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A CUC Triplet Confers Leucine-Dependent Regulation of the *Bacillus subtilis ilv-leu* Operon

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Regulation of the *ilv-leu* operon probably involves interaction of a $tRNA_{GAG}$ with leader region mRNA. Conversion of a CUC (Leu) triplet located within the leader region to UUC (Phe), CGC (Arg), or UAC (Tyr) converted reporter gene expression to control by corresponding amino acids. Conversion of the CUC triplet to CUU (Leu) decreased expression and disrupted regulation. The results suggested that other tRNAs can substitute for $tRNA^{Leu}$ but that interactions in addition to pairing of the anticodon with the CUC triplet are important for proper control.

The *ilv-leu* operon of *Bacillus subtilis* is composed of seven genes involved in synthesis of branched-chain amino acids (Fig. 1). Previous results showed that transcription of the structural genes of the operon was induced by limitation for leucine but not by limitation for isoleucine or valine. Transcription is controlled by an attenuation mechanism involving a factor-independent terminator located within a 482-bp leader region (5). Studies of mutant *ilv-leu* leader constructs identified regions essential for leucine-dependent control, including stem 1 and the antiterminator (Fig. 1). In vitro transcription experiments using the mutant templates demonstrated that the antiterminator stem prevented termination and that sequences upstream of the antiterminator prevented formation of the antiterminator. A potential stem-loop (the protector) in this upstream region overlaps the antiterminator and may be involved in sequestering the upstream portion of the antiterminator stem, thereby allowing the terminator to form (Fig. 1).

Grundy and Henkin pointed out that the leader RNAs from several attenuation-controlled operons of gram-positive bacteria can be folded into structures that resemble one another (6). These operons include *ilv-leu*, *cysE-cysS*, and the tRNA synthetase genes *tyrS*, *leuS*, *pheS*, *thrS*, *tyrZ*, and *thrZ* of *B. subtilis* and *valS*, *trpS*, and *tyrS* of *Bacillus stearothermophilus* (1, 6). They also found similar potential structures in the *trp* and *valS* operons of *Lactobacillus casei*, the *trp* and *his* operons of *Lactococcus lactis*, and the *argS* gene of *Corynebacterium glutamicum* (7, 9). All of these operons contain a stem 1, antiterminator, terminator, and a conserved sequence within the antiterminator called a T box (Fig. 1). Stem 1 has a bulge containing a nucleotide triplet (specifier) that corresponds to the amino acid associated with the function of the operon (6, 7). Grundy and Henkin proposed a model for *tyrS* regulation which we have adapted for *ilv-leu* regulation (Fig. 1). When intracellular leucine is low, the proportion of uncharged $tRNA^{Leu}$ increases, and uncharged $tRNA^{Leu}_{GAG}$ binds directly to the leader mRNA at the CUC specifier in stem 1. An interaction between the 3′ end of the uncharged tRNA and the T box stabilizes the antiterminator. The specificity of the interaction is determined primarily by the pairing of the anticodon with the specifier triplet.

Garrity and Zahler showed that a mutation in *leuG*, encoding $tRNA_{GAG}$, prevented repression of the *ilv-leu* operon by leucine (2). The phenotype of the *leuG1* strain may result from decreased charging of the mutant tRNA because of decreased recognition by tRNA synthetase. Surprisingly, wild-type *leuG* was dominant to *leuG1*, suggesting that charged tRNA may compete with uncharged tRNA for binding to the *ilv-leu* leader mRNA (Fig. 1). This idea was further supported by the results of Putzer et al. which showed that overexpression of threonine tRNA synthetase from a gene on a plasmid caused a reduction in expression of the *thrS* operon (12).

In this report we demonstrate that the CUC specifier of the *ilv-leu* operon is required for leucine-dependent control. Changing the specifier to UUC (Phe), UAC (Tyr), or CGC (Arg) resulted in at least partial regulation by phenylalanine, tyrosine, or arginine, respectively. Furthermore, CUU (Leu) in the specifier position does not confer control by leucine.

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study are listed in Table 1. Strains BG61 (*trpC2 leuB6 pheA2 argA2 amy*::*erm*) and BG82 (*trpC2 tyrA1 amy*::*erm*) were constructed by transforming strain CU233 (*trpC2 leuB6 pheA2 argA2*) and CU218 (*trpC2 tyrA1*) with DNA isolated from strain MO199 (*pheA amy*::*erm*) and selecting for resistance to 1 mg of erythromycin and 25 mg of lincomycin per liter. Strain CU3369 (*liv3*::Tn*917 leuB6 trpC2*) contains Tn*917* in the *ilvN* gene (15). Strains BG108 and BG111 were constructed by transforming strains BG83 and BG84 with DNA from strain CU3369 (*liv3*::Tn*917 leuB6 trpC2*), selecting for resistance to erythromycin and lincomycin, and screening for leucine auxotrophy. Other conditions were as described previously (4).

Site-directed mutagenesis and measurement of *lacZ* expression. Site-directed mutagenesis was performed on plasmid pACB1 using the Clontech Transformer kit (Fig. 2) (5, 10). All sequences were confirmed by DNA sequencing (13). Fusion constructs were prepared as described previously (4). Regulation of expression of the *lacZ* gene was measured as follows. A single colony from a Luria broth plate was cultured in minimal medium containing the required amino acids. When the cells were in logarithmic growth, an aliquot of each culture was transferred to new medium of the same composition. This culture was grown to mid-logarithmic phase and split into

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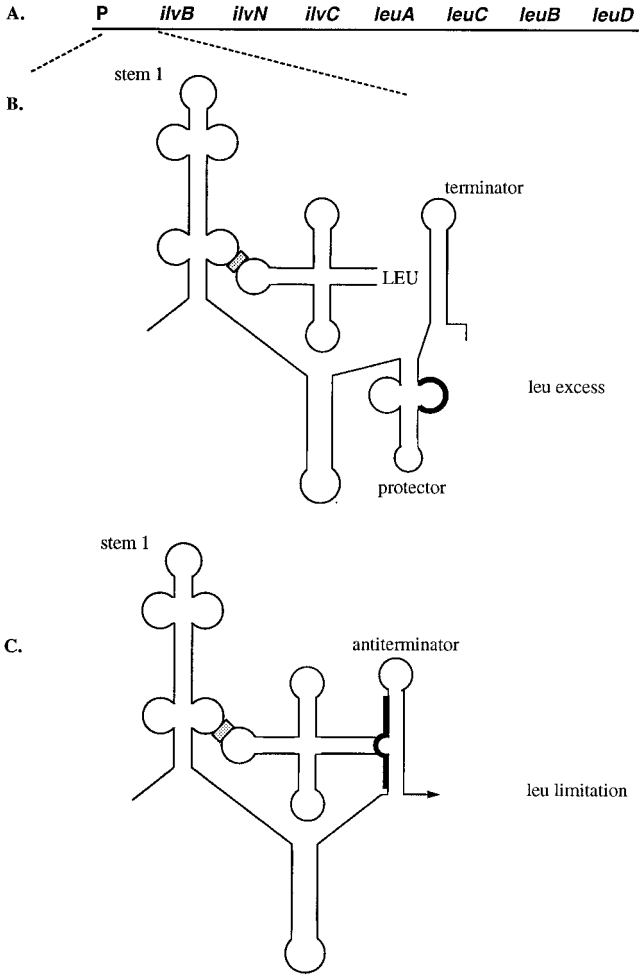

FIG. 1. Modified Grundy-and-Henkin model for regulation of the *ilv-leu* operon. (A) Structure of the *ilv-leu* genes. P, *ilv-leu* promoter. (B) Structure of *ilv-leu* leader mRNA formed when leucine is in excess. Charged leucine $tRNA_{GAG}$ binds to the CUC specifier triplet (stippled box) but does not interact with the T box (black box). As a result, the terminator can form and expression of the *ilv-leu* genes is low. (C) Structure of *ilv-leu* leader mRNA when cells are starved for leucine. Uncharged $tRNA_{GAG}$ binds to the specifier and to the T box. The antiterminator stem is favored and leads to increased transcription beyond the terminator.

equal portions. The cells were pelleted by centrifugation and suspended in new medium lacking one of the required amino acids. Growth of all cultures ceased upon suspension in the new medium. Samples of cell cultures were removed at various times after suspension in the final medium, and the activity of β-galactosidase was determined for each time point as described previously (4). The level of derepression by amino acid starvation was calculated by using the β-galactosidase activity measured at the time point when activity was highest, usually 4 h after transfer to the medium lacking one of the required amino acids.

Regulation of transcription by the wild-type *ilv-leu* control region. Expression of β-galactosidase in a strain containing the wild-type *ilv-leu* control region fused to *lacZ* (BG74 or BG108) was 12- to 15-fold higher in cells that were starved for leucine than in cells that were starved for phenylalanine or arginine or tyrosine (Table 2). In previous reports, leucine limitation resulted in a 24-fold increase in expression of the *lacZ* reporter gene during leucine limitation (4). The difference between

TABLE 1. Strains used in this study

Strain	Genotype
MO199	*trpC2 pheA amy*::*erm*[a]
CU218	*trpC2 tyrA1*
CU233	*trpC2 leuB6 pheA2 argA2*
CU3369	*trpC2 liv3*::Tn*917 leuB6*
BG61	*trpC2 leuB6 pheA2 argA2 amy*::*erm*
BG72	*trpC2 leuB6 pheA2 argA2 amy*::*M10 lacZ*[b]
BG73	*trpC2 leuB6 pheA2 argA2 amy*::*M11 lacZ*
BG74	*trpC2 leuB6 pheA2 argA2 amy*::*LD100 lacZ*
BG75	*trpC2 leuB6 pheA2 argA2 amy*::*M17 lacZ*
BG76	*trpC2 leuB6 pheA2 argA2 amy*::*M18 lacZ*
BG77	*trpC2 leuB6 pheA2 argA2 amy*::*M19 lacZ*
BG82	*trpC2 tyrA1 amy*::*erm*
BG83	*trpC2 tyrA1 amy*::*M17 lacZ*
BG84	*trpC2 tyrA1 amy*::*LD100 lacZ*
BG108	*trpC2 tyrA1 liv3*::Tn*917 leuB6 amy*::*LD100 lacZ*
BG111	*trpC2 tyrA1 liv3*::Tn*917 leuB6 amy*::*M17 lacZ*

[a] *amy*::*erm* represents insertion of the erythromycin resistance gene into *amyE*.
[b] Strain contains *lacZ* fusion to *ilv-leu* leader construct M10 integrated into *amyE* in place of *erm*.

results reported here and those reported previously is probably due to differences in the method of leucine limitation used. In our previous work, cells were limited for leucine by growing the culture in medium containing *N*-acetyl leucine, which is slowly converted to leucine. With this protocol, the leucine-fed and -deprived cultures grew logarithmically. For work presented here, we wanted to compare the effect of leucine starvation with the effects of starvation for other amino acids and we did not add *N*-acetyl leucine to leucine-starved cultures.

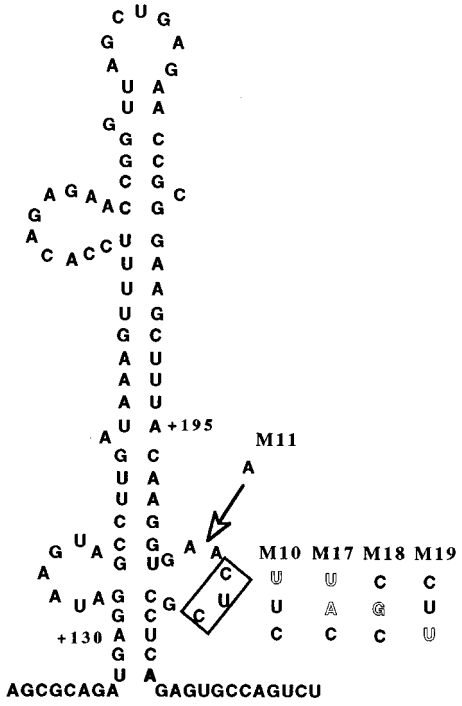

FIG. 2. Secondary structure of stem 1 of the *ilv-leu* operon and mutations in the specifier region. Insertion of an adenine nucleotide in construct M11 (arrow), specifier triplet (boxed), and nucleotide replacements introduced in the specifier region (outlined letters) are indicated.

TABLE 2. Expression of *lacZ* reporter gene in strains containing mutant *ilv-leu* leader regions

Strain	Relevant genotype	Specifier region[a]	β-Galactosidase specific activity[b] (Miller units) in cultures starved for:			
			Leu	Phe	Arg	Tyr
BG74	*leuB6 pheA2 argA2 amy*::*LD100 lacZ*	AA**CUC** (Leu)	260 (2)	21 (2)	16 (2)	ND[c]
BG108	*tyrA1 leuB6 amy*::*LD100 lacZ*	AA**CUC** (Leu)	260 (6)	ND	ND	17 (6)
BG73	*leuB6 pheA2 argA2 amy*::*M11 lacZ*	AA**ACUC** (Leu)	270 (7)	53 (5)	19 (2)	ND
BG77	*leuB6 pheA2 argA2 amy*::*M19 lacZ*	AA**CUU** (Leu)	7 (8)	3 (6)	2 (2)	ND
BG72	*leuB6 pheA2 argA2 amy*::*M10 lacZ*	AA**UUC** (Phe)	70 (7)	240 (5)	30 (2)	ND
BG76	*leuB6 pheA2 argA2 amy*::*M18 lacZ*	AA**CGC** (Arg)	46 (8)	33 (2)	120 (6)	ND
BG111	*tyrA1 leuB6 amy*::*M17 lacZ*	AA**UAC** (Tyr)	23 (8)	ND	ND	69 (8)

[a] Specifier is shown in boldface type.
[b] The values shown are the averages from multiple trials. Numbers in parentheses are the numbers of trials.
[c] ND, not determined.

Mutations that changed the specifier triplet changed the specificity of *ilv-leu* regulation. To determine if the CUC triplet at the putative specifier position within the *ilv-leu* leader is required for leucine-dependent control, we prepared site-directed mutations within the specifier region and constructed fusions of these mutant leaders to a *lacZ* reporter gene. These constructs were introduced in single copy into the *B. subtilis amyE* locus, and β-galactosidase activity was measured in cultures of cells that were maintained under conditions of amino acid starvation. Construct M10 contained a mutation that changed the CUC (Leu) specifier to UUC (Phe). When cultures of strain BG72 (*trpC2 leuB6 pheA2 argA2 amy*::*ilv-leu M10 lacZ*) were starved for leucine or arginine, expression of the reporter gene was low (Table 2). In contrast, when BG72 was starved for phenylalanine, a four- to eightfold induction of *lacZ* expression was observed. The specifier of the *C. glutamicum argS* was proposed to be a CGC triplet located in the bulge region of stem 1 (7). The CUC specifier of *ilv-leu* was converted to CGC, and the construct containing this mutation (M18) was integrated into *amyE* to form strain BG76. Expression of *lacZ* in cultures of BG76 that were starved for arginine was three- to fourfold higher than in cultures starved for leucine or phenylalanine (Table 2).

To determine if the *ilv-leu* operon could be converted to tyrosine-specific control, the CUC specifier was converted to UAC (Tyr) and the construct containing this mutation was integrated into *amyE* of *B. subtilis* to produce strain BG111 (*trpC2 tyrA leuB6 liv3*::Tn*917 amy*::*M17*). When cultures of strain BG111 were starved for leucine, β-galactosidase activity was low, whereas starvation for tyrosine resulted in a threefold increase in β-galactosidase activity (Table 2). To compare expression of the M10 construct with expression of the wild-type (LD100) construct, we constructed strain BG108 (*trpC2 tyrA leuB6 liv3*::Tn*917 amy*::*LD100 lacZ*). Expression of the reporter gene in cultures of strain BG108 was 15-fold higher when the cells were suspended in medium lacking leucine than when the cells were suspended in medium lacking tyrosine (Table 2). This was similar to the level of derepression seen with strain BG74 (*trpC2 leuB6 pheA2 argA2 amy*::*LD100 lacZ*).

Conversion of the specifier to a different leucine triplet. We converted the CUC specifier to CUU to produce construct M19 and measured the effect of this mutation on leucine-dependent control of expression. Expression of the reporter gene in cultures of strain BG77 (*trpC2 leuB6 pheA2 argA2 amy*::*ilv-leu M19 lacZ*) was about 10-fold lower than in cultures of strain BG74 (*trpC2 leuB6 pheA2 argA2 amy*::*LD100 lacZ*) under all culture conditions tested (Table 2). The twofold increase in β-galactosidase activity in strain BG77 upon leucine starvation may be due to induction of the *ilv-leu* promoter that we noted in previous reports (5).

Disruption of a potential reading frame. The results seen with the specifier could be explained by postulating that the CUC codon is part of a short open reading frame that is important for regulation. To determine whether translation of the CUC codon is required for leucine-specific control, a single nucleotide was inserted immediately upstream of the CUC codon to produce construct M11 (Fig. 2). Regulation of reporter gene expression in BG73 (*trpC2 leuB6 pheA2 argA2 amy*::*M11 lacZ*) was almost identical to that of the wild-type strain, suggesting that the CUC specifier is not part of a short open reading frame (Table 2).

Concluding remarks. The results presented here showed that the CUC triplet at positions 205 to 207 of the *ilv-leu* leader confers specificity of regulation of this operon and provided support for the model developed by Grundy and Henkin (Fig. 1). This regulatory mechanism is apparently unique to gram-positive bacteria and is different from the attenuation mechanisms proposed for regulation of amino acid biosynthesis operons in enteric bacteria, which require translation of a short open reading frame (11).

Conversion of the *ilv-leu* specifier to UUC (Phe) destroyed most of the leucine-dependent regulation and conferred phenylalanine-dependent regulation to the *ilv-leu* leader region. β-Galactosidase activity in the strain containing the wild-type *ilv-leu* leader was increased 12- to 15-fold by leucine starvation. When the specifier was replaced by UUC (Phe), β-galactosidase activity was increased eightfold by phenylalanine starvation (Table 2). Likewise, Grundy and Henkin reported that the *tyrS* operon was converted to phenylalanine-dependent control by changing the *tyrS* specifier to UUC (6). β-Galactosidase activity in the strain containing the wild-type *tyrS* leader fused to *lacZ* was 10-fold increased upon tyrosine starvation, whereas starvation for phenylalanine of the strain containing a UUC specifier in the *tyrS* leader region resulted in a 5-fold increase in β-galactosidase activity (6). Since the $tRNA^{Phe}$ that contains a GAA anticodon ($tRNA^{Phe}_{GAA}$) is suitable for partial regulation of both *tyrS* and *ilv-leu*, we suspected that the sites of contact between tRNA and the leader regions of *ilv-leu* and *tyrS* might be similar and that $tRNA^{Tyr}_{GUA}$ would therefore function in regulation of the *ilv-leu* operon. We tested this hypothesis by converting the *ilv-leu* specifier to UAC (Tyr) and found that tyrosine starvation of the strain containing this construct led to threefold-higher expression of the reporter gene than did starvation for leucine. These results suggested that $tRNA^{Tyr}_{GUA}$ can function as a regulator of *ilv-leu*,

although this tRNA does not apparently function as well as $tRNA^{Leu}_{GAG}$ does.

We showed that conversion of the CUC specifier to CGC (Arg) conferred three- to fourfold regulation by arginine, but conversion of the specifier to CUU (Leu) did not confer regulation by leucine. This suggested that $tRNA^{Arg}_{GCG}$ can function in *ilv-leu* regulation but that $tRNA^{Leu}_{AAG}$ cannot (Table 2). In protein synthesis, $tRNA_{GAG}$ can pair with a CUU codon because the 5′ G of the anticodon can pair with the U in the 3′ position of the codon. The finding that CUU did not substitute for CUC as a specifier for the *ilv-leu* operon supported the suggestion that the third position of the specifier is important for specifier-anticodon interaction and suggested that this interaction is more stringent than that of a codon-anticodon. Consistent with this, changing the ACC specifier of *thrS* to ACA (Thr) resulted in loss of regulation by threonine, and conversion of the UAC of *tyrS* to UAU (Tyr) resulted in loss of regulation of *tyrS* by tyrosine (9, 12). All of the operons that are believed to be regulated by the tRNA-dependent mechanism, except the *valS* operon of *L. casei*, contain specifiers that have a C or G in the third position (9).

The ability for a particular tRNA to function as an antitermination factor probably depends on structural features of the tRNA that allow it to bind efficiently to the leader mRNA. One such feature was shown by Grundy and Henkin to be the discriminator base located upstream of the CCA at the 3′ end of tRNA (8). They showed that the discriminator base probably pairs with a nucleotide preceding the UGG that is conserved within the T box. $tRNA^{Phe}_{GAA}$ and $tRNA^{Tyr}_{GUA}$ contain the same discriminator base as $tRNA^{Leu}_{GAG}$ does (3, 16). The finding that the UUC and UAC specifiers confer regulation by phenylalanine and tyrosine, respectively, is consistent with the conclusion that the discriminator is important for tRNA binding. However, these specifiers conferred only partial regulation by the corresponding amino acids, suggesting that there may be other contacts in addition to the specifier and the T box that are important for binding of the tRNA to the mRNA. The discriminator for $tRNA_{GCG}$ is a G, which is predicted to be able to pair with the U preceding the UGG of the T box (14). This pairing, however, would not be as stable as the A-U base pair that is believed to form when the Leu, Tyr, or Phe tRNA interacts with the *ilv-leu* T box.

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