dramatically alter the level of aminoacylation. If both the analyses of attenuation and the assumption that tRNA is not limiting (quality, aminoacylation, etc.) are correct, then the question arises of how repression is mediated by the leader-attenuator of the *ilvGMEDA* operon. This seems to imply that the decrease in gene expression must result from subtler changes affecting the attenuator than are required for increased expression.

In this study, the role of the leader-attenuator in repression has been evaluated more thoroughly. A set of recombinant DNA plasmids in which the *ilv* regulatory sequences were fused to the *galK* gene were used. Our data clearly indicate that the decrease in expression of the *ilv* genes (upon the addition of ILV) is not modulated at transcription

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TABLE 1. Plasmids

Plasmid	Description	Reference
pDR720	*trpEp-galK* expression vector	42
pKO4, pKO6	*galK* fusion vectors	31
pT7/T3-18	T7 and T3 RNAP expression vector	
pRL137	700-bp *Hae*III-*Alu*I restriction fragment extending from 50 bp upstream of *ilvGp2* through the initial portion of *ilvG* inserted into pKO6 (Fig. 1)	37
pMO139	1,100-bp *Alu*I restriction fragment extending 450 bp upstream of transcription initiation site (*ilvGp2*) through the initial 650 bp of *ilvG* inserted into pKO6 (Fig. 1)	37
pRL149	170-bp *Sau*3AI-*Rsa*I restriction fragment including the attenuator (*ilvGa*) inserted into pDR720 between *trpEp* and *galK* (Fig. 1)	This study
pMO167	180-bp *Hae*III-*Sau*3A restriction fragment including *ilvGp2* and the *ilv* leader (*ilvGe*) inserted into pKO4 (Fig. 1)	37
pKB212	300-bp *Hae*III-*Hin*fI restriction fragment including *ilvGp2* and the attenuator (*ilvGa*), inserted into pKO6 (Fig. 1)	This study
pCR276	600-bp *Taq*I-*Alu*I restriction fragment including *ilvGe*, *ilvGa*, and proximal portion of *ilvG* inserted into pDR720 between *trpEp* and *galK* (Fig. 1)	This study
pDM287	Identical to pRL137 except for the mutation *ilvGA951*, G → A transition at bp 177 (Fig. 1)	21
pMW333	Identical to pRL137 except for the mutation *ilvGe952*, T → C transition at bp 34 (Fig. 1)	21

initiation but occurs at the leader-attenuator. The addition of all three amino acids to minimal medium results in a five- to sevenfold reduction of galactokinase expression when the *galK* gene is fused to the *ilv* regulatory region. Furthermore, analysis of both the initiation of RNA synthesis and steady-state levels of *galK* mRNA indicate that the decrease in gene expression is mediated by the leader-attenuator. Thus, unlike the *trp* operon, one mechanism (attenuation) serves to modulate expression of the *ilvGMEDA* operon in response to either an excess of the amino acids or limitation for one of them.

MATERIALS AND METHODS

Bacteria, plasmids, and medium. The *E. coli* K-12 strain used in this study was M152 [*galK2 recA3 rpsL200* IN(*rrnD-rrnE*)*1*], which was obtained from the *E. coli* Genetic Stock Center. Plasmid pT7/T3-18 was obtained from Bethesda Research Laboratories, Inc., and the other plasmids used in this study are described in Table 1 (with reference to Fig. 1). M63 minimal medium was prepared as described by Miller (32) and contained ampicillin (100 μg/ml). The amino acids isoleucine and leucine were added to a final concentration of 0.5 mM, while the concentration of valine was 1.0 mM.

Enzymes and biochemicals. Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were obtained from New England BioLabs, Inc. S1 nuclease and [α-^{32}P]UTP were obtained from New England Nuclear Corp. T3 and T7 DNA-dependent RNAP were obtained from Bethesda Research Laboratories, Inc. Calf alkaline phosphatase came from Boehringer Mannheim Biochemicals. Nucleoside triphosphates were supplied by P-L Biochemicals, Inc. [γ-^{32}P]ATP was obtained from ICN Pharmaceuticals, Inc. D-[1-^{14}C]galactose was obtained from Amersham Corp. All other reagents were obtained from Sigma Chemical Co.

Determination of galactokinase and threonine deaminase. Galactokinase was assayed for the conversion of galactose

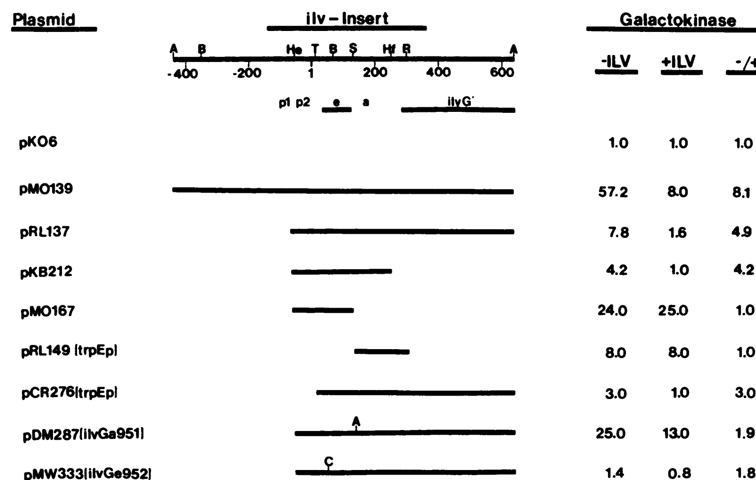

FIG. 1. Deletion analysis of repression of the *ilvGMEDA* operon by using *ilv-galK* fusions. A partial restriction site map of the *ilv* regulatory region is presented, and the segment inserted into each construct is indicated by a line. Distances within the *ilv* regulatory region are indicated in base pairs. Restriction sites indicated: A, *Alu*I; B, *Bst*NI; He, *Hae*III; Hf, *Hin*fI; R, *Rsa*I; S, *Sau*3AI; T, *Taq*I. The promoters *ilvGp1* and *ilvGp2* are indicated as p1 and p2, respectively. The leader (*ilvGe*) is indicated by e, and the attenuator (*ilvGa*) is shown by a, in accordance with the nomenclature proposed by Bachmann et al. (3). The primary promoter for the *trp* operon is designated *trpEp*. The two *ilv* mutations are *ilvGpa951*, which changes bp 177 from G to A, disrupting the attenuator stem, and *ilvGe952*, which changes the translation initiation codon from AUG to ACG at bp 34 (21). Galactokinase units are in nanomoles of galactose 1-phosphate formed per minute per milligram of protein per femtomole of plasmid and are shown with (+) and without (−) the addition of ILV; the −ILV/+ILV ratio is also shown (−/+).

to galactose 1-phosphate, as measured by the quantity of [^{14}C]galactose bound to DEAE filters. The galactokinase assay and the determination of plasmid copy number were done as described previously (37). Threonine deaminase was assayed as described by Lawther and Hatfield (24), in an assay that measures the conversion of threonine to α-ketobutyrate by reaction with 2,4-dinitrophenylhydrazine. Specific activities are the average of at least three independent assays of each plasmid construct. Protein was determined by the method of Bradford (5) with a reagent obtained from Bio-Rad Laboratories.

Quantitation of transcription initiation by S1 nuclease analysis. Cellular RNA was prepared as described by Salser et al. (43). The RNA concentration was determined by measuring the A_{260}. The 416-base-pair (bp) *Bst*NI restriction fragment (from −356 to +60 bp, Fig. 1) was isolated by standard procedures. The DNA fragment was 5′-end labeled with T4 polynucleotide kinase and then treated with exonuclease III to generate single-stranded probe (28, 33). The DNA probe was hybridized with cellular RNA and treated with S1 nuclease as previously described (28). The products of the S1 digestion were resolved by electrophoresis on an 8 M urea–20% polyacrylamide gel, visualized by autoradiography, and quantitated by densitometric analysis.

Quantitation of *galK*-specific mRNA by RNA dot blot hybridization. To generate labeled RNA for use as hybridization probes, the pT7/T3-18 derivative pJL286 (Table 1) was transcribed in vitro. The vector plasmid insert resides between the T7 and T3 bacteriophage promoters, and thus complementary hybridization probes can be generated. To yield RNA complementary only to the insert, the plasmid was linearized by digestion with the appropriate restriction endonuclease. Transcription reactions were conducted in vitro in a 10-μl volume at 37°C for 1 h. Each reaction mix contained 250 ng of template DNA, 5 mM dithiothreitol, 400 μM each ATP, GTP, and CTP, 10 μM [α-^{32}P]UTP (specific activity, 800 Ci/mmol), the reaction buffer [40 mM Tris hydrochloride (pH 8.0), 8 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine-$(HCl)_3$], and 20 U of either T7 or T3 RNAP. The reactions were terminated by the addition of 1 μl of 100 mM EDTA. The RNA products were precipitated from 0.3 M sodium acetate (pH 5.5) with 2.5 volumes of 100% ethanol by incubating in a dry ice-ethanol bath for 20 min. The RNA was then collected by centrifugation in a microcentrifuge (15,000 × *g*, 15 min).

Cellular RNA was filtered onto Nytran with a Schleicher & Schuell Minifold filtration apparatus. Two sheets of dry 3MM Whatman filter paper placed underneath the Nytran served to draw the nucleic acid solution by capillary action through the Nytran that was preequilibrated with 6× SSPE (20× SSPE is 3 M NaCl, 20 mM EDTA, 0.2 M $NaPO_4$ [pH 7.7]). RNA was denatured in 100 μl of filter-sterilized 6% formaldehyde–30 mM $NaPO_4$ (pH 6.8)–1 M NaCl by incubating at 65°C for 5 min. Once denatured, the RNA was immediately filtered onto the membrane, the apparatus was disassembled, and the blot was soaked in 6× SSPE until the aroma of the formaldehyde could no longer be detected. The blots were annealed in vacuo at 80°C for 2 h and stored at room temperature.

Prehybridization of the filter was accomplished by overnight incubation at 65°C in 50% formamide–5× SSPE, 5× Denhardt reagent (50× Denhardt reagent is 2% Ficoll 400, 2% polyvinylpyrrolidone, 2% bovine serum albumin) or 1% sodium dodecyl sulfate (SDS)–100 μg of denatured calf thymus DNA per ml. The prehybridization solution was then replaced with fresh hybridization solution, and the RNA probe was added. The blot was hybridized overnight at 65°C and then washed twice for 20 min in 2× SSPE–0.1% SDS at room temperature, followed by two washes for 20 min each in 0.1× SSPE–0.1% SDS at 65°C.

RESULTS

Effect of the addition of amino acids on expression of galactokinase from *ilv-galK* fusions. To analyze the multivalent regulation of the *ilvGMEDA* operon by ILV, a series of plasmids were constructed by inserting different segments of the *ilv* regulatory region into the pKO *galK* expression vector system (Table 1, Fig. 1). Each plasmid was transfected into M152 (*galK2*), and the specific activity of galactokinase was determined for cells grown in M63 minimal medium in the absence or presence of ILV. This analysis indicated that the ILV-modulated decrease in expression required the sequences that include the *ilvGMEDA* leader-attenuator.

As described previously (1, 26, 37), the DNA sequences upstream of the site for the initiation of transcription in vivo (*ilvGp2*, Fig. 1) enhance transcription from *ilvGp2* by roughly eightfold. This is evidenced in Fig. 1 by a comparison of the specific activity of galactokinase for M152 transfected with pMO139 compared with that for M152 containing pRL137 (deletion of upstream DNA). Because these DNA sequences influence expression from *ilvGp2*, it is possible that they are involved in the amino acid-specific regulation of the operon. The data in Fig. 1 demonstrate that the DNA upstream of *ilvGp2* did not serve a role in amino acid-specific regulation. A similar reduction in the specific activity of galactokinase was observed for both pRL137- and pMO139-containing bacteria upon the addition of ILV (Fig. 1). Because the upstream region was not involved in amino acid-mediated regulation, it was not included in any subsequent plasmid constructs. Comparison of the decrease in expression of galactokinase from pRL137 with that from pKB212 indicated that the decrease in expression (upon the addition of ILV) did not require the DNA sequences downstream of the *ilv* attenuator (*ilvGa*). The reduced expression observed from the plasmid pKB212 relative to that from pRL137 was a translational effect (i.e., polar), on *galK* expression due to the deletion of the *ilvG* translation initiation site. This conclusion is based upon the results of inserting into pKB212 an oligonucleotide that included the *ilvG* translation initiation site (R. P. Lawther and K. Bell, unpublished observations). Thus, comparison of galactokinase expression from the plasmids pMO139, pRL137, and pKB212 indicates that the reduced expression upon the addition of ILV requires sequences that include *ilvGp2* through the attenuator.

The absence of ILV repression of galactokinase expression from the plasmids pMO167 and pRL149 demonstrates that ILV-specific regulation requires an intact *ilv* regulatory region (Fig. 1). Plasmid pMO167 contains a restriction fragment that includes *ilvGp2* through the carboxy terminus of the leader peptide. Expression of galactokinase from this plasmid was unaffected by the addition of ILV to the medium. Plasmid pRL149 was constructed from plasmid pDR720 by inserting the attenuator (i.e., terminator), *ilvGa*, downstream of the *trp* promoter (*trpEp*). As shown in Fig. 1, the presence of ILV had no effect on expression of galactokinase from pRL149. Galactokinase expression from plasmid pCR276 demonstrated that *ilvGp2* was not required for ILV-specific regulation (Fig. 1). This plasmid was constructed by inserting a fragment from +10 through +645 bp

into pDR720 (Table 1 and Fig. 1). Thus, this plasmid is analogous to pRL137 except that *ilvGp2* has been replaced by the *trp* operon promoter, *trpEp*. Although the extent of repression was reduced from approximately fivefold to threefold, the observed differences are inconsistent with ILV regulation being mediated at *ilvGp2*. Together, all the data indicate that ILV repression requires that the *ilvGMEDA* leader-attenuator be intact.

Analysis of the regulation of *galK* expression from the two plasmids pDM287 and pMW333 (containing point mutations [21]) further supports the role of the leader-attenuator in ILV-specific repression. The stem of the attenuator, *ilvGa*, contains five contiguous G residues. In plasmid pDM287, the central G has been replaced by an A (*ilvGa951*), which reduces the stability of the attenuator (21). The unrepressed level of galactokinase for pDM287 was identical to that for pMO167, in which the attenuator is totally absent. Plasmid pMW333 contains a mutation (*ilvGe952*) of the initiation codon for the leader peptide, changing the AUG codon to ACG. For each of these plasmids there was slightly less than a twofold effect on expression after the addition of ILV. For pMW333, the low level of expression that remained after the translation initiation codon was altered (AUG to ACG) has in the case of other operons been ascribed to a continued low level of translation initiation (20). The reduction of expression from pDM287 upon the addition of ILV appears to indicate that substitution of A for G in the stem of the attenuator limits its effectiveness as a terminator but that under repressing conditions it retains some effectiveness as a terminator of transcription.

Analysis for deattenuation. Previous studies of the effect of the addition of ILV on gene expression have usually assayed transaminase B (*ilvE*) and threonine deaminase (*ilvA*), the third and fifth genes of the operon, respectively (23). Those studies demonstrated two- to threefold ILV repression of the operon. Therefore, the possibility existed that the five- to sevenfold decrease observed with the *ilv-galK* fusions was the result of a combination of the two aspects of ILV-specific regulation of the *ilvGMEDA* operon. Thus, the five- to sevenfold differential would be due to both the effect of an excess of the three amino acids (i.e., the twofold repression observed for the chromosomal operon) and, in part, a limitation for one of the three amino acids (because of the presence of the plasmids) causing reduced transcription termination of the attenuator. Whether a limitation for isoleucine, leucine, or valine explains why the repression of the *ilv-galK* fusions was greater than expected (i.e., fivefold instead of twofold) was examined by two approaches. If the specific activity of galactokinase observed in the absence of ILV is the function of a limitation for one of the three amino acids or an aminoacylated tRNA, then the effect should occur in *trans* and expression of the *ilvGMEDA* operon should increase. The specific activity of threonine deaminase in M152 grown on unsupplemented minimal medium was 95 nmol/min per mg of protein, and the introduction of either the parental vector pKO6 or the plasmid pRL137 did not alter the specific activity of threonine deaminase (specific activities of 99 and 95 nmol/min, respectively). Additionally, the response of threonine deaminase to the presence of an excess of ILV or limitation for one of the branched-chain amino acids was unaffected by the presence of the plasmids (data not shown). Together, these observations indicate that amino acid limitation (caused by the introduction of plasmids) does not explain the five- to sevenfold effect on *galK* expression.

As an alternative to the analysis of threonine deaminase,

TABLE 2. Analysis of repression of the *ilv-galK* fusions as single-copy DNA inserted into the *E. coli* K-12 genome

Bacteriophage (equivalent plasmid)	Galactokinase sp act (nmol/min per mg of protein)		
	−ILV	+ILV	−ILV/+ILV ratio
λ65 (pKO6)	UD[a]	UD	
λ250 (pRL137)	1.3	0.15	8.3
λ305 (pMO167)	7.0	6.2	1.1

[a] UD, Undetectable.

the *ilv-galK* fusions were introduced as single-copy DNA into M152. This was achieved by using λ65 a derivative of λgt4 (9) into which *npt* (the gene for kanamycin resistance from transposon Tn*5*) and *galK* had been introduced. The bacteriophage (λ65) was constructed with the *npt* and *galK* genes adjacent to one another but oriented in opposite directions without promoters (i.e., npt^-, $galK^-$). To facilitate the transfer of an *ilv-galK* construct from a plasmid to λ65, a functional *npt* gene was inserted into the plasmid in opposite orientation to *galK* by using a *Bam*HI cassette. The *ilv-galK* fusions were then transferred to the virus by homologous recombination between the *npt* and *galK* genes of the virus and the plasmid. The recombined viral product was retrieved from lysates by selection of kanamycin-resistant lysogens of M152. The virus was purified by standard techniques (9), and the structures of the bacteriophage and their lysogens were analyzed by digestion with restriction endonucleases (R. Pereira and R. P. Lawther, unpublished observations). M152 was lysogenized with the viruses corresponding to the plasmid vector (λ65, i.e., no promoter preceding *galK*), pRL137 (λ250), and pMO167 (λ305). The lysogenized bacteria were grown in M63 medium with and without the addition of ILV. The addition of ILV resulted in about an eightfold decrease in *galK* expression when it was fused to an intact leader-attenuator (λ250), whereas the addition of ILV had little effect if the attenuator was deleted (λ305) (Table 2). These results further support the plasmid analysis results and indicate not only that the degree of repression is not due to an amino acid limitation but also that it is not a function of the overall structure, conformation, or copy number of the plasmids.

Analysis of transcription initiation from *ilvGp2*. The data obtained by analysis of galactokinase expression are most consistent with ILV regulation being modulated via the leader-attenuator. To determine whether this regulation is attenuator-modulated requires knowing whether transcription initiation remains unchanged relative to the level of *galK* expression. To achieve this, transcription initiation was quantitated by S1 nuclease analysis. The 416-bp *Bst*NI restriction fragment, from −356 bp upstream to 60 bp downstream of the in vivo transcription initiation site (Fig. 1), was labeled with T4 polynucleotide kinase and converted to single-stranded probe by digestion with exonuclease III (28, 33). RNA was prepared from M152 transfected with plasmid pRL137, pMO167, or pDM287. Each strain was grown in M63 minimal medium with or without ILV. The single-stranded DNA probe was hybridized with the RNA and subsequently treated with S1 nuclease. The products of digestion were separated by electrophoresis on an 8 M urea–20% polyacrylamide gel and localized by autoradiography. The amount of protected product was quantitated by densitometric analysis.

The addition of ILV to M63 minimal medium had only a slight effect on initiation of transcription from *ilvGp2* as

TABLE 3. Effect of ILV on initiation from *ilvGp2* determined by S1 nuclease analysis

Plasmid	Quantity of RNA (cpm/μg of RNA)		
	−ILV	+ILV	−ILV/+ILV ratio
pRL137	716	627	1.14
pMO167	445	362	1.2
pDM287	673	548	1.21

measured by using S1 nuclease to quantitate the 5′ terminus of the RNA (Table 3). Since the product of S1 digestion corresponded to the previously determined 5′ terminus of the *ilv* leader RNA (2, 37), it seems reasonable to conclude that the S1 analysis measures initiation. The addition of ILV caused a fivefold decrease in the specific activity of galactokinase in M152 containing pRL137, while *galK* expression originating from pMO167 was unaffected. However, the ratio of transcription initiation without and with ILV (−ILV/+ILV) for *galK* (as measured by S1 nuclease analysis) was roughly 1.2 for both plasmids. Similarly, the attenuator mutation *ilvGa951* (pDM287) yielded the same ratio. Thus, analysis of the initiation of transcription results is consistent with the analysis of galactokinase activity results, i.e., repression is not mediated at transcription initiation.

Quantitation of *galK*-specific mRNA. As described above, the data presented for the expression of the *ilv-galK* fusions indicate that the ILV-specific decrease in gene expression is modulated through the leader-attenuator. If the decrease in expression reflects alterations in transcription downstream of the attenuator, then the quantity of *galK* mRNA should fluctuate accordingly. Galactokinase mRNA was quantitated by dot blot hybridization. To facilitate this analysis, a unique plasmid was constructed by using the vector pT7/T3-18. A 418-bp *AluI* restriction fragment that includes DNA sequences encoding the amino-terminal portion of galactokinase was inserted into the *BamHI* restriction site of pT7/T3-18 to yield pJL286. The vector contains two promoters on opposite sides of a polylinker. One promoter is only recognized by T3 RNAP, while the second is only recognized by T7 RNAP. Transcription of pJL286 with T3 RNAP yielded an RNA molecule with a sequence identical to that of the galactokinase mRNA. Transcription of the plasmid with T7 RNAP yielded an RNA with a sequence complementary to galactokinase mRNA. T3 and T7 RNAP-directed transcripts were labeled with [α-^{32}P]UTP. These RNAs were hybridized to Nytran filters spotted with 0.5, 1.0, and 2.0 μg of RNA extracted from M152 transfected with the plasmids indicated in Table 4. The RNA-RNA hybrids were localized by autoradiography, and the radioactivity was determined for each spot by liquid scintillation counting.

No cellular RNA was detected with the probe generated by using T3 RNAP (sequence identical to *galK* mRNA). The data obtained with the probe generated by using T7 RNAP (sequence complementary to *galK* mRNA) are presented in Table 4. The RNA extracted from M152 transfected with pRL137 indicated that the addition of ILV resulted in nearly a fourfold decrease in *galK*-specific RNA (a fivefold difference was observed for galactokinase activity, Fig. 1). RNA extracted from M152 containing pDM287 showed a twofold difference without and with ILV, similar to the change observed in galactokinase specific activity. RNA extracted from M152 containing pRL149 or pMO167 showed no differences without or with ILV, in agreement with the observations for galactokinase. The result for pMO167 was especially gratifying because the −ILV/+ILV ratio was identical whether transcription initiation (Table 3) or *galK* mRNA levels (Table 4) were measured. It should also be noted that in the absence of ILV, the *trp* promotor construct pRL149 yielded approximately one-half the quantity of mRNA obtained from the *ilv* plasmid (pRL137) but both plasmids yielded similar quantities of galactokinase (compare Table 1 with Table 4). This difference may be related to observations made for *trpE* (8) and the bacteriophage T7 (36), in which nucleotide sequences upstream of the translation initiation site affect the efficiency of translation of an mRNA.

TABLE 4. Quantitation of *galK*-specific RNA by hybridization

Plasmid	Quantity of *galK* RNA (cpm/μg of RNA)		
	−ILV	+ILV	−ILV/+ILV ratio
pRL137	220	60	3.7
pDM287	745	320	2.3
pMO167	1,110	950	1.2
pRL149	120	100	1.2

DISCUSSION

As described in the Introduction, the *ilvGMEDA* operon responds in two ways to ILV-specific regulation. Limitation for one of the three branched-chain amino acids causes an increase in expression, while the concomitant addition of all three amino acids causes a decrease in expression. Both the structure of the leader-attenuator and the known effect of a decrease in aminoacylation of ILV tRNA indicate that the effect of amino acid limitation is mediated by attenuation. The data discussed in this report demonstrate that the leader-attenuator also has a functional role in the response of the operon to an excess of the three amino acids. As shown in Fig. 1, the addition of ILV resulted in a five- to sevenfold decrease in galactokinase activity, and this effect required that the leader-attenuator but not *ilvGp2* be intact. The data presented in Table 4 demonstrate that transcription initiation is unaffected by the addition of ILV while transcription distal to the attenuator decreases in response to the addition of ILV. What is especially gratifying is that the effect of the addition of ILV on both the initiation of transcription and *galK*-specific mRNA levels was identical for pMO167 (which was not affected by ILV). Together these observations fulfill the classic criteria for regulation by attenuation or transcription termination, i.e., initiation of transcription remains unaffected while expression of a gene (*galK*) downstream of the leader-attenuator changes in response to a physiological signal (the addition of ILV).

An unexpected aspect of these experiments was the five- to sevenfold decrease in expression of the *ilv-galK* fusions compared with the twofold change previously observed for *ilvE* and *ilvA* in the chromosome (23). The analysis of the specific activity of both threonine deaminase and galactokinase (for the *ilv-galK* fusions inserted into the chromosome, Table 2) indicates that this difference does not occur because the plasmid-containing bacteria are limited for aminoacylated tRNA. A further indication that the five- to sevenfold decrease in *galK* expression caused by ILV is not likely to be due to an alteration of the aminoacylation of isoleucyl-, leucyl-, or valyl-tRNA is that addition of the three amino acids did not alter the rate of growth of M152. This is contrary to the expectation that if the aminoacylation of the tRNA of one of these amino acids was limiting (i.e., resulting in a decrease in attenuation), the rate of growth of the

bacteria would be stimulated upon its addition. These observations on the absence of an effect on the rate of growth of the bacteria are consistent with the cells' not being limited for one of the three amino acids, but they do not preclude the possibility that the aminoacylation of the tRNA could be reduced sufficiently to affect attenuation but not the rate of growth of the bacteria. Thus, the observed five- to sevenfold decrease is not due to a combination of limitation for one amino acid and an excess of all three. The limited change in expression observed for the wild-type operon probably results from two aspects of its structural organization. First, as a result of a frameshift site within *ilvG*, expression of *ilvEDA* from *ilvGp2* is reduced due to polarity (22, 29). Second, an internal promoter, *ilvEp*, lies within *ilvM* (28, 48) and accounts for a substantial fraction of the expression of *ilvEDA* (29). Since the effect of the addition of ILV occurs at the leader-attenuator, the combination of polarity on expression from *ilvGp2* and the presence of *ilvEp* limits the extent of the effect of the ILV addition to the distal portion of the operon. Thus, the difference (five- versus twofold) observed in change in gene expression for the *ilv-galK* fusions versus previous analysis of the chromosomal operon is due to the structure of the wild-type operon.

The subject of this study is the manner or mechanism by which ILV-specific decrease in gene expression occurs. The data presented indicate that the nucleic acid sequences that constitute the leader-attenuator modulate this physiological response. Two obvious models exist, i.e., the binding of an ILV-specific repressor or modulation via attenuation. As discussed, no ILV-specific regulatory protein which accounts for multivalent regulation has been identified, and currently the existence of such a protein seems unlikely. Thus, the most probable mechanism for mediating the effect of an excess of ILV is attenuation. Investigations of the regulation of amino acid biosynthesis demonstrate that several factors can alter the efficiency of attenuation. Besides the physiological state of tRNA (6), these include translation of the leader RNA (20), the coupling of translation to transcription (20), and the secondary structure of the leader RNA (20).

The importance of tRNA in the regulation of amino acid biosynthesis was first indicated by the demonstration that reduced aminoacylation of tRNA caused an increase in gene expression (10). These observations were extended by the isolation of mutations that caused an increase in the expression of the *his* operon due to alterations in the metabolism of $tRNA^{His}$. Mutant strains (27, 41) demonstrated that expression of the *his* operon was affected by alteration of (i) the percentage or level of tRNA aminoacylation, (ii) the quantity of tRNA, and (iii) tRNA modification. Presumably, the effect of tRNA on gene expression results from an alteration in the rate of translation of the leader RNA (38). Changing either the quantity or quality of the tRNA alters the progression of the ribosome relative to RNAP and thus affects leader RNA secondary structure and/or the coupling of transcription with translation of the leader RNA. Our recognition of the importance of the coupling of transcription and translation was confirmed by identification of sites at which RNAP pauses during transcription of the leader (20). It appears that the pausing of RNAP assists in the coupling of translation with transcription, and thus the overall efficiency of attenuation is increased as a result of the effect of the ribosome on leader RNA secondary structure.

The addition of exogenous ILV results in decreased expression of the *ilvGMEDA* operon (i.e., in some manner increases the efficiency of attenuation; Table 4). This could be achieved by any of the alterations in tRNA metabolism first described for the *his* operon (i.e., percent aminoacylation, quantity, or tRNA structure). One obvious option would be that the regulatory codons (i.e., ILV codons) for translation of the leader be rare or unusual (19), because then a minor tRNA species or subset of tRNA could be either more completely aminoacylated or modified, reducing ribosome step time (i.e., increase the rate of translation) with a minimal effect on overall cellular metabolism. This is unlikely to be the entire explanation, because with the exception of one rare leucine codon (CUA), the leader region contains the more common ILV code words. However, a role for the single rare leucine codon in the regulation of the operon was demonstrated by Hsu et al. (17) and Harms and Umbarger (14). The leader of *E. coli* has an adjacent pair of leucine codons (CUUCUA), while in *Seratia marcescens* there is a single CUA codon. Using biochemical and physiological studies, Hsu et al. (17) concluded that the single CUA codon affected expression of the *S. marcescens* operon. Harms and Umbarger (14) investigated the role of this codon by changing the CUA to the more common leucine codon CUG and to the proline codon CCG. Both of these changes resulted in reduced derepression of the operon when fully repressed cultures were compared with cultures growth-limited for leucine. While these studies demonstrate the role of this codon in derepression of the operon, they do not necessarily yield insight into the mechanism of repression.

Both theoretical (30, 46, 47) and experimental (20) analyses of the relationship between attenuation and changes in aminoacylation of tRNA indicate that relatively large changes (i.e., 40 to 50%) in the degree of aminoacylation would be required to achieve the five- to sevenfold modulation of gene expression observed upon the addition of ILV. Analysis of bulk ILV tRNA from *E. coli* K-12 growing on minimal medium indicates very high levels of aminoacylation. Unfortunately, the published determinations of the percent aminoacylation of isoleucyl-, leucyl-, and valyl-tRNAs isolated from different strains of *E. coli* grown in the absence or presence of ILV are sufficiently inconsistent to evaluate any relationship between repression, aminoacylation and attenuation (7, 12, 15–17, 39, 40, 49, 51). Furthermore, the results obtained by Freundlich et al. (12) studying leucine tRNA make it doubtful that the measurement of the percent aminoacylation of bulk leucyl-, isoleucyl-, and valyl-tRNAs will yield insight into this process. As discussed, no change was observed in the rate of growth of *E. coli* K-12 (strain M152) upon the addition of ILV, which is also consistent with the idea that biosynthesis of these amino acids does not limit the growth of the bacteria. Thus, if the more abundant ILV tRNAs are altered, any major change in the rate of translation must be contextual (13, 44, 45), occurring only over short segments of mRNA (i.e., in response to specific nucleotide sequences). Alternatively, the leader of the *ilvGMEDA* operon has an unusual secondary structure (25, 26, 35) which may be in part responsible for the observed regulation. Further understanding of the mechanism of regulation in response to an excess of ILV will require detailed mutational analysis of the leader and of the effect of exogenous ILV on the appropriate tRNA species. Careful analysis of those phenomena will increase our understanding of how the degree of attenuation or other translation-dependent termination events are modulated via the relative movements of RNAP and ribosomes through the leader.

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LITERATURE CITED

1. **Adams, C. W., and G. W. Hatfield.** 1984. Effects of promoter strengths and growth conditions on copy number of transcription-fusion vectors. J. Biol. Chem. **259:**7399–7403.
2. **Adams, C. W., M. Rosenberg, and G. W. Hatfield.** 1985. Analysis of *in vivo* RNA transcription products of the *ilvGEDA* attenuator region of *Escherichia coli* K12. J. Biol. Chem. **260:**8538–8544.
3. **Bachmann, B. J., and K. B. Low.** 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. **44:**1–56.
4. **Bennett, D. C., and H. E. Umbarger.** 1984. Isolation and analysis of two *Escherichia coli* K-12 *ilv* attenuator deletion mutants with high-level constitutive expression of an *ilv-lac* fusion operon. J. Bacteriol. **157:**839–845.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brenchley, J. E., and L. S. Williams.** 1975. Transfer RNA involvement in the regulation of enzyme synthesis. Annu. Rev. Microbiol. **29:**251–274.
7. **Calvo, J. M., M. Freundlich, and H. E. Umbarger.** 1969. Regulation of branched-chain amino acid biosynthesis in *Salmonella typhimurium*: isolation of regulatory mutants. J. Bacteriol. **97:**1272–1282.
8. **Cho, K.-O., and C. Yanofsky.** 1988. Sequence changes preceding a Shine-Dalgarno region influence *trpE* mRNA translation and decay. J. Mol. Biol. **204:**51–60.
9. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Eidilic, L., and F. C. Neidhardt.** 1965. Role of valyl-sRNA synthetase in enzyme repression. Proc. Natl. Acad. Sci. USA **53:**539–543.
11. **Freundlich, M., R. O. Burns, and H. E. Umbarger.** 1962. Control of isoleucine, valine and leucine biosynthesis. I. Multivalent repression. Proc. Natl. Acad. Sci. USA **48:**1804–1808.
12. **Freundlich, M., J. Trela, and W. Peng.** 1971. Evidence that the majority of leucine transfer ribonucleic acid is not involved in repression in *Salmonella typhimurium*. J. Bacteriol. **108:**951–953.
13. **Gutman, G. A., and G. W. Hatfield.** 1989. Nonrandom utilization of codon pairs in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86:**3699–3703.
14. **Harms, E., and H. E. Umbarger.** 1987. Role of codon choice in the leader region of the *ilvGMEDA* operon of *Serratia marcescens*. J. Bacteriol. **169:**5668–5677.
15. **Harris, C. L., and L. Lui.** 1981. Cysteine and growth inhibition of *Escherichia coli*: derepression of the *ilvGEDA* operon. Biochem. Biophys. Res. Commun. **101:**1145–1151.
16. **Harris, C. L., L. Lui, S. Sakallah, and R. DeVore.** 1983. Cysteine starvation, isoleucyl-tRNA^{Ile}, and the regulation of the *ilvGEDA* operon of *Escherichia coli*. J. Biol. Chem. **258:**7676–7683.
17. **Hsu, J.-H., E. Harms, and H. E. Umbarger.** 1985. Leucine regulation of the *ilvGEDA* operon of *Serratia marcescens* by attenuation is modulation by a single leucine codon. J. Bacteriol. **164:**217–222.
18. **Hughes, J., and G. Mellows.** 1978. Inhibition of isoleucyl-transfer ribonucleic acid synthetase in *Escherichia coli* by pseudomonic acid. Biochem. J. **176:**305–318.
19. **Konigsberg, W., and G. N. Godson.** 1983. Evidence for use of rare codons in the *dnaG* gene and other regulatory genes of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **80:**687–691.
20. **Landick, R., and C. Yanofsky.** 1987. Transcription attenuation, p. 1276–1301. *In* F. C. Neidhardt, J. L. Ingraham, K. Brooks, B. Magasanik, M. Schaecter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
21. **Lawther, R. P.** 1989. Point mutations in the regulation region of the *ilvGMEDA* operon of *Escherichia coli* K-12. J. Bacteriol. **171:**1188–1191.
22. **Lawther, R. P., D. H. Calhoun, C. W. Adams, C. A. Hauser, J. Gray, and G. W. Hatfield.** 1981. Molecular basis of valine resistance in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **78:**922–925.
23. **Lawther, R. P., and G. W. Hatfield.** 1977. Biochemical characterization of an *Escherichia coli hisT* strain. J. Bacteriol. **130:**552–557.
24. **Lawther, R. P., and G. W. Hatfield.** 1978. A site of action for tRNA-mediated regulation of the *ilvOEDA* operon of *Escherichia coli* K12. Mol. Gen. Genet. **167:**227–234.
25. **Lawther, R. P., and G. W. Hatfield.** 1980. Multivalent translational control of transcription termination of attenuator of *ilvGEDA* operon of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **77:**1862–1866.
26. **Lawther, R. P., R. C. Wek, J. M. Lopes, R. Pereira, B. E. Taillon, and G. W. Hatfield.** 1987. The complete nucleotide sequence of the *ilvGMEDA* operon of *Escherichia coli* K-12. Nucleic Acids Res. **15:**2137–2155.
27. **Lewis, J. A., and B. N. Ames.** 1972. Histidine regulation in *Salmonella typhimurium* XI. The percentage of transfer RNA^{His} charged *in vivo* and its relation to the repression of the histidine operon. J. Mol. Biol. **66:**131–142.
28. **Lopes, J. M., and R. P. Lawther.** 1986. Analysis and comparison of the internal promoter pE of the *ilvGMEDA* operons from *Escherichia coli* K-12 and *Salmonella typhimurium*. Nucleic Acids Res. **14:**2779–2798.
29. **Lopes, J. M., N. Soliman, P. K. Smith, and R. P. Lawther.** 1989. Transcription polarity enhances the contribution of the internal promoter, *ilvEp*, in the expression of the *ilvGMEDA* operon in wild-type *Escherichia coli* K12. Mol. Microbiol. **3:**1039–1051.
30. **Manabe, T.** 1981. Theory of regulation by attenuation mechanism: stochastic model for the attenuation of the *Escherichia coli* tryptophan operon. J. Theor. Biol. **91:**527–544.
31. **McKenney, K. H., H. Shimatake, D. Court, U. Schmeissner, C. Brady, and M. Rosenberg.** 1981. A system to study promoter and terminator signals recognized by *E. coli* RNA polymerase, p. 383–415. *In* J. Chirikijian and T. Papos (ed.), Gene amplification and analysis, vol. II. Elsevier/North-Holland Publishing Co., New York.
32. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Miller, K. G., and B. Sollner-Webb.** 1981. Transcription of mouse rRNA genes by RNA polymerase I: *in vitro* and *in vivo* initiation and processing sites. Cell **27:**165–174.
34. **Morse, D. E., and A. N. C. Morse.** 1976. Dual-control of the tryptophan operon is mediated by both tryptophanyl-tRNA synthetase and the repressor. J. Mol. Biol. **103:**209–226.
35. **Nargang, F. E., C. S. Subrahmanyam, and H. E. Umbarger.** 1980. Nucleotide sequence of the *ilvGEDA* operon attenuator region of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77:**1823–1827.
36. **Olins, P. O., and S. H. Rangwala.** 1989. A novel sequence element derived from bacteriophage T7 mRNA acts as an enhancer of translation of the *lacZ* gene in *Escherichia coli*. J. Biol. Chem. **264:**16973–16976.
37. **Ortuno, M. J., and R. P. Lawther.** 1987. Effect of the deletion of upstream DNA sequences on expression from the *ilvGp2* promoter of the *ilvGMEDA* operon of *Escherichia coli* K-12. Nucleic Acids Res. **15:**1521–1542.
38. **Palmer, D. T., P. H. Blum, and S. W. Artz.** 1983. Effects of the *hisT* mutation of *Salmonella typhimurium* on translation elongation rate. J. Bacteriol. **153:**357–363.
39. **Rizzino, A. A., R. S. Bresalier, and M. Freundlich.** 1974. Derepressed levels of the isoleucine-valine and leucine enzymes in *hisT1504*, a strain of *Salmonella typhimurium* with altered leucine transfer ribonucleic acid. J. Bacteriol. **117:**449–455.
40. **Rogerson, A. C., and M. Freundlich.** 1970. Control of isoleucine, valine and leucine biosynthesis VIII. Mechanism of growth

inhibition by leucine in relaxed and stringent strains of *Escherichia coli* K-12. Biochim. Biophys. Acta **208**:87–98.

41. **Rudd, K. E., and R. Menzel.** 1987. *his* operons of *Escherichia coli* and *Salmonella typhimurium* are regulated by DNA supercoiling. Proc. Natl. Acad. Sci. USA **84**:517–521.
42. **Russell, D. R., E. A. Auger, P. S. Vermersch, and G. N. Bennett.** 1984. Role of DNA regions flanking the tryptophan promoter of *Escherichia coli*: insertion of synthetic oligonucleotides. Gene **32**:337–348.
43. **Salser, W., R. F. Gesteland, and A. Bolle.** 1967. *In vitro* synthesis of bacteriophage lysozyme. Nature (London) **215**: 588–591.
44. **Shpaer, E. G.** 1986. Constraints on codon context in *Escherichia coli* genes: their possible role in modulating the efficiency of translation. J. Mol. Biol. **188**:555–564.
45. **Sørensen, M. A., C. G. Kurland, and S. Pedersen.** 1989. Codon usage determines translation rate in *Escherichia coli*. J. Mol. Biol. **207**:365–377.
46. **Suzuki, H., T. Kunisawa, and J. Otsuka.** 1986. Theoretical evaluation of transcriptional pausing effect on the attenuation in *trp* leader sequence. Biophys. J. **49**:425–436.
47. **von Heijne, G.** 1982. A theoretical study of the attenuation control mechanism. J. Theor. Biol. **97**:227–238.
48. **Wek, R. C., and G. W. Hatfield.** 1986. Examination of the promoter, P_E, in the *ilvGMEDA* operon of *E. coli* K-12. Nucleic Acids Res. **14**:2673–2777.
49. **Williams, L., and M. Freundlich.** 1969. Control of isoleucine, valine and leucine biosynthesis. VII. Role of valine transfer RNA in repression. Biochim. Biophys. Acta **186**:305–316.
50. **Yanofsky, C., R. L. Kelley, and V. Horn.** 1984. Repression is relieved before attenuation in the *trp* operon of *Escherichia coli* as tryptophan starvation becomes increasingly severe. J. Bacteriol. **158**:1018–1024.
51. **Yegian, C. D., and G. S. Stent.** 1969. Differential aminoacylation of three species of isoleucine transfer RNA from *Escherichia coli*. J. Mol. Biol. **39**:59–71.