

SURVEY AND SUMMARY

Roles of 5-substituents of tRNA wobble uridines in the recognition of purine-ending codons

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Received July 22, 2003; Revised September 10, 2003; Accepted September 20, 2003

ABSTRACT

Many tRNA molecules that recognize the purine-ending codons but not the pyrimidine-ending codons have a modified uridine at the wobble position, in which a methylene carbon is attached directly to position 5 of the uracil ring. Although several models have been proposed concerning the mechanism by which the 5-substituents regulate codon-reading properties of the tRNAs, none could explain recent results of the experiments utilizing well-characterized modification-deficient strains of *Escherichia coli*. Here, we first summarize previous studies on the codon-reading properties of tRNA molecules with a U derivative at the wobble position. Then, we propose a hypothetical mechanism of the reading of the G-ending codons by such tRNA molecules that could explain the experimental results. The hypothesis supposes unconventional base pairs between a protonated form of the modified uridines and the G at the third position of the codon stabilized by two direct hydrogen bonds between the bases. The hypothesis also addresses differences between the prokaryotic and eukaryotic decoding systems.

INTRODUCTION

During protein biosynthesis, the ribosomes select correct aminoacyl-tRNA molecules one-by-one by recognizing the anticodon triplet of the tRNA molecule that fits to the A-site codon triplet. According to the wobble hypothesis (1), when a tRNA molecule is recognized as a correct one, the third and second nucleosides of the anticodon (positions 36 and 35, respectively) form Watson–Crick base pairs with the first and second nucleosides of the codon, respectively, and the nucleoside at the first position of the anticodon (position 34)

forms a Watson–Crick or a wobble base pair with the nucleoside at the third position of the codon (position III) (1). The base pairs allowed between position 34 and position III were assumed to be only those that could form two or more than two direct hydrogen bonds between them with a small displacement from the position for the Watson–Crick base pair. A U–G pair with the U at position 34 could be formed with a small displacement of the uracil base toward the major groove side, while it was known at that time that uridines at position 34 are quite often post-transcriptionally modified.

Modified uridines found at position 34 of naturally occurring tRNA species are classified into two groups (Fig. 1) (2). A modified uridine with a methylene carbon directly bonded to the C5 atom (xm^5U) (Fig. 1a) is often found in tRNA species that recognize only the purine-ending codons. The xm^5U nucleosides are often thiolated at position 2 (xm^5s^2U) or methylated at the ribose 2'-hydroxyl group (xm^5Um). A modified uridine with an oxygen atom directly bonded to the C5 atom of the uracil ring (xo^5U) (Fig. 1b) is often found in tRNA species that recognize the U-, A- and G-ending codons.

In the present paper, we use an asterisk to represent any substituent. For example, U^* represents any of the naturally occurring modified and unmodified uridines. In the same way, xm^5U^* stands for any of the xm^5U derivatives including xm^5U , xm^5s^2U and xm^5Um . Parts of the nucleoside symbols, such as xm^5 and s^2 , may also be used to stand for the substituents, as in Figure 1. 5'-Nucleotides may be symbolized such as pxo^5U . The position of a codon nucleoside may be shown in parentheses in Roman numerals, such as G(III), and the position of the anticodon nucleoside may be shown in the same way in Arabic numerals, such as U(34).

The puckering equilibrium of the ribose ring of the nucleosides in RNA molecules is generally biased to the C3'-endo form instead of the C2'-endo form, which is required for the formation of the typical A-form helices. This bias is also observed in mononucleotides and nucleosides. In xm^5U^* , the ribose puckering is biased to the C3'-endo conformation to a greater extent than in unmodified U (3–6). On the other hand, the puckering equilibrium of pxo^5U is much shifted to the

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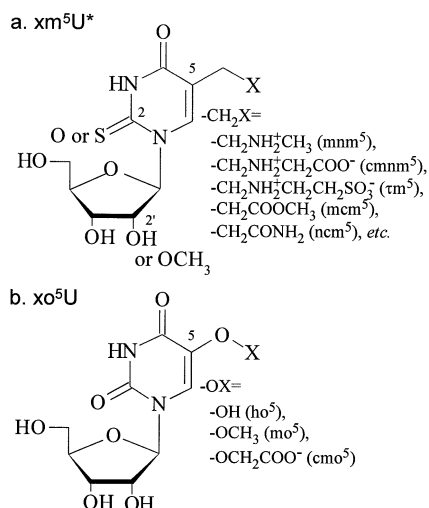


Figure 1. Chemical structures of modified uridines found at position 34 of tRNA species. Symbols of the 5-substituents are shown in parentheses.

C2'-endo form (3). It was also shown that a U in the C2'-endo conformation could basepair with another U through two direct base–base hydrogen bonds by a model building study (3). Therefore, it was proposed that the modifications in xm^5U^* restricts, and the xo^5 modification promotes, the formation of the $U^*(34)$ – $U(III)$ pair (3). It has been shown that the substitution of $U(34)$ of the unmodified form of *Escherichia coli* $tRNA_1^{Ser}$ by $mo^5U(34)$ enhances the *in vitro* reading of the UCU codon (7).

It is noteworthy that this theory of the regulation of codon recognition at the level of the dynamic conformation of the nucleotides is based on the wobble hypothesis proposed by Crick (1): two direct hydrogen bonds are required between the bases at positions 34 and III. A mechanism that does not require the two direct base–base hydrogen bonds has also been proposed to contribute to the codon reading and is named as the ‘two out of three’ mechanism (8,9) (see below).

Recently, the physicochemical effects of the mnm^5 and s^2 modifications were elucidated in detail by NMR structural analyses of anticodon stem–loop (ASL) oligonucleotides from *E. coli* $tRNA^{Lys}$ (10). The comparison of different ASLs with different modifications showed that the s^2 modification enhances the stacking of the bases in positions 35 and 36 onto the 3' side of the anticodon to elevate the interaction of these bases with the first and second bases of the codon. The mnm^5 modification also reduces the flexibility of the anticodon and contributes to ‘preorganize’ the anticodon into an A-form structure ready to interact with the codon in collaboration with the s^2 modification. This clearly explained the effects of each modification on the misreading of the AAU/C codons by the tRNA observed *in vivo* under an Asn starvation condition (11), with the assumption that the misreading primarily depends on the ‘two out of three’ mechanism. Therefore, in this case, the ‘two out of three’ mechanism may dominate over the wobble mechanism. It is reasonable that, in such cases, the decoding properties of the modification-deficient tRNAs could not be predicted from the conformational properties of the nucleotide at position 34.

On the other hand, the *in vivo* effects of the lack of each modification on the reading of the GAA/G codons by *E. coli* $tRNA^{Glu}$ with an mnm^5s^2U at position 34 were also measured with the modification mutants (12). However, the data could not be explained completely even with the dynamic 3D structures of the ASLs (10), as described in detail below. This may mean that some unknown mechanism, different from the conformational regulation, has some contribution to the codon reading by the tRNAs with $mnm^5s^2U(34)$.

In the present paper, we first summarize the known experimental results and theories on the effects of the xm^5 modification and on other related subjects. Then, we propose a physicochemical model of $xm^5U(34)$ – $G(III)$ pairing that could explain the *in vivo* effects of the mnm^5 and s^2 modifications on the reading of the purine-ending codons.

EXPERIMENTAL FACTS AND THEORIES

Views from tRNA composition

Distribution and properties of $xm^5U^(34)$.* In *E. coli*, $mnm^5s^2U(34)$ is found in $tRNA^{Lys}$, $tRNA^{Glu}$ and one of the two $tRNA^{Gln}$ (13). $tRNA_4^{Leu}$ and $tRNA_4^{Arg}$ have $cmnm^5Um(34)$ and $mnm^5U(34)$, respectively (6,14). Many eubacteria also have the $cmnm^5$ or mnm^5 modification in Leu (UUA/G), Gln, Lys, Glu and Arg (AGA/G) tRNA species (13). At least *E. coli* $tRNA^{Lys}$, $tRNA^{Glu}$ and $tRNA_4^{Leu}$ could read the G-ending codons: $tRNA^{Lys}$ and $tRNA^{Glu}$ are the single tRNA species for the amino acids (15), and a *su6* strain in which only $tRNA_4^{Leu}$ could read the UUG codon grows very well (6,16). $tRNA_4^{Arg}$ was suggested to read the AGG codon only weakly (17), although the experimental results are not very conclusive: overproduction of $tRNA_4^{Arg}$ might have caused undermodification, which might have led to overproduction of tRNA molecules that do not recognize the AGG codon; and the tRNA species that compete with $tRNA_4^{Arg}$ in their frameshifting assay were different between the assay of the AGA and AGG codons, which made direct comparison of the activities on the different codons difficult. Instead, the above *in vivo* experiment clearly showed that the $tRNA^{Glu}$ mutant with $mnm^5U(34)$ could read the GAG codon efficiently (12). Human mitochondrial $tRNA^{Leu}$ (UUA/G) and $tRNA^{Lys}$ are also the single tRNAs for the codons and have $tm^5U(34)$ and $tm^5s^2U(34)$, respectively (18).

Difference in prokaryotic and eukaryotic systems. The substituents in the xm^5U derivatives in prokaryotes and eukaryotes are different. Prokaryotic tRNAs have derivatives of mnm^5U , and eukaryotic tRNAs have those of mcm^5U or ncm^5U (13). Although many bacteria dispense with some tRNAs with C(34) that would read the CAG, AAG or GAG codon, all eukaryotes so far investigated have the C(34)-containing tRNAs for these codons (19). Therefore, it is possible that the eukaryotic xm^5U derivatives do not pair with G(III). It has also been suggested that eukaryotes cannot decode G-ending codons with tRNAs having a U derivative at position 34, based on the fact that they have at least a copy of an Ile tRNA gene with a T at the first position of the anticodon (20), although the post-transcriptional modifications of the Us are unknown (the tRNA would insert Ile for the Met codon if it could read the G-ending codon). This difference between

prokaryotes and eukaryotes could be ascribed to the difference in the ribosomes as well as the difference in the tRNA modifications. As for prokaryotes, we focus on the modifications in eubacteria, as information on archaeobacterial modifications is limited.

Undiscriminating reading by mitochondria and mycoplasma tRNA species with U(34). In mitochondria and mycoplasmas, many family codon boxes (a codon box is a set of four different codons that have the first two bases in common, and if it specifies a single amino acid in the genetic code, it is a family box) are each translated by only one tRNA species with unmodified U(34) (21–24). Therefore, it was proposed that this kind of undiscriminating codon reading is based on the ‘two out of three’ mechanism. In some cases, this undiscriminating codon reading was shown to be less significant in the split codon boxes (a split codon box is a codon box that specifies more than one amino acid) than in family boxes (8,9,25).

Unmodified U

In vitro translation assay and properties of undiscriminating tRNA species with unmodified U(34). It is important to understand the codon-reading properties of tRNA species with an unmodified U(34) before discussing the properties of the modified species. Most studies on such tRNA molecules utilize an *in vitro* translation system. It is well known that the discrimination of the third bases of the codons will be ambiguous if only one aminoacyl-tRNA species is used in excess to introduce radiolabeled amino acid into proteins (26). This could be so even in a split codon box (27). It is also known that the accuracy of *in vitro* translation is affected by the reaction conditions. For example, pH and the concentrations of magnesium ions and polyamines could affect the fidelity of translation (28). Therefore, it is necessary to control the experimental conditions carefully. However, such assays have been used successfully to determine the relative efficiency of a tRNA species in reading a codon as compared with that of another competing tRNA species.

tRNA^{Gly} from *Mycoplasma mycoides* with U(34) is a single tRNA species for the four Gly codons (29). This tRNA reads all the four Gly codons even in an *in vitro* translation system from *E.coli*. It has also been demonstrated that this undiscriminating codon reading requires a C at the first position of the anticodon loop (position 32) of the tRNA (30–33). However, C(32) is very often found in tRNA molecules. Therefore, it also seems essential that the interaction of the ‘two out of three’ is strong enough to support such ambiguous reading (25). These results also suggested that tRNAs with U(34) behave differently in different situations concerning the discrimination of the third bases of the codons.

On the other hand, *Mycoplasma capricolum* has two Thr tRNAs with A(34) and U(34) (24). Although the tRNA with U(34) reads all the four Thr codons, the reading of the ACC codon is weak when the tRNA with A(34) is competing in an *in vitro* translation system from *M.capricolum* (34). The readings of the ACU and ACG codons by the tRNA with U(34) are also weaker than those by the tRNA with A(34). Thus, codon preferences could be observed in this case. Therefore, some interaction between U(34) and the third base of the codon contributes to the undiscriminating reading.

As it seems that there is some confusion in some of the literature concerning the use of the terms ‘wobble mechanism’ and ‘two out of three’ mechanism, we define the meanings within this paper as follows. The wobble mechanism is a class of those mechanisms by which two or more direct hydrogen bonds are formed between the bases at positions 34 and III while two Watson–Crick base pairs are formed between the last two positions of the anticodon and the first two positions of the codon. The ‘two out of three’ mechanism is a class of those mechanisms by which less than two direct base–base hydrogen bonds are formed between positions 34 and III while two Watson–Crick base pairs are formed at the other two positions. A ‘two out of three’ mechanism is one that satisfies the criteria for this particular mechanism. Therefore, a ‘two out of three’ mechanism may involve some interaction without two direct base–base hydrogen bonds between positions 34 and III. A ‘base pair’ in this paper means a pair of bases with two or more than two direct hydrogen bonds between them, unless mentioned otherwise. Some researchers use the term ‘four-way wobble’. However, we do not use this term because it is for the mechanism of the ambiguous recognition of the four different codons but is not for the mechanism of recognition of individual codons.

Unexpected inefficiency in the reading of G-ending codons by unmodified tRNAs with U(34). Artificial unmodified tRNAs with U(34) have also been investigated with an *in vitro* translation system. The *in vitro* transcript of *E.coli* tRNA_{1^{Ser}} reads the UCA codon half as efficiently as the fully modified molecules, but does not read the UCU and UCG codons (the UCU codon may be recognized weakly) (35). A sample of the same tRNA prepared by chemical synthesis followed by enzymatic ligation had the same codon-reading properties, and the substitution of the U(34) by an mo⁵U enhanced the reading of the UCU and UCG codons (7). The transcripts with base changes at the second and third positions of the anticodon into AA, UC and CU, respectively, also read the A-ending codons and did not read the G-ending codons (36). Thus, the U(34)–G(III) interaction is very weak at least with the structural context of the tRNA. As all of these unmodified tRNAs discriminate well the UCX codons, irrespective of C(32) that the tRNAs have, it is unlikely that any of the codon readings depends on a ‘two out of three’ mechanism. Therefore, U(34), which primarily assumes the C3'-endo form, cannot form a base pair with G(III). Something other than the ribose puckering equilibrium should be different between U(34) and xm⁵U*(34).

In vitro analyses of the effects of the xm⁵U* modifications

A-site binding of ASL. Oligonucleotide-dependent ribosome-binding experiments have been used most conveniently for the determination of codon-specificity of tRNAs. However, researchers should choose an experimental condition that gives reasonable results, as some tRNAs bind strongly and the others bind weakly. Nevertheless, many researchers have obtained reasonable results. Recently, 17mer ASL oligonucleotides were found to bind to the ribosomes. Although they mainly bind to the P site in the original techniques, the binding to the A site could also be measured as the tetracycline-sensitive binding (37). With this technique, the effects of the

mnm^5s^2U modifications were investigated (37). The results clearly showed that the mnm^5 and s^2 modifications enhance the A-site binding to the AAA and AAG codons, and that the efficiency of the A-site binding is always in parallel with that of the P-site binding. The results from the P-site binding assay showed that the ASLs with U(34) bind only in the cases where the codon is from a family codon box. It is noteworthy that, although the GUG codon could bind the corresponding ASL with U(34) with a low affinity, the GCG, UCG and CCG codons did not bind the ASLs with a measurable affinity. This is consistent with the inefficiency observed for the reading of the G-ending codons by the unmodified tRNAs with U(34) during the *in vitro* translation assay mentioned above. Anyway, the modifications are required for the $U^*(34)$ -containing ASLs to bind to the G-ending codons.

xm⁵U from eukaryotes.* An early work has shown that a yeast tRNA^{Glu} specifically translates the GAA codon in an *in vitro* translation system from rabbit reticulocytes (38). This tRNA has $mcm^5s^2U(34)$, and this may fit to the above idea that eukaryotes do not use the $U^*(34)$ -G(III) wobbling (20). This tRNA was also investigated by the conventional ribosome-binding method (38). The results were consistent with those from the *in vitro* translation assay, while the source of the ribosomes was *E.coli*. Therefore, it may be the difference in tRNA but not that in the ribosomes that causes the difference in the codon specificity between eukaryotes and eubacteria at least in this case. It was also shown that yeast tRNA₃^{Arg} with $mcm^5U(34)$ does not recognize the G-ending codon either (39). Therefore, the inability to recognize G(III) in tRNA^{Glu} may be independent of the s^2 modification.

***In vivo* experiments using modification mutant strains**

Rates of translation of the GAA/G codons. The effects of the mnm^5 and s^2 modifications on the rates for the translation of the GAA and GAG codons were measured with the use of well-characterized *E.coli* mutants of the mnm^5s^2U modifications (12). The rates at which the GAG codon was translated in the strains with $mnm^5s^2U(34)$, $s^2U(34)$ and $mnm^5U(34)$ were 7.7, 1.9 and 6.2 codons/s, respectively, and the rates of the reading of the GAA codon were 18, 47 and 4.5 codons/s, respectively. Therefore, the s^2 modification of mnm^5U elevates the reading of the GAA codon and has only a small effect on the reading of the GAG codon, and the mnm^5 modification of s^2U restricts the reading of the GAA codon and enhances the reading of the GAG codon. Although the level of the available undermodified aminoacyl-tRNA species in each mutant was not clear, the elongation rates during the translation of the whole *lacZ* coding sequence were almost the same for these mutants. Therefore, we believe that the results are highly reliable. However, these results cannot be explained completely by the 3D structures of the ASL variants as described in detail below.

Results from frameshift assays. Brierley *et al.* (40) also utilized the modification mutants of *E.coli* to determine the *in vivo* correlation between the mnm^5s^2U modification and frameshifting efficiency at a coronavirus frameshift site. In their assay, the frameshift efficiencies at the AAA/G codons in the modification mutant strains were measured. They tried to interpret the results with the assumption that the frameshift

efficiency should be negatively correlated with the stability of the codon binding by the tRNA^{Lys} species with different modifications. However, the frameshift efficiency may also be affected by the efficiency of the tRNA molecules to shift to the AAA codon in the -1 frame, and, if this was the rate-limiting step of the whole process, the frameshift efficiency should have reflected the relative efficiency to bind to the -1 frame codon as compared to the 0 frame codon. Therefore, interpretation of these results as related to the efficiency of the A-site codon binding is quite difficult, as pointed out in other papers (10,12).

Misreading of pyrimidine-ending codons. The same *E.coli* strains were used to analyze the efficiency of the misreading of the AAU/C Asn codons by the tRNA^{Lys} modification mutants under an Asn starvation condition (11). The results indicate that both mnm^5 and s^2 modifications enhance the misreading. These results were unexpected, because the modifications were thought to restrict wobbling at that time (3), but were rationalized when the NMR structures of the wild type and mutant ASLs were revealed in detail (10) (see below).

Physicochemical aspects

Conformational preferences of uridine derivatives and the expected basepairing pattern. As described above, the conformation of xm^5s^2U is biased to the C3'-endo form (3,4,41). This conformational preference is mainly due to the s^2 modification, which would enhance steric repulsion between the O2' and the atom at position 2 in the C2'-endo form. Therefore, 2'-O-methylation was also suggested to stabilize the C3'-endo form. The xm^5 modification also contributes to the stabilization of the C3'-endo form (5,10). By contrast, pxo^5U is much more in its C2'-endo form than pU. Although the mechanism of the preference is not clear, it was suggested to be due to the interaction between the 5'-phosphate and the oxygen atom of the xo^5 substituent (3). With the C2'-endo form, $U^*(34)$ could basepair with U(III) if the codon and the second and third positions of the anticodon are in the A form, as shown by a model building study. Therefore, it was proposed that the conformational restriction into the C3'-endo form in $xm^5U^*(34)$ should prevent mispairing with U(III) and stabilize the correct pair with A(III) (3).

As long as the A-type RNA is assumed in the other parts of the codon-anticodon duplex, U(34) could not form a base pair with C(III) because of steric hindrance (41,42). Therefore, the C2'-endo form could not explain the reading of the C-ending codons by mitochondrial and mycoplasma tRNAs with U(34). Although the anticodon loop of *E.coli* tRNA^{Lys} was recently shown to have a remarkable flexibility (10), it is well known that the 2'-hydroxyl groups of the five nucleosides of the codon-anticodon duplex except the first one of the anticodon are hydrogen-bonded to the ribosome at the A site (43). Thus, it is reasonable to consider that these five nucleosides should be in the C3'-endo form on the A site, no matter how flexible the conformation of the unbound anticodon loop is.

In the original hypothesis (3), the $U^*(34)$ -G(III) was thought to be possible with both of the C2'-endo and C3'-endo forms. However, the above *in vitro* translation experiments (4,35,36) showed that the U(34)-G(III) pair with the C3'-endo form of the U should be weak. Thus, the

$xm^5s^2U(34)-G(III)$ pair should be very weak, because the C2'-endo form of xm^5s^2U should be less stable than that of U and the S...H-N hydrogen bond required for the base pair should be weaker than the O...H-N bond required for the U-G pair. Therefore, the reading of the AAG and GAG codons by *E.coli* tRNA^{Lys} and tRNA^{Glu}, respectively, could not be rationalized by this theory, as pointed out in an excellent review (44).

NMR structures of the modification variants of the tRNA^{Lys} ASL. As described above, the physicochemical effects of the modifications in mnm^5s^2U in the tRNA^{Lys} ASL have been elucidated by NMR analyses (10). The s^2 modification enhances the stacking of the 'two out of three' onto the 3'-side of the anticodon, and the mnm^5 modification decreases the flexibility of the loop.

As described above, the stabilization of the A-form structure of the anticodon resulted in the elevated misreading of the AAU/C Asn codons during the Asn starvation. This enhancement of the misreading could not be predicted from the conformational properties of the nucleotides at position 34 (11). The prediction implied that the misreading should depend primarily on the wobble mechanism with the U*(34)-U(III) base pair, instead of the 'two out of three' mechanism. The fact that the AAC codons were also misread under the starvation condition indicates that at least the misreading of the AAC codons depended on the 'two out of three' mechanism. It is likely that the conformational restriction into the C3'-endo form by the modifications reduced the efficiency of the misreading by the wobble mechanism to the extent that it was lower than that by the 'two out of three' mechanism.

The s^2 modification could be predicted to enhance the reading of the GAA codon from its structural effects, and it did in the *in vivo* experiment. In the case of the GAG codon, the substitution of O2 with a sulfur atom would destabilize the wobble base pair because the O2...H-N hydrogen bond would be substituted by a weak S2...H-N hydrogen bond, if any, while the stacking enhancement should more or less compensate for the destabilization (though this point is not described explicitly in the published material). Therefore, the small effect on the reading of the GAG codon observed in the *in vivo* experiment could be rationalized. On the other hand, the mnm^5 modification should stabilize the interaction with A(III), as it should stabilize the 'preorganized' conformation. This contradicts to the *in vivo* data. As for G(III), if the $mnm^5s^2U(34)-G(III)$ base pair is formed with the C2'-endo form of the mnm^5s^2U , then the mnm^5 modification should reduce the reading of the G-ending codon because it should destabilize the C2'-endo form, which is again contradictory to the *in vivo* result. This may mean that the $mnm^5s^2U(34)-G(III)$ pair with the C3'-endo form of the mnm^5s^2U is stabilized by the mnm^5 modification through some unknown mechanism.

Thermodynamic analyses. Stabilities of RNA duplexes could be estimated by measuring the melting profiles of the duplexes. Many oligonucleotides containing modified nucleosides have been studied by this method. It should be noted that the contribution of a single base pair to the stability of the duplex could not be defined because a base pair should affect

the neighboring base pair interactions. However, a sum of free energy parameters for all the pairs of neighboring two base pairs in the duplex, plus the parameters for the terminal base pairs and other constants, could be a good estimation of the stability of the duplex (45,46). Substitution of an A-U pair in the middle of an RNA duplex by a G-U pair would usually destabilize the duplex, and the free energy difference could be estimated easily if the neighboring base pairs are known. In the same way, the effect of the substitution of a terminal A-U pair by a G-U pair could be estimated. However, this substitution turns out to be non-destabilizing or even stabilizing, in general, when the A and G are at the 5'-ends of the duplexes and the Us are at the 3'-ends (46). Codon-anticodon duplexes with U(III) are in a similar situation to the latter case, as position 34 is at the 5'-end of the codon-anticodon duplex. It has also been observed that the substitution of A(34) by a G in a series of unmodified tRNAs raises, or does not change, the efficiencies to read the U-ending codons in an *in vitro* translation system (36). Therefore, the effects of a modification of U(34) could not be estimated from the stabilities of RNA duplexes with the modified uridines in the middle or at the 3'-end of the duplexes. There have been no experiments in which the effects of the 5-substitution of uridines at the 5'-end of RNA duplexes are estimated, although even this type of experiment would not necessarily be promising in terms of the estimation of the effects of the modification on tRNA codon recognition.

Model-building study (Lim's model). Lim and coworkers have proposed a model that explains the codon-reading patterns as related to the properties of the nucleosides at position 34 (42,47). In the model, U(34) could interact with U(III) and C(III) through water bridges, and a tRNA with U(34) reads all the four bases at position III. Therefore, the model may be useful to predict codon preferences when the family codons are not fully discriminated. The mnm^5 substitution would break some bonds needed for the water-bridged pairs, and this loss of the stabilizing interaction would not be compensated. Therefore, the modification should restrict the formation of the water-bridged pairs. However, it is obvious that this model does not take into account that the strength of the interaction between the first two codon positions and the last two anticodon positions could be changed by the s^2 and xm^5 modifications (10). Therefore, the model could not predict the *in vivo* effects of the modification (11,12).

A MODEL OF THE $xm^5U(34)-G(III)$ BASEPAIRING

Here, we propose a model of the $xm^5U^*(34)-G(III)$ pairing, by which the *in vivo* effects of each modification on the codon-reading rates and the prokaryote/eukaryote difference of the wobble rule concerning the U*(34)-G(III) pairing could be rationalized. The xm^5U nucleosides from prokaryotes and mitochondria are derivatives of 5-aminomethyluridine (xnm^5U^*), while those from eukaryotes are not (13). As for xnm^5U^* , the 5-substituent is likely to lower pK_a at the N3 position of the uracil ring, because the positively charged nitrogen atom of the substituent should withdraw electrons from the uracil ring. Thus, xnm^5U^* may partially ionize under the physiological condition. The ionized form of the nucleoside (xnm^5U^{*-}) could base pair with G(III) in two different

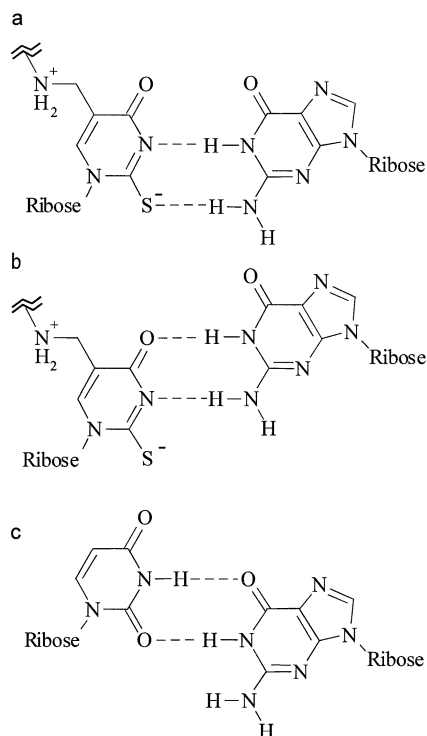


Figure 2. The proposed base pairs between a deprotonated modified U and a G proposed in the present study (a and b) and the conventional wobble U–G pair (c). (a) The proposed xnm^5U^*-G pair with the Watson–Crick configuration; (b) the alternative xnm^5U^*-G pair with the xnm^5U^* displaced toward the minor groove side from the Watson–Crick configuration; (c) the conventional wobble U–G pair. The sulfur atom in (a) and (b) could be substituted with an oxygen atom, and the negative charge could be delocalized within the π -electron system.

configurations (Fig. 2a and b). In the case of the 2-thiolated uridines, the ionization could confer a negative charge on the sulfur atom and convert it to an efficient proton acceptor. The ionized form would be able to pair only with G(III), and the neutral form, which may be symbolized here as xnm^5U^{*0} , could pair only with A(III). Both pairs could be formed with the C3'-endo conformation. Thus, the stabilization of the C3'-endo form by the modifications would stabilize both pairs. We suppose that the ionization should be partial. The relative efficiency in the reading of the G-ending codon to that of the A-ending codon would not only depend on the degree of the ionization, but would also depend on the difference in the intrinsic stabilities between the $xnm^5U^*(34)-G(III)$ and $xnm^5U^{*0}(34)-A(III)$ pairs. Therefore, an xnm^5U^* could pair with G(III) more efficiently than with A(III) even when the neutral form is the major species. In the case of mnm^5s^2U , the pairing in the neutral form with A(III) may be still more efficient, in total, than the pairing in the ionized form with G(III). The ionized modified uridine would not pair with U(III) or C(III). As the eukaryotic xm^5 substituents do not withdraw electrons as xnm^5 may do, the eukaryotic tRNAs do not recognize the G-ending codons.

HOW THE MODEL FITS TO THE KNOWN FACTS AND THEORIES

Hammett equation and the known pK_a values for uridine and uracil derivatives. The pK_a values of a series of substituted

compounds could be well predicted using the Hammett equation (48), as described in many organic chemistry textbooks. pK_a of a substituted molecule is predicted to be lower than the unsubstituted one by σ times ρ , where σ is a constant specific to the substituent and its position, and ρ is a constant specific to the core acid. As judged from the known pK_a values for several uracil derivatives (U, 9.3; m^5U , 9.7; and 1-methyl-5-bromouracil, 7.8) (49) and the σ values for the *meta* position (methyl, -0.07 and bromo, 0.39), ρ for uracil should be positive (and ~ 5).

The effects of substitutions at aromatic rings are mainly ascribed to two factors: inductive electron withdrawal and the resonance electron donation by the substituent. In the case of the xnm^5 substituent, the resonance effect may be small because of the methylene group directly attached to the uracil ring, and the inductive effect may be large because the nitrogen atom is protonated and is positively charged. Therefore, it is expected that the inductive effect dominates over the resonance effect, and the pK_a of mnm^5U should be significantly lower than that of U. In fact, the pK_a values of $cmnm^5Um$ and $cmnm^5U$ have been measured to be 8.3 and 8.2, respectively (6). As the negative charge of the carboxyl group of the $cmnm^5$ substituent may somewhat neutralize the electron withdrawing effect of the charged nitrogen, it is expected that the pK_a value for mnm^5U is not ≤ 8.2 . Furthermore, as pK_a for s^2U (8.8) is lower than that for U by ~ 0.5 , it is expected that pK_a for mnm^5s^2U should be < 8 .

pK_a values for the uracil N3 position are lower in nucleosides than in the corresponding nucleotides, in general, because of the negative charge of the phosphate group. In fact, the measured values (pU, 9.7; pm^5U , 10.1; 5-bromouridine 5'-monophosphate, 8.1) (50) are higher than those of the corresponding nucleosides by ~ 0.4 . We have to be careful when comparing pK_a values measured under different conditions because the effect of the charge of the phosphate may depend on the ion strength and probably on the conformational preference of the nucleotide. However, it is still reasonable to assume that the pK_a value for $pmnm^5s^2U$ may be higher than that of the nucleoside by ~ 0.4 .

The environment around the uracil ring on the decoding site of the ribosome may also affect the pK_a values. However, from the recent results of the X-ray crystallographic analyses of the ribosome (51), we could see that the charged group nearest to the first base of the anticodon of the A-site-bound tRNA is its 5'-phosphate, and that the distance of the phosphate from the uracil ring may be almost the same as in the free nucleotide. Therefore, there is no reason at present to consider that the pK_a values on the ribosome are much higher than those in the corresponding nucleotides.

If the pK_a value of a molecule is 8.4, for instance, a 9% fraction of the molecule is ionized under pH 7.4. Therefore, it is reasonable to assume that a considerable fraction of mnm^5s^2U in tRNA is ionized under the physiological condition.

Two possible configurations. Two different configurations might be possible for the pair between $xnm^5U^*(34)$ and G(III) (Fig. 2a and b). We expect that the one with the Watson–Crick configuration (a) should be more stable than the one with the wobble configuration in which the xnm^5U^* is displaced toward the minor groove side (b). However, it is still possible

that the latter configuration could contribute significantly. The pair may be possible with the same ribose-phosphate conformation as in the G(34)–U(III) wobble pair, which could be stabilized by the stacking onto the neighboring base pair (2), and was shown to be no less stable than the A(34)–U(III) pair in a cell-free translation assay (36), as stated above. Thus, the wobble form of the $xm^5U^*(34)$ –G(III) pair might be stabilized by the same mechanism. In addition, as the sulfur atom of the wobble $xm^5s^2U^*(34)$ –G(III) pair should not participate in the hydrogen bonding interactions, the pair could be more stable than the pair with the Watson–Crick configuration. The possibility of the dual-mode base pairing itself might also contribute to the enhancement of the reading of the G-ending codon.

Physicochemical and biological effects of the $mnm^5s^2U(34)$ modifications. As mentioned above, it is expected that the s^2 modification of xnm^5U decreases the pK_a value. Therefore, the modification should promote the deprotonation. In addition, the s^2 modification should promote the stacking of the anticodon as in tRNA^{Lys} (10) and stabilize the C3'-endo form of the nucleoside. If, for example, the conformational effect stabilizes the pair by 5-fold and the fraction of the ionized form increased by the thiolation is 20%, then the total effect should be stabilization by 4-fold (though this may be an oversimplification). Therefore, the xnm^5U -to- xnm^5s^2U modification should enhance the reading of the A-ending codon. This is consistent with the case of *E. coli* tRNA^{Glu} (12). As for the G-ending codon, the xnm^5s^2U –G pair could be weaker in itself than the xnm^5U –G pair, because at least one of the two possible base pair configurations in the former (Fig. 2) needs a hydrogen bond that involves the sulfur atom. Therefore, it is possible that this effect cancels the conformational effects. This is not contradictory to the fact that the s^2 modification of tRNA^{Glu} had only a small effect on the reading of the GAG codon *in vivo* (12). The experimental results also indicate that the reading of the GAG codon by the tRNA with $mnm^5U(34)$ is more efficient than the reading of the GAA codon (12), and this could be reasoned if we assume that the mnm^5U –G pair might be intrinsically much more stable than the mnm^5U^0 –A pair.

The mnm^5 modification of s^2U would cause the partial ionization, which would destabilize the pair with A(III) and stabilize the pair with G(III), and the restriction of the flexibility of the anticodon, which would stabilize both pairs through stabilizing the C3'-endo form. Therefore, the pair with G(III) should be stabilized, while the prediction of the effects on the pair with A(III) is more difficult. The conformational effect might be smaller for the s^2U -to- mnm^5s^2U modification than for the U -to- mnm^5U modification, as the s^2U -containing anticodon is already much biased to the preorganized conformation (10). On the other hand, the decrease in the fraction of the neutral form required for the pairing with A(III) by the lowering of pK_a should be larger in the presence of the s^2 modification, as s^2U has a lower pK_a value than U . Thus, the mnm^5 modification might be more destabilizing as for the pair with A(III) in thiolated uridines than in non-thiolated uridines. In the case of tRNA^{Glu} (12), the effect through the deprotonation may have been larger than the conformational effect. As xnm^5U^* could form no base pair with U(III) or C(III), the observed misreading of the AAU/C codons by

tRNA^{Lys} during Asn starvation (11) should not have been due to the ionized form.

2-Selenouridine derivatives. Bacteria modifies $mnm^5s^2U(34)$ into 5-methylaminomethyl-2-selenouridine (mnm^5Se^2U), when selenium is available (52). The pK_a value for this nucleoside is ~ 7.1 , and a glutamate tRNA with $mnm^5Se^2U(34)$ binds efficiently to the GAG codon (53). Thus, it has been proposed that the ionized form is responsible for the base pairing with G(III) (53). It seems that the low pK_a was considered to be mainly due to the 2-seleno substitution, but the mnm^5 substitution might also contribute.

xo^5U . $xo^5U(34)$ has been proposed to recognize G(III) with the C2'-endo conformation (3,7). If the pK_a value for pxo^5U is low enough, it would be also possible that $xo^5U(34)$ could also be partially ionized. In fact, the pK_a value for pmo^5U has been measured to be 8.96 (50). The pK_a values for the xo^5U nucleosides could also be predicted based on Hammett equation: pK_a for mo^5U and ho^5U could be predicted to be ~ 8.7 , using the σ values for the methoxy and hydroxyl substituents at the *meta* position (0.12) and the above-estimated ρ value for uracil. Therefore the predicted value is consistent considering the effect of the 5'-phosphate. In cmo^5U , the pK_a could be higher than in mo^5U because of the negative charge in the carboxyl group. Thus, only a small fraction could be ionized in these nucleotides under the physiological condition. In addition, the C3'-endo conformation is destabilized in these nucleotides. Therefore, it may be reasonable to consider that the primary mechanism for the G(III) recognition should be the formation of the neutral $xo^5U(34)$ –G(III) pair with the C2'-endo form of xo^5U .

Eukaryotic xm^5U^ nucleosides.* In mcm^5U^* and ncm^5U^* found in eukaryotic tRNAs (13,54), the substituents are unlikely to withdraw electrons as well as the xnm^5 group may do. Therefore, it could be predicted that the tRNAs with the nucleoside could not pair efficiently with G(III). The experimental facts are as described above, and the G-ending codons in eukaryotes seem to be recognized by tRNAs with C(34) in general (19,54). This could be an explanation for the prokaryote/eukaryote difference of the wobble rule, if any, though it is still possible that the ribosomes are also different. It is possible that the tRNA modifications and ribosome functions have coevolved to optimize the translational function. If the present model is correct, it would mean that the eukaryotic ribosomes dispense with the C2'-endo conformation of $U^*(34)$ and a wobble pair with the anticodon base shifted toward the major groove, as eukaryotes do not have $xo^5U(34)$ (13). Thus, it is possible that the eukaryotic ribosomes have lost the ability to accept eubacterial tRNAs with $xo^5U(34)$ for the reading of the U- and G-ending codons.

TESTING THE MODEL

An obvious experimental test of the model is to measure the pK_a values of the modified uridines. This could disprove the model if the pK_a values for mnm^5U^* or τm^5U^* are not lower than ~ 8.5 , which is unlikely considering the values for $cmnm^5U$ and $cmnm^5Um$.

Another possible experiment may be to measure the pH dependence of the relative efficiency of the U*(34)-containing tRNA in the reading of the G-ending codon as compared to the efficiency of the tRNA with C(34) (35). However, the experiment could be difficult because the overall fidelity in cell-free translation from *E.coli* depends on pH (28). Conventional ribosome-binding experiments and other advanced A-site binding methods may also be possible (37,55).

It is known that mnm⁵s²U(34) in tRNA molecules could be reversibly oxidized by iodine (56). Therefore, it may be possible to test the pH sensitivity of this reaction using prokaryotic and eukaryotic tRNA molecules, as the fraction of the ionized species should directly correlate with the reactivity. It may also be possible to estimate the pK_a values of xnm⁵U* in tRNA molecules by measuring the pH-dependence of the aminoacylation reactions catalyzed by the corresponding aminoacyl-tRNA synthetases. Fortunately, bacterial Lys-, Glu- and Gln-tRNA synthetases are in direct contact with position 34 when complexed with the cognate tRNA molecules (57–59).

It may also be possible to measure the efficiency of the reading of a codon with an inosine at the third position. If the displacement of the uracil ring of the modified uridine to the major groove side is possible, it is expected that the I(III)-containing codon also could be recognized (see Fig. 2c). On the other hand, if the ionization is responsible for the pairing with G(III), the codon with I(III) could not be recognized. Some other methods may also be possible that could determine whether the 2-amino group of G(III) participates in the base pair hydrogen bonding.

The 3D structure of the decoding site is emerging in detail in these years (43,51,60,61). However, the position of the base of G(34) in the crystal was somewhat deviated from the normal wobble configuration. Therefore, it may still take some time to localize the uracil ring of some of the modified uridines on the A site precisely enough to tell the base pair configuration.

ACKNOWLEDGEMENTS

We thank Dr Mitsuo Sekine (Tokyo Institute of Technology) and Dr Kensaku Sakamoto (Tokyo University) for discussion. This work was supported in part by the Sumitomo Foundation, Tokyo, Japan (no. 020764).

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