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Misacylation of Yeast Amber Suppressor tRNA^{Tyr} by *E. coli* Lysyl-tRNA Synthetase and Its Effective Repression by Genetic Engineering of the tRNA Sequence

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Through an exhaustive search for *Escherichia coli* aminoacyl-tRNA synthetase(s) responsible for the misacylation of yeast suppressor tRNA^{Tyr}, *E. coli* lysyl-tRNA synthetase was found to have a weak activity to aminoacylate yeast amber suppressor tRNA^{Tyr} (CUA) with L-lysine. Since our protein-synthesizing system for site-specific incorporation of unnatural amino acids into proteins is based on the use of yeast suppressor tRNA^{Tyr}/tyrosyl-tRNA synthetase (TyrRS) pair as the "carrier" of unusual amino acid in *E. coli* translation system, this misacylation must be repressed as low as possible. We have succeeded in effectively repressing the misacylation by changing several nucleotides in this tRNA by genetic engineering. This "optimized" tRNA together with our mutant TyrRS should serve as an efficient and faithful tool for site-specific incorporation of unnatural amino acids into proteins in a protein-synthesizing system *in vitro* or *in vivo*.

Key words: aminoacyl RNA synthesis, misacylation, protein synthesis, transfer RNA, translation.

Abbreviations: aaRS, aminoacyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase, EC 6.1.1.6; TyrRS, tyrosyl-tRNA synthetase, EC 6.1.1.1; EGFP, enhanced green fluorescent protein; DHFR, dihydrofolate reductase, EC 1.5.1.3.

We have been trying to utilize a yeast tRNA^{Tyr}/tyrosyl-tRNA synthetase (TyrRS) pair as a "carrier" of non-canonical (*i.e.* being not specified in the genetic code) amino acid(s) in an *Escherichia coli* translation system. The rationale for this is as follows. (i) Yeast tRNA^{Tyr} has a unique identity element, a C1-G72 base pair at the end of the acceptor stem (*1*, *2*), which is not found in *E. coli* tRNAs except for tRNA^{Pro}. (ii) Since the size of the variable arm in yeast tRNA^{Tyr} is completely different from that of *E. coli* tRNA^{Tyr}, yeast tRNA^{Tyr} cannot be aminoacylated by *E. coli* TyrRS, and nor can *E. coli* tRNA^{Tyr} by yeast TyrRS (*3*, *4*). (iii) Yeast tRNA^{Tyr} can be easily converted to an amber or ochre suppressor tRNA that can function *in vitro* or *in vivo* (*5*, *6*). (iv) We have succeeded in constructing by genetic engineering a mutant of yeast TyrRS that can aminoacylate yeast tRNA^{Tyr} (or its suppressor derivative) with non-canonical amino acids, such as 3-substituted tyrosine analogues, instead of L-tyrosine (*7*).

In a previous paper (*8*), we developed an efficient system for co-expression of yeast amber suppressor tRNA^{Tyr} (anticodon; CUA) and wild-type TyrRS in *E. coli* cells. Analyses of suppression patterns using several sets of *E. coli* and λ phage mutants suggested that the expressed yeast suppressor tRNA^{Tyr} was aminoacylated only with tyrosine by its cognate yeast TyrRS and not by *E. coli* TyrRS or other aminoacyl-tRNA synthetases (aaRSs). Thus, this extra tRNA/TyrRS pair seemed to be a promising bridgehead for developing a system for incorporating unnatural amino acids site-specifically into proteins. However, at least two papers published since then have commented that yeast amber suppressor tRNA^{Tyr} could be aminoacylated by *E. coli* aaRS(s) in some circumstances, although the misacylated amino acid has not been identified and the aminoacylation level is quite low (*9*, *10*). We have also detected a trace amount of misacylation depending on the balance of tRNA/aaRS levels when this tRNA was used in an *in vitro* protein-synthesizing system. Such misacylation could cause a "double-meaning" in the amino acid assignment for the amber UAG codon and eventually lead to inhomogeneity of product proteins in which unnatural amino acid is incorporated at the amber-mutated site.

Therefore, we addressed the following three questions in this study. (i) Is yeast amber suppressor tRNA^{Tyr} (CUA) actually misacylated by any of the endogenous aaRS(s) contained in the *E. coli* cell–free protein-synthesizing system? (ii) If so, which aaRS is responsible for the misacylation? (iii) Is it possible to optimize the nucleotide sequence of yeast amber suppressor tRNA^{Tyr} (CUA) so that it is not recognized by any other aaRSs except for yeast TyrRS? We report here that *E. coli* lysyl-tRNA synthetase (LysRS) does have a weak activity to aminoacylate yeast amber suppressor tRNA^{Tyr} (CUA) with L-lysine, but the misacylation can be effectively repressed by changing several nucleotides in this tRNA by genetic engineering.

EXPERIMENTAL PROCEDURES

General—Oligonucleotides for PCR primer were obtained from Hokkaido System Science Co. Non-radioactive amino acids (20 species) were purchased

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from Peptide Institute Inc. L-[^{14}C]-tyrosine (14.65 GBq/mmol) and L-[^{14}C]-lysine (11.77 GBq/mmol) were obtained from Amersham Biosciences and L-[^{14}C]-leucine (11.32 GBq/mmol) from Moravek Biochemicals. Other chemicals were obtained from Wako Pure Chemicals. Restriction enzymes, *Taq* DNA polymerase and T4 DNA ligase were obtained from MBI Fermentas, Takara Shuzo and Nippon gene, respectively. T7 RNA polymerase was prepared according to (*11*). Creatine phosphate and creatine kinase were obtained from Roche Diagnostics. Aminoacyl-tRNA synthetases were prepared as described previously; yeast TyrRS (*7*) and *E. coli* aminoacyl-tRNA synthetases (including LysRS) (*12*). *T. thermophilus* EF-Tu and EF-Ts were prepared according to (*13*).

Preparation of tRNAs—Several tRNA transcripts of yeast tRNA^{Tyr} or its derivatives and *E. coli* tRNA^{Lys} were used in this study. Since yeast tRNA^{Tyr} has a 5′-terminal sequence unfavorable to transcription with T7 RNA polymerase, we developed an efficient method for preparation of yeast tRNA^{Tyr} or its derivatives by utilizing additional 5′-terminal precursor sequence and processing with RNase P as described previously (*14*). Transcripts of yeast amber suppressor tRNA^{Tyr} (anticodon; CUA) and its derivatives and wild-type tRNA^{Tyr} (GUA) were prepared according to this method. Transcripts of *E. coli* tRNA^{Lys} (CUU) and tRNA^{Lys} (UUU) were prepared by conventional *in vitro* transcription using T7 RNA polymerase.

Assay for Ternary-Complex Formation with T. thermophilus *EF-Tu*—The reaction mixtures (10 μl each) containing 50 mM Tris-HCl (pH 7.6), 7 mM $MgCl_2$, 50 mM KCl, 50 mM NH_4Cl, 5 mM 2-mercaptoethanol, 2 mM ATP, 1 mM GTP, 10 μM *T. thermophilus* EF-Tu, 1 μM *T. thermophilus* EF-Ts, 7 μM individual *E. coli* aaRS and 7 μM yeast amber suppressor tRNA^{Tyr} (CUA) were incubated at 37°C for 20 min. Aliquots were subjected to electrophoresis on 6% native PAGE according to Hornung *et al.* (*15*). Protein bands were visualized by CBB staining and RNA bands by methylene blue staining.

Aminoacylation Assay—The reaction mixture for tyrosylation or lysylation contained 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 40 mM KCl, 4 mM ATP, 20 μM L-[^{14}C]-tyrosine or lysine, 0.25 A_{260} unit tRNA, and 0.3 μg of yeast TyrRS or 12.5 μg of *E. coli* LysRS (total volume 50 μl). The reaction mixture was incubated at 30°C (tyrosylation) or 37°C (lysylation). Aliquots (10 μl each) were withdrawn at appropriate time intervals and acid-insoluble radioactivities were measured. For determination of the kinetic constants of tyrosylation, the initial rates of reaction were determined by using various concentrations of tRNA transcripts at fixed concentrations of tyrosine (55 μM), ATP (4 mM) and suitable concentrations of yeast TyrRS.

Construction of the Template Plasmid DNAs for Cell-Free Protein Synthesis—The plasmid for EGFP (pHSGEGFPht) was constructed as follows. First, the coding region of EGFP in a plasmid pEGFP (Clontech) was amplified by PCR to create 5′ *Nco*I and 3′ *Xho*I sites by using the primers 5′-GCC ACC ATG GTG AGC AAG GGC GAG GAG CT-3′ and 5′-GGG GCT CGA GCT TGT ACA GCT CGT CCA-3′, and inserted into pET29-a(+) (Novagen). The resultant EGFP gene has the S-tag sequence at the 5′ end and the hexa-histidine tag sequence at the 3′ end. Second, the region from the T7 promoter to terminator sequences was PCR-amplified by using a T7 promoter primer and a T7 terminator primer and inserted into the *Pvu*II site of a high copy plasmid, pHSG299 (Takara Shuzo) generating the plasmid pHSGEGFPht. The plasmid for amber mutant EGFP (pHSGambEGFPht) was constructed by the same procedure as above except that the PCR primer, 5′-GCC ACC ATG GTG AGC TAG GGC GAG GAG CT-3′ was used instead of the former PCR primer. The use of this primer introduces an in-frame amber codon (underlined) at the 31st codon of the S-tagged EGFP gene.

The plasmid for wild-type DHFR (pETDHFR) was constructed as described previously (*12*), and the substitution of the amber codon for the 128th codon in the DHFR gene by the QuikChange® method (*16*) resulted in the plasmid pETDHFR128amb.

These plasmid DNAs were purified by using Genopure Plasmid Maxi Kit (Roche Diagnostics) and used as templates for cell-free protein synthesis.

Cell-Free Protein Synthesis—E. coli cell–free extract was prepared according to Kigawa *et al.* (*17*) with slight modifications as follows. *E. coli* Q13 (Hfr: *met pnp rna tyr*) was used to prepare the cell extract and cells were harvested by centrifugation when cell density reached an A_{600} of 0.37. The cells were washed and resuspended in 20 mM Hepes-KOH (pH 7.5), 10 mM $Mg(OAc)_2$, 20 mM NH_4OAc and 10 mM 2-mercaptoethanol. After homogenization in a MSK cell homogenizer (B. Braun), the cell debris was removed by centrifugation at 30,000 × *g* for 30 min, and the supernatant was used as the cell extract. Cell-free protein synthesis reaction was performed basically according to (*17*) except that the reaction was done at 30°C for 2 h, and 2 μM yeast TyrRS, 1.6 μM yeast amber suppressor tRNA^{Tyr} or its mutant (U4C/AS G-Cr) and 24 μg/ml template plasmid DNA (pHSGEGFPht or pHSGambEGFPht for EGFP synthesis and pETDHFR or pETDHFR128amb for DHFR synthesis) were added when they were needed. After synthesizing EGFP, the reaction mixture was loaded onto a tiny column (50 μl) of Ni-NTA Superflow (Qiagen) and the synthesized EGFP was purified by the manufacturer's protocol. The purified EGFP was concentrated with Microcon 10 (Millipore) and subjected to electrophoresis on 10% native PAGE. The synthesized EGFP was detected as green fluorescent bands under UV light (366 nm).

In the case of DHFR synthesis, the reaction was carried out similarly except that L-[^{14}C]-leucine was added to the reaction mixture. After the reaction, the synthesized polypeptides in 15-μl aliquots of the reaction mixture were precipitated with 300 μl of acetone. The precipitates were centrifuged, rinsed with 150 μl of fresh acetone and subjected to 15% SDS PAGE. The synthesized peptides were detected by autoradiography using a BAS-2500 phosphoimager (Fujifilm). Suppression efficiency was calculated as: [^{14}C]-count incorporated into the full-length DHFR band/([^{14}C]-count incorporated into the full-length DHFR band + [^{14}C]-count incorporated into the imperfectly synthesized polypeptides interrupted at the amber codon).

RESULTS AND DISCUSSION

Identification of the Enzyme Responsible for Misacylation of Yeast Amber Suppressor tRNA^{Tyr}—To confirm that yeast amber suppressor tRNA^{Tyr} (anticodon; CUA) is actually misacylated by an *E. coli* aaRS and to

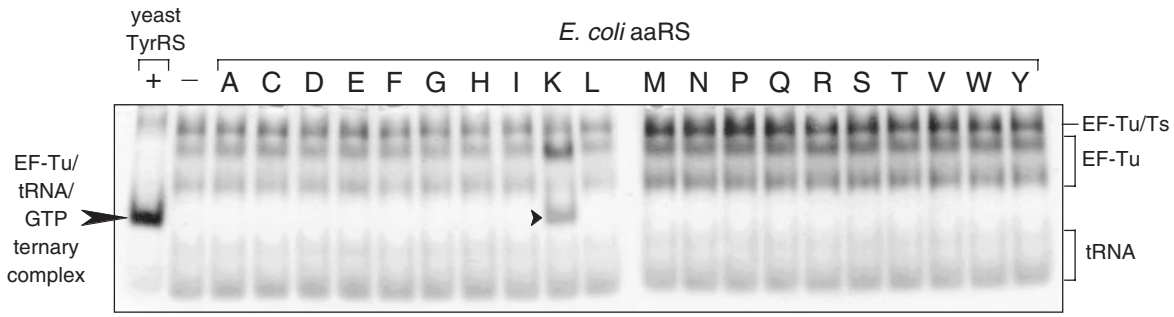

Fig. 1. **Search for the enzyme responsible for the misacylation of yeast amber suppressor tRNA^{Tyr} by ternary complex formation with EF-Tu and GTP.** After incubation of yeast amber suppressor tRNA^{Tyr} (CUA) with one each of the purified *E. coli* aaRSs in the presence of *T. thermophilus* EF-Tu, EF-Ts, GTP and a mixture of 20 amino acids, aliquots were subjected to electrophoresis on 6% native PAGE according to Hornung *et al.* (*15*). Protein bands were visualized by CBB staining and RNA bands by methylene blue staining. The capital letter at the top of each lane is the single-letter code for each amino acid and represents here the corresponding aaRS (*e.g.*, K stands for LysRS). Lane + is a positive control (yeast TyrRS added) and—a negative control (no aaRS added). Positions of EF-Tu/Ts and suppressor tRNA^{Tyr} (CUA) are marked on the right and positions of EF-Tu/aminoacyl-tRNA/GTP ternary complex are indicated by arrowheads.

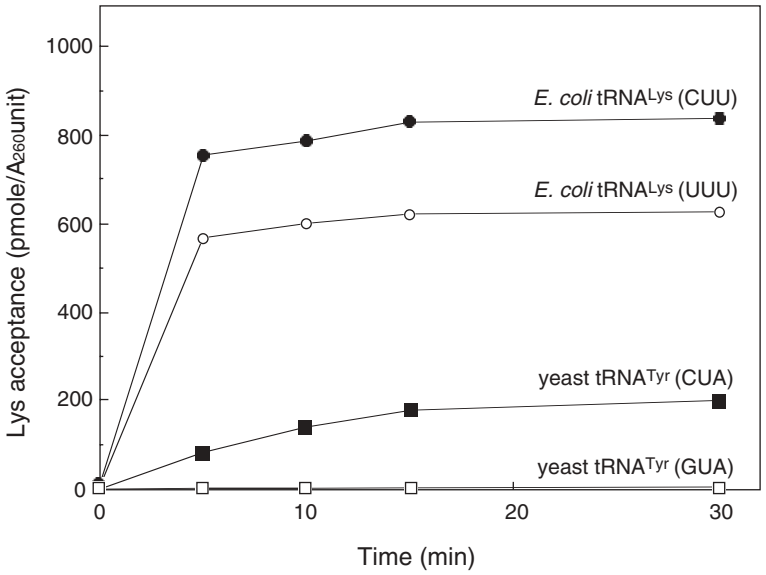

Fig. 2. **Lysine acceptance of several tRNAs by *E.* coli LysRS.** Lysylation reaction was carried out at 37°C in a reaction mixture (50 μl) containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 40 mM KCl, 4 mM ATP, 20 μM L-[^{14}C]-lysine, 0.25 A_{260} unit of tRNA, and 12.5 μg of *E. coli* LysRS. Aliquots (10 μl each) were withdrawn at the time intervals indicated and acid-insoluble radioactivities were counted.

identify which aaRS is responsible for the misacylation, we took advantage of our collections of purified *E. coli* aaRSs and peptide elongation factors (*12*). Yeast amber suppressor tRNA^{Tyr} (CUA) was incubated with each of the purified *E. coli* aaRSs in the presence of *T. thermophilus* EF-Tu and EF-Ts and GTP and a mixture of 20 amino acids. Since only the aminoacylated tRNAs can form stable ternary complexes with EF-Tu and GTP, they should have lower electrophoretic mobility on polyacrylamide gels than the non-acylated tRNAs (*15*). Thus, the enzyme responsible for the misacylation should be identified by the band shift of the tRNA. Figure 1 shows a typical result of such analyses using 20 species of purified *E. coli* aaRSs and yeast TyrRS as a positive control. This result clearly shows that *E. coli* LysRS (lane K in Fig. 1) has the activity to misacylate yeast amber suppressor tRNA^{Tyr} (CUA). Similar assays using varied aaRS concentrations gave similar results, suggesting that aaRSs other than LysRS are not responsible for the misacylation. However, the possibility remains that tRNAs misacylated with another amino acid (for example, glutamic acid or aspartic acid) escaped detection by this method due to their intrinsic weak binding, since the binding affinities between EF-Tu/GTP and aminoacyl-tRNAs could be variable depending on the chemical nature of the charged amino acids and the sequence of tRNA bodies (*18*, *19*). However, even if such a "misacylated" tRNA should be formed in the reaction mixture, it would not interfere with our present purpose of incorporating unnatural amino acid(s) into proteins, as long as it was poorly bound to EF-Tu/GTP and thus unable to participate in protein synthesis.

In Vitro *Lysylation of Yeast Amber Suppressor tRNA^{Tyr} by* E. coli *LysRS*.—Since the gel-shift assay of EF-Tu/GTP/aminoacyl-tRNA ternary complex suggested that *E. coli* LysRS is the enzyme responsible for the misacylation of yeast amber suppressor tRNA^{Tyr} (CUA), lysine-accepting activities of several tRNAs were measured quantitatively by the conventional radioisotopic method using L-[^{14}C]-lysine and precipitation with trichloroacetic acid.

Time-courses of lysine-acceptance are shown in Fig. 2. This figure clearly shows that yeast amber suppressor

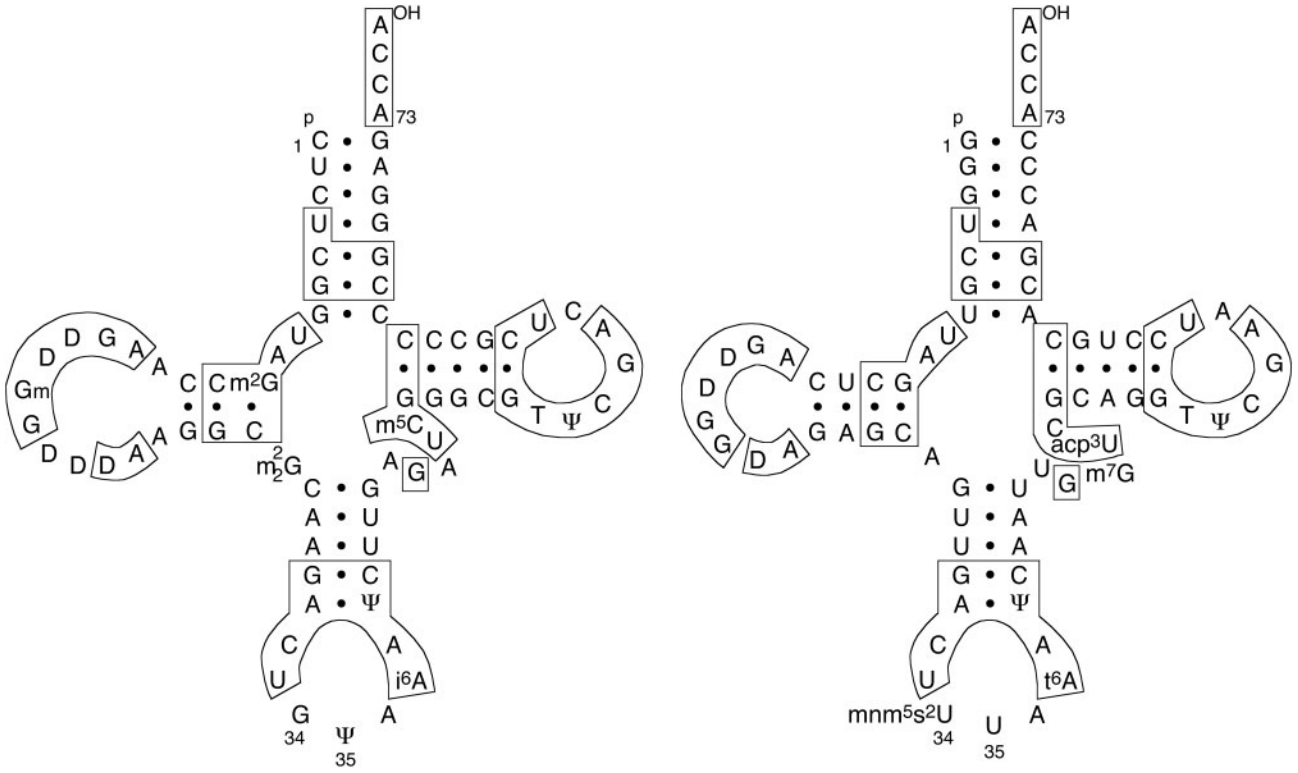

Fig. 3. **The nucleotide sequences of yeast tRNA^{Tyr} (left) and *E.* coli tRNA^{Lys}** (right) arranged in the clover-leaf secondary structure. The nucleotide sequences common to both tRNAs are boxed.

tRNA^{Tyr} (CUA) is actually charged with L-lysine to a maximal extent of about 200 pmol A_{260} unit of tRNA by the action of *E. coli* LysRS, although an unusually high concentration of the enzyme (12.5 μg in 50 μl of reaction mixture) was used in this assay. Interestingly, yeast wild-type tRNA^{Tyr} (GUA) did not accept L-lysine at all under the same conditions. This means that the misacylation of yeast amber suppressor tRNA^{Tyr} (CUA) by *E. coli* LysRS was caused by the base substitution from G34 to C34 in the course of transformation from wild-type to an amber suppressor tRNA^{Tyr}. Parallel relationship is observed between *E. coli* tRNA^{Lys} (CUU) and tRNA^{Lys} (UUU) (Fig. 2). The reason for the preference of *E. coli* LysRS for C34 is not yet clear, but the discussions based on the NMR and X-ray analyses of the interaction between *E. coli* LysRS and tRNA^{Lys} (*20–22*) might be instructive; hydrogen-bonding between the α NH of lysine132 of *E. coli* LysRS and the N3 of pyrimidine ring of the 34th nucleotide in the anticodon loop of tRNA^{Lys} is observed in the LysRS/tRNA^{Lys} co-crystals. C34 is more suitable to this situation than unmodified U34 (or other nucleotides). Tamura *et al.* reported that the substitution of G34 for U34 in *E. coli* tRNA^{Lys} would result in the total loss of the aminoacylation activity, and U35 is most important for the anticodon recognition by *E. coli* LysRS (*23*). Unfortunately, the transcript of yeast amber suppressor tRNA^{Tyr} (CUA) has both of these C34 and U35 residues.

Design of Suppressor tRNA^{Tyr} Mutants to Prevent the Misaminoacylation—Many of the amber suppressor tRNAs so far studied are known to be more or less mischarged with lysine (*24–26*), but tRNAs that have the CUA anticodon are not necessarily charged with lysine. Therefore, there must be one or more determinants in the nucleotide sequence of yeast amber suppressor tRNA^{Tyr} (CUA) that are used for recognition as a substrate by *E. coli* LysRS. If they could be identified and then mutated to different sequences, it should be possible to protect the tRNA from misacylation by *E. coli* LysRS. This mutation must be such that it would not affect the original aminoacylation activity of yeast TyrRS and would effectively repress the misacylation activity of *E. coli* LysRS. Otherwise, the primary purpose to utilize the tRNA as a "carrier" of unnatural amino acid (tyrosine derivatives) would not be achieved.

The native nucleotide sequences of yeast tRNA^{Tyr} (GΨA) and *E. coli* tRNA^{Lys} (mnm^{5}s^{2}UUU) are compared in Fig. 3. Nucleotide sequences commonly found in both tRNAs are boxed. Although these are possible targets for mutation, many of these (especially, those found in the D- and T-loops and some of those in the single-stranded regions) should not be changed since they are the 'conserved' or 'semi-conserved' nucleotides among almost all the tRNAs and are thought to be related to the intrinsic structure or function of the tRNAs. The identity elements for *E. coli* tRNA^{Lys} are reported to be the discriminator A73 and the anticodon U34U35U36 (*23*, *27*). But these cannot be mutated since the discriminator A73 and U35 are also identity elements for yeast tRNA^{Tyr} (*2*, *28*, *29*), and C34 is indispensable for amber suppressor tRNA as described above.

It has been speculated that the nucleotide sequence of the acceptor stem is one of the determinants for lysine-acceptability of tRNAs (CUA) in *E. coli* cells (*30*).

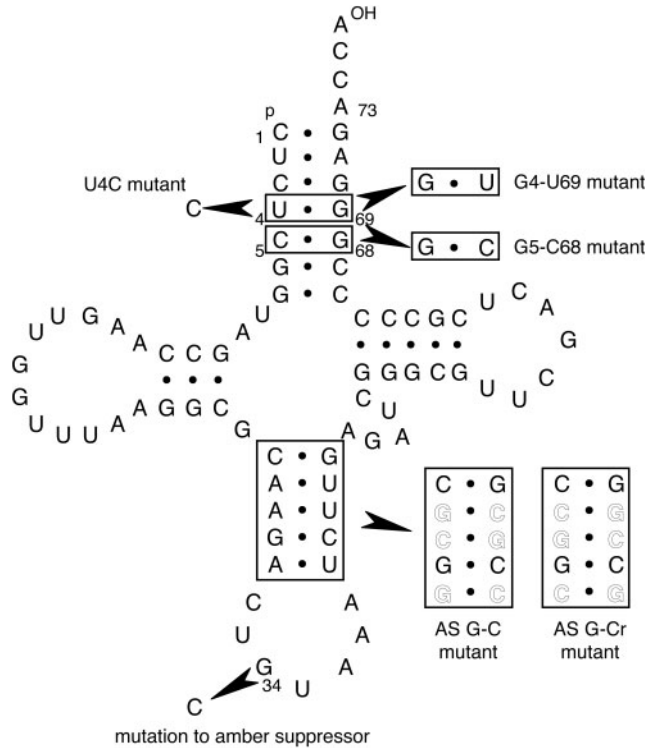

Fig. 4. **Mutants of yeast tRNA^{Tyr} used in this study.** Yeast amber suppressor tRNA^{Tyr} (CUA) was made from the wild-type tRNA^{Tyr} (anticodon; GUA) by introducing G34 to C34 mutation. Five mutants derived from this "original" amber suppressor tRNA^{Tyr} (CUA) are shown by arrowheads. Two more mutants carrying double mutations, U4C/AS G-C mutant and U4C/AS G-Cr mutant, were also made. All of the suppressor tRNAs listed were synthesized by transcription with T7 RNA polymerase and processing with RNase P according to our method (*14*).

It is also known that the G3–U70 wobble base pair in the acceptor stem of tRNA^{Ala} is a critical identity element of this tRNA (*31–33*). In the previous paper, we showed that the replacement of the U4–G69 wobble base pair in the acceptor stem of yeast tRNA^{Tyr} by C4–G69 Watson-Crick type normal base pair resulted in improvement of its tyrosine acceptance (*14*). These observations together seem to suggest that the local conformational change associated with the wobble base pair in the acceptor stem might be related to this misacylation. We therefore introduced in this region three mutations: U4 to C4 mutation (U4C mutant), change of U4–G69 base pair to G4–U69 base pair (G4–U69 mutant), and change of C5–G68 base pair to G5–C68 base pair (G5–C68 mutant) (Fig. 4).

In the anticodon arm region, C34 and U35 cannot be changed by the reason described above. The 'conserved' or 'semi-conserved' nucleotides like C32, U33 or A38 were kept unchanged. The number of A-U base pairs in the anticodon stem of *E. coli* tRNA^{Lys} (three: U28–A42, U29–A41, and A31–U39) is higher than that of usual *E. coli* tRNAs. On the other hand, yeast tRNA^{Tyr} also has three A-U base pairs (A28–U42, A29–U41, and A31–U39) at the same positions as *E. coli* tRNA^{Lys}. We reasoned that this pattern of A-U base pairs in the anticodon stem region might be one of the factors causing the misacylation by *E. coli* LysRS. We therefore constructed two mutants containing five G-C base pairs in tandem in the anticodon stem region (AS G-C mutant and AS G-Cr mutant). The sequences of the C and G stretch were chosen such that they would not hybridize easily to other part of the tRNA molecule (*e.g.*, the T-stem region). Double mutants carrying both U4C and AS G-C mutations (U4C/AS G-C mutant) or both U4C and AS G-Cr mutations (U4C/AS G-Cr mutant) were also constructed to examine the additive effects of the mutations. All the mutants of yeast amber suppressor tRNA^{Tyr} (CUA) were prepared by our method, *i.e.*, transcription as pre-tRNA and processing by RNase P as described previously (*14*), and are illustrated in Fig. 4.

Aminoacylation Properties of suppressor tRNA^{Tyr} Mutants—Eight species of amber suppressor tRNA^{Tyr} and its derivatives were prepared in this study and all of these have the anticodon CUA (corresponding to the amber codon UAG). These include the original amber suppressor tRNA^{Tyr} (CUA) and its derivatives carrying one of the following seven mutations; U4C, G4–U69, G5–C68, AS G-C, U4C/AS G-C, AS G-Cr, or U4C/AS G-Cr. Kinetic parameters for these tRNAs in the tyrosylation (tyrosyl-tRNA formation) reaction were determined as described in the "EXPERIMENTAL PROCEDURES" section and are summarized in Table 1.

We previously reported that the tyrosine-accepting activity of tRNA^{Tyr} transcripts (composed only of unmodified nucleotides) was greatly decreased compared to that of native tRNA^{Tyr} (containing modified nucleotides) and that the replacement of the U4–G69 wobble base pair in the acceptor stem of yeast tRNA^{Tyr} by C4–G69 Watson-Crick type base pair (U4C mutation) has an improving effect (about two fold) on its tyrosine-acceptance (*14*). Interestingly, this improving effect by U4C mutation is also observed in the double mutants carrying both U4C and AS G-C mutations in the anticodon stem (U4C/AS G-C mutant and U4C/AS G-Cr mutant), indicating that the effects of these mutations are additive, as expected. Although the G5–C68 mutation had no substantial effect on the tyrosine-accepting activity of the original suppressor tRNA^{Tyr} (CUA), the G4–U69 mutant had a slightly lower activity than the original suppressor tRNA^{Tyr} (CUA). The local conformational difference around the wobble base pair at the middle (4th) position in the acceptor stem seems likely to be important for the recognition of yeast tRNA^{Tyr} by TyrRS.

Determination of kinetic parameters for these tRNAs in the misacylation (lysyl-tRNA formation) reaction was difficult because the misacylation level itself was not so high (see Fig. 2).

Therefore the plateau levels of misacylation with lysine were compared among these tRNAs, taking the value of the "original" amber suppressor tRNA^{Tyr} (CUA) (about 200 pmol lysine/A_{260} unit) as 100%. These values and those for tyrosylation levels are summarized in Table 2. The plateau levels of tyrosylation broadly coincided with the relative values of kinetic constants for tyrosylation (Table 1). Transfer RNA mutants carrying U4C mutation (U4C mutant, U4C/AS G-C mutant and U4C/AS G-Cr mutant) had about 25% higher plateau levels of tyrosylation than tRNAs without this mutation (the amber suppressor, AS G-C mutant and AS G-Cr mutant). The

Table 1. Kinetic parameters for tyrosylation of yeast suppressor $tRNA^{Tyr}$ (CUA) and its derivatives.

Suppressor tRNAs[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	Aminoacylation efficiency (χ-fold)[b]
Original amber suppressor $tRNA^{Tyr}$ (CUA)	1.45	0.013	8.97	1
U4C mutant	0.62	0.010	16.1	1.8
G4–U69 mutant	3.30	0.014	4.24	0.47
G5–C68 mutant	1.80	0.013	7.22	0.80
AS G-C mutant	1.53	0.012	7.84	0.87
U4C/AS G-C mutant	0.90	0.015	16.7	1.86
AS G-Cr mutant	1.38	0.015	10.9	1.21
U4C/AS G-Cr mutant	0.70	0.016	22.9	2.55

[a]All the tRNAs listed were synthesized by transcription with T7 RNA polymerase and processing with RNase P according to our method (*14*), and all have the anticodon CUA. [b]Aminoacylation efficiencies are expressed relative to the k_{cat}/K_m value for original amber suppressor $tRNA^{Tyr}$ (CUA).

Table 2. **Amino acid acceptance levels of yeast suppressor $tRNA^{Tyr}$ (CUA) and its derivatives.**

Suppressor tRNAs[a]	Amino acid acceptance (plateau level)[b]	
	Tyrosine	Lysine
Original amber suppressor $tRNA^{Tyr}$ (CUA)	100 (%)	100 (%)
U4C mutant	124.0	42.3
G4–U69 mutant	44.5	40.4
G5–C68 mutant	73.1	36.4
AS G-C mutant	97.1	12.7
U4C/AS G-C mutant	125.0	3.3
AS G-Cr mutant	105.7	4.8
U4C/AS G-Cr mutant	126.5	<1.9

[a]All the tRNAs listed are the same as in Table 1. [b]Amino acid acceptance of each tRNA is expressed as the percentage of the plateau value for original amber suppressor $tRNA^{Tyr}$ (CUA). Actual values (100%) are 800 pmol/A_{260} unit for tyrosine acceptance and 200 pmol/A_{260} unit for lysine acceptance.

G4–U69 mutant had the lowest plateau value and the G5-C68 mutant had an intermediate value.

Mutations within the acceptor stem (U4C mutation, G4–U69 mutation and G5–C68 mutation) partially prevented the misacylation with lysine, while mutations within the anticodon stem (AS G-C mutation and AS G-Cr mutation) had a more drastic effect. The double mutants carrying both the U4C mutation and the AS G-C or AS G-Cr mutation (U4C/AS G-C mutant and U4C/AS G-Cr mutant) were superior (Table 2). These results clearly indicate that the change of three A-U base pairs in the anticodon stem of yeast $tRNA^{Tyr}$ to three G-C or C-G base pairs had virtually no effect on the recognition by yeast TyrRS but greatly repressed the recognition by *E. coli* LysRS. In earlier studies, Schultz's group employed *in vivo* selection from pools of *M. jannaschii* $tRNA^{Tyr}$(CUA) mutants to generate "orthogonal" $tRNAs^{Tyr}$ that are not recognized by *E. coli* endogenous synthetases to any meaningful degree, and still function efficiently with *Methanococcus jannaschii* TyrRS to translate the amber codon with tyrosine (*34*, *35*). Interestingly, their "orthogonal" $tRNAs^{Tyr}$ (CUA) derived from *M. jannaschii* $tRNA^{Tyr}$ also have four G-C or C-G base pairs in the anticodon stem. This coincidence may suggest that an anticodon stem rich in G-C base pairs is one of the essential features needed for tRNAs to escape recognition by *E. coli* LysRS.

In Vitro *Protein Synthesis Using the "Optimized" $tRNA^{Tyr}$ Mutant*—As the tyrosine-acceptance of U4C/AS G-Cr mutant is rather improved and the misacylation with lysine is repressed to less than 2% of the original suppressor $tRNA^{Tyr}$ (CUA), this mutant tRNA is the best candidate for use as the "carrier" tRNA presently available. To examine the suppressor activity of this "optimized" suppressor $tRNA^{Tyr}$ mutant, *in vitro* protein syntheses based on an *E. coli* transcription/translation coupled system were carried out using this $tRNA^{Tyr}$ mutant and template DNAs for S-tagged EGFP or DHFR (Fig. 5). In EGFP-synthesis (Fig. 5, upper panel), the green fluorescent bands shown in the figure correspond to the synthesized full-length (read-through) EGFP protein. Since the amber mutation was introduced at the 31st codon of the S-tagged EGFP gene, these bands should be seen only if the suppression of the amber codon is effective. Similar levels of EGFP-synthesis are observed in lane 1 (positive control; the EGFP gene has no amber mutation), lane 2 (original amber suppressor $tRNA^{Tyr}$ + yeast TyrRS) and lane 4 ("optimized" U4C/AS G-Cr mutant + yeast TyrRS). In lane 3 (original amber suppressor $tRNA^{Tyr}$ in the absence of yeast TyrRS), a faint band can be seen at the full-length protein region, indicating that an amino acid (probably lysine) has been mischarged to the suppressor $tRNA^{Tyr}$ by an endogenous *E. coli* aaRS (probably LysRS). No such read-through product is detected when the "optimized" U4C/AS G-Cr mutant is used (lane 5), suggesting that this tRNA is no longer misacylated with lysine under the reaction conditions used and could function as a "carrier" of non-canonical amino acids in an *E. coli* protein-synthesizing system. In similar experiments, no fluorescent band (full-length EGFP suppressed at the amber codon) was observed in the lanes using the "optimized" U4C/AS G-Cr mutant in the absence of yeast TyrRS. Therefore, the improvement of misacylation level was not estimated quantitatively in this case.

A basically similar result was obtained when the amber mutation was introduced at the 128th codon of the DHFR gene and the incorporation of L-[^{14}C]-leucine into the DHFR synthesized was measured, instead of mere observation of the fluorescent bands. Figure 5 lower panel shows a typical result of these experiments together with the values for the suppression efficiency and standard deviations (average of three experiments). Although complete disappearance of the read-through product in the absence of yeast TyrRS was not achieved, the use of U4C/AS G-Cr mutant (lane 5) substantially improved the misacylation of original amber suppressor $tRNA^{Tyr}$ (lane 3).

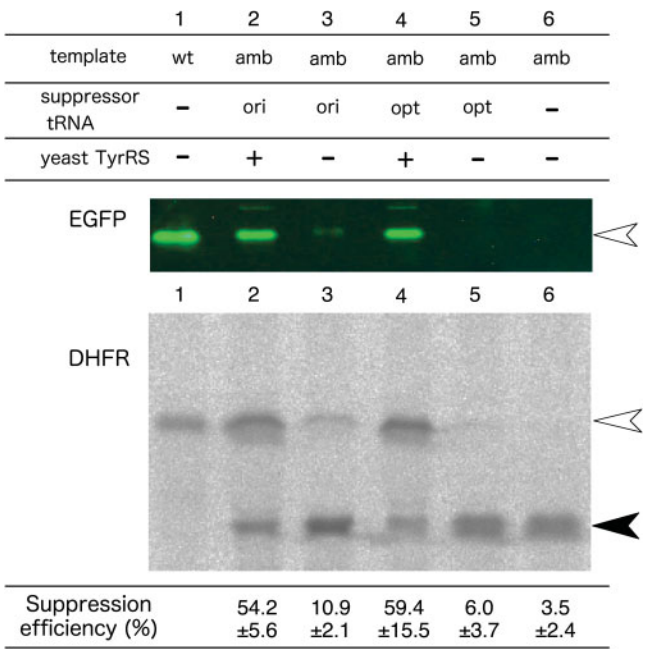

Fig. 5. ***In vitro* suppression of amber codons in EGFP (upper panel) or DHFR (lower panel) using the original and "optimized" suppressor tRNAs in an *E.*** coli transcription/translation coupled system. Cell-free protein synthesis was performed basically according to Kigawa *et al.* (*17*) except that the reaction was done at 30°C for 2 h, and 2 μM yeast TyrRS, 1.6 μM yeast amber suppressor $tRNA^{Tyr}$ or its mutant (U4C/AS G-Cr) and 24 μg/ml template plasmid DNA were added when they were needed. The following plasmid DNAs were used as the templates: pHSGEGFPht (lane 1, upper panel) or pHSGambEGFPht (lanes 2–6, upper panel) for EGFP synthesis and pETDHFR (lane 1, lower panel) or pETDHFR128amb (lanes 2–6, lower panel) for DHFR synthesis. Lane 1, positive control (the template gene has no amber mutation); lanes 2 and 3, yeast original amber suppressor $tRNA^{Tyr}$ added; lanes 4 and 5, "optimized" suppressor $tRNA^{Tyr}$ (U4C/AS G-Cr mutant) added; lane 6, no suppressor tRNA added. The reaction was done in the presence (lanes 2 and 4) or absence (lanes 1, 3 and 5, 6) of yeast TyrRS. Open arrowheads indicate the position of full-length (read-through) protein products, and the filled arrowhead indicates the position of imperfectly synthesized polypeptides interrupted at the amber codon. Figures at the bottom of the lower panel represent the values for the suppression efficiency and standard deviations of DHFR synthesis (average of three experiments).

Thus, we think that the U4C/AS G-Cr mutant is one of the best candidates now available to us for use as the "carrier" of non-canonical tyrosine analogue(s) in an *E. coli* protein-synthesizing system, although there is still room for improvement by elucidating the precise molecular mechanism for recognition by yeast TyrRS and *E. coli* LysRS. Site-specific incorporation of 3-substituted tyrosine analogues into proteins by utilizing this U4C/AS G-Cr mutant tRNA and genetically mutated yeast TyrRSs is now underway in our laboratory and will be published elsewhere. On the other hand, several "orthogonal" tRNA-synthetase pairs have been generated for use in eukaryotes. For example, *E. coli* TyrRS mutants were used with *Bacillus stearothermophilus* suppressor $tRNA^{Tyr}$ in mammalian CHO cells (*36*) or with *E. coli* suppressor $tRNA^{Tyr}$ in a wheat germ cell-free system (*37*) to incorporate 3-iodo-L-tyrosine into proteins in response to an amber codon. A pair consisting of *E. coli* suppressor $tRNA^{Tyr}$ and *E. coli* TyrRS has been engineered to incorporate more than 10 unnatural amino acids into proteins in response to an amber codon in *Saccharomyces cerevisiae* (*38*, *39*). By utilizing these and other new "orthogonal" pairs, the structural and chemical diversity of unnatural amino acids that can be incorporated into proteins will certainly be expanded, allowing the generation of proteins having enhanced or unprecedented properties.

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