J Struct Func Genom (2006) 7:31–36
DOI 10.1007/s10969-006-9007-y

ORIGINAL PAPER

Translation of 'rare' codons in a cell-free protein synthesis system from *Escherichia coli*

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Received: 27 September 2005 / Accepted: 3 April 2006 / Published online: 16 May 2006

Abstract We analyzed the effect of nine 'rare' codons (AGA, AGG, AUA, CCC, CGA, CGG, CUA, GGA, and UUA) on gene expression in an *Escherichia coli* coupled transcription/translation cell-free system, in comparison with a cell-based expression system. Each reporter gene contained five consecutive repeats of a rare codon, or in some experiments, three consecutive repeats. The cell-free expression of the genes bearing the codons CGA, CUA, GGA, and UUA was not affected, although these codons, except for GGA, were inefficiently translated in *E. coli* cells. Translation of the remaining five codons (AGA, AGG, AUA, CCC, and CGG) was severely reduced in both systems, and was remarkably facilitated in the cell-free system based on an S30 extract from the *E. coli* cells overproducing 'minor' tRNAs for these codons.

Keywords Cell-free protein synthesis · Codon usage · Consecutive codons · Minor tRNA · Glutathione *S*-transferase

Abbreviation
GST glutathione *S*-transferase

Introduction

Cell-free translation systems are useful tools for protein expression. This technology has been employed to prepare proteins for biochemical studies, to incorporate stable isotopes into proteins for structural determinations [1–4], to produce 'alloproteins' with nonnatural amino acids [5, 6], and to express integral membrane proteins [7, 8]. Moreover, functional and structural proteomics has increased the importance of this technology, which is suitable for the high-throughput expression of a large variety of different proteins [9].

The usefulness of cell-free protein expression has been enhanced by the optimization of experimental conditions, including the reaction conditions [1–3, 10] and the protocol for cell-extract preparation [11, 12], and also by the development of continuous-flow and semi-continuous flow systems [13, 14]. In addition to these improvements specific to cell-free synthesis, the factors affecting cell-based protein expression should be examined to expand the applicability of cell-free expression.

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Biased codon usage is a phenomenon widely observed among unicellular and multicellular organisms [15, 16]. The codon usage of *Escherichia coli* as a host cell reportedly has serious effects on the yields and quality of recombinant proteins [17]. In this organism, some of the synonymous codons are preferentially used in highly expressed genes, while others are rarely used in such genes [18]. The frequently used codons are translated by the tRNA species abundant in *E. coli* cells, while most of the 'rare' codons are translated by 'minor' tRNAs, which are expressed at low levels [19]. Thus, the frequent occurrence of rare codons in a recombinant gene reduces the expression level of this gene, because of the limited availability of the corresponding tRNAs.

In the highly expressed genes from *E. coli*, the strongest bias in codon usage is found with arginine, glycine, isoleucine, leucine, and proline codons [20]. None of the arginine codons AGA, AGG, and CGG accounts for more than 1% of the arginine codons used in these genes. The isoleucine codon AUA also accounts for less than 1% of the isoleucine codons, while the codons CCC (proline), CGA (arginine), CUA (leucine), GGA (glycine), and UUA (leucine) account for only 1–4% of their synonymous codons in the highly expressed genes. These codons are used rarely among not only their respective synonymous codons but also all of the codons in the *E. coli* genome; each of these 9 codons occurs less than once in a 100 codons [17]. Translation of some of these codons, the codons AGA, AGG, CGG, and AUA, has reportedly been facilitated by the overproduction of corresponding minor tRNA species from a plasmid in a host cell [21–25].

In the present study, we examined the effect of the nine rarest codons on the expression of a reporter gene in an *E. coli* cell-free system, in comparison with cell-based expression. Eight of these codons actually reduced the expression of the reporter gene in the cell, whereas only five exhibited this deleterious effect in the cell-free translation. The cell-free reaction mixture was then supplemented with the corresponding minor tRNAs, in two different manners, to facilitate the translation of these five codons.

Materials and methods

Expression of GST in *E. coli* cells and assay of the transferase activity of GST

We added a leader sequence, *GGATCC*GGGCGC-TAACTCTXG TAACTAAGAATTCT, to the 5′ end of the wild-type *Schistosoma japonicum* GST gene from a commercially available plasmid, pGEX (Amersham Biosciences). This sequence contains a *Bam*HI site (in italics), a site for the incorporation of consecutive rare codons (denoted as X), and two stop codons in the −1 and +1 reading frames (underlined). This GST gene was cloned between the *Bam*HI and *Xho*I sites of the expression vector pET21b (Novagen) to generate the pET21b-GST plasmid, where the resulting GST gene has an additional sequence of 23 amino acids at the N-terminus and a cluster of rare codons is to be incorporated after the 18th codon. pET21b-GST was used to transform the *E. coli* BL21(DE3) strain (Novagen) to express GST variants.

The GST activity was determined by using the GST detection module (Amersham Biosciences) for purified GST variants, extracts from *E. coli* cells, and cell-free translation mixtures. GST(Arg3), GST(Arg5), GST(Gly5), GST(Ile3), GST(Ile5), GST(Leu5), GST(Pro3), and GST(Pro5) were purified by using the GST purification module (Amersham Biosciences) from *E. coli* cells expressing GST(AGA3), GST(AGA5), GST(GGA5), GST(AUA3), GST(AUA5), GST(CUA5), GST(CCC3), and GST(CCG5), respectively, under the condition that minor tRNAs were overproduced in the cells.

Cell-free expression of GST

E. coli S30 cell extracts were prepared from the BL21(DE3) strain and that harboring the plasmid pMINOR (described below), according to the previously reported methods [12, 26–28]. The synthesis of GST was performed at 30 °C overnight in a coupled transcription/translation cell-free system with dialysis [3]. The internal reaction mixture (30 μL) consisted of 58 mM Hepes-KOH buffer (pH 7.5), containing 1.2 mM ATP, 0.8 mM each of GTP, CTP and UTP, 1.7 mM DTT, 0.64 mM 3′,5′-cyclic AMP, 200 mM potassium glutamate, 27.5 mM ammonium acetate, 10.7 mM magnesium acetate, 80 mM creatine phosphate, 250 μg/mL creatine kinase, 500 μM of each of the 20 amino acids, 4.0% PEG8000, 25 mM phospho*enol*pyruvate, 35 μg/mL L(−)-5-formyl-5,6,7,8-tetrahydrofolic acid, 0.05% sodium azide, a tRNA mixture (170 μg/mL), the pET21b-GST plasmid (4 μg/mL) as the template, S30 extract (9 μL), and T7 RNA polymerase (66.6 μg/mL), which was prepared according to Zawadzki and Gross [29]. The external solution (300 μL) contained all of the components of the internal solution except for creatine kinase, tRNA, the template plasmid, T7 RNA polymerase, and S30 extract. The dialysis membrane, MWCO 50,000 Spectra/Por, was from Spectrum.

Preparation of minor tRNA transcripts and amino-acid sequence analysis

The minor tRNAs, $tRNA_3^{Arg}$, $tRNA_4^{Arg}$, $tRNA_5^{Arg}$, and $tRNA_2^{Ile}$, were prepared by run-off transcription with T7 RNA polymerase, as described previously [30].

Amino-acid sequencing was performed on a Perkin-Elmer/Applied Biosystems Protein Sequencer (Procise 494) according to the manufacturer's instructions.

Construction of the plasmid pMINOR

The *tyrT* promoter [31] and *rrnC* terminator [32] were placed at the 5′ and 3′ ends, respectively, of a tRNA cluster, which includes $tRNA_2^{Ile}$, $tRNA_3^{Arg}$, $tRNA_2^{Pro}$, and $tRNA_4^{Arg}$ in this order, with appropriate intervening sequences of several bases. This tRNA operon was cloned between the *Hin*dIII and *Eco*RV sites of the vector pACYC184, to generate the plasmid pMINORpre1. The $tRNA_5^{Arg}$ gene, to be transcribed from the *tyrT* promoter to the *rrnC* terminator, was cloned between the*Eag*I and *Nru*I sites of pMINORpre1, to generate pMINORpre2. Then, three copies of the $tRNA_2^{Ile}$ gene, with the *lpp* promoter [32] and*rrnC* terminator, were cloned between the *Sph*I and *Sal*I sites of pMINORpre2 to generate pMINORpre3. The *proL* gene [33] was finally cloned within the *Bam*HI site of pMINORpre3, to generate the plasmid pMINOR.

Results

Translation of five consecutive rare codons

In order to investigate the effect of rare codons on gene expression, clusters of rare codons have been inserted into reporter genes [22, 25, 34–38]. We inserted three or five consecutive repeats of rare codons into an additional sequence at the N-terminus of the glutathione *S*-transferase (GST) gene, and expressed this gene in *E. coli* cells and an *E. coli* coupled transcription/translation cell-free system. The expression levels of these GST variants with rare codons were compared with that of the control GST, which contains five consecutive copies of the major proline codon, CCG, in place of the consecutive rare codons.

Since the GST variants have a different amino acid sequence at the N-terminus according to the inserted rare codons, we first determined the specific activities (the transferae activities for the same amount of purified enzyme) of the variants relative to that of the control GST. The GST variants with three and five consecutive repeats of arginine, or GST(Arg3) and GST(Arg5), showed the relative activities of 0.82 and 1.1, respectively, while the relative activities of GST(Gly5), GST(Ile3), GST(Ile5), GST(Leu5), and GST(Pro3) were determined to be 1.0, 1.1, 0.82, 0.79, and 0.93, respectively. In the following experiments, the expression levels of GST variants were compared with that of the control GST by determining the transferase activities of *E. coli* cell extracts or cell-free translation mixtures, which are corrected by the specific activities of the variants.

In *E. coli* cells, the rare codons, except for the codon GGA, actually reduced the expression levels of these GST variants (white bars, Fig. 1). In particular, the codons AGA, AGG, AUA, and CCC reduced the GST expression severely; the yields of the GST variants with these codons were less than 5% of that of the control GST. These findings are consistent with the previous reports with *E. coli* cells [22, 25, 34–38], except that the effects of the clusters of GGA and UUA had not been reported.

In the cell-free system (grey bars, Fig. 1), GST(CGA5), GST(CUA5), GST(GGA5), and GST(UUA5) were synthesized efficiently; their yields relative to that of the control GST were higher than 1. In addition, the relative yield of GST(CGG5), determined as 0.3, was 10 times higher than that in the cell. Thus, for the codons CGA, CGG, CUA, and UUA, the cell-free translation had an advantage over the cell-based expression. The remaining codons, AGA, AGG, AUA, and CCC, were poorly translated also in the

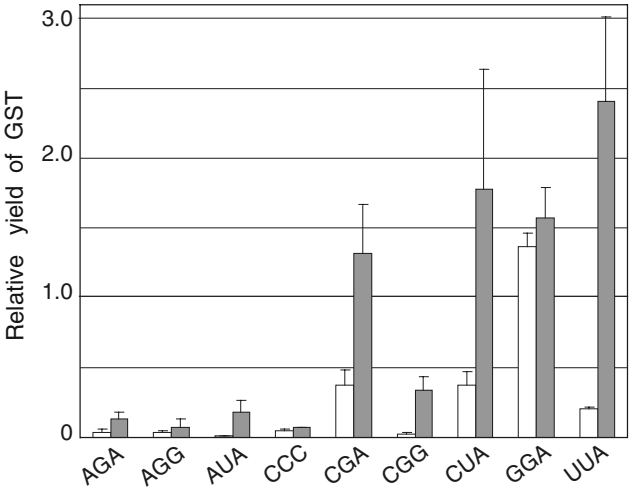

Fig. 1 Cell-based and cell-free expressions of the GST variants with five consecutive rare codons. The white bars indicate the yields of GST variants relative to that of the control GST, both expressed in *E. coli* cells. The grey bars indicate the yields of GST variants relative to that of the control GST, both expressed in the cell-free system

cell-free system, although the relative yields of the GST variants with these codons were appreciably higher in the cell-free system than in the cells.

Addition of the T7 transcripts of minor tRNA species to the cell-free system

According to Zubay's protocol, the *E. coli* cell-free translation mixture used in the above experiments was supplemented with 170 μg/mL of bulk tRNA. To facilitate the translation of the codons AGA, AGG, AUA, and CCC, we increased the amount of the tRNA mixture up to 1.7 mg/mL, but the yields of the GST variants with these rare codons were barely affected (data not shown). Then, minor tRNAs prepared in vitro were added to the cell-free translation at concentrations of 8.5 and 170 μg/mL; $tRNA_3^{Arg}$, $tRNA_4^{Arg}$, $tRNA_5^{Arg}$, and $tRNA_2^{Ile}$ for codons CGG, AGA, AGG, and AUA, respectively, have G at the 5′ end, and can be transcribed in vitro by T7 RNA polymerase. The in vitro transcript of *E. coli* $tRNA^{Arg}$ is almost as active as the native molecule with post-transcriptional modifications [39], while $tRNA_2^{Ile}$ requires a modification on the first nucleotide of the anticodon to be aminoacylated with isoleucine as well as to recognize AUA codons [40].

As shown in Fig. 2 (grey and shaded bars), the yields of GST(AGA5), GST(AGG5), and GST(CGG5) were remarkably increased by the addition of $tRNA_4^{Arg}$, $tRNA_5^{Arg}$, and $tRNA_3^{Arg}$, respectively; the 8.5 μg/mL concentration was sufficient for these tRNAs. On the other hand, the addition of $tRNA_2^{Ile}$ up to 170 μg/mL only slightly increased the yield of GST(AUA5). An amino-acid sequence analysis revealed that the five consecutive AUA codons in the GST(AUA5) thus obtained were actually translated into isoleucines, indicating that the T7 transcript of $tRNA_2^{Ile}$ was properly modified in the *E. coli* cell-free system.

To analyze the effect of tRNA addition further, the number of consecutive rare codons was reduced from five to three. In the absence of the added minor tRNA, the GST variant with three consecutive AUA codons, GST(AUA3), as well as GST(CGG3), was produced almost as efficiently or as efficiently as the control GST (Fig. 2). By contrast, lower amounts of GST(AGA3) and GST(AGG3) were produced. In the presence of added minor tRNAs, the yields of GST(AGA3), GST(AGG3), GST(AUA3) and GST(CGG3) all exceeded that of the control GST.

Cell-free translation of rare codons using an S30 extract from E. coli cells overproducing minor tRNA species

To overproduce minor tRNAs in *E. coli*, we created a multicopy plasmid, pMINOR (Figure 3), which expresses their genes from heterologous promoters, the *tyrT* and *lpp* promoters, and thus facilitates abundant expression in *E. coli*. The plasmid bears two *tyrT* promoters, with one directing the co-transcription of

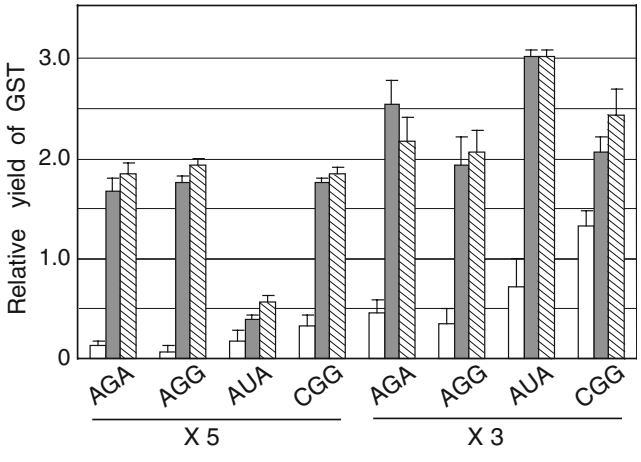

Fig. 2 Cell-free expression of the GST variants with three and five consecutive rare codons, in the absence and presence of minor tRNA transcripts. White bars indicate the relative yields of the GST variants with five consecutive rare codons (the same data in Fig. 1) and those with three consecutive rare codons. Grey and shaded bars indicate the relative yields of GST variants synthesized in the presence of a minor tRNA transcript, corresponding to the rare codon in the variant, at 8.5 and 170 μg/mL concentrations, respectively

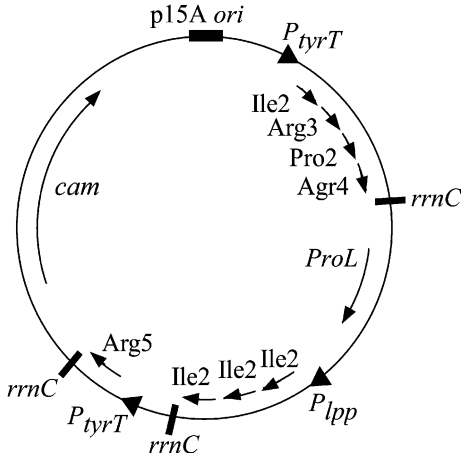

Fig. 3 Arrangement of the tRNA genes in plasmid pMINOR. The genes for $tRNA_3^{Arg}$, $tRNA_4^{Arg}$, $tRNA_5^{Arg}$, $tRNA_2^{Ile}$, and $tRNA_2^{Pro}$ are indicated by Arg3, Arg4, Arg5, Ile2, and Pro2, respectively. *ProL* is the gene for $tRNA_2^{Pro}$ with the native promoter. A black triangle represents the *tyrS* or *lpp* promoter, and a black rectangle represents the *rrnC* terminator or the replication origin of the plasmid. The gene coding for chloramphenicol actyltransferase is indicated by *cam*. The arrows indicate the direction of the genes (from the 5′ to 3′ end)

$tRNA_2^{Ile}$, $tRNA_3^{Arg}$, $tRNA_2^{Pro}$ (for codon CCC), and $tRNA_4^{Arg}$ in this order, and the second directing the transcription of $tRNA_5^{Arg}$. To enhance the expression of $tRNA_2^{Ile}$ and $tRNA_2^{Pro}$, this plasmid also carried three copies of $tRNA_2^{Ile}$, expressed from the *lpp* promoter, and the *proL* gene, the gene encoding $tRNA_2^{Pro}$, with the native promoter.

We first determined if an S30 extract from the *E. coli* cell harboring pMINOR had the same productivity as the S30 cell extract used in the above experiments, because the overproduction of a translation component, such as tRNA, might affect the translation process. The yield of the control GST was compared between these two extracts and was found to be almost the same; the GST yield for the *E. coli*(pMINOR) cell extract was higher that for the other extract by a factor of 1.05, with a standard deviation of 0.18.

Then, the GST variants with five consecutive rare codons, as well as those with three consecutive rare codons, were subjected to cell-free translation with the *E. coli*(pMINOR) cell extract. All of these GST variants, except for GST(AUA5) and GST(CCC5), were synthesized efficiently and produced yields higher than that of the control GST (grey bars, Fig. 4). As compared to the cell extract without the overproduced minor tRNA, the relative yields of GST(AUA5) and GST(CCC5) were increased significantly, to 0.6 and 0.2, respectively.

Discussion

The effects of rare codons on gene expression in *E. coli* cells have been analyzed using various experimental systems [22–25, 34–38]. We studied nine rare codons in a comparative manner with an assay system, and determined the relative strengths of their effects on gene expression. In addition, the cell-based and cell-free systems were compared to each other. This systematic approach revealed a sharp contrast between these systems with respect to the effects of the rare codons CGA, CGG, CUA, and UUA. The demonstrated advantage of the cell-free system in the translation of rare codons is probably due to the addition of bulk tRNA to the cell extract, according to the standard protocol and/or the enhanced energy source regeneration system, which may facilitate the turnover of minor tRNAs during polypeptide elongation. The bulk tRNA may provide sufficient additional cognate tRNA to overcome the inhibition, or could even facilitate near-cognate codon recognition by more common isoacceptors for the same amino acid.

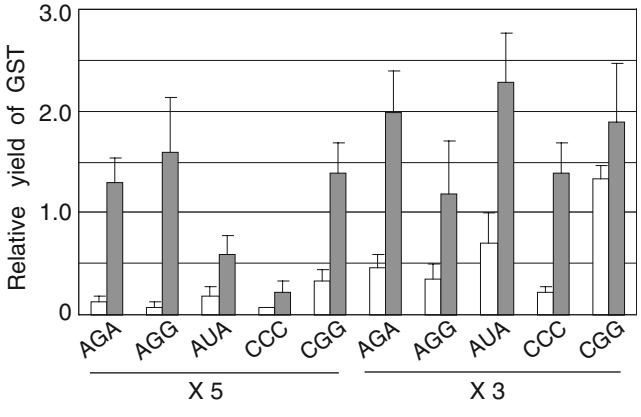

Fig. 4 Cell-free expression of GST variants with three and five consecutive rare codons, using an S30 extract of *E. coli* cells harboring pMINOR. The white bars indicate the relative yields of GST variants with five and three consecutive rare codons (the same data in Fig. 2). The grey bars indicate the yields of GST variants relative to that of the control GST, both expressed in the cell-free system based on *E. coli*(pMINOR)

The arginine codon CGA is unique among the rare codons, in that it is recognized by a major tRNA species. It has been suggested that the inosine in the first position of the major $tRNA^{Arg}$ anticodon can only inefficiently base pair with adenosine, as the codon's third letter [38]. The reduced rate of CGA translation in *E. coli* cells indicates that this inefficient base pairing is not overcome by an abundance of the recognizing tRNA. On the other hand, this problem is circumvented in the cell-free system, in which this codon is efficiently translated.

An increase in the abundance of a minor tRNA has been shown to alleviate the deleterious effect of the corresponding rare codon in *E. coli* cells [21–25]. This is also true in the cell-free translation system. The rare arginine codons, AGA, AGG, and CGG, were efficiently translated by the addition of the corresponding minor tRNAs, transcribed in vitro, as well as by using an extract from *E. coli* overproducing these minor tRNAs. For the codons AUA and CCC, the use of this cell extract boosted the yield of GST(AUA5) to a level almost comparable to that of the control GST, and increased the yield of GST(CCC5) significantly.

This yield of GST(CCC5), however, was comparatively low. Since the synthesis of GST(CCC3) was remarkably facilitated by using the extract from the cells overproducing minor tRNAs, the amount of $tRNA_2^{Pro}$ was probably increased in this extract. Therefore, the low yield of GST(CCC5) was probably not due to a lack of tRNA. The CCC codon is reportedly 'shifty', and causes frameshifting in *E. coli* cell-free translation [41] as well as whole cells [42]. This frameshifting problem has not been suppressed completely by the addition of the mature $tRNA_2^{Pro}$ to the cell-free reaction mixture [41]. In our assay system, a

frameshift at the site of the consecutive rare codons will abort the translation at the stop codon in the −1 or +1 reading frame downstream of this site, and will thus reduce the yield of GST.

It has been reported that a cluster of rare codons led to mRNA cleavage on the ribosome, followed by the *ssrA* tagging [43]. This cleavage is caused by the scarcity of the cognate tRNA species in the cell or cell extract. The low yields of GST variants with rare codons may have been exacerbated by this mechanism in the absence of supplemented minor tRNAs. Another mechanism of mRNA cleavage on the ribosome, which is codon-specific and involves the RelE protein, may have reduced the expression level of the control GST, because CCG codon is a potential target of this cleavage [44]. This could explain why the yields of some GST variants exceeded that of the control GST.

In conclusion, the *E. coli* cell-free system is free of the deleterious effect of some of the rarest codons of *E. coli*, and the use of an S30 extract from *E. coli* cells overproducing minor tRNAs is a practical solution to circumvent the translational impairments due to the other rare codons. Such S30 extracts may also be prepared from the commercially available *E. coli* strains overproducing minor tRNAs, such as Rosetta2 (Novagen) and BL21-Codonplus (Stratagene).

Acknowledgements We thank Dr. Takashi Yabuki and Mr. Takayoshi Matsuda for technical advice and valuable discussions. This work was supported by the RIKEN Structural Genomics/Proteomics Initiative (RSGI), the National Project on Protein Structural and Functional Analyses and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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