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Codon-reading specificity of an unmodified form of *Escherichia coli* $tRNA_1^{Ser}$ in cell-free protein synthesis

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ABSTRACT

Unmodified tRNA molecules are useful for many purposes in cell-free protein biosynthesis, but there is little information about how the lack of tRNA post-transcriptional modifications affects the coding specificity for synonymous codons. In the present study, we prepared an unmodified form of *Escherichia coli* $tRNA_1^{Ser}$, which originally has the cmo^{5}UGA anticodon (cmo^{5}U = uridine 5-oxyacetic acid) and recognizes the UCU, UCA and UCG codons. The codon specificity of the unmodified tRNA was tested in a cell-free protein synthesis directed by designed mRNAs under competition conditions with the parent $tRNA_1^{Ser}$. It was found that the unmodified tRNA with the UGA anticodon recognizes the UCA codon nearly as efficiently as the modified tRNA. The unmodified tRNA recognized the UCU codon with low, but detectable efficiency, whereas no recognition of the UCC and UCG codons was detected. Therefore, the absence of modifications makes this tRNA more specific to the UCA codon by remarkably reducing the efficiencies of wobble reading of other synonymous codons, without a significant decrease in the UCA reading efficiency.

INTRODUCTION

Cell-free protein biosynthesis is becoming more useful in applications for biochemical and molecular biological studies (1–3). This method is free of many of the limitations associated with *in vivo* protein production systems. For example, engineered tRNA molecules can be added to the cell-free protein synthesis system. Proteins with an unnatural amino acid residue at a specific position can be synthesized by the use of an engineered suppressor tRNA molecule charged with the unnatural amino acid through chemical aminoacylation (2,4).

In general, however, it is not easy to purify and engineer large amounts of naturally occurring tRNA molecules. In contrast, *in vitro* transcription with T7 RNA polymerase (5) is a powerful method for the preparation and engineering of tRNA molecules. By this method, a large amount of homogeneous tRNA of the desired sequence can easily be obtained. The tRNA molecules generated by this method are not natural, as they lack post-transcriptional modifications, which are considered to have important roles in codon reading (6,7). Nevertheless, unmodified tRNAs have been shown to insert natural (8–10) and unnatural (11,12) amino acids into polypeptide chains.

Comparison of the codon reading specificity between the unmodified and naturally modified forms have been done for *Escherichia coli* $tRNA^{Phe}$ (9) and *Mycoplasma mycoides* $tRNA^{Gly}$ (8). These studies indicated that the modifications had minimal effects on the specificity concerning the third codon bases. Note that the natural *E.coli* $tRNA^{Phe}$ and *M.mycoides* $tRNA^{Gly}$ have no modified nucleoside in the anticodon. In contrast, many other tRNAs have a modified nucleoside at the first position of the anticodon (position 34) (13), which significantly contributes to codon recognition (6). Therefore, for these anticodon-modified tRNAs, it is interesting to examine the codon reading specificities of the unmodified forms.

Uridines substituted at position 5 by an oxygen atom (xo^{5}U) have been identified at position 34 of many tRNAs that correspond to a four-codon box (a set of four codons with the first two bases in common, which specify the same amino acid) (14–16). Such tRNAs recognize the codons ending with A, G and U. For example, *E.coli* $tRNA_1^{Ser}$ has uridine 5-oxyacetic acid (cmo^{5}U), and the cmo^{5}UGA anticodon reads the UCU, UCA and UCG triplets on ribosomes (17,18). On the other hand, in mitochondria and in *Mycoplasmas*, a single tRNA species with an unmodified uridine at the first position of the anticodon recognizes all four cognate codons (19–23). Thus, it has been argued that the xo^{5}U-type modifications restrict the reading of codons ending with C (24). In contrast, it has also been proposed that the xo^{5}U-type modifications are responsible for increasing the reading efficiency for U, but not for C, to levels nearly as high as those for A and G (16).

In the present study, we investigated the activity and specificity of an unmodified form of *E.coli* $tRNA_1^{Ser}$ in the reading of codons UCN (N = U, C, A and G). Direct comparison between the modified and unmodified molecules revealed the codon reading properties of the unmodified tRNA molecules, and the roles of the modifications.

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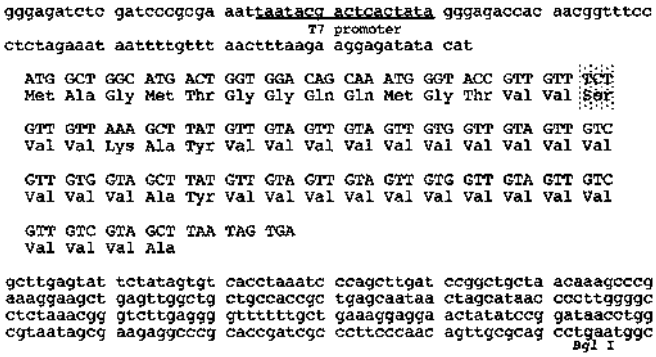

Figure 1. A partial nucleotide sequence of pART23GGC3TCT and the encoded amino acid sequence. The capitalized region was prepared by chemical synthesis and PCR. The small letters show the nucleotides derived from pGEMEX-1, the T7 promoter of which is indicated. The shadowed codon is the test codon, which has been changed, by PCR, to TCC in pART23GGC3TCC, TCA in pART23GGC3TCA, TCG in pART23GGC3TCG and GAG in pART23GGC3GAG.

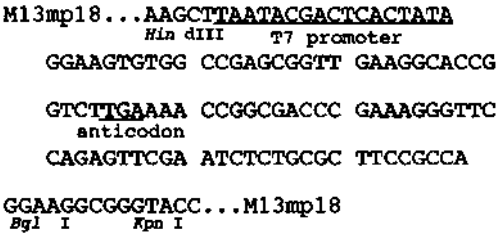

Figure 2. Nucleotide sequence of T7Ser1TGA. The T7 promoter and anticodon are underlined. The recognition sequences of the restriction enzymes are indicated.

MATERIALS AND METHODS

Chemicals

[^{14}C]Serine (5.98 GBq/mmol) was purchased from New England Nuclear. [^{3}H]Serine (1.07 TBq/mmol) was from Amersham, and was concentrated with a centrifugal concentrator to 8.0 mCi/ml (295 GBq/ml).

Preparation of S30 extract

The preparation of the S30 extract was as described (25), except that an MSK cell homogenizer (B. Braun) was used for lysis of the cells. The glass beads were removed by centrifugation at 2300 *g* immediately after lysis.

Preparation of mRNA

The mRNAs were designed and prepared on the basis of the method used in analyses of codon assignment in *Mycoplasma capricolum* (26,27). A 156-bp fragment (capital letters in Fig. 1) was chemically synthesized, amplified by PCR, and substituted for a 0.9 kb *Nde*I–*Hin*dIII fragment (the major part of the coding region of T7 gene *10*) of pGEMEX-1 (Promega). Then, the whole DNA fragment shown in Figure 1 was prepared by *Sma*I and *Bgl*I digestion, and substituted for a 0.3 kb *Pvu*II–*Bgl*I region of pUC118 (pART23GGC3TCT). The coding region has a single test codon, TCT for Ser (shadowed in Fig. 1), in the middle of 48 non-Ser codons; the amino terminal 11 amino acid residues are derived from those of the gene *10* product except that the third codon, AGC, for Ser has been mutated to GGC for Gly. Four variants in terms of the test codon were also prepared by PCR mutagenesis of TCT to TCC, TCA, TCG and GAG (pART23GGC3TCC, pART23GGC3TCA, pART23GGC3TCG and pART23GGC3GAG respectively).

For the preparation of mRNA, the template plasmid (10 µg) was first digested completely with *Bgl*I, and was transcribed with T7 RNA polymerase, prepared as described (28). The reaction contained 40 mM HEPES–KOH, pH 7.5, 16 mM magnesium chloride, 40 mM potassium chloride, 4 mM each of ATP, CTP, GTP and UTP, 5 mM dithiothreitol (DTT), 2 mM spermidine, 80 U/ml RNase inhibitor (Toyobo, Japan), 0.2 mg/ml bovine serum albumin and 70–140 µg/ml T7 RNA polymerase. After an incubation for 1 h at 37°C, an equal amount of the enzyme was added, and the reaction was continued for another hour. The samples were extracted with phenol and dialyzed against water. The mRNA with the UCU test codon, for example, is designated as mRNA(UCU) hereafter in the text. mRNA(GAG) was prepared as the control mRNA having no Ser codon.

Preparation of unmodified tRNAs

The sequences of the unmodified tRNAs were constructed on the M13mp18 vector (T7Ser1TGA, Fig. 2). The single-stranded DNA of T7Ser1TGA was used as the template for a polymerase chain reaction with the primers 5′-ACGACGTTGTAAAAC-GACGGCCAG-3′ and 5′-ATTTCACACAGGAAACAGCTAT-GAC-3′ both of which hybridize outside the multi-cloning site. The product was digested with the restriction enzyme *Bgl*I, which produces the proper CCA end of the template, and was purified with a standard polyacrylamide gel. This DNA was used for the transcription reaction containing 20 mM GMP, in addition to the components in the mRNA preparation described above. After transcription, the sample was purified with 15% polyacrylamide gel containing 8 M urea.

Preparation of $tRNA_1^{Ser}$

The $tRNA_1^{Ser}$ from *E.coli* A-19 (29) was prepared essentially as described (18). A modified nucleoside, 2′-*O*-methylcytidine, occurs at position 32 of $tRNA^{Ser}_1$ from *E.coli* B (30). In our sample, this position is only partially modified, or is nearly completely unmodified, as judged from the facts that it was cleaved efficiently between positions 32 and 33 with RNase A, and that the 3′-terminal nucleoside of the resulting 5′-oligonucleotide was an unmodified C (data not shown).

Preparation of seryl-tRNA synthetase (SerRS) crude fraction from *E.coli*

The DNA sequence containing the *serS* gene (31) was amplified by PCR from the chromosomal DNA of *E.coli* strain A-19, using the phosphorylated primers 5′-pTGACGTGCCGAATTCATTT-GCGTAATG-3′ and 5′-pCTCACGATTGAATTCCAGTAAC-AAA-3′ (each with an *Eco*RI recognition site). The resulting DNA fragment was directly inserted into the plasmid pBR322. The *Eco*RI fragment, which was considered to encompass the *serS* gene, was obtained from one clone and was transferred into plasmid pUC119 (pUCSRS). *Escherichia coli* MV1184 harboring

pUCSRS was cultured overnight in 2× TY medium (4 l) containing 150 μg/ml ampicillin. The cells were lysed and the lysate was separated on a column (2.5 × 25 cm) of DE52 (Whatman) with a linear gradient of ammonium chloride concentration from 50 to 300 mM in buffer A [50 mM Tris–HCl, pH 7.9, 10 mM magnesium acetate, 5 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. The fractions with high activity were pooled. Solid ammonium sulfate was added to 60% saturation, and the precipitate was dialyzed against buffer A without PMSF. Glycerol was added to a final concentration of 50%. This crude SerRS (~2 mg protein/ml or 0.1–0.2 mg/ml SerRS) was stored at –20°C and was used for the preparation of Ser-tRNA.

Preparation of aminoacyl-tRNAs

We prepared [^{14}C]Ser-tRNA(modified), [^{14}C]Ser-tRNA(unmodified), [^{3}H]Ser-tRNA(modified) and [^{3}H]Ser-tRNA(unmodified) as follows.

For the preparation of Ser-tRNA, tRNA (2 A_{260} U/ml or 3.4 μM) was incubated at 37°C for 15–25 min with 50 mM HEPES–KOH, pH 7.2, 16 mM $MgCl_2$, 2 mM ATP, potassium salt, 10 mM KCl, 1/10 vol of the crude SerRS described above and 16–36 μM ^{3}H- or ^{14}C-labeled serine. The reaction was stopped by the addition of 1/4 vol of 1 M potassium acetate, pH 4.5. The Ser-tRNA was extracted with phenol, and was precipitated with ethanol. The precipitate dissolved in aminoacyl-tRNA buffer (10 mM acetic acid, 4 mM magnesium acetate, pH 4.5). An aliquot was used to measure the radioactivity using ReadyCap™ (Beckman). It should be noted that Tris inhibits SerRS, although it has been used in many published experiments.

The nonradioactive aminoacyl-tRNA mixture (cold aminoacyl-tRNA^{Mix}) was prepared by incubating 2 mg/ml of the tRNA crude fraction from *E.coli* with a mixture of 0.55 mM threonine, alanine, glycine, tyrosine, methionine, glutamine and lysine hydrochloride and 1.1 mM valine, in the presence of 1/10 vol of the S100 extract (32), in the same manner as in the serylation reaction described above. The cold aminoacyl-tRNA^{Mix} is not essential for the protein synthesis reaction, but it shortens the initial lag period of the reaction.

The principle of the measurement of relative codon reading efficiencies through cell-free protein synthesis

The method utilized in the present study is a modification of that for the relative codon-reading efficiency analysis (33) according to the translational error analysis method (34). For a given test mRNA, cell-free protein synthesis reactions were performed with [^{14}C]Ser-tRNA(modified) and [^{3}H]Ser-tRNA(unmodified) and also with [^{14}C]Ser-tRNA(unmodified) and [^{3}H]Ser-tRNA(modified). During translation of the mRNA, the test codon, UCU, UCC, UCA, UCG or GAG, was recognized, in competition, by the Ser-tRNAs, for example, [^{14}C]Ser-tRNA(modified), [^{3}H]Ser-tRNA(unmodified) and/or other intrinsic, nonradioactive aminoacyl-tRNAs. We added rather large amounts of the radioactive Ser-tRNAs so that they could recognize codons in competition with nonradioactive Ser-tRNAs present in the reaction. In order to prevent transfer of the radioactive serine from the preaminoacylated Ser-tRNAs to other tRNAs, we used a large amount of nonradioactive serine in the reaction (see below).

Cell-free protein synthesis

Four micrograms of cold aminoacyl-tRNA^{Mix}, 1.9×10^4 c.p.m. (~60 pmol) of [^{14}C]Ser-tRNA(modified or unmodified), 8.5×10^5 c.p.m. (~60 pmol) of [^{3}H]Ser-tRNA(unmodified or modified) and 0.6 A_{260} unit (~0.16 nmol) of one of the five test mRNAs were first mixed in 0.2 M potassium acetate, pH 4.5, and precipitated with ethanol. The precipitate was rinsed with ethanol, dried *in vacuo*, and dissolved in 20 μl of the aminoacyl-tRNA buffer (RNA mixture). The protein synthesis reaction (240 μl) was incubated at 37°C and contained 55 mM HEPES–KOH, pH 7.5, 1.7 mM DTT, 275 μM GTP, 26 mM phosphoenolpyruvate, potassium salt (Boehringer Mannheim), 1.2 mM ATP, 1.9% (w/v) polyethyleneglycol 8000 (Sigma), 34 μg/ml folinic acid, calcium salt (Sigma), 6.9 mM ammonium acetate, 1 mM spermidine, 7.5 mM magnesium acetate, 210 mM potassium glutamate (Sigma), 0.74 mM valine, 1.1 mM serine, 0.37 mM each of threonine, alanine, glycine, tyrosine, methionine, glutamine and lysine hydrochloride, 1/6 vol of S30 extract, and the RNA mixture. The reaction was started by mixing the RNA mixture with the other reaction components.

Aliquots (50 μl) were transferred into 50 μl of 0.2 N NaOH at 0, 4, 8 and 12 min, and were incubated at 37°C for 30–60 min. Aliquots (10 μl) were transferred to 90 μl of chilled 5% TCA at 2, 6 and 10 min. These samples (100 μl) were placed on the Whatman 3MM filter disks, and were washed three times with ice-cold 5% TCA. The retained radioactivity on the filter disk was counted using a standard liquid scintillator with an LSC-700 liquid scintillation counter (Aloka, Japan). The ^{14}C and ^{3}H radioactivities incorporated into the polypeptide chain were measured separately in c.p.m. The amounts of the surviving, labeled Ser-tRNA at $(2n + 2)$ min ($n = 0, 1, 2$) also in c.p.m. were estimated as those of the alkali-sensitive, TCA-insoluble matter by subtracting the average of the amounts of the alkali-resistant, TCA-insoluble matter at $2n$ and $(2n + 4)$ min from the amount of the total TCA-insoluble matter at $(2n + 2)$ min. Codon reading efficiencies of the unmodified tRNA relative to the modified tRNA were calculated as described in detail in the Results and Discussion. Note that the calculation does not require the values of the counting efficiencies for ^{14}C and ^{3}H, because they were each canceled out upon comparison between modified and unmodified tRNAs.

RESULTS AND DISCUSSION

Cell-free protein synthesis under competition conditions with modified and unmodified tRNAs

First, we prepared [^{14}C]Ser-tRNA(modified), [^{14}C]Ser-tRNA(unmodified), [^{3}H]Ser-tRNA(modified) and [^{3}H]Ser-tRNA(unmodified). The unmodified tRNA was serylated efficiently with the crude SerRS. The recovery of Ser-tRNA was ~60% for the unmodified tRNA and ~90% for the modified tRNA. As *E.coli* SerRS recognizes characteristic secondary and tertiary structures of serine tRNAs during aminoacylation (35), we concluded that the unmodified tRNA was properly folded into such characteristic structures.

We then measured the incorporation of labeled serine into the polypeptide during a protein synthesis reaction containing mRNA(UCA) in the presence of the [^{14}C]Ser-tRNA(modified) and [^{3}H]Ser-tRNA(unmodified). The recognition of codon UCA by the tRNAs is through three Watson–Crick-type base pairs.

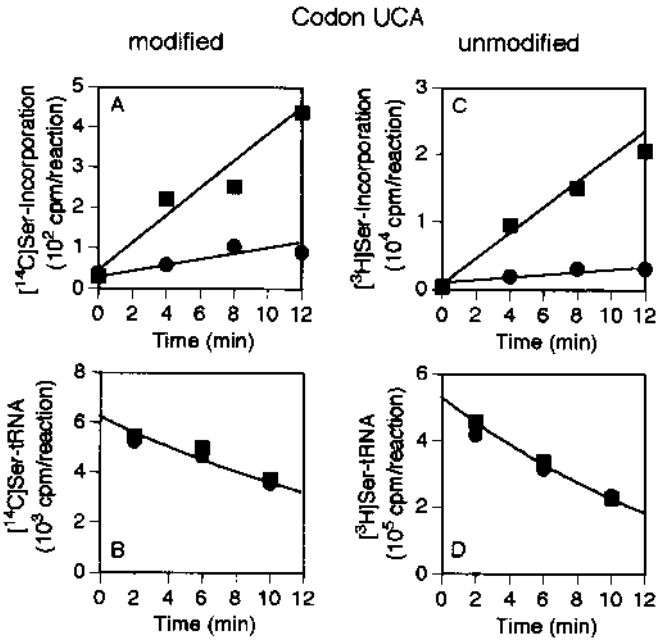

Figure 3. Cell-free protein synthesis assay for the UCA codon with [^{14}C]Ser-tRNA(modified) and [^{3}H]Ser-tRNA(unmodified). Profiles of the test reaction containing mRNA(UCA) (■) and of the control reaction with mRNA (GAG) (●) are shown. (**A**) [^{14}C]serine incorporation to the polypeptide; (**B**) the levels of surviving [^{14}C]Ser-tRNA(modified); (**C**) [^{3}H]serine incorporation into the polypeptide; (**D**) the levels of surviving [^{3}H]Ser-tRNA(unmodified).

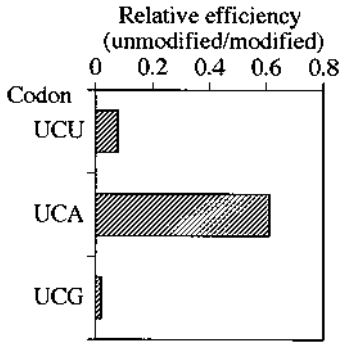

Figure 4. Relative codon reading efficiencies of the unmodified tRNA. Shown are the relative efficiencies of the unmodified tRNA relative to the modified tRNA. See text for details.

As the synthesized polypeptide contained many valine residues, it was easily precipitated in 5% TCA. As shown in Figure 3, [^{14}C]serine was efficiently incorporated from the [^{14}C]Ser-tRNA(modified) into the acid insoluble fraction (Fig. 3A, ■). As a control, we performed, in parallel, a reaction containing mRNA(GAG), which does not contain any serine codons; the incorporation was expectedly inefficient (Fig. 3A and C, ●).

During the incubation, the amount of [^{14}C]Ser-tRNA decreases by alkaline hydrolysis, even if there is no corresponding codon in the mRNA. Actually, we measured the level of surviving, labeled Ser-tRNAs. Figure 3B shows that the amounts of the [^{14}C]Ser-tRNA(modified) in c.p.m. decreased during the course of the test (■) and control (●) reactions. The decrease in the [^{14}C]Ser-tRNA level was much faster than the apparent incorporation of [^{14}C]serine into polypeptide, and therefore attributed primarily to alkaline hydrolysis. In fact, the difference in the levels of [^{14}C]Ser-tRNA(modified) between the test and control reactions was small. Note that radioactive serine released by hydrolysis would rarely be transferred to any other tRNAs, as mentioned above. In order to correct the effect of such hydrolysis, the radioactivity incorporated into polypeptide was normalized with that of the surviving [^{14}C]Ser-tRNA(modified), resulting in the apparent efficiency of incorporation. The difference between the apparent incorporation efficiencies for mRNA(UCA) and mRNA(GAG) gave the UCA-dependent [^{14}C]Ser-incorporation efficiency for the modified tRNA.

In the same manner, we also obtained the UCA-dependent [^{3}H]Ser-incorporation efficiency for the unmodified tRNA using the ^{3}H data (Fig. 3C and D). Furthermore, we performed the second reaction set with [^{14}C]Ser-tRNA(unmodified) and [^{3}H]Ser-tRNA(modified) (data not shown), and obtained the UCA-dependent [^{14}C]Ser- and [^{3}H]Ser-incorporation efficiencies for the unmodified and modified tRNAs respectively. By dividing the efficiency for [^{14}C]Ser-tRNA(unmodified) by that for [^{14}C]Ser-tRNA(modified), we obtained the efficiency of the unmodified tRNA relative to that of the modified tRNA. This value is independent of the counting efficiencies and recovery of the polypeptide or Ser-tRNA. From the ^{3}H data, we also obtained the corresponding value. Then, we calculated the geometric mean of these two values. Finally, we obtained the efficiency of the unmodified tRNA relative to the modified tRNA in the reading of the UCA codon (61%).

We performed the reactions also for mRNA(UCU), mRNA(UCC) and mRNA(UCG). The relative codon reading efficiencies for the UCU and UCG codons were certainly determined as shown in Figure 4. The UCU codon was recognized efficiently by the modified tRNA, while it was only weakly recognized by the unmodified tRNA. The UCG codon was also recognized efficiently by the modified tRNA, but the reading by the unmodified tRNA was not detectable. In contrast, as shown in Figure 5, both tRNAs exhibited only minimal Ser-incorporation for mRNA(UCC) over mRNA(GAG). Accordingly, we concluded that neither tRNA recognized the UCC codon, and therefore did not calculate the relative efficiencies.

The unmodified tRNA reads the UCA codon almost as efficiently as the modified tRNA

The results indicated that the Watson–Crick type recognition of the A of the UCA codon was only slightly affected by the lack of modifications in the present case of $tRNA_1^{Ser}$. Two other unmodified tRNA molecules have been compared with their parent tRNAs for their codon reading activities (8,9). As compared with the modified $tRNA^{Phe}$, the unmodified $tRNA^{Phe}$ was weaker (~70%) in the formation of a ternary complex with elongation factor Tu, but was more efficient (~260%) in the dipeptide formation rate per ternary complex (9). An unmodified form of *M.mycoides* $tRNA^{Gly}$ is as active as the naturally modified form (8). All these results indicate that the unmodified tRNA is, in general, nearly as efficient as the modified tRNA.

Actually, $tRNA_1^{Ser}$ is different from $tRNA^{Phe}$ and $tRNA^{Gly}$, in that it is modified at position 34, while the other tRNAs are not. However, the modification does not change the arrangement of the hydrogen donor–acceptor pattern at positions 2, 3 and 4 of the pyrimidine ring. Thus, it is not likely that it dramatically changes

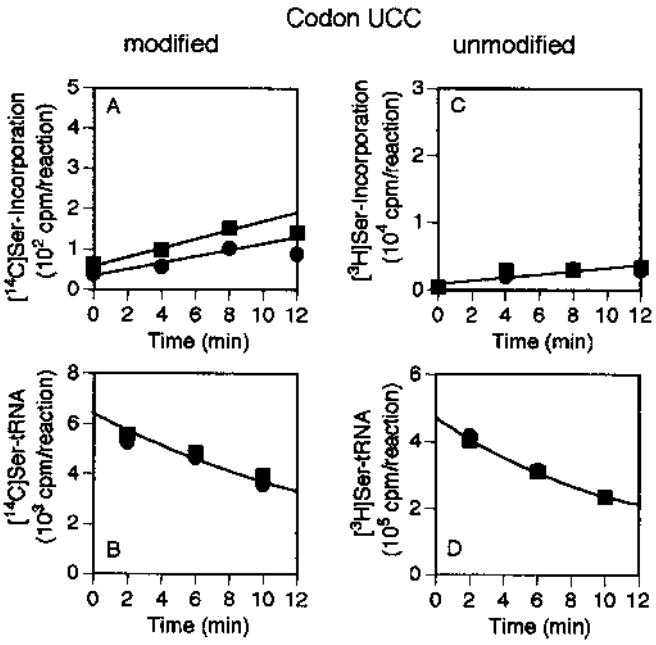

Figure 5. Cell-free protein synthesis assay for the UCC codon with [^{14}C]Ser-tRNA(modified) and [^{3}H]Ser-tRNA(unmodified). Profiles of the test reaction containing mRNA(UCC) (■) and of the control reaction with mRNA (GAG) (●) are shown. The arrangement of the figures is the same as in Figure 3.

the efficiency of the formation of the Watson–Crick base pair with A. The effects of the xo^{5}U modification are being analyzed in our laboratories by using two tRNA molecules that are different only in the modification at position 34.

The fact that the unmodified tRNA had a comparable codon-reading activity with the modified tRNA indicates that the tRNA was properly recognized by EF-Tu•GTP and ribosome. The serylation reaction was also efficient as described above. To be recognized both by EF-Tu and SerRS, the tRNA should be folded into the characteristic secondary and tertiary structure of tRNA molecules (35,36). Thus, the unmodified tRNA that we prepared here appears to have a conformation required for the proper recognition and discrimination of codons on ribosomes.

Reading of the UCU and UCG codons

The U•U base pair, in which the N3s of both U residues serve as the proton donors, and the O4 of one and the O2 of the other accept the protons, was considered in the wobble hypothesis, but it was concluded that this type of U•U base pair was unlikely to be formed on ribosomes, because it would cause misreading of the genetic information (37). Afterwards, however, we indicated that the U•U base pair is stably formed when the ribose moiety of the uridine of the anticodon takes the C2′-endo conformation (16). In addition to this Crick-type wobble U•U base pair, a water-bridged base pair was also proposed (38). In any case, the detectable, but weak, reading of the UCU codon by the unmodified UGA anticodon may be ascribed to some unusual structural features of the U•U base pair.

As for the modified tRNA, it was clearly shown that the cmo^{5}U•U pair is much more efficiently formed than the U•U pair ('M•N' means hereafter that the first nucleoside of the anticodon is M and the third nucleoside of the codon is N). This efficient base pairing can be explained by the characteristic conformational properties of cmo^{5}U, which prefers the C2′-endo form to the C3′-endo form (16), but not by a formation of a water-bridged base pair (38).

To our surprise, the unmodified tRNA only slightly reads the UCG codon; the G at the third position of the codon cannot be recognized efficiently by the unmodified U at the first position of the anticodon. This is probably because the U of the putative U•G base pair cannot stack well onto its 3′ neighbor (39). Such a loss of stacking may also be the case for cmo^{5}U, at the first position of the anticodon. On the other hand, this modified uridine has another peculiar feature, in that the C2′-endo form is unusually stable as compared with the C3′-endo form. In this context, our model building study has revealed that the formation of the cmo^{5}U•G pair is possible, without any steric hindrance, with both the C2′-endo and C3′-endo conformations of the cmo^{5}U (16). Therefore, the present results point out the possibility that the cmo^{5}U(C2′-endo) •G pair is very stable.

The UCC codon cannot be read by the unmodified tRNA

As in the ribosome binding experiment (18), the naturally-modified tRNA$_1^{Ser}$ exhibited no detectable recognition of the UCC codon in the present model mRNA translation. Furthermore, the unmodified form of tRNA$_1^{Ser}$ was found to have very little, if any, activity to read the UCC codon in the present system. The UCC codon was properly read by tRNA$_2^{Ser}$ with the GGA anticodon (data not shown). Therefore, it is concluded that neither the unmodified U nor the cmo^{5}U recognizes C. On the other hand, it has been proposed that an unmodified U at the first position of the anticodon can form a base pair with C, through a water bridge, as efficiently as those with A, G and U, and that the xo^{5}U modification selectively abolishes this recognition of C without affecting the recognition of the other three bases (24). It is obvious that this model is incorrect, at least for *E.coli* tRNA$_1^{Ser}$.

In contrast, in systems such as mitochondria and *Mycoplasmas*, most tRNA species with an unmodified U at the first position of the anticodon can recognize all four of the cognate codons terminating in A, G, U and C to the same extent (27,40). However, it has been found recently that the *M.capricolum* threonine tRNA with the UGU anticodon is an exception: the efficiency of U•C recognition is much weaker than those of the other three, probably because of the t^{6}A modification at position 37 (27). Accordingly, some structural feature outside the wobble position of tRNA is responsible for the efficient reading of C by U. In fact, the *M.mycoides* tRNA^{Gly} with the UCC anticodon, but not an unmodified version of the *E.coli* tRNA^{Gly} with the same anticodon, can read the GGC codon as efficiently as the other three Gly codons on the *E.coli* ribosome (40). Furthermore, a single mutation of U to C, at position 32 of the unmodified *E.coli* tRNA^{Gly}, was sufficient to switch its reading property to that of *M.mycoides* tRNA^{Gly} (41), and vice versa (42). Thus, in the absence of particular structural features, the unmodified U at position 34 of tRNA^{Gly} cannot recognize the C at the third position of the codon. In contrast, the unmodified form of *E.coli* tRNA$_1^{Ser}$ has C32, like *M.mycoides* tRNA^{Gly}, but it did not recognize the UCC codon. This difference between *E.coli* tRNA$_1^{Ser}$ and *M.mycoides* tRNA^{Gly} in the recognition of C at the third position of codon may be ascribed to the difference in the stability of the base pairs between the first and second positions

of the codon and the second and third positions of the anticodon, respectively: two G•C pairs for $tRNA^{Gly}$, and one A•U pair and one G•C pair for $tRNA_1^{Ser}$ (43,44).

Use of unmodified tRNA molecules in a cell-free translation system

In summary, the unmodified form of $tRNA_1^{Ser}$ primarily reads the UCA codon, weakly reads the UCU codon, and hardly reads the UCC and UCG codons. This means that the specificity is higher for the unmodified form than the modified form. This property is advantageous for use in a cell-free translation system, particularly for the introduction of unnatural amino acids into specific positions of proteins. An unnatural amino acid can be introduced specifically into the positions encoded by UCA codons with the unmodified tRNA, but not with the modified tRNA.

The higher specificity of the unmodified $tRNA_1^{Ser}$ is mainly due to the lack of the wobble-extending, xo^5U-type modification at position 34. This type of modified nucleoside occurs in many other tRNAs, such as a $tRNA^{Val}$ and a $tRNA^{Ala}$ from *E.coli* (13). The unmodified forms of such tRNAs may be more specific than the modified molecules, and would be useful for cell-free protein synthesis.

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