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Role of the ribosome in suppressing transcriptional termination at the *pyrBI* attenuator of *Escherichia coli* K-12

(attenuation control/pyrimidine gene expression/coupled transcription–translation)

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ABSTRACT **Pyrimidine-mediated regulation of *pyrBI* operon expression in *Escherichia coli* K-12 occurs primarily by an attenuation control mechanism. Previous studies have suggested a model for attenuation control in which low intracellular levels of UTP cause close coupling of transcription and translation within the *pyrBI* leader region. This close coupling apparently prevents transcriptional termination at an attenuator (a ρ-independent transcriptional terminator) located 23 base pairs before the *pyrBI* structural genes within an open reading frame for a 44-amino acid leader polypeptide. Presumably, a ribosome involved in the synthesis of the leader polypeptide disrupts or precludes the formation of the attenuator-encoded RNA hairpin, which is required for transcriptional termination. In this study, we examined the role of the ribosome in inhibiting transcriptional termination at the *pyrBI* attenuator. Using oligonucleotide-directed mutagenesis, we systematically introduced termination codons into the reading frame for the leader polypeptide to determine the distance a ribosome must translate to suppress transcriptional termination. These mutations were incorporated individually into a *pyrB*::*lacZ* gene fusion, which was then introduced into the *E. coli* chromosome. The resulting fusion strains were used to measure the effect of each mutation on *pyrB*::*lacZ* expression. The results show that a ribosome must translate to within 14–16 nucleotides of the attenuator-encoded RNA hairpin to inhibit transcriptional termination efficiently, which indicates a direct interaction between the ribosome and the termination hairpin sequence as proposed in the present model. Additional results indicate that factors not included in the present model for attenuation control contribute to the expression and regulation of the *pyrBI* operon.**

The *pyrBI* operon of *Escherichia coli* K-12 encodes the catalytic (*pyrB*) and regulatory (*pyrI*) subunits of the pyrimidine biosynthetic enzyme aspartate transcarbamoylase (ATCase; carbamoyl-phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2). Expression of this operon is negatively regulated over a several hundredfold range by pyrimidine availability. This regulation occurs, at least in large part, by a UTP-sensitive attenuation control mechanism (1, 2). Previous studies have indicated that transcriptional termination at the *pyrBI* attenuator, which is a ρ-independent transcriptional terminator located 23 base pairs (bp) upstream of the *pyrB* structural gene (see Fig. 1), is regulated by the relative rates of transcription and translation within the *pyrBI* leader region (1, 3–5).

According to the present model for attenuation control of *pyrBI* operon expression, low intracellular levels of UTP cause RNA polymerase to stall at UTP-sensitive pause sites (uridine-rich regions in the leader transcript) preceding the attenuator (see Fig. 1). This pausing allows time for a ribosome to initiate translation of a 44-amino acid leader polypeptide and translate up to the stalled RNA polymerase. When RNA polymerase eventually transcribes the attenuator, the adjacent translating ribosome will disrupt or preclude the formation of the attenuator-encoded RNA hairpin, which is necessary for transcriptional termination. The disruption of the termination hairpin permits RNA polymerase to continue transcription into the structural genes. The ribosome then completes the translation of the leader polypeptide open reading frame, which terminates three nucleotides before the *pyrB* cistron. When intracellular levels of UTP are high, RNA polymerase does not pause during the transcription of the leader region. Without this pausing, there is not enough time for a ribosome to initiate translation of the leader transcript and catch up to RNA polymerase before it transcribes the attenuator and terminates transcription before the structural genes.

In this study, we examined the central regulatory role of the ribosome proposed in the model for attenuation control. In particular, we determined the extent of translation within the leader region required to suppress transcriptional termination at the attenuator. The results indicate that the ribosome does in fact inhibit transcriptional termination by physically preventing the formation of the attenuator-encoded hairpin as suggested in the model. The results also indicate that previously uncharacterized factors are involved in controlling the level of *pyrBI* expression.

MATERIALS AND METHODS

Bacterial Strains and Bacteriophage. The bacterial strains used in this study are *E. coli* K-12 derivatives. Strain CLT42 [Δ(*argF-lac*)*U169 rpsL150 thiA1 relA1 deoC1 ptsF25 flbB5301 rbsR car-94*] was constructed in this laboratory (1). Strain N3030 (*gal-76*::Tn*10*) was obtained from the Coli Genetic Stock Center (CGSC 6659). Phage λRZ5 (see Fig. 2) was constructed by Robert Zagursky [E. I. duPont, Wilmington, DE (unpublished)].

DNA Preparations. Plasmid DNA (1), DNA from phages M13 (1) and λ (6), and DNA restriction fragments (1) were prepared as previously described. Oligonucleotides were synthesized using an Applied Biosystems model 380A DNA synthesizer and were purified by gel electrophoresis (1).

Restriction Digests, Ligations, and Transformations. Restriction digests, ligations, and transformations were performed as previously described (1).

DNA Sequence Analysis of Oligonucleotide-Directed Mutations. The presence of each ochre mutation introduced into the *pyrBI* leader region by *in vitro* oligonucleotide-directed mutagenesis was confirmed by DNA sequence analysis (7). Single-stranded DNA used as a sequencing template was prepared from the recombinant M13 phage in which the

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Abbreviations: ATCase, aspartate transcarbamoylase; bp, base pair(s).

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mutation was initially introduced. The sequence of the entire *pyrBI* promoter-regulatory region (i.e., the sequence shown in Fig. 1) was determined in each case to verify that additional changes were not introduced by the mutagenesis procedure.

Transfer of *pyrB*::*lacZ* Gene Fusions from Plasmids to the *E. coli* Chromosome. The wild-type and mutant *pyrB*::*lacZ* gene fusions constructed in this study and carried on plasmids were individually transferred in single copy to the chromosome of strain CLT42 by using the following protocol. For each transfer, a transformant of strain CLT42 carrying a *pyrB*::*lacZ* fusion plasmid was infected with phage λRZ5 (Fig. 2), and the resulting lysate was used to transduce strain CLT42 (6). Ampicillin-resistant (Ap^r), Lac^+ lysogens were isolated by plating onto LB plates containing ampicillin at 10 μg/ml and 5-bromo-4-chloro-3-indolyl β-D-galactoside (8). Phage were prepared from these lysogens (6) and used to reinfect strain CLT42 at a multiplicity of infection of 0.01. The resulting Ap^r Lac^+ lysogens were used to prepare phage DNA, which was subjected to restriction enzyme analysis to confirm the presence of the *pyrB*::*lacZ* fusion. The lysogens (at least six isolates) were then screened for the presence of a single copy of the recombinant λRZ5 by measuring the activity of phage-encoded β-galactosidase and β-lactamase. The lysogens displaying the lowest enzyme levels were presumed to carry a single prophage. In selected cases, the presence of a single prophage was confirmed by Southern hybridization as described below. All single lysogens constructed in this study had the same β-lactamase activity.

Test for Single Lysogens by Southern Hybridization. Chromosomal DNA was isolated (9) from a lysogen carrying a recombinant λRZ5 prophage and digested with *Pvu* I. The digested DNA was prepared for hybridization and probed as described by Maniatis *et al.* (10). The radiolabeled probe (11) was the 1313-bp *Pst* I–*Nde* I fragment of plasmid pMLB1034 (6), which contains the promoter-distal end of the *bla* gene (see Fig. 2). Single lysogens showed only a 2.1-kilobase pair band corresponding to a *Pvu* I fragment that contains phage and chromosomal sequences. Polylysogens had an additional 8.6-kilobase pair band that contains only phage sequences.

Cotransductional Mapping of Recombinant λRZ5 Prophage. Phage P1-mediated cotransductional mapping was used to show the integration of recombinant λRZ5 at the λ attachment site in the *E. coli* chromosome. Phage P1 *vir* was grown on strain N3030 (*gal-76*::Tn*10*), and the resulting lysate was used to transduce a lysogenic derivative of strain CLT42 with selection for tetracycline resistance. Transductants were screened for the loss of λ by cross-streaking against λ *c*I and λ *vir* (6).

Media and Culture Methods. Cells used for enzyme assays were grown in N^-C^- medium (12) supplemented with 10 mM NH_4Cl, 0.4% glucose, 0.015 mM thiamine hydrochloride, 1 mM arginine, and either 1 mM uracil or 0.25 mM UMP. Cultures (25 ml in a 125-ml flask) were grown at 30°C with shaking.

Enzyme Assays and Protein Determinations. Cultures were grown to an A_{650} of 0.5, and 15-ml samples were taken. Cells were collected by centrifugation (4°C), washed with ice-cold 50 mM sodium phosphate (pH 7.0), and stored at −70°C for up to several days without loss of enzymatic activity. Cells were then suspended in 5 ml of 50 mM sodium phosphate (pH 7.0) and disrupted by sonic oscillation at 0°C. Extracts were centrifuged at 17,000 × *g* for 30 min at 4°C to remove cell debris, and the supernatants were used for enzyme and protein assays. ATCase (13), β-galactosidase (8), and β-lactamase (14) activities were measured as previously described, except that β-lactamase assays were done at 30°C. Protein concentrations were measured by the method of Lowry *et al.* (15), using crystalline bovine serum albumin as the standard.

RESULTS

Construction of *pyrB*::*lacZ* Gene Fusion Plasmids Containing Premature Translational Terminational Codons in the *pyrBI* Leader Region. The first step in determining the extent of translation required to suppress transcriptional termination at the *pyrBI* attenuator was to construct a set of *pyrB*::*lacZ* gene fusion plasmids each containing an ochre mutation at a different position in the open reading frame for the *pyrBI* leader polypeptide (Fig. 1). The mutations were constructed by *in vitro* oligonucleotide-directed mutagenesis essentially as previously described (1). Briefly, each mutation was introduced individually into a recombinant M13 phage that contains a segment of the *pyrBI* operon. The mutations, which were confirmed by DNA sequence analysis, were excised from replicative form phage DNA as part of a 692-bp *Alu* I restriction fragment that includes the entire *pyrBI* promoter-regulatory region and the first 38 codons of the *pyrB* structural gene (Fig. 1). Each *Alu* I fragment was then inserted into the unique *Sma* I site of plasmid pMLB1034 (6). Plasmid pMLB1034 contains the *E. coli lacZ* gene lacking a promoter, a ribosome binding site, and the first eight codons for β-galactosidase. Recombinant plasmids were isolated in which the *Alu* I fragment was inserted in an orientation that created an open reading frame joining the *pyrB* codons to the ninth codon of *lacZ*. In all constructions the orientation and size of the insert were checked by restriction mapping.

Transfer of the *pyrB*::*lacZ* Gene Fusions to the *E. coli* Chromosome. Before the effect of each ochre mutation on *pyrB*::*lacZ* expression was measured, the mutant gene fusions were transferred from the recombinant plasmids to the chromosome of *E. coli* strain CLT42 [Δ(*argF-lac*)*U169 car-94*]. The transfers were done because previous results (1) had indicated that pyrimidine-mediated regulation of *pyrB*::*lacZ* (or *pyrBI*) expression is severalfold greater when the gene fusion (or operon) is present as a single, chromosomal copy compared to when it is carried on a multicopy plasmid like pMLB1034. Initially, the gene fusion in each plasmid was transferred to phage λRZ5 by homologous recombination *in vivo* as described in Fig. 2 and *Materials and Methods*. The resulting recombinant phage were used to construct single lysogens of strain CLT42. Single lysogens were identified by Southern blotting and by phage-encoded β-lactamase levels. They were shown to carry the prophage in the λ attachment site by phage P1-mediated cotransductional mapping.

An additional single lysogen of strain CLT42 that carries a wild-type *pyrB*::*lacZ* gene fusion was constructed as described above. This strain was made using a previously constructed plasmid, pBHM19 (1), in which the 692-bp *Alu* I fragment of the wild-type *pyrBI* promoter-regulatory region has been inserted into plasmid pMLB1034.

Effect of Premature Translational Termination in the *pyrBI* Leader Region on *pyrB*::*lacZ* Expression. The effect of the ochre mutations on *pyrB*::*lacZ* expression was determined by measuring the levels of ATCase catalytic subunit/β-galactosidase fusion protein synthesis in the single lysogens (Table 1). The strains, which are pyrimidine auxotrophs because the *car-94* mutation inactivates a pyrimidine biosynthetic enzyme, were grown in a glucose-minimal salts medium containing either uracil or UMP as the pyrimidine source. Growth on uracil causes repressed *pyr* gene expression, whereas growth on UMP, which is only slowly utilized by the cells, results in derepressed expression. In the strains carrying an ochre mutation at codon 6, 14, 23, or 24, *pyrB*::*lacZ* expression was strongly inhibited. Fusion protein synthesis was reduced to 13–22% of the wild-type level under repressing conditions and 4–6% of the wild-type level under derepressing conditions (Fig. 3). The level of pyrimidine-mediated regulation was also reduced severalfold. In contrast, in the strains carrying an ochre mutation at codon 25,

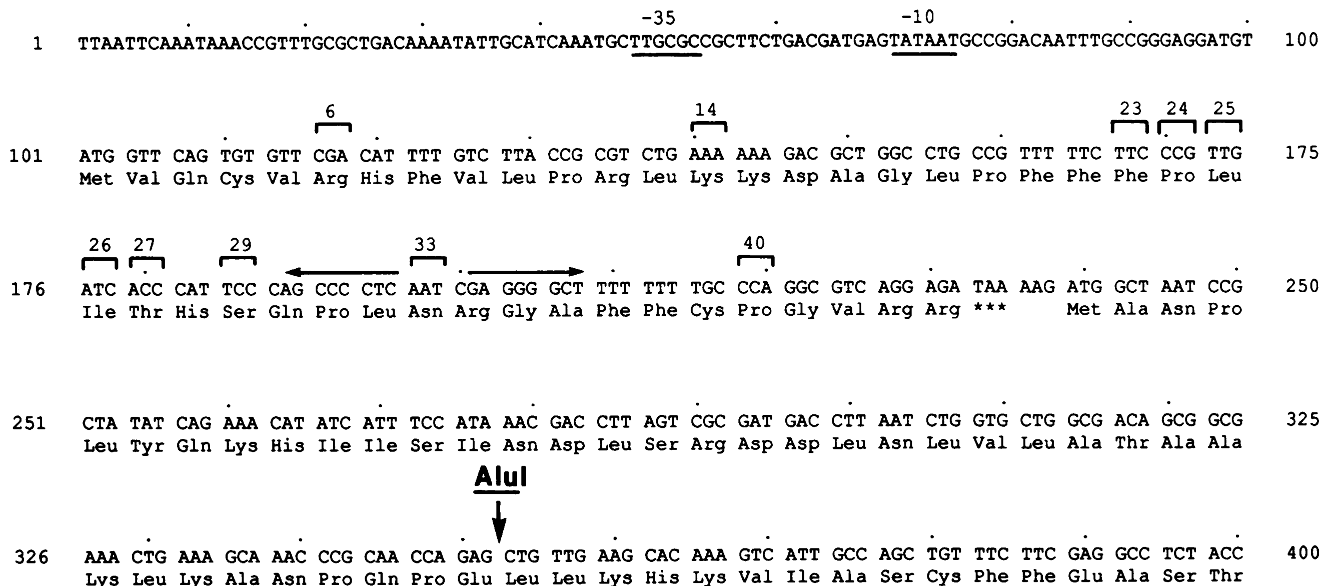

FIG. 1. Nucleotide sequence and encoded polypeptides of the *pyrBI* promoter-regulatory region. Only the antisense strand is shown; numbering is from the 5′ end. The −10 and −35 regions of the *pyrBI* promoter, previously designated promoter P_2 (3), are underlined and labeled. Approximately 99% of *pyrBI* transcripts are initiated at this promoter *in vivo* (J. Donahue and C.L.T., unpublished data). The leader polypeptide is encoded by bp 101 through bp 232, and the *pyrB* structural gene begins at bp 239. Codons where UAA (ochre) substitutions were made are numbered and indicated by brackets. The hyphenated dyad symmetry of the *pyrBI* attenuator (bp 189 through bp 215) is indicated by horizontal arrows. The *Alu* I site shown indicates the position at which the *pyrB* gene was joined to *lacZ* in the construction of the gene fusions described in the text.

26, 27, 29, or 33, the levels of *pyrB*::*lacZ* expression were much closer to that in the wild-type fusion strain. Under repressing conditions, fusion protein synthesis steadily increased from 31% to 123% of the wild-type level as the ochre mutation was moved downstream from codon 25 through codon 33. Under derepressing conditions, fusion protein synthesis was 50–91% of the wild-type level (Fig. 3). In four of these five strains, pyrimidine-mediated regulation was similar to that in the wild-type strain. In one of these strains, CLT5007, the fold derepression of fusion protein synthesis was twice as high as the wild-type level. These results define a sharp boundary between codon 24 and codon 25 through which a translating ribosome must pass to permit high levels of *pyrB*::*lacZ* expression and near wild-type levels of regulation.

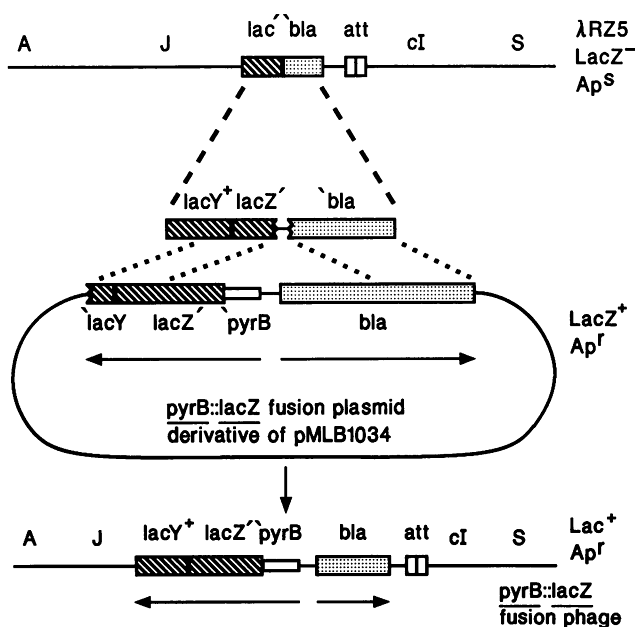

FIG. 2. Transfer of *pyrB*::*lacZ* gene fusions from plasmids to λRZ5: recombination within the homologous regions of the *lac* and *bla* genes carried by the *pyrB*::*lacZ* fusion plasmid and λRZ5 results in the transfer of the gene fusion, including mutations in the *pyrBI* leader region, to the phage. The arrows indicate direction of transcription. The phenotypes of strains carrying the *pyrB*::*lacZ* fusion plasmid or the indicated phage are shown at the right.

The level of fusion protein synthesis was also measured in strain CLT5012, which carries an ochre mutation at codon 40 (Table 1). In this strain, translation can proceed through the entire attenuator-encoded region of the leader transcript and terminates only five codons before the wild-type termination codon. Unexpectedly, fusion protein synthesis was only half of the wild-type level under repressing conditions and just 29% of the wild-type level under derepressing conditions. A

Table 1. Effect of premature termination of *pyrBI* leader polypeptide synthesis on *pyrB*::*lacZ* fusion protein synthesis

Strain	Location of ochre codon*	β-Galactosidase activity, $nmol \cdot min^{-1} \cdot (mg\ of\ protein)^{-1}$		
		Repressed†	Derepressed‡	Fold derepression
CLT5000§	45	164	27,700	169
CLT5002	6	21.7	1,670	77
CLT5003	14	32.6	1,320	40
CLT5005	23	36.6	1,190	33
CLT5006	24	20.9	1,550	74
CLT5007	25	51.1	17,800	348
CLT5008	26	71.7	14,900	208
CLT5009	27	95.6	13,800	144
CLT5010	29	104	21,000	202
CLT5011	33	201	25,100	125
CLT5012	40	89.2	8,120	91
CLT5004	6+14	19.6	1,630	83

Doubling times for all strains were 65 ± 1 and 115 ± 1 min on uracil and UMP, respectively.
*Number of the codon in the open reading frame for the *pyrBI* leader polypeptide at which UAA has been substituted.
†Cells grown on uracil; mean of two experiments.
‡Cells grown on UMP; mean of two experiments.
§Strain CLT5000 carries the wild-type *pyrB*::*lacZ* fusion.

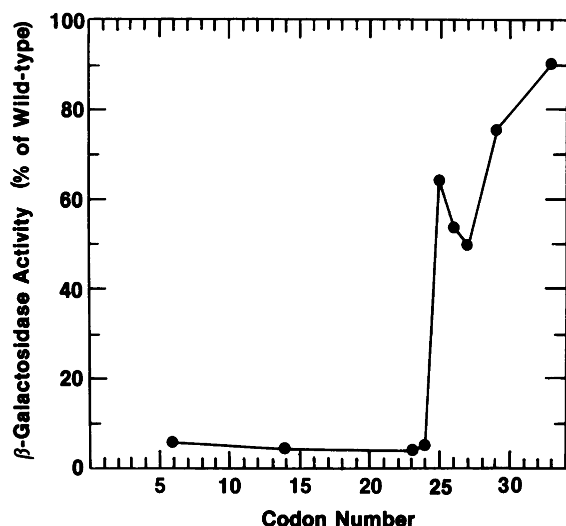

FIG. 3. Effect of premature termination of *pyrBI* leader polypeptide synthesis on *pyrB::lacZ* fusion protein synthesis. The levels of β-galactosidase activity in the strains carrying an ochre mutation at the indicated codon in the open reading frame for the 44-amino acid *pyrBI* leader polypeptide are plotted as a percentage of the activity in the wild-type strain. Enzyme activities are for cells grown on UMP, the pyrimidine-limiting (derepressing) growth condition.

possible reason for this large reduction in *pyrB::lacZ* expression is discussed below.

In the strains carrying an ochre mutation at codon 6, 14, 23, or 24, the level of pyrimidine-mediated regulation of *pyrB::lacZ* expression (i.e., the fold derepression) was reduced to a much lesser extent than the level of fusion protein synthesis (Table 1). This result suggests that some read-through translation may be occurring at the ochre codons. To test this possibility, we constructed, exactly as described above, a lysogen that carries an ochre mutation at codon 6 and at codon 14. Fusion protein synthesis in this double mutation strain was measured, and it was found to be essentially identical to that in the lysogen that carries a single ochre mutation at codon 6 (Table 1). This result indicates that in-frame read-through translation does not contribute to the observed residual regulation.

In addition to fusion protein levels, the levels of ATCase encoded by the intact chromosomal *pyrBI* operon of each lysogen were measured in the experiments summarized in Table 1. The ATCase levels provide an internal control for pyrimidine availability and the intracellular signals controlling *pyrBI* expression. For every strain, the ATCase activity was the same within experimental error for cells grown under repressing [17.0 ± 1.2 nmol·min^{-1}·(mg of protein)$^{-1}$] or derepressing [5400 ± 110 nmol·min^{-1}·(mg of protein)$^{-1}$] conditions. The fold derepression for ATCase synthesis was 320 ± 18, which is twice that for fusion protein synthesis in the wild-type lysogen. The reason for this difference is not clear. It may be due to the different chromosomal locations or genetic environments of the *pyrBI* operon and the *pyrB::lacZ* gene fusion, but presumably it does not reflect a fundamental difference in the mechanisms of expression and regulation.

DISCUSSION

The experiments described here were designed to examine the role of the ribosome in inhibiting transcriptional termination at the *pyrBI* attenuator. The results define a sharp boundary between codon 24 and codon 25 of the open reading frame for the *pyrBI* leader polypeptide beyond which a ribosome must translate to permit near wild-type levels of *pyrB::lacZ* (equivalent to *pyrBI*) expression. Premature translational termination at or before codon 24 reduces expression to approximately 5% of the wild-type level under derepressing conditions. The boundary between codon 24 and codon 25 also marks the position beyond which a ribosome must translate to interact directly with the attenuator-encoded termination hairpin in the leader transcript. Previous studies have shown that a ribosome in a 70S initiation complex will protect up to 15 nucleotides downstream of the initiation codon from ribonuclease digestion (16, 17). Assuming the maximum length of protected sequence defines the size of the ribosome binding site, translational termination at codon 24 would not permit a ribosome to bind the attenuator-encoded sequence, which is 16 nucleotides downstream. Translational termination at codon 25, however, would allow contact between the ribosome and the two nucleotides at the 5′ end of the attenuator-encoded sequence. This interaction should have a large destabilizing effect on the termination hairpin (18) and result in a high level of read-through transcription (19). These data provide strong evidence that a ribosome involved in synthesizing the 44-amino acid leader polypeptide prevents transcriptional termination at the attenuator by physically blocking the formation of the attenuator-encoded RNA hairpin as proposed in the current model for attenuation control of *pyrBI* expression.

The general effect of moving the site of translational termination downstream in the leader transcript from codon 25 through codon 33 is to increase *pyrB::lacZ* expression. Apparently, the further downstream the ribosome is permitted to translate, the more effective it is at disrupting the attenuator-encoded hairpin. In the strain carrying an ochre mutation at codon 33, which is in the middle of the attenuator-encoded sequence (Fig. 1), expression was actually slightly higher than that in the wild-type strain under repressing conditions and only 9% less than the wild-type level under derepressing conditions. Based on the data shown in Fig. 3, however, there clearly are other factors in addition to the position of the translating ribosome that affect the level of *pyrB::lacZ* expression in the mutant strains. There is a small but reproducible decrease in the level of fusion protein synthesis under derepressing conditions when the ochre mutation is moved from codon 25 to either codon 26 or codon 27. This result suggests that in addition to causing premature translational termination, the introduction of an ochre mutation can change some other property of the leader transcript (e.g., transcript stability or potential to form secondary structures) that influences the level of *pyrB::lacZ* expression. Based on recent studies (K. Mayne, J. Donahue, and C.L.T., unpublished data), it is unlikely that any of the ochre mutations would significantly affect UTP-sensitive transcriptional pausing.

The most unexpected result in this study was the low level of *pyrB::lacZ* expression in strain CLT5012, which carries an ochre mutation at codon 40. The presence of an ochre mutation at this position in the leader transcript would allow a ribosome to translate through the entire attenuator-encoded sequence exactly as occurs in the wild-type strain. However, fusion protein synthesis in strain CLT5012 was reduced to 29% of the wild-type level under derepressing conditions, which is much less than that in any other strain with an ochre mutation downstream of codon 24. This large reduction in the level of expression indicates that the mutation at codon 40 interferes with an important step in the expression of the read-through transcript. One possible explanation for this effect is that the mutation results in a large reduction in the half-life of the transcript. The ochre mutation at codon 40 changes two bases within a region of the leader transcript that is complementary to the ribosome binding site for the leader polypeptide. It has been suggested that these two comple-

mentary sequences form a secondary structure in the *pyrBI* mRNA that prevents multiple rounds of leader polypeptide synthesis (20). The formation of this secondary structure may also protect the mRNA from nucleolytic degradation (21). The destabilization of this secondary structure by the mutational change at codon 40 could therefore make the *pyrB*::*lacZ* transcript more susceptible to degradation, which would decrease the level of fusion protein synthesis. Clearly more experiments are needed to test this hypothesis as well as other possible explanations for the low level of expression in strain CLT5012.

The substantial residual regulation of *pyrB*::*lacZ* expression observed in the strains carrying an ochre mutation early in the leader region (e.g., at codon 6) (Table 1) indicates that some pyrimidine-mediated control can occur in the absence of translation of the open reading frame encoding the 44-amino acid leader polypeptide. The residual regulation does not appear to be due to in-frame read-through translation past the ochre mutations. Recent experiments have shown that part of the residual regulation (approximately 6-fold) is due to an as yet uncharacterized, attenuator-independent control mechanism (C.L. and C.L.T., unpublished data). The remainder of the residual regulation, however, appears to be attenuator-dependent. A possible mechanism for this regulation is translation of the leader region in a different reading frame than that of the 44-amino acid leader polypeptide. The leader region does in fact contain a second long open reading frame (bp 97 through bp 243 in Fig. 1) that could encode a 49-amino acid polypeptide. This second open reading frame includes the entire open reading frame for the 44-amino acid leader polypeptide. Preliminary experiments indicate that this second open reading frame can be translated *in vivo* at approximately 2% of the level of the open reading frame for the 44-amino acid leader polypeptide (K.L.R. and C.L.T., unpublished data). It seems reasonable, therefore, to propose that translation of this second open reading frame could account for at least some of the residual attenuator-dependent regulation.

In addition to elucidating the mechanism controlling *pyrBI* expression in *E. coli*, the results discussed above provide important information about the proposed control mechanisms for the *E. coli pyrE* gene (22) and the *pyrBI* operon of *Salmonella typhimurium* (23). In both cases, regulation of gene expression apparently occurs through UTP-sensitive attenuation control mechanisms that are essentially the same as that described for the *E. coli pyrBI* operon. The major difference in the three mechanisms is the site of translational termination within the leader region. Translation is terminated five nucleotides upstream of the attenuator-encoded sequence in the case of the *pyrE* gene and within the attenuator-encoded sequence of the *S. typhimurium pyrBI* operon (at a position equivalent to codon 34 in Fig. 1). Based on the data presented here, the sites of translational termination in all three leader regions are essentially equivalent in that they all would allow a translating ribosome to disrupt the signal for transcriptional termination at the respective attenuators.

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