Inhibition of Translation by Consecutive Rare Leucine Codons in *E. coli*: Absence of Effect of Varying mRNA Stability

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Consecutive homologous codons that are rarely used in *E. coli* are known to inhibit translation to varying degrees. As few as two consecutive rare arginine codons exhibit a profound inhibition of translation when they are located in the 5' portion of a gene in *E. coli*. We have previously shown that nine consecutive rare CUA leucine codons cause almost complete inhibition of translation when they are placed after the 13th codon of a test message (although they do not inhibit translation when they are placed in the middle of the message). In the present work, we report that five consecutive rare CUA leucine codons exhibit approximately a threefold inhibition of translation when they are similarly placed after the 13th codon of a test message, compared to five consecutive common CUG leucine codons, in a T7 RNA polymerase-driven system. Further, by removing RNase III processing sites at the 3' ends of the mRNAs, we have manipulated the stability of the mRNAs encoding the test and control messages to see if decreasing mRNA stability might have an effect on the extent of translation inhibition by the rare leucine codons. However, the inhibition with the less stable mRNAs was similar to that with the stable mRNAs, approximately 3.4-fold, indicating that mRNA stability per se does not have a major influence on the effects of rare codons in this system.

Key words: CUA leucine codon; Codon bias; Inhibition by rare codons; T7 expression systems; RNase III and mRNA stability

INTRODUCTION

Expression of heterologous recombinant genes in *E. coli* sometimes proves to be difficult, if not impossible (15,19,27). A number of strategies for overcoming this problem have been explored ([reviewed in (20, 21,36,44)]. In many of the cases where this occurs, the difficulty can be overcome by resynthesizing the gene using the preferred synonymous codons of *E. coli* (26,32,33,40,48,50). Another approach that is often successful is to introduce a plasmid in the *E. coli*

cells that overexpresses one or more tRNA genes corresponding to the rare (in *E. coli*) codons present in the heterologous sequence to be expressed (1–3,8,9, 24,28,29,31,37,49). This approach has become commercially available in recent years (e.g., from Strata-gene and Novagen).

This laboratory has previously studied the inhibition of translation in *E. coli* by consecutive rare AGG arginine (43) and CUA leucine (17) codons in a T7 system where translation is, in effect, uncoupled from transcription (18), because the T7 RNA polymerase

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is highly processive (25) and the mRNA produced is highly stable due to T7-derived RNase III processing sites at the 3' ends of the mRNAs (39). While we varied the number of AGG codons, and found inhibitory effects with as few as two, we only published a test of a string of nine CUAs and observed almost complete inhibition when these codons were located following the 13th codon of the message. For reasons still not understood, these codons did not inhibit expression when they were placed after the 223rd codon of the 313 codon mRNA; hence, the inhibition after the 13th codon has been described as "5'-translational blockage" (17).

Modeling of protein synthesis suggested the possibility that the extent of translational inhibition by impediments such as rare codons might be influenced by the stability of the mRNA, such that less stable messages might show greater inhibitory effects of the rare codons compared to more stable messages (16). The logic behind this proposition was that less stable messages will not reach a "steady-state" condition (in which the rate-limiting step, whether initiation or translation through a string of rare codons, governs the yield), and therefore the effect of translational impediments would be exacerbated.

In the present study, we report that a string of five consecutive rare CUA leucine codons (placed near the 5' end of the mRNA) has about a threefold inhibitory effect on translation compared to five consecutive common CUG leucine codons. We tested the effects of these strings of codons in both the original T7 system and in a modification of the system such that the mRNA was destabilized. However, no major difference was observed in the extent of translation inhibition between the stable and unstable mRNAs.

MATERIALS AND METHODS

Plasmid Vectors

Vectors pCT1 and pCT1.1, diagramed in Figure 1, have been previously described (23,43) and their features are discussed in the Results section; pCT1.3 (6), which is discussed but not used in this work, is the same as pCT1.4 in Figure 1 with the exception that the 2.4 kb spacer in pCT1.3 was replaced with a different spacer, 2.0 kb, in pCT1.4 (see below).

Construction of pCT1.4, pCT1.4(CUG)₅, and pCT1.4(CUA)₅

Vector pCT1.4 was designed to substitute the 2.4 kb spacer fragment separating the test and control genes in pCT1.3 with another fragment, 2.0 kb in length, from the same source plasmid, pJC27 (7). The

main reason for doing this was because the 2.4 kb spacer contained an *EcoRI* site, which interferes with use of the cloning site in the pCT vectors. An additional reason is that the 2.4 kb spacer contains an origin of replication that is expected to make the plasmid incompatible with pLysS (included to reduce basal expression). The 2.0 kb fragment was obtained by amplifying another part of pJC27, and introducing a new *BglIII* site at the 5' end of the 2.0 kb fragment to facilitate its insertion into the pCT1.1 vector.

The portion of the pJC27 plasmid that was amplified is comprised almost entirely from the coding sequence of the *lacZ* gene in the vector, starting at nucleotide (nt) 1465 and ending at nt 3472, which is the cutting point (a/gatct) of one of the *BglIII* sites in pJC27. Nt 1465 in pJC27 corresponds to nt 1129 in the sequence coding for β -galactosidase (not counting the start codon), and is located at the 377th codon. The end point, at nt 3472, is 58 nt downstream of the last of the three TAA stop codons at the end of the *lacZ* gene. Because there are no known independent translation start signals in the open reading frame of the 2.0 kb fragment, it is not expected to be translated, although even if it were, this should not affect the comparison of test and control protein expression.

Two primers, P1 and P2, were designed to amplify the 2.0 kb fragment from pJC27, and *BglIII* sites in these primers allowed insertion of the fragment into the middle *BglIII* site of pCT1.1. Following *BglIII* cutting and insertion of the fragment, primer P1 added 4 nt (for the restriction site) at the 5' end of the 2.0 kb fragment. The sequences of P1 and P2 (*BglIII* sites underlined) are: P1, 5'-GGAAGATCTGCTGATGA AGCAGAACAACTTTAACG-3'; P2, 5'-CGGCGTAGAGCAGATCTGAAATAGTAC-3'.

The 2.0 kb fragment amplified from pJC27 was digested with *BglIII*. pCT1.1 was partially digested with *BglIII*, and a 6 kb fragment was recovered from an agarose gel, treated with phosphatase, and then ligated with the *BglIII*-digested 2.0 kb fragment. Colony PCR (polymerase chain reaction) with primers P3 and P4 was used to screen transformants to obtain pCT1.4. This screen worked because when the insertion is in the left or right *BglIII* site, PCR amplifies 438 nt, but when the insertion is in the middle *BglIII* site, PCR amplifies 2449 nt. The approximate locations in pCT1.4 corresponding to these primers are indicated by the arrows above the pCT1.4 schematic diagram in Figure 1. The sequences of P3 and P4 are: P3, 5'-CGTAAACAGTGTTCATTGA-3'; P4, 5'-GGGAAACCGTTGTGGTCTC-3'.

Two constructions with five CTGs and five CTAs in frame at the *BamHI-EcoRI* (B-E) cloning site of pCT1.4 are called pCT1.4(CUG)₅ and pCT1.4(CUA)₅.

Figure 1. Arrangement of elements in the pCT1 series of vectors used in this work. $\phi 10$ represents a promoter for T7 RNA polymerase, *s10* is a strong translational start site, and CJ is a 17-bp sequence from the T7 concatemer junction. The test and control genes are derived from T7 gene 9, with a 45-codon in-frame deletion from the test gene to derive the control gene. A *Bam*HI-*Eco*RI site in the test gene allows insertion of nucleotides after codon 13. R represents an RNase III cleavage site, and T ϕ is a transcriptional terminator for T7 RNA polymerase. *bla* represents the plasmid β -lactamase gene, and *ori* represents the plasmid's ColE1 origin of replication. The 2 kb fragment is derived from plasmid pJC27, as described in Materials and Methods. The arrows labeled P3 and P4 above pCT1.4 represent the primers used to identify the construct. Restriction sites: Bg, *Bgl*III; X, *Xba*I; Nd, *Nde*I; N, *Nhe*I; N/X, *Nhe*I-*Xba*I fusion; B-E, *Bam*HI-*Eco*RI cloning site.

Two pairs of complementary oligonucleotides (oligo1/oligo2 and oligo3/oligo4) were synthesized, annealed, and inserted into pCT1.4 pre-cut with *Bam*HI and *Eco*RI. Colony PCR with primers oligo1 and P4 or oligo3 and P4 were used to screen transformants for the presence of insertions. The oligo sequences are: oligo1, 5'-GATCCGCTACTACTACTAG-3'; oligo2, 5'-AATTCTAGTAGTAGTAGTACG-3'; oligo3, 5'-GATCCGCTGCTGCTGCTGCTGG-3'; oligo4, 5'-AATTCAGCAGCAGCAGCAGCG-3'. The final vectors were placed in *E. coli* strain DH5 α for maintenance, and in strain BL27(DE3)pLysS for expression experiments. The junction sequences of plasmids pCT1.4, pCT1.4(CUG)₅, and pCT1.4(CUA)₅ were sequenced from the maintenance strain by the New Jersey Medical School Molecular Resource Facility.

Protein Expression Experiments

The host strain for expression experiments, BL27(DE3)pLysS (43), contains a chromosomally

integrated gene for T7 RNA polymerase controlled by the *lacUV5* promoter. [BL27(DE3) is a *recA* derivative of BL21(DE3).] All cells were grown in minimal medium containing 25 μ g/ml chloramphenicol (to maintain pLysS); media for cells containing any of the codon test vectors also included 50 μ g/ml ampicillin.

Cultures were grown shaking at 37°C to mid-log phase. Portions (50 μ l) of each culture were labeled with [³⁵S]methionine (Amersham) at 0 time (uninduced samples). The remainder of the cultures had IPTG added, and were similarly labeled with [³⁵S]methionine at the indicated times after induction.

Incorporation was stopped by addition of 25 μ l of 3 $\times$ MOPS cracking buffer, vortexing, and placing the samples on ice. The 3 $\times$ MOPS cracking buffer contains 60 mM MOPS, 24 mM sodium acetate, 3% SDS, 6 mM EDTA, 30% glycerol, and 0.15% each of bromophenol blue and xylene cyanole FF; it is stored at room temperature and 3% (v/v) β -mercaptoethanol is added before use. Samples were placed in

a boiling water bath for 2 min in capped Eppendorf tubes, briefly spun to minimize volume loss, stored at -20°C , and boiled again for 1 min in capped tubes before loading an aliquot on 10% sodium-dodecyl-sulfate polyacrylamide gels for electrophoresis (SDS-PAGE). Gels were dried and bonded to Whatmann 3MM paper, subjected to autoradiography on X-ray film, and quantitated by phosphorimaging or densitometry, as indicated.

RESULTS

Vector System to Test Effects of Rare Codons

The pCT (codon test) series of vectors was originally designed to permit a standardized and controlled vehicle for assessing the extent of inhibition, if any, of rare codons on translation. To this end, the system utilized transcription by T7 RNA polymerase and mRNA 3' end processing by RNase III, essentially uncoupling translation from transcription by creating stable mRNAs whose synthesis is not subject to polarity.

The original system (43), diagramed in Figure 1, consisted of a plasmid vector containing a "test" and "control" gene (T7 gene 9) in tandem on one transcription unit, controlled by a T7 RNA polymerase promoter ($\phi 10$) and terminator (T ϕ). Both genes were fused to common strong translation start signals (S10), and both were flanked (and separated) by single-cut RNase III processing sites (R). The control gene has actually the same sequence as the test gene, except for a 45-codon (in-frame) deletion to permit distinguishing by size both test and control protein and mRNA in the same cell. In the complete system as originally constructed, the test gene had inserted three alternate cloning sites, either 13 codons from the ATG start in vector pCT1 (5' end), 223 codons downstream from the start in vector pCT2 (internal), or 307 codons downstream, which is six codons from the TAA stop in vector pCT3 (3' end). Because of results showing that the most translational inhibition by rare codons occurs when they are inserted near the 5' end of a message (5,17,38,43), we have not depicted in Figure 1 vectors pCT2 and pCT3.

The test and control genes contain a typical high-usage codon composition characteristic of highly expressed genes in *E. coli*. The plasmid is ordinarily inserted in a strain harboring a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter (46); hence, induction with IPTG turns on synthesis of the T7 RNA polymerase, which in turn begins transcription of the test and control genes on the vector. Basal transcription in the absence of induction is usually restricted by addition

of a compatible plasmid constitutively expressing T7 lysozyme, an inhibitor of T7 RNA polymerase (45). Insertion of low- or synonymous high-usage codons into the test gene cloning site permits a direct and controlled comparison of translation rates. Expression of test protein is always compared to expression of control protein (an internal standard), minimizing effects of experimental variation from sample to sample.

Because of our interest in whether differences in mRNA stability could alter the effects of rare codons on expression, modification of pCT1 was previously undertaken: a 17-bp element from the T7 concatemer junction sequence (CJ), reported to be a weak transcription terminator for T7 RNA polymerase, was introduced at the 3' ends of the test and control genes, replacing pCT1's RNase III processing sites as well as the T7 terminator, and a new T7 promoter was also introduced to drive the downstream (control) gene (23). While these modifications did indeed destabilize the mRNA, this vector, pCT1.1 (Fig. 1), unexpectedly showed inhibition of the downstream control gene expression (23). This inhibition was reversed by introduction of a 2.4 kb spacer between the test and control genes, in vector pCT1.3 (6). However, the 2.4 kb spacer happened to also contain an additional *EcoRI* restriction site, complicating its ease of use for testing insertions at the cloning site, which also depends on an *EcoRI* restriction site.

In the present work, the 2.4 kb spacer of pCT1.3 was replaced by a 2 kb spacer from the same source plasmid, to create pCT1.4 (Fig. 1). Because this vector no longer had the extra *EcoRI* restriction site, it was now easy to use for testing insertion of rare codons.

Inhibition of Translation by Rare Leucine Codons

Five rare CTA leucine codons, or common CTG leucine codons, were inserted into the cloning site of the test gene in pCT1, creating vectors pCT1(CUA)₅ and pCT1(CUG)₅, respectively. These vectors were tested in Gao's Ph.D. thesis (13), and the results are shown in Figure 2. Compared to pCT1(CUG)₅ [5'-(CUG)₅ in Fig. 2], the expression of test protein (normalized to control protein) in pCT1(CUA)₅ [5'-(CUA)₅ in Fig. 2] was three- to fourfold less at all time points; as expected, in cases of middle insertions in pCT2 [m-(CUA)₅/m-(CUG)₅], and 3' insertions in pCT3 [3'-(CUA)₅/3'-(CUG)₅], test/control ratios were essentially 1:1, indicating that there was no inhibition by the CUAs at these downstream positions. The quantitations were rough estimates from densitometry of autoradiograms (see legend to Fig. 2).

In further experiments, pCT1(CUA)₅ and pCT1

Figure 2. Effect of five consecutive CUA codons on translation of test protein. Plasmids with 5', middle (m-), and 3' insertions are indicated; numbers above the wells are time in minutes after induction. Cultures were grown in MOPS medium [described in (14)] with antibiotics (see Materials and Methods) to $\sim 3 \times 10^8$ cells/ml at 37°C. Cultures were induced by adding IPTG to 0.4 mM. At the indicated times after induction, portions of the cultures were removed to fresh Eppendorf tubes containing [35 S]methionine (50 Ci/mmol, 20 μ Ci/ml) (Amersham). Incorporation was stopped 3 min later by mixing the samples with 1/3 volume of 3 $\times$ MOPS cracking buffer (see Materials and Methods). Uninduced cultures (0 time) were labeled in parallel. Following SDS-PAGE, autoradiograms were subjected to densitometry, which yielded the following ratios of normalized (i.e., test/control) expression of (CUG) $_5$ to (CUA) $_5$. Insertions at the 5' end: 2.72–4.44; insertions at the middle: 0.95–0.96; insertions at the 3' end: 0.97–1.01.

(CUG) $_5$ were retested and again found to show approximately threefold inhibition by the CUAs, this time with quantitation on a phosphorimager. *E. coli* strain BL27(DE3)pLysS containing either no codon test plasmid, pCT1 alone, or pCT1 producing mRNAs with five CUAs or five CUGs were grown to mid-log phase and induced with IPTG at 0 time. Because the T7 RNA polymerase is insensitive to the *E. coli* RNA polymerase inhibitor rifampicin, portions of the cultures were treated with 100 μ g/ml rifampicin at 15 min, to visualize expression from the T7 RNA polymerase-derived mRNAs in the absence of host background synthesis. (Note, however, that because the T7 RNA polymerase in this system is itself synthesized from an *E. coli* RNA polymerase promoter, further T7 RNA polymerase production is also inhibited when rifampicin is added.) At 30 min postinduction, all samples were pulse-labeled with [35 S]methionine for 10 min, subjected to SDS-PAGE, and visualized in a phosphorimager.

The results of this experiment are shown in the right panel of Figure 3. The migrations of the test and control genes are indicated by arrows. As previously observed and described for this system, for reasons apparently connected to the molecular architecture of the T7 gene 9 protein, any insertion encoding more than two residues after the 13th codon of the test protein slows the mobility of the protein in the gel, even under the denaturing conditions of SDS-PAGE, compared to that of the vector without insertions (17, 18,43).

Background host synthesis is shown in lane 10 of

Figure 3, which looks quite similar to uninduced synthesis in the pCT1 vector shown in lane 1, with the exception that one can see basal expression of the control gene with the vector present. Following 30-min induction of the vector, a substantial amount of both test and control protein was made (lane 2), and background host synthesis was virtually eliminated in the presence of rifampicin (lane 3), leaving only the test and control proteins visible.

When the string of five CUGs (common leucine codon) or five CUAs (rare leucine codon) was inserted into the vector's mRNA, the uninduced samples (lanes 4 and 7, respectively) also looked quite similar to the uninduced vector sample (lane 1). Following 30-min induction, synthesis of both test and control proteins was evident, but there was visibly more relative test protein with the CUGs (lane 5) versus CUAs (lane 8). Because of the slower mobility with the insertions after residue 13, the test bands in these samples migrated close to the position of a background band seen in the "no plasmid" (lane 10) and uninduced (lanes 1, 4, and 7) samples. However, when rifampicin was added, host background synthesis was eliminated, and the identification of these bands as test protein was evident (lanes 6 and 9, respectively), again showing somewhat greater synthesis of test protein with the CUGs versus CUAs.

Further, a characteristic of T7 systems such as the one used here is that background host synthesis is also inhibited simply by induction of the T7 promoters, as all the cell's resources are diverted to T7 RNA polymerase-driven expression due to the processive

Figure 3. Expression of test and control proteins from pCT1 and pCT1.4 with insertion of five consecutive CUA or CUG leucine codons. Ten-milliliter cultures were grown in M9 minimal medium shaking at 37°C to 2×10^8 cells/ml (50 klett units or $A_{600} = 0.4$). Portions (50 μ l) of each culture were labeled with [35 S]methionine (Amersham) (20 μ Ci/ml, 1 Ci/mmol) for 10 min at 0 time (uninduced samples). The remainder of the cultures had IPTG added to 1 mM, and were similarly labeled with [35 S]methionine at 30 min after induction. For those samples also treated with rifampicin, at 15 min, 5 ml from the culture flask was transferred to another flask containing 50 μ l of 10 mg/ml rifampicin in DMSO (final concentration 100 μ g/ml). Incorporation was stopped at 10 min by the addition of 25 μ l of 3 $\times$ MOPS cracking buffer, and 20 μ l of the samples was subjected to SDS-PAGE as described in Materials and Methods. The dried gels were subjected to phosphorimaging on a Molecular Dynamics Typhoon 9410 Phosphorimager. Lanes 1–9 were samples based on vector pCT1, and lanes 11–19 were based on pCT1.4. Lanes 10 and 20 did not contain any codon test vector. Lanes 1–3 and 11–13 contained the respective vectors alone, without insertion of any leucine codons at the cloning site. Lanes 4–6 and 14–16 contained the respective vectors with insertion of five consecutive CUG leucine codons at the cloning site, while lanes 7–9 and 17–19 had insertion of five consecutive CUA leucine codons. Rifampicin (rifamp) was added to lanes 3, 6, 9, 13, 16, 19, and 20. Minutes indicated reflect either uninduced samples at 0 time (lanes 1, 4, 7, 11, 14, 17) or all the other samples that were induced (i.e., exposed to 1 mM IPTG) for 30 min. Migrations of test and control proteins are indicated by arrows, but as explained in the text, the mobility of the test protein shifts to a slightly slower migration when additional residues are inserted at the cloning site.

and high rate of transcription and the stable mRNAs produced. This can be seen in Figure 3 as well, by comparing overall synthesis of host bands in the induced samples (lanes 2, 5, and 8) versus the uninduced samples (lanes 1, 4, and 7). Quantitation of this effect shows that there was ~50% reduction in background level of synthesis after 30 min of induction (see legend to Table 1).

Quantitation of the test and control bands was performed on a phosphorimager. Rectangles of the same size encompassing the test and control bands in each sample were drawn and the amount of radioactivity (“volume”) was reported by the phosphorimager’s Image Quant software. Results of this analysis are shown in Table 1. To obtain values specific for synthesis of test and control proteins, the volumes reported for the uninduced (0 time) samples were subtracted from the values for the induced samples. Because of nonspecific general reduction of host background when pCT1 vectors are induced, the subtracted values for these samples were normalized for

the overall nonspecific inhibition, as described in the legend to Table 1.

Lines 3–6 of Table 1 show the uninduced and induced values observed with pCT1(CUA)₅ and pCT1(CUG)₅, respectively. The ratio of synthesis of test to control proteins for the insertion of five CUAs was 0.6 (line 4) whereas that for the five CUGs was 1.8 (line 6), indicating that the inhibitory effect of the rare codons was about threefold. Lines 1 and 2 show that the ratio of synthesis of test to control protein for the vector without inserted codons was about 1.5.

In the presence of rifampicin, the ratio of test to control proteins for the five CUAs was 0.5 (Table 1, line 15) while for the five CUGs it was 1.0 (line 16). [The vector alone in the presence of rifampicin gave a ratio of 1.2 (line 14).] Therefore, the overall inhibitory effect in the presence of rifampicin was twofold (rather than the threefold seen without rifampicin). However, it should be noted that the absence of competing host synthesis due to rifampicin will also remove any competing demands for the rare cognate

TABLE 1
EFFECT OF FIVE CUAs ON EXPRESSION OF TEST PROTEIN

Line	Plasmid	Time (min)	Volume Reported*		Uninduced Values† Subtracted			Fold Effect
			Test	Control	Test	Control	Ratio	
1	pCT1	0	-2677	1484				
2		30	13243	10503	14582	9761	1.5	
3	pCT1(CUA) ₅	0	3218	2272				
4		30	4689	6681	3080	5545	0.6	3.0
5	pCT1(CUG) ₅	0	3790	2862				
6		30	8427	5056	6532	3625	1.8	
7	no plasmid	30	1005	94				
8	pCT1.4	0	-78	888				
9		30	9280	3185	9358	2297	4.1	
10	pCT1.4(CUA) ₅	0	2471	409				
11		30	3490	2607	1019	2198	0.5	3.4
12	pCT14(CUG) ₅	0	1389	497				
13		30	5501	2891	4112	2394	1.7	
					"No Plasmid" Values Subtracted			
Rifampicin treated					Test	Control	Ratio	
14	pCT1	30	18728	15225	18375	15242	1.2	
15	pCT1(CUA) ₅	30	1837	2975	1484	2992	0.5	2.0
16	pCT1(CUG) ₅	30	2188	1745	1835	1762	1.0	
17	no plasmid	30	353	-17				
18	pCT1.4	30	3085	1189	2732	1206	2.3	
19	pCT14(CUA) ₅	30	2300	1531	1947	1548	1.3	2.0
20	pCT14(CUG) ₅	30	4969	1742	4616	1759	2.6	

*Quantitation of test and control bands from the SDS-PAGE gels in Figure 3. Samples at 0 time were uninduced and labeled with [³⁵S]methionine for 10 min in parallel and at the same time as samples subjected to IPTG induction. The induced samples were similarly labeled at 30 min after addition of IPTG. Volume = local median values reported by the ImageQuant software (v. 5.2) following exposure of the gel in a Molecular Dynamics Phosphorimager (see legend to Fig. 3). Because the software automatically corrects for signal-to-noise backgrounds, three of the values with low signals were reported as negative numbers.

†100% of uninduced values for pCT1.4 samples, 50% for pCT1 samples. The uninduced values subtracted for pCT1 samples were normalized for general inhibition of expression when pCT1 was induced. The normalization value was obtained from taking four random regions (not shown) of the uninduced and induced pCT1 sample lanes (Fig. 3, lanes 1 and 2) and quantitating these regions on the phosphorimager. The average of the four regions showed a nonspecific reduction in expression of $\sim 50 \pm 10\%$ when pCT1 was induced with IPTG; therefore, 50% of the uninduced values were subtracted as background from the induced pCT1 samples. By contrast, pCT1.4 does not cause a general inhibition of expression following induction; therefore, 100% of the uninduced values were subtracted as background from the induced pCT1.4 samples.

tRNA-Leu species that decode rare CUA codons, which may explain why the inhibition is not as great in the rifampicin-treated samples.

Absence of Change in Inhibition by Rare Codons With Destabilized mRNAs

To test whether the effect of rare leucine codons on expression was altered by reduced mRNA stability, the mRNAs in this system were destabilized by removing the RNase III processing sites at the 3' ends of the test and control genes (Fig. 1). Vector pCT1.4 contains the same genes for the test and control proteins, but the mRNAs are expected to have much shorter half-lives. We have previously demonstrated

that removal of the RNase III processing elements from the pCT1 vector [and replacement of these with the T7 concatemer junction sequence element (CJ)] results in destabilization of the mRNA from the vector (6,23). For example, Figure 3 in Cheng and Goldman (6) shows the immense reduction in observable mRNA in the vectors where the RNase III sites have been replaced by the CJ element. This was shown directly for the parent vectors pCT1.1 (23) and pCT1.3 (6).

Because the change from pCT1.3 to pCT1.4 was relatively minor (only swapping the spacer sequence between the test and control genes), we did not measure mRNA stability directly in this work; however, the loss of mRNA stability can be inferred by exam-

ining the patterns of expression of pCT1.4 in Figure 3. For one thing, there is no loss of host background synthesis upon induction of T7 expression (compare lanes 11 and 12), in contrast to standard T7 systems such as that of pCT1 (compare lanes 1 and 2). This is likely because the destabilized mRNA is not able to expropriate the biosynthetic capacity of the host cell the way stable T7 mRNAs do.

Another indication that the mRNA is destabilized in the pCT1.4 samples comes from the rifampicin-treated samples (lanes 13, 16, and 19), where we can observe a smear, and even a few discreet bands, of lower molecular weight products, which are absent in the rifampicin-treated pCT1 samples (lanes 3, 6, and 9). We also know that these lower molecular weight products are specific to the T7-induced mRNAs, because host cells containing no vector treated with rifampicin do not show any such products (lane 20). Because these lower molecular weight products are not due to instability of the test and control proteins, which remain intact in the rifampicin-treated pCT1 samples (lanes 3, 6, and 9), we are left with the plausible explanation that these lower molecular weight products must be derived from aborted synthesis on destabilized and/or truncated mRNAs in pCT1.4.

Lines 10–13 of Table 1 show the uninduced and induced values observed with pCT1.4(CUA)₅ and pCT1.4(CUG)₅, respectively. The ratio of synthesis of test to control proteins for the insertion of five CUAs was 0.5 (line 11) whereas that for the five CUGs was 1.7 (line 13), indicating that the inhibitory effect of the rare codons was about 3.4-fold. Lines 8 and 9 show that the ratio of synthesis of test to control protein for the vector without inserted codons was about 4.1. [The dominance of test over control synthesis in the pCT1.4 vector may reflect some residual inhibition of the downstream gene, as reported for pCT1.1 (23).]

In the presence of rifampicin, the ratio of test to control proteins for the five CUAs was 1.3 (line 19) while for the 5 CUGs it was 2.6 (line 20). [The vector alone in the presence of rifampicin gave a ratio of 2.3 (line 18).] Therefore, the overall inhibitory effect in the presence of rifampicin was twofold (rather than the 3.4-fold seen without rifampicin), the same value obtained for the pCT1 vectors (lines 15 and 16).

DISCUSSION

In this work, we have shown that insertion of five consecutive rare CUA leucine codons into a test gene mRNA has approximately a threefold inhibitory effect on the production of the protein compared to insertion of five consecutive common CUG leucine co-

dons. Further, this value does not change much, only to a 3.4-fold inhibition, when the mRNA encoding the test gene was destabilized by removal of the 3' end stability elements, RNase III processing sites.

Other workers have reported that even a single CUA codon near the 5' end of a gene was inhibitory to expression under certain conditions, and that addition of the cognate tRNA-Leu abolished the inhibition (30). Earlier studies on bacterial attenuators also had suggested that CUA codons near the translation start had a significant inhibitory effect on translation rates *in vivo* (4), even in the case of a single CUA codon (22). It has been argued that selective charging of different tRNA-Leu isoacceptors accounts for these phenomena (10).

Although we reported evidence for premature termination of translation with rare arginine codons inserted at the 5' end of the test message, we did not observe the same with the rare CUA leucine codons (14). The basis for inhibition of translation by consecutive CUA leucine codons may involve some kind of switch to nonproductive attachment of ribosomes to mRNA in the absence of acceptable charged tRNA. Indeed, it has been reported that ribosomes can bypass codons that are deprived of cognate charged tRNA, resuming protein synthesis further downstream (11,12,35), and that this occurs spontaneously even in unstressed cells, with a frequency correlated to the abundance of the cognate tRNA in the cells (34).

In *E. coli*, when a ribosome comes to the end of a truncated mRNA, a tRNA-like molecule, tmRNA (also called SsrA RNA or 10Sa RNA), enters the A site of the ribosome, bringing an attached Ala to accept the peptide from peptidyl tRNA in the P site. This same tmRNA then encodes, via "trans-translation," a decapeptide, ending with a stop codon, so that the truncated protein can be properly terminated; the C-terminal peptide tag targets the truncated protein for proteolysis [reviewed in (47)]. Ribosomes paused at consecutive rare Arg codons AGA or CGA are also subject to the SsrA tagging system (42), which may explain our previous results of premature termination of translation with rare AGG Arg codons (14). However, there is no evidence to our knowledge that this mechanism has any role with rare Leu codons.

In the system used in this work, the other component (besides the RNase III sites) that uncouples translation from transcription in these vectors was the use of T7 RNA polymerase, which was not varied in these experiments. Thus, it remains possible that the effects of the rare codons could still be exacerbated in an *E. coli* RNA polymerase-driven system, because of potential polarity triggered by translational

stalling at the rare codons. In this view, polarity [i.e., termination of RNA polymerase transcription when translation is blocked; reviewed in (41)] would cause a much stronger deprivation of mRNA than mRNA degradation due to instability. Nevertheless, we can conclude from these experiments that at least to a first approximation, mRNA stability per se is not by itself a major factor modulating the extent of inhibition of rare codons on expression.

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REFERENCES

1. Acosta-Rivero, N.; Sanchez, J. C.; Morales, J. Improvement of human interferon HUIFNalpha2 and HCV core protein expression levels in *Escherichia coli* but not of HUIFNalpha8 by using the tRNA(AGA/AGG). *Biochem. Biophys. Res. Commun.* 296:1303–1309; 2002.
2. Brinkmann, U.; Mattes, R. E.; Buckel, P. High-level expression of recombinant genes in *Escherichia coli* is dependent on the availability of the *dnaY* gene product. *Gene* 85:109–114; 1989.
3. Calderone, T. L.; Stevens, R. D.; Oas, T. G. High-level misincorporation of lysine for arginine at AGA codons in a fusion protein expressed in *Escherichia coli*. *J. Mol. Biol.* 262:407–412; 1996.
4. Carter, P. W.; Bartkus, J. M.; Calvo, J. M. Transcription attenuation in *Salmonella typhimurium*: The significance of rare leucine codons in the leu leader. *Proc. Natl. Acad. Sci. USA* 83:8127–8131; 1986.
5. Chen, G. F.; Inouye, M. Suppression of the negative effect of minor arginine codons on gene expression; preferential usage of minor codons within the first 25 codons of the *Escherichia coli* genes. *Nucleic Acids Res.* 18:1465–1473; 1990.
6. Cheng, L.; Goldman, E. Reversal of inhibition by the T7 concatemer junction sequence on expression from a downstream T7 promoter. *Gene Expr.* 9:257–264; 2001.
7. Curran, J. F.; Yarus, M. Use of tRNA suppressors to probe regulation of *Escherichia coli* release factor 2. *J. Mol. Biol.* 203:75–83; 1988.
8. Del Tito, Jr., B. J.; Ward, J. M.; Hodgson, J.; Gershater, C. J.; Edwards, H.; Wysocki, L. A.; Watson, F. A.; Sathe, G.; Kane, J. F. Effects of a minor isoleucyl tRNA on heterologous protein translation in *Escherichia coli*. *J. Bacteriol.* 177:7086–7091; 1995.
9. Dieci, G.; Bottarelli, L.; Ballabeni, A.; Ottonello, S. tRNA-assisted overproduction of eukaryotic ribosomal proteins. *Protein Expr. Purif.* 18:346–354; 2000.
10. Elf, J.; Nilsson, D.; Tenson, T.; Ehrenberg, M. Selective charging of tRNA isoacceptors explains patterns of codon usage. *Science* 300:1718–1722; 2003.
11. Gallant, J.; Bonthuis, P.; Lindsley, D. Evidence that the bypassing ribosome travels through the coding gap. *Proc. Natl. Acad. Sci. USA* 100:13430–13435; 2003.
12. Gallant, J. A.; Lindsley, D. Ribosomes can slide over and beyond “hungry” codons, resuming protein chain elongation many nucleotides downstream. *Proc. Natl. Acad. Sci. USA* 95:13771–13776; 1998.
13. Gao, W. Studies of translational accuracy in *Escherichia coli*: Editing and methionyl-tRNA synthetase; uncharged tRNA and a programmed translational frameshift; low-usage codons and ribosome release. Ph.D. thesis, University of Medicine & Dentistry of New Jersey, Newark; 1996.
14. Gao, W.; Tyagi, S.; Kramer, F. R.; Goldman, E. Messenger RNA release from ribosomes during 5′-translational blockage by consecutive low-usage arginine but not leucine codons in *Escherichia coli*. *Mol. Microbiol.* 25:707–716; 1997 (Erratum 27:669; 1998).
15. Gerchman, S. E.; Graziano, V.; Ramakrishnan, V. Expression of chicken linker histones in *E. coli*: Sources of problems and methods for overcoming some of the difficulties. *Protein Expr. Purif.* 5:242–251; 1994.
16. Goldman, E. Modeling protein synthesis: Elongation delays have greater impact on short-lived messages, and “slowest clearing time” determines output. Las Vegas, NV: American Society for Microbiology Meeting; 1994:229.
17. Goldman, E.; Rosenberg, A.; Zubay, G.; Studier, F. W. Consecutive low-usage leucine codons block translation only when near the 5′ end of a message in *Escherichia coli*. *J. Mol. Biol.* 245:467–473; 1995.
18. Goldman, E.; Zubay, G. Investigating codon usage in *E. coli*. *Methods Mol. Genet.* 3:159–178; 1994.
19. Griswold, K. E.; Mahmood, N. A.; Iverson, B. L.; Georgiou, G. Effects of codon usage versus putative 5′-mRNA structure on the expression of *Fusarium solani* cutinase in the *Escherichia coli* cytoplasm. *Protein Expr. Purif.* 27:134–142; 2003.
20. Gustafsson, C.; Govindarajan, S.; Minshull, J. Codon bias and heterologous protein expression. *Trends Biotechnol.* 22:346–353; 2004.
21. Hannig, G.; Makrides, S. C. Strategies for optimizing heterologous protein expression in *Escherichia coli*. *Trends Biotechnol.* 16:54–60; 1998.
22. Harms, E.; Umbarger, H. E. Role of codon choice in the leader region of the *ilvGMEDA* operon of *Serratia marcescens*. *J. Bacteriol.* 169:5668–5677; 1987.
23. Harvey, B.; Korus, M.; Goldman, E. The T7 concatemer junction sequence interferes with expression from a downstream T7 promoter *in vivo*. *Gene Expr.* 8:141–149; 1999.

24. Imamura, H.; Jeon, B.; Wakagi, T.; Matsuzawa, H. High level expression of *Thermococcus litoralis* 4-alpha-glucanotransferase in a soluble form in *Escherichia coli* with a novel expression system involving minor arginine tRNAs and GroELS. *FEBS Lett.* 457: 393–396; 1999.
25. Iost, I.; Guillerez, J.; Dreyfus, M. Bacteriophage T7 RNA polymerase travels far ahead of ribosomes in vivo. *J. Bacteriol.* 174:619–622; 1992.
26. Jana, S.; Deb, J. K. Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 67:289–298; 2005.
27. Kane, J. F. Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* 6:494–500; 1995.
28. Kapust, R. B.; Routzahn, K. M.; Waugh, D. S. Processive degradation of nascent polypeptides, triggered by tandem AGA codons, limits the accumulation of recombinant tobacco etch virus protease in *Escherichia coli* BL21(DE3). *Protein Expr. Purif.* 24:61–70; 2002.
29. Kleber-Janke, T.; Becker, W. M. Use of modified BL21(DE3) *Escherichia coli* cells for high-level expression of recombinant peanut allergens affected by poor codon usage. *Protein Expr. Purif.* 19:419–424; 2000.
30. Kuhar, I.; van Putten, J. P.; Zgur-Bertok, D.; Gastra, W.; Jordi, B. J. Codon-usage based regulation of colicin K synthesis by the stress alarmone ppGpp. *Mol. Microbiol.* 41:207–216; 2001 (Erratum 42:1385; 2001).
31. Laine, S.; Salhi, S.; Rossignol, J. M. Overexpression and purification of the hepatitis B e antigen precursor. *J. Virol. Methods* 103:67–74; 2002.
32. Lakey, D. L.; Voladri, R. K.; Edwards, K. M.; Hager, C.; Samten, B.; Wallis, R. S.; Barnes, P. F.; Kernodle, D. S. Enhanced production of recombinant *Mycobacterium tuberculosis* antigens in *Escherichia coli* by replacement of low-usage codons. *Infect. Immun.* 68: 233–238; 2000.
33. Li, Y.; Chen, C. X.; von Specht, B. U.; Hahn, H. P. Cloning and hemolysin-mediated secretory expression of a codon-optimized synthetic human interleukin-6 gene in *Escherichia coli*. *Protein Expr. Purif.* 25:437–447; 2002.
34. Lindsley, D.; Gallant, J.; Doneanu, C.; Bonthuis, P.; Caldwell, S.; Fontelera, A. Spontaneous ribosome bypassing in growing cells. *J. Mol. Biol.* 349:261–272; 2005 (Erratum 351:248; 2005).
35. Lindsley, D.; Gallant, J.; Guarneros, G. Ribosome bypassing elicited by tRNA depletion. *Mol. Microbiol.* 48:1267–1274; 2003.
36. Makrides, S. C. Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol. Rev.* 60:512–538; 1996.
37. McNulty, D. E.; Claffee, B. A.; Huddleston, M. J.; Kane, J. F. Mistranslational errors associated with the rare arginine codon CGG in *Escherichia coli*. *Protein Expr. Purif.* 27:365–374; 2003.
38. Olivares-Trejo, J. J.; Bueno-Martinez, J. G.; Guarneros, G.; Hernandez-Sanchez, J. The pair of arginine codons AGA AGG close to the initiation codon of the lambda int gene inhibits cell growth and protein synthesis by accumulating peptidyl-tRNAArg4. *Mol. Microbiol.* 49:1043–1049; 2003.
39. Panayotatos, N.; Truong, K. Cleavage within an RNase III site can control mRNA stability and protein synthesis in vivo. *Nucleic Acids Res.* 13:2227–2240; 1985.
40. Peng, L.; Xu, Z.; Fang, X.; Wang, F.; Yang, S.; Cen, P. Preferential codons enhancing the expression level of human beta-defensin-2 in recombinant *Escherichia coli*. *Protein Pept. Lett.* 11:339–344; 2004.
41. Platt, T. Transcription termination and the regulation of gene expression. *Annu. Rev. Biochem.* 55:339–372; 1986.
42. Roche, E. D.; Sauer, R. T. SsrA-mediated peptide tagging caused by rare codons and tRNA scarcity. *EMBO J.* 18:4579–4589; 1999.
43. Rosenberg, A. H.; Goldman, E.; Dunn, J. J.; Studier, F. W.; Zubay, G. Effects of consecutive AGG codons on translation in *Escherichia coli* demonstrated with a versatile codon test system. *J. Bacteriol.* 175:716–722; 1993.
44. Sorensen, H. P.; Mortensen, K. K. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J. Biotechnol.* 115:113–128; 2005.
45. Studier, F. W. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.* 219:37–44; 1991.
46. Studier, F. W.; Rosenberg, A. H.; Dunn, J. J.; Dubendorff, J. W. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60–89; 1990.
47. Withey, J. H.; Friedman, D. I. A salvage pathway for protein structures: tmRNA and trans-translation. *Annu. Rev. Microbiol.* 57:101–123; 2003.
48. Wu, X.; Jornvall, H.; Berndt, K. D.; Oppermann, U. Codon optimization reveals critical factors for high level expression of two rare codon genes in *Escherichia coli*: RNA stability and secondary structure but not tRNA abundance. *Biochem. Biophys. Res. Commun.* 313:89–96; 2004.
49. Zdanovsky, A. G.; Zdanovskaia, M. V. Simple and efficient method for heterologous expression of clostridial proteins. *Appl. Environ. Microbiol.* 66:3166–3173; 2000.
50. Zhou, Z.; Schnake, P.; Xiao, L.; Lal, A. A. Enhanced expression of a recombinant malaria candidate vaccine in *Escherichia coli* by codon optimization. *Protein Expr. Purif.* 34:87–94; 2004.