Multiple incorporation of non-natural amino acids into a single protein using tRNAs with non-standard structures

Takashi Ohtsuki, Taishi Manabe, Masahiko Sisido*

Department of Bioscience and Biotechnology, Okayama University, 3-1-1 Tsushimanaka, Okayama 700-8530, Japan

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Abstract The ability to introduce non-natural amino acids into proteins opens up new vistas for the study of protein structure and function. This approach requires suppressor tRNAs that deliver the non-natural amino acid to a ribosome associated with an mRNA containing an expanded codon. The suppressor tRNAs must be absolutely protected from aminoacylation by any of the aminoacyl-tRNA synthetases in the protein synthesizing system, or a natural amino acid will be incorporated instead of the non-natural amino acid. Here, we found that some tRNAs with non-standard structures could work as efficient four-base suppressors fulfilling the above orthogonal conditions. Using these tRNAs, we successfully demonstrated incorporation of three different non-natural amino acids into a single protein.

Keywords: tRNA; Non-natural amino acid; Mitochondrial tRNA; Four-base codon; Orthogonal tRNA

1. Introduction

Non-natural amino acids can be incorporated into proteins by the delivery of an aminoacylated suppressor tRNA to a ribosome that is associated with a mRNA containing an expanded codon/anticodon pair (reviewed in [1–4]). In *Escherichia coli* translation systems, several four-base codon/anticodon pairs have been used to incorporate non-natural amino acids [1] and some have been used to introduce multiple kinds of non-natural amino acids [5]. Only a few four-base suppressor tRNAs which are totally protected from aminoacylation by endogenous aminoacyl-tRNA synthetase (ARS) have been reported [6–8]. To expand the availability of the four-base codon strategy, more suppressor tRNAs having different backbone sequences and different four-base anticodons need to be devised.

Since tRNAs of non-standard structures seem to easily escape recognition by *E. coli* ARSs in comparison with tRNAs of standard cloverleaf structures and their mutants, we screened for efficient four-base suppressor tRNAs from various non-standard tRNAs, specifically to determine if such non-standard tRNAs can evade recognition by *E. coli* ARSs and act as efficient carriers of non-natural amino acids.

Almost all tRNAs in the prokaryotic and eukaryotic cytoplasm have well-conserved cloverleaf secondary structures bearing a 7-bp acceptor stem, a 5-bp anticodon stem, a 5-bp T stem, and a 4-bp D stem. However, some exceptions have been found [9], primarily in animal mitochondria. For example, bovine mitochondrial (mt) $tRNA^{Ser}_{GCU}$ lacks the entire D stem [10], most nematode mt tRNAs lack the entire T stem [11], and the bovine mt $tRNA^{Ser}_{UGA}$ has a long anticodon stem and short connector regions between the acceptor and D stems and the anticodon and T stems [10]. A few exceptional secondary structures have been found in cytoplasmic tRNA species, such as eukaryotic $tRNA^{[Ser]Sec}$, which bears a 9-bp acceptor stem, a 4-bp T stem and a 6-bp D stem [12], and *Methanosarcina barkeri* $tRNA^{Pyl}$ [13], which has the same structural features as bovine mt $tRNA^{Ser}_{UGA}$. These non-standard tRNAs seem to be a good starting point to develop four-base suppressor tRNAs as carriers of non-natural amino acids.

2. Materials and methods

2.1. Preparation of tRNAs possessing a four-base anticodon

To generate DNA templates for transcription, primer extension was performed using two primers (1 μM each) in a 100-μL reaction mixture containing 0.2 mM dNTPs, 25 units KOD Dash DNA polymerase (Toyobo, Japan), and 10 μL of 10× Buffer #1, with the following temperature program: 94 °C for 60 s, and 3 cycles of 94 °C for 30 s, 55 °C for 2 s, and 72 °C for 30 s. The primers were designed to complement each other at each 3′ region (about 20 nucleotides). The resultant double-stranded DNA was collected by precipitation with 2-propanol. The transcription reaction was performed at 37 °C for 4 h in a reaction mixture that contained 40 mM Tris–HCl (pH 8.0), 24 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 0.01% Triton X-100, 10 mM GMP, 2 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP, 1.8 units/mL pyrophosphatase (Sigma), 750 units/mL T7 RNA polymerase (Takara), and 200 nM DNA template. The tRNA transcripts were purified in a 10% denaturing polyacrylamide gel. Non-natural amino acid (Xaa)-tRNAs were prepared by ligating transcribed tRNA without the 3′-CA sequence and the aminoacyl dinucleotide (pdCpA) with T4 RNA ligase as described [14,15].

2.2. In vitro translation and Western blot analysis

Mutated streptavidin mRNAs with four-base codons were prepared as described [16]. A T7-tag sequence was also encoded at the N-terminus of this streptavidin mRNA so that the protein could be detected with an anti-T7 antibody. In vitro translation and Western blot analysis were performed as described [16]. Briefly, an in vitro translation mixture (10 μL) containing 2 μL *E. coli* S30 Extract for Linear Templates (Promega), 55 mM HEPES–KOH (pH 7.5), 210 mM potassium

*Corresponding author. Fax: +81 86 251 8219.
E-mail address: sisido@cc.okayama-u.ac.jp (M. Sisido).

Abbreviations: ntrPhe, L-*p*-nitrophenylalanine; acdAla, L-2-acridonylalanine; 2napAla, L-2-naphthylalanine; 2antAla, L-2-anthrylalanine; Xaa, non-natural amino acid; ARS, aminoacyl-tRNA synthetase; mt, mitochondrial; Sec, selenocysteine; Pyl, pyrrolysine; EF-Tu, elongation factor Tu

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glutamate, 6.9 mM ammonium acetate, 1.7 mM dithiothreitol, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% polyethyleneglycol-8000, 35 μg/mL folinic acid, 12 mM magnesium acetate, 0.1 mM of each amino acid, 8 μg mRNA, and 0.1 nmol aminoacyl-tRNA was incubated at 37 °C for 1 h. The reaction mixture was separated using a 15% SDS–polyacrylamide gel. Proteins were transferred to a PVDF membrane (Bio-Rad) and Western blotted using an anti-T7-tag monoclonal antibody (Novagen) and the Proto-Blot II AP system (Promega). The efficiency of the four-base decoding was estimated by comparing the band intensity of the full-length product with those of serial dilutions of wild-type streptavidin expressed in vitro. The band intensity was evaluated using ImageJ Ver.1.34s (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). The efficiency was measured at least three times and is expressed as mean ± S.D.

3. Results and discussion

The sequences and secondary structures of these non-standard tRNA candidates for four-base suppressors are shown in Fig. 1. As the first generation of suppressor tRNAs, we synthesized non-standard $tRNA_{CCCG}$s in which only the anticodons were mutated to a CCCG anticodon. The main bodies of the tRNAs were taken from mouse $tRNA^{Sec}$ [12], *Methanosarcina acetivorans* $tRNA^{Pyl}$ [17], human mt $tRNA^{Leu}_{UAA}$ [18], and bovine mt $tRNA^{Phe}$, $tRNA^{Ser}_{UGA}$, and $tRNA^{Ser}_{GCU}$ [10]. Mouse $tRNA^{Sec}$ and *M. acetivorans* $tRNA^{Pyl}$ are tRNAs for the natural 21st selenocysteine (Sec) and 22nd pyrrolysine (Pyl) amino acids. For second generation suppressor tRNAs, the main bodies were mutated to replace mismatch pairs with cognate ones in the stem structures of mouse $tRNA^{Sec}_{CCCG}$, human mt $tRNA^{Leu}_{(UAA)CCCG}$, bovine mt $tRNA^{Phe}_{CCCG}$ and $tRNA^{Ser}_{(UGA)CCCG}$, because the mismatches seemed to cause low suppression efficiency observed in the first generation tRNAs. The mutated positions are indicated by arrows in Fig. 1.

The $tRNA_{CCCG}$'s designed and synthesized above were first examined to determine if they are protected from aminoacylation by endogeneous ARSs. Various deacyl-$tRNA_{CCCG}$s were added to an *E. coli* translation system for expressing streptavidin from an mRNA having a CGGG codon at the 83rd position of streptavidin (Fig. 2A). The yields of full-length streptavidin was used as the measure of suppression efficiency (Fig. 2B). If deacyl-$tRNA_{CCCG}$s are totally protected from endogenous ARSs, no decoding of the CGGG codon will take place, resulting in the absence of full-length protein. Fig. 2B shows that none of the non-standard tRNAs examined in this study produced full-length streptavidin, only truncated proteins were formed. These results indicate either that these tRNAs are absolutely protected from endogenous ARSs or they are totally ignored in the *E. coli* translation system. In these experiments, yeast $tRNA^{Phe}_{CCCG}$ was used as a control. A small amount of full-length streptavidin was produced in the

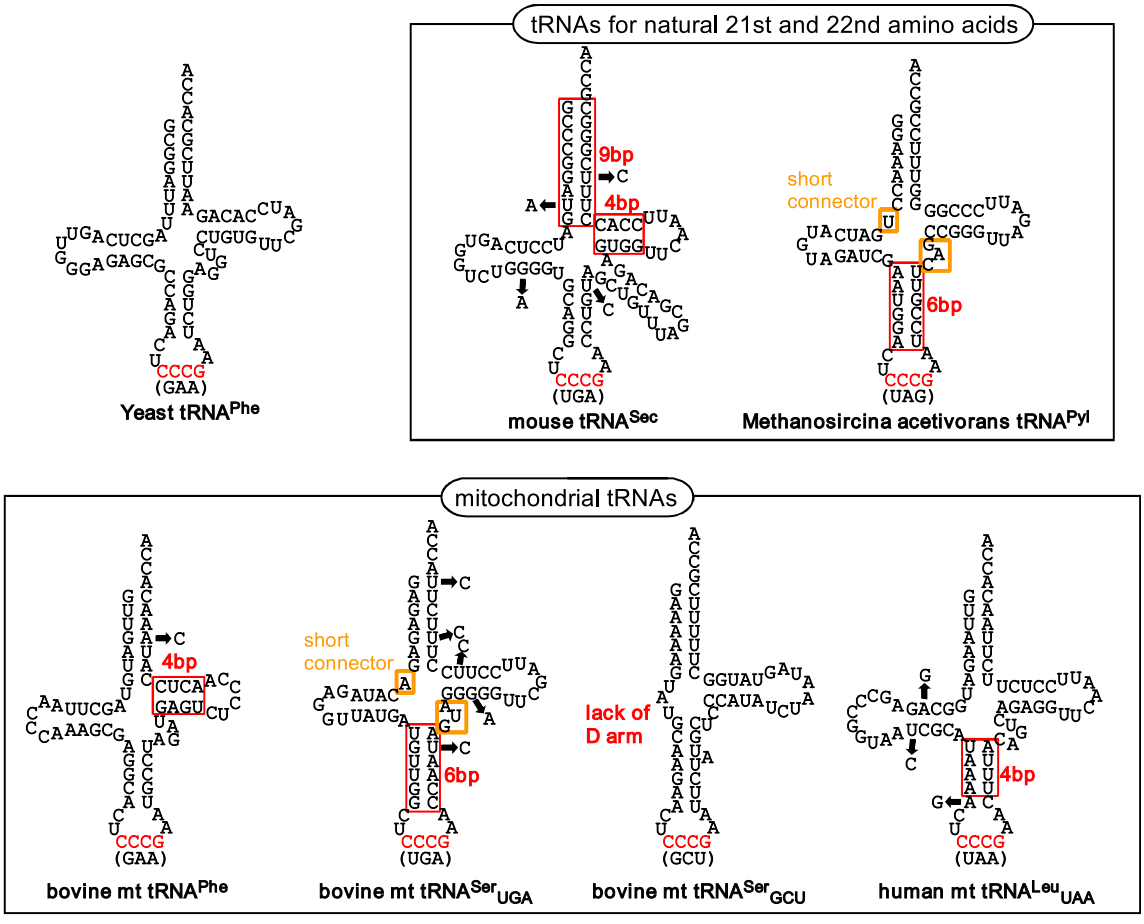

Fig. 1. Sequences and secondary structures of suppressor tRNAs bearing CCCG anticodons. Parentheses show the original anticodon sequences. Characteristic secondary structures are shown by red boxes and letters and unique short connector regions are shown by orange boxes. Arrows indicate mutations introduced to replace mismatch pairs by cognate ones in the stem regions.

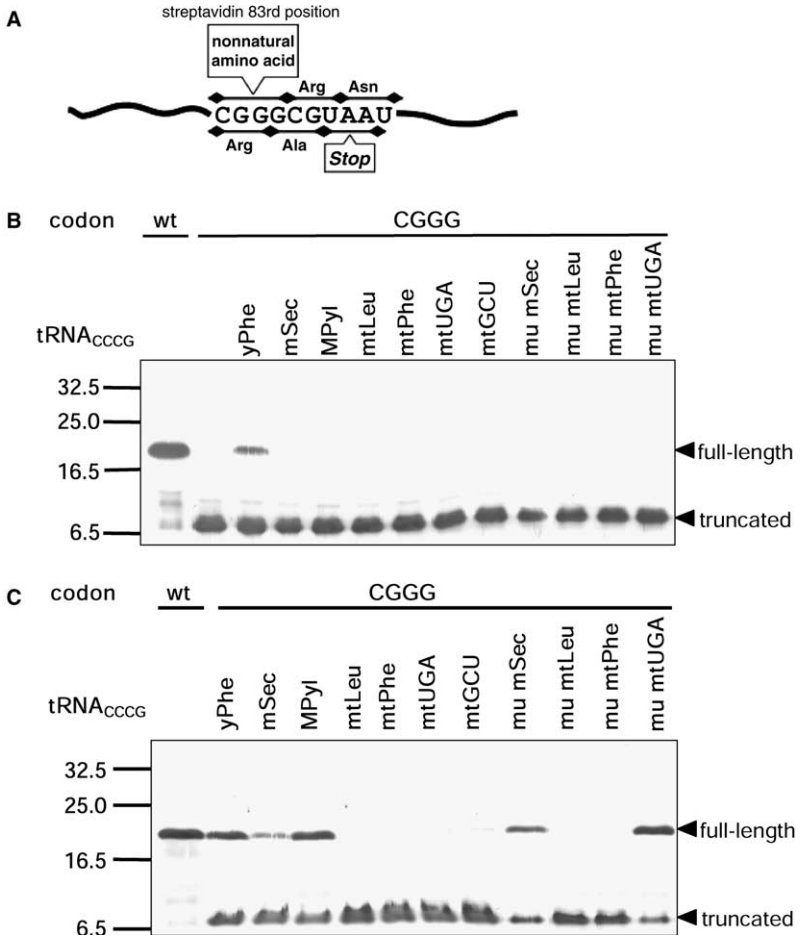

Fig. 2. Four-base suppression experiment using various tRNAs$_{CCCG}$. (A) The mRNA sequence of a mutated streptavidin and its associated amino acids (positions 83–85). When the CGGG four-base codon is translated with an aminoacyl-tRNA$_{CCCG}$, the correct reading frame is maintained. When the CGGG sequence is decoded as a CGG triplet by an endogenous arginyl-tRNA, the translation will be stopped at the UAA codon. (B, C) Western blot analysis of translation products using suppressor tRNA$_{CCCG}$s derived from yeast tRNA^{Phe} (yPhe); mouse tRNA^{Sec} (mSec); *M. acetivorans* tRNA^{Pyl} (MPyl); human mt tRNA$^{Leu}_{UAA}$ (mtLeu); bovine mt tRNA^{Phe} (mtPhe), tRNA$^{Ser}_{GCU}$ (mtGCU), and tRNA$^{Ser}_{UGA}$ (mtUGA); and four mutants with mismatches removed (mu mSec, mu mtLeu, mu mtPhe, and mu mtUGA). Translation using suppressor tRNA$_{CCCG}$s was performed using the mutant streptavidin mRNA with the CGGG codon shown in (A). Wild-type streptavidin was also translated in vitro using wild-type mRNA (wt), which is identical to the mutant mRNA except for the 83rd codon. Western blotting analysis was performed as described in Section 2. (B) Translation using deacyl-tRNA$_{CCCG}$s. (C) Translation using ntrPhe-tRNA$_{CCCG}$s.

presence of this control tRNA, indicating that the tRNA was recognized by some endogenous ARSs. The aminoacyl-tRNA prepared by the chemical aminoacylation method [14,15] bears 75dC which prevents most undesired aminoacylation [15]. However, tRNAs with no deoxynucleosides are preferred when other aminoacylation methods are employed. The undesired aminoacylation of yeast tRNA$^{Phe}_{CCCG}$ took place primarily by arginyl-tRNA synthetase [19]. The inertness of the non-standard tRNAs in Fig. 2B, whether this indicates full protection from ARSs or that they are ignored by the translation system machinery, makes them a good starting point for finding efficient and orthogonal tRNAs as carriers of non-natural amino acids.

A streptavidin mRNA having a ^{83}CGGG codon was translated in vitro with various tRNA$_{CCCG}$s chemically aminoacylated with *p*-nitrophenylalanine [14,15]. The translation products were analyzed by Western blotting (Fig. 2C). The efficiency of CGGG suppression by each tRNA$_{CCCG}$ was determined by comparing the band intensity of the full-length streptavidin to the wild-type streptavidin expressed in vitro (Table 1). Most of the non-standard tRNAs showed no suppression efficiency even when chemically aminoacylated, indicating that they are totally ignored in the *E. coli* system. But, *M. acetivorans* tRNA$^{Pyl}_{CCCG}$ showed high suppression efficiency (about 70% compared to wild-type streptavidin). Mouse tRNA$^{Sec}_{CCCG}$ showed less efficient but definite suppression. Because they are protected from ARSs, these tRNAs can be used as four-base suppressor tRNAs to incorporate non-natural amino acids in the *E. coli* translation system. Some tRNAs with mismatches in their stem regions became efficient suppressors when the mismatches were removed (Fig. 1). For example, the mouse tRNA$^{Sec}_{CCCG}$ mutant showed 15% suppression, slightly higher efficiency than the original sequence. More surprisingly, very high suppression efficiency was observed in the mismatch-removed bovine mt tRNA$^{Ser}_{(UGA)CCCG}$ mutant (80%), although no detectable suppression was observed in the original sequence.

Bovine mt tRNA$^{Ser}_{UGA}$ has a long anticodon stem which is structurally compensated by its short connector regions to generate a tertiary structure similar to standard tRNAs

Table 1
Efficiency of CGGG codon suppression using deacyl-tRNA or nitrophenylalanyl-tRNA (%)

	deacyl-$tRNA_{CCCG}$	ntrPhe-$tRNA_{CCCG}$
Yeast $tRNA^{Phe}$	20 (±10)	70 (±10)
mouse $tRNA^{Sec}$	ND[a]	11 (±5)
M. acetivorans $tRNA^{Pyl}$	ND	68 (±7)
Human mt $tRNA^{Leu}$	ND	ND
Bovine mt $tRNA^{Phe}$	ND	ND
Bovine mt $tRNA^{Ser}_{UGA}$	ND	ND
Bovine mt $tRNA^{Ser}_{GCU}$	ND	ND
Mouse $tRNA^{Sec}$ mutant	ND	15 (±1)
Human mt $tRNA^{Leu}$ mutant	ND	ND
Bovine mt $tRNA^{Phe}$ mutant	ND	ND
Bovine mt $tRNA^{Ser}_{UGA}$ mutant	ND	80 (±15)

The efficiency was defined as the relative yield of full-length mutant product to the wild-type streptavidin expressed in vitro. The data were obtained from at least three measurements.
[a]ND; not detected (<3%).

[20,21]. *M. acetivorans* $tRNA^{Pyl}$ has the same secondary structure as bovine mt $tRNA^{Ser}_{UGA}$. The above experimental results suggest that tRNAs with this secondary structure work efficiently in the *E. coli* translation system. Successful four-base decoding with mouse $tRNA^{Sec}_{CCCG}$ is somewhat surprising, because *E. coli* aminoacyl-$tRNA^{Sec}$ cannot interact efficiently with the *E. coli* elongation factor Tu (EF-Tu) [22]. The mouse tRNA may work in this system because mouse $tRNA^{Sec}$ does not have an antideterminant for prokaryotic EF-Tu (C7-G66, G49-U65, and C50-G64) [23]. A T stem that is 1 bp shorter than normal seems to be allowed in the *E. coli* system, as demonstrated by suppression of the CGGG codon by mouse $tRNA^{Sec}_{CCCG}$. However, in this $tRNA^{Sec}$, the shorter T stem may be compensated by a longer acceptor stem, which forms a consecutive co-axial helix with the T stem.

The three four-base suppressor tRNAs that specifically decoded the CGGG codon, i.e., *M. acetivorans* $tRNA^{Pyl}_{CCCG}$, mismatch-removed mutants of mouse $tRNA^{Sec}_{CCCG}$ and bovine mt $tRNA^{Ser}_{(UGA)CCCG}$, were discovered in the above experiment. The $tRNA^{Pyl}_{CCCG}$ and the mutated $tRNA^{Ser}_{(UGA)CCCG}$ were quite efficient. To demonstrate the availability of these two tRNAs, the same tRNAs bearing other four-base anticodons, AGAG and ACCC, were synthesized and decoding efficiencies of these tRNAs were evaluated. The $tRNAs_{AGAG}$ and $tRNAs_{ACCC}$ were again completely protected from endogenous ARSs. Supplementary Fig. 1 shows that L-*p*-nitrophenylalanine (ntrPhe)-incorporation efficiencies of these tRNAs were 43–90%, consistent with previously reported four-base suppressor tRNAs [6,8]. Supplementary Fig. 1 indicates that each tRNA probably has preferences in codon/anticodon sequence and codon position (i.e., neighboring sequence of the codon in an mRNA). For example, the $tRNA^{Pyl}_{CCCG}$ was the most efficient in suppressing the ^{54}CGGG codon, but the $tRNA^{Ser}_{(UGA)ACCC}$ was the most efficient in suppressing ^{120}GGGU. Thus, incorporation of multiple non-natural amino acids into a protein requires multiple kinds of optimal suppressor tRNAs to their respective codons.

As a demonstration of the high efficiency of the present tRNAs, we incorporated three different non-natural amino acids into a single protein using *M. acetivorans* $tRNA^{Pyl}_{CCCG}$, mutated bovine mt $tRNA^{Ser}_{(UGA)ACCC}$, and the mutated *E. coli* $tRNA^{Asn}_{AGAG}$ originally reported by Murakami et al. [8] (Fig. 3). The streptavidin mRNA used in this experiment in-

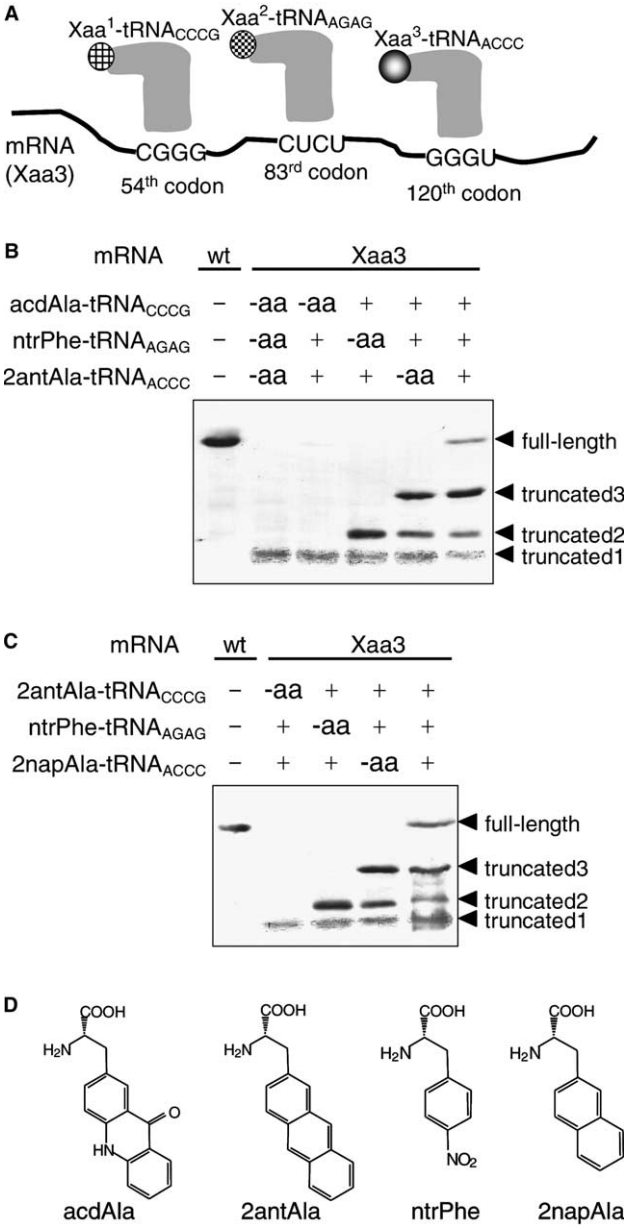

Fig. 3. Incorporation of three non-natural amino acids into a single protein using efficient four-base suppressor tRNAs. (A) A schematic representation of the mRNA of a streptavidin mutated at three positions (Xaa3 mRNA). Three aminoacyl-tRNAs bearing CCCU, AGAG and ACCC anticodons are depicted at the corresponding codon positions. Hatched and gradated circles illustrate non-natural amino acids (Xaa^1, Xaa^2 and Xaa^3). (B, C) Western blot analysis of translation products using three four-base suppressor tRNAs charged with non-natural amino acids. *M. acetivorans* $tRNA^{Pyl}_{CCCG}$, mutated *E. coli* $tRNA^{Asn}_{AGAG}$, and mutated bovine mt $tRNA^{Ser}_{(UGA)ACCC}$ were used in these experiment. Wild-type streptavidin was also translated in vitro using wild-type mRNA (wt), which is identical to the mutant mRNA except for the 54th, 83rd and 120th codons. Western blotting analysis was performed as described in Section 2. Twice as much Xaa3-mRNA translation mixture was loaded on the gel as the wild-type reaction mixture. [-aa] means the use of the deacyl-tRNAs. Truncated 1, 2 and 3 indicate the translation products truncated at the 54th, 83rd and 120th positions of streptavidin, respectively. (B) Translation using acdAla-$tRNA_{CCCG}$, ntrPhe-$tRNA_{AGAG}$, and 2antAla-$tRNA_{ACCC}$. (C) Translation using 2antAla-$tRNA_{CCCG}$, ntrPhe-$tRNA_{AGAG}$, and 2napAla-$tRNA_{ACCC}$. (D) Chemical structures of non-natural amino acids used in this study.

cludes three four-base codons; ^{54}CGGG, ^{83}CUCU and ^{120}GGGU (Fig. 3A). When these quadruplet codons are translated as triplet codons, a stop codon appears at each position (Supplementary Fig. 2). Translation stopped at these positions when a deacyl-tRNA was added instead of the aminoacyl-tRNA (Fig. 3B and C), indicating that no readthrough occurred with deacyl-tRNAs. Full-length mutant streptavidin including L-2-acridonylalanine (acdAla) at the 54th, ntrPhe at the 83th, and L-2-anthrylalanine (2antAla) at the 120th position (Fig. 3B), and a mutant including 2antAla at the 54th, ntrPhe at the 83th, and L-2-naphthylalanine (2napAla) at the 120th position (Fig. 3C) were successfully synthesized by this system. The incorporation of the three non-natural amino acids was confirmed by TOF-mass spectroscopy (Supplementary Fig. 3).

By adding efficient four-base suppressor tRNAs to the four-base decoding system, we can incorporate multiple non-natural amino acids into a single protein without misincorporation of any natural amino acid. In this study, incorporation of three different non-natural amino acids into a protein was demonstrated for the first time. Multiple incorporation of non-natural amino acids will expand the scope of the non-natural mutagenesis from a simple technique for fluorescence labeling, to the fabrication of complex protein systems where a variety of chemical functions are built in, for example, a protein including one functional probe, and two fluorescent probes that undergo FRET. The four-base suppressor tRNAs found in this work will be applicable for in vivo purposes as well, by importing suppressor tRNAs aminoacylated in vitro into a living cell [24,25], or by using an artificial ARS created from natural ARSs [6,7,26] or non-protein artificial ARSs (aminoacyl-peptide nucleic acid [27] or the ribozyme [28]).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.11.010.

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