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Specificity of Attenuation Control in the *ilvGMEDA* Operon of *Escherichia coli* K-12

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Three different approaches were used to examine the regulatory effects of the amino acids specified by the peptide-coding region of the leader transcript of the *ilvGMEDA* operon of *Escherichia coli* K-12. Gene expression was examined in strains carrying an *ilvGMED'-lac* operon fusion. In one approach, auxotrophic derivatives were starved of single amino acids for brief periods, and the burst of β-galactosidase synthesis upon adding the missing amino acid was determined. Auxotrophic derivatives were also grown for brief periods with a limited supply of one amino acid (derepression experiments). Finally, prototrophic strains were grown in minimal medium supplemented with single and multiple supplements of the chosen amino acids. Although codons for arginine, serine, and proline are interspersed among the codons for the three branched-chain (regulatory) amino acids, they appeared to have no effect when added in excess to prototrophs or when supplied in restricted amounts to auxotrophs. Deletions removing the terminator stem from the leader removed all *ilv*-specific control, indicating that the attenuation mechanism is the sole mechanism for *ilv*-specific control.

The specific control of expression of the *ilvGMEDA* operon in the enteric bacteria is characterized by repression of the operon when the three branched-chain amino acids are in ample supply but derepression when the supply of any one of the three is limited (14, 30). This *ilv*-specific regulation appears to be exclusively a consequence of an attenuation mechanism (Fig. 1) (4, 32, 38). In principle, the attenuation mechanism controlling the *ilv* operon appears to be quite similar to that found to control operons involved in tryptophan (35), leucine (19), phenylalanine (61), histidine (3), and threonine (17) biosynthesis: the leader region specifies a short peptide containing a disproportionate frequency of the amino acids that are found to regulate the operon. Thus, the rate at which the leader can be translated serves to sense whether the regulatory amino acid is in short or ample supply.

As emphasized by Landick and Yanofsky (31), an important feature of the attenuation mechanism is the pausing of RNA polymerase at a certain site in the leader region. The pause allows time for the ribosome to initiate translation, and as translation proceeds, the base pairing in the protector (1:2 stem, Fig. 2) is progressively disrupted. Should ribosome movement be retarded, presumably by stalling at a regulatory codon, long enough for the bases in the upstream arm of the terminator (bases 157 to 164) to emerge on the nascent transcript, these bases can pair with those in the downstream arm of the protector (bases 98 to 105) in the antiterminator (2:3 stem, Fig. 3). It is assumed that, in the absence of ribosome stalling (repressing conditions), translation proceeds rapidly enough that the bases in the downstream arm of the protector will also be covered by the ribosome or otherwise be sterically prevented from antiterminator formation and the terminator will be formed instead. Although there seems to be no fundamental difference between attenuation of the *ilv* operon and that of the much better known *trp* operon, a comparison of the proposed secondary structures of the two leaders suggests a subtle difference.

The 3′ terminus of the protector stem proposed for the *ilv* leader is eight bases upstream of the pause site and is base paired with the middle base of the initiation codon. According to the model of Yager and von Hippel (58) for hairpin-induced polymerase pausing, pausing occurs whenever the 12 most recently linked nucleotides are not paired with the template DNA strand in the "transcription bubble." Accordingly, we picture the protector stem, which presumably grows from a nucleation site in the nascent RNA chain and proceeds at a rate even faster than does the polymerase, as having caused the pause by stripping five nucleotides from the RNA-DNA hybrid in the transcription bubble. Upon assembly of the ribosome at the initiation codon, these five nucleotides would be released from the protector stem, the RNA-DNA hybrid would re-form, and transcription would resume immediately. (We assume that the ribosome covers 13 bases beyond the P site at which the AUG codon is bound [46].) Indeed, the newly assembled ribosome would also release the four guanine residues (bases 102 to 105, Fig. 2) that are considered important in initiating antiterminator formation (Fig. 3) by pairing with bases 157 to 160. However, these guanine residues would probably immediately join in an alternative base-paired structure (not shown) that, although relatively weak, might delay these bases from antiterminator formation except during very slow movement or stalling of the ribosome. This alternative protector would become progressively weaker and be lost when the A site on the ribosome has reached codon 7. Thereafter, pairing of these guanines in the antiterminator structure could be prevented only if translation was rapid enough for the translating ribosome to cover guanines 102 to 105 before cytosines 157 to 160 emerged from the transcription bubble and became available for base pairing and formation of the antiterminator.

In contrast to the *ilv* leader, we assume that a ribosome initiating translation of the *trp* leader would have to form six

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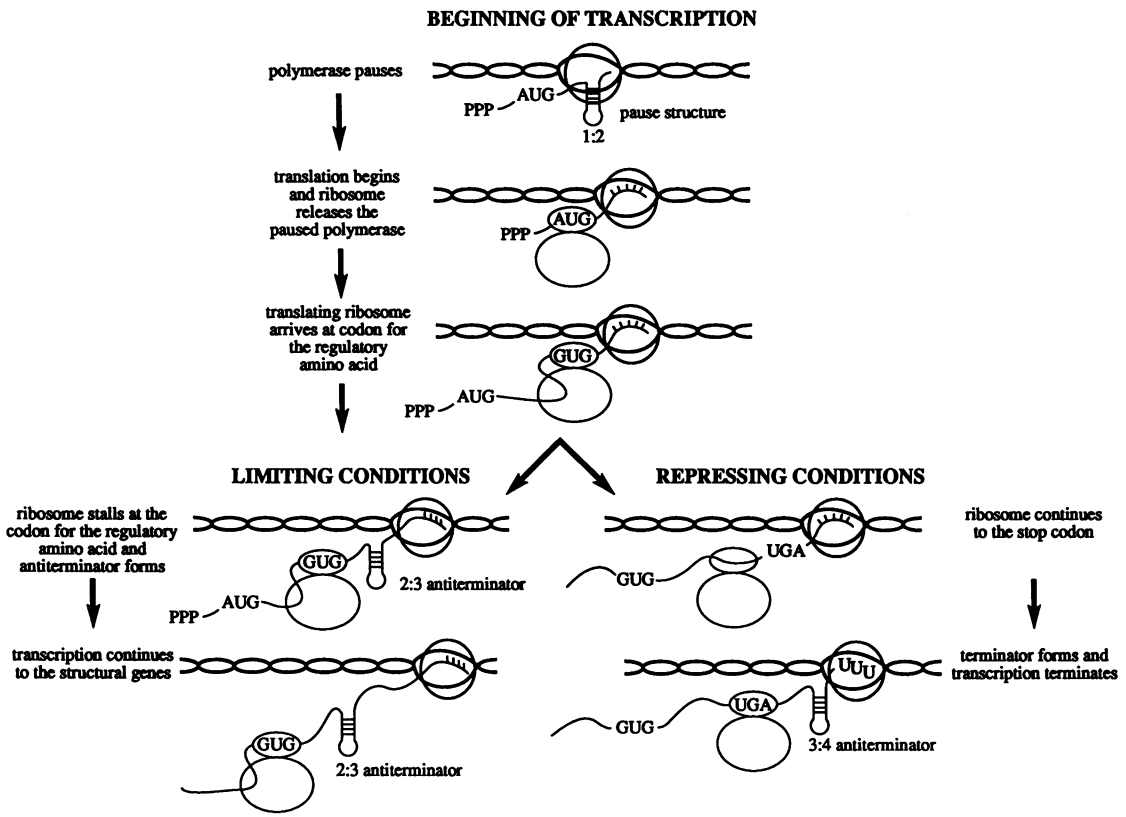

FIG. 1. Model for attenuation control of the *ilvGMEDA* operon. RNA polymerase rapidly transcribes the leader region until it is made to pause by the rapidly forming 1:2 base pairing (protector) in its transcript that has disrupted the RNA-DNA hybrid essential for continued transcription. Transcription is reinitiated when a ribosome is assembled, disrupting part of the protector and restoring the RNA-DNA hybrid region on the polymerase. Under derepressing conditions (for example, valine limitation), 2:3 base pairing occurs (antiterminator formation). When the ribosome proceeds rapidly enough to cover the upstream bases of the antiterminator before the downstream bases emerge from the polymerase as part of the nascent chain (repressing conditions), the 3:4 stem (terminator) is free to form. Adapted from the model for *trp* operon attenuation described by Landick and Yanofsky (31).

or seven peptide bonds before the 12-base transcription bubble on the paused polymerase could be restored. Thereafter, the race between the ribosome covering the upstream bases in the potential antiterminator (leader segment 2) and the polymerase releasing the downstream bases (leader segment 3) appears to be tilted much more in favor of terminator formation than we believe occurs in the *ilv* operon. Nevertheless, the overall pattern of attenuation is similar in the two systems.

This apparently greater sensitivity of the *ilv* leader may contribute to the fact that the relatively short peptide it specifies (32 amino acid residues) can have sensing sites for three different amino acids. Recall that the leader of the histidine operon specifies a peptide containing a run of seven histidine residues (3); that of the *pheA* gene specifies seven phenylalanine residues, but they are arranged in two runs of three and a single residue separated by single nonregulatory amino acids (61). In contrast, the peptide specified by the *ilv* leader (M-T-A-L-L-R-V-I-S-L-V-V-I-S-V-V-V-I-I-I-P-P-C-G-A-A-L-G-R-G-K-A) responding to regulation by three amino acids, all of which are far more common than either histidine or phenylalanine, is employed in a sensing mechanism that appears to be far more sensitive. Indeed, the *ilv* leader from *Serratia marcescens* can allow response to limiting leucine by virtue of but a single leucine residue, albeit that the efficiency of the control is strongly dependent on that single leucine codon being a rarely used one (23, 26). Because the single leucine codon at position 10 in the leader region of *S. marcescens* can alone account for an apparently efficient control by leucine, what is the significance of the two leucines at positions four and five of the *Escherichia coli* leader? If these are important, what would be the effect of ribosome stalling at position 6, where there is an arginine codon? In the run of 14 codons between positions 7 and 20, there are 12 branched-chain amino acid codons and two serine codons. Could control of the operon be affected by the supply of serine? Questions such as these are addressed in this report.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The bacterial strains used in this study and their sources are described in Table 1. The bacteriophages used are described in Table 2.

Auxotrophic derivatives lysogenic for phages λd*ilv-lac*11, λd*ilv-lac*12, and λd*ilv-lac*13 (4) were prepared so they could be grown with any one of the branched-chain amino acids limiting. For this purpose, strain CU838, carrying the *ilvG-C-rep-2049* deletion, was doubly infected with a (1:1) mixture of wild-type λ and one of the defective (*c*I857) phages. IlvE$^+$ Lac$^+$ transductants were selected at 37°C. Transductants that were resistant to λ*c*I857 at 42°C were converted to Leu$^-$ by introducing a *leu*::Tn*10* marker. Parallel experi-

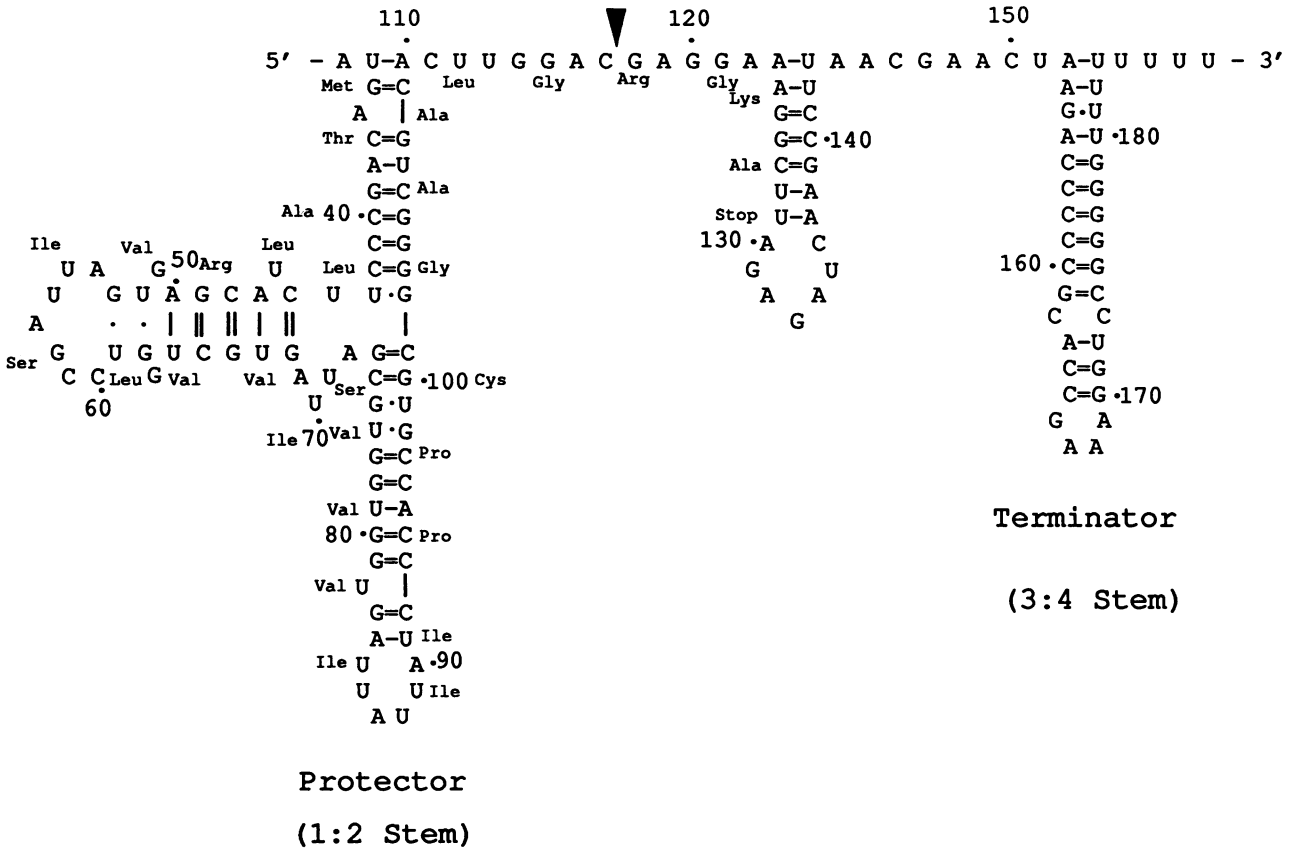

FIG. 2. Proposed secondary structure of the leader transcript of the *ilvGMEDA* operon. Bases 1 to 32 are not shown. The structures were deduced with the FOLD program of Zuker and Steigler (60). The calculated free energies were −31.3 kcal for the protector and −15.9 kcal for the terminator. The small stem and loop formed by bases 123 through 143 shown above is thought to contribute to the formation of the antiterminator as well as to its stability and might serve to seed the development of the antiterminator stem (see Fig. 3). Its calculated free energy of formation is −25.8 kcal. Also included in the diagram is the position of a ribosome with a valine codon (Val-16) occupying the empty A site on the ribosome. The ribosome is assumed to cover an additional 10 bases downstream of the valine codon and 21 bases upstream of the codon (Val-15) occupying the P site, which would be filled with a peptidyl-tRNA^{Val} in this case. Thus, the bases required for protector formation are prevented from doing so.

ments with the three types of phage yielded CU1677, CU1678, and CU1679, respectively.

The plasmids used and their sources or modes of preparation are described in Table 3.

Media. The minimal medium was that of Davis and Mingioli (11) modified by the omission of citrate and an increase in glucose concentration to 0.5%. The supplements of amino acids were, when indicated as being supplied in excess, isoleucine and leucine, 0.4 mM; valine, 0.8 mM; proline, 0.5 mM; and arginine, 1.0 mM. The limiting concentrations were 1/20th the excess concentrations. The rich medium was L broth (6). Ampicillin, kanamycin, tetracycline, spectinomycin, and chloramphenicol, when used, were used in concentrations of 50, 50, 12.5, 50, and 5 μg/ml, respectively.

Reagents. Restriction endonucleases and other enzymes used in DNA treatment were obtained from Bethesda Research Laboratories, Boehringer Mannheim Biochemicals, and Pharmacia-PL and used as the manufacturers recommended.

Enzyme assays. β-Galactosidase activity in sodium dodecyl sulfate (SDS)-chloroform-treated cells was determined by the method of Miller (37). This assay was used in the starvation experiments (see below) involving auxotrophic cells and in those involving the addition of amino acids to the medium of prototrophic cells. When activities of auxotrophic cells were compared after growth under repressing conditions or conditions of amino acid limitation, sonically disrupted cell extracts were examined. Cell extracts were also assayed for β-galactosidase activity by the method of Miller (37), except that SDS and chloroform were omitted from the assay and cell extracts rather than cell suspensions were used. Transaminase B activity was also determined when cell extracts were examined. The method of Duggan and Wechsler (12) was used. Protein was determined by the dye binding assay of Bradford (7). Specific activities of β-galactosidase are expressed in micromoles per minute per milligram of protein, and those of transaminase B are expressed in nanomoles per minute per milligram of protein.

Amino acid limitation. Amino acid restriction signals were generated in two ways. One, modeled after the experiments of Wasmuth and Umbarger (54), is referred to as starvation experiments. The inoculum was from an overnight culture. To keep the culture as viable as possible, the cells were grown in excess medium again until they reached the log phase. The centrifuge and solutions were all prewarmed to 25 to 30°C to prevent temperature shock. Cell samples were removed from the starved suspension at 0, 3, 5, and 8 min and stored in an ice bath. At 10 min, the missing amino acid

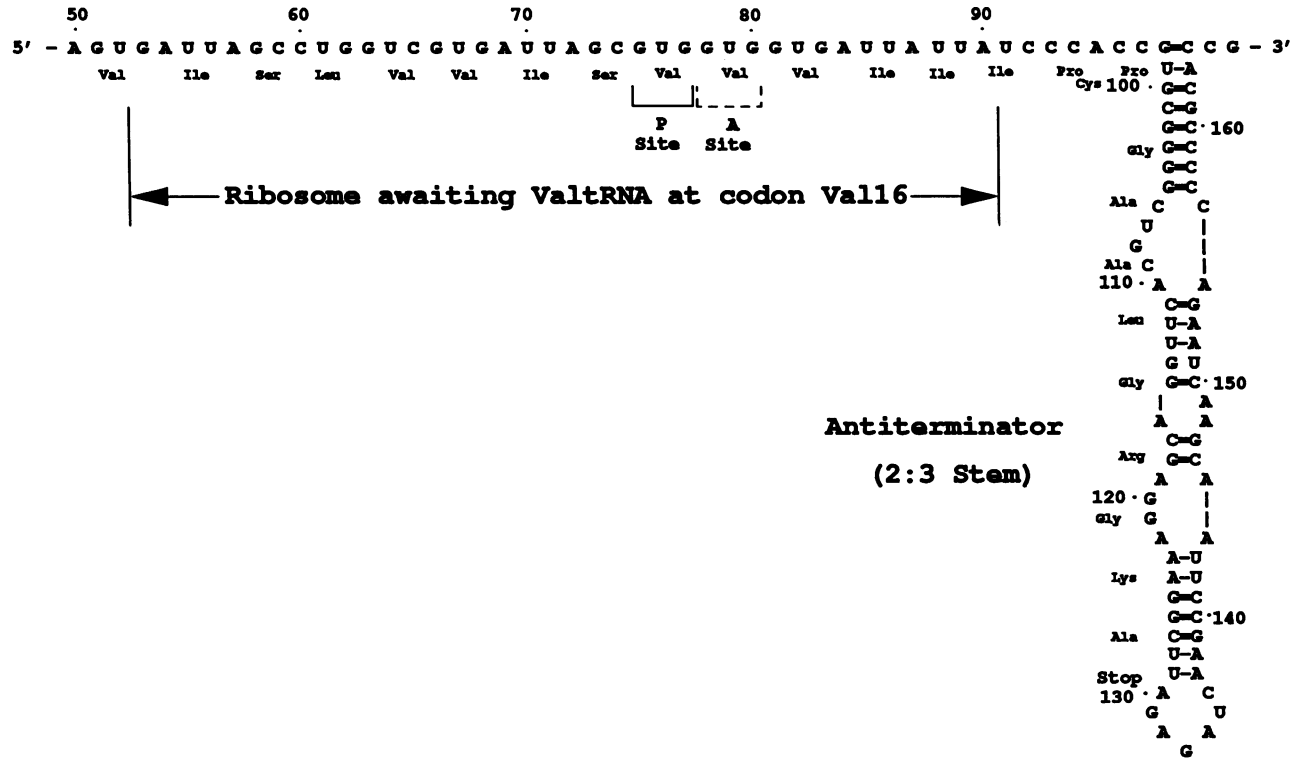

FIG. 3. Proposed secondary structure of the 2:3 stem (antiterminator) which is formed by the pairing of bases 98 to 105 with bases 157 to 165 and bases 111 to 115 with bases 150 to 154.

was added, and samples were removed at 3, 5, 8, and 10 min. The starvation period allowed the accumulation of a steady-state level of enzyme-forming potential from any genes controlled by that amino acid but allowed very little translation. Enzyme-forming potential can be pictured as some undefined mixture of initiated messages containing translating ribosomes backed up behind a lead ribosome stalled at a codon for the restricted amino acid. This pool of message was then translated upon addition of the missing amino acid, and further message formation was rapidly curtailed as the repression signal was restored, but messages already initiated would have been completed and presumably translated. β-Galactosidase activity was then measured in the harvested samples. This procedure was used to examine the effects of valine, isoleucine, leucine, arginine, and proline limitation.

Because deprivation of serine with the serine auxotrophs examined did not result in an immediate cessation of growth, an alternative procedure was used for observing the effect of serine deprivation. The strain was suspended in a medium supplemented with all its required amino acids plus 1.28 mM DL-serine hydroxamate for the first 10-min period. This analog has been shown by Tosa and Pizer (50) to result in a reduced seryl-tRNA level by virtue of its inhibitory effect on seryl-tRNA synthetase. Absorbancy increase of the culture was markedly retarded (to less than 30% of the normal growth rate) during this period. Serine deprivation was terminated after 10 min by adding 1.0 mM L-serine to the culture. Samples were removed at 0, 3, 5, 8, and 10 min during the serine-supplemented period and chilled. The chilled samples were assayed for β-galactosidase formation.

Although the relative increase in β-galactosidase activity in this type of experiment results in much less than a doubling in activity, the initial rate of synthesis upon adding the missing amino acid can represent up to a 30-fold derepression (54).

The other way of examining derepression was that described by Gayda et al. (18), in which enzyme levels in cells grown with all the amino acid supplements in excess were compared with the levels in cells harvested after 2 to 3 h of growth in a medium with one of the amino acids limiting (limitation experiments). As discussed previously (23), this procedure does not provide a quantitative test of maximal derepression but only determines whether or not derepression occurs when the amino acid under examination is limiting.

Repression experiments. The procedures described above allowed a comparison between enzyme activities under repressing conditions and those under derepressing conditions. Prototrophic derivatives were required to compare enzyme activities under repressing and nonrepressing conditions. For this purpose, an episome that complemented both the Ilv$^-$ and Pro$^-$ markers was selected. Palchaudhuri et al. (42) reported that the chromosomal DNA on two different F′ factors could be joined into a single F′ factor by selection for the presence of the markers on both F′ factors after mating the donor strain carrying one F′ factor with an F$^-$-phenocopied strain bearing the second F′ factor.

CU946 was first conjugated with CU137, which carries F′ *pro*$^+$ Δ*lacZ*(*H119*). The resulting conjugant, CU1287, was crossed with CU564, which carries the F′ 16 *ilv*$^+$ factor. Selection for Lac$^+$, Pro$^+$, and Ilv$^+$ gave the prototrophic strain CU1288, which carries the fused episome (F′ *pro*$^+$)::(F′ 16 *ilv*$^+$). To test whether these two F′ factors have been indeed fused into a single genetic element, we conjugated the prototrophic derivative, CU1288, with an F$^-$ *pro ilv* strain, CU1257. The conjugants were selected on minimal

TABLE 1. Bacterial strains used

Strain	Also called	Genotype	Source and description
CU137	CSH15	F′ Δ*lacZ*(*H119*) *proA*$^+$ *proB*$^+$/Δ(*pro-lac*) *thi supE*	Cold Spring Harbor Laboratory (37)
CU152	CSH26	F$^-$ *ara* Δ(*pro-lac*) *thi*	Cold Spring Harbor Laboratory (37)
CU564		F′ 16 *ilv*$^+$/Δ(*pro-lac*) *ara thi ilvE2050*	Watson et al. (55)
CU838		Δ(*pro-lac*) *ara thi* Δ*ilvGMEDAYC2049*	Watson et al. (55)
CU925		Δ(*pro-lac*) *ara thi* Δ*ilvGME2130*	Gayda et al. (18)
CU946		Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209)	Leathers et al. (34)
CU1088		Δ(*pro-lac*) *ara thi* Δ*ilvGME2130 ilvD2139*::-ΔMu-::λp1 (209)	Gayda et al. (18)
CU1132	NK5304	HfrPO45 *srlC300*::Tn*10 recA56 ilv-318 thr-300 thi-1 rel-1 rpsE300*	N. Kleckner
CU1247	IT1022	F$^-$ *ilvY864*::Tn*10 his-871 relA1 rpsL181 gal-3*	Tessman et al. (49)
CU1248		*ilvY864*::Tn*10 ara* Δ(*pro-lac*) *thi*	CU152 transduced with lysate of CU1247 by J. Falk
CU1256	PS2018	*araD139* Δ*lacU169 thi-1 rpsL arg*::Tn*10*	M. Levinthal
CU1257		Δ(*pro-lac*) *ara thi* Δ*ilvGMEDAYC2049 arg*::Tn*10*	CU838 transduced with P1 lysate of CU1256; selected for Tet^r, screened for Arg$^-$
CU1259		Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *arg*::Tn*10*	CU946 transduced with P1 lysate of CU1256; selected for Tet^r, screened for Arg$^-$
CU1287		F′ Δ*lacZ* (*H119*) *proA*$^+$ *proB*$^+$/Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209)	CU946 mated with CU137; selected for Pro$^+$ Lac$^+$, screened for Ilv$^-$
CU1288		(F′ Δ*lacZ* (*H119*) *proA*$^+$ *proB*$^+$)::(F′ 16 *ilv*$^+$)/ Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::gp1 (209)	CU1287 mated with CU564; selected for Ilv$^+$ Pro$^+$
CU1289		(F′ Δ*lacZ*(*H119*) *proA*$^+$ *proB*$^+$)::(F′ 16 *ilv*$^+$)/Δ(*pro-lac*) *ara thi arg*::Tn*10* Δ*ilvGMEDAYC2049*	CU1257 mated with CU1288; selected for Ilv$^+$ Pro$^+$ Tet^r
CU1294		Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *srlC300*::Tn*10 recA56*	CU946 transduced with P1 lysate of CU1132; selected for Tet^r, screened for UV^s Srl$^-$
CU1296		(F′ Δ*lacZ*(*H119*) *proA*$^+$ *proB*$^+$)::(F′ 16 *ilv*$^+$)/Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *srlC300*::Tn*10 recA56*	CU1294 mated with CU1289; selected for Pro$^+$ Ilv$^+$ Lac$^+$
CU1318	CGSC5913, W5449	λ$^-$ *leuB6 proA2 trpB9579 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 hsdR18 tsx-33 tonB56 supE44 recB21 recC22 sbcB15*	B. Bachmann; Sgaramella et al. (45)
CU1451	NK6039	HfrH Δ(*pro-lac*)XIII *thi leu*::Tn*10*	N. Kleckner
CU1609	CGSC5398, AA100	F$^-$ *thr-1 leuB6 thi-1 hisG1 argH1 trp-1 rbsK3 mtl-2 xyl-7 malA1 ara-13 gal-6 lacY1 rpsL9 tonA2 supE44* λ$^-$ λ^r	B. Bachmann; Anderson and Cooper (2)
CU1674		λd*ilv-lac*11/Δ(*pro-lac*) *ara thi* Δ*ilvGMEDAYC2049*	CU838 transduced with wild-type λ and λd*ilv-lac*11; selected for Lac$^+$ at 37°C
CU1675		λd*ilv-lac*12/Δ(*pro-lac*) *ara thi* Δ*ilvGMEDAYC2049*	CU838 transduced with wild-type λ and λd*ilv-lac12*; selected for Lac$^+$ at 37°C
CU1676		λd*ilv-lac*13/Δ(*pro-lac*) *ara thi* Δ*ilvGMEDAYC2049*	CU838 transduced with wild-type λ and λd*ilv-lac*13; selected for Lac$^+$ at 37°C
CU1677		λd*ilv-lac*11/Δ(*pro-lac*) *ara thi* Δ*ilvGMEDAYC2049 leu*::Tn*10*	CU1674 transduced with P1 lysate of CU1451; selected for Tet^r, screened for Leu$^-$
CU1678		λd*ilv-lac*12/Δ(*pro-lac*) *ara thi* Δ*ilvGMEDAYC2049 leu*::Tn*10*	CU1675 transduced with P1 lysate of CU1451; selected for Tet^r, screened for Leu$^-$
CU1679		λd*ilv-lac*13/Δ(*pro-lac*) *ara thi* Δ*ilvGMEDAYC2049 leu*::Tn*10*	CU1676 transduced with P1 lysate of CU1451; selected for Tet^r, screened for Leu$^-$
CU1690		λ$^-$ *leuB6 proA2 trpB9579 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 hsdR18 tsx-33 tonB56 supE44 recB21 recC22 sbcB15 ilvY864*::Tn*10*	CU1318 transduced with P1 lysate of CU1248; selected for Ilv$^+$, screened for Rbs$^-$ Tet^s
CU1693		λ$^-$ *leuB6 proA2 trpB9579 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 hsdR18 tsx-33 tonB56 supE44 recB21 recC22 sbcB15 rbsK3* (Pro$^+$)	CU1690 transduced with P1 lysate of CU1609; selected for Ilv$^+$, screened for Rbs$^-$ Tet^s; spontaneous Pro$^+$ revertant
CU1694		λ$^-$ *leuB6 proA2 trpB9579 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 hsdR18 tsx-33 tonB56 supE44 recB21 recC22 sbcB15* (Pro$^+$) Δ*ilvGME2130*	CU1693 transduced with P1 lysate of CU925; selected for Rbs$^+$, screened for Ilv$^-$
CU1699		λ$^-$ *leuB6 proA2 trpB9579 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 hsdR18 tsx-33 tonB56 supE44 recB21 recC22 sbcB15* (Pro$^+$) *ilvG2257*::*aph*$^+$ *ilvG2200* Δ*ilvGe2225*	CU1694 transformed with *Eco*RI/*Bam*HI-digested pPU335; selected for Kan^r, screened for Ilv$^+$ Amp^s
CU1700		λ$^-$ *leuB6 proA2 trpB9579 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 hsdR18 tsx-33 tonB56 supE44 recB21 recC22 sbcB15* (Pro$^+$) *ilvG2257*::*aph*$^+$ *ilvG2200*	CU1694 transformed with *Eco*RI-*Bam*HI-digested pPU336; selected for Kan^r, screened for Ilv$^+$ Amp^s

Continued on following page

TABLE 1—*Continued*

Strain	Also called	Genotype	Source and description
CU1701		Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *ilvG2257*::*aph*$^+$ *ilvG2200* Δ*ilvGe2225*	CU1088 transduced with P1 lysate of CU1699; selected for Kan^r blue colonies on X-Gal[a] plate, screened for KA$^+$[b]
CU1702		Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *ilvG2257*::*aph*$^+$ *ilvG2200*	CU1088 transduced with P1 lysate of CU1700; selected for Kan^r blue colonies on X-Gal plate, screened for KA$^+$
CU1705		Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *ilvG2257*::*aph*$^+$ *ilvG2200* Δ*ilvGe2225 leu*::Tn*10*	CU1701 transduced with P1 lysate of CU1451; selected for Tet^r, screened for Leu$^-$
CU1706		Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *ilvG2257*::*aph*$^+$ *ilvG2200 leu*::Tn*10*	CU1702 transduced with P1 lysate of CU1451; selected for Tet^r, screened for Leu$^-$
CU1707		Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *ilvG2257*::*aph*$^+$ *ilvG2200* Δ*ilvGe2225 srlC300*::Tn*10 recA56*	CU1701 transduced with P1 lysate of CU1132; selected for Tet^r, screened for UV^s, Srl$^-$
CU1708		Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *ilvG2257*::*aph*$^+$ *ilvG2200 srlC300*::Tn*10 recA56*	CU1702 transduced with P1 lysate of CU1132; selected for Tet^r, screened for UV^s Srl$^-$
CU1709		F′ 16 *ilv*$^+$/Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *ilvG2257*::*aph*$^+$ *ilvG2200* Δ*ilvGe2225 srlC300*::Tn*10 recA56*	CU1707 mated with CU564; selected for Ilv$^+$ Tet^r
CU1710		F′ 16 *ilv*$^+$/Δ(*pro-lac*) *ara thi ilvD2139*::-ΔMu-::λp1 (209) *ilvG2257*::*aph*$^+$ *ilvG2200 srlC300*::Tn*10 recA56*	CU1708 mated with CU564; selected for Ilv$^+$ Tet^r

[a] X-Gal, 5-bromo-4-chloro-3-indolyl-β-galactoside.
[b] KA$^+$; Requirement for isoleucine and valine satisfied by corresponding α-keto acids.

agar plates supplemented with proline or three branched-chain amino acids and then tested for Pro$^+$ or Ilv$^+$, respectively. All the colonies tested carried both markers from the fused episome. Subsequent transfer of the fused episome from the conjugant, CU1289, to its parental derivative, CU946, also exhibited 100% cotransfer.

To avoid any recombination between the episomal and chromosomal genes, the fused episome was transferred to a *recA* derivative of CU946, CU1294, and the resulting conjugant, CU1296, was used for the study of repression experiments and as the donor of the fused episome for the examination of the effects of the site-specific *ilv* leader mutations on the expression of *ilvGMED′-lac* operon under repressing conditions.

Insertion of selectable marker upstream of the *ilv* gene cluster. The transfer of the 15-bp deletion mutations in the *ilv* leader or its wild-type counterpart from one strain to another was conveniently done with a selectable marker linked to the *ilv* region. For this purpose, the *Hin*dIII-*Sma*I fragment from pBR322::Tn*5* containing the *aphA* gene (Kan^r) from λTn*5* was inserted into the *Sma*I site upstream of *ilvG* in pPU24 after blunting the *Hin*dIII end of the fragment to yield pPU333. Modified forms of pPU333, pPU336, and pPU335 (Table 3) were cleaved with *Eco*RI and *Bam*HI and used to transform the *recBC* strain CU1694 to yield strains containing the Kan^r marker upstream of either the wild-type or the deletion attenuator. The Kan^r marker allowed direct transfer of both kinds of *ilv* leader to strains containing the *ilv-lac* fusion. From the absence of any effect of the Kan^r marker on gene expression in the *ilv* cluster (unpublished observations), it appears the *aphA*$^+$ gene had been inserted well upstream of any sequences that influence expression of the *ilvGMEDA* operon.

Nucleotide sequence analysis. Nucleotide sequence analysis of the leader region in pPU125 was performed by the chain termination method with Sequenase, which was used according to the supplier's directions (United States Biochemical Corp.). The templates were prepared in M13mp18 and M13mp19 derivatives carrying inserts of the *ilv* DNA carrying the *ilv* leader region and flanking sequences. The *ilv*-specific primers were prepared by Phillip Andrews in the Purdue Laboratory for Macromolecular Structure with an ABI synthesizer (model 380A). They were 5′ C-T-A-T-G-G-T-A-A-C-T-C-T-T-T-A-G-G-C 3′ for sequence determination (in M13mp19) beginning 19 bases upstream of the leader region and 5′ T-T-C-A-T-T-G-T-C-T-G-C-T-C-C-T-C-G-G 3′ for sequence determination (in M13mp18) beginning 35 bases downstream of the string of thymidine residues of the leader region.

RESULTS

Loss of *ilv* regulation resulting from two leader deletion mutations. High-level constitutive expression of the *ilvGMED′-lacZ* operon in two attenuator deletion mutants, Δ*ilvG2216* and Δ*ilvGM2219*, has been reported previously by

TABLE 2. Bacteriophages used

Phage	Genotype or description	Reference
λd*ilv-lac*11	Δ(*Z-aatP*) [*rrfC-ilvGMEDAYC* Δ*ilv*(′*G-C*′)*2217* Ω(*ilv′GMED′ trp′BA′ lacZoZYA′* λ′)*2218*] c1857 *S*am7	Bennett and Umbarger (4)
λd*ilv-lac*12	λd*ilv-lac*11 Δ*ilv*(′*Ga-′G*)*2216*	Bennett and Umbarger (4)
λd*ilv-lac*13	λd*ilv-lac*11 Δ*ilv*(′*Ga-′M*)*2219*	Bennett and Umbarger (4)
M13mp18	Wild-type M13 with multiple cloning sites	Norrander et al. (39)
M13mp19	Wild-type M13 with multiple cloning sites	Norrander et al. (39)

TABLE 3. Plasmids used

Plasmid	Genotype[a]	Source and description
pBR322::Tn*5*	Tn*5* inserted at the hot spot 1 of pBR322	Berg et al. (5)
pPU5	pBR313 Ω[0.375 kb: λh*80dilv ilvGMEDAYC* −2.4/10.7 kb (−)]5	Subrahmanyam et al. (47)
pPU24	pBR322 Ω[0.029 kb: λh*80dilv ilvG2200 ilvE2201 ilvGME'* −2.1/2.437 kb (−)]13	Subrahmanyam et al. (47)
pPU34	pBR313 Ω[0.375 kb: λh*80dilv ilvGMEDAYC* −2.4/10.7 kb (−)]5 Δ[*ilv'GMEDAYC'* 1.476/ 8.413 kb]1 Ω[2.42 kb: λ*pilv-lac*3 *ilv'GMED' trp'*AB'*lac'ZoZYA'* λ' 7.34 (K-12)/17.28 (λ) kb (−)]7	Subrahmanyam et al. (48)
pPU119	pPU24 Δ[λh*80dilv* −2.0/−0.46 kb; pBR322 *tet* 0.29 − 2.065 kb]1	Digestion of pPU24 with *Pvu*II followed by religation and selection for Val^r and Amp^r
pPU125	pPU119 Δ[*ilv'Ge'* 0.060/0.075 kb]1	BAL 31 treatment of pPU119 cleaved by *Bst*NI followed by ligation
pPU316	pPU315 Δ[0.235/0.443 kb]1 Ω[0.234 kb: pPU314 *lac* 0.235/0.442; *ilv* −0.8/−2.1; *aadA*$^+$ (Spc^r-Str^r) 2.76 kb]1	Omega fragment (Spc^r/Str^r) flanked by DNA from upstream of the *ilv* regulatory region and from the *'ilvYC'* region inserted into the multicloning site of pUC18. Harms et al. (22)
pPU331	pUC8 Δ[0.404/0.426 kb]10 Ω[0.404 kb: pBR322::Tn*5* *aph*$^+$ 1.321 kb (+)]11	*Hin*dIII-*Sma*I *aph*$^+$ fragment of pBR322::Tn*5* was cloned into *Hin*dIII-*Sma*I sites of pUC8
pPU333	pPU24 Ω[3.6 kb: pPU331 *aph*$^+$ 1.321 kb (−)]2	*Hin*dIII-*Sma*I *aph*$^+$ fragment of pPU331 was blunted with Klenow and cloned into *Sma*I site upstream of *ilvGpeaGME'* in pPU24. The kanamycin resistance marker is designated as *ilvG2257*::*aph*$^+$
pPU334	pPU125 Ω[0.029 kb: pPU34 *ilv'ED'* 1.659 kb (−)]1	*Hin*dIII *ilv'ED'* fragment of pPU34 was cloned into *Hin*dIII site of pPU125
pPU335	pPU333 Δ[8.8/9.4 kb 0.001/3.1 kb]1 Ω[8.8 kb: pPU334 Δ*ilvGe2225 ilvG2200 ilvGMED' bla'* 5.4 kb (-)]1	The larger *Pvu*I fragment of pPU333 was replaced with the larger *Pvu*I fragment of pPU334
pPU336	pPU333 Ω[0.029 kb: pPU334 *ilv'ED'* 2.438/ 4.097 kb (−)]2	A 1.7-kb *Hin*dIII fragment of pPU334 containing *ilv'ED'* was inserted into pPU333 partially digested with *Hin*dIII. Selection was for IlvE$^+$
pUC8	*bla*$^+$ *lacZpZoZ'*	Vieira and Messing (53)
pUC18	*bla*$^+$ *lacZpZoZ'*	Yanisch-Perron et al. (59)
pUC19	*bla*$^+$ *lacZpZoZ'*	Yanisch-Perron et al. (59)

[a] The standard conventions used earlier (22) are used for genotype designation except that the *ilv* DNA coordinates, where indicated, have been renumbered so that the +1 base is the first base in the primary leader transcript. Negative coordinates are thus bases upstream of the transcription initiation site.

Bennett and Umbarger (4). Both deletions begin in the distal portion (base 153, Fig. 2) of the *ilvGMEDA* attenuator region. The former ends in the *ilvG* gene (immediately before the codon for His-494), and the latter ends in the *ilvM* gene (disrupting the codon for Met-36).

In the earlier study, the λ phages carrying the *ilvGMED'-lacZ* operon were carried in a prototrophic strain, and only the effect of an isoleucine limitation could be examined. To examine the effect of limiting each of the branched-chain amino acids, we transferred the phages to a strain bearing a deletion of the *ilv* gene cluster. The transductants were converted to Leu$^-$ by introducing a *leu*::Tn*10* marker. As indicated in Table 4, 30- and 60-fold increases in β-galactosidase activity under repressing conditions over that of the wild-type counterpart, CU1677 (λd*ilv-lac*11), were observed for strains CU1678 (λd*ilv-lac*12) and CU1679 (λd*ilv-lac*13), respectively. Complete loss of the response to the limitation of isoleucine, leucine, and valine was observed.

The contrasting quantitative responses of β-galactosidase and transaminase B activities to limitation by the three branched-chain amino acids are strikingly illustrated by the data in Table 4. It has been repeatedly observed in all experiments of this kind that the derepression obtained with β-galactosidase when leucine is limiting is less than that seen

TABLE 4. High-level constitutive expression of the *ilvGMED'-lacZ* operon caused by two attenuator deletion mutations in the *ilvGMEDA* leader region of a λ prophage carrying an *ilv-lac* fusion in strains deleted for the entire *ilvGMEDAYC* gene cluster

Growth condition	CU1677 (λd*ilv-lac*11)				CU1678 (λd*ilv-lac*12)				CU1679 (λd*ilv-lac*13)			
	β-Galactosidase		Transaminase B		β-Galactosidase		Transaminase B		β-Galactosidase		Transaminase B	
	Sp act[a]	%	Sp act[a]	%	Sp act	%	Sp act	%	Sp act	%	Sp act	%
Repressing	0.19 ± 0.03	100	9 ± 2	100	6.12 ± 0.70	100	526 ± 105	100	12.16 ± 1.36	100	732 ± 81	100
Limiting isoleucine	0.83 ± 0.05	437	25 ± 3	278	5.64 ± 0.53	92	657 ± 157	125	11.08 ± 0.42	91	1,114 ± 33	152
Limiting leucine	0.40 ± 0.06	211	45 ± 2	500	4.00 ± 0.80	65	893 ± 117	170	8.76 ± 1.00	72	1,300 ± 148	177
Limiting valine	0.59 ± 0.01	311	25 ± 4	278	6.39 ± 0.75	104	980 ± 130	186	10.73 ± 0.96	88	1,131 ± 104	154

[a] See text for units.

TABLE 5. Comparison of the accumulation of β-galactosidase-forming potential during starvation of strains carrying a wild-type *ilv-lac* fusion

Amino acid restricted	% Increase in β-galactosidase activity[a]	
	CU946 (Ilv⁻ Pro⁻)	CU1259 (Ilv⁻ Pro⁻ Arg⁻)
Isoleucine	46 ± 5*	30 ± 3**
Leucine	56 ± 5***	63 ± 7
Valine	20 ± 3	32 ± 2
Proline	−20 ± 4	−3 ± 1
Serine[b]	−2 ± 1	−1 ± 1
Arginine	ND[c]	−1 ± 1

[a] Enzyme activity was determined in chloroform-SDS-treated cells. All the values shown were the average of two independent experiments, except: *, 14 experiments; **, 7 experiments; and ***, 4 experiments.
[b] Serine starvation was achieved by adding serine hydroxamate.
[c] ND, Not done.

TABLE 6. Expression of wild-type *ilvGMED'-lacZ* operon under limiting regulatory and nonregulatory amino acids in CU1259

Growth condition	β-Galactosidase[a]	
	Sp act	%
Repressing	0.15 ± 0.02	100
Limiting isoleucine	0.69 ± 0.08	460
Limiting leucine	0.23 ± 0.01	153
Limiting valine	0.38 ± 0.04	253
Limiting proline	0.13 ± 0.01	87
Limiting arginine	0.15 ± 0.01	100
Limiting serine	0.14 ± 0.02	93

[a] Enzyme activities were determined in cell extracts. Units are described in the text.

when either isoleucine or valine is limiting. This difference is thought to reflect the different proportion of leucine residues in the two enzymes (9.4% for β-galactosidase, 6.8% for transaminase B) relative to the proportions of isoleucine and valine in the two enzymes (3.8% isoleucine and 6.3% valine for β-galactosidase versus 6.5% isoleucine and 8.1% valine for transaminase B).

These observations clearly indicate that the leader region is essential for regulation of the *ilvGMEDA* operon. In view of the earlier demonstration by Wek et al. (57) of a rho-dependent transcriptional polarity occurring when translation of *ilvG* mRNA is blocked (as occurs in the wild-type K-12 strain), it must be assumed that these deletions have either removed the polarity sites or reduced their effectiveness. Indeed, the endpoint of the *ilvGM2219* deletion is beyond the third polarity site (4, 57). The endpoint of the other deletion, Δ*ilvG2216*, is about 130 bp upstream of the first of the three rho-dependent termination sites, a distance that may interfere with the rho-dependent termination (44). It is of interest, nevertheless, that the expression in the construct with Δ*ilvG2216* is only about one-half that in the construct with the three rho-dependent terminators deleted. This removal of the polarity site (by deletion *ilvGM2219*) and apparent reduction of the polar effect (by deletion *ilvG2216*) probably account for the much greater effect of the deletion than is ever found upon deattenuation by branched-chain amino acid limitation.

Comparison of starvation for regulatory and nonregulatory amino acids. In view of the proximity of serine and arginine codons to codons for the branched-chain amino acids in the *ilv* leader, it was of interest to determine whether there was any derepression of the *ilvGMEDA* operon when these nonregulatory amino acids were limited. Because of the greater ease in determining the activity of β-galactosidase than the enzymes of the isoleucine pathway, a strain containing an *ilvD-lac* operon fusion was initially chosen (40). This strain, CU1259, was auxotrophic for arginine, proline, isoleucine, and valine and, although not completely blocked in leucine biosynthesis, was nevertheless bradytrophic for leucine and could therefore be used to examine the effect of restricting the leucine supply. Table 5 shows the results of an experiment in which derepression was examined by the method of Wasmuth and Umbarger (54), i.e., by the burst in β-galactosidase formation upon addition of the missing amino acid after a 10-min starvation. The serine restriction was imposed by the addition of DL-serine hydroxamate as described in Materials and Methods. As Table 5 shows, there was a burst in synthesis only when one of the three branched-chain amino acids was omitted. Although the arginine codon at position 6 and the two serine codons at positions 9 and 14 were placed between codons for branched-chain amino acids which signal derepression when in limited supply, no effect of either serine or arginine could be demonstrated. Neither was there an effect of starvation for proline. This amino acid restriction was chosen as a control, since both proline codons are immediately downstream of the last of the branched-chain amino acid codons that are thought to be important in *ilv* regulation and thus might be too far along the putative peptide-coding region to be involved in *ilv* regulation.

Effect of limitation for regulatory and nonregulatory amino acids. The effect of providing a limited supply of the required amino acids on the expression of the *ilvGMED'-lacZ* operon was also examined in strain CU1259. An exponentially growing culture was harvested and transferred to a medium to which a limited amount of one required amino acid was added, and the cells were allowed to grow under such limiting conditions for 3 h or one doubling. Both β-galactosidase and transaminase B activities were assayed in the sonically disrupted cell extracts.

Approximately 4.6-fold-greater β-galactosidase activity was observed when isoleucine was limited, and 2.5-fold-greater activity was observed for valine limitation (Table 6). That CU1259 still has an active *ilvE* gene which can convert excess valine into the precursor of leucine biosynthesis, α-ketoisovalerate, may account for the poor response of the *ilv* regulatory region in CU1259 to the limitation of leucine. As observed in the starvation experiments, limitation of the nonregulatory amino acids proline and arginine did not derepress the *ilvGMED'-lacZ* operon.

Effect of supplementing the medium of a prototrophic strain with regulatory and nonregulatory amino acids. Observations on the regulation of the *thr* operon have suggested that the addition of nonregulatory amino acids to an otherwise complete medium resulted in a repression of the *thr* operon (10). This result indicated that the nonregulatory amino acids did indeed influence the rate of ribosome passage down the leader transcript and that they were, presumably, somewhat limiting during normal growth. Thus, in the *ilv* operon studied here, the level of *ilv* gene expression in a prototrophic strain growing in a minimal medium might be as high as it is because either arginine or serine is limiting. If so, the addition of the limiting amino acid would result in some repression. This possibility was examined in a strain containing the same *ilvD-lac* fusion but rendered prototrophic

TABLE 7. Effect of adding amino acids found in the *ilv* leader peptide on the expression of *lacZ* controlled by the wild-type *ilvGMEDA* regulatory region in a prototropic derivative, CU1296

Addition(s) to medium[a]	% of β-galactosidase activity[b] in:	
	Minimal medium	Minimal medium + Ile, Leu, Val
None	100	33 ± 2
Isoleucine	55 ± 2	
Leucine	163 ± 3	
Proline	98 ± 1	31 ± 0
Arginine	100 ± 3	34 ± 3
Serine	177 ± 17	35 ± 3
Proline, arginine, serine	185 ± 3	36 ± 4
Methionine	113 ± 1	35 ± 0
Threonine	70 ± 1	34 ± 1
Cysteine	154 ± 2	34 ± 0
Alanine	111 ± 4	38 ± 2
Glycine	99 ± 1	30 ± 1
Methionine, threonine, cysteine, alanine, glycine	63 ± 1	20 ± 2
As above plus proline, arginine, serine	76 ± 1	26 ± 2

[a] All supplements were added in excess (see Materials and Methods). The effect of adding valine alone could not be tested because of the valine sensitivity of CU1296.

[b] Enzyme activity was determined on chloroform-SDS-treated cells.

by introducing an F′ factor carrying both *ilv*$^+$ and *pro*$^+$ genes (see Materials and Methods). The results are shown in Table 7. Interestingly, the single addition of isoleucine resulted in a repression to about 60% of the minimal (nonrepressed) medium level of activity. This result would imply that the intracellular concentration of isoleucine is less than optimal in prototrophic K-12 derivatives and is in accord with the results of Jackson and Umbarger (27), who reported a similar effect several years ago. In contrast, the addition of leucine resulted in a derepression. This result is also similar to earlier observations. One possibility is that the derepression results from the repression of the *ilvIH* genes (acetohydroxy acid synthase III) by leucine with the result that the formation of both isoleucine and valine must occur via the *ilvBN* genes, which specify acetohydroxy synthase I. The effect of a single addition of valine could not be tested, since the strain was valine sensitive (i.e., lacked acetohydroxy acid synthase II). There was no effect of leucine addition when isoleucine and valine were also present as would be expected if acetohydroxy acid synthase activity was limiting when leucine alone was present. Another amino acid causing a derepression when added was serine. This derepression may well have been due to interference of threonine deaminase activity by serine, which is also a substrate for threonine deaminase. As Table 7 shows, this effect also was suppressed by the addition of the three branched-chain amino acids (or even by adding isoleucine alone [data not shown]).

Thus, no evidence was found for any role of these nonregulatory codons in the regulation of attenuation. In the proposed secondary structure of the attenuated transcript (presumably the structure that would be formed if protein synthesis was completely blocked), the second serine codon is considered to add stability to the protector stem (Fig. 1), and the arginine codon is considered to contribute stability to a relatively weak stem that may be of no importance in regulation (see below). Thus, it may be that these codons play a structural role in the various secondary structures that are specified by the leader sequence.

Effect of a deletion removing four branched-chain amino acid codons from the *ilv* leader region. In the course of generating some deletion mutations by brief BAL 31 treatments of the plasmid, pPU119, cleaved with *Bst*NI in its *ilv* leader insert (3a), one shortened product was isolated that had retained a *Bst*NI site. Determination of the nucleotide sequence established that BAL 31 digestion had occurred in

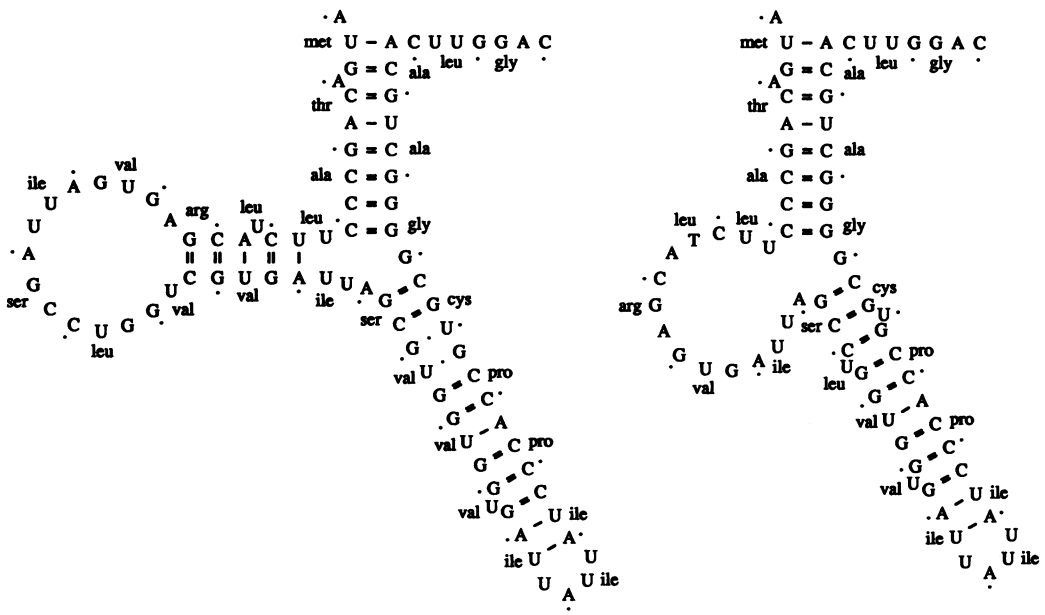

FIG. 4. Comparison of proposed structure of the protector stem of the wild-type *ilvGMEDA* leader transcript (left) with that proposed for the leader that lacks five codons (right). Only that portion of the leader extending from the AUG codon to the site of polymerase pausing (25) is shown. The structures were deduced with the program FOLD of Zuker and Steigler (60). The calculated free energies for the structures shown were ΔG = −31.3 kcal for the wild-type protector and −23.8 kcal for the mutant protector.

TABLE 8. Effect of the 15-bp *ilv* leader deletion, Δ*ilvGe2225*, on the expression of *ilvGMED'-lacZ* operon under repressing and limiting conditions

Strain and growth condition	β-Galactosidase		Transaminase B	
	Sp act	%	Sp act	%
CU1705 (Δ*ilvGe2225*)				
Repressing	0.75 ± 0.06	100	88 ± 12	100
Limiting isoleucine	4.08 ± 0.52	544	369 ± 43	419
Limiting leucine	1.42 ± 0.13	189	310 ± 37	352
Limiting valine	1.51 ± 0.2	201	190 ± 25	216
CU1706 (*ilvGe*$^+$)				
Repressing	0.84 ± 0.09	100	104 ± 9	100
Limiting isoleucine	4.57 ± 0.49	544	466 ± 51	448
Limiting leucine	1.82 ± 0.21	217	407 ± 45	391
Limiting valine	1.51 ± 0.17	180	268 ± 37	258

[a] Enzyme activity was determined in cell extracts. See text for units.

only one direction and had removed 15 bases in the leader region (Fig. 4) such that the reading frame in the leader transcript was maintained but a new *Bst*NI site had been generated and three valine codons, one isoleucine codon, and one serine codon had been removed. The leucine codon specified by the *Bst*NI site was regenerated in the new *Bst*NI site. That the leader transcript still specified a short open reading frame suggested that it still played a regulatory role. However, the question was whether that regulatory role would be less effective than it is with the wild-type leader. To answer the question, the *ilv* region containing the deletion was transferred to a *recBC* strain (CU1694) and then by P1 transduction to a strain carrying the *ilvD*::λp1 (209) *ilv-lac* fusion. The transfer was facilitated by inserting the kanamycin resistance gene (*aphA*) of Tn*5* upstream of the *ilv* promoter in the original plasmid and extending its *ilv* region to include all of the *ilvE* gene to yield pPU335 (Table 3). Introduction of the *leu*::Tn*10* lesion (CU1705) allowed limitation by all three branched-chain amino acids, whereas introduction of the *ilv*$^+$ F′ 16 factor (CU1709) allowed a comparison of repressed and nonrepressed enzyme levels. A pair of isogenic strains (CU1706 and CU1710) containing the wild-type leader region were prepared in a parallel fashion and used in the limitation and repression experiments, respectively.

Limitation for any of the branched-chain amino acids gave equivalent results for both the strain containing the wild-type leader (CU1706) and that containing a leader with Δ*ilvGe2225* (CU1705) (Table 8). Comparison of the two prototrophic strains, CU1709 and CU1710 (Table 9), revealed that the addition of the branched-chain amino acids to the minimal medium either singly or in combination also failed to reveal any difference between regulation with the wild-type leader region and regulation with the leader region bearing the 15-bp deletion.

Other experiments, in which a short-term starvation was followed by a burst in synthesis upon addition of the missing amino acid, also failed to reveal any differences between the two strains (data not shown). It is concluded, therefore, that the 15-bp deletion had no effect on *ilv*-specific regulation.

TABLE 9. Effect of 15-bp *ilv* leader deletion, Δ*ilvGe2225*, on expression of *ilvGMED'-lacZ* operon under repressing and nonrepressing conditions

Addition to minimal medium	CU1709 (Δ*ilvGe2225*)		CU1710 (*ilvGe*$^+$)	
	β-Galactosidase sp act[a]	%	β-Galactosidase sp act[a]	%
None	1.20 ± 0.08	100	1.01 ± 0.09	100
Isoleucine	0.97 ± 0.08	81	0.78 ± 0.09	77
Leucine	1.63 ± 0.20	136	1.22 ± 0.15	121
Valine	1.69 ± 0.18	141	1.21 ± 0.18	120
All of the above	0.43 ± 0.05	36	0.42 ± 0.06	42

[a] Enzyme activity was determined on chloroform-SDS-treated cells. See text for units.

DISCUSSION

The earlier study by Bennett and Umbarger (4) reported strong evidence that the major if not the sole mechanism of control over the expression of the *ilvGMEDA* operon by the branched-chain amino acids was attenuation of transcription at the end of the leader region. The same general conclusion comes from the more recent report of Lawther et al. (33), who showed, in addition, that attenuation was dependent only on the intact leader region and not on either the *ilv* promoter itself or on those sequences upstream of the promoter that increased promoter strength.

The earlier experiments were based on the fact that two deletions that removed the region specifying the termination stem and loop of the leader resulted in a derepressed level of expression that seemed not significantly increased when isoleucine was limiting, nor did the level seem to be repressed when all three branched-chain amino acids were added to the medium (4). Derepression of the operon was judged by the derepression of the *lacZ* gene under control of the *ilv* regulatory region. Since the strains examined were prototrophic, it was only possible to bring about an isoleucine limitation by virtue of the sensitivity of the strain to valine.

In this study, the deletion mutations studied earlier were examined in Ilv$^-$ Leu$^-$ strains which allowed growth of the strain with any one of the three branched-chain amino acids limiting. Whereas a derepression of β-galactosidase was observed upon limiting the supply of any of the three branched-chain amino acids with the strain containing the *ilv-lac* operon with a wild-type *ilv* leader region, there was no derepression when any of the three amino acids was limited with the strains containing the mutant leader. Furthermore, the level of expression was severalfold higher in the mutants than even the most derepressed level obtained with the wild-type leader. As emphasized by Wek et al. (57) and by Lopes et al. (36), there is a region of polarity in the *ilvG* gene that reduces *ilvEDA* expression in *E. coli* K-12 stocks that carry the naturally occurring frameshift in *ilvG*. This polar effect would have influenced the expression of the *lacZ* gene in strains carrying the wild-type leader region. That the strains carrying the deletion had such a high level of expression indicated not only that was there strong readthrough from the leader but also that the polarity effect was overcome. In strain CU1676, the deletion extended well beyond the sites of rho-dependent transcription termination (57), so that the level of gene expression is probably as high as can be expected for the *ilv* promoter. In strain CU1675, the deletion did not extend that far but did apparently extend far enough to have strongly reduced the polar effect. Recall that rho-dependent termination is believed to be dependent on a region of unstructured as well as untranslated message upstream of the termination site (44).

The absence of regulation of the *ilvGMEDA* operon by the availability of the three branched-chain amino acids when

the attenuation function is lost does not indicate that other factors do not serve to modulate *ilv* gene expression. For example, integration host factor in concert with DNA gyrase is known to be stimulatory for *ilvGMEDA* expression (15, 16, 52). Its action is presumably due to binding to a region upstream of the promoter that had earlier been shown to be needed for maximal expression (41, 52). Interestingly, binding at this site appears to prevent transcription from occurring at an upstream (perhaps fortuitous) *ilv* promoter that is detected only in vitro (1, 43). ppGpp has also been shown to stimulate transcription of the *ilvGMEDA* operon in vitro (40). In vitro transcription experiments have revealed a marked reduction in transcript formation initiated from both *ilvG* promoters upon relaxation of supercoiled templates (1a).

All the above factors are clearly global controls affecting the *ilvGMEDA* operon, in contrast to the specific control by the branched-chain amino acids. A claim for a specific repressor protein specified by the *ileR* gene has been made (28, 29, 56), but these studies did not address the question of whether regulation by the IleR protein is modulated by any of the branched-chain amino acids or is itself a component of some global control affecting *ilv* gene expression rather than one specifically affecting the *ilv* operon (1, 28, 29, 41, 43, 52, 56).

One of the major questions that led to this study was whether the non-branched-chain amino acids in the putative peptide specified by the *ilv* leader could play a regulatory role in *ilv* expression. Of particular interest were the two serine and single arginine codons interspersed among the branched-chain amino acid codons in the coding region of the leader. The experiments point quite clearly to the lack of any derepression resulting from limiting arginine, serine, or proline (the proline codons were beyond the last of the regulatory amino acid codons and should not have been expected to have had a regulatory effect). This observation is in accord with the results of Tsui and Freundlich (51) in their study of the *ilvBN* operon but in contrast to those of Hauser and Hatfield (24) who found a regulatory response of that same operon upon limiting threonine and alanine, neither of which can be considered products of the *ilvBN* operon. In the former study, enzyme activity was measured (as in this study), whereas in the latter, pre- and postattenuation message was measured.

In view of the results reported by Daniel and Saint-Girons (10), indicating that the nonregulatory amino acids specified by the *thr* leader would reduce expression of the *thr* operon when added to a minimal medium, it was felt that making single additions of serine, arginine, and proline should be examined. Such an experiment was performed with a prototrophic strain. In other words, the question asked was whether *ilv* gene expression was as high as it was because arginine (or serine) was already partially limiting. From previous work, it had been known that for *E. coli* K-12 prototrophic strains, isoleucine was somewhat limiting (27). Indeed, the single addition of isoleucine results in a significant decrease in *ilv* gene expression, as previously observed. Almost as effective in repressing *ilv* expression was the precursor to isoleucine, threonine, indicating that the isoleucine limitation in the K-12 strain may not be due entirely to restricted acetohydroxybutyrate formation owing to the absence of acetohydroxy acid synthase II. It may be due in part to a limitation in the aspartate family pathway. In contrast, single or combined addition of arginine, serine, or proline failed to exert any repressing effect.

Serine addition, although not causing a repression, did cause a significant derepression when added singly. This effect we attribute to an interference with isoleucine biosynthesis in part, at least to interference with threonine deamination since L-serine is a substrate for this enzyme. The other amino acid that caused a derepression of operon expression was leucine. Two factors might have come into play: one, leucine is an inhibitor of threonine deaminase, mimicking, to a limited extent, the inhibitory effect of isoleucine; and, two, leucine addition leads to repression of acetohydroxy acid synthase III. As shown by Gollop et al. (20), isozyme III is more effective in forming acetohydroxybutyrate than isozyme I, the only other isozyme present in this strain. Thus, the addition of leucine may accentuate the relative isoleucine deficiency in these strains. Both the serine- and leucine-invoked derepressions are counteracted by the simultaneous addition of isoleucine (data not shown).

The experiments with the wild-type *ilv* leader region reported here reveal that the response of the *ilvGMEDA* operon is remarkably specific and efficient. The placement of an arginine codon and two serine codons among those for the branched-chain amino acids appears not to interfere in any way with operon control. It may be that the limitation conditions chosen did not sufficiently reduce the charging levels of the tRNA species responding to these particular codons or that these tRNAs can be mischarged at a rate precluding their involvement in attenuation control. It is interesting in this respect that the pathways to arginine and serine in *E. coli* are not regulated by attenuation. Are these tRNAs, when uncharged, unable to participate in attenuation? That such an explanation seems unlikely is attested to by the fact that arginine starvation was almost as effective as tryptophan starvation in bringing about transcription beyond the *trp* leader region (62).

The difference between the results obtained with the *trp* operon and those obtained with the *ilv* operon appear even more paradoxical when it is recalled that the arginine codon in the *trp* leader (CGC) is one that can be misread (13) and therefore could be less effective in ribosome stalling, whereas that in the *ilv* leader (CGA) is one for which the tRNA is less efficiently charged (21). The effect of arginine starvation on *trp* operon expression had been measured by formation of hybridizable message, i.e., a measure of functional transcription beyond the attenuator. In this study, the effect of arginine starvation on *ilv* operon expression was by accumulation of translatable message and the level of repression and derepression. In the accompanying report (9a), evidence will be presented indicating that a strong signal for ribosomal stalling at the position normally occupied by the arginine codon (i.e., one generated by a UGA codon) does indeed lead to readthrough at the attenuator. Thus, for some reason, there was apparently no ribosome stalling when the cells were starved for arginine.

A factor that should be considered is that reducing the growth rate by limiting the arginine supply may not ensure that the ribosomes will stall at every arginine codon. The reduced growth rate could be due to arginine restriction at certain codons in messages for very critical proteins. Thus, the specific context for the arginine codon in the *ilv* leader might be one that allows very rapid translation of the amino acid coding region of the *ilv* leader. That rapid translation is necessary to allow for termination rather than readthrough was emphasized in the Introduction. Indeed, inspection of the transcript (Fig. 2) allows us to estimate that the ribosome would have to form 19 peptide bonds (to enable it to cover the guanine-rich sequence in the protector) before the released polymerase has incorporated an additional 55 nucle-

otides (which would result in emergence of the cytosine-rich sequence in the terminator) if attenuation under conditions of excess amino acid supply is to occur. The calculations of Bremer and Dennis (8) suggest that the mRNA chain elongation rate is 50 nucleotides per s and, for cells with a 60-min doubling time, that the average peptide chain elongation rate is 16 amino acid residues per s. Thus, unless the polymerase incorporates those 55 nucleotides more slowly than average, the coding segment of the *ilv* leader transcript must be translated considerably faster than the average protein message.

One unexplained observation in this study is the absence of any detectable effect of the 15-bp deletion that removed three valine codons, one isoleucine codon, and one serine codon. Indeed, a shortened peptide-coding region would be expected to allow the ribosome to reach and cover the bases that enter into antiterminator formation and actually increase attenuation. However, no difference in growth rate or effectiveness of *ilv* operon regulation could be detected. A short-term (~50 generations) evolution experiment failed to provide any evidence for superiority of the wild type. (This experiment was performed with Tn*10* inserted into an *arg* gene in the strain with the wild-type leader in one case and in the strain with the 15-base deletion leader in the other. In each case, the Tn*10*-containing strain outgrew the other, similar to the earlier findings of Chao et al. [9].) Thus, the question remains why these 15 bases have been preserved in the *ilv* leader.

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