Single synonymous codon substitution eliminates pausing during chloramphenicol acetyl transferase synthesis on *Escherichia coli* ribosomes in vitro

Vasanthi Ramachandiran[a], Gisela Kramer[a,*], Paul M. Horowitz[b], Boyd Hardesty[a]

[a] *Department of Chemistry and Biochemistry, University of Texas, Austin, TX, USA*
[b] *Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX, USA*

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Abstract The coding sequence for chloramphenicol acetyl transferase (CAT) contains several rare codons; three of them are ATA encoding isoleucine in positions 13, 84 and 119 of the amino acid sequence. Expression of CAT on *Escherichia coli* ribosomes in vitro results in mostly full-length product but also distinct smaller polypeptides from less than 3 kDa to over 20 kDa. As reported earlier, the smaller polypeptides are the predominant products, if translation is initiated with fluorophore-Met-tRNA$_f$. All this translational pausing is eliminated when the first ATA codon is mutated to ATC, a frequently used codon for isoleucine in *E. coli*. Addition of large amounts of *E. coli* tRNA to the coupled transcription/translation reaction does not reduce the number of pause-site peptides seen in the expression of wild-type CAT. Thus we hypothesize that the mRNA structure may be an important determinant for translational pausing. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Rare codon; In vitro protein synthesis; Nascent polypeptide; Translational pausing

1. Introduction

Non-random size distribution of nascent peptides generating a specific pattern for a given coding sequence was observed during coupled transcription/translation on *Escherichia coli* ribosomes (exemplified and reviewed in [1]). Possible reasons for translational pausing as presented in the literature were briefly reviewed [2]. The main two aspects discussed in this regard are either mRNA secondary structure or rare codons and low abundance of the corresponding tRNA. It has been well substantiated that a strong correlation exists between the frequency of codon usage and the level of its cognate tRNA [3]. The differential population of isoaccepting tRNA molecules has been suggested to be the key factor in regulating the relative translational rate of synonymous codons [4–6]. It has been observed earlier that in the absence of appropriate acylated rare tRNA species, ribosomes would pause at a specific site [7–11]. The effect of the rare arginine codons AGG and AGA [12,13] and the rare isoleucine codon AUA [14] on protein translation has been characterized. It has also been reported that rare codons located in the slow regions of the mRNA tend to form higher order secondary structures that might provide an additional time pause for the ribosome to move through the critical region [15,16].

We noticed enhanced low mass nascent polypeptides when chloramphenicol acetyl transferase (CAT) synthesis was initiated with fluorophore-Met-tRNA$_f$ [2,17]. It was hypothesized that a hydrophobic residue like coumarin at the N-terminus of CAT may interfere with the passage of the nascent peptide through the tunnel in the large ribosomal subunit [2]. Pyrene covalently attached to the N-terminal methionine had even a greater effect on translational pausing when CAT was synthesized than the other fluorophores tested [17]. Translational pausing of CAT was also enhanced, when the amount of cell extract used in the coupled transcription/translation assay was reduced [2]. In a separate study, translational pausing as observed on *E. coli* ribosomes was not seen, when the same coding sequences were expressed either in the wheat germ or rabbit reticulocyte lysate [18]. CAT was included in this study.

Here we demonstrate that translational pausing during CAT synthesis in the cell-free *E. coli* system can be affected by replacing a rare codon for isoleucine at position 13 of the amino acid sequence of the protein. The silent mutagenesis, changing one nucleotide in this codon without changing the amino acid sequence, eliminates all pause-site peptides observed during synthesis of wild-type CAT when initiated with pyrene-Met-tRNA.

2. Materials and methods

2.1. Materials

N-(1-Pyrene)-maleimide was from Molecular Probes, Inc. (Eugene, OR, USA). tRNA$_f^{Met}$, rifampicin and all other biochemicals were from Sigma (St. Louis, MO, USA). [^{35}S]Methionine was purchased from NEN Life Science Products (Boston, MA, USA). [^{14}C]L-Amino acids (52 mCi/mmol C atom) and [^{3}H]acetyl-coenzyme A (4.9 Ci/mmol) were bought from Amersham Pharmacia Biotech (Boston, MA, USA). Low molecular weight protein standards for SDS–PAGE were from Promega (Madison, WI, USA). *E. coli* tRNA from MRE-600 was purchased from Boehringer Mannheim (Germany).

2.2. Methods

2.2.1. Plasmid constructs. Using the wild-type CAT sequence cloned into pGEM-3Z as template, silently mutated CAT was generated using the primers (5′ to 3′) TCA GGA GCT CAA GGA AGC TAA AAT GGA GAA AAA AAT CAC TGG ATA TAC CAC CGT TGA TAT CTC CCA ATG GCA TC and M13 as a reverse primer.

*Corresponding author. Fax: (1)-512-471 4491.
E-mail address: g.kramer@mail.utexas.edu (G. Kramer).

Abbreviations: CAT, chloramphenicol acetyl transferase

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The PCR product was digested with *Sac*I and *Hin*dIII, then ligated into pGEM-3Z that had been cut with the same enzymes. The mutated CAT sequence was confirmed by sequencing carried out at the UT sequencing center, Institute for Cellular and Molecular Biology, at Austin, TX, USA.

2.2.2. Synthesis of fluorophore-Met-tRNA$_f$ species. Pyrene-succinimido-thiopropionate-Met-tRNA$_f$ (designated as pyrene-Met-tRNA in the text) was synthesized as described previously [17].

2.2.3. The cell-free system. The in vitro coupled transcription/translation assays were performed as described in [17]. Pyrene-[^{35}S]Met-tRNA (2500 Ci/mol) or [^{14}C]L-amino acids (52 mCi/mmol C atom) were used to initiate or trace protein synthesis. In the latter case, the missing five amino acids (not included in the mixture of 15 radioactive amino acids) were added in unlabeled form each at 0.3 mM final concentration. Reaction mixtures contained 5 μl of A19 S30 unless stated otherwise. Incubation was for the indicated length of time. Then an aliquot was withdrawn to determine the amount of polypeptides synthesized. In the absence of plasmid, trichloroacetic acid-insoluble material was less than 5% of the polypeptides formed in the presence of plasmid and these blank values were subtracted. Another aliquot was prepared for SDS–PAGE in Tricine buffer according to Schägger and von Jagow [19]. When CAT activity was determined, 1 μl of the reaction mixture after coupled transcription/translation was used.

2.2.4. CAT enzyme assay. CAT enzyme activity was assayed by the method of Sleigh [20]. The assays were performed at 30°C for 10 min. The specific enzymatic activities were calculated from the amount of protein synthesized, the percentage of full-length product formed and the units of enzyme activity obtained.

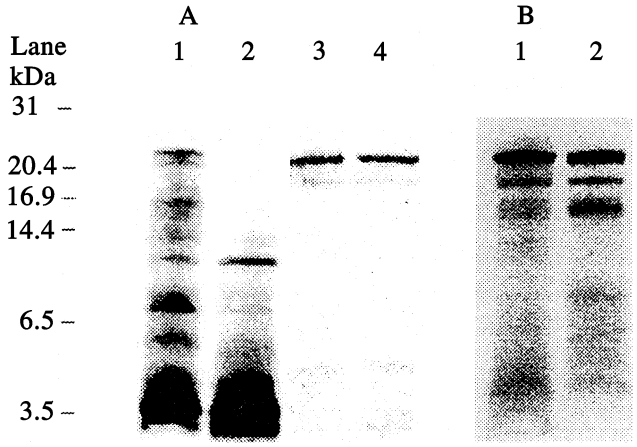

Fig. 1. Pause-site peptide pattern of CAT or silently mutated CAT. A: CAT (lanes 1 and 2) or CAT from mutated mRNA (lanes 3 and 4) was synthesized using pyrene-[^{35}S]Met-tRNA as the initiator tRNA with either 5 μl (lanes 1 and 3) or 2.5 μl (lanes 2 and 4) of S30/30 μl of reaction mixture. These were incubated at 37°C for 30 min, then an aliquot was analyzed by SDS–PAGE and phosphorimaging. The positions of the molecular weight markers are indicated. B: The coding sequences for CAT (lane 1) or mutated CAT (lane 2) were expressed for 30 min at 37°C using 5 μl of S30/30 μl of reaction mixture in the presence of a mixture of [^{14}C]amino acids (see Section 2.2). The translation products were analyzed by SDS–PAGE and phosphorimaging.

3. Results

3.1. Silent mutation of the first rare isoleucine codon eliminates translational pausing

When CAT was synthesized in the *E. coli* cell-free system, a distinct ladder of pause-site peptides was observed in size from less than 3.0 kDa migrating close to the front of the gel to near full size of 25 kDa [1,2,17,18]. Accumulation of the smallest nascent peptide was pronounced, when protein synthesis was initiated with fluorophore-[^{35}S]Met-tRNA [2,17]. Attempts were made to isolate this smallest peptide. By mass spectral analysis, its molecular mass was found to correspond to the first 12 amino acids of the CAT sequence (Ramachandiran and Linse, unpublished results, manuscript in preparation). Isoleucine, located at position 13 of the sequence, is coded by a rare AUA codon which has been classified as the fifth rarest codon in *E. coli* mRNA occurring at a frequency of 0.4% [21]. There are a total of 14 rare codons in CAT of which three are the rare isoleucine ATA codons. In general, CAT translational pause sites could not be correlated with the positions of rare codons (data not shown). But the correlation between the first ATA codon and the smallest of the nascent pause peptides is more intriguing in light of its accumulation (60–70% of the total protein produced) when synthesized with pyrene-[^{35}S]Met-tRNA.

To investigate whether this rare ATA codon at position 13 has an effect on translational pausing, site-directed mutagenesis was carried out to silently mutate this codon to ATC. The procedure is described in Section 2.2. The PCR product was ligated into the same plasmid in the same position as the wild-type CAT coding sequence. Mutant and wild-type coding sequences were expressed by coupled transcription/translation with pyrene-[^{35}S]Met-tRNA to initiate protein synthesis. The translation products were analyzed by SDS–PAGE followed by phosphorimaging. The results are presented in Fig. 1A. They indicate that with the mutant CAT coding sequence, over 90% of the synthesized polypeptides represent the full-length protein (lane 3), whereas in the case of wild-type CAT, the usual pattern of pause-site peptides is observed with less than 10% full-length product (lane 1). When protein synthesis was initiated with pyrene-Met-tRNA, reduction of the amount of S30 in the assay had a strong effect on the synthesis of wild-type CAT polypeptides (lane 2); however, the pattern seen with the mutated CAT coding sequence did not change (lane 4). It should be pointed out that the bonds between pyrene and the derivatized methionine as well as between pyrene-Met and the next amino acid are stable (unpublished results, Odom, Kramer and Hardesty). Thus the N-terminal (radioactive) Met cannot be hydrolyzed by a methionine aminopeptidase as would occur after deformylation

Table 1
Enzymatic activity of CAT protein expressed from wild-type or mutated coding sequence

Protein from coding sequence	Amount synthesized (ng)	Full-length (%)	Enzymatic activity	
			($U \times 10^{-6}$)	Protein (U/mg)
Wild-type CAT	15.4	80	523.1	41.2
Mutant CAT	16.7	70	373.6	31.8

Wild-type or silently mutated CAT mRNA was expressed by coupled transcription/translation under optimal conditions (using 5 μl S30/30 μl reaction mixture) in the presence of a mixture of [^{14}C]amino acids as described in Section 2.2. An aliquot was withdrawn after 30 min of incubation at 37°C, precipitated with trichloroacetic acid and its radioactivity determined. A second aliquot was analyzed by SDS–PAGE (see Fig. 1B) and phosphorimaging. Another aliquot was used to measure enzymatic activity.

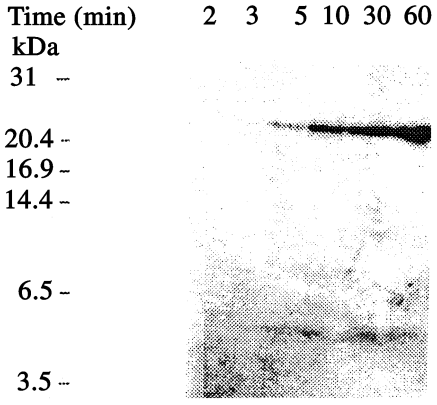

Fig. 2. Kinetics of CAT synthesis from the mutated mRNA initiated with pyrene-[^{35}S]Met-tRNA. CAT synthesis was initiated with pyrene-[^{35}S]Met-tRNA under optimal conditions. Reaction mixtures were incubated, and at indicated times aliquots were withdrawn and analyzed by SDS–PAGE and phosphorimaging.

of a formyl group when the peptide is initiated with fMet-tRNA. In other experiments, the CAT polypeptides were labeled during protein synthesis with a mixture of 15 [^{14}C]L-amino acids (see Section 2.2). These results are shown in Fig. 1B for comparison and in relation to Table 1 below, where specific enzymatic activity is presented. Under these conditions, full-length CAT is the prominent product.

3.2. Kinetics of expression of silently mutated CAT

It has been shown previously that nascent CAT peptides initiated with coumarin-Met-tRNA could be shifted to full-length product to some extent though pause-site peptides of distinct sizes remained visible [2]. To eliminate the possibility that the mutant form of the CAT coding sequence would generate the same pause-site peptides but its mRNA was translated much faster than that for the wild-type protein, the kinetics of CAT synthesis were investigated. The results are shown in Fig. 2. When CAT synthesis from the mutated mRNA was initiated with pyrene-[^{35}S]Met-tRNA, the full-length product appeared after 5 min of incubation. Accumulation of the low molecular mass peptides was not observed. These were the more predominant products after 10 and 30 min of incubation in the case of wild-type CAT (see [17], figure 4C).

3.3. Enzymatic activity of CAT

To investigate whether CAT synthesized from the mutated coding sequence is folded correctly, its enzymatic activity was measured and compared to that of wild-type CAT. Both wild-type and mutated CAT coding sequences were expressed under identical conditions in the presence of a mixture of 15 [^{14}C]L-amino acids with the missing five amino acids added in unlabeled form (see Section 2.2). Incubation was for 30 min at 37°C. Then CAT enzymatic activity was determined using [^{3}H]acetyl-coenzyme A to quantitate the product of the reaction. The SDS–PAGE analysis of an aliquot of the reaction mixtures after coupled transcription/translation is shown in Fig. 1B. The percentage of full-length polypeptide was estimated. The total amount of protein synthesized was determined from incorporation of radioactive amino acids into trichloroacetic acid-precipitable polypeptides. The results are shown in Table 1. They indicate a reduced specific enzymatic activity for CAT synthesized from the mutated CAT mRNA sequence. We hypothesize that this might be due to a faster synthesis of CAT from this mRNA.

3.4. Possible cause for pause-site peptides and its elimination

Based on the results presented above in Section 3.1 and shown in Fig. 1A, one might assume that the concentration of the rare isoleucine tRNA species might be one of the major factors in regulating ribosomal pausing at the codon for amino acid 12 in the case of wild-type CAT. This was tested by adding total *E. coli* tRNA to the coupled transcription/translation reaction mixture. Exogenously added tRNA up to 60 μg/30 μl reaction mixture had no effect on the pause-site pattern of wild-type CAT expression initiated with pyrene-Met-tRNA except that overall protein synthesis was reduced by about 20% (data not shown).

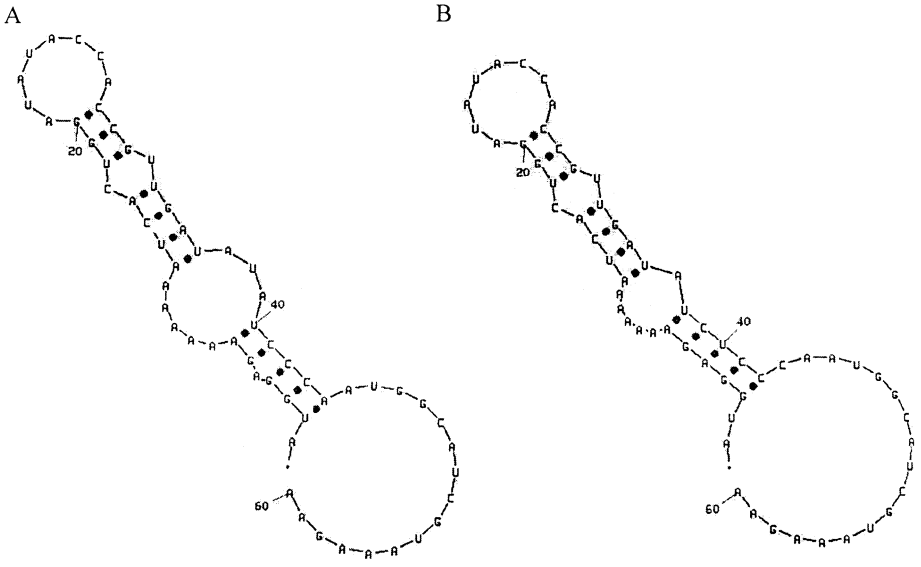

Fig. 3. Predicted secondary structures for the first 60 nucleotides of wild-type and mutated CAT mRNA coding sequence. A = wild-type mRNA; B = mutated mRNA.

The secondary structures of the 5′ 60 nucleotides of the two CAT coding sequences were compared as predicted from the mfold program [22]. This analysis (Fig. 3) suggests that the secondary structures formed by the wild-type and the mutant CAT mRNAs are slightly different with a change in the stem structure formed with nucleotides 2–7 of the wild-type mRNA and the size of the first loop. An increase in the overall stability of the shown structure of the mutated CAT sequence was predicted (−9.7 kcal/mol versus −7.3 kcal/mol for the wild-type).

4. Discussion

When synthesis of CAT was initiated with pyrene-Met-tRNA, accumulation of a low molecular weight nascent peptide was observed (Fig. 1A, lanes 1 and 2). The translational pause site has been identified as the rare ATA encoding isoleucine in position 13 of the CAT amino acid sequence. As the ATA codon for isoleucine occurs at a frequency of 0.4% in *E. coli* cells [21] and since there exists a correlation between frequency of codon usage and the level of its cognate tRNA, we assumed that a large amount of exogenously added tRNA would alleviate the translational pausing. This was not the case. Up to 60 μg *E. coli* tRNA was added to a 30 μl reaction mixture containing 5 μl S30 fraction. This amount of S30 contains about 38 pmol of ribosomes of which maximally 20% are active in protein synthesis in vitro. That means about 7.6 pmol is translating ribosomes. The tRNA added should contain about 9.6 pmol of the rare $tRNA^{Ile}$ (the product of the *ileX* gene) – theoretically enough to provide Ile for the rare AUA codon of the mRNA.

Based on this calculation with its assumptions, we favor the hypothesis that the change in mRNA structure due to the mutation is the cause that eliminates the translational pause site. We present this hypothesis though it is not evident to us how the predicted change in mRNA structure (with a slight increase in stability) could eliminate the pause site. Recently, it has been proposed that ribosomes themselves have the ability to unwind mRNA secondary structure by an unknown mechanism and the possible roles of the proteins S3, S4 and S5 of the 30S subunit in binding and unwinding of an mRNA helix have been suggested [23]. A small change in mRNA structure may greatly facilitate this helicase action.

The puzzling observation is that the rare ATA codon close to the 5′ end of the CAT coding sequence has such a strong effect on translational pausing and only when protein synthesis is initiated with fluorophore-Met-tRNA (Fig. 1A). As the nascent CAT peptide with a bulky hydrophobic N-terminus has to feed through relatively narrow passages in the ribosomal tunnel of the 50S subunit [24], a feature in mRNA structure may be a secondary determinant that affects the elongation rate of the growing peptide chain.

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