Incorporation of Nonnatural Amino Acids into Proteins by Using Various Four-Base Codons in an *Escherichia coli* in Vitro Translation System

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ABSTRACT: Incorporation of nonnatural amino acids into proteins is a powerful technique in protein research. Amber suppression has been used to this end, but this strategy does not allow multiple incorporation of nonnatural amino acids into single proteins. In this article, we developed an alternative strategy for nonnatural mutagenesis by using four-base codons. The four-base codons AGGU, CGGU, CCCU, CUCU, CUAU, and GGGU were successfully decoded by the nitrophenylalanyl-tRNA containing the complementary four-base anticodons in an *Escherichia coli* in vitro translation system. The most efficient four-base decoding was observed for the GGGU codon, which yielded 86% of the full-length protein containing nitrophenylalanine relative to the wild-type protein. Moreover, highly efficient incorporation of two different nonnatural amino acids was achieved by using a set of two four-base codons, CGGG and GGGU. This work shows that the four-base codon strategy is more advantageous than the amber suppression strategy in efficiency and versatility.

Nonnatural mutagenesis, which allows us to introduce nonnatural amino acids into proteins, is a powerful technique for studying protein structures and functions (*1*−*8*). Most researchers have been employing the amber suppression method to this end, despite some serious drawbacks. One is competition with release factors, which reduces the suppression efficiency. Recently, Hecht and co-workers reported that a mutant *Escherichia coli* that expresses a thermosensitive release factor provides an improved in vitro translation system, in which some nonnatural amino acids were incorporated more efficiently through amber suppression (*9*). The other drawback is a limitation of the number of nonnatural amino acids that can be introduced into a single protein, because only an amber codon has been practically used for the suppression. Chamberlin and co-workers reported that ochre and opal suppressors are effective in a rabbit reticulocyte lysate (*10*), but Schultz's group reported that a readthrough was the major route for the opal codon in an *E.coli* S30 extract (*11*). Bain et al. reported that an artificial codon−anticodon pair containing isoC and isoG could direct a specific tRNA[1] to the codon without any competition with release factors and endogenous tRNAs (*12*). This strategy may overcome the limitation of the genetic code, but the chemical synthesis of the artificial RNAs restricts its wide application.

It is known that naturally occurring tRNAs with extended anticodons suppress frameshift mutations. Hardesty and co-workers reported that a synthetic $tRNA_{ACCU}$ aminoacylated enzymatically with alanine could decode an AGGU codon (*13*). Recently, we have found that four-base codons such as AGGU (*14*) and CGGG (*15*) work well for the incorporation of nonnatural amino acids. This frameshift suppression strategy may have two advantages over amber suppression. The first is that competition between the suppressor tRNA and the endogenous release factors is avoided. Because AGG and CGG are minor codons in *E. coli*, the concentration of tRNA containing the CCU or CCG anticodon is very low in the cell or in the cell extract (*16, 17*). This implies that the four-base codons such as AGGU and CGGG are decoded by the tRNAs containing the complementary four-base anticodons without serious competition from endogenous tRNAs. In fact, we have observed that the efficiency of frameshift suppression by using CGGG−CCCG codon−anticodon pair is very high (*15*). The second advantage is that more than two nonnatural amino acids can be incorporated into a single protein by using two independent four-base codons. We have previously shown that two different nonnatural amino acids were introduced into a single protein independently by using AGGU and CGGG four-base codons (*18*), though the efficiency was quite low.

In this study, we explored a wide variety of four-base codons in an *E. coli* in vitro translation system. Four-base codons derived from stop codons were also examined, as well as stop codons themselves. Effects of the substitution of the fourth letter of the codons on the four-base decoding were also investigated. Finally, a highly efficient double incorporation of nonnatural amino acids was demonstrated by using a novel set of the four-base codons.

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[1] Abbreviations: tRNA, transfer ribonucleic acid; mRNA, messenger ribonucleic acid; Tyr, tyrosine; Phe, phenylalanine; nitroPhe, nitrophenylalanine; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); SD, standard deviation; Xaa, nonnatural amino acid; GlyRS, glycyl-tRNA synthetase; napAla, 2-naphthylalanine; IgG, immunoglobulin G.

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EXPERIMENTAL PROCEDURES

Plasmid. Synthetic streptavidin gene purchased from R&D Systems Europe had been cloned into a T7 tag (MASMTGGQQMG) fusion vector containing T7 promoter (*15*). Substitutions of various four-base codons for UAU codon at a Tyr83 position were carried out by using mutagenic oligonucleotide primers CGCATTACG-XXXX-GTTGTTTTTCC, in which XXXX indicates a complementary sequence of one of the four-base codons. Synthetic yeast $tRNA^{Phe}$ gene containing ACCU anticodon had been constructed in pUC18 (*15*). Substitutions of various four-base anticodons for the ACCU anticodon were carried out by using mutagenic oligonucleotide primers GCCAGACT-XXXX-AATCTGGA, in which XXXX indicates one of the four-base anticodons.

In Vitro Translation. The mutated streptavidin mRNAs and the nitrophenylalanyl-tRNAs containing four-base anticodons were prepared as previously reported (*15*). The reaction mixture for in vitro translation (10 μL) contained 2 μL of *E. coli* S30 Extract for Linear Templates (Promega), 55 mM Hepes-KOH (pH 7.5), 210 mM potassium 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% poly(ethylene glycol)-8000, 35 μg/mL folinic acid, optimum concentration (12 mM) of magnesium acetate, 0.1 mM each of amino acids, 8 μg of mRNA, and 0.1 nmol of aminoacyl-tRNA. The reaction mixture was incubated at 37 °C for 1 h and then cooled to 0 °C.

Western Blot Analysis. The reaction mixture of the in vitro translation (2 μL) was mixed with gel loading buffer (38 μL), and 5 μL of the resulting solution was applied to an SDS−15% polyacrylamide gel. Western blotting was carried out on a PVDF membrane (Bio-Rad) by using anti-T7-tag monoclonal antibody (Novagen) and ProtoBlot 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 (1, 0.7, 0.5, 0.35, 0.25, 0.18, 0.12, 0.09, 0.06, and 0.03) of wild-type streptavidin expressed in vitro. The band intensity was evaluated by using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The efficiency was measured at least three times and expressed as mean ± SD.

RESULTS AND DISCUSSION

Design of Four-Base Codons. The incorporation of a nonnatural amino acid through four-base decoding was evaluated by using a streptavidin expressing system previously described (*15*). Four-base codons were designed taking the codon usage and the number of tRNA gene copies in the *E. coli* genome (*17*) into account. Arginine is coded by six codons, i.e., CGN (N indicates one of the four bases) and AGR (R indicates A or G), but only CGU and CGC are used primarily. The corresponding $tRNA_{ICG}$ has four copies of its gene in the genome. On the other hand, CGA, CGG, AGA, and AGG are rarely used, and they each have a single gene copy, although CGA is decoded by $tRNA_{ICG}$. The above consideration implies that four-base codons derived from the CGG, AGA, and AGG codons can circumvent competitive triplet decoding by endogenous arginyl-tRNAs. In this study, U was added to these codons, and the resulting four-base codons were tested for introducing *p*-nitrophenylalanine into streptavidin in the *E. coli* in vitro translation system. For comparison, CGCU derived from a major CGC codon was also examined. It should be noted that the CGC codon in the streptavidin gene was replaced by the CGU codon when the CGCU codon was tested.

Other possibilities for codon extension from those of leucine, serine, proline, threonine, and glycine were also examined. Leucine is coded by six codons as is the case for arginine, of which the CUG codon decoded by the $tRNA_{CAG}$ (four gene copies in the genome) is the most frequently used. The most scarcely used CUA codon and other infrequently used CUC and UUG codons were chosen for the four-base codon extension. These codons are decoded by tRNAs that are coded by single-copy genes. Serine is also coded by six codons that are used with about the same frequency, from which UCG was picked in this study. Proline and glycine are each coded by four codons, CCN and GGN, respectively, from which the most scarcely used CCC and GGG codons were chosen. Threonine is also coded by four codons, ACN. ACA is the most scarcely used one, but two ACA codons are present in the streptavidin gene. Therefore, ACG decoded by a single-copy $tRNA_{CGU}$ was chosen. Valine and alanine are also coded by four codons, but they were not tested because all of them are frequently used.

Analysis of Four-Base Decoding. To the *E. coli* in vitro translation system, streptavidin mRNA containing one of the four-base codons and nitrophenylalanyl-tRNA containing the complementary four-base anticodon were added. The streptavidin gene was fused with T7-tag and His-tag sequences at the N- and C-termini, respectively. Various four-base codons were introduced into the Tyr83 position of the streptavidin, because nitrophenylalanine incorporated into the Tyr83 position does not cause loss of the activity (*15*). From the resulting gene, mRNA was synthesized by T7 RNA polymerase. The synthetic gene encoding a $tRNA_{ACCU}$ derived from yeast $tRNA^{Phe}$ under a T7 promoter had been constructed (*15*), and the ACCU anticodon was replaced by various four-base anticodons. The tRNA lacking two nucleotides at the 3′ terminus was synthesized by T7 RNA polymerase and linked with the nitrophenylalanyl-pdCpA by T4 RNA ligase (*15*, *19*). The mRNA and the aminoacyl-tRNA were added to the *E. coli* in vitro translation system. The reaction mixture was incubated at 37 °C for 60 min and then applied to SDS−PAGE, followed by Western blotting with anti-T7-tag antibody. The yield of the wild-type streptavidin under this experimental condition had been determined to be 10 μg/mL by a fluorescence polarization assay (*15*).

As illustrated in Figure 1, when the four-base codon is decoded by the tRNA containing the complementary four-base anticodon and the nonnatural amino acid (Xaa-tRNA), the four-base codon will be translated into the nonnatural amino acid and the correct reading frame will be maintained. However, when the first three bases of the four-base codon are decoded as a triplet codon by an endogenous aminoacyl-tRNA, the reading frame will shift and then the translation will stop at a subsequent stop codon. In consequence, the four-base decoding will yield a full-length protein incorporated with the nonnatural amino acid at the Tyr83 position, whereas the undesired three-base decoding will result in a

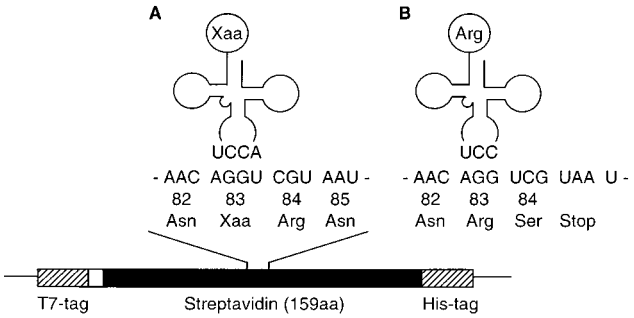

FIGURE 1: Nucleotide and amino acid sequences of 82−85 region of a mutated streptavidin containing a four-base codon like AGGU. (A) A $tRNA_{ACCU}$ aminoacylated with a nonnatural amino acid (Xaa) decodes the four-base codon AGGU, and in consequence, the correct reading frame is maintained. (B) When the AGGU sequence is decoded as an AGG triplet by an endogenous arginyl-$tRNA_{CCU}$, the translation will be stopped at the UAA stop codon downstream.

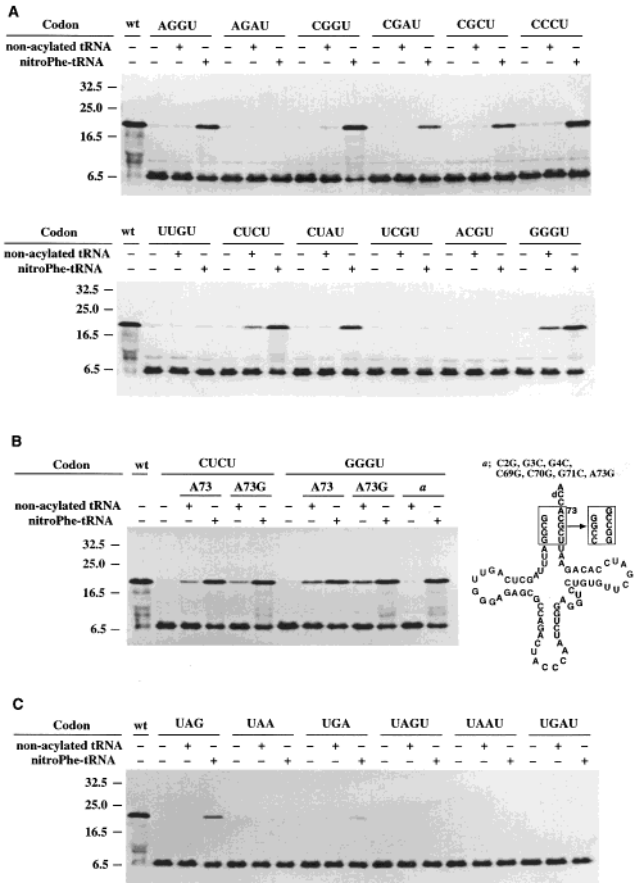

FIGURE 2: Western blot analysis of the incorporation of *p*-nitrophenylalanine into the Tyr83 position of the streptavidin through the four-base decoding (A and B) and the nonsense and frameshift suppression (C). Translation of the mutated streptavidin mRNA containing various four-base codons or nonsense codons at the Tyr83 position was carried out in the absence of tRNA, in the presence of nonaminoacylated tRNA (nonacylated tRNA) or in the presence of nitrophenylalanyl-tRNA (nitroPhe-tRNA). The full-length and truncated streptavidin were detected by using an anti-T7-tag antibody and an alkaliphosphatase-labeled anti-mouse IgG.

premature termination of translation after the four-base codon.

In Figure 2A, the results of the decoding of various four-base codons by the corresponding tRNAs are shown. The first lane contained a wild-type streptavidin of 19 kDa expressed in vitro. The next three lanes showed the case of AGGU. In the absence of $tRNA_{ACCU}$, no full-length streptavidin was detected; instead a truncated protein of about 8 kDa appeared, consistent with termination of translation at the UAA codon downstream of the four-base codon by two triplets. In the presence of the nitrophenylalanyl-$tRNA_{ACCU}$, however, the full-length streptavidin was successfully synthesized, indicating that the AGGU is correctly decoded by the nitrophenylalanyl-$tRNA_{ACCU}$, and in consequence, the reading frame is maintained to the end. In the presence of the nonaminoacylated $tRNA_{ACCU}$, no full-length streptavidin was detected, suggesting that the tRNA is not aminoacylated by any endogenous aminoacyl-tRNA synthetases in the in vitro system.

In a similar manner, we found that the four-base codons CGGU, CGAU, CGCU, CCCU, and CUAU gave the full-length streptavidin. On the other hand, the four-base codons AGAU, UUGU, UCGU, and ACGU did not. In the cases of CUCU and GGGU codons, however, addition of the nonaminoacylated tRNA gave the full-length streptavidin, indicating that these tRNAs are aminoacylated by some endogenous aminoacyl-tRNA synthetase(s).

It has been reported that *E. coli* glycyl-tRNA synthetase (GlyRS) recognizes the cognate tRNA with G1-C72, C2-G71, G3-C70, C35, and C36 as identity determinants in addition to the U73 discriminator base (*20*). Because the $tRNA_{ACCC}$ contains these identity determinants (except for U73), the $tRNA_{ACCC}$ might be aminoacylated with glycine by the GlyRS. To avoid this undesired aminoacylation, C2-G71 and G3-C70 were replaced by G2-C71 and C3-G70, respectively. In addition, a weak identity determinant G4-C69 (*21*) was replaced by C4-G69. Because it is important for the T7 RNA polymerase-mediated transcription, G1-C72 was kept unchanged. A73 of $tRNA_{ACCC}$ was replaced by G on the basis of the finding that replacement of A73 by G decreases the glycine acceptability by 3-fold (*20*). As a result, the nonaminoacylated $tRNA_{ACCC}$ with the G2-C71, C3-G70, C4-G69, and G73 mutations (marked *a* in Figure 2B) gave no full-length streptavidin, whereas the same $tRNA_{ACCC}$ aminoacylated with the nitrophenylalanine gave the full-length streptavidin (Figure 2B). It should be noted that the G73-mutated $tRNA_{ACCC}$ was still aminoacylated enzymatically. In the case of the $tRNA_{AGAG}$, the discriminator base A73 is the same as that of the *E. coli* $tRNA^{Leu}$. It has been reported that the replacement of the A73 by G decreases the leucine acceptability down to 0.6% (*22*). However, addition of the nonaminoacylated G73-mutated $tRNA_{AGAG}$ gave full-length streptavidin (Figure 2B). This result suggests that the $tRNA_{AGAG}$ is aminoacylated with some amino acids other than leucine.

The stop codons and the four-base codons derived from them were also examined for nitrophenylalanine incorporation (Figure 2C). The UAG codon gave the full-length streptavidin in the presence of the nitrophenylalanyl-$tRNA_{CUA}$, but its band on Western blotting was much weaker than those of most four-base codons. The UAA, UGA, UAGU, UAAU, and UGAU codons gave absent or negligible band of the full-length streptavidin. These results may be due to a strong competition with the release factors. This is consistent with the finding that a UAGU is hardly decoded by a $tRNA_{ACUA}$ that was aminoacylated enzymatically with alanine (*13*). On the other hand, a UAGA codon can be decoded by a $tRNA_{NCUA}$ with a 13−26% efficiency in an *E.*

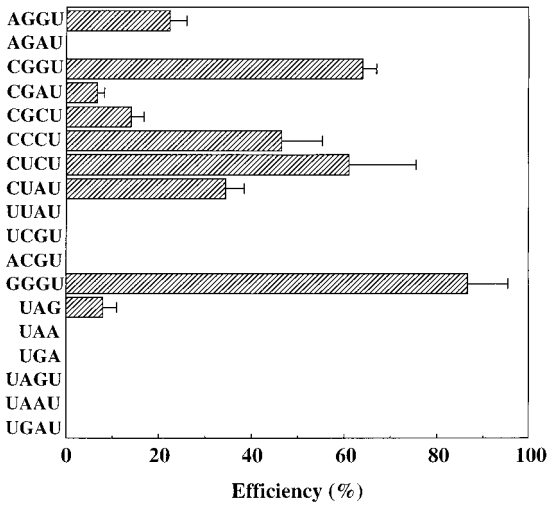

FIGURE 3: Efficiency of the incorporation of the nitrophenylalanine through four-base decoding. The efficiency was determined as a relative yield of the full-length product to the wild-type streptavidin expressed in vitro. The data (mean ± SD) were measured at least three times.

coli MRA8, which has a temperature-sensitive release factor 1 (*23*). This relatively higher efficiency may reflect the lower activity of the mutated release factor 1.

The efficiency of the incorporation of nitrophenylalanine was determined by comparing the band intensity of the full-length product on Western blotting with those of serial dilutions of the wild-type streptavidin expressed in vitro (Figure 3). The most efficient incorporation was observed in the case of the GGGU (86%). This value is higher than that of the CGGG (68%), which had been used as the most efficient four-base codon (*24*, *25*). The efficiencies of the CCCU, CUCU, and CUAU codons were 46%, 61%, and 34%, respectively, which seem to be high enough for practical applications. It should be noted that the efficiency of the CUCU codon contains a small contribution from a byproduct due to an enzymatic aminoacylation of the $tRNA_{AGAG}$. On the other hand, the CGCU (14%) and CGAU (7%) codons were rather inefficient. The amber codon UAG gave 8%, consistent with the previously reported value (*1*). This result indicates that the four-base codon strategy is much more efficient than the amber suppression strategy in conventional *E. coli* in vitro translation systems.

The efficiency of the four-base decoding may be influenced by two major factors. First, the four-base decoding would be influenced by the concentration of endogenous competitive tRNAs. For example, higher incorporation with the CGGU than the CGCU and CGAU may reflect lower concentration of the $tRNA_{CCG}$ than that of the $tRNA_{ICG}$. Inability of the AGAU, UUGU, UCGU, and ACGU codons may suggest that relatively large amounts of the competitive tRNAs exist in the *E. coli* extract. The second factor is strength of the interaction between four-base codons and four-base anticodons. The CGAU and CGCU compete for the same $tRNA_{ICG}$, but the efficiency of the CGCU is higher than that of the CGAU. This difference may be simply due to a different strength of the interaction at the third position.

Effect of the Fourth Letter on the Four-Base Decoding. For the four-base codons AGGU, CGGU, CUCU, and GGGU, the fourth letter U was replaced by other bases. To avoid a possible enzymatic aminoacylation, the G2-C71, C3-G70, C4-G69, and G73 mutations were introduced into the

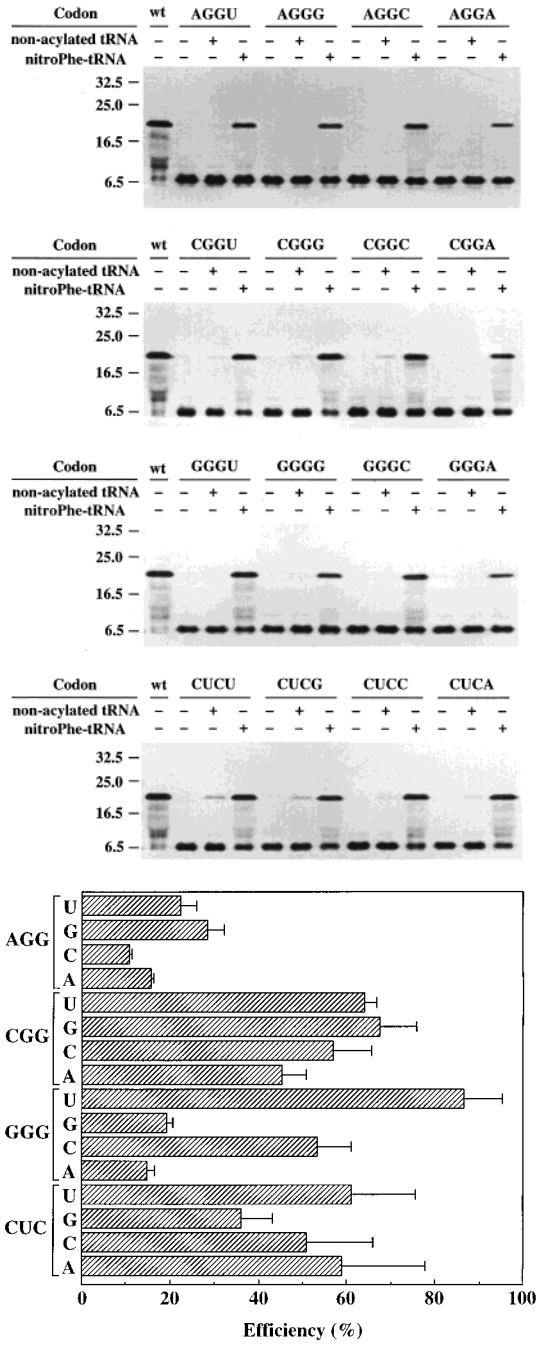

FIGURE 4: Effect of the fourth letter of the four-base codons on the four-base decoding: Western blot analysis and efficiency of incorporation of the nitrophenylalanine. The $tRNA_{NCCC}$ contained G2-C71, C3-G70, C4-G69, and G73 mutations.

$tRNA_{NCCC}$, and the G73 mutation was introduced into the $tRNA_{NGAG}$. The result of Western blotting (Figure 4, upper panels) showed that all bases were accepted as the fourth letter but the efficiency was influenced by the replacements (Figure 4, lower panel). In the case of the AGGN, the AGGG codon was slightly more efficient. Similarly, the CGGG codon was slightly better in the CGGN series. In the case of GGGN, however, the GGGU codon was the best but the GGGG codon was much less effective, possibly due to a higher-order structure of the GGGG sequence.

As described above, the $tRNA_{AGAG}$ is subjected to a subtle aminoacylation by endogenous aminoacyl-tRNA synthetases. However, the $tRNA_{UGAG}$ and $tRNA_{GGAG}$ were hardly aminoacylated enzymatically. It is interesting that the recognition

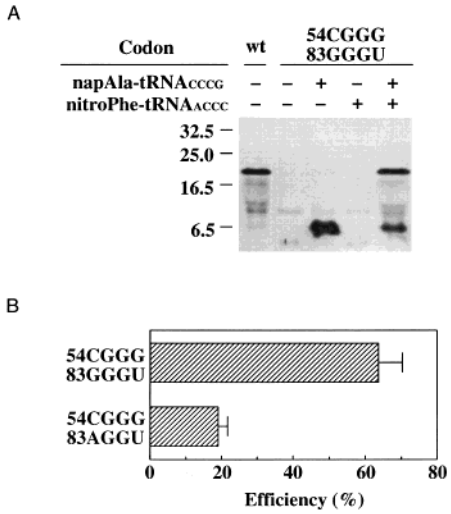

FIGURE 5: Incorporation of two nonnatural amino acids into a single streptavidin. (A) Western blot analysis of the expression of the streptavidin containing CGGG at the Tyr54 and GGGU at the Tyr83 in the absence or presence of 2-naphthylalanyl-tRNA$_{CCCG}$ (napAla-tRNA$_{CCCG}$) and nitrophenylalanyl-tRNA$_{ACCC}$ (nitroPhe-tRNA$_{ACCC}$). The tRNA$_{ACCC}$ contained G2-C71, C3-G70, C4-G69, and G73 mutations. (B) Efficiency of the incorporation of the naphthylalanine and nitrophenylalanine into the Tyr54 and Tyr83 positions of the streptavidin by using sets of CGGG and GGGU codons and CGGG and AGGU codons.

of the aminoacyl-tRNA synthetases is very sensitive to the first letter of the four-base anticodon.

In many cases examined, U was generally preferable as the fourth letter of the four-base codons. This justifies the use of U as the fourth letter in the initial screening described above.

Efficient Incorporation of Two Different Nonnatural Amino Acids into a Single Protein. We recently reported that two different nonnatural amino acids were successfully introduced into a single protein by using two independent four-base codons, CGGG and AGGU (*18*). The yield of the double mutant protein, however, was quite low because of the inefficient decoding of the AGGU codon. Here we demonstrate that the double nonnatural mutagenesis becomes a practical tool by using two highly efficient four-base codons, CGGG and GGGU. The streptavidin mRNA containing the CGGG at the Tyr54 position and the GGGU at the Tyr83 position was prepared and expressed in the presence of the tRNA$_{CCCG}$ and the mutated tRNA$_{ACCC}$ aminoacylated with 2-naphthylalanine and nitrophenylalanine, respectively. As shown in Figure 5, the full-length streptavidin was successfully synthesized only in the presence of the two aminoacyl-tRNAs, indicating that the two nonnatural amino acids were incorporated into the directed positions. The yield of the full-length protein was estimated to be 64% relative to the wild-type protein, which was in excellent agreement with the predicted product (65%) of the efficiency of the decoding of the CGGG codon by the 2-naphthylalanyl-tRNA$_{CCCG}$ (76%) and that of the GGGU codon by the nitrophenylalanyl-tRNA$_{ACCC}$ (86%). When the same experiment was carried out by using the CGGG and AGGU codons, the yield of the full-length mutant streptavidin was only 19%. The marked improvement clearly reflects the higher efficiency of the GGGU codon than the AGGU codon.

Advantages of the Four-Base Codons. The four-base codon strategy is more advantageous than the nonsense suppression strategy for its efficiency and for its ability for multiple incorporation. The present result allows us to use the double nonnatural mutagenesis technique in practical applications. Especially, the double nonnatural mutagenesis will be useful to introduce donor and acceptor pairs for energy transfer and electron transfer to measure structural changes of proteins. The four-base codon strategy has a drawback that the corresponding triplet codon must be eliminated from the mRNA. A wide repertoire of the four-base codons, however, allows us to choose an appropriate four-base codon according to the codon usage of each gene.

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