Transcription attenuation in *Salmonella typhimurium*: The significance of rare leucine codons in the *leu* leader

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**ABSTRACT** The leucine operon of *Salmonella typhimurium* is controlled by a transcription attenuation mechanism. Four adjacent leucine codons within a 160-nucleotide *leu* leader RNA are thought to play a central role in this mechanism. Three of the four codons are CUA, a rarely used leucine codon within enteric bacteria. To determine whether the nature of the leucine codon affects the regulation of the leucine operon, we used oligonucleotide-directed mutagenesis to first convert one CUA of the leader to CUG and then convert all three CUA codons to CUG. CUG is the most frequently used leucine codon in enteric bacteria. A mutant having (CUA)3CUGCUC in place of (CUA)3CUC has an altered response to leucine limitation, requiring a slightly higher degree of limitation to effect derepression. Changing (CUA)3CUC to (CUG)3CUC has more dramatic effects upon operon expression. First, the basal level of expression is lowered to the point that the mutant grows more slowly than the parent in a minimal medium lacking leucine. Second, the response of the mutant to a leucine limitation is dramatically altered such that even a strong limitation elicits only a modest degree of derepression. If the mutant is grown under conditions of leucyl-tRNA limitation rather than leucine limitation, complete derepression can be achieved, but only at a much higher degree of limitation than for the wild-type operon. These results provide a clear-cut example of codon usage having a dramatic effect upon gene expression.

Expression of the leucine operon of *Salmonella typhimurium* varies over a 40-fold range depending on the intracellular concentration of leucine (1). The major mechanism by which expression of this operon is controlled by leucine is transcription attenuation (2). According to a model proposed by Gemmill et al. (3), formation of alternative secondary structures in a leader RNA determines whether or not premature termination of transcription occurs at a site 160 bases downstream from the point of transcription initiation (Fig. 1). Formation of a terminator stem-and-loop at this site in the leader RNA causes premature termination, whereas prior formation of an alternative stem-and-loop—the preemptor—precludes formation of the terminator, thereby allowing transcription of the operon. Which stem-and-loop structure forms is dependent on the progress of a ribosome that translates the leader RNA. The preemptor stem-and-loop can form only when a ribosome stalls at one of four tandem leucine codons in the leader RNA; therefore, the structural genes of the operon are transcribed at a high rate only during a limitation for charged leucine tRNA. This model is similar to one developed by Yanofsky and coworkers to explain regulation of the trp operon of *Escherichia coli* (4).

We demonstrated previously that the four adjacent leucine codons in the leader RNA are necessary for regulation: a strain having four threonine codons in place of four leucine codons is regulated by threonine but not by leucine (5). Here we investigate the nature of the leucine codons selected during evolution to play a role in attenuation control. In the *S. typhimurium* *leu* leader, three of the four tandem leucine codons are CUA codons (3), and in the *leu* leader of the closely related organism *Escherichia coli*, all four of the tandem leucine codons are CUA (6). These findings are particularly interesting in view of the fact that CUA is a rarely used leucine codon in *E. coli*. Among 52 *E. coli* genes, only 30 of 1645 leucine codons (1.8%) are CUA (7). Is there any significance to the use of CUA as a control codon in attenuation? To answer this question, we used oligonucleotide-directed mutagenesis first to convert one CUA of the *S. typhimurium* leader to CUG and then to convert all three CUA's to CUGs. CUG is the most frequently used leucine codon in *E. coli* (7). Analysis of these mutants showed that the nature of the leucine control codons, indeed, can significantly affect the regulation of the *leu* operon. Specifically, converting CUA's to CUG's reduces both the basal level of operon expression and the sensitivity with which the operon responds to a leucine limitation.

**MATERIALS AND METHODS**

Oligonucleotide-Directed Mutagenesis. Mutations were constructed by using the following strategy and methods. DNA from single-stranded phage PS15 was used as a template for oligonucleotide-primer DNA synthesis. Bacteriophage PS15 contains a 5.1-kilobase fragment of *S. typhimurium* DNA carrying *leuPLABCD* (*D*' means that part of *leuD* is missing; ref. 8) in the unique *EcoRI* site of bacteriophage f1R229. In *vitro* DNA synthesis, ligation, and treatment with S1 nuclelease were done as described by Gilmour and Smith (9). Transfection, growth of phage from plaques, and binding of phage DNA to nitrocellulose filters were as described by Miyada et al. (10). The nitrocellulose filters were screened with 32P-labeled oligonucleotide (11), and then the DNA was sequenced to verify the presence of the desired mutation (12). The transfer of mutations from f1 phage to λ phage by recombination, hybridization against 32P-labeled oligonucleotide to verify the presence of the mutation, and construction of single λ phage lysogens were as described by Carter et al. (5). The two oligonucleotides used in this study, tetradecamer CGTTGAGCAGTAGT [used to make λPC-III, (CUA)3CUGCUC] and octadecamer TGAGCAGCAGCACGGCCAG [used to make λPC-IV, (CUG)3CUC], were purchased from Applied Biosystems. λPC-0 contains *leu-PLABCD* with the wild-type sequence (CUA)3CUC.

**Limitations for Leucine and Leucyl-tRNA.** Starvation for leucine was achieved by growing leucine auxotrophs in a chemostat under conditions of leucine limitation. In these experiments, conditions for starvation and for assay of 3-isopropylmalate dehydrogenase (EC 1.1.1.85) were as described by Carter et al. (5). Starvation for leucyl-tRNA was achieved by growing strains containing a temperature-sensitive leucyl-tRNA synthetase (*leuS5I*) mutation (13) at elevated temperatures. The *leuS* mutation was transferred from *E. coli* strain CP79-118 (Tn10 10% cotransducible with...
leuS31) to recipients by transduction with bacteriophage P1vir, selection being for resistance to tetracycline.

**Determination of leu Operon Expression.** Expression of the leu operon in mutant and wild-type strains was determined by measuring the specific activity of the leuB gene product, 3-isopropylmalate dehydrogenase. Cells were grown at 20°C in SS medium (1) containing 0.2% D-glucose, 5 μg of thiamine·HCl per ml, and 50 μg of L-leucine per ml to an OD₆₀₀ of 1.0 and then diluted 1:20 into the same prewarmed medium containing, in addition, 50 μl of L-isoleucine and 100 μg of L-valine per ml and incubated at an elevated temperature in a New Brunswick reciprocating waterbath. Samples (5 ml) were taken, centrifuged, and resuspended in 1 ml of 0.05 M potassium phosphate buffer (pH 7.2) and stored at 4°C prior to being assayed. Cells were diluted to 2.5 × 10⁶ per ml in the same buffer and permeabilized with 10 μl of chloroform and 50 μl of 1% sodium deoxycholate per ml. The permeabilized cells were assayed for 3-isopropylmalate dehydrogenase activity by a modification (unpublished data) of the method of Parsons and Burns (14).

**RESULTS**

**Mutations Altering leu Operon Control Codons.** Two oligonucleotide-directed mutations were created in the leader region of the S. typhimurium leu operon. The first construction converted the third of the four tandem leucine codons from CUA to CUG (Fig. 1C). The second construction converted all three CUA codons to CUG codons. The mutations were transferred to bacteriophage λ, and single

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**Fig. 1.** Diagrammatic representation of the control region of the leu operon. (A) Partial nucleotide sequence of the S. typhimurium leu leader RNA, folded to emphasize the preemper pair region (Left) or the protector and terminator pair regions (Right). Numbering begins at the first nucleotide of the RNA. Leucine control codons are in boldface, and the mutations giving rise to bacteriophage APC-III (single filled arrow) and APC-IV (open arrows) are indicated. The most stable secondary structures predicted by the algorithm of Williams and Tinoco (15) differ from that shown in A Right as follows: for the single CUA-to-CUG change, 57–63 pairs with 82–88 and 67–71 pairs with 77–80; for the (CUA)₂-to-(CUG)₂ change, 58–66 pairs with 94–102 and 68–71 pairs with 79–82. The overall stabilities calculated for the wild type, CUΑ-to-CUG, and (CUΑ)₂-to-(CUG)₂ mutant leader RNAs are −66.7, −68.3, and −75.0 kilocalories (1 cal = 4.18 J), respectively. (B) mRNA secondary structures postulated to play a role in the control of transcription termination. The solid and open rectangles, representing parts of the leader RNA shown in A that form double-stranded regions (16), are aligned over the structures in C so that their positions relative to the control codons and a ribosome can easily be seen. (C) Cartoons depicting events during translation of the leu leader RNA. Boxes represent translatable codons within the leader. The four leucine control codons are hatched. I, II, III, and IV represent predicted positions of a ribosome (large rectangle with an oval signifying the ribosome A site) on the leu leader when RNA polymerase reaches the termination site (vertical arrow). I, wild-type leu leader with cells growing with excess leucine; II, wild-type leader with mild leucine limitation; III, mutant leu leader [(CUG)₂CUΑ] with excess leucine; IV, mutant leu leader with mild leucine limitation.
lysogens were formed in E. coli strain CSH73 (Δara-leu, Δlac, thi) yielding strains CV752 [with λPC-III, (CUA)₃CUC] and CV755 [with λPC-IV, (CUG)₃CUC]. Strain CV745, which is strain CSH73 carrying a prophage with the wild-type operon [λPC-0, (CUA)₃CUC], was used in control experiments (5).

leu Operon Expression in Strains Starved for Leucine. Strains CV752, CV755, and CV745 are leucine auxotrophs because their chromosomes have a leu operon deletion, and the leuPLABCD prophages they carry are missing part of leuD. These strains were grown in a chemostat under conditions of leucine limitation. Fig. 2 shows operon expression as measured by 3-isopropylmalate dehydrogenase activity as a function of increasing generation time (that is, increasing leucine starvation). The curve for the control strain [CV745, (CUA)₃CUC] is similar to what had previously been determined for a S. typhimurium strain having the leucine operon at its normal position in the chromosome (1). The curve for the mutant having the single CUA-to-CUG change, [CV752 (CUA)₃CUC]CUC, is not very different from that for the control, but it is perceptibly shifted to the right, indicating that the mutant operon requires slightly greater leucine starvation in order for derepression of the operon to occur.

In contrast, the strain having three CUA changes to CUGs [CV755, (CUG)₃CUC] exhibited a dramatic difference in response to leucine limitation (Fig. 2). Most expression in the mutant was repressed during mild-to-moderate leucine starvations, and even severe starvation (170-min generation time) did not elicit extensive derepression. Clearly, the nature of the leucine codons occupying the control position can have a significant effect on regulation.

leu Operon Expression in Strains Limited in Leucyl-tRNA Synthesis. In the chemostat experiments described above, reduced availability of leucine presumably resulted in lower endogenous levels of leucyl-tRNA. We altered leucyl-tRNA levels more directly in the following manner. The LeuS31 allele encodes a temperature-sensitive leucyl-tRNA synthetase and results in temperature-sensitive growth. This allele was transduced into control strain CV745 [(CUA)₃CUC] and strain CV755 [(CUG)₃CUC], yielding strains CV868 [leuS31, (CUA)₃CUC] and CV869 [leuS31, (CUG)₃CUC], respectively.

Two problems were encountered when we attempted to carry out experiments with CV868 and CV869 grown in a minimal medium containing leucine (these strains are leucine auxotrophs). First, a relatively high growth temperature (about 40°C) was required to markedly reduce the growth rate; and second, a relatively long time (several hours) was required to achieve a constant level of operon expression when the cells were shifted from one temperature to another. Both of these problems presumably result from the fact that leucine protects the mutant leucyl-tRNA synthetase from inactivation (17). We circumvented these problems by growing the strains in a medium containing excess isoleucine and valine, amino acids that are known to compete with leucine for entry into cells (18). Excess endogenous isoleucine and valine effectively lower the internal concentration of leucine,

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**Fig. 2.** Leucine operon expression as a function of leucine starvation in strains having wild-type and mutant leu leader regions. Cells were grown in a chemostat containing leucine at 8 μg/ml at the indicated generation times. Generation time is the time required to replace the culture volume multiplied by the natural logarithm of 2. Specific activity is μmol of product formed per hr per mg of protein. ○, CV745 [(CUA)₃CUC]; ◦, CV752 [(CUA)₃CUC]; ■, CV755 [(CUG)₃CUC].

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**Fig. 3.** Differential rate of synthesis of 3-isopropylmalate dehydrogenase in strains CV745 [leuS₅, (CUA)₃CUC] (A) and CV869 [leuS₅, (CUG)₃CUC] (B). Data for strain CV745 [leuS₅, (CUA)₃CUC] grown at 20, 25, and 37°C (-----) are included in both panels. Inocula were grown at 20°C, diluted 20-fold (at arrow) into a medium containing isoleucine and valine, and incubated at the same or elevated temperature. 3-Isopropylmalate dehydrogenase activity is ΔA₄₅₀ per 15 min. The cell mass is calculated as the OD₆₀₀ of the culture multiplied by 20 (the dilution factor of the inoculum). Doubling times for CV745 were 240 min at 20°C, 138 min at 25°C, and 57 min at 37°C. Doubling times for CV868 were 264 min at 20°C, 162 min at 25°C, 126 min at 30°C, 150 min at 33°C, and 360 min at 37°C. Doubling times for CV869 were 240 min at 20°C, 156 min at 25°C, 120 min at 30°C, and 324 min at 37°C. ○, 20°C; △, 25°C; □, 30°C; ○, 33°C; ▲, 37°C.
thereby lessening its protective effect on leucyl-tRNA synthetase.

The results of shifting strains CV868 [(CUA)]CUC and CV869 [(CUG)]CUC from minimal medium at 20°C to minimal medium containing isoleucine and valine at a variety of temperatures are illustrated in Fig. 3. The data are presented as differential rates of synthesis of 3-isopropylmalate dehydrogenase. Note that under the conditions of these experiments, there was no derepression of the leu operon in wild-type strain CV749 [leuS* (CUA)]CUC grown at 20°C or 30°C or 37°C (dashed lines in Fig. 3A and B). In the strain carrying a leuS11 mutation and a wild-type leu operon (CV868), the leu operon was already substantially derepressed at a growth temperature of 20°C (63% of the maximum observed at higher temperatures) and growth at temperatures higher than 25°C resulted in maximal derepression (Fig. 3A). In strain CV869 [leuS11, (CUG)]CUC, on the other hand, the leu operon was only slightly derepressed during growth at 20°C, and maximal derepression required growth at temperatures > 33°C (Fig. 3B). These data indicate that the mutant operon having (CUA)₃CUC in place of (CUA)₃ can be derepressed to the same extent as the wild-type operon, but only under a more severe leucine limitation.

The (CUA)₃-to-(CUG)₃ Mutation Affects the Basal Level of Operon Expression. Because we wanted to measure the effect of the (CUA)₃-to-(CUG)₃ change on both enzyme levels and growth rates, we introduced, separately, phage λ carrying (CUA)₃CUC (PC-0) and (CUG)₃CUC (PC-IV) into E. coli strain CGSC3004, which contains a leuB point mutation. The resulting lysogens contain all four genes necessary for the synthesis of leucine, but the only functional leuB gene is under the control of the leu promoter and leader on the prophage. The basal level of leu operon expression in strain CV757 [(CUG)]CUC was lower than that in control strain CV749 [(CUA)]CUC for strains grown either in minimal medium or minimal medium containing leucine (Table 1). The reduction in the basal level of expression was of sufficient magnitude to cause a reduction in the growth rate of the mutant in the absence of leucine (Table 1).

DISCUSSION

Changing three of the four control codons in the leader RNA from CUA to CUG codons has two obvious effects upon leu operon expression: it reduces the basal level of operon expression and it changes the operon response to leucine starvation such that a higher degree of starvation is required to elicit a given response. We can imagine two ways in which the mutations might affect operon expression: by affecting leader RNA secondary structures and/or by affecting the rate at which ribosomes traverse the four leucine control codons. leu leader RNA secondary structures were analyzed with the aid of a computer using the algorithm of Williams and Tinoco (15). The mutations do not affect the preemperor terminator stem-and-loops but do lead to predicted alterations in the region of the protector stem-and-loop (see the legend to Fig. 1A). We will return to this point later in the discussion.

The rate at which ribosomes traverse the leu leader RNA is likely to be faster with CUG control codons than with CUA control codons for the following two reasons: (i) There are more tRNAs that can decode CUG than CUA. According to wobble rules, tRNAaac (3' GAC 5') should decode only CUG whereas tRNAau (3' GAU 5') should decode both CUA and CUG (19). (ii) The concentration of charged tRNAaa is probably higher than the concentration of tRNAeu (20). The steady-state concentration of charged tRNAaa and tRNAeu depends upon a number of factors, including the concentration of leucine, the concentration of individual tRNAaa species, and the Km of leucyl-tRNA synthetase for these species. For cells grown with excess leucine, >90% of tRNAeu is charged (20). Although the degree of charging of individual tRNAaa species is not known, it seems likely that they are about equally charged because the Km values of the synthetase for the five isoaccepting tRNAaa species are about the same (21). On the other hand, the total amounts of tRNAaa and tRNAeu per cell are very different. tRNAaa is the most-abundant tRNA, whereas tRNAeu is among the least-abundant tRNAs within cells (22). The possibility should also be considered that the rate constants for ribosomes decoding CUA and CUG differ. The average step time of ribosomes is about 0.07 sec per codon (23), but the actual step times at individual codons are not known. Thus, it could take longer for a ribosome to insert leucine into a growing polypeptide in response to a CUA codon than in response to a CUG codon. Also, there is some evidence that the efficiency of translation of a given codon can be affected by the identity of neighboring codons, perhaps because of interactions between the tRNAs in the A and P sites of the ribosome (24–26). Possibly then, the CUA-CUA codon pair is translated more slowly than the CUG-CUG codon pair.

To summarize, for cells grown in excess leucine, replacing three CUA codons by three CUG codons likely results in a shorter time required for a ribosome to traverse the four control codons. This shorter time probably results from a higher concentration of leucyl-tRNAeu and/or a shorter step time.

In discussing further the effects of the mutations upon the basal level of operon expression, it is necessary to describe some alternative ways in which the basal level of expression might be established. In one such model, a large fraction of ribosomes translating the leader RNA terminate translation at the in-phase UAA stop codon and fall off the message. By this model, the basal level of expression is established by the relative proportions of messages that adopt the preemperor and protector stem-and-loops. The (CUA)₃-to-(CUG)₃ change could shift the ratio in favor of the protector stem-and-loop either because that structure is more stable in the mutant (by about 8 kilocalories) or because a more rapid rate of ribosome movement across CUG codons causes the ribosome to fall off the message before the preemperor stem-and-loop is synthesized. An alternative model, and one that we favor, is depicted by the cartoons shown in Fig. 1C. In the wild-type operon, under conditions of excess leucine, most ribosomes traverse the four leucine control codons (Fig.

### Table 1. Growth rates and leu operon expression in strains CV749 and CV757

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Growth condition*</th>
<th>Generation time, min</th>
<th>Specificity activity of 3-isopropylmalate dehydrogenase†</th>
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</thead>
<tbody>
<tr>
<td>CV749</td>
<td>leu* A·B C·D*</td>
<td>leucine</td>
<td>60</td>
<td>0.70 ± 0.25 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>leucine</td>
<td>60</td>
<td>1.18 ± 0.15 (4)</td>
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<tr>
<td></td>
<td></td>
<td>leucine</td>
<td>60</td>
<td>0.24 ± 0.04 (3)</td>
</tr>
<tr>
<td>CV757</td>
<td>leu* A·B·C·D*</td>
<td>leucine</td>
<td>60</td>
<td>0.56 ± 0.05 (3)</td>
</tr>
</tbody>
</table>

*For growth with leucine, minimal medium contained leucine at 50 μg/ml.
†Specific activity is μmol of product formed per hour per mg of protein. The numbers in parentheses indicate the number of separate determinations.
1C, row I) and enough downstream codons so that the formation of the preemtpor stem-and-loop is not possible. Transcription termination results in such cases. However, because of statistical fluctuations in the rates of movement of individual ribosomes, at low frequency a ribosome may be translating a control codon when the RNA polymerase is at the attenuation site (Fig. 1C, row II). The predicted outcome of such situations is transcription read-through. For the mutant containing three CUGs, more rapid translation of the control codons would result in a more generally distal ribosome location (Fig. 1C, rows III and IV) and, as a consequence, more frequent transcription termination. The idea that rare codons can cause RNA polymerase to move ahead of ribosomes, thereby influencing the probability of transcription termination, was suggested by Bonekamp et al. in their studies of pyrE regulation (27).

The experiments involving operon expression under conditions of leucine or leucyl-tRNA limitation can be similarly interpreted. For the wild-type operon, even a mild limitation will lead to a higher frequency of ribosomes paused over control codons (Fig. 1C). By contrast, only a strong limitation, such as that produced by growing the leuS31 mutant at high temperatures, will slow translation of CUG codons sufficiently to effect complete derepression.

It is probably significant that, while most of the other operons controlled by attenuation do not have rare codons as control codons, they do have more than four control codons. The his operon has 7 control codons (28), phe has 7 (29), thr has 12 (30), ilvGEDA has 14 (31, 32), and ilvB has 11 (33). For these cases, a small limitation in endproduct may result in substantial derepression because a short pause at each control codon is summed over 7–14 codons. The trp operon, with two control codons, would appear to be an exception. However, the trp operon is controlled by both attenuation and repression. Moreover, repression is relieved by tryptophan limitation before attenuation is relieved (34) and, therefore, the attenuation mechanism may not provide a sensitive response in this case. Of particular relevance to this discussion is the recent finding of Hsu et al. that control of the ilvGEDA operon of Serratia marcescens involves multiple isoleucine and valine control codons but only a single CUA leucine control codon (35). Thus, the leu operon of S. typhimurium and the ilvGEDA operon of S. marcescens may be unusual in that sensitivity is achieved through the use of a relatively small number of rare control codons.

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