Regions of the *Bacillus subtilis* *ilv-leu* Operon Involved in Regulation by Leucine

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The *ilv-leu* operon of *Bacillus subtilis* is regulated in part by transcription attenuation. The *cis*-acting elements required for regulation by leucine lie within a 683-bp fragment of DNA from the region upstream of *ilvB*, the first gene of the operon. This fragment contains the *ilv-leu* promoter and 482 bp of the *ilv-leu* leader region. Spontaneous mutations that lead to increased expression of the operon were shown to lie in an imperfect inverted repeat encoding the terminator stem within the leader region. Mutations within the inverted repeat of the terminator destroyed most of the leucine-mediated repression. The remaining leucine-mediated repression probably resulted from a decrease in transcription initiation. A systematic analysis of other deletions within the *ilv-leu* leader region identified a 40-bp region required for the derepression that occurred during leucine limitation. This region lies within a potential RNA stem-and-loop structure that is probably required for leucine-dependent control. Deletion analysis also suggested that alternate secondary structures proximal to the terminator are involved in allowing transcription to proceed beyond the terminator. Additional experiments suggested that attenuation of the *ilv-leu* operon is not dependent on coupling translation to transcription of the leader region. Our data support a model proposed by Grundy and Henkin (F. J. Grundy and T. M. Henkin, Cell 74:475–482, 1993) in which uncharged tRNA acts as a positive regulatory factor to increase gene expression during amino acid limitation.

The *ilv-leu* operon of *Bacillus subtilis* contains seven genes encoding enzymes for the biosynthesis of isoleucine, valine, and leucine (Fig. 1). Transcription of this operon is repressed by leucine but not by isoleucine or valine (21). In work published previously we showed that transcription is initiated 482 bp upstream of the start codon for *ilvB*, the first gene in the operon (4).

In vitro transcription studies defined a strong transcription termination site at position +405, immediately downstream from an extended inverted repeat. Ninety percent of transcripts initiated at the promoter in vitro were terminated at this site. Analysis of mRNA levels in vivo suggested that repression by leucine occurs by attenuation of transcription within the 482-bp leader region and that the transcription terminator within the *ilv-leu* leader region is involved in the mechanism of leucine control (4).

The *ilv* and *leu* genes in enteric bacteria are regulated mostly by translation-dependent attenuation, which relies on translation of a short open reading frame (ORF) within the leader RNA upstream of the structural genes. In the case of the *leu* operon, limitation for leucine results in stalling of the ribosome at leucine control codons within the leader. Stalling at these sites prevents formation of a terminator stem and loop by promoting formation of an antiterminator stem, leading to readthrough (10). When leucine is in excess, the ribosome does not stall and formation of the terminator is favored because

another stem and loop, the protector, prevents formation of the antiterminator. This kind of attenuation requires an ORF containing control codons as well as overlapping pairing regions in the leader region that can form alternate structures. A good ribosome binding site for *B. subtilis* was not found in the leader region of the *ilv-leu* operon, suggesting that it may be regulated by translation-independent attenuation.

The *trp* operon of *B. subtilis* is regulated by a translation-independent mechanism that is most likely different from that involved in regulation of the *ilv-leu* operon. In contrast to the case with *ilv-leu*, transcription in vitro with the *trp* promoter and leader region as template led to 90% readthrough (16). A *trans*-acting regulatory protein called the *trp* attenuation protein was shown to bind to the *trp* leader RNA and to promote termination (1, 12). Binding of the *trp* attenuation protein to the *trp* leader RNA is increased in the presence of tryptophan. The finding that most transcripts terminated within the *ilv-leu* leader region in vitro indicates that the mechanism of regulation requires a positively acting transcription factor instead of a negatively acting factor like the *trp* attenuation protein.

Grundy and Henkin recently presented evidence to support their model for translation-independent regulation of the *B. subtilis* *tyrS* gene (5). In this model, uncharged tyrosine tRNA acts as a positive regulatory factor and directly interacts with leader mRNA at a cognate triplet located in the bulge region of a specific stem and loop. The interaction of uncharged tRNA with the triplet and with a conserved "T box," located within the antitermination stem, was postulated to result in readthrough transcription. The *ilv-leu* leader RNA and the leader RNAs from the *B. subtilis* tRNA synthetase genes *thrS*, *valS*, *trpS*, *leuS*, and *pheS* share conserved sequences and secondary structures believed to be involved in the regulatory mechanism and were proposed to be regulated similarly to *tyrS* (5).

We have carried out an extensive mutational analysis of the

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FIG. 1. (A) Organization of the *ilv-leu* operon of *B. subtilis*. (B) Nucleotide sequence of the promoter and regulatory regions. Boxed areas, -10 and -35 regions of promoter; +1, startpoint of transcription; inverted arrows over sequence, pairing regions of terminator stem; dots over sequence, ribosome binding site for *ilvB*; hatched box, putative start codon for *ilvB*. Restriction sites are indicated over the DNA sequence, and the numbering at right is from the start point of transcription.

leader region of the *ilv-leu* operon of *B. subtilis*. Here we provide further evidence that this operon is controlled by transcription attenuation by showing that mutations in the *ilv-leu* terminator result in a substantial loss of leucine-dependent repression. We also identified a region of the *ilv-leu* leader required for derepression in response to leucine limitation and show that the attenuation mechanism probably involves formation of three different secondary structures that are analogous to protector, antiterminator, and terminator stem-and-loop structures. As we point out in Discussion, the *ilv-leu* operon of *B. subtilis* shows many of the features of operons regulated by translation-dependent attenuation, but the weight of evidence supports the model of Grundy and Henkin (5).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used are presented in Table 1. *B. subtilis* CU4609 (*trpC2 leuB16 ilvN::Tn917 lacZ erm*) was used for selection of leucine-resistant mutants. This strain was derived from a strain containing Tn917 integrated into the *ilvN* gene and was constructed by first replacing the transposon by recombination with linearized pTV21Δ2 and replacing the resulting construct by recombination with linearized pTV32 (13, 20). *B. subtilis* CU4846 (*trpC2 leuB16 amyE::erm*), used for integration of single-copy *lacZ* fusions, was constructed by transforming strain CU257 (*trpC2 leuB16*) with DNA from *B. subtilis* MO199 (*trpC2 pheA amyE::erm*) and selecting for macrolide-lincosamide-streptogramin (MLS) resistance. *Escherichia coli* strains used were DH5α [*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1*] and XL1 Blue [*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* (F' *proAB lacI^q lacZΔM15 Tn10*)] (Stratagene). Complex medium was LB broth containing 50 μg of ampicillin per ml when required. Minimal medium contained 0.5% glucose, 1 μg of biotin per ml, 20 μg of tryptophan per ml, 35 μg of isoleucine per ml, 70 μg of valine per ml, and salts to produce a final concentration of 2 g of (NH₄)₂SO₄, 13.8 g of K₂HPO₄·3H₂O, 6 g of

TABLE 1. Strains used in this study

Strain	Genotype
CU4609.....	<i>trpC2 leuB16 ilvN::Tn917 lacZ erm</i>
CU4846.....	<i>trpC2 leuB16 amyE::erm</i>
CU257.....	<i>trpC2 leuB16</i>
CU4806.....	<i>trpC2 leuB16 ilvN::Tn917 azlA115</i>
CU4807.....	<i>trpC2 leuB16 ilvN::Tn917 azlA152</i>
CU4808.....	<i>trpC2 leuB16 ilvN::Tn917 azlA153</i>
CU1065.....	<i>trpC2</i>
CU4928.....	<i>trpC2 leuB16 amyE::ACwt</i>
CU4885.....	<i>trpC2 leuB16 amyE::101</i>
CU4886.....	<i>trpC2 leuB16 amyE::102</i>
CU4929.....	<i>trpC2 leuB16 amyE::103</i>
CU4887.....	<i>trpC2 leuB16 amyE::201</i>
CU4891.....	<i>trpC2 leuB16 amyE::202</i>
CU4888.....	<i>trpC2 leuB16 amyE::203</i>
CU4889.....	<i>trpC2 leuB16 amyE::204</i>
CU4890.....	<i>trpC2 leuB16 amyE::205</i>
CU4901.....	<i>trpC2 leuB16 amyE::207</i>
CU4908.....	<i>trpC2 leuB16 amyE::302</i>
CU4966.....	<i>trpC2 leuB16 amyE::303</i>
CU4880.....	<i>trpC2 leuB16 amyE::304</i>
CU4881.....	<i>trpC2 leuB16 amyE::305</i>
CU4882.....	<i>trpC2 leuB16 amyE::306</i>
CU4897.....	<i>trpC2 leuB16 amyE::500</i>
CU4900.....	<i>trpC2 leuB16 amyE::501</i>
CU4902.....	<i>trpC2 leuB16 amyE::502</i>
CU4903.....	<i>trpC2 leuB16 amyE::503</i>
CU4904.....	<i>trpC2 leuB16 amyE::504</i>
CU4893.....	<i>trpC2 leuB16 amyE::TF1</i>
CU4894.....	<i>trpC2 leuB16 amyE::TF2</i>
CU4892.....	<i>trpC2 leuB16 amyE::M3</i>
CU4967.....	<i>trpC2 leuB16 amyE::M4</i>
CU4968.....	<i>trpC2 leuB16 amyE::M5</i>

KH₂PO₄, 0.2 mg of MnCl₂·4H₂O, 1 g of sodium citrate, and 0.2 g of MgSO₄ per liter (17). Cultures were incubated at 37°C and were aerated by shaking in baffled flasks. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used on plates at 80 μg/ml. Selection for MLS resistance was performed on LB plates containing 1 μg of erythromycin and 25 μg of lincomycin per ml.

Plasmid construction and site-directed mutagenesis. The vector used for integration of *lacZ* fusions into the *amyE* locus of *B. subtilis* was pDH32 (provided by Dennis Henner). This plasmid contains the proximal and distal portions of the *amyE* gene flanking the chloramphenicol acetyl transferase gene, a cloning site containing *EcoRI* and *BamHI* sites, and *lacZ* containing the *spoVG* ribosome binding site. Plasmid pDH32 has a ColE1 replication origin and the β-lactamase gene used for selection in *E. coli*.

Plasmid pACB1 is a derivative of pBluescript containing a 785-bp fragment of the *ilv-leu* upstream region cloned into the *SmaI* site (4). Site-directed mutagenesis was used to introduce an *XhoI* site in pACB1 at position +21 of the *ilv-leu* leader to produce pXhoI (9). This destroyed the *HindIII* site at this position. The oligonucleotide used in the mutagenesis was 5'-TATTTACGTTTCGAGCTCTTCGCTTTTAG-3'. The 800-bp *EcoRI-BamHI* fragment of pXhoI was cloned into *EcoRI-BamHI*-digested pDH32 to yield pLD100. Plasmids pM3 and pM4 were produced starting with pACB1 by the same method. The oligonucleotides used to produce these plasmids were 5'-ATCCGCTTTAGTTACGAGTGAATCAAG-3' and 5'-ATATTTCTATCCGCTTAAAAACGAGTGAATCAA-3'.

The 800-bp *EcoRI-BamHI* fragment of pACB1 containing 785 bp of the upstream region of the *ilv-leu* operon was cloned

TABLE 2. Primers, templates, and restriction enzymes used for construction of plasmids

Construct	PCR product prepared from:		Vector	Enzymes used to digest vector and PCR product
	Primers ^a	Template		
pLD101	F-DSA1	pLD100	pLD100	<i>XhoI</i> - <i>Bam</i> HI
pLD102	F-DSA2	pLD100	pLD100	<i>XhoI</i> - <i>Bam</i> HI
pLD201	DSB1-DH32R	pLD101	pLD100	<i>XhoI</i> - <i>Bam</i> HI
pLD203	DSB3-DH32R	pLD101	pLD100	<i>XhoI</i> - <i>Bam</i> HI
pLD204	DSB4-DH32R	pLD101	pLD100	<i>XhoI</i> - <i>Bam</i> HI
pLD205	DSB5-DH32R	pLD101	pLD100	<i>XhoI</i> - <i>Bam</i> HI
pLD207	DSB7-DH32R	pLD101	pLD100	<i>XhoI</i> - <i>Bam</i> HI
pLD301	DH32L-DSC1	pACwt	pLD201	<i>Eco</i> RI- <i>Xho</i> I
pLD302	DH32L-DSC2	pACwt	pLD201	<i>Eco</i> RI- <i>Xho</i> I
pLD303	DSB2-DH32R	pLD101	pLD302	<i>XhoI</i> - <i>Bam</i> HI
pLD304	DH32L-DSC2.5	pACwt	pLD303	<i>Eco</i> RI- <i>Xho</i> I
pLD305	DH32L-DSC2.7	pACwt	pLD303	<i>Eco</i> RI- <i>Xho</i> I
pLD306	DH32R-DSB1.5	pLD101	pLD303	<i>XhoI</i> - <i>Bam</i> HI
pTF1	F-TF1	pLD100	pJG10	<i>Xho</i> I- <i>Sal</i> I
pTF2	F-TF2	pLD100	pJG10	<i>Xho</i> I- <i>Sal</i> I

^a The sequences of the primers used are given in Table 3.

into *Eco*RI-*Bam*HI-digested pDH32 to produce pACwt. pLD202 was made by deleting the *Hind*III fragment of pACwt.

Plasmids pLD500 and pLD103 were made by digesting pLD100 with *Hind*III-*Eag*I and *Bam*HI-*Xho*I, respectively, filling in the ends with the Klenow fragment of DNA polymerase (Klenow fragment), and ligating the resulting blunt ends.

pLD501 was constructed by digesting pLD101 with *Xho*I-*Sac*II, filling in the *Xho*I site with reverse transcriptase, filling in the *Sac*II site with Klenow fragment, and ligating the resulting blunt ends. pLD502 was constructed by digesting pLD101 with *Hind*III, filling in with reverse transcriptase, and ligating the resulting blunt ends. pLD503 was constructed by digesting pLD101 with *Hind*III-*Sac*II, filling in with Klenow fragment, and ligating the blunt ends. pLD504 was constructed by digesting pLD101 with *Xho*I-*Eag*I, filling in with reverse transcriptase, and ligating the blunt ends.

Plasmids constructed by polymerase chain reaction (PCR) and primers used in the amplification reaction are listed in Tables 2 and 3, respectively. As an example, pLD101 was

TABLE 3. Sequences of primers used for construction of plasmids

Primer	Sequence
DH32R.....	5'-TTCCACAGTAGTTTACCACC-3'
DH32L.....	5'-TCTTATCTTGATAATAAGGG-3'
F.....	5'-TTTGGATCCTATGAGTTCAACAAAAGATA-3'
DSA1.....	5'-TTTGGATCCCTTTTCATTAGCTAGATAG-3'
DSA2.....	5'-TTTGGATCCCTGCGATAGCTAAAAGGGG-3'
DSB1.....	5'-TTTCTCGAGGCGGATAGAAATATCCATGAG-3'
DSB1.5.....	5'-TTTCTCGAGTGATTAAGTCTGTTACTAAAG-3'
DSB2.....	5'-TTTCTCGAGTAGGACTTGGCCCGG-3'
DSB3.....	5'-TTTCTCGAGTTTTCACAGAGAACCGGGT-3'
DSB4.....	5'-AAACTCGAGGCTGAATATGAAAAGCGCAG-3'
DSB5.....	5'-ATATCTCGAGCAACGACCTTCTTGAACAGC-3'
DSB7.....	5'-TTTCTCGAGATTAAACAGGCGTAAACAAG-3'
DSC1.....	5'-TTTCTCGAGAACGAGTGAATCAAGT-3'
DSC2.....	5'-TTTCTCGAGTTTTCAGGCTGGCACTCT-3'
DSC2.5.....	5'-AAACTCGAGTGAGGCGAGTTCACCTTG-3'
DSC2.7.....	5'-TTTCTCGAGGCTTCGCGGTTCTCAGC-3'
TF1.....	5'-TTTGTGACTATCCGCTTTAGTAACAG-3'
TF2.....	5'-TTTGTGACGCTCATCGTCTGTGTACAT-3'

FIG. 2. Construction of plasmid pLD101. Primers used for PCR are indicated by bent lines. Restriction sites incorporated into the PCR products are indicated at the ends of the primers. All plasmids are derivatives of pDH32. P, *ilv-leu* promoter; inverted arrows, transcription terminator sequence.

constructed by using primers F and DSA1 with pLD100 as template. PCR conditions were as follows: 92°C for 1 min, 50°C for 1 min, and 72°C for 1 min, for 25 cycles, and 72°C for 5 min for 1 cycle. The PCR product and the vector (pLD100) were digested with *Xho*I and *Bam*HI. The digested vector was treated with calf intestinal phosphatase and phenol extracted. The PCR product and vector were mixed and ligated with T4 DNA ligase, and the ligation mix was used to transform *E. coli* DH5 α . Transformants were plated on LB medium containing 50 μ g of ampicillin per ml and X-Gal. Blue colonies were picked, and plasmids were isolated and screened for the correct insert by restriction enzyme digestion (Fig. 2).

For construction of translational fusions, pLD100 was modified to remove a *Sal*I site. Plasmid pLD100 was digested with *Eco*NI and the ends were filled in with reverse transcriptase and ligated together to yield pJG10. PCR products were synthesized with *Sal*I sites at the downstream end and were cloned into *Xho*I-*Sal*I-digested pJG10. This produced an in-frame fusion to the eighth codon of the *lacZ* coding region in pJG10.

β -Galactosidase assays. β -Galactosidase assays were done as described by Platko et al. (14), except that the A_{600} of the culture was used without correction.

Primer extension. Primer extension was performed as described previously (4).

Transcription in vitro. Templates were purified by digesting plasmid DNA with *Eco*RI-*Bam*HI, separating the fragments by electrophoresis on an agarose gel, and purifying the appropriate fragment with GeneClean (Bio 101). Ends were filled in by using reverse transcriptase. All other conditions were as described previously (4).

DNA sequencing. All plasmid constructions were confirmed by DNA sequencing with a Sequenase kit (United States Biochemical). PCR amplification of the *ilv-leu* leader region in strains CU4806, CU4807, and CU4808 was accomplished by using primers F (Table 3) and G. The sequence of primer G was 5'-GGGCTGCAGGTCCCCATTTAGTTCTCC-3'. PCR products were sequenced by the method of Casanova et al., using a primer-to-template ratio of 20, an annealing temperature of -70°C, and a labeling time of 45 s (3). For sequencing of the chromosomal copy of the *ilv-leu* leader region in the

strain containing construct 503 at the *amy* locus, PCR was performed with primers F and DH32R and the product was sequenced by using the *fmol* DNA Sequencing System (Promega). Primer DH32R hybridized within the *lacZ* gene, ensuring that the *ilv-leu* leader region amplified in the PCR was from the *lacZ* fusion construct integrated into the *amy* locus.

RESULTS

Identification of mutations that increase expression of *ilv-leu*. A procedure was devised for selecting mutants with increased transcription of the *ilv-leu* operon during growth with excess leucine (21a). The selection strategy relied on the fact that a Tn917 insertion in *ilvN* results in growth inhibition by leucine when isoleucine and valine are not provided in the medium. The phenotype, termed leucine sensitivity, may arise because the Tn917 insertion in *ilvN* results in decreased activity of acetohydroxy acid synthase, the product of the *ilvB* and *ilvN* genes. *IlvB* alone can likely carry out catalysis without the *IlvN* subunit, but its activity is probably greatly reduced (22). Repression of the *ilv-leu* operon by leucine reduces the amount of *IlvB*, causing starvation for isoleucine and valine. Without added leucine, the operon is derepressed and the cells produce enough *IlvB* to synthesize isoleucine and valine. Mutations that lead to decreased repression by leucine allow the cells to grow without isoleucine and valine when leucine is present. We call such mutant strains leucine resistant.

B. subtilis CU4609 (*ilvN*::Tn917 *lacZ erm trpC2 leuB16*) was plated on minimal medium containing 100 µg of leucine per ml. Colonies that grew on this medium and that were blue on LB agar plus X-Gal were purified and studied further. When the *ilv-leu* operon is regulated normally, colonies containing *ilvN*::Tn917 *lacZ* are white on LB-X-Gal because of the high leucine content of the medium. DNA was isolated from the mutant strains and used to transform CU1065 (*trpC2*) to MLS resistance. Linkage of the mutation to *erm* was determined by estimating the fraction of MLS-resistant transformants that were blue on LB containing X-Gal. Of 16 isolates tested, all were >94% linked to *erm*.

DNA encompassing the leader region was amplified by PCR and sequenced in the *ilv-leu* leader region. Three mutations that led to increased expression were found. The *azlA115* and *azlA153* mutations changed single bases within the predicted terminator stem (Fig. 3). The leader regions of these mutant strains contained no other mutations. The third mutation, *azlA152*, had a 10-bp deletion that affects most of the GC base pairs in the lower portion of the predicted stem (Fig. 3). We sequenced only the terminator region of this allele, but the mutation was found in two independent leucine-resistant isolates. It is therefore unlikely that the phenotype of these alleles was caused by a second mutation elsewhere. One previously identified mutation with a similar phenotype, *azlA102*, is also shown in Fig. 3.

The effects of the *azlA115*, *azlA152*, and *azlA153* mutations on *ilv-leu* transcription were determined by comparing β-galactosidase activities in the wild-type and mutant strains under conditions of leucine limitation and leucine excess (4). Cell cultures were limited for leucine by substituting *N*-acetyl leucine for leucine. Each of the three newly identified mutations led to an increase in expression during growth with excess leucine (Table 4). Expression in cells limited for leucine was not affected. Expression of *lacZ* was repressed 30-fold by leucine in the wild-type strain, whereas expression in each of the mutant strains was repressed only about 3-fold. These results suggest that a small perturbation in the base pairing of

FIG. 3. Proposed secondary structure of the *ilv-leu* terminator located in the leader region upstream of *ilvB*. Changes found in mutant strains are indicated by arrows. The heavy line indicates nucleotides deleted in *azlA152*. Numbering is from the start of transcription.

the stem of the terminator stem and loop has as much effect on regulation as does the 10-bp deletion.

Preparation of *ilv-leu* leader-*lacZ* fusions. To analyze effects of mutations in the *ilv-leu* leader region, we constructed transcriptional fusions of this leader region to *lacZ* and integrated the fusions into the *amyE* locus of the *B. subtilis* chromosome. The parent plasmid, pDH32, contains the proximal and distal portions of *B. subtilis amyE* flanking a *cat* gene, a cloning site, and a *lacZ* gene containing the *spoVG* ribosome binding site. A fragment of DNA from position -248 to +545 relative to the transcription start site of *ilv-leu* was cloned into pDH32 to yield pACwt. This plasmid was linearized with *ScaI* and used to transform *B. subtilis* CU4846 (*trpC2 leuB16 amyE::erm*) to chloramphenicol resistance. Integration of the

TABLE 4. β-Galactosidase specific activity in leucine-resistant *ilvN*::*lacZ* strains

Strain	Relevant allele	β-Galactosidase sp act (Miller units) in medium containing ^a :		Fold repression by leucine
		L	N	
CU4609	Wild type	36	1,065	30
CU4806	<i>azlA115</i> (G-362→A)	402	1,408	3.5
CU4807	<i>azlA152</i> (Δ345-354)	423	1,168	2.7
CU4808	<i>azlA153</i> (G-382→T)	423	1,402	3.3

^a Cells were cultured in minimal medium containing either leucine, isoleucine, and valine (L) or *N*-acetyl leucine, isoleucine, and valine (N).

FIG. 4. Schematic representation of *ilv-leu* leader deletions integrated into the *amyE* locus and β -galactosidase activity in *lacZ* fusion strains. These constructs contain the *spoVG* ribosome binding site upstream of the *lacZ* gene. Heavy lines, DNA remaining after deletions were made; numbers above top line, nucleotide positions relative to transcription start; numbers in parentheses at right, number of repetitions performed for each strain; arrows above top line, inverted repeat regions; boxed letters above inverted repeat regions, proposed stem-and-loop structures corresponding to stem I (s I), protector (p), antiterminator (a), and terminator (t) of Fig. 5; B, coding region for *ilvB*; *XhoI* above constructs 100 and 303, restriction site introduced for cloning PCR fragments as indicated in Table 1; L, cells grown in medium containing leucine, isoleucine, and valine; N, cells grown in medium containing *N*-acetyl leucine, isoleucine, and valine; N/L, ratio of activity in N medium to that in L medium. Specific activity was the slope of the line from a graph of β -galactosidase activity per milliliter versus A_{600} of the culture.

cat gene and *lacZ* fusion into the *amyE* gene by a double recombination event results in loss of the *erm* gene from the chromosome. Transformants were screened for MLS sensitivity to ensure that the desired region of the plasmid had integrated by double recombination into *amyE*. A chloramphenicol-resistant, MLS-sensitive transformant containing construct ACwt was purified and designated strain CU4928. β -Galactosidase expression in this strain was measured under conditions of leucine limitation and leucine excess. As expected for the wild type, reporter gene expression was high under conditions of leucine limitation and low under conditions of excess leucine (Fig. 4).

Regulation of *lacZ* expression by leucine was very similar to that seen for the *lacZ* gene carried on the Tn917::ilvN insertion in strain CU4609 (Table 4). We conclude that regulation of β -galactosidase expression in our *lacZ* fusion strain is similar to that seen for the normal *ilv-leu* operon.

An *XhoI* site at position +26 was introduced by site-directed mutagenesis into plasmid pACwt to facilitate construction of

leader deletions. The resulting construct, pLD100, when transferred to the chromosome in single copy, behaved identically with respect to reporter gene expression as did the parent construct (Fig. 4). Note that for both pACwt and pLD100, a short section of *ilvB* containing a Shine-Dalgarno sequence and translational start is fused to a *lacZ* gene, also containing a Shine-Dalgarno sequence and translational start. To determine whether the short section of *ilvB* affected *lacZ* expression, a derivative of pLD100, pLD101, that lacked that section was prepared. As shown in Fig. 4, all three constructs, pACwt, pLD100, and pLD101, behaved similarly with respect to reporter gene expression. All of the mutations described in the next section are derivatives of these three plasmids.

Effects of deleting various parts of the leader region upon *ilv-leu* expression. Most of the mutations introduced into the leader region resulted in greatly reduced levels of expression. Leader sequences within the constructs were always verified by sequencing, but we worried that additional mutations, for example, in *lacZ*, might have inadvertently been introduced

and that low expression was caused by these latter putative mutations. We investigated this possibility with some of the constructs. For example, deletion of a *Hind*III fragment within the leader region caused greatly reduced reporter gene expression (Fig. 4, construct 202), but addition of the same *Hind*III fragment back to the construct led to normal regulated expression (data not shown).

If leucine-mediated repression in this system resulted solely from transcription attenuation at position +405, then deletion of the termination site should have eliminated repression. In fact, it did not. Deletion of DNA from +373 to +545, which removed all of the downstream pairing region of the terminator stem, did not remove all of the leucine-mediated repression (Fig. 4; compare constructs 101 and 102). Further, deletion of almost the entire leader region did not eliminate leucine-mediated repression (Fig. 4; compare constructs 101 and 103). The deletion in construct 103 caused an overall twofold increase in expression, but repression by leucine was still fourfold. We conclude from this that (i) most of the regulation by leucine is accounted for by the transcription attenuation control and (ii) a smaller fraction of repression by leucine occurs by some other mechanism, presumably one involving the initiation of transcription by the *ilv-leu* promoter.

In a strain containing construct 101, limitation for leucine led to about 1,700 U of β -galactosidase, whereas in a similar strain containing construct 103 (which lacks most of the leader region), expression was increased to 5,800 U. This suggests that leucine limitation of a strain that has a fully functional leader region did not completely prevent transcription termination. Moreover, the finding that expression in the strain containing construct 103 was almost twofold higher than that in the strain containing construct 102, whether or not the cells were limited for leucine, suggests that some termination within the leader region may occur at a site other than +405. An alternative possibility is that differences in mRNA stability account for the different results with constructs 102 and 103.

In the series of constructs 205, 204, and 203, the size of the deletion was progressively increased by removing increasing amounts of DNA downstream from the *Xho*I site at position +26 (Fig. 4). The effects of these deletions on expression and regulation were determined as described above. Deletion of 40 bp (construct 205) had no effect on regulation by leucine. Deletion of 80 bp (construct 204) caused a 5-fold decrease in expression, but leucine still repressed expression 24-fold. When 120 bp was deleted (construct 203), expression of *lacZ* was no longer derepressed when the cells were limited for leucine. These results indicate that a region required for leucine control lies between positions +106 and +146 of the leader region.

We identified a potential stem and loop (stem I) that could form by pairing of nucleotides between positions +128 and +213 ($\Delta G = -25$ kcal (ca. -100 kJ)/mol) (Fig. 5). Deletion of the bottom half of the upstream side of stem I (construct 203) resulted in loss of leucine-mediated regulation, but basal levels of expression (expression in medium containing excess leucine) remained near normal. Surprisingly, there was no regulation by leucine in the strain containing construct 203. We expected a two- to fourfold effect because of regulation due to the effect of leucine on promoter activity, as observed for construct 103. A possible reason for this finding is discussed below. A deletion that almost totally eliminated stem I (construct 202) led to levels of reporter gene activity that were barely detectable, suggesting that the upper part of stem I is necessary for substantial expression of this operon (Fig. 4). Additional evidence for the importance of the upper part of stem I came from an analysis of construct M5, which contains

two single-base substitutions within this region (A-181 $\rightarrow$ T and A-183 $\rightarrow$ T) (Fig. 5). These changes, which should weaken base pairing at the top of stem I, resulted in the loss of leucine-mediated control and reduced basal expression (Fig. 4).

The low expression associated with the deletion in construct 202 is due to an increase in transcription termination efficiency rather than to an effect on *ilv-leu* promoter activity. To determine whether the very low expression observed with construct 202 was due to an effect on *ilv-leu* promoter activity or to an increase in transcription termination efficiency, we used primer extension to measure mRNA levels upstream and downstream of the terminator in vivo. Total RNA was isolated from the strains containing construct 202 or ACwt grown in medium with excess leucine or limiting leucine. The experiment was complicated by the fact that these strains had two copies of the *ilv-leu* leader region. One copy was at the normal *ilv-leu* locus, and a second copy was fused to *lacZ* at the *amyE* locus (Fig. 6A). Two primers were used for primer extension. Primer B, complementary to a region upstream of the terminator, was used to measure upstream mRNA, and primer Z, complementary to *lacZ* mRNA, was used to measure mRNA downstream of the terminator. Primer B hybridized to the mRNA produced from the normal *ilv-leu* operon and to mRNA produced from the *ilv-leu* leader-*lacZ* fusion. Primer Z hybridized only to mRNA produced from the *ilv-leu*-*lacZ* fusion (Fig. 6A). Figure 6B shows that with RNA from the strain containing construct ACwt as template, primer B produced a cDNA product that was about 310 nucleotides (nt) long, indicating that transcription started at the +1 nucleotide identified previously (Fig. 6B, lane 1, arrow A) (4). Extension of primer Z produced a cDNA about 200 nt long, corresponding to an endpoint just downstream of the proposed stem and loop of the terminator (Fig. 6B, lane 2, arrow B). This result was reported previously and is probably caused by the inability of reverse transcriptase to read past the stable structure of the stem-and-loop terminator (4).

When RNA isolated from the strain containing construct 202 was used as template for primer extension, primer B produced two major cDNA bands (Fig. 6B, lane 5, arrows A and C). One was a 310-nt cDNA corresponding to primer extension from the normal copy of the *ilv-leu* operon (Fig. 6A). Extension of primer B also produced a cDNA of about 145 nt (Fig. 6B, lanes 5 and 6, arrow C), a size expected for primer extension with mRNA from construct 202 as template. When primer Z was used with the RNA from the strain containing construct 202, no cDNA was detected (Fig. 6B, lane 7). This result indicates that the low β -galactosidase expression from construct 202 was not due to inactivation of the *ilv-leu* promoter and that deletion of nt +24 to +193 prevented synthesis of detectable amounts of mRNA downstream of the terminator.

The transcription termination site within the *ilv-leu* leader region is very efficient in vivo. Comparison of the expression from construct 501 with that from construct 103 indicates that the 107-bp region of DNA containing the terminator decreased expression dramatically. With construct 103, the promoter and first 26 bp of the leader region were fused directly to the *lacZ* gene, and expression was 5,800 U. Construct 501 was similar to 103 but contained the terminator region between the promoter and *lacZ*. Expression of β -galactosidase from construct 501 was undetectable, indicating that the terminator completely blocked transcription of the *lacZ* gene.

Deletions designed to test whether secondary structures predicted to form in the *ilv-leu* leader mRNA are involved in regulation of the operon. A computer search for potential stem-and-loop structures in the *ilv-leu* leader yielded many

FIG. 5. Secondary structures of *ilv-leu* leader mRNA. Arrows, deletion endpoints of constructs indicated. Nucleotide changes in the M5 mutant construct are indicated by arrows. The inverted structure is an alternative potential stem and loop within the region 305 to 375. The heavy line indicates the location of the T box region, the boxed area indicates the possible specifier codon proposed by Grundy and Henkin (5), and asterisks indicate nucleotides conserved in *ilv-leu* and tRNA synthetase leader regions (see Discussion).

possible pairing regions, two of which were just upstream of the terminator (Fig. 5). These regions, along with the terminator, possess overlapping sequences analogous to attenuators found in amino acid biosynthetic operons such as *trp*, *leu*, and *ilv* of enteric bacteria (10). In the regulatory regions of these latter operons, three pairing regions are arranged in an overlapping fashion such that formation of one prevents formation of its downstream neighbor. In the absence of protein factors, transcription terminates at the terminator because the first stem (the protector) forms efficiently and protects the terminator by preventing the antiterminator from forming (8, 10).

To investigate the importance of some of these pairing regions, constructs 201, 504, 207, 501, 500, and 503, containing deletions into the putative protector and antiterminator, were prepared (Fig. 4). The deletion in construct 201 removed up to nt +276, leaving the protector pairing region intact. Expression from this construct was almost undetectable. The deletion in construct 504 removed 23 bp more than that in 201 and deleted part of the protector stem. Expression in the strain containing construct 504 remained low, but deletion of an additional 5 bases (construct 207) led to markedly higher expression (Fig. 4). The effect of deletion of these additional 5 bp was also seen when templates from constructs 504 and 207

were used as templates in *in vitro* transcription studies, suggesting that base pairing in this region prevents terminator formation by affecting secondary structure (see below). The results with construct 207 may be understood in terms of disruption of a stem consisting of the top 4 bp of the protector stem in Fig. 5, allowing formation of the antiterminator stem. Similar results were obtained by Stroynowski and Yanofsky in their studies of the *Serratia marcescens trp* operon (18).

A strain having construct 500 had relatively high expression, and that expression was relatively insensitive to leucine (Fig. 4). These results would seem to be at odds with those obtained with construct 504 (very low expression), because 500 and 504 share the same right endpoint. However, the high expression exhibited by construct 500 is likely caused by the fact that the nucleotides near position 300 are not available for protector formation because they fortuitously pair with nucleotides in the region upstream of the deletion (nt 180 to 186) (Fig. 5). In construct 501, the deletion extended through one arm of the antiterminator. The results with construct 501, i.e., very low expression of the reporter gene, support the idea that the antiterminator is necessary for readthrough beyond the terminator.

Since constructs 501 and 503 share the same right endpoint, we expected only low expression for 503. In fact, construct 503

FIG. 6. (A) Origin of cDNAs produced in the primer extension experiment shown in panel B. Oval, *B. subtilis* chromosome; P, promoter; inverted arrows, terminator; cDNA, cDNAs produced by primer extension with primer B or Z (expected sizes indicated); mRNA, terminated (downward arrow) or readthrough (rightward arrow) transcripts; *, ^{32}P label at 5' ends of primers. (B) Steady-state levels of mRNA upstream and downstream of the *ilv-leu* terminator. cDNAs produced by primer extension of mRNA transcripts in strains ACwt and 202 were analyzed by electrophoresis on a polyacrylamide gel. Primers B and Z are indicated in panel A. Cells were grown in minimal medium containing leucine, isoleucine, and valine (L) or with *N*-acetyl leucine substituted for leucine (N). Bands A, B, and C are described in the text. Numbers to the right indicate sizes (in base pairs) of molecular weight standards (*Msp*I-digested pBR322).

supported a modest basal level of expression, and that expression was elevated in response to a limitation for leucine. These results would appear to demand that leucine-mediated control involve sequences upstream of position +193. This point is considered further in Discussion.

Deletions affecting regions between stem I and the protector stem. Construct 303, containing an *Xho*I site at position +229, behaves like the parent construct, 101 (Fig. 4). Short deletions extending from position +229, both leftward and rightward (constructs 302, 306, 304, and 305), lowered basal expression and eliminated leucine-mediated repression. With the exception of construct 305, the deletions in these constructs did not affect any of the stem-and-loop structures shown in Fig. 5. Possibly the region around +229 is involved in some RNA secondary structure other than those shown in Fig. 5 and this secondary structure is important for function. Alternatively, the region around +229 may interact directly with some factor that is necessary for transcription readthrough.

Transcription in vitro. To address the question of whether perturbations of secondary structure might be responsible for the effects seen with some of the deletions, transcription in vitro was performed with purified *B. subtilis* RNA polymerase (σ^A). Linear templates for these reactions were purified following digestion of appropriate plasmids with *Eco*RI and *Bam*HI. Transcripts synthesized in vitro were labeled by including [32 P]UTP in the reaction mixture. Products were fractionated by electrophoresis and quantitated with a Beta-scope blot analyzer (Betagen). Two classes of transcripts were analyzed: those whose synthesis was terminated at position +405 (T) and those whose synthesis terminated at the end of the template (readthrough RNA [RT]). After correction of radioactivity for differences in sizes of transcripts, the percentage of transcripts representing transcription to the end of the template was expressed as the percent readthrough $[(RT \times 100)/(RT + T)]$.

We previously showed that transcription in vitro from a wild-type template resulted in 10% readthrough (4). Similar results were obtained with templates prepared from pACwt, pLD100, and pLD101, constructs that showed normal leucine-dependent control in vivo (Fig. 4 and 7 and Table 5). Two prominent bands of unknown origin were seen in several of the lanes (arrows in Fig. 7). To determine whether these transcripts originated from the *ilv-leu* promoter, a control reaction was run with a template containing a promoter with two point mutations in the -10 region (-10 BglII) (4). The reaction in which the -10 BglII mutant template was used produced few terminated or runoff-size transcripts yet produced the two unknown bands (Fig. 7). We conclude that these bands do not represent transcripts initiated from the *ilv-leu* promoter and did not consider them further.

We next investigated some deletions that decreased transcription readthrough in vivo. The deletions in constructs 202 and 504 resulted in β -galactosidase expression that was greatly reduced from the repressed levels seen with the wild type (Fig. 4). When a template prepared from either of these constructs was used for transcription in vitro, the result was only about 2% readthrough (Fig. 7 and Table 5). This result contrasts with the 6 to 9% readthrough seen with wild-type templates derived from pACwt, pLD100, and pLD101. In other experiments the percent readthrough for the wild-type template was 10%, whereas the percent readthrough for the 202 deletion was never above 2% (3b). Since this experiment was carried out in vitro with purified template and RNA polymerase, it is likely that the effects of the deletions are due to alterations in the secondary structure of the transcript. The results suggest that the decrease in transcription readthrough in vivo observed with

FIG. 7. Transcription in vitro from a wild type *ilv-leu* leader template and from templates containing deletions and point mutations. Templates isolated from the plasmids indicated at the top of each lane were incubated for 30 min with purified *B. subtilis* RNA polymerase (σ^A) and nucleoside triphosphates. Arrows, bands of unknown origin described in the text; x, transcript of size predicted if transcription terminated at terminator; o, transcript of size predicted for readthrough transcription to the end of the template.

constructs 202 and 504 was due to disruption of secondary structure in the *ilv-leu* leader mRNA. In the case of construct 202, this is particularly interesting because it suggests that secondary structure upstream of the putative protector stem can affect formation of the terminator stem. It is tempting to speculate that upstream secondary structures may overlap the proposed protector stem and thus affect formation of the terminator by inhibiting formation of the protector.

The deletions introduced into the *ilv-leu* leader to produce constructs 207 and 500 resulted in three- to fivefold-higher levels of expression than in the wild type during growth on excess leucine (Fig. 4). When the leader region of these deletion constructs was used as the template for transcription in vitro, transcription readthrough was between 50 and 60% (Fig. 7 and Table 4), an increase of fivefold over the 10%

TABLE 5. Quantitation of radioactivity in transcripts from transcription in vitro

Template plasmid ^a	Counts (10 ³) in:		% Readthrough
	Terminated transcript	Readthrough transcript (corrected) ^b	
pACwt	64	3.8	6
pLD202	75	1.3	2
pLD100	56	5.6	9
pLD101	154	15	9
pLD207	17	19	52
pLD504	42	0.87	2
pLD500	25	36	59
pM3	43	5.1	11
pM4	111	7.2	6

^a Templates were *EcoRI*-*Bam*HI fragments purified from the plasmids indicated.

^b Counts in the readthrough transcript were corrected for the difference in uridine content between terminated and readthrough transcripts. The formula used was as follows: Corrected counts in readthrough transcript = actual counts × (number of uridines in terminated transcript/number of uridines in readthrough transcript).

readthrough seen with the construct 100 as the template. These results suggest that the increased expression observed with these constructs in vivo is due to disruption of secondary structure within the *ilv-leu* leader region.

Transcriptional pausing in the *ilv-leu* leader region. For the *trp* operon of *S. marcescens*, a prominent feature of the attenuation mechanism is pausing of RNA polymerase at specific sites upstream of the terminator stem (15). To determine whether pausing occurs in the *ilv-leu* leader region, we carried out transcription in vitro for short periods of time. Reaction mixtures were incubated for 30 s, 1 min, 2 min, and 5 min, and reactions were stopped by phenol extraction. If pausing occurred during transcription, bands of smaller size than the terminated and readthrough bands were expected to accumulate at early time points and perhaps disappear at a later point. We observed no such bands with the ACwt template, which suggests that RNA polymerase does not pause at any specific sites under the conditions used for our reactions (data not shown).

Is translation of a short ORF involved in regulation of the *ilv-leu* operon? We considered the possibility that regulation is dependent on translation of an ORF within the leader region as has been demonstrated for several amino acid biosynthetic operons of *E. coli* and *Salmonella typhimurium* (10). The 482-bp *ilv-leu* leader RNA contains many short ORFs, but none of them are preceded by sequences that match closely to a Shine-Dalgarno sequence. It should be noted that for *B. subtilis*, efficient translation initiation requires a close match to the Shine-Dalgarno consensus (11). The closest match that we found ($\Delta G = -8.4$ kcal [ca. -35 kJ]) is centered at position

FIG. 8. (A) Translational fusions of ORF1 and *ilvB* to *lacZ* and β -galactosidase activity measured in strains containing translational fusions integrated at the *amyE* locus. In these fusions, the *spoVG* ribosome binding site was deleted, and β -galactosidase expression is dependent on either the ORF1 ribosome binding site (TF1) or the *ilvB* ribosome binding site (TF2). A diagram of the wild-type *ilv-leu* leader is included at top to indicate locations of fusions. (B) Transcriptional fusions containing ORF1 mutations and effect of these mutations on regulation of the *ilv-leu* operon. Mutations were prepared by site-directed mutagenesis of leader DNA, and transcriptional fusions to *lacZ* were constructed in pDH32-derived plasmids and integrated into the *amyE* locus. Changes in the leader are indicated above each construction. Translation of β -galactosidase mRNA was dependent on the *spoVG* ribosome binding site in these constructs. Cells were cultured as for Fig. 4. Large boxes, ORFs; heavy vertical lines, potential ribosome binding sites (RBS); in top line, leucine codons; other symbols and abbreviations are as described in the legend to Fig. 4.

+238, near the middle of a 47-codon ORF called ORF1 (Fig. 8A). A GUG potential translational start codon is located 10 bases downstream from the Shine-Dalgarno sequence, and this is followed by three closely spaced Leu codons. A ribosome stalled at these leucine codons could prevent formation of the putative protector stem.

We prepared two constructs having translational fusions to *lacZ*. Fusion TF1 contains the 8th codon of *lacZ* joined to the 36th codon of ORF1 (Fig. 8A). Construct TF2, used as a control in these experiments, contained the 8th codon of *lacZ* fused to the 19th codon of *ilvB*. DNA sequencing of these constructs confirmed that ORF1 and the β -galactosidase gene were in the same reading frame.

The effect of leucine on expression of β -galactosidase was measured in strains containing these constructs integrated into the *amyE* locus. In the strain containing the TF2 construct, β -galactosidase was expressed and regulated normally by leucine, although expression was about fivefold lower than for the strain containing the wild-type transcriptional fusions discussed above (compare Fig. 8A, construct TF2, with Fig. 4, construct 100). The difference in expression may be due to differences in translational efficiencies of the two messages, differences in message stability, or differences in specific activities of the fusion proteins. In the strain containing the TF1 fusion, β -galactosidase expression was not detected, indicating that ORF1 is probably not translated in vivo.

Next we determined the effect of two site-directed mutations that changed leucine codons in ORF1. In mutation M3, a single T-to-A change at position 269 introduced a UAA stop codon in place of the UUA leucine codon at position 32 in ORF1. In mutation M4, a 3-base substitution (A-270→T, C-271→T, and A-273→T) converted the leucine codons at positions 32 and 33 to phenylalanine codons (Fig. 8B). On the basis of results seen with the *leu* operon of *S. typhimurium*, we expected that changing leucine control codons to phenylalanine codons would lead to expression levels similar to those in the wild type grown with excess leucine (2). Mutations M3 and M4 were transferred into the chromosome at the *amyE* locus in single copy, and β -galactosidase assays were performed under conditions of leucine limitation and leucine excess. Both mutations decreased β -galactosidase expression in leucine-containing medium to below that seen with the wild-type leader (8-fold for M3 and 21-fold for M4) and markedly reduced leucine-mediated repression (Fig. 8B; compare M3 and M4 constructs with Fig. 4, ACwt). An additional mutation was prepared by PCR. In this construct the Leu-32 and Leu-33 codons were changed to Phe and Ser, respectively. Expression of β -galactosidase in this mutant was in the same range as seen with the M3 and M4 mutants (not shown).

Transcription in vitro was performed with templates containing mutations M3 and M4. The extent of readthrough with these templates (11% for M3 and 6% for M4) was about the same as that seen with the wild-type template (6 to 10%) (Table 5). This finding was surprising because expression of β -galactosidase from these mutants in vivo was decreased significantly from the wild-type level (17-fold for M3 and 43-fold for M4) (Fig. 4 and 8B).

A frameshift mutation was introduced into ORF1 by digesting at the *Hind*III site at position +192, filling in, and ligating the blunt ends. The resulting 4-bp insertion introduced a frameshift mutation at the 7th codon of ORF1, resulting in a UGA stop codon at position 11 in the reading frame. This mutant construct, 502, when introduced at the *amyE* locus, behaved similarly to the parent construction. The frameshift mutation had no effect on expression or regulation by leucine (compare 100 in Fig. 4 with 502 in Fig. 8B). This result suggests

that if translation of ORF1 is involved in regulation of the *ilv-leu* operon, translation initiation occurs downstream of codon 11.

DISCUSSION

Evidence presented previously demonstrated that the *ilv-leu* operon of *B. subtilis* is controlled by transcription attenuation (4). Here we present further evidence to support this contention and show that the terminator stem-and-loop structure located in the *ilv-leu* leader region is required for most of the regulation by leucine. Point mutations (*azlA102*, *azlA115*, and *azlA153*) and deletions (*azlA152* and construct 102) affecting the terminator resulted in elevated expression of the operon. None of these mutations, however, caused a complete loss of leucine-dependent control. A three- to fivefold repression of β -galactosidase expression by leucine was seen in all of these mutants. This residual regulation by leucine was also seen with a deletion that removed all leader DNA except that upstream of position +26 (Fig. 4, construct 103). We conclude that, in addition to its effect on transcription termination, leucine probably decreases the rate of initiation of transcription at the *ilv-leu* promoter.

Henkin et al. noted that the *ilv-leu* leader RNA shares conserved structural features with leader RNAs of several *B. subtilis* tRNA synthetase genes, including a 14-bp conserved region which they called the T box (6). Grundy and Henkin proposed a model in which the leader regions of these operons fold in such a way that three consecutive nucleotides, termed the specifier, are exposed on a bulge region of the secondary structure (5). They presented evidence that the specifier codon of *tyrS* is essential for regulation of this operon by tyrosine and showed that this requirement is not due to involvement of this triplet in translation. They proposed that the anticodon of an uncharged tRNA recognizes and binds to the specifier. The other end of the uncharged tRNA was postulated to stabilize the antiterminator stem by interacting with the T box and possibly another regulatory protein that recognizes the T box (Fig. 5). Stabilization of the antiterminator should lead to transcription through the terminator site and thus to elevated operon expression. Charging of the tRNA, it was postulated, prevents its action as a positive regulator by preventing the interaction with the T box. Some of the secondary structures we proposed in Fig. 5 (stem I, the antiterminator stem, and the terminator) agree well with those postulated by Grundy and Henkin. Stem I contains a CUC triplet in a bulge region that may provide the specificity required for sensing leucine starvation. This CUC triplet may interact with the GAG anticodon of one of the leucine tRNAs. The gene coding for the tRNA^{Leu} that decodes CUC has recently been identified, and several mutations in this gene that lead to elevated constitutive expression of the *ilv-leu* operon have been found (3a). An extensive mutant search also identified a mutation in the *leuS* gene that caused increased expression of the *ilv-leu* operon (19).

Our data indicate that stem I is important for regulation. A deletion that removed a large portion of the potential pairing region of stem I led to complete loss of leucine-dependent control (Fig. 4 and 5, construct 203). Further, a deletion that removed almost all of the stem I pairing region resulted in complete loss of reporter gene expression (Fig. 4 and 5, construct 202) but did not inactivate the *ilv-leu* promoter (Fig. 6B). These results suggest that the basal level of transcription past the terminator stem is dependent on the region between positions +146 and +193. The mRNA from this region is predicted to form a secondary structure containing two loop

areas which contain several nucleotides that are conserved between *ilv-leu* and all of the tRNA synthetase leader regions (Fig. 5) (5). Furthermore, two point mutations in this region of stem I resulted in loss of most of the leucine-dependent control (Fig. 4 and 5, construct M5). This suggests that the upper part of stem I may be important for the stability of stem I or for interaction of the upper portion of the stem with a *trans*-acting positive regulatory factor. We are not certain why the two- to fourfold promoter-dependent regulation seen with other constructs was not observed with constructs 203 and 305. Perhaps these deletions affect RNA structure in such a way that binding of a regulatory factor during leucine limitation stabilizes the terminator structure instead of destabilizing it, thereby increasing termination and compensating for the effect of the increase in promoter activity on reporter gene expression.

Grundy and Henkin (5) did not comment on whether the antiterminator is capable by itself of interfering with formation of the termination stem and loop. Our results suggest that it is. The antiterminator can preempt formation of the terminator stem and loop but ordinarily is prevented from doing so by sequences further upstream. The evidence for this is as follows. Construct 207 contains a deletion that removes DNA from position +26 to a point just upstream of the antiterminator, including all of the sequences required for formation of stem I. This mutation caused fivefold-elevated expression of the reporter gene in cells grown with excess leucine and a fivefold increase in transcription readthrough in vitro (Fig. 4 and 5 and Table 5, construct 207). These in vitro transcription results strongly suggest that the antiterminator is capable of causing readthrough without the aid of stabilizing factors. Furthermore, since high-level reporter gene expression occurred in vivo with construct 207, stabilization of the antiterminator by direct action of a regulatory factor is not necessarily a requirement for readthrough during leucine limitation.

What are the upstream sequences that interfere with the action of the antiterminator? The results with construct 504 suggest an answer to this question. Construct 504 is identical to construct 207 except that it contains an additional five nucleotides. Four of these nucleotides, plus nucleotide 25, which becomes attached when the deletion endpoints are joined, form the strong GC-rich stem that is at the top of the protector stem and loop (Fig. 5). One can easily imagine this GC-rich stem preempting formation of the antiterminator. The wild-type operon contains a more extensive stem-loop in this region that we call the protector (Fig. 5). We suggest that the protector prevents formation of the antiterminator, thereby promoting terminator formation. A protector stem-and-loop structure was not postulated by Grundy and Henkin (5) for the tRNA synthetase genes, but our results suggest that it is a critical part of the mechanism for the *ilv-leu* operon. A search for secondary structures capable of precluding antiterminator formation in the leader regions of *tyrS*, *thrS*, *valS*, *trpS*, and *leuS* indicated that such structures may exist in these leaders but not in the *pheS* leader. To determine whether such stems can form will require further analysis.

The mutations in constructs M3 and M4 were intended to disrupt leucine codons in ORF1 of the *ilv-leu* leader. Unexpectedly, both mutations reduced readthrough in vivo to well below the basal level (Fig. 8B) but did not decrease the amount of readthrough in vitro (Table 5). The M3 and M4 mutations lie in a CGUUA sequence of the *ilv-leu* leader (Fig. 5, 265 to 270) that Grundy and Henkin (5) have identified as conserved among the tRNA synthetase leaders. Perhaps this sequence is a site of contact for uncharged tRNA or another factor involved in antitermination.

The results with one construct are inconsistent with the

model of Grundy and Henkin (5) and indeed with other models that we have considered. Construct 503 lacks both the specifier codon and the T box region, yet it shows significant regulation by leucine (Fig. 4). The experimental results seem firm because the sequence of the 503 construct in the chromosome was verified and the assays were repeated many times. At this point, we cannot explain the results with construct 503.

Without the insights provided by the model of Grundy and Henkin (5), we had difficulty reconciling our data with any of the current models of transcription termination regulation. Mutations in both *leuS* (leucyl tRNA synthetase) (19) and *lerA* (tRNA^{Leu} that decodes CUC) lead to elevated constitutive expression of the *ilv-leu* operon. These facts are not readily explained by an attenuation mechanism of the type described for the *E. coli* *bgl* operon (7) or the *B. subtilis* *trp* operon (16), but they are easily reconciled with a ribosome-dependent model of antitermination. The presence of distinct protector, antiterminator, and termination pairing regions with the *ilv-leu* leader is also consistent with this latter model. However, another major feature of this model, a good ribosome binding site followed by an ORF containing multiple control codons, was not easily discerned. Moreover, the analysis of several translational fusions to the leader region did not uncover any evidence of translation of the leader.

In summary, almost all of the information that we have is consistent with the *ilv-leu* operon being controlled by a mechanism similar to that proposed by Grundy and Henkin (5). However, it should be noted that many of the features of a translation-dependent mechanism and an uncharged-tRNA-dependent mechanism are the same, namely, the involvement of overlapping RNA secondary structures (protector, antiterminator, and terminator), tRNA synthetase, and tRNA. Definitive evidence that the *ilv-leu* operon is controlled by an uncharged-tRNA-dependent mechanism will require experiments demonstrating that the CUC triplet in stem I indeed determines the specificity of the regulatory mechanism and that translation is not important to its function.

ACKNOWLEDGMENTS

We thank Tina Henkin for communication of unpublished results, Dennis Henner for providing plasmid pDH32, Patricia Elliot for performing β -galactosidase assays and sequencing, Patricia Grandoni for help with computer analysis, John Helmann for purified *B. subtilis* RNA polymerase, and Robert Switzer and Donald Holzschu for critical comments on the manuscript.

This work was supported by National Institutes of Health grant GM43970 to S.A.Z. and Hatch grant NYC181411, USDA.

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