Mutations Replacing the Leucine Codons or Altering the Length of the Amino Acid-Coding Portion of the *ilvGMEDA* Leader Region of *Escherichia coli*

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The specificity of regulation by attenuation of the *ilvGMEDA* operon of *Escherichia coli* was examined by making alterations in the peptide-coding portion of the leader region. The effects of the alterations on attenuation control were monitored by operon fusions with the *lacZ* or *cat* gene. Substitution of the tandem leucine codons with arginine codons did not result in arginine control of attenuation even though the altered leader transcripts contained three consecutive arginine codons. Substitution of the single leucine codon with a proline codon at position 10 of the putative peptide, which had been shown to be important in the regulation of the *Serratia marcescens* *ilv* operon, did not result in control of attenuation by proline. Since the formation of neither proline nor arginine biosynthetic enzymes is regulated by attenuation control, the effect of tandem phenylalanine codons in place of the tandem leucine codons was examined and found not to result in control by phenylalanine supply. The latter failure may have been due to a configuration in the secondary structure of the protector stem of the leader transcript different from that of the wild-type transcript. The results of the study favored the idea that the lead ribosome does not initiate translation of the leader transcript until after the RNA polymerase has reached the pause site (117 bases into the leader region).

In *Escherichia coli*, the multivalent regulation of the *ilvGMEDA* operon by the three branched-chain amino acids appears to be achieved exclusively via an attenuation mechanism (2, 21, 22, 26). In the preceding report (4), the specificity of the *ilvGMEDA* regulatory region was examined with respect to the amino acids that, upon limitation, would lead to derepression or when added singly to minimal medium-grown cells would lead to repression. Although the 32-amino-acid peptide-coding region of the *ilv* leader transcript contains 14 branched-chain amino acid codons between positions 4 and 20, there are interspersed among them, at position 6, a codon for arginine, and at positions 9 and 14, two codons for serine. Neither limitation of these non-branched-chain amino acids in an auxotrophic strain nor their supplementation in a prototrophic strain had any effect on *ilv* operon control as measured by β -galactosidase activity expressed from an *ilv-lac* operon fusion.

Here we report experiments to address this specificity by site-specific mutagenesis of the *ilv* leader region. The experimental results support the concept that translation of the leader transcript is delayed until a significant portion of the transcript has been formed and the polymerase has reached the pause site (15). They also raise the possibility that the nascent leader peptide can influence the efficiency of the attenuation mechanism.

MATERIALS AND METHODS

Bacterial strains. The strains used initially for these experiments are described in Table 1. Strain CU1644 deserves special mention, since it provided a readily transferred

deletion of nearly the entire *ilv* gene cluster. Although it did not extend into *ilvC*, it did prevent *ilvC* expression, since it disrupted the positive control gene, *ilvY*. It thus did not extend into *rep* as do the other commonly used *ilv* deletions, $\Delta ilvDAC115$ and $\Delta ilvG-C2049$. The deletion itself was prepared as described by Harms et al. (13). The set of isogenic derivatives containing the wild-type and mutated *ilv* leader regions controlling the *ilvGMED'-lacZ* operon were prepared as shown in Fig. 1 and are described further in Table 4 (see below).

Growth of cells. The media employed and the methods of growing the cells under conditions of excess and limiting amino acid supplements were those described in the preceding report (4).

Preparation of cell extracts. Crude extracts of cells grown under limiting conditions were prepared from 200-ml cultures. The cell pellet was resuspended in 5 to 10 ml of cold disruption buffer consisting of 0.05 M potassium phosphate (pH 7.0), 0.1 mM magnesium sulfate, 0.5 mM dithiothreitol, and 5% glycerol. The cell suspensions were disrupted with a Branson Sonifier (model W185) at 4°C. After centrifugation, the clear, viscous supernatant fluid was transferred to a clean test tube and placed in ice for immediate determination of enzyme activity and later determination of protein concentration.

Assay of enzyme activity. Transaminase B activity in cell extracts was assayed by measuring the amount of α -ketoisovalerate formed from α -ketoglutarate and valine by the method of Duggan and Wechsler (5). Specific activity was expressed as nanomoles of product per milligram of protein per minute. β -Galactosidase activity was measured in cell extracts and in cells treated with sodium dodecyl sulfate (SDS) and chloroform by the method of Miller (25). Specific activity was expressed as micromoles of product per milligram of protein per minute. Protein in cell extracts was

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TABLE 1. Bacterial strains used

Strain	Also called	Genotype	Source and description
CU838		$\Delta(\text{ilvG-Y})2049 \Delta(\text{pro-lac}) \text{ara thi}$	Watson et al. (33)
CU1088		$\Delta(\text{pro-lac}) \text{ara thi} \Delta\text{ilvGME2130 ilvD2139::}\Delta\text{Mu::}\lambda\text{p1 (209)}$	Gayda et al. (9)
CU1132	NK5304	HfrPO45 <i>srlC300::Tn10 recA56 ilv-318 thr-300 thi-1 rel-1 rpsE300</i>	N. Kleckner
CU1230		$\Delta\text{ilvGME1230 ara} \Delta(\text{pro-lac}) \text{thi recA56 hsdR}$	Harms et al. (14)
CU1256	PS2018	<i>araD139</i> $\Delta\text{lacU169 thi-1 rpsL arg::Tn10}$	M. Levinthal
CU1257		$\Delta(\text{ilvG-Y})2049 \Delta(\text{pro-lac}) \text{ara thi arg::Tn10}$	CU838 transduced with P1 lysate of CU1256; selected for Tet ^r , screened for Arg ⁻
CU1259		$\Delta(\text{pro-lac}) \text{ara thi ilvD2139::}\Delta\text{Mu::}\lambda\text{p1 (209) arg::Tn10}$	Chen et al. (4)
CU1289		(F' $\Delta\text{lacZ(H119) proA}^+ \text{proB}^+$)::(F' 16 <i>ilv</i> ⁺)/ $\Delta(\text{pro-lac}) \text{ara thi arg::Tn10}$ $\Delta\text{ilvGME2130}$	Chen et al. (4)
CU1318	CGSC5913, W5449	$\lambda^- \text{leuB6 proA2 trpB9579 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 hsdR18 tsx-33 tonB56 supE44 recB21 recC22 sbcB15}$	B. Bachmann; Sgaramella et al. (29)
CU1330		$\Delta(\text{ilvG-Y})2049 \text{leu-455 metE zig-117::Tn10 polA1 galT12}$	Hsu et al. (16)
CU1447	NK5935	<i>pro endA hsdR ilv::Tn5</i>	N. Kleckner
CU1450	NK6018	HfrH $\Delta(\text{pro-lac})\text{XII thi leu::Tn5}$	N. Kleckner
CU1451	NK6039	HfrH $\Delta(\text{pro-lac})\text{XIII thi leu::Tn10}$	N. Kleckner
CU1644		$\Delta(\text{ilvG-Y})2243::\text{aadA}^+$ (omega fragment, Str ^r Spc ^r (<i>leuB6 proA2 trpB9579 his-4 argE3 thi-1 hsdR18 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 tonB56 supE44 recB21 recC22 sbcB15</i> λ^-	Harms et al. (13)
CU1662		$\Delta(\text{ilvG-Y})2243::\text{aad}^+$ (omega fragment, Str ^r Spc ^r) $\Delta(\text{pro-lac}) \text{ara thi arg::Tn10}$	CU1257 transduced with P1 lysate of CU1644; selected for Spc ^r , screened for Ilv ⁻
CU1694		$\lambda^- \text{leuB6 proA2 trpB9579 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 hsdR18 tsx-33 tonB56 supE44 recB21 recC22 sbcB15 (pro}^+$ $\Delta\text{ilvGME2130}$	Chen et al. (4)
CU1695		$\Delta(\text{pro-lac}) \text{ara thi} \Delta\text{ilvGME2130 ilvD2139::}\Delta\text{Mu::}\lambda\text{p1 (209) arg::Tn10}$	CU1088 transduced with P1 lysate of CU1256; selected for Tet ^r , screened for Arg ⁻
CU1696		$\Delta(\text{pro-lac}) \text{ara thi} \Delta\text{ilvGME2130 ilvD2139::}\Delta\text{Mu::}\lambda\text{p1 (209) arg::}\Delta\text{Tn10}$	CU1695 selected on Bochner plate; screened for Tet ^s Arg ⁻
CU1697		$\Delta(\text{pro-lac}) \text{ara thi} \Delta\text{ilvGME2130 ilvD2139::}\Delta\text{Mu::}\lambda\text{p1 (209) arg::}\Delta\text{Tn10 leu::Tn10}$	CU1696 transduced with P1 lysate of CU1451; selected for Tet ^r , screened for Leu ⁻
CU1698		$\Delta(\text{pro-lac}) \text{ara thi} \Delta\text{ilvGME2130 ilvD2139::}\Delta\text{Mu::}\lambda\text{p1 (209) arg::}\Delta\text{Tn10 leu::}\Delta\text{Tn10}$	CU1697 selected on Bochner plate; screened for Tet ^s Leu ⁻ Arg ⁻
CU1708		$\Delta(\text{pro-lac}) \text{ara thi ilvD2139::}\Delta\text{Mu::}\lambda\text{p1 (209) ilvG2257::aph}^+ \text{ilvG2200 srlC300::Tn10 recA56}$	Chen et al. (4)
CU1737		$\Delta(\text{pro-lac}) \text{ara thi arg::}\Delta\text{Tn10 leu::}\Delta\text{Tn10 ilvD2139::}\Delta\text{Mu::}\lambda\text{p1 (209) ilvG2260::aph}^+ \text{ilvG2200 ilvGe2266}$	CU1698 transduced with P1 lysate of CU1736 (see Table 4); selected for Kan ^r blue colonies on X-gal plate, screened for KA ⁺ ^a
CU1738	SP1001	<i>trpB8::Tn10 pheA1-1 Zfh(URF2)::</i> ($\Omega(\text{Spc}^r \text{Str}^r)$)	G. P. Zhao
CU1766		$\Delta(\text{ilvG-Y})2243::\text{aad}^+$ (omega fragment, Str ^r Spc ^r) $\Delta(\text{pro-lac}) \text{ara thi arg::Tn10}$	CU1622 transduced with P1 lysate of CU1450; selected for Kan ^r , screened for Leu ⁻
JM103		F' <i>traD36 proAB⁺ lacI^a Δ15/</i> ($\Delta(\text{pro-lac}) \text{supE44 thi rbsL sbcB15 endA hsdR4}$	Yanisch-Perron et al. (36)

^a KA⁺, Isoleucine and valine requirement satisfied by the corresponding α -keto acids.

measured by the dye binding assay of Bradford (3) with reagent supplied by Bio-Rad Laboratories. Chloramphenicol acetyltransferase activity was measured as described earlier (13).

Plasmids and bacteriophages. The plasmids used initially for these experiments are described in Table 2. The bacteriophages used initially are described in Table 3. The set of plasmids carrying the *ilv* leader mutations was prepared as shown in Fig. 1. The plasmid and bacteriophage derivatives carrying these mutations are described further in Table 4 (see below).

Site-specific mutagenesis of *ilv* leader region and transfer of the mutations to plasmids and to *ilv-lac* operon fusions. Two of the mutant leader regions were generated by the general method of Zoller and Smith (37). The template was bacteriophage M13JW12, an M13mp18 derivative carrying the

2.1-kb *SmaI-SphI* fragment from pPU24 (Fig. 1A). The oligonucleotide primers used in the mutagenesis were synthesized in the Purdue Laboratory for Macromolecular Structure by Philip Andrews with an ABI synthesizer (model 380A). One of these primers, a 19-mer, 5' C-A-C-T-C*-G-T-C*-G-A-C-G-G-G-C-T-G-T-C 3' (the substituted bases are marked with asterisks), allowed the formation of a leader in which two arginine codons replaced the two leucine codons at positions 4 and 5 of the open reading frame in the leader region. The mutation could be identified by the fact that a new *SalI* site (G-T-C-G-A-C) had been generated. A second primer, a 21-mer, 5' C-T-A-A-T-C-A-C-G-C*-C-C-G*-G-G-C-T-A-A-T-C 3', allowed the formation of a leader in which proline and glycine codons replaced the leucine and valine codons at positions 10 and 11. This mutation could be identified by the fact that a new *SmaI* site (C-C-C-G-G-G)

FIG. 1. Construction of M13 derivatives, plasmids, and strains used to generate and transfer the modified *ilvGMEDA* leader regions to the auxotrophic and prototrophic *ilv::lac* fusion strains, recorded in Table 4. (A) Mutations were generated by synthetic oligonucleotide-primed, site-specific mutagenesis or restriction enzyme digestion of an M13 derivative carrying a 2.1-kb *Sma*I-*Sph*I *ilvGpeaG'* fragment derived from pPU24. This fragment also included the *ilvG2200* mutation which restored *ilvG* function in the parental plasmid. (B) The mutated *ilvG2200* fragment was transferred to an intermediate pUC19 derivative that carries a 3.4-kb *Pst*I-*Kpn*I '*aph* *ilvGpeaG'*' fragment. (C) The *Bgl*II-*Kpn*I *ilvGpeaG'* fragment from the pPU351 derivative was transferred to a pBR322 derivative that carries the *aph*⁺ gene at the *Hpa*I site upstream of the *ilv* gene (*ilvG2260::aph*⁺) and the *Hind*III *ilvGpeaGMED'* fragment. (D) The linear *Bam*HI-*Eco*RI *ilvG2260::aph*⁺ *ilvGpeaGMED'* fragment was used to transform a *recBC sbcB* Δ *ilvGME2130* strain, CU1694. Selection for Kan^r and screening for Ilv⁺ and Amp^s would indicate the transformants that have the mutated *ilvGpea* integrated on the chromosome. *ilvGpea*, Wild-type leader region. (E) The P1 lysate prepared from the *recBC sbcB* derivative was used to transduce the *ilv::lac* fusion strain, CU1698. Selection for blue colonies on the minimal X-Gal (5-bromo-4-chloro-3-indolyl- β -galactoside) plates supplemented with kanamycin and an excess of arginine, proline, isoleucine, leucine, and valine and screening for IlvE⁺ resulted in an auxotrophic derivative used for the limitation experiments. (F) The same P1 lysate was used to transduce another *ilv-lac* fusion strain, CU1088. Blue colonies on minimal X-Gal plates supplemented with kanamycin and excess proline, isoleucine, and valine were selected and scored for IlvE⁺. (G) IlvE⁺ colonies shown in panel F were made *recA* by P1 transduction. (H) The *recA* derivative was conjugated with CU1289, which carries a fused episome, (F' *pro*⁺):(F' 16 *ilv*⁺). The prototrophic conjugants were used for the repression experiments.

had been generated. The resulting double-stranded DNA was used to transform strain CU1645 (Δ *ilvG-Y'*::*aad*⁺). Transformants were examined for phage DNA replicating form (RF) in which a new *Sal*I or *Sma*I site had been generated. That the intended base changes had indeed occurred was verified by nucleotide sequence analysis.

The mutagenized *ilv* leader regions were excised from the RF of the mutant M13mp18 derivatives with *Sph*I and *Sma*I and inserted into similarly cut pPU351 (Fig. 1B), a pUC19 derivative containing the 3.4-kb *Bgl*II-*Kpn*I portion of the same 6.4-kb *Hind*III fragment that had been placed in pPU353 (Fig. 1C) (to yield pPU354 and pPU356, respectively), as shown in Fig. 1 and Table 4. The new plasmids were again identified by the *Sal*I and *Sma*I sites and cut with *Bgl*II

and *Kpn*I and mixed with similarly cut pPU353, ligated, and used to transform CU1230 (Δ *ilvGME2130*) to yield IlvE⁺ Kan^r strains. These plasmids, containing the *aphA* gene upstream of *ilvGMED'* (see below), were cut with *Eco*RI and *Bam*HI and used to transform CU1694, a *recBC* strain containing Δ *ilvGME2130*, to yield strains CU1711 and CU1716 (Fig. 1D and Table 4). P1 phage grown on these strains was then used to transduce strain CU1698 to yield strains CU1712 and CU1717 (Fig. 1E and Table 4). These strains thus contained the modified *ilv* leader regions upstream of the *ilvD::lac* fusion in strains that were auxotrophic not only for the three branched-chain amino acids but also for arginine and proline. They could therefore be used to compare repressing and derepressing conditions. For

TABLE 2. Plasmids used

Plasmid	Genotype ^a	Source or description
pPU24	pBR322 Ω [0.029 kb: λ h80 <i>ilv</i> <i>ilvG2200</i> <i>ilvE2201</i> <i>ilvGME'</i> -2.1/2.37 kb (-)]13	Formerly pCS7, Subrahmanyam et al. (32)
pPU331	pUC8 Δ [0.404 - 0.426 kb]10 Ω [0.404 kb: pBR322::Tn5 <i>aph</i> ⁺ 1.321 kb (+)]11	<i>Hind</i> III- <i>Sma</i> I <i>aph</i> ⁺ fragment of pBR322::Tn5 was cloned into <i>Hind</i> III- <i>Sma</i> I sites of pUC8; Kan ^r
pPU336	pPU333 Ω [0.029 kb: pPU334 <i>ilv'ED'</i> 2.438/4.097 kb (-)]2	Chen et al. (4)
pPU339	pUC18 Δ [0.404 - 0.436 kb]6 Ω [0.404 kb: pPU24 3.6/5.1 kb (+)]7	1.5-kb <i>Hind</i> III- <i>Sma</i> I <i>ilv</i> upstream fragment of pPU24 was cloned into <i>Hind</i> III- <i>Sma</i> I sites of pUC18
pUC18	<i>bla</i> ⁺ <i>lacZpZoZ'</i>	Yanisch-Perron et al. (36)
pUC19	<i>bla</i> ⁺ <i>lacZpZoZ'</i>	Yanisch-Perron et al. (36)

^a The standard conventions used earlier (15) were 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.

a comparison of nonrepressing conditions (growth in minimal medium) with repressing conditions, prototrophic counterparts with the same mutant leader regions were prepared.

The prototrophic derivatives were prepared by using phage lysates grown on the CU1694 derivatives (CU1711 and CU1716) to transduce strain CU1088, an Arg⁺ Leu⁺ strain bearing the *ilvD-lacZ* operon fusion (Fig. 1F and Table 4). Kanamycin-resistant transductants were selected and scored for Ilv⁺ and retention of the *ilvD::lac* fusion. The resulting transductants which were Ilv⁻ Pro⁻ were rendered prototrophic by converting to *recA* (Fig. 1G) by P1 transduction with strain CU1132 as the donor and by conjugal transfer of the *ilv*⁺-*pro*⁺-*lac*⁻ hybrid F factor (see below) from strain CU1289 to yield strains CU1715 and CU1720 (Fig. 1H).

During the oligonucleotide-directed mutagenesis designed to introduce a new *Sma*I site into the leader region, an unexpected mutant phage was isolated. This phage, M13JW-30, upon nucleotide sequence analysis of its pBR322 counterpart, pPU359, was shown to have a 9-base repeat in addition to the new *Sma*I site. The origin of the repeat is not known, but the fact that the same three codons appear both before and after the modified codons may have been a factor. Furthermore, the single-stranded template would have had the same tendency for base pairing as that proposed for the leader transcript. The mutation was designated mutation VI.

Because cutting at the *Sal*I site that had been generated during the change in the tandem leucine codons in the leader to two additional arginine codons resulted in four 5' overhanging bases, it was possible to change the reading frame in the leader by the filling in with Klenow polymerase or by removing the overhanging bases with mung bean nuclease. Both procedures were used to produce the leaders that would be expected to give rise to truncated leader peptides. The manipulations were performed on the RF of the original M13mp19 mutant phage (M13JW27) containing the new *Sal*I site (Leu-Leu $\rightarrow$ Arg-Arg). These derivatives, carrying the frameshifts, were then transferred to pPU351 as shown in

Fig. 1, and in a series of manipulations parallel to those used to produce the auxotrophic and prototrophic strains containing the original site-directed leader mutations, strains suitable for derepression and repression experiments were prepared. The strains specifying the peptide truncated by inserting 4 bases (mutation IV) into the leader region at the *Sal*I site were designated CU1727 (auxotrophic) and CU1730 (prototrophic). Those specifying the peptide truncated by mung bean nuclease digestion at the *Sal*I site (mutation V) were designated CU1732 (auxotrophic) and CU1735 (prototrophic) as shown in Table 4.

A third oligonucleotide primer, 5' C-A-C-T-C-G-G*-A*-A*-G*-A*-A*-G-G-C-T-G-T-C 3', was used to generate a mutant leader specifying a peptide in which the leucine residues at positions 4 and 5 were replaced by phenylalanine residues. Because this oligonucleotide did not generate a restriction site, the template used was the M13mp19 derivative specifying the leader containing the string of three arginine codons, M13JW27 (mutation I). Although the new oligonucleotide primer differed in only 4 bases from the wild-type leader region, the primer template hybrid actually used had six mismatched base pairs (indicated by stars) because the template contained the *ilvGe2261* allele. It was thus possible to identify the desired RF DNA by the loss of the unique *Sal*I site. Again, the mutant leader was incorporated into the chromosome upstream of the *ilvD-lac* fusion by a series of manipulations as described above for the other mutations. The resulting prototrophic derivative was designated CU1742, and the auxotrophic derivative was designated CU1739. (The latter also contained the *pheA1* mutation.) The mutant development is also summarized in Fig. 1 and Table 4.

Generation of *ilv* leader mutations and their transfer to operon fusions lacking internal promoter *ilvEp*. The fusions described above were prepared in strains and plasmids in which the internal promoter (*ilvEp*) had been retained. Since this promoter serves to damp the regulatory response of the

TABLE 3. Bacteriophages used

Phage	Genotype or description	Source or reference
M13mp18	M13-based cloning vector	Yanisch-Perron et al. (36)
M13mp19	M13-based cloning vector	Yanisch-Perron et al. (36)
M13JW12	M13mp18 Δ [6.249-6.279] Ω [6.249 kb: pPU24 <i>ilvGpea</i> ⁺ <i>ilvG2200</i> <i>ilvG'</i> 2.1 kb (+)]	2.1-kb <i>Sma</i> I- <i>Sph</i> I <i>ilvGpea</i> <i>ilvG'</i> fragment of pPU24 ligated with M13mp18 RF cut with <i>Sma</i> I and <i>Sph</i> I
P1cm100clr	Transducing phage	Rosner (28)
λ RZ5	λ <i>plac'</i> ZY ' <i>bla</i>	Grove and Gunsalus (12)

TABLE 4. M13 derivatives, plasmids, and strains prepared to generate and transfer the modified *ilvGMEDA* leader regions to auxotrophic and prototrophic *ilv::lac* fusion strains

Allele	Mutation	A ^a (M13mp18 derivatives)	B (pUC19 derivatives)	C (Km ^r <i>ilvE</i> ⁺ pBR322 derivatives)	D (Km ^r <i>ilvE</i> ⁺ CU1694 derivatives)	E (Km ^r <i>ilvE</i> ⁺ CU1698 derivatives)	F (Km ^r <i>ilvE</i> ⁺ CU1088 derivatives)	G (<i>recA</i> derivatives)	H (<i>pro</i> ⁺ <i>ilv</i> ⁺ conjugants)
<i>ilvGe</i> ⁺	Wild type	M13JW12	pPU351	pPU353	CU1743	CU1744	CU1746	CU1747	CU1748
<i>ilvGe2261</i>	I (Leu-4-Leu-5 to Arg-4-Arg-5)	M13JW27	pPU354	pPU355	CU1711	CU1712	CU1713	CU1714	CU1715
<i>ilvGe2262</i>	II (Leu-10-Val-11 to Pro-10-Gly-11)	M13JW32	pPU356	pPU357	CU1716	CU1717	CU1718	CU1719	CU1720
<i>ilvGe2266</i>	III (Leu-4-Leu-5 to Phe-4-Phe-5)	M13JW57 ^a	pPU364	pPU365	CU1736	CU1739 ^b	CU1740	CU1741	CU1742
<i>ilvGe2264</i>	IV (UAG codon position 10)	M13JW34	pPU360	pPU361	CU1726	CU1727	CU1728	CU1729	CU1730
<i>ilvGe2265</i>	V (UGA codon position 6)	M13JW35	pPU362	pPU363	CU1731	CU1732	CU1733	CU1734	CU1735
<i>ilvGe2263</i>	VI (Leu-10-Val-11 to Pro-10-Gly-11 + 9-bp insertion)	M13JW30	pPU358	pPU359	CU1721	CU1722	CU1723	CU1724	CU1725

^a A to H, Refer to Fig. 1 for details.

^b CU1739 also contains the *pheA1-l* mutation introduced by transduction of CU1737 by a P1 lysate of CU1738, selection for Spc^r, and screening for Phe⁻.

attenuation system, it seemed worthwhile to generate leader mutations that would not be affected by the internal promoter. Two kinds of fusion operons were examined in this way. One was an *ilv-cat* fusion, and the other was an *ilv-lac* fusion. The same oligonucleotide primers described above were used independently to generate mutations I and II.

The template for mutagenesis was an M13mp18 derivative carrying a 1.4-kb *AccI* (filled in)-*SphI* *ilv* fragment inserted into its *SmaI* and *SphI* sites. Site-specific mutagenesis was performed in exactly the same way as described above. The leader mutations so obtained as well as the unaltered wild-type leader were transferred to pUC18 derivatives by excising the *EcoRI*-*HindIII* fragments from the M13mp18 derivatives and inserting into *EcoRI*-*HindIII*-digested pUC18. As shown in Fig. 2 and described in Table 5, the wild-type and

mutated *ilv* leader regions were inserted into pPU191 to generate *ilv-cat* fusions. Since these plasmid derivatives carried *ilvB* gene DNA, it was possible to insert them into the chromosome of CU1330, a *polA* strain deleted for the entire *ilv* gene cluster (16). The resulting strains, CU1793, CU1794, and CU1795, were used to assess *ilv*-specific regulation by the activity of chloramphenicol acetyltransferase.

The alternative approach was to generate *ilv-lac* fusions with the operon fusion vector pRS415 (30). The 1-kb *ilv* fragments derived from *EcoRV* digests of M13mp18 RF containing the wild-type and mutated *ilv* leader regions, described above, were inserted into the *SmaI* site of pRS415 to generate Lac⁺ Amp^r. The *ilv-lac* fusions in the desired orientation were then transferred to λRZ5 by homologous recombination as described by Grove and Gunsalus (12).

FIG. 2. Construction of *ilv-cat* operon fusions containing the wild-type and mutant *ilvGMEDA* promoter-leader regions. The wild-type leader region inserted in M13mp18 was altered by site-specific mutagenesis to yield mutations I and II as indicated in Table 5 and transferred as shown to the *cat* expression vector pPU191, which contained a fragment of the *ilvB* gene, allowing integration into the chromosome.

TABLE 5. M13 derivatives, plasmids, and strains used to examine the effects of mutations I and II on *ilv* fusions lacking the internal promoter, *ilvEp*

Allele	Mutation	M13mp18 derivatives	pUC18 derivatives	pPU191 derivatives	CU1330 <i>polA</i> derivatives	pRS415 derivatives	λ RZ5 <i>ilv-lac</i> derivatives	CU1766 <i>ilv-lac</i> lysogens
<i>ilvGe</i> ⁺	Wild type	M13EH1	pUCEH1	pPU368	CU1793	pPU371	λ RZ5- <i>ilv1</i>	CU1796
<i>ilvGe2280</i>	Leu-4-Leu-5 to Arg-4-Arg-5	M13EH2	pUCEH2	pPU369	CU1794	pPU372	λ RZ5- <i>ilv2</i>	CU1797
<i>ilvGe2281</i>	Leu-10-Val-11 to Pro-10-Gly-11	M13EH3	pUCEH3	pPU370	CU1795	pPU373	λ RZ5- <i>ilv3</i>	CU1798

Lysogens of the λ RZ5 derivatives containing the *ilv-lac* fusions and a reconstituted *bla* gene were retained as CU1796 (wild-type leader), CU1797 (leader mutation I), and CU1798 (leader mutation II).

Nucleotide sequence analysis. Nucleotide sequence analysis of the mutant leader region was performed by the chain termination method with Sequenase used according to the supplier's directions (United States Biochemical Corp.). The templates were the pBR322 or the M13mp18 and M13mp19 derivatives carrying the various leader mutations (Tables 4 and 5). The templates were hybridized with the primers flanking the leader region described in the preceding report (4).

RESULTS

Examination of the *ilv::lac* fusion controlled by wild-type and mutant *ilv* leader regions. As described in Fig. 1 and Table 4, the auxotrophic strain, CU1744, was constructed to compare the expression of the *ilv-lac* fusion that was controlled by wild-type *ilv* leader under limiting and repressing conditions, whereas the prototrophic strain, CU1748, was used for comparing repressing and nonrepressing conditions. The expression of the wild-type *ilvGMED'-lacZ* operon under limiting conditions in the auxotrophic strain is shown in Table 6 and that under repressing conditions in the prototrophic strain is shown in Table 7.

Limitation of isoleucine increased the β -galactosidase and transaminase B activities approximately sevenfold. Under limiting valine, both enzyme activities increased 3.5-fold. As was mentioned in the preceding report (4) and was observed consistently, the fold derepression of transaminase B under limiting leucine was always about twice that of β -galactosi-

dase. Fourfold derepressed β -galactosidase activity and sevenfold derepressed transaminase B activity were observed when leucine was limited in the auxotrophic *ilv::lac* strain with the wild-type leader. Limitation of proline did not derepress the *ilvGMED'-lacZ* operon. Neither did the limitation of arginine.

A fivefold-greater β -galactosidase activity was observed in CU1744 under repressing conditions than had been observed for another strain with a wild-type *ilv* control region, CU1259, studied previously (4). This difference is due to the frameshift mutation, *ilvG2200* (previously designated as *ilvO*), which was brought into the chromosome along with the *aph*⁺ gene during the homologous recombination between the linear plasmid DNA and chromosome. The lysogen of λ *dilv-lac11*, CU1677, described in the preceding report (4), also lacked the frameshift mutation and therefore exhibited less β -galactosidase activity than did strain CU1744.

The *ilvG2200* mutation also enables the cells to make an active Val^r acetohydroxy acid synthase II, which made the study of the repressing effect of the single addition of valine possible. The lower sensitivity of this wild-type prototrophic derivative to leucine inhibition than that of CU1259 is probably also due to the presence of the *ilvG2200* mutation, as will be discussed below. However, the inhibition of cell growth and derepression of the *ilvGMED'-lacZ* operon by serine were still observed. In other words, the *ilv-lac* region used in this study exhibited the same highly specific response to the three branched-chain amino acids when gene expression was controlled by the wild-type leader as did the *ilv-lac* region in the previous study. The presence of the *ilvG* mutation restoring the reading frame in that gene only increased gene expression.

TABLE 6. Effects of several *ilv* leader mutations on the expression of the *ilvGMED'-lacZ* operon under repressing and limiting conditions

Limiting amino acid	Wild type (<i>ilvGe</i> ⁺) (CU1744)				I (<i>ilvGe2261</i>) (Leu-4-Leu-5→Arg-4-Arg-5) (CU1712)				II (<i>ilvGe2262</i>) (Leu-10-Val-11→Pro-10-Gly-11) (CU1717)			
	β -Galactosidase ^a		Transaminase B ^a		β -Galactosidase		Transaminase B		β -Galactosidase		Transaminase B	
	Sp act ^b	%	Sp act ^c	%	Sp act	%	Sp act	%	Sp act	%	Sp act	%
None	0.74 ± 0.06	100	74 ± 6	100	0.75 ± 0.14	100	87 ± 5	100	0.99 ± 0.08	100	69 ± 8	100
Isoleucine	5.51 ± 0.84	745	517 ± 33	699	3.10 ± 0.14	413	313 ± 10	360	2.56 ± 0.08	259	176 ± 27	255
Leucine	2.95 ± 0.29	399	547 ± 57	739	0.65 ± 0.03	87	109 ± 9	125	1.03 ± 0.10	104	183 ± 24	265
Valine	2.61 ± 0.2	353	262 ± 63	354	1.46 ± 0.04	195	162 ± 16	186	1.07 ± 0.10	108	125 ± 23	181
Arginine	0.66 ± 0.10	89	102 ± 20	138	0.63 ± 0.13	84	100 ± 4	115				
Proline	0.63 ± 0.11	85	98 ± 35	132					0.68 ± 0.03	69	75 ± 12	109
Phenylalanine												

^a The enzyme activities were determined in sonically disrupted cell extracts. All values given are the average of three independent experiments.

^b Micromoles per milligram of protein per milliliter.

^c Nanomoles per milligram of protein per milliliter.

The addition of isoleucine to the prototrophic strain, CU1748, repressed the expression of the *ilvGMED'-lacZ* operon to 61% of the value at nonrepressing level. The repression was further enhanced by the addition of the two other branched-chain amino acids, to 35% of the value under nonrepressing conditions. The addition of proline, serine, or phenylalanine alone or in conjunction with the three branched-chain amino acids did not have any significant effect on the expression of the *ilvGMED'-lacZ* operon.

Figure 3 compares the predicted secondary structures of the protector regions of the wild-type leader with those resulting from the several mutations. The new arginine codons in mutant leader I are presumed to have the same relative position in the protector stem and loop as did the leucine codons in the wild-type leader. Moreover, there are three consecutive arginine codons in this leader. In contrast, the new phenylalanine codons in mutant III leader are presumed to be base paired in a way that probably would not liberate bases that interfere with protector stem formation if the ribosome stalled at one of those codons (Fig. 3D). Tables 6 and 7 give the results of derepression and repression experiments, respectively, with the strains carrying the various mutant leader regions.

The auxotrophic CU1698 derivative, CU1712, and the prototrophic CU1088 derivative, CU1715, prepared as shown in Table 4 and Fig. 1, were used to determine the effect of mutation I on the expression of the *ilvGMED'-lacZ* operon under limiting conditions and under repressing conditions, respectively.

Mutation I almost completely eliminated the response to the limitation of leucine, but the response to isoleucine and valine limitation was reduced to approximately 60% of that with the wild-type leader. The striking feature of this mutation is that, although there are three tandem arginine codons in the leader transcript, the limitation of arginine did not derepress the *ilvGMED'-lacZ* operon. Thus, it appears that the pattern of derepression in the strain containing mutation I cannot be explained solely on the basis of the proposed secondary structure or placement of the various codons in that structure. The addition of isoleucine to the medium of CU1715, the prototrophic derivative with mutation I, repressed the β -galactosidase to 62% of the value at the nonrepressing level, approximately the same as observed with the wild-type leader. The single additions of valine or leucine led to small increases in activity, owing perhaps to an accentuation of the isoleucine limitation as detected with the wild-type leader (4). The addition of all three branched-chain amino acids to the prototrophic strain with mutation I

repressed it to 47%, which is probably not significantly less repression than that observed with the wild-type leader (Table 7).

In mutant leader II, the Leu-10 and Val-11 codons were replaced by the codons for proline and glycine, respectively. Figure 3C shows the probable secondary structure of the protector region of the transcript resulting from mutation II, which is not unlike that proposed for the wild-type and mutation I leaders. The effect of mutation II on the expression of the *ilvGMED'-lacZ* operon under limiting conditions in the auxotrophic strain (CU1717) is shown in Table 6 and that under repressing conditions in the prototrophic strain (CU1720) is shown in Table 7.

When leucine was limiting, transaminase B activity had increased to about 2.7 times the repressed level, but β -galactosidase activity was about the same as the repressed level. Just as was reported in the preceding paper (see Table 8 [4]) there are consistent differences between the responses of transaminase B and β -galactosidase to limiting leucine. Thus, compared with the regulation observed with the wild-type leader (Table 6), the substitution of this single leucine codon has greatly reduced the response of the *ilvGMED'-lacZ* operon to the limitation of leucine. However, the loss seemed to be less than that caused by mutation I. If the slightly higher value obtained with mutation II could be interpreted as the residual leucine control, the Leu-10 codon might be less crucial than Leu-4 and Leu-5 in the regulation of the *ilvGMED'-lacZ* operon.

Although only one valine codon had been altered, the response of the *ilvGMED'-lacZ* operon to the limitation of valine was completely abolished judging from the β -galactosidase activity, whereas approximately the same extent of response, an 81% increase, in transaminase B was observed as with mutation I. The response to isoleucine limitation was reduced to one-third of the value in the strain with the wild-type leader and was less than the response of that with mutation I. Thus, both mutations replacing only leucine codons had a detrimental effect on the response to the isoleucine and valine availability as well as to the leucine availability. The limitation of proline did not have any derepressing effect.

In the prototrophic strain, the addition of isoleucine repressed the expression of the *ilvGMED'-lacZ* operon to 76% of the level under nonrepressing conditions. The addition of all three branched-chain amino acids still indicated some degree of multivalent control. The replacement of Leu-10 and Val-11 with Pro and Gly, respectively, was accompanied by 15% less repression when adding isoleucine and 20% less

TABLE 6—Continued

III (<i>ilvGe2266</i>) (Leu-4-Leu-5→Phe-4-Phe-5) (CU1739)				IV (<i>ilvGe2264</i>) (UAG codon at position 10) (CU1727)				V (<i>ilvGe2265</i>) (UGA codon at position 6) (CU1732)			
β -Galactosidase		Transaminase B		β -Galactosidase		Transaminase B		β -Galactosidase		Transaminase B	
Sp act	%	Sp act	%	Sp act	%	Sp act	%	Sp act	%	Sp act	%
0.86 $\pm$ 0.23	100	82 $\pm$ 6	100	3.71 $\pm$ 0.24	100	334 $\pm$ 14	100	3.45 $\pm$ 0.49	100	336 $\pm$ 12	100
7.02 $\pm$ 0.34	816	719 $\pm$ 150	877	2.69 $\pm$ 0.09	73	265 $\pm$ 26	79	2.39 $\pm$ 0.35	69	225 $\pm$ 16	67
0.87 $\pm$ 0.10	101	311 $\pm$ 2	379	2.08 $\pm$ 0.06	56	336 $\pm$ 9	101	1.68 $\pm$ 0.17	49	306 $\pm$ 29	91
3.96 $\pm$ 0.64	460	595 $\pm$ 21	726	2.04 $\pm$ 0.12	55	255 $\pm$ 29	76	1.27 $\pm$ 0.08	37	210 $\pm$ 11	63
				3.29 $\pm$ 0.26	89	363 $\pm$ 16	109	2.84 $\pm$ 0.08	82	340 $\pm$ 15	101
0.86 $\pm$ 0.03	100	97 $\pm$ 21	118								

Continued on following page

repression when adding all three branched-chain amino acids relative to that found with the wild-type leader. The addition of proline had no significant effect.

Because the biosynthetic pathways of proline and arginine are not regulated by a transcription attenuation mechanism in *E. coli*, it may be that the cells cannot efficiently respond to the limitation of proline and arginine in the way they do for those amino acids with biosynthetic pathways regulated by attenuation mechanisms. A replacement of the tandem leucine codons with those for an amino acid that is formed in a biosynthetic pathway regulated by attenuation was thought to be indicated. The codons chosen for examination were those for phenylalanine.

Figure 3D shows the probable secondary structure of the region of the leader specified by mutation III in which the tandem leucine protector codons were replaced by those for phenylalanine. This proposed structure places codons 4 and 5 in a configuration unlike that postulated for the other leader transcripts but, rather, one very similar to that predicted in the previous report (4) to be secondarily formed upon binding of the ribosome to the initiation codon. This structure also raises the question whether ribosome stalling at these codons could be expected to interfere with terminator formation. An alternative structure like that shown for the other leaders could form but would be thermodynamically less favorable. The effect of mutation III on the expression of the *ilvGMED'-lacZ* operon under limiting conditions in the auxotrophic strain is shown in Table 6, and that under repressing conditions in the prototrophic strain is shown in Table 7.

The limitation of phenylalanine, which replaced leucine in the leader peptide, was without any regulatory effect. This result was initially surprising in view of the fact that phenylalanine limitation can lead to derepression of the genes for chorismate mutase-prephenate dehydratase and phenylalanyl-tRNA synthetase. It is unclear whether the absence of derepression in this case is due to there being only two codons for phenylalanine or to the peculiar position of the phenylalanine codons in the probable secondary structure of the protector region of the leader. It might well be that a ribosome stalled at either of the phenylalanine codons would not disrupt the secondary structure of the transcript sufficiently to result in derepression.

It is particularly striking that the strain carrying leader mutation I, which has the alteration at the identical place except that the replacement was arginine instead of phenylalanine, showed significant reduction in the regulatory effects of isoleucine and valine, whereas there was not such a difference between the strain carrying leader mutation III

TABLE 6—Continued

VI (<i>ilvGe2263</i>) (9-base insertion) (CU1722)			
β-Galactosidase		Transaminase B	
Sp act	%	Sp act	%
0.62 ± 0.08	100	43 ± 21	100
2.86 ± 0.77	461	202 ± 86	470
0.95 ± 0.21	153	125 ± 69	291
1.09 ± 0.21	176	86 ± 30	200
0.38 ± 0.05	61	46 ± 7	107

TABLE 7. Effect of several *ilv* leader mutations on β-galactosidase formation from the *ilvGMED'-lacZ* operon under repressing and nonrepressing conditions^a

Addition(s) to minimal medium	No mutation (<i>ilvGe</i> ⁺) (wild type) (CU1748)		I (<i>ilvGe2261</i>) (Leu-4- Leu-5→Arg-4-Arg-5) (CU1715)		II (<i>ilvGe2262</i>) (Leu-10- Val-11→Gly-10-Pro- 11)(CU1720)		III (<i>ilvGe2266</i>) (Leu-4- Leu-5→Phe-4-Phe-5) (CU1742)		IV (<i>ilvGe2264</i>) (UAG codon at position 10) (CU1730)		V (<i>ilvGe2265</i>) (UGA codon at position 6) (CU1735)		VI (<i>ilvGe2263</i>) (9-base insertion) (CU1725)	
	Sp act ^b	%	Sp act	%	Sp act	%	Sp act	%	Sp act	%	Sp act	%	Sp act	%
None	1.17 ± 0.18	100	1.23 ± 0.07	100	0.72 ± 0.05	100	0.75 ± 0.06	100	1.08 ± 0.09	100	1.55 ± 0.05	100	0.52 ± 0.02	100
Isoleucine	0.71 ± 0.11	61	0.76 ± 0.06	62	0.55 ± 0.01	76	0.57 ± 0.01	76	1.06 ± 0.01	98	1.45 ± 0.06	94	0.36 ± 0.01	69
Leucine	1.28 ± 0.22	109	1.17 ± 0.02	95	0.67 ± 0.03	93	1.35 ± 0.17	180	0.90 ± 0.02	83	1.44 ± 0.09	93	0.76 ± 0.07	146
Valine	1.34 ± 0.19	115	1.46 ± 0.04	119	0.85 ± 0.03	118	1.20 ± 0.16	160	1.08 ± 0.06	100	1.45 ± 0.05	94	0.73 ± 0.01	140
Proline	1.19 ± 0.15	102			0.78 ± 0.02	108							0.57 ± 0.02	110
Arginine	1.30 ± 0.18	111	1.17 ± 0.10	95					1.11 ± 0.05	103	1.35 ± 0.01	87		
Serine	2.11 ± 0.26	180												
Phenylalanine	1.02 ± 0.00	87	0.58 ± 0.07	47	0.40 ± 0.02	56	0.85 ± 0.05	113	0.94 ± 0.03	87	1.28 ± 0.01	83	0.24 ± 0.01	46
Isoleucine, valine, leucine	0.41 ± 0.06	35					0.44 ± 0.00	59						
All of the above	0.45 ± 0.04	38	0.54 ± 0.07	44	0.40 ± 0.02	56	0.48 ± 0.02	64	0.94 ± 0.01	87	1.34 ± 0.01	86	0.23 ± 0.01	44

^a The enzyme activities were determined on chloroform-SDS-treated cells, and all values are given as the average of two independent experiments.

^b Micromoles per minute per milligram of protein.

FIG. 3. Proposed secondary structures for the wild-type and mutant protector stems of the *ilvGMEDA* leader transcripts. Only the portion of each leader extending from the AUG codon to the site of polymerase pausing (15) is shown. The structures were deduced by the program FOLD of Zuker and Steigler (38). The calculated free energies for the structures shown are given in parentheses. (A) Wild type ($\Delta G = -31.3$ kcal); (B) mutation I, Leu-4-Leu-5 to Arg-4-Arg-5 ($\Delta G = -30.5$ kcal); (C) mutation II, Leu-10-Val-11 to Pro-10-Gly-11 ($\Delta G = -29.1$ kcal); (D) mutation III, Leu-4-Leu-5 to Phe-4-Phe-5 ($\Delta G = -27.4$ kcal); (E) mutation IV, UAG at position 10 ($\Delta G = -31.4$ kcal); (F) mutation V, UGA at position 6 ($\Delta G = -28.0$ kcal); (G) mutation VI, 9-base insertion ($\Delta G = -31.4$ kcal).

and that with the wild-type leader. The significance of these differences will be considered later.

In the prototrophic derivative carrying mutation III, there was again a decrease in activity when isoleucine was supplied (indicating a slight isoleucine deficiency) and an increase in activity upon adding leucine or valine (indicating an accentuation of the isoleucine deficiency). Most striking, however, is the absence of any effect of adding phenylalanine either alone or in addition to the branched-chain amino acids.

Two additional leader mutations that were examined in auxotrophic and prototrophic strains are described in Tables 6 and 7. They were both frameshift mutations that resulted in early translational stops in the peptide-coding regions of the leader transcripts. One, mutation IV, was generated from mutation I by cleaving the RF of the M13mp18 derivative carrying the unique *SaI* site and filling in the recessed ends with Klenow enzyme. The other, mutation V, was generated by trimming the overhanging ends with mung bean nuclease. Mutation IV resulted in a stop codon at position 10 (Fig. 3). If we assume, as have Landick et al. (19), that a ribosome is rather slowly released from the transcript upon reaching a stop codon, this mutation would have simulated the stalling of a ribosome at the Leu-10 codon. Mutation V would have simulated stalling of the ribosome at codon 6, normally occupied by a codon for arginine. The enzyme activity measurements of the strains bearing these mutations (strains CU1727 and CU1732, respectively) indicated that ribosome stalling at these positions does indeed lead to a constitutively expressed operon and a loss of branched-chain amino acid regulation (Table 6).

These same frameshift mutations were also examined in prototrophic derivatives (Table 7). Although the β -galactosidase activities in strain CU1730 (mutation IV) were about two-thirds of those in strain CU1735 (mutation V) under both repressing and nonrepressing conditions, there was no significant repressibility of β -galactosidase activity upon addition of the branched-chain amino acids to the minimal medium. Thus, as indicated by the limitation experiments (Table 6), these mutations abolished *ilv*-specific control. The greater activity in strain CU1735 compared with strain CU1730 is probably due to some subtle difference in these strains, which had been presumed to be isogenic, since no such difference was observed with the same mutations in the auxotrophic strains (CU1732 and CU1727, Table 6).

The results with mutation V are particularly interesting, since they revealed that a severe ribosome stalling (due to a nonsense codon) at codon 6 of the leader does lead to deattenuation. Thus, the stalling of the lead ribosome at this same codon by restricting the arginine supply was not sufficiently effective to cause deattenuation. This paradox will be discussed further below.

Regulation of *ilv* operon expression in the absence of the internal (*ilvEp*) promoter. The *ilv-lac* fusions described above were linked to the *ilvG2200* mutation, which allowed complete translation of the *ilvG* transcript as well as that of the translationally coupled *ilvM* gene. Such conditions reduced considerably the basal level of transcription arising from the internal promoter (*ilvEp*) lying within the *ilvM* gene (24). Nevertheless, under repressing conditions some of the transcription into the *ilvE* gene and the *ilvD'-lac* fusion probably arose at that internal promoter. To demonstrate

TABLE 8. Effect of *ilv* leader mutations I and II on the expression of *ilvG'*-*cat* and *ilvG'*-*lacZ* operons under repressing and limiting conditions

Limiting amino acid	No mutation (<i>ilvGe</i> ⁺)				I (<i>ilvGe2280</i>) (Leu-4-Leu-5→Arg-4-Arg-5)				II (<i>ilvGe2281</i>) (Leu-10-Val-11→Pro-10-Gly-11)			
	CU 1793 (chloramphenicol acetyltransferase)		CU1796 (β-galactosidase)		CU 1794 (chloramphenicol acetyltransferase)		CU1792 (β-galactosidase)		CU1795 (chloramphenicol acetyltransferase)		CU1798 (β-galactosidase)	
	Sp act ^a	%	Sp act ^b	%	Sp act	%	Sp act	%	Sp act	%	Sp act	%
None	2	100	0.51	100	4	100	0.58	100	3	100	1.15	100
Isoleucine	35	1,750	5.18	1,016	22	675	3.30	569	23	767	2.31	201
Leucine	21	1,050	1.26	247	15	375	0.42	72	11	367	0.88	77
Valine	45	2,250	5.37	1,052	30	750	4.87	839	16	533	2.17	188
Arginine			0.38	75			0.34	59			0.45	39
Proline			0.37	73			0.31	53			0.65	57

^a Percent chloramphenicol converted to acetylchloramphenicol per 15 minutes per microgram of protein.

^b Micromoles per milligram of protein per minute. The activities were determined in SDS-chloroform-treated cells.

that such transcription would not alter the general conclusions drawn from the experiments reported in Table 6, we examined the effects of the wild-type and mutant leaders in operon fusions that lacked the internal promoter. These fusions contained either the *cat* gene or the *lacZ* gene, each with its own ribosomal start site fused early in the *ilvG* gene as described in Materials and Methods, and were also examined as single-copy constructs.

The results obtained with the two kinds of fusion are shown in Table 8. In general, the results indicate effects of mutations I and II similar to those shown in Table 6. The control by limiting leucine was reduced (relative to that by limiting valine or limiting isoleucine) by the mutations. Furthermore, derepression by limiting isoleucine or limiting valine was also lower with the mutant leaders than with the wild type, a feature that will be considered in the Discussion.

As previously noted, when the effect of limiting leucine on β-galactosidase activity was examined in the presence of either a wild-type or a mutant leader, the values were low, reflecting perhaps the higher frequency of leucine residues in β-galactosidase than in bulk *E. coli* protein. In contrast, the leucine content of chloramphenicol acetyltransferase is about like that in *E. coli* protein. In these experiments, the response to leucine limitation was reduced about the same extent by either of the two mutations. The fact that the limitation signals were more effective in the experiments described in Table 8 than in those in Table 6 (particularly when valine was limiting) may indicate differences in the relative amino acid uptake for the three branched-chain amino acids in the two genetic backgrounds.

Effect of a 9-base insertion into the amino acid-coding region of the *ilv* leader. In the previous report (4), a deletion was described in which a deletion of 15 bp in the leader region had occurred. Surprisingly, this deletion, in which three valine, one isoleucine, and one serine codons had been removed, appeared to have no effect on the specificity of attenuation control by the branched-chain amino acids. In view of this result, it was of interest to examine an unexpected leader mutation that was generated during the same site-directed mutagenesis experiment that gave rise to mutation II. The unexpected mutation was one in which the same Pro-Gly substitution for Leu-Val (generating a new *Sma*I site) had occurred, but in addition, the three following codons for valine, isoleucine, and serine were duplicated and inserted.

The 9-base insertion mutation was recovered from an M13

derivative (M13JW30) and transferred, as shown in Fig. 1 and Table 4, to the auxotrophic and prototrophic strains CU1722 and CU1725, respectively. The auxotrophic strain was examined as were the other mutants for the effect of branched-chain amino acid limitation on expression of the *ilvGMED'*-*lac* operon. As Table 6 shows, regulation was affected by this mutant leader much like that by mutant leader II. Examination of the prototrophic strain showed (Table 7) that repressibility by the branched-chain amino acids was also about like that in the strain with mutation II. Thus, combined with the results of the 15-base deletion studied earlier, it appears that an effective attenuator can function in the *E. coli ilv* operon even though the leader can vary by as many as eight codons. It may be significant that this variability does not extend into the major stem of the protector region, which appears to be essential for RNA polymerase pausing (1).

DISCUSSION

In earlier studies, it has been shown that the *ilvGMEDA* leader of *E. coli* is remarkably efficient in the few amino acid codons needed to signal a deficiency of three such frequently used amino acids as the branched-chain amino acids (16, 21, 26). In contrast, the operons for leucine, phenylalanine, and histidine have leaders with longer runs of consecutive codons specifying the regulatory amino acid (18). In the preceding report (4), it was shown that the *ilvGMEDA* leader is also remarkably specific in its role of detecting a branched-chain amino acid deficiency and in controlling attenuation or readthrough at the end of the leader. Although one arginine and two serine codons are interspersed between the short runs of branched-chain amino acid codons, restriction of neither was effective in causing a derepression of the *ilvGMEDA* operon. Because in each case these were single codons rather than runs of several, the study reported here was undertaken to determine whether a run of three arginine codons encompassing the sites in the leader at which two leucine codons were thought to be very important in the *E. coli* leader might convert arginine to a regulatory amino acid for the *ilv* operon.

Two approaches were taken in this analysis. In one, the *ilv-lac* fusion used in the preceding report (4) was used with the modification that the *ilv-lac* strains in this study carried an *ilvG* mutation that corrected the frameshift that prevents the expression of this gene in most *E. coli* K-12 strains (20).

In all these fusions, the internal promoter lying within the *ilvM* gene, *ilvEp*, was present (24, 34). Although this promoter would tend to damp the apparent derepressibility by allowing internal transcription initiation under repressing conditions, the absence of the frameshift in the *ilvG* gene would reduce the effect. However, the results in Tables 6 and 7 may have been affected by *ilvEp*. Therefore, two of the leader mutations were also examined in fusions of *ilvG* upstream of the site of the frameshift with the *cat* gene and with the *lac* operon. That the results (Table 8) are comparable indicates that the presence of *ilvEp* has not strongly perturbed the results in Tables 6 and 7.

The failure of the leader with three arginine codons to be effective in allowing deattenuation when arginine was limiting was unexpected in view of the report by Zurawski et al. (40) that a significant derepression of the *trp* operon occurred when *E. coli* was limited for arginine. The single arginine codon in the *trp* leader occurs immediately after the tandem *trp* codons, analogous to the position of the single arginine codon that follows the tandem leucine codons in the *ilv* leader. Furthermore, two of the three consecutive arginine codons were CGA, a codon that is recognized more poorly by arginyl-tRNA than are most codons by their aminoacyl-tRNAs (11). On the other hand, when ribosome stalling was made extremely effective at the position of the naturally occurring arginine codon (at codon 6 in the peptide-coding region of the leader) by placing a nonsense codon at that point, constitutive, derepressed expression of the *ilv* operon occurred. Thus, if the arginine limitation had been effective enough, the *ilv* operon should have responded with derepressed expression.

Although the experiments described here might be faulted for not being conducted with a more severe mode of derepression (such as might be obtained by prolonged derepression in a chemostat), we wanted to compare arginine limitation under conditions more similar to those that are effective with branched-chain amino acid limitation. The question arises whether this failure to obtain arginine regulation of the *ilv* operon in such a facile manner may be related to the fact that none of the genes of the arginine regulon are regulated by attenuation in *E. coli*. Is there something about tRNA^{Arg} that prevents it from allowing ribosome stalling? Is it more readily mischarged, can its codon be skipped when arginyl-tRNA is limiting, or is the arginyl-tRNA pool too slowly reduced to be responsive to the kind of changes needed to control operon expression through an attenuation mechanism? Similar questions or reservations can be raised concerning seryl-tRNA, which does not participate in regulation of the serine biosynthetic enzymes in *E. coli*.

In view of these questions, a substitution of the two leucine codons with those for another hydrophobic amino acid known to be involved in attenuation control, phenylalanine, was examined. Again, unexpectedly, no control of the *ilv* operon was obtained by altering the phenylalanine supply. Either or both of two factors might account for the failure to obtain control of the *ilv* operon with this phenylalanine-containing leader. One is that there are only two phenylalanine codons in the mutation III leader transcript. In contrast, the leader transcripts for the *pheA* gene and the *pheST* operon have seven and five phenylalanine codons, respectively (31, 39). The number of consecutive codons could be a factor. The second factor, mentioned earlier, is that the predicted secondary structure for the paused transcript, pictured in Fig. 3D, has the two phenylalanine codons in a configuration different from that predicted for the corresponding leucine codons in the wild-type transcript.

This structure, shown in Fig. 3D, is dependent on the three terminal bases in the paused transcript. A transcript 3 bases shorter would have nearly the same secondary structure as that of the wild-type transcript (Fig. 3A). The base pairing postulated in Fig. 3D differs from the others in the figure in that it extends farther into the transcription bubble and thereby more severely disrupts the 12-base RNA-DNA hybrid thought to be essential for continued procession by the polymerase (39) (see below). The initiation of translation of such a paused transcript and translation as far as the phenylalanine codons would lead to disruption of the RNA-RNA base pairing in the region of the transcription bubble, restoration of the RNA-DNA hybrid, and resumption of transcription. Even if the ribosome did stall, owing to lack of phenylalanyl-tRNA, it might not disrupt the protector stem sufficiently to allow preemptor (or antiterminator) formation. Although there is no direct evidence for this scenario, it seems like a plausible one. Implicit in the idea is the assumption that the paused transcript is already formed before translation by the lead ribosome is initiated. Some support for the formation of the paused transcript before initiation of translation is found in the better response to limiting leucine with mutation III (Fig. 3D, Tables 6 and 7) than with mutation I (Fig. 3B, Tables 6 and 7). Both leaders contain only a single leucine codon. Unless the structures of the transcripts were different at the time of initiation, there should be no difference between the transcripts with mutations I and III under leucine-limiting conditions. However, as discussed below, there is another potential difference between these two mutations that should be considered.

The model of Yager and von Hippel (35) for hairpin-induced pausing assumes that the 3'-terminal 12 bases of the nascent transcript must be paired with the corresponding bases on the DNA template for transcription to proceed. In the structure proposed for the paused transcript formed as a result of mutation III, 11 of these 12 bases within the transcription bubble would be stripped away from the template and paired with upstream bases in an extended stem-and-loop structure. For the other structures, however, only 5 of the 12 terminal bases are proposed to be unpaired with template bases, suggesting that the polymerase is less likely to pause on a wild-type template than on a mutation III template. Experiments currently being conducted by Jennie Williams, using an in vitro transcription system, have indicated that pausing persists much longer with a supercoiled template containing mutation III than with one containing a wild-type leader region. In contrast, mutant leader I or II had no apparent effect on the persistence of pausing. It is interesting that the effect of this apparent different degree of RNA-DNA hybrid disruption is on the duration of the pause and not on the length of the paused transcript (indeed, no difference between the sizes of any of the paused transcripts, except that with the 9-base insertion, could be detected on the electrophoretic gels).

Still another feature of the effects of mutations I (Leu-Leu to Arg-Arg) and II (Leu-Val to Pro-Gly) is apparent from the data in Table 6. Although both mutations would have been expected to have decreased the derepression observed with limiting leucine, we did not expect them to have affected that with isoleucine or valine (only one of six valine codons was removed by mutation II). However, derepression was reduced with these amino acid limitations as well. It is assumed that for both isoleucine and valine starvation the entire coding portion of the transcript up to about 10 bases beyond the waiting codon would be covered by the ribosome or would be sterically prevented from pairing with any of the

bases in the preemtor stem-and-loop structure. The stalled ribosome-transcript complex should thus be like that with the wild-type leader.

Two questions arise, however. One is whether that stalled complex would be formed as quickly as it is with the wild-type leader, and the second is whether stalling persists long enough for antitermination to occur. Our *in vitro* transcription experiments until now have revealed no differences between the wild-type leader region and those of mutation I or II with respect to rate of polymerase travel, length of the paused transcript, or the length of the pause period. We assume, therefore, that the secondary effects of mutations I and II on the response to valine and isoleucine limitation could be related to either of two intervals. One is the interval between the time the ribosome initiates translation (thereby restoring the 12-base RNA-DNA hybrid in the transcription bubble and allowing resumption of transcription) and the time it reaches a point that disrupts the newly formed protector stem and loop sufficiently to preempt termination. The other is the length of time the ribosome remains stalled at isoleucine or valine codons and is thus delayed from reaching the point at which antiterminator formation is blocked.

It may be that there is a unique context effect that arose from placing the arginine codons where we did that has made translation through this region so slow that the deattenuation arising from valine and isoleucine limitation was restricted and, under our conditions of enhancing or limiting the arginine supply, no effect on deattenuation or readthrough could be detected. It would be difficult to pose a similar argument for the leader with the Pro-Gly for Leu-Val substitution because, by the time the Pro-10 codon is encountered, the bases leading to antiterminator formation have already been released from the protector stem.

On the other hand, decreased antitermination could also result if the ribosome passed by valine and isoleucine codons more rapidly than it did on the wild-type leader. A more rapid transit would presumably be context related and might arise from either more efficient translation of the isoleucine or valine codons even when isoleucyl- or valyl-tRNA levels were reduced or even a skipping of these codons as postulated for the translation of the T4 gene 60 protein (17).

Another difference would be the nature of the nascent peptide itself. That from mutation I would bear two additional positive charges and be more polar than that of the wild type, whereas the proline-containing peptide specified by mutation II would be bent. Indeed, when the nascent 12-amino-acid peptide specified by the wild-type leader is examined by the prediction scheme of Garnier et al. (8), it is observed to have a greater propensity for helix formation than does either of those specified by the two mutants. In contrast, the nascent peptide containing two phenylalanine residues in place of the two leucines resembles that of the wild type. This difference may be reflected in the much better response to limiting isoleucine and valine, as revealed in Table 6.

Inspection of the peptides specified by the leader regions of the other genes or operons regulated by the level of charged aminoacyl-tRNAs reveals that the pattern of hydrophobicity or helical propensity in the early few amino acid residues is not a uniform feature. Hydrophobic residues predominate in at least the early part of the peptides specified by the *his* (1a), *trp* (23), *pheST* (31), and *leu* (10) leaders, and of these, the first three leaders specify peptides in which the early amino acids exhibit a helical tendency (data not shown). In contrast, the peptide specified by the *thr* operon leader was neither helical nor hydrophobic (7). However, the hydrophilic character is kept relatively low by the intersper-

sion of isoleucine residues among the first few threonine residues. It would be interesting to know whether the change of the isoleucine codons at positions 4 and 8 of the *thr* operon leader to serine codons (A→G) (changes that would not affect the important alternative secondary structures) would affect the regulation of the operon by threonine.

The idea that the nature of a short nascent peptide might affect the response of a ribosome is, indeed, a highly speculative point, but it might also be considered in the examination of context effects in codon misreading (6). A question might be whether the context effect on codon misreading can be explained solely on the basis of the codon immediately preceding or immediately following the misread codon. It is interesting that Parker (27) has documented one context effect on the misreading of the phenylalanine codons UUC and UUU by leucyl-tRNA. Such misreading occurs in position 8 of the *E. coli* ornithine transcarbamylase peptide but not at position 3.

The fact that the peptide-coding portion of the *ilv* leader region between bases 61 and 76 (between codons for Leu-10 and Val-15) can be lengthened by three codons or shortened by five codons (4) without materially affecting the *ilv*-specific regulation was another unexpected finding in this study. In the previous report (4), it was suggested that for termination rather than readthrough to occur at the *ilv* leader termination site, progression of the translating ribosome at least as far as the first proline codon (Pro-21) would have to be very rapid to prevent antiterminator formation. It would be expected that the lengthening of the peptide-coding region would delay even more the arrival of the ribosome to the critical point. In contrast, shortening of the peptide-coding region would be expected to give the ribosome a greater advantage in the race with the polymerase. However, after the initial binding of the ribosome and subsequent resumption of transcription, a new but weaker protector structure could be formed, different from that shown in Fig. 3A but similar to that proposed for the paused transcript of the mutation III *ilv* leader (Fig. 3D). This secondary protector may be of some importance in further delaying antiterminator formation. Secondary structure predictions (data not shown) suggest that the relative stabilities of the weak protectors that form after translation disrupts those shown in Fig. 3 actually compensate for the effects that leader length alone might produce. Thus, the shorter leader would form a weak protector considerably less stable than the wild type, and the longer leader would form a weak protector considerably more stable than the wild type, factors that might nullify the size advantage or disadvantage.

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