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Comprehensive screening of amber suppressor tRNAs suitable for incorporation of non-natural amino acids in a cell-free translation system

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ABSTRACT

Incorporation of non-natural amino acids into proteins in response to amber or four-base codons is a useful technology for protein research. In the case of the amber codon, however, release factor 1 can competitively decode the same codon, and consequently inhibit the incorporation of non-natural amino acids. To improve amber codon-mediated incorporation, we carried out a comprehensive screening of amber suppressor tRNAs derived from all tRNAs encoded in the genomes of *Escherichia coli* K12 and *Mycoplasma capricolum*. The amber suppressor tRNAs were synthesized from synthetic genes, aminoacylated with a fluorescent non-natural amino acid, and added to an *E. coli* cell-free translation system. Fluorescent SDS–PAGE analysis indicated that Trp tRNAs showed high suppressor activity in both organisms. Further mutagenesis and screening revealed that *M. capricolum* Trp_1 tRNA with G1C72A73 mutation is the most suitable for efficient and specific incorporation of non-natural amino acids into proteins in response to the amber codon.

The incorporation of non-natural amino acids into proteins is a useful technique for engineering proteins with artificial functions and for analyzing the structure and function of proteins. An amber suppression method and a four-base codon method have been developed for the position-specific incorporation of non-natural amino acids in cell-free translation systems [1–4]. In these methods, amber suppressor or frameshift suppressor tRNAs that are aminoacylated with non-natural amino acids are used to decode the amber or four-base codons into non-natural amino acids. However, endogenous factors such as release factor 1 (RF-1) and triplet tRNAs can competitively decode the same codon, and consequently inhibit the incorporation of non-natural amino acids. We have revealed that four-base codons derived from rarely used triplet codons in *Escherichia coli* such as CGG and GGG allow much more efficient incorporation than the amber codon [4]. This can be explained by the fact that the competitive decoding of the triplet tRNAs corresponding to the rarely used codons, whose concentrations are low in the translation system, is less efficient than that of RF-1 for the amber codon. The efficient four-base codons enable us to introduce two non-natural amino acids into single proteins [4,5]. Improvement of the amber suppression method will enhance the multiple incorporation of non-natural amino acids by combination with four-base codons. Elimination of RF-1 from the cell-free translation reaction mixture [6] and addition of antibodies or aptamers against RF-1 to the cell-free mixture [7,8] have been investigated as means of enhancing amber suppression.

Improvement of amber suppressor tRNAs is another strategy for increasing suppression efficiency. Several types of amber suppressor tRNAs derived from *E. coli* tRNAs have been examined for the incorporation of non-natural amino acids, and suppression efficiency was found to differ depending on the type of tRNA [9]. In addition, amber suppressor tRNAs derived from tRNAs of other organisms, such as *Tetrahymena* and *Methanococcus*, have been used for non-natural amino acid incorporation in an *E. coli* cell-free translation system [10,11]. These results suggest that a variety of tRNAs may potentially have amber suppressor activity in an *E. coli* translation system.

Here we carried out a comprehensive screening of amber suppressor tRNAs derived from all tRNAs encoded in the genomes of *E. coli* K12 and *Mycoplasma capricolum*. *E. coli* tRNAs are expected to be active in an *E. coli* cell-free translation system. *M. capricolum* was chosen because it has a very small number of tRNAs (29 types) with few modified nucleotides [12]. This fact raises the possibility that tRNAs with low translation activity may have been excluded during evolution in *M. capricolum* and that tRNAs could maintain high translation activity without modified nucleotides. The lack of modified nucleotides is advantageous because the suppressor tRNAs used in cell-free translation are usually prepared using T7 RNA polymerase, a method that does not permit the introduction of modified nucleotides.

In this study, the amber suppressor activity of tRNAs was evaluated using a fluorescent non-natural amino acid, BODIPYFL-

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aminophenylalanine (BFLAF). BFLAF has been found to be efficiently incorporated into proteins in response to a four-base codon [13] and facilitates the quantification of suppressor activity of tRNAs by fluorescent SDS–PAGE analysis of cell-free translation products.

Escherichia coli and *M. capricolum* tRNAs were synthesized from synthetic genes containing the CUA anticodon under the control of the T7 promoter. These tRNAs were screened for their ability to introduce BFLAF into streptavidin in response to the UAG codon in an *E. coli* cell-free translation system. The tRNAs that showed high suppression efficiency were then mutated to render them free from aminoacylation by any *E. coli* aminoacyl-tRNA synthetase. Because of the similarity of the results obtained from *E. coli* and *M. capricolum*, Trp tRNAs derived from various bacteria were then examined, and the most effective suppressor tRNA was identified.

Materials and methods

Materials. Synthetic oligonucleotides were obtained from Texas Genomics Japan (Tokyo, Japan). KOD Dash DNA polymerase was purchased from Toyobo Biochemicals (Osaka, Japan). MinElute PCR purification kit was from Qiagen (Hilden, Germany). T7 RNA polymerase was from New England Biolabs (Beverly, MA). T4 RNA ligase and ribonuclease inhibitor were from Takara Bio (Otsu, Japan). *E. coli* S30 extract for linear templates was obtained from Promega (Madison, WI), inorganic pyrophosphatase from Sigma–Aldrich (St. Louis, MO), and the anti-T7 tag antibody from Novagen (San Diego, CA).

Preparation of synthetic tRNA genes. DNA sequences of *E. coli* K12 tRNAs and *M. capricolum* tRNAs were obtained from the literatures [14,15]. DNA sequences of bacterial Trp tRNAs were obtained from the Genomic tRNA Database (http://lowelab.ucsc.edu/GtRNAdb/). For each tRNA, three oligonucleotides were designed. The first oligonucleotide had a 5′ fragment containing a T7 promoter and the 1–33 nucleotide sequence of the tRNA. The second had an anticodon fragment containing a sequence complementary to positions 24–46 with the CUA anticodon. The third had a 3′ fragment containing a sequence complementary to positions 37–74 without the 3′-terminal C and A. The primer extension reaction mixture contained a 5′ fragment (25 pmol), an anticodon fragment (25 pmol), 0.2 mM dNTPs, and 0.6 units of KOD Dash DNA polymerase in 25 μL. The mixture was incubated for seven cycles of 15 s at 98 °C, 2 s at 55 °C, and 20 s at 74 °C. Using the resulting reaction mixture (1 μL) as a template, PCR was performed using the T7 promoter primer CTAATACGACTCACTATA (50 pmol) and the 3′ fragment (50 pmol) in 100 μL. The reaction mixture was incubated for 25 cycles of 15 s at 98 °C, 2 s at 55 °C, and 20 s at 74 °C. The PCR product was purified with the MinElute PCR purification kit, eluted with 50 μL of elution buffer, and subjected to the following transcription reaction. Mutated tRNA genes were synthesized in a similar manner using oligonucleotides containing the corresponding mutations.

Preparation of fluorescent aminoacyl-tRNAs. The transcription reaction mixture contained 40 mM Tris–HCl (pH 8.0), 20 mM $MgCl_2$, 5 mM DTT, 4 mM NTP, 2 mM spermidine, 10 μg/mL BSA, 40 units of ribonuclease inhibitor, 1 unit of inorganic pyrophosphatase, 400 units of T7 RNA polymerase, and 40 μL of the purified PCR product in 100 μL. The reaction mixture also contained 20 mM nucleoside monophosphate (GMP, CMP, AMP, or UMP) corresponding to the 5′ nucleotide of the tRNAs. After incubation at 37 °C for 18 h, the product was purified on a DEAE-cellulose column. The resulting tRNA lacking the 3′ terminal dinucleotide was then ligated with phosphodeoxycytidyladenosine (pdCpA), which was aminoacylated with BFLAF [13]. The ligation reaction contained 0.25 nmol of tRNA lacking the 3′ terminal dinucleotide, 2.2 nmol BFLAF-pdCpA in 1 μL DMSO, 55 mM HEPES-Na (pH 7.5), 1 mM ATP, 15 mM $MgCl_2$, 3.3 mM DTT, 20 μg/mL BSA, and 15 units of T4 RNA ligase in 10 μL. After incubation at 4 °C for 2 h, potassium acetate (pH 4.5) was added to a final concentration of 0.3 M. The product was isolated by extraction with phenol/chloroform and chloroform, and ethanol precipitation. The precipitate was dissolved in 1 μL of prechilled 1 mM potassium acetate (pH 4.5) just prior to addition to the cell-free translation reaction.

Cell-free translation and evaluation of suppressor activity. Each BFLAF-tRNA was added to an *E. coli* cell-free translation reaction together with T7-tagged streptavidin mRNA containing a UAG codon at the Tyr83 position as previously described [4]. The translation reaction mixture (1 μL) was mixed with SDS–PAGE sample buffer (19 μL), and 5 μL of the resulting solution was applied to 15% SDS–PAGE after boiling. The SDS–PAGE gel was visualized and quantified using a fluorescence image analyzer (FMBIO III; Hitachi Software Engineering) with excitation at 488 nm and emission at 520 nm. Aminoacylation of tRNAs by endogenous *E. coli* aminoacyl-tRNA synthetases was evaluated by adding non-aminoacylated tRNA, prepared by ligation of tRNA with non-aminoacylated pdCpA, to the translation reaction. The translation product was applied to 15% SDS–PAGE and analyzed by Western blotting using an anti-T7 tag antibody. The yield of the full-length streptavidin was quantified by comparing the band intensity with those for serial dilutions of the full-length streptavidin expressed from a wild-type mRNA. Incorporation of valine, glutamic acid, and tyrosine was evaluated in a similar manner using the corresponding aminoacyl-pdCpA [16].

Results

Screening of E. coli tRNAs

First, we examined amber suppressor tRNAs derived from 46 species of *E. coli* K12 tRNAs [14]. The experiment was carried out as shown in Fig. 1A. Three oligonucleotides were designed for each tRNA and connected using a primer extension reaction and PCR to generate tRNA genes. The resulting tRNA genes were transcribed by T7 RNA polymerase to obtain tRNAs lacking the 3′ terminal dinucleotide. The successful synthesis of the tRNA genes and the truncated tRNAs was confirmed by PAGE analysis. Aminoacylated tRNAs were then obtained by ligation of the truncated tRNAs with a dinucleotide pdCpA aminoacylated with BFLAF. A streptavidin gene containing a T7 tag and a His tag at the N- and C-termini, respectively, and a UAG codon at the Tyr83 position was used to evaluate the incorporation of BFLAF. The streptavidin mRNA was expressed in an *E. coli* cell-free translation system in the presence of each BFLAF-tRNA. The translation products were separated by SDS–PAGE and the gel was visualized with a fluorescence image analyzer.

The fluorescence image of the SDS–PAGE gel (Fig. 1B) shows that Trp tRNA (Fig. 1C) gave a distinct fluorescent band corresponding to the full-length streptavidin. This indicates that Trp tRNA successfully introduced BFLAF into streptavidin in response to the UAG codon. Thr_4 tRNA also gave a fluorescent band, although the intensity was weaker than that for Trp tRNA. An amber suppressor tRNA derived from yeast Phe tRNA had very low activity in introducing BFLAF in response to the UAG codon. The relative incorporation efficiency of BFLAF for Trp tRNA was however about a half of that for yeast Phe tRNA in response to the CGGG four-base codon (Fig. 1D and E).

For the specific incorporation of non-natural amino acids, suppressor tRNAs are required not to be aminoacylated with any natural amino acids by aminoacyl-tRNA synthetases. To evaluate the enzymatic aminoacylation of suppressor tRNAs, the cell-free translation was carried out in the presence of non-aminoacylated tRNAs,