A single uridine modification at the wobble position of an artificial tRNA enhances wobbling in an *Escherichia coli* cell-free translation system

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Received 4 January 1999; received in revised form 15 February 1999

Abstract 5-Methoxyuridine was introduced into the first position of the anticodon of the unmodified form of $tRNA_1^{Ser}$ from *Escherichia coli*. The codon reading efficiencies of this tRNA (tRNA(5-methoxyuridine UGA)) relative to those of the unmodified counterpart (tRNA(UGA)) were measured in a cell-free translation system. tRNA(5-methoxyuridine UGA) was more efficient than tRNA(UGA) in the reading of the UCU and UCG codons and was less efficient in the reading of the UCA codon. Thus, the single modification of U to 5-methoxyuridine can enhance the wobble readings.

Key words: 5-Methoxyuridine; Codon recognition; Post-transcriptional modification; Cell-free protein synthesis; tRNA selection; *Escherichia coli*

1. Introduction

Genetic information is translated through codon-anticodon interactions on ribosomes. The codon triplet recognizes the anticodon of a cognate aminoacyl-tRNA molecule, and the anticodon recognizes the codon, by three consecutive base pairs. The first and second nucleotides of the codon bind to the third and second nucleotides, respectively, of the anticodon by standard Watson-Crick base pairs and the third nucleotide of the codon (position III) binds to the first nucleotide of the anticodon (position 34 of the tRNA) by either a Watson-Crick or a wobble base pair [1].

Post-transcriptionally modified nucleosides are found in most naturally occurring tRNA species. Position 34 is one of the most frequent sites of modification in natural tRNA species and the modification of this position is generally believed to affect the codon reading properties of the tRNA [2]. In particular, many bacterial tRNA species specific to Val, Ser, Pro, Thr and Ala have a 5-oxyuridine derivative (xo^5U) [3,4]. While U(34) was predicted to basepair with A(III) and G(III) in the original wobble hypothesis [1], tRNAs with an xo^5U(34) generally recognize three codons ending with U(III), A(III) or G(III) [5,6] (in this paper, we describe a codon with U(III) as U(III)-codon, a tRNA with U(34) as U(34)-tRNA, etc.).

$tRNA_1^{Ser}$ from *Escherichia coli* has an xo^5U-type nucleoside, uridine 5-oxyacetic acid or 5-carboxymethoxyuridine (cmo^5U), at position 34 [7,8]. In a previous study, we measured the relative codon reading efficiencies of this tRNA as compared to the unmodified counterpart prepared by T7 RNA polymerase-mediated transcription [9]. We found that the modified form reads the UCU and UCG codons far more efficiently than the unmodified form. These results suggested that the enhancement of these wobble readings is primarily attributable to the cmo^5U modification. However, it was still unclear whether the xo^5U-type modification by itself can affect the codon reading specificities without any supporting effects from the modifications at the other positions of the tRNA molecule. To clarify this point, it is necessary to compare directly a set of two tRNA molecules that have a common sequence except for the modification at position 34.

In the present study, we measured the effects of a single modification of U(34) to 5-methoxyuridine (mo^5U, Fig. 1) on the codon reading efficiencies. mo^5U is a xo^5U-type nucleoside that occurs in natural tRNAs, such as a valine tRNA from *Bacillus subtilis* that reads the GUU, GUA and GUG codons [3]. The nucleoside can easily be synthesized by a chemical method [10,11] and can be introduced into RNA molecules by a standard phosphoramidite procedure [12]. We prepared the synthetic tRNA with the sequence of *E. coli* $tRNA_1^{Ser}$ but without any modified nucleosides (tRNA(UGA)) and that with the same sequence but with mo^5U(34) (tRNA(mo^5UGA)) (Fig. 1). The relative efficiencies of these tRNAs in the reading of the UCN (N = U, C, A or G) codons were measured.

2. Materials and methods

2.1. Synthesis of oligoribonucleotides

Dry solvents for the synthesis of the amidite unit of mo^5U were prepared as follows. Pyridine was distilled twice from *p*-toluenesulfonyl chloride and from CaH_2 and was then stored over activated 4-A molecular sieves. THF was continuously refluxed over sodium/benzophenone and was distilled prior to use. CH_2Cl_2 was distilled from P_2O_5 and was stored over activated 4-A molecular sieves. DMF and triethylamine were freshly distilled from CaH_2. CH_3CN was distilled twice from P_2O_5 and from CaH_2 and was then stored over activated 4-A molecular sieves.

Thin layer chromatography (TLC) was carried out on Merck Kieselgel $60F_{254}$ plates, which were developed with CH_2Cl_2-MeOH (95:5). Silica gel chromatography was performed on a BW127zH column (100 mesh) (Fuji Davison). Ultraviolet spectra were recorded on a Shimadzu UV-2200A spectrometer. ^{31}P-NMR spectra were measured at 160.1 MHz using a Bruker AMX 400 spectrometer. Chemical shifts are given in ppm (δ) relative to 80% H_3PO_4 as the external standard. ^{1}H-NMR spectra were measured at 400 MHz using the same instrument as for ^{31}P-NMR spectra. The chemical shifts for ^{1}H-NMR are given in ppm (δ) relative to tetramethylsilane.

The mo^5U nucleoside was prepared according to the procedure of Vörbruggen [10,11] and was converted to 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(tert-butyldimethylsilyl)-mo^5U by the procedures of Hakimelahi

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PII: S0014-5793(99)00255-0

et al. [12]. R_f 0.82 (System B), ^{1}H NMR (DMSO-d_6) 11.64 (s, 1H, NH), 6.89–7.41 (m. 14H, Ar-H, H6), 5.87 (d, 1H, H-1′), 5.10 (d, 1H, H2′), 4.28 (d, 1H, H-3′), 4.01 (s, 1H, H-4′), 3.96 (d, 2H, H-5′), 3.74 (s, 6H, ArOMe), 3.24 (s, 3H, OMe-5), 0.85 (s, 9H, t-butyl), 0.05 and 0.01 (6H, 2Me, $SiMe_2$).

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(tert-butyldimethylsilyl)-mo^{5}U 3′-*O*-((*N,N*-diisopropylamino)-(2-cyanoethyl))-phosphoramidite was prepared as follows. 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(tert-butyldimethylsilyl)-mo^{5}U (1.17 g, 1.69 mmol) was dried by successive evaporations with dry pyridine and THF and was redissolved in THF (10 ml). To this solution, *N*-ethyldiisopropylamine (2.70 ml, 15.5 mmol) was added on a dry ice-methanol bath and this mixture was stirred for 5 min. 2-Cyanoethyl-*N,N*-diisopropylaminochlorophosphine (1.75 ml, 7.87 mmol) was added and the reaction mixture was stirred for 3 h at room temperature. The reaction was monitored by TLC (System B, R_f 0.8). The solvent was evaporated in vacuo and the residue was cooled on ice and dissolved in ethyl acetate (30 ml). The solution was washed with saturated NaCl solution and water, dried with Na_2SO_4 and evaporated. The phosphoramidite unit was purified on the silica gel column and dried in vacuo: yield 1.15 g, 1.3 mmol (76%): ^{31}P NMR ($CDCl_3$) d 149.46 and 151.70 ppm.

Oligoribonucleotides were synthesized on an Applied Biosystems Model 392 DNA/RNA automatic synthesizer on a 1 μM scale with ribonucleotide phosphoramidite reagents. Standard phosphoramidite chemistry was employed. The oligoribonucleotides were base-deprotected by an incubation in 3 ml of aqueous concentrated ammonia (28%) for 12 h at 55°C in a screw-cap glass vial. After complete removal of the solvent by evaporation, the residue was taken up in 0.4 ml of 1.1 M tetrabutylammonium fluoride in THF and left at room temperature overnight. The salts were removed using an Oligo-PakSP reversed phase cartridge (Millipore). The desalted RNA was separated by 20% polyacrylamide gel electrophoresis, visualized by UV shadowing, excized and eluted in a Biotrap electroelution apparatus (Schleicher and Schuell). The eluent was desalted by ethanol precipitation.

2.2. Preparation of tRNA molecules

The 5′ half RNAs (37mer, positions 1–36) of tRNA(UGA) and tRNA(mo^{5}UGA) and the 3′ half RNA (51mer, positions 37–76) were synthesized and purified as described above. These RNAs were phosphorylated by T4 polynucleotide kinase, purified by gel electrophoresis and electroelution, joined by heat annealing and ligated with each other using T4 RNA ligase. The ligated RNAs were separated on standard polyacrylamide gels with 8 M urea and were recovered by electroelution.

2.3. Measurement of relative codon reading efficiencies

The relative codon reading efficiencies of tRNA(mo^{5}UGA) as compared with tRNA(UGA) were measured according to the procedure and the assay conditions described in the previous paper (Fig. 2) [9], except that different tRNAs were used. A series of mRNAs (Fig. 3) [9], each with a single test codon (UCU, UCC, UCA, UCG or GAG) in a 49 codon encoding sequence, were used to direct cell-free protein synthesis (the mRNAs are hereafter symbolized with the test codon in parentheses, such as mRNA(UCU), etc.) (Fig. 2). [^{14}C]Ser-tRNA(mo^{5}UGA) and [^{3}H]Ser-tRNA(UGA) (approximately 60 pmol each) were added to the translation reaction (240 μl) directed by the mRNA with the test codon of interest (UCU, UCC, UCA or UCG). The rates of radioactivity incorporation into the protein in cpm/min (A(^{14}C, mo^{5}U) and A(^{3}H, U), respectively) and the radioactivities of the surviving aminoacyl-tRNAs in cpm (B(^{14}C, mo^{5}U) and B(^{14}C, mo^{5}U), respectively) were measured with the use of a scintillation counter. In parallel, the background incorporation rates were measured in a control reaction directed by mRNA(GAG) (A'(^{14}C, mo^{5}U) and A'(^{3}H, U), respectively). B(^{14}C, mo^{5}U), B(^{3}H, U), A(^{14}C, mo^{5}U)−A'(^{14}C, mo^{5}U), and A(^{3}H, U)−A'(^{3}H, U) correspond to a, b, c and d, respectively, in Fig. 2. The relative efficiency of tRNA(mo^{5}UGA) to tRNA(UGA) (E_1) was calculated as (A(^{14}C, mo^{5}U)−A'(^{14}C, mo^{5}U))/(A(^{3}H, U)−A'(^{3}H, U))×B(^{3}H, U)/B(^{14}C, mo^{5}U). The corresponding value (E_2) was also obtained from the reactions containing [^{3}H]Ser-tRNA(mo^{5}UGA) and [^{14}C]Ser-tRNA(UGA). The relative codon reading efficiency of tRNA(mo^{5}UGA) as compared to tRNA(UGA) was obtained as the geometric mean of E_1 and E_2. This indicates the activity of Ser-tRNA(mo^{5}UGA) in reading the codon of interest as compared to that of Ser-tRNA(UGA), when the concentrations of these Ser-tRNAs are the same [9].

3. Results and discussion

3.1. Codon reading preferences and relative efficiencies of tRNA(UGA) and tRNA(mo^{5}UGA)

The codon reading preferences of tRNA(UGA), as judged from the radioactivity incorporation rates, were similar to those of the T7 RNA polymerase-mediated transcript with

Table 1
The codon reading efficiencies of tRNA(mo^{5}UGA) as compared with tRNA(UGA)

	Codon			
	UCU	UCC	UCA	UCG
A. Incorporation of radioactivity from:				
tRNA(mo^{5}UGA)	+	−	+	+
tRNA(UGA)	±	−	+	−
B. Relative codon reading efficiency				
tRNA(mo^{5}UGA)/tRNA(UGA)	3.5	−[1]	0.5	>20
C. Rates of radioactivity incorporation and amounts of Ser-tRNA at 6 min				
C-1.[^{14}C]Ser-tRNA(mo^{5}UGA) and [^{3}H]Ser-tRNA(UGA)				
[^{14}C]Ser-tRNA(mo^{5}UGA) (10^3 cpm)[2,6]	5.3	5.6	5.0	7.0
[^{3}H]Ser-tRNA(UGA) (10^5 cpm)[3,6]	2.9	2.5	2.6	3.2
[^{14}C]Ser incorporation (10 cpm/min)[4,7]	4.5	−	1.5	1.7
[^{3}H]Ser incorporation (10^3 cpm/min)[4,7]	1.1	−	2.2	0.3
C-2. [^{3}H]Ser-tRNA(mo^{5}UGA) and [^{14}C]Ser-tRNA(UGA)				
[^{3}H]Ser-tRNA(mo^{5}UGA) (10^5 cpm)[2,6]	2.1	2.8	2.9	3.5
[^{14}C]Ser-tRNA(UGA) (10^3 cpm)[3,6]	7.1	6.8	7.0	7.7
[^{3}H]Ser incorporation (10^3 cpm/min)[4,7]	13	−	0.5	0.3
[^{14}C]Ser incorporation (10 cpm/min)[5,7]	7.7	−	2.0	−

[1]The relative efficiency was not calculated because neither tRNA(mo^{5}UGA) nor tRNA(UGA) had a measurable activity.
[2]a in Fig. 2.
[3]b in Fig. 2.
[4]c in Fig. 2.
[5]d in Fig. 2.
[6]Aminoacyl-tRNAs were measured at 6 min as alkali sensitive, cold 5% trichloroacetic acid insoluble material.
[7]The incorporation of the radioactivity was measured as alkali resistant, cold 5% trichloroacetic acid insoluble material. The background incorporation rate obtained from the control reaction has been subtracted.

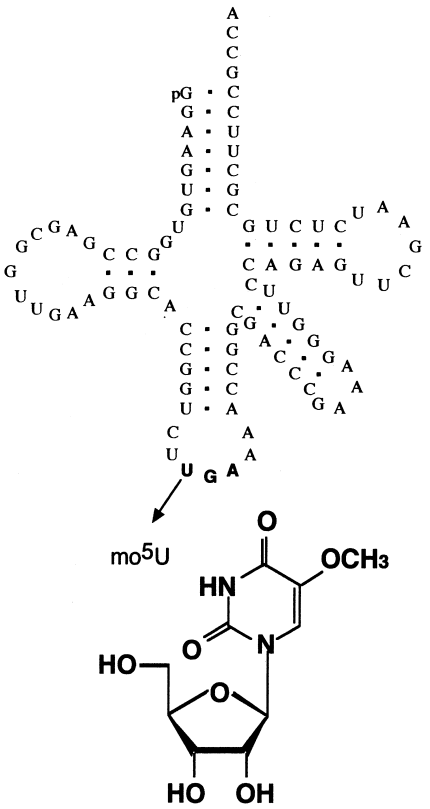

Fig. 1. The sequence of the unmodified form of *E. coli* $tRNA_1^{Ser}$ (tRNA(UGA)) and the chemical structure of mo^5U.

the same sequence [9]. The radioactivity was incorporated efficiently for the UCA codon, marginally for the UCU codon and minimally for the UCC and UCG codons (Table 1A and C). The radioactivity from Ser-tRNA(mo^5UGA) was incorporated in response to the UCU, UCA and UCG codons, but not in response to the UCC codon (Table 1A and C). These preferences are essentially the same as those of the naturally modified form of *E. coli* $tRNA_1^{Ser}$ [8,9].

In addition to the Ser-tRNAs of interest, one or more competing Ser-tRNA species recognize the test codon and insert non-radioactive serine. In the case of the UCC codon, neither Ser-tRNA(UGA) nor Ser-tRNA(mo^5UGA) incorporated radioactive serine into the protein. This means that the activities of these tRNA species to read the UCC codon were both much lower than that of the natural species, $tRNA_2^{Ser}$, which read the codon and inserted non-radioactive serine efficiently. The incorporation rates for mRNA(UCU) are higher than those for mRNA(UCA) and mRNA(UCG) (Table 1C). The activity of the competing natural tRNA species to read the UCU codon may be low in this system.

As the activity of the competing Ser-tRNA species may differ from codon to codon, the incorporation rate does not represent the absolute codon reading activity. Alternatively, the relative codon reading efficiencies of tRNA(mo^5UGA) to tRNA(UGA) (Table 1B) represent the effects of the modification of U into mo^5U. A relative efficiency of one means that these tRNAs read the codon of interest with equal efficiency. If the relative efficiency is larger than one, tRNA(mo^5UGA) recognizes the codon more efficiently than tRNA(UGA). Thus, tRNA(mo^5UGA) reads the UCU and UCG codons more efficiently, and the UCA codon less efficiently, than tRNA(UGA). In the case of the UCC codon, the relative efficiency was not calculated because neither of these tRNAs had a measurable incorporation rate.

3.2. The $mo^5U(34)$ modification enhances the reading of the UCU and UCG codons

The readings of the UCU and UCG codons are enhanced by the $mo^5U(34)$ modification. This is the first direct observation that a single modification of U(34) to mo^5U, without the aid of any other modification, can transform the tRNA so that it efficiently wobbled to read the U(III) and G(III) codons. mo^5U is present at position 34 of many tRNAs that correspond to a four codon family (a set of four codons that have common nucleotides in the first and second positions and correspond to the same amino acid), such as $tRNA^{Val}$, $tRNA^{Pro}$, $tRNA^{Thr}$ and $tRNA^{Ala}$ from *B. subtilis* [3]. These tRNA species read the corresponding U(III), A(III) and G(III) codons and not the C(III) codons. These properties are in common with those of tRNA(mo^5UGA). Thus, the characteristic codon reading properties of these mo^5U-containing, naturally occurring tRNAs are primarily caused by the modification of U(34) into mo^5U.

3.3. Conformational properties and wobbling

According to Crick's wobble hypothesis [1], the reading of a U(III) codon by a tRNA molecule with a U derivative (U*) at position 34 requires a close U*(34)•U(III) base pair. NMR studies on the conformational properties of nucleotides containing an xo^5U nucleoside unit have shown that the ribose moieties of the xo^5U nucleoside units prefer the C2′ endo conformation to the C3′ endo conformation [2,6]. Since the close U*•U pair is possible only when the U* is in the C2′ endo conformation, it was concluded that the ability of the xo^5U to assume the C2′ endo form is responsible for the efficient reading of U(III) codons by $xo^5U(34)$-containing tRNAs. The $xo^5U(34)$•G(III) pair was originally suggested to be possible with both the C2′ endo and the C3′ endo conformations of the xo^5U [6]. The comparison of the UCG reading activities of fully modified *E. coli* $tRNA_1^{Ser}$ and its

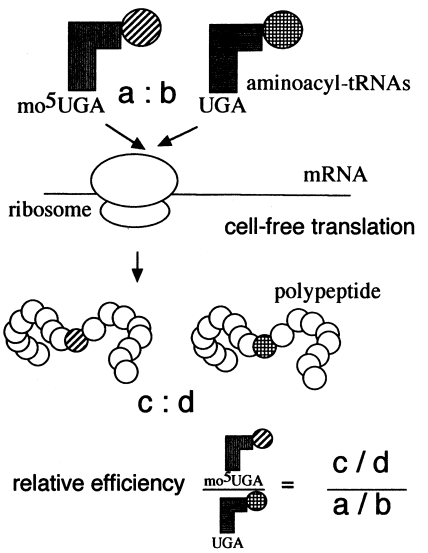

Fig. 2. Schematic illustration of the measurement of the relative codon reading efficiencies [9].

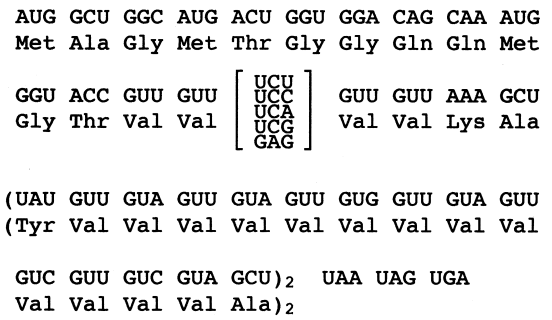

Fig. 3. Coding sequences of test mRNAs [9]. The codons in brackets are the codons of interest.

unmodified transcript suggested that the C2′ endo conformation mainly contributes to the reading [9]. C(III) codons are not read by either the U(34)-tRNAs or the $xo^5U(34)$-tRNAs, as C(III) cannot basepair with either U(34) or $xo^5U(34)$, due to steric hindrance [2,13]. The present results are consistent with this hypothesis and these observations.

3.4. The reading of the UCA codon is reduced by the $mo^5U(34)$ modification

The reading of the UCA codon by tRNA(mo^5UGA) was less efficient than that by tRNA(UGA) (Table 1B). The probability of having a conformation that is required for basepairing with the A(III) may be lower in $mo^5U(34)$ than in U(34), as mo^5U is more flexible in the ribose conformation. In other words, the formation of the $mo^5U(34)$•A(III) pair with mo^5U in the C2′ endo form may be restricted. The mo^5U modification enhances the wobble reading of the U(III) and G(III) codons at the cost of the Watson-Crick reading of the A(III) codon.

3.5. xo^5U modification and usage of U(III) codons in highly expressed genes

In the case of *E. coli*, most of the U(III) codons recognized by only a single tRNA species with G(34) or Q(34) (Q is queuosine, a G derivative) are less frequently used than the corresponding C(III) codons, probably because the tRNAs can decode the U(III) codons less efficiently than the C(III) codons [14]. By contrast, all three of the U(III) codons (GUU, UCU and GCU) known to be read by the $cmo^5U(34)$-containing tRNAs, as well as by the G(34)-tRNAs, occur most frequently among the synonymous codons in highly expressed genes [15]. These frequencies are still higher than the sums of the frequencies of the C(III) and A(III) codons, which may be the most efficient codons to the G(34)-tRNAs and the $cmo^5U(34)$-tRNAs, respectively. Therefore, the decoding of the U(III) codons may be more efficient, in total, than the decoding of the other codons. Thus, the large gain in total efficiency that was caused by the evolution of the xo^5U modifications may outweigh the loss of the efficiency in the reading of the A(III) codons.

Acknowledgements: This work was supported in part by the 'Research for Future' Program (Project number JSPS-RFTF96100306) of the Japan Society for the Promotion of Science.

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