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In vitro selection of tRNAs for efficient four-base decoding to incorporate non-natural amino acids into proteins in an *Escherichia coli* cell-free translation system

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ABSTRACT

Position-specific incorporation of non-natural amino acids into proteins is a useful technique in protein engineering. In this study, we established a novel selection system to obtain tRNAs that show high decoding activity, from a tRNA library in a cell-free translation system to improve the efficiency of incorporation of non-natural amino acids into proteins. In this system, a puromycin–tRNA conjugate, in which the 3′-terminal A unit was replaced by puromycin, was used. The puromycin–tRNA conjugate was fused to a C-terminus of streptavidin through the puromycin moiety in the ribosome. The streptavidin–puromycin–tRNA fusion molecule was collected and brought to the next round after amplification of the tRNA sequence. We applied this system to select efficient frameshift suppressor tRNAs from a tRNA library with a randomly mutated anticodon loop derived from yeast $tRNA^{Phe}_{CCCG}$. After three rounds of the selection, we obtained novel frameshift suppressor tRNAs which had high decoding activity and good orthogonality against endogenous aminoacyl-tRNA synthetases. These results demonstrate that the *in vitro* selection system developed here is useful to obtain highly active tRNAs for the incorporation of non-natural amino acid from a tRNA library.

INTRODUCTION

Position-specific incorporation of non-natural amino acids into proteins in a cell-free translation system is a powerful technique for structural and functional analyses of proteins and for design and synthesis of engineered proteins with artificial functions (1–6). A majority of researchers has been using an amber codon for assigning the positions of non-natural amino acids. In this case, a UAG codon is decoded by an amber suppressor tRNA that has been aminoacylated with a non-natural amino acid. The amber suppression method, however, has a disadvantage that the incorporation of a non-natural amino acid must compete with a release factor that binds to the amber codon and terminates the protein synthesis. Alternatively, we have developed a four-base codon strategy and successfully incorporated a wide variety of non-natural amino acids into proteins (7–9). The four-base decoding also must compete with a three-base decoding by endogenous tRNAs. But the competition becomes in favor by the use of four-base codons derived from rarely used codons such as CGG and GGG. For efficient incorporation of non-natural amino acids, it is also important to use tRNAs that show high affinity to a protein biosynthetic machinery, especially to ribosome. There are several attempts to find efficient tRNAs suitable for the amber suppression method from naturally occurring tRNAs (10,11). The screening process is, however, time-consuming and inefficient, because each tRNA has to be synthesized, aminoacylated and evaluated, separately. An improved screening has been reported using *in vivo* selection of tRNAs, selection of tRNAs, in which tRNAs recognized by any of aminoacyl-tRNA synthetases are selected from randomly mutated tRNA library (12,13). In their *in vivo* system, however, tRNAs that are not recognized by aminoacyl-tRNA synthetases cannot be selected. The selection of tRNA for non-natural amino acid incorporation should be carried out independently from the aminoacylation by any of aminoacyl-tRNA synthetases.

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Here, we propose a novel strategy to find efficient tRNAs that are appropriate for *in vitro* four-base decoding to incorporate non-natural amino acids. This strategy enables us to select tRNAs that show high affinity to translational machinery from a tRNA library in a cell-free translation system. For this strategy, a tRNA containing a puromycin moiety at 3′-terminus was used. The puromycin–tRNA conjugate will be fused to the C-terminus of an elongating peptide chain in a ribosomal system. The efficiency of the fusion synthesis will depend on relative population of tRNAs in the A site, that is determined by the affinity of each tRNA to the translational machinery. As a consequence, tRNAs with high decoding activity are selected by collecting the tRNA–peptide fusion. In this study, we developed the selection system of tRNA and then applied it to the selection of efficient frameshift suppressor tRNAs for the non-natural amino acid incorporation.

MATERIALS AND METHODS

Materials

Synthetic oligonucleotides were obtained from PROLIGO JAPAN. 5′-Phospho-deoxycytidyl-phospho-puromycin (pdCp-puromycin) and a fluorescently labeled primer BFL-YF515 were from Japan Bio Services. QIAquick PCR Purification kit and MinElute PCR Purification kit were purchased from QIAGEN. KOD Dash DNA polymerase was from TOYOBO. T7 RNA polymerase and Vent DNA polymerase were from New England BioLabs. T4 RNA ligase, *Bca*BEST RNA PCR kit ver1.1, GelStar Nucleic Acid Stain and ribonuclease inhibitor were from TaKaRa BIO. *Escherichia coli* S30 Extract System for Linear Templates and pGEM-T vector were from Promega. Inorganic pyrophosphatase and biotinamido-caproyl labeled BSA was from Sigma–Aldrich. Dynabeads M-280 Streptavidin was from DYNAL. Pellet Paint NF Co-precipitant and anti-T7 tag antibody were from Novagen.

Preparation of tRNA(-CA)

A yeast $tRNA^{Phe}$ containing a CCCG anticodon and lacking a 3′-terminal CA dinucleotide was prepared as described previously (8). The PCR mixture contained 0.1 nmol of an M13 Forward primer (CAACATTTTGCTGCCGGTCA), 0.1 nmol of a 3′-CA primer (GTGCGAATTCTGTGGATCGA), 0.1 μg of a plasmid encoding a yeast $tRNA^{Phe}$ gene under the control of the T7 promoter in pUC18 (8), 0.2 mM dNTPs, 2 U of Vent DNA polymerase in 100 μl of Vent buffer. After the PCR, the product was isolated by QIAquick PCR Purification kit. The PCR product was then supplied to a run-off transcription reaction containing 40 mM Tris–HCl (pH 8.0), 20 mM $MgCl_2$, 5 mM DTT, 4 mM NTP, 20 mM GMP, 2 mM spermidine, 10 μg/ml BSA, 40 U of ribonuclease inhibitor, 1 U of inorganic pyrophosphatase, 400 U of T7 RNA polymerase and 10 μg of template DNA in 100 μl. After incubation at 37°C for 18 h, the product was purified on a DEAE-cellulose column.

Construction of tRNA(-CA) library

A template DNA for the transcription of a tRNA library, in which the 32nd, 33rd, 37th and 38th positions of the anticodon loop were randomly mutated, was prepared using a primer extension reaction and PCR. A tRNA-5′ primer ATACGACTCACTATAGCGGATTTAGCTCAGTTGGG AGAGCGCCAGA containing a T7 promoter sequence, and a tRNA-AC primer GACCTCCAGANNCGGGNNTCTGGCGCTC containing a randomized anticodon loop sequence (NNCCCGNN, in which N indicates 1 of 4 nt) were added to a primer extension reaction containing KOD Dash buffer, 0.2 mM dNTP, 50 pmol tRNA-5′ primer, 50 pmol tRNA-AC primer, 1.25 U of KOD Dash DNA polymerase in a 50 μl reaction. Using the resulting reaction mixture (1 μl) as a template, PCR was carried out with a T7 promoter primer CTAATACGACTCACTATAG and a tRNA-3′ primer GTGCGAATTCTGTGGATCGAACACAGGACCTCCAGA in 50 μl. The PCR product was purified with MinElute PCR Purification kit and then subjected to a transcription reaction using T7 RNA polymerase as described above.

Preparation of puromycin–tRNA

Puromycin–tRNA was prepared by ligating pdCp-puromycin to the tRNA(-CA) with T4 RNA ligase. The ligation reaction mixture contained 55 mM HEPES-Na (pH 7.5), 10% DMSO (v/v), 1 mM ATP, 15 mM $MgCl_2$, 3.3 mM DTT, 20 μg/ml BSA, 0.5 nmol of tRNA(-CA), 1 nmol of pdCp-puromycin and 30 U of T4 RNA ligase. After incubation at 4°C for 6 h, the reaction mixture was extracted once with phenol/chloroform and once with chloroform, precipitated with ethanol, and then resolved in 10 μl of water. The ligation reaction was confirmed by 12% PAGE with 8 M urea, and the yield was estimated to be 80%.

Preparation of a streptavidin mRNA containing CGGG at C-terminus

A linker and CGGG sequence shown in Figure 2A was added by PCR to a streptavidin gene that had been constructed (8). The coding region of the resulting gene was amplified by PCR using T7 promoter and T7 terminator primers, and then transcribed to mRNA with T7 RNA polymerase as described previously (8).

Expression of streptavidin–tRNA fusion in a cell-free translation system

The expression of the streptavidin–tRNA fusion was performed by the addition of the puromycin–tRNA and the mRNA into an *E.coli* cell-free translation system. The translation reaction mixture contained 55 mM HEPES–KOH (pH 7.5), 210 mM potassium glutamate, 6.9 mM ammonium acetate, 1.7 mM DTT, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% poly(ethylene glycol)-8000, folinic acid at 35 μg/mL, 12 mM magnesium acetate, 0.1 mM each amino acids, 8 μg of mRNA, 100 pmol of puromycin–tRNA, and 2 μl of *E.coli* S30 extract in 10 μl. The reaction mixture was incubated at 37°C for 20 min, and then 4 μl of 0.5 M EDTA was added to dissociate the streptavidin–tRNA fusion from ribosome complex.

Collection of the streptavidin–tRNA fusion

The streptavidin–tRNA fusion was collected with biotin-coated magnetic beads as follows. Biotinamido-caproyl

(10 μg) labeled BSA was added to 20 μl of Dynabeads M-280 Streptavidin, and gently shaken at room temperature for 20 min. The beads were washed three times with 200 μl of TBS (20 mM Tris–HCl, pH 7.5 and 150 mM NaCl) to remove unbound biotinamido-caproyl BSA. The EDTA-treated translation mixture (14 μl) was added to the beads, and gently shaken at room temperature for 20 min. The beads were subsequently washed once with 200 μl of TBS, twice with 8 M urea, once with 5 M NaCl, twice with TBST (TBS + 0.1% Tween20), and finally once with TBS. The streptavidin–tRNA fusion bound onto the magnetic beads was eluted in 50 μl of TESUB (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1% SDS, 7 M urea and 8 mM biotin) by heating at 95°C for 10 min. The collected streptavidin–tRNA fusion was ethanol-precipitated with Pellet Paint NF Co-precipitant, and the pellet was resuspended in 6 μl of water and subjected to reverse transcription.

Reverse transcription and real-time quantitative PCR

Reverse transcription and real-time quantitative PCR were performed using *Bca*BEST RNA PCR kit ver1.1. The reaction mixture (20 μl) contained Bca first buffer, 0.5 mM dNTPs, 22 U of *Bca*BEST polymerase, 0.5 μM YF315 primer GTGCGAATTCTGTGG complementary to the 3′ region of the tRNA and 2.5 μl of the recovered streptavidin–tRNA fusion. After incubation at 65°C for 30 min, 6 μl portion was applied to real-time quantitative PCR (30 μl) containing Bca 2nd buffer, 0.75 U of Bca-optimized *Taq*, 0.2 μM YF315 and 0.2 μM BFL-YF515 primer that is complementary to the 5′ region of the tRNA and contains BODIPY FL through a C nucleotide at its 5′ end (BODIPY FL-CGCGGATTTAGCTCAG). The fluorescence intensity was monitored using SmartCycler System (Cepheid) with a pre-PCR heat step of 4 min at 94°C, followed by 50 cycles of 30 s at 94°C, 30 s at 48°C, 30 s at 72°C. The cycle number at a defined fluorescence intensity was determined for each sample, and the amount of the recovered tRNA was calculated using a calibration curve prepared from a series of non-mutated tRNA(-CA) solutions. The RT–PCR product was confirmed by 10% PAGE with GelStar staining.

Regenerative PCR

The RT–PCR products were purified on a 10% PAGE, and then amplified by overlap-extension PCR to regenerate the tRNA(-CA) gene. A 5′ primer T7YF526 which contained a T7 promoter sequence and a complementary sequence to 26 nt of the 5′ region of the tRNA (CTAATACGACTCACTATAGCGGATTTAGCTCAGTTGGGAGAGCG), and a 3′ primer YF331 complementary to 31 nt of the 3′ region of the tRNA(-CA) (GTGCGAATTCTGTGGATCGAACACAGGACCT) were used. The PCR product was purified with MinElute PCR Purification kit and used as a template for the tRNA pool for the next round of selection.

Evaluation of selected tRNAs

The selected tRNA genes were identified by TA-cloning and sequencing. To evaluate the activity of the selected tRNAs for the incorporation of non-natural amino acid, each clone was transcribed to tRNA(-CA), and then ligated with a pdCpA aminoacylated with a fluorescently labeled amino acid, BODIPY FL *p*-aminophenylalanine (14). The resulting BODIPY FL *p*-aminophenylalanine-tRNA was added to the *E.coli* cell-free translation system together with a streptavidin mRNA containing a CGGG four-base codon at the Tyr83 position. The expression of the streptavidin containing BODIPY FL *p*-aminophenylalanine was confirmed by 15% SDS–PAGE. The fluorescence image was measured by a fluorescence image analyzer (FMBIO III; Hitachi software engineering), and the band intensity of the fluorescent streptavidin was quantified. Using the SDS–PAGE gel, western blotting was carried out using anti-T7 tag antibody.

To test the aminoacylation of the selected tRNAs by endogenous aminoacyl-tRNA synthetases, each tRNA(-CA) was ligated with non-aminoacylated pdCpA and then added to the cell-free translation system together with the streptavidin mRNA containing CGGG codon at the Tyr83 position. The translation product was analyzed by western blotting using anti-T7 tag antibody, and the relative yield of the full-length streptavidin was quantified by comparing the band intensity with those of serial dilutions of the wild-type streptavidin.

RESULTS AND DISCUSSION

Strategy of *in vitro* selection of tRNA

In order to take up tRNAs that show high affinity to translational machinery from a tRNA library, it is necessary to collect tRNAs that are successfully incorporated into ribosome. For this purpose, we designed puromycin–tRNA that contained a puromycin moiety in place of a 3′-terminal aminoacyl-adenosine unit (Figure 1A). The puromycin–tRNA with a CCCG anticodon is expected to bind to ribosomal A site, when a CGGG four-base codon comes to the A site. In the present case, a streptavidin mRNA containing a four-base codon CGGG at the 3′-terminus of streptavidin gene was added to a cell-free translation system. When the CGGG is decoded by the puromycin–$tRNA_{CCCG}$, a peptide bond will be formed between the amino group of the puromycin moiety and the carboxyl group of the C-terminus of streptavidin. The resulting streptavidin–puromycin–tRNA may be translocated to the P-site. In this case, the next aminoacyl-tRNA binds to the vacant ribosomal A site, but can not react with the streptavidin–puromycin–tRNA because the corresponding carbonyl group of the growing streptavidin is linked to the $tRNA_{CCCG}$ through an amide bond instead of an ester bond. Consequently, a covalently-linked streptavidin–tRNA fusion will remain seated in the ribosome–mRNA complex. This complex can be dissociated by the addition of EDTA, and the covalently-linked streptavidin–tRNA fusion will be released from the complex. The streptavidin–tRNA fusion can be collected by biotin-immobilized materials, like biotin-coated magnetic beads. Although an enzymatically aminoacylated tRNA containing 3′-amino-3′-deoxyadenosine at its 3′-terminus had been utilized for ribosome display (15), we independently designed the puromycin–tRNA conjugate and utilized it for *in vitro* selection of tRNA.

Using the puromycin–tRNA conjugate and streptavidin gene, selection of tRNAs is performed according to a scheme

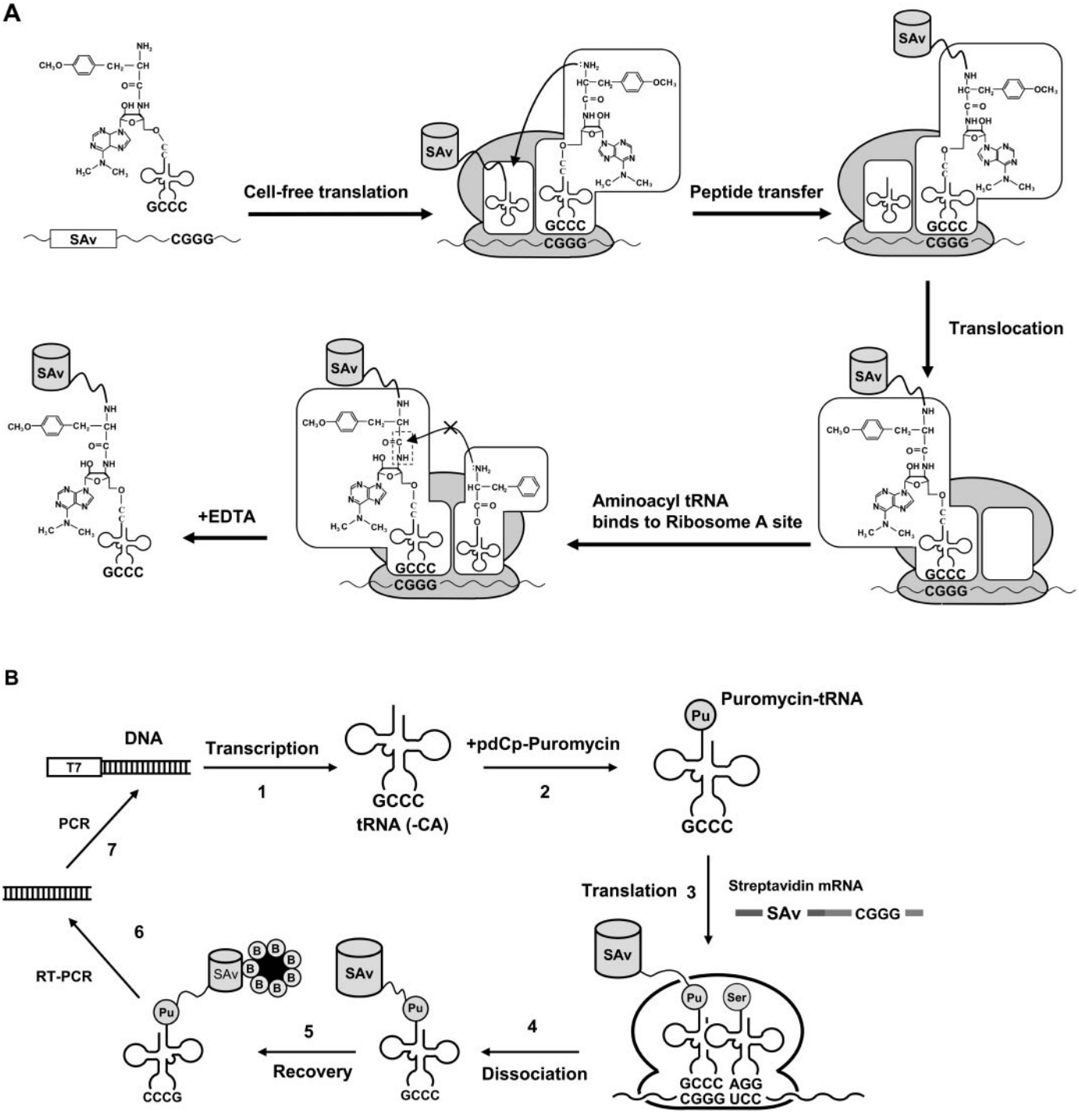

Figure 1. (**A**) Schematic illustration of the formation of streptavidin–tRNA fusion using puromycin–tRNA, which contains a puromycin moiety in the place of 3′ terminal aminoacyl-adenosine and a four-base anticodon CCCG. The puromycin–tRNA binds to ribosomal A site and accepts a streptavidin polypeptide chain as an analog of aminoacyl-tRNA in response to a four-base CGGG codon at 3′ terminus of streptavidin mRNA in a cell-free translation. The resulting streptavidin–puromycin–tRNA may be translocated to the P-site. In this case, the next aminoacyl-tRNA binds to the vacant ribosomal A site, but can not accept the polypeptide chain because of the amide bond of puromycin–tRNA. The resulting streptavidin–tRNA fusion is released from the ribosome complex by the addition of EDTA. (**B**) Schematic illustration of the *in vitro* selection system of tRNAs. Step 1, a DNA pool encoding tRNAs containing a four-base anticodon CCCG is transcribed by T7 RNA polymerase to tRNA(-CA) pool. Step 2, the tRNA(-CA) pool is ligated with pdCp-Puromycin by T4 RNA ligase to generate puromycin–tRNA. Step 3, a streptavidin mRNA containing a four-base CGGG codon at C-terminus is translated in an *E.coli* cell-free translation system in the presence of the puromycin–tRNA. Puromycin–tRNAs that successfully decode the CGGG codon form ribosome–mRNA–streptavidin–tRNA complex. Step 4, the streptavidin–tRNA fusion is dissociated from the complex by the addition of EDTA. Step 5, the streptavidin–tRNA fusion is recovered with biotin-coated magnetic beads. Step 6, the streptavidin–tRNA fusion is dissociated from the beads, and then the tRNA moiety is subjected to RT–PCR. Step 7, the tRNA genes are regenerated by overlap-extension PCR with a T7 promoter primer, which are used as template DNAs in the next round of selection.

of Figure 1B. First, a DNA pool encoding T7 promoter and tRNAs containing a four-base anticodon CCCG is designed and constructed. The DNA pool is then transcribed by T7 RNA polymerase to give a pool of tRNAs lacking 3′-terminal dinucleotide. The tRNA(-CA) pool is then ligated with 5′-phosphodeoxycytidyl-phospho-puromycin (pdCp-puromycin) by T4 RNA ligase to obtain a puromycin–tRNA conjugate pool. The puromycin–tRNA conjugates are added to a cell-free translation system together with a streptavidin mRNA containing a four-base CGGG codon at the C-terminus. The puromycin–tRNA conjugates that were successfully incorporated into the ribosome form streptavidin–tRNA fusions in a ribosome complex. The streptavidin–tRNA fusions are dissociated from the complex by the addition of EDTA, and recovered with biotin-coated magnetic beads. A pool of the recovered streptavidin–tRNA fusion is subjected to RT–PCR. After overlap-extension PCR to regenerate a DNA pool encoding tRNAs under the control of the T7 promoter, a pool of tRNAs is prepared and applied to the next round of selection. Multiple cycles of selection will enrich the tRNAs that show high decoding activity in the translation system.

Formation of streptavidin–tRNA fusion

Formation of the fusion of $tRNA_{CCCG}$ and streptavidin was first examined. A modified streptavidin gene containing five CGGG codons (CGGGTC CGGGCA CGGGTG CGGGCT CGGGTC) at the downstream end of streptavidin gene through a linker sequence was designed and constructed (Figure 2A). If the first CGGG was decoded as a CGG three-base codon to arginine, then the second CGGG four-base appears. If the second CGGG was also decoded as a three-base codon, then the third appear, and so on. So, the repeated CGGG codons will markedly enhance the efficiency of the four-base decoding by the puromycin–$tRNA_{CCCG}$ and decoding of any of the five CGGG codons by the puromycin–$tRNA_{CCCG}$ will give the streptavidin–tRNA fusion. On the other hand, if all of the five CGGG codons are translated by endogenous arginyl-$tRNA_{CCG}$, free streptavidin that does not contain the puromycin–tRNA conjugate will be produced by a UAA stop codon at the 3′ end. The linker peptide sequence GlyGlySerGlyGlySer will serve to help tetramerization of streptavidin. The DNA encoding the streptavidin–5×CGGG gene was prepared by overlap-extension PCR, and the corresponding

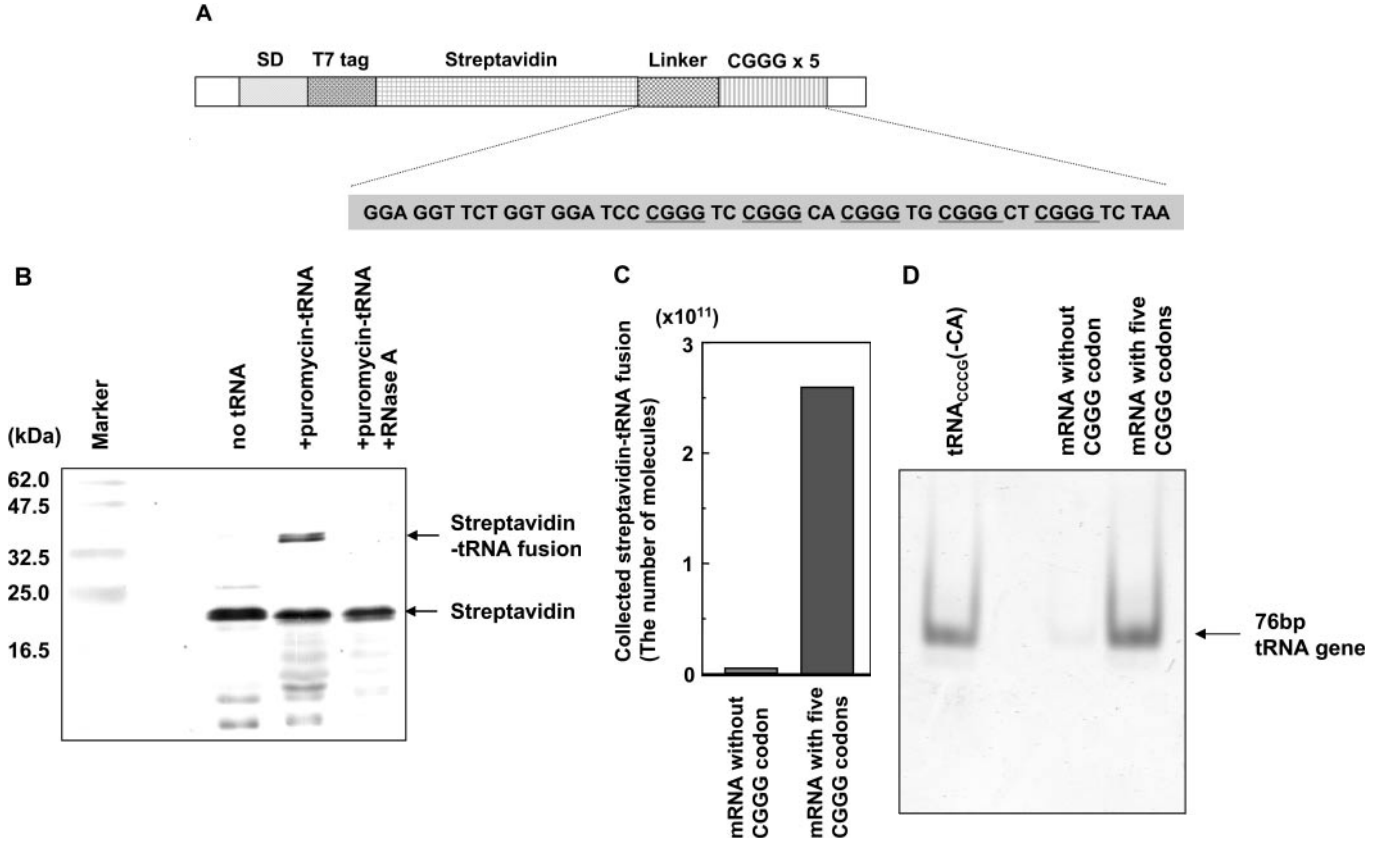

Figure 2. Formation of streptavidin–tRNA fusion and recovery of the corresponding tRNA gene using yeast $tRNA^{Phe}_{CCCG}$. (**A**) An mRNA sequence used for the formation of streptavidin–tRNA fusion. The streptavidin gene contains T7 tag at N-terminus for the detection by western blot analysis, and five CGGG codons at C-terminus through a linker sequence consisted of GlyGlySerGlyGlySer sequence. (**B**) Western blot analysis of the formation of streptavidin–tRNA fusion by adding the streptavidin mRNA to a cell-free translation system in the absence of the puromycin–tRNA, in the presence of the puromycin–tRNA, and in the presence of the puromycin-tRNA and after treatment with RNaseA. (**C**) The amount of tRNA recovered with biotin-coated magnetic beads as streptavidin–tRNA fusion was determined by quantitative PCR analysis. The tRNAs were recovered from the cell-free translation products obtained in the presence of mRNA without CGGG codon, and with five CGGG codons at C-terminus. (**D**) PAGE analysis of the RT–PCR product of the recovered tRNAs obtained in the presence of mRNA without CGGG codon, and with five CGGG codons at C-terminus. The RT–PCR product of the yeast $tRNA^{Phe}_{CCCG}$(-CA) was applied as a positive control.

mRNA was synthesized by T7 RNA polymerase as previously described. Puromycin–tRNA conjugate was prepared by a chemical aminoacylation method (16) using pdCp-puromycin and a synthetic yeast $tRNA^{Phe}$ lacking 3′ CA dinucleotide and containing a CCCG four-base anticodon.

The puromycin–yeast $tRNA^{Phe}_{CCCG}$ conjugate and the mRNA were added to an *E.coli* cell-free translation system. The translation product was analyzed by western blot using an anti-T7 tag monoclonal antibody to detect T7-tagged translation products. As shown in Figure 2B, in the presence of the puromycin–tRNA conjugate, a band was observed around 43 kDa corresponding to the expected molecular weight of the streptavidin–tRNA fusion. On the other hand, in the absence of the puromycin–tRNA, only free streptavidin was observed around 20 kDa. When the translation product obtained in the presence of the puromycin–tRNA was treated with RNaseA, the band around 43 kDa disappeared. These results suggest that the covalently-linked tRNA–streptavidin fusion is successfully obtained as the 43 kDa band. When a streptavidin mRNA containing one CGGG sequence was used, only a weak band was observed around 43 kDa, indicating the effectiveness of the five CGGG sequences.

Recovery of yeast $tRNA^{Phe}_{CCCG}$ and quantitative RT–PCR

Next, selective recovery of the streptavidin–yeast $tRNA^{Phe}_{CCCG}$fusion by biotin-coated magnetic beads was investigated. To the cell-free translation mixture, EDTA was added to release the covalently-linked streptavidin–tRNA fusion from ribosome–mRNA complex. The streptavidin–tRNA fusion was collected by biotin-coated magnetic beads, and was used as a template of RT–PCR. Reverse transcription was performed at 65°C using a thermostable RT. Under this condition, tRNA molecules could be unfolded to facilitate the reverse transcription.

The quantitative PCR was then performed according to a method reported by Kurata *et al.* (17), in which a primer containing BODIPY FL and cytosine at 5′ end was used. When the BODIPY FL-labeled primer becomes double-stranded by PCR amplification, the fluorescence of BODIPY FL will be quenched by a guanine unit on the opposite strand. The PCR process was followed by the decrease of the fluorescence of BODIPY FL. The cycle number at which the fluorescence intensity reached a defined value was determined, and the initial amount of tRNA was calculated from the cycle number using a calibration curve prepared from a series of tRNA(-CA) solutions. As shown in Figure 2C, 2.6×10^{11} molecules of tRNA were found to be recovered from the puromycin–tRNA conjugate after *in vitro* translation in the presence of streptavidin mRNA containing five CGGG sequences. The RT–PCR product was analyzed on 10% PAGE, and a band of 76 bp corresponding to the tRNA gene was observed (Figure 2D). The recovered tRNA genes were then cloned, and 20 clones were randomly picked up and sequenced. All of the 20 clones were identical to the yeast $tRNA^{Phe}_{CCCG}$. On the other hand, after the *in vitro* translation in the presence of mRNA that did not contain the CGGG sequence, only 2.0×10^9 molecules of tRNA were recovered. The very small amount of tRNA may be resulted from a non-specific adsorption of the puromycin–tRNA conjugates to the magnetic beads. These results demonstrate that the puromycin–$tRNA_{CCCG}$ fused to streptavidin is recovered by the biotin-coated magnetic beads, and that the tRNA can be successfully reverse-transcribed despite of a large streptavidin moiety that is fused. In addition, the result supports that the puromycin–$tRNA_{CCCG}$ conjugate is incorporated into ribosome in response to the four-base codon CGGG, and that the unreacted puromycin–$tRNA_{CCCG}$ conjugate can be removed from the beads by washing.

The amount of the recovered tRNA (2.6×10^{11} molecules) corresponds to 0.4% of the added puromycin–tRNA (6.0×10^{13} molecules). Because of the limited amount of mRNA and ribosome in the cell-free translation system, and limited productivity of the cell-free system, not all of the puromycin–tRNA initially added will form the streptavidin–tRNA fusion. The recovery rate (0.4%) is thought to be sufficient for the effective selection.

Selection of tRNAs from a tRNA library with randomly mutated anticodon loops

The *in vitro* selection system for tRNAs was then utilized to obtain tRNAs that are effective for the four-base decoding from a tRNA library with randomly mutated anticodon loops. In this work, random mutations were carried out at the 32nd, 33rd, 37th and 38th positions in the anticodon loop of yeast $tRNA^{Phe}_{CCCG}$(Figure 3A). Because one additional nucleotide unit has been inserted to the anticodon loop of the $tRNA_{CCCG}$, optimization of the anticodon loop will be essential for finding efficient tRNAs. A pool of template DNA was synthesized by PCR with three synthetic oligonucleotides including the random mutations. The sequence analysis of 20 tRNA clones from the non-selected pool indicates the 32nd, 33rd, 37th and 38th positions are randomly mutated. Then a pool of puromycin–$tRNA_{CCCG}$ conjugates was prepared and added to the cell-free translation system together with the streptavidin mRNA containing five CGGG codons at the C-terminus. Puromycin–tRNA conjugates that show high affinity to the translational machinery were expected to form streptavidin–tRNA fusions preferentially. The streptavidin–tRNA fusion was recovered with the biotin-coated magnetic beads and subjected to quantitative RT–PCR. The RT–PCR product was then used as a template for the next round of selection.

The amount of recovered tRNA in each round of selection is shown in Figure 3B. At the first round, only a very small amount of tRNA was recovered. The recovered tRNA, however, increased markedly and reached saturation level after the second round. Western blot analysis of the cell-free translation product also indicated that the streptavidin–tRNA fusion was successfully obtained after the second and third round of selection, whereas only very small amount of the fusion was obtained after the first round (Figure 3C). These results suggest that the tRNAs with high decoding activity are accumulated rapidly from the randomized pool.

The tRNA genes recovered after the third round were cloned and sequenced. Of 43 clones picked up, 8 CU-CCCG-AA, 5 CU-CCCG-AU, 4 CA-CCCG-AA, 3 UC-CCCG-AC, 2 UU-CCCG-AC and AU-CCCG-AC, and 19 single clones were found (Table 1). The anticodon loop sequence CU-AA and UU-AC are naturally occurring in *E.coli* tRNAs (*E.coli* $tRNA^{Ala1B}$ has a UU-UGC-AC anticodon loop), but others are

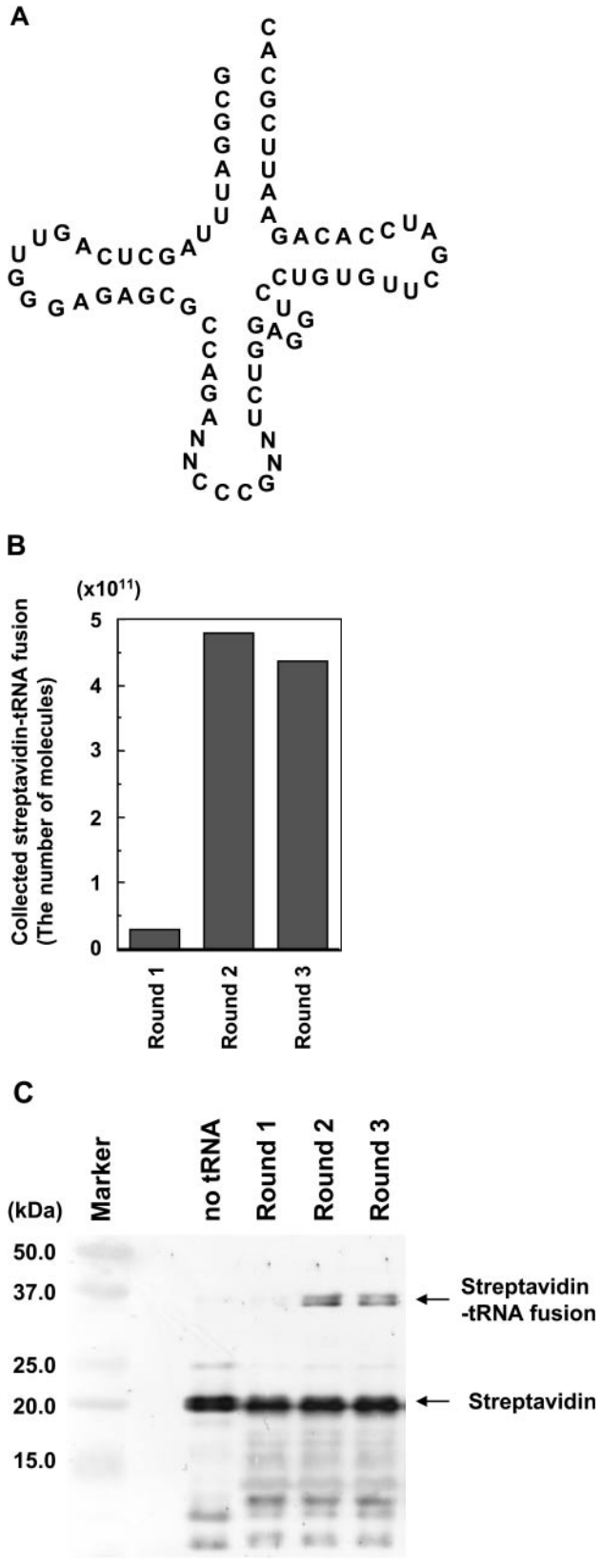

Figure 3. Selection of tRNA from a random pool library of yeast $tRNA^{Phe}$ containing NN-CCCG-NN in anticodon loop. (**A**) Structure of tRNA(-CA), in which N indicates A, G, C or U. (**B**) The amount of recovered tRNA determined by quantitative PCR in each round of selection. (**C**) Western blot analysis of the streptavidin–tRNA fusion obtained in the each round of selection.

Table 1. The sequences of anticodon loop of the selected tRNAs

Anticodon loop sequence	Number of clones
CU-CCCG-AA	8
CU-CCCG-AU	5
CA-CCCG-AA	4
UC-CCCG-AC	3
UU-CCCG-AC	2
AU-CCCG-AC	2
Others	19

not. The result indicates that the selection system gives tRNAs of non-natural sequences which differ from natural ones, and some of them may have high decoding activity in the cell-free translation system.

Activity of the selected tRNAs for the translation of a CGGG four-base codon to a non-natural amino acid

Activity of the above six clones for the incorporation of non-natural amino acid was evaluated by using a fluorescently labeled non-natural amino acid, BODIPY FL *p*-aminophenyl-alanine. This non-natural amino acid has been found to be incorporated into proteins in moderate efficiency, and allows us to quantify the decoding activity of tRNAs. The six tRNA genes were transcribed to tRNA(-CA)s, and latters were chemically aminoacylated with BODIPY FL-aminophenylalanine. The aminoacyl-tRNAs were added to the *E.coli* cell-free translation system together with a streptavidin mRNA containing a CGGG codon at the Tyr83 position. The translation product was subjected to SDS–PAGE and analyzed on a fluorescence image analyzer and by western blotting. As shown in Figure 4A, the full-length streptavidin was observed both in a fluorescence image of the SDS–PAGE and in a western blotting for all clones. On the other hand, tRNAs with CC-CCCG-CC, GG-CCCG-GG, UU-CCCG-UU anticodon loop sequences, which were not obtained in the third round of selection, gave no fluorescent band, supporting the specificity of the selection system. The band intensities of the fluorescence image were quantified, and shown in Figure 4B. These results indicate that all of the selected tRNAs are active for non-natural amino acid incorporation. Particularly the tRNAs with CU-CCCG-AA and CU-CCCG-AU anticodon loops show very high activity. The activity of the tRNAs with CU-CCCG-AU sequence is comparable to that of the original yeast $tRNA^{Phe}$ with CU-CCCG-AA sequence. The result demonstrates that the present *in vitro* selection system for tRNA is working effectively to obtain novel tRNA sequences that are appropriate for decoding CGGG four-base codon and for incorporating a non-natural amino acid. Further random mutagenesis at various regions of the tRNA will enhance the possibility of increasing the decoding activity of the tRNA.

Orthogonality of the selected tRNAs against endogenous aminoacyl-tRNA synthetases in an *E.coli* system

Transfer RNAs for the incorporation of non-natural amino acids should not be recognized by any endogenous aminoacyl-tRNA synthetases in a cell-free translation system, otherwise its aminoacylation with any naturally occurring amino acid will result in the incorporation of the natural

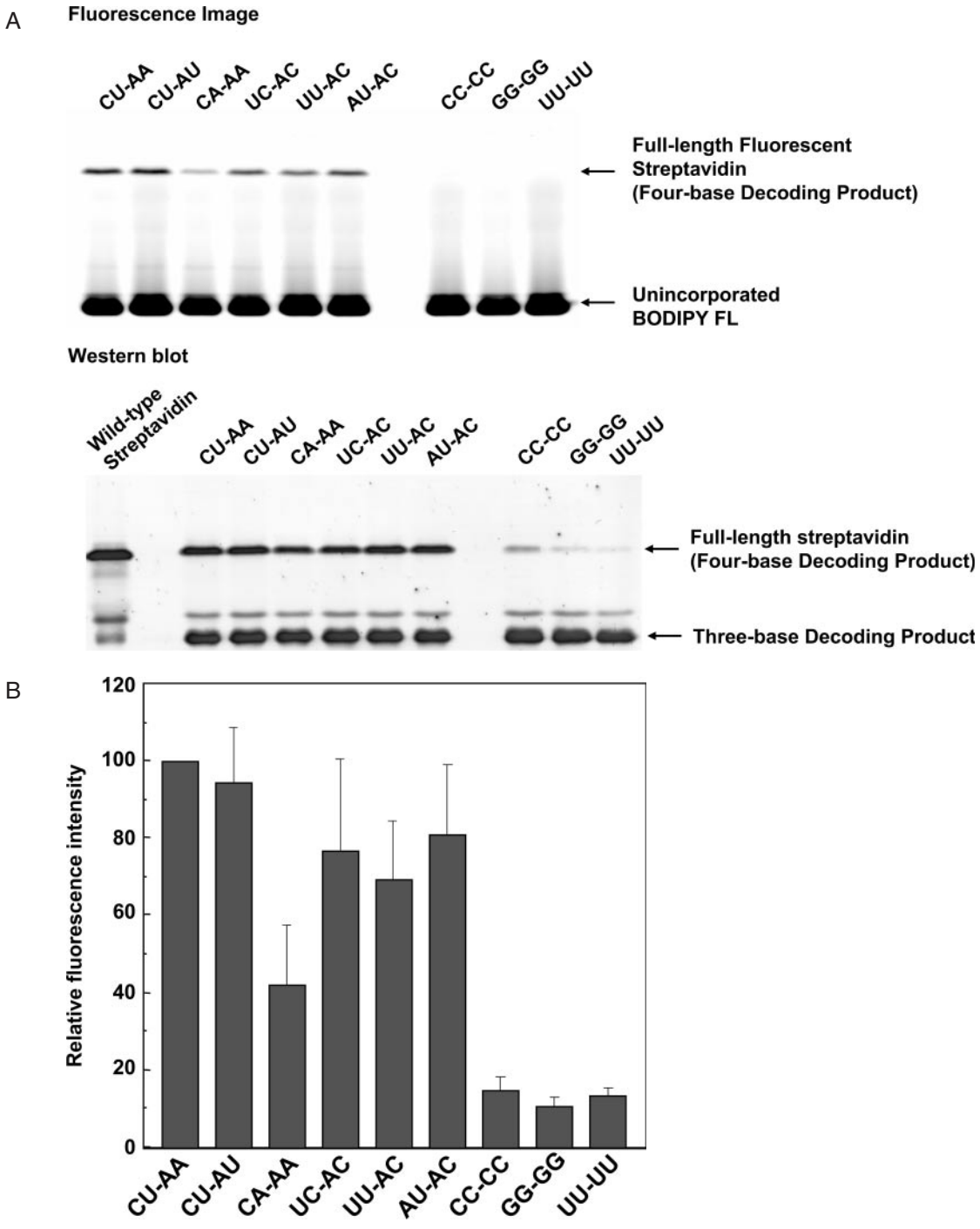

Figure 4. Evaluation of selected tRNAs. (**A**) Fluorescence image of SDS–PAGE and western blot analysis of the incorporation of a fluorescently labeled non-natural amino acid into Tyr83 position of streptavidin by using the selected and unselected tRNAs. (**B**) Relative fluorescence intensity of the band of streptavidin. The data were represented as mean ± SD of six assays.

one. To test the aminoacylation of the selected tRNAs by endogenous aminoacyl-tRNA synthetases, translation was performed in the presence of non-aminoacylated tRNAs. The latters were prepared by ligation with non-aminoacylated pdCpA by T4 RNA ligase, and added to the cell-free translation system together with the mRNA containing a CGGG four-base codon at the Tyr83 position. The translation products were analyzed by western blotting and the yield of the full-length product was quantified by densitometric analysis. The results (Figure 5) showed the selected tRNAs gave smaller amount of the full-length product than the original tRNA with CU-CCCG-AA anticodon loop. This result suggests that the selected five types of tRNAs have better orthogonality against endogenous *E.coli* aminoacyl-tRNA synthetases than the original yeast $tRNA^{Phe}_{CCCG}$ that is slightly aminoacylated by some endogenous synthetases. The strict orthogonality and

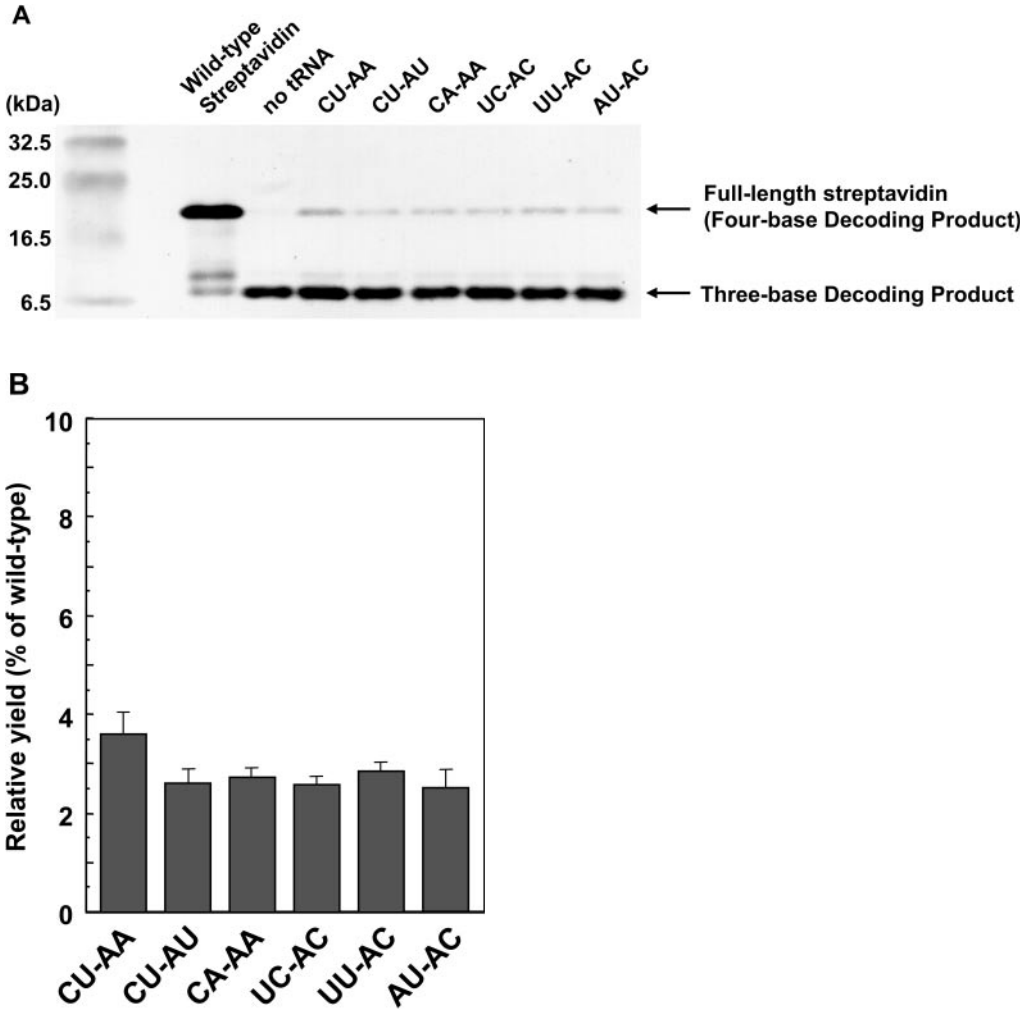

Figure 5. (**A**) Western blot analysis of the expression of full-length streptavidin in the presence of non-aminoacylated tRNAs to examine orthogonality of the tRNAs against endogenous *E.coli* aminoacyl-tRNA synthetases. (**B**) Relative yield of the full-length streptavidin. The data were represented as means ± SD of three assays.

high decoding activity of the tRNA with CU-CCCG-AU anticodon loop makes it more suitable for incorporation of non-natural amino acids in response to a CGGG four-base codon than the original yeast $tRNA^{Phe}$.

CONCLUSION

We developed a novel selection system for tRNAs that are efficient in incorporating non-natural amino acids into proteins through four-base decoding. We demonstrated that the genes of tRNAs that facilitate the incorporation of non-natural amino acids in a cell-free translation system were specifically recovered from a library of puromycin–tRNA conjugates. Several novel tRNAs with different sequences in the anticodon loop obtained by selecting from a tRNA library with a randomly mutated anticodon loop. Particularly, the tRNA with CU-CCCG-AU sequence showed high efficiency of non-natural amino acid incorporation and strict orthogonality against any endogenous aminoacyl-tRNA synthetases.

The most important advantage of the *in vitro* selection strategy is that desired clones could be selected from a very large library. In this system, it is possible to input 6.0×10^{13} molecules of tRNAs with randomized sequences into a 10 μl of the cell-free translation reaction. Novel tRNAs that are superior to yeast $tRNA^{Phe}_{CCCG}$ in the orthogonality and the activity for the incorporation of non-natural amino acids may be selected from a much larger library. We selected here frameshift suppressor tRNAs decoding a four-base codon CGGG in an *E.coli* cell-free translation system. However, this selection system will be applicable to other tRNAs such as nonsense suppressor tRNAs and conventional triplet tRNAs in other cell-free translation systems such as rabbit reticulocyte and wheat germ systems.

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