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REVIEW

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Celebrating wobble decoding: Half a century and still much is new

Paul F. Agris[a,b,c], Emily R. Eruysal[b], Amithi Narendran[b], Ville Y. P. Väre[b], Sweta Vangaveti[a], and Srivathsan V. Ranganathan[a]

[a]The RNA Institute, State University of New York, Albany, NY, USA; [b]Department of Biology, State University of New York, Albany, NY, USA; [c]Department of Chemistry, State University of New York, Albany, NY, USA

ABSTRACT

A simple post-transcriptional modification of tRNA, deamination of adenosine to inosine at the first, or wobble, position of the anticodon, inspired Francis Crick's Wobble Hypothesis 50 years ago. Many more naturally-occurring modifications have been elucidated and continue to be discovered. The post-transcriptional modifications of tRNA's anticodon domain are the most diverse and chemically complex of any RNA modifications. Their contribution with regards to chemistry, structure and dynamics reveal individual and combined effects on tRNA function in recognition of cognate and wobble codons. As forecast by the Modified Wobble Hypothesis 25 years ago, some individual modifications at tRNA's wobble position have evolved to restrict codon recognition whereas others expand the tRNA's ability to read as many as four synonymous codons. Here, we review tRNA wobble codon recognition using specific examples of simple and complex modification chemistries that alter tRNA function. Understanding natural modifications has inspired evolutionary insights and possible innovation in protein synthesis.

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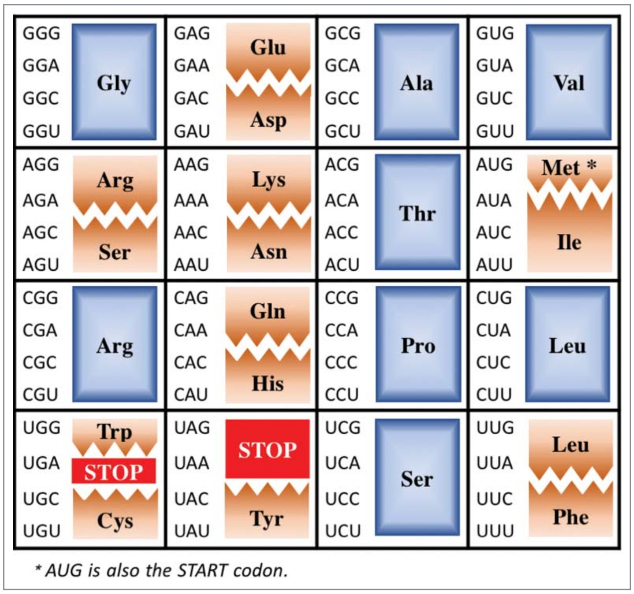
Introduction

Translation of the Universal Genetic Code (Fig. 1) into the amino acid sequence of proteins requires accurate and efficient decoding of mRNA (mRNA) on the ribosome by tRNA (tRNA). Sixty-one of 64 3-nucleoside codons of the mRNA, N1N2N3, are decoded in frame with a complementary sequence of the tRNA anticodon, $N_{34}N_{35}N_{36}$. Three codons are recognized by protein factors and correspond to translation termination signals. Complementariness of base pairing between tRNA's anticodon and the mRNA codon, A•U, U•A, G•C and C•G where • denotes canonical Watson-Crick hydrogen bonding, does not explain how the 61 amino acid codons are decoded by far fewer tRNAs. Fifty years ago, Francis Crick published the Wobble Hypothesis.[1] At the time there was evidence to suggest that the first two positions, N1N2, of mRNA's 3-nucleoside codons were uniquely identified by the tRNA with some ambiguity in the third position. Crick offered the idea that uridine (U) at the first position of the anticodon, position 34 in tRNA (Fig. 2), would base pair with guanosine (G) and that inosine (I), which at the time had only recently been found at position 34 in yeast $tRNA^{Ala}$,[2] would base pair with uridine, cytidine (C) and adenosine (A). Thus, canonical, Watson-Crick base pairing with tRNA and mRNA on the ribosome was supplemented with non-canonical hydrogen bonding, 'wobble' base pairing, including that of a purine with another purine. Though conceding that the wobble position base pairing of two purines would widen the anticodon-codon double helix at the point of an $I_{34}{\circ}A3$ base pair where A3 is the third nucleoside of the codon and ◦ denotes non-canonical hydrogen bonding, Crick excluded the possibility of a wobble position pyrimidine-pyrimidine base pairing. He argued that the narrowing of the helix would be too dramatic in comparison to the neighboring canonical purine-pyrimidine distances at the first and second positions.

The post-transcriptional modification of tRNA, the original soluble RNA, has been known for over 50 years pioneered by the extraction and characterization methods of Ross Hall in the 1960s.[3] At the time the Universal Genetic Code was unveiled,[4] there were several RNAs known to contain modified nucleosides, but their sequence locations and functions were a mystery before the evolution of RNA sequencing methods. By 1991, 25 years later, a sufficient number of modified nucleosides had been found to occupy the wobble position 34 of tRNA's anticodon that a Modified Wobble Hypothesis was advanced.[5] Biophysical and biochemical experiments had suggested and continue to support the principle that some position 34 modifications structure the architecture of the anticodon stem and loop (ASL) to counter wobble whereas other modifications shape the ASL to enable a tRNA to decode three or even four synonymous codons (codons differing only in the wobble position N3).[6-10]

Of what importance are these ubiquitous and highly conserved anticodon modified nucleosides to the decoding of mRNA codons? In general, modified nucleosides of tRNA's anticodon stem and loop (ASL) domain are found within the stem at positions 27, 28, 31, 39, and 40 and at loop positions 32, 34, 35, 37, and 38 (Fig. 2).[11-13] These ten nucleoside positions of the ASL are not simultaneously modified in any one tRNA, rather a set of 3–5 specific nucleosides are found to be

CONTACT Paul F. Agris pagris@albany.edu The RNA Institute, 1400 Washington Ave. Albany, NY 12222, USA.



GGG	Gly	GAG	Glu	GCG	Ala	GUG	Val
GGA		GAA		GCA		GUA	
GGC		GAC	Asp	GCC		GUC	
GGU		GAU		GCU		GUU	
AGG	Arg	AAG	Lys	ACG	Thr	AUG	Met *
AGA		AAA		ACA		AUA	Ile
AGC	Ser	AAC	Asn	ACC		AUC	
AGU		AAU		ACU		AUU	
CGG	Arg	CAG	Gln	CCG	Pro	CUG	Leu
CGA		CAA		CCA		CUA	
CGC		CAC	His	CCC		CUC	
CGU		CAU		CCU		CUU	
UGG	Trp	UAG	STOP	UCG	Ser	UUG	Leu
UGA	STOP	UAA		UCA		UUA	
UGC	Cys	UAC	Tyr	UCC		UUC	Phe
UGU		UAU		UCU		UUU	

** AUG is also the START codon.*

Figure 1. The Universal Genetic Code. The Universal Genetic Code is shown in an atypical array to highlight those codons and their decoding by tRNAs discussed here. Fully degenerate codon boxes are shown in blue, split codon boxes in brown and stop codons in red.

modified in an individual tRNA. Many times in the sequencing of a tRNA species one finds that as many as three nucleoside positions of the seven residue loop are modified. Considering that the ASL of tRNA constitutes 17 of the molecule's ~76 nucleosides and that the stem constitutes 5 base pairs, with 10 nucleosides, the modifications within the ASL loop represent a dense population of altered nucleoside chemistries. Often the modifications at wobble position 34 and 3′-adjacent to the anticodon at position 37 are the most chemically complex of all RNA modifications and are composed of hydrophobic aliphatic chains or aromatic substituents, or of highly hydrophilic or even charged functional groups participating in translational efficiency and fidelity.[11] Thus, posttranscriptional modifications of the ASL nucleosides emulate the chemistries of amino acid side chains.[14] Even what appears to be one of the simplest of modifications, such as the substitution of a sulfur atom for an oxygen, a thioketone for a carbonyl group, or the deamination of adenosine to inosine (I), takes on significance in the decoding of mRNA. The modification of wobble position U_{34} to s^2U_{34} alters tRNA's ability to wobble to G3;[5] the modification of C_{32} to s^2C_{32} negates the ability of tRNAs with I_{34} to decode A3 of the codon.[6]

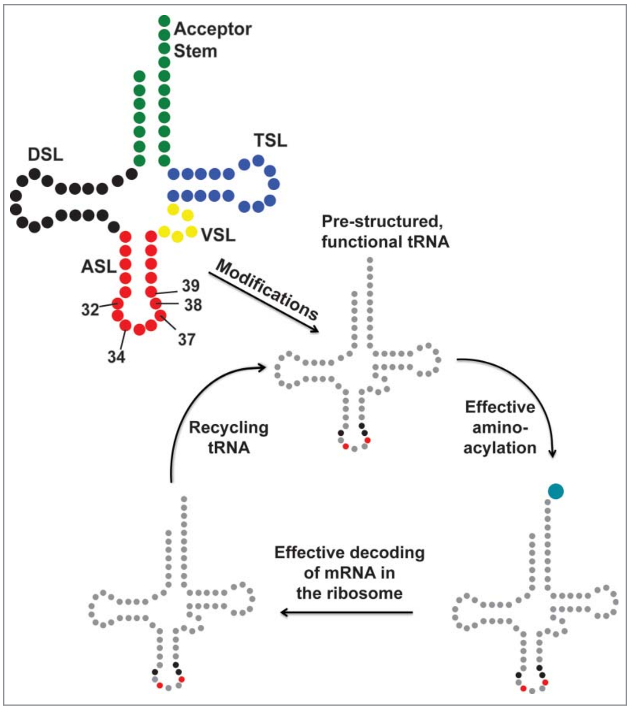


Figure 2. The tRNA journey. The secondary structure of tRNA with its constituent domains marked in different colors (top left): Acceptor Stem (green); Dihydrouridine Stem and Loop, DSL (black); Anticodon Stem and Loop, ASL (red); Variable Stem and Loop, VSL (yellow); Thymidine Stem and Loop, TSL (blue). tRNA transcripts are processed by sizing and modification, some are spliced, before functioning in translation. Modification of tRNAs, particularly the anticodon stem and loop (ASL) domain at positions 32, 34, 37, 38 and 39, is an important step toward achieving functional chemistry and architecture. The wobble nucleoside, first of the anticodon, is position 34. Red and black highlights of mature tRNA after modification indicate the locations in the ASL where it is heavily modified.

The importance of being modified

The high percentage of anticodon modification and their composite chemistries provides different functionalities to tRNA in its role in decoding mRNA (Fig. 3). Individual anticodon domain modified nucleosides are identity determinants for protein recognition, particularly aminoacylation,[15-17] increase accuracy and efficiency in codon recognition,[18-24] and prestructure the ASL for translation.[9,25-30] Each of the modified nucleosides contribute distinct chemistries, nucleoside conformations and dynamics, and their contributions to decoding have been studied extensively over decades and most recently reviewed.[20,22,24,28,31-38] However, there is significant evidence that a combination of two or three anticodon domain modifications play a synergistic role in tRNA function where modification of a wobble position U is crucial.[9,20,24,39-46] Today, we know that certain modifications of U_{34} enable expansion of codon from NNA/G recognition to synonymous codons ending in pyrimidines, N1-N2-Pyr, where N is any of the 4 nucleosides and Pyr is either U or C.[29] Yet, the anticodon domain of some tRNA species lack modification and can be totally devoid of modified nucleosides. Bacteriophage T4 $tRNA^{Gly}$ is an early example of a tRNA lacking modification.[47] The unmodified U_{34} nucleosides of native mitochondrial alanine, leucine, threonine and valine tRNA species *in vivo*, and that of a totally unmodified tRNA transcript *in vitro* were shown to read codons ending in U3 and C3, as well as A3 and G3.[48,49] To understand the forces that maintained and propagated tRNAs' anticodon domain modifications throughout all life, first we will discuss the limited number of examples revealed over many years to have unmodified Us and As at wobble position 34, and the influence of position 32 nucleosides on decoding.

Unmodified wobble position 34 and the importance of position 32

Bacteria, archaea and eukaryotes have some 40 tRNA species decoding the Universal Genetic Code. However, many times we find that in mammalian and yeast mitochondria, in chloroplasts, and in *Mycoplasma ssp* only a single tRNA species decodes all 4

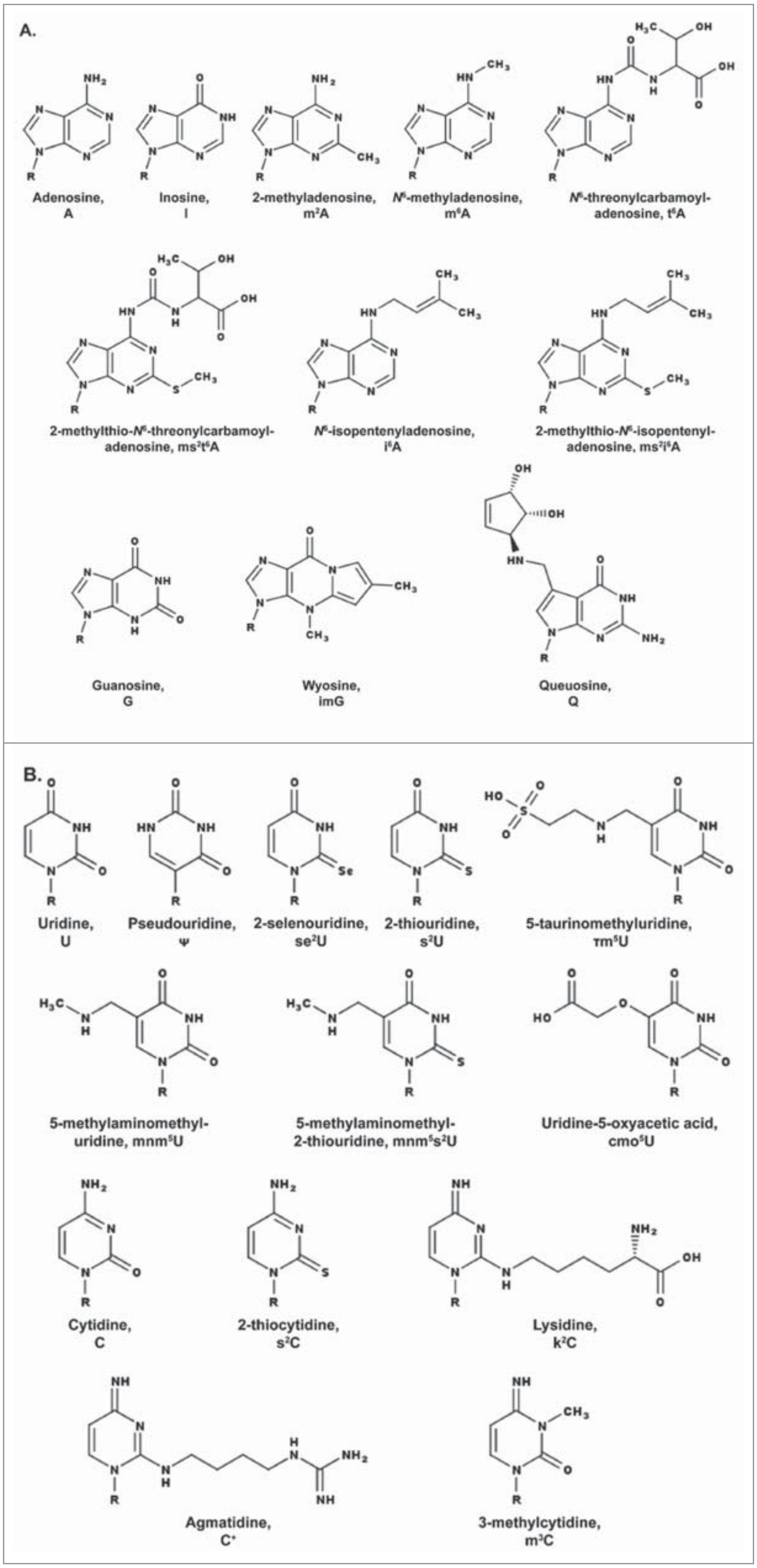


Figure 3. Modifications present in tRNA's anticodon stem and loop domain (ASL) featured in the text and displayed in their neutral state. A. Modified purines, adenosine (A) and guanosine (G). B. Modified pyrimidines, uridine (U) and cytidine (C). R = ribose.

codons of a fully degenerate codon box of the Universal codes (Fig. 1).[49-57] Wobble codon recognition is at its extreme in these circumstances and has been referred to as 'superwobble'.[58] An unmodified U in tRNA's wobble position 34 facilitates the use of far fewer tRNAs in organelles than the over 40 cytoplasmic species typically reading the 61 amino acid codes. Fewer tRNAs is certainly an advantage for the small genomes of the organelles and the minimal single cell organism, mycoplasma. An unmodified U_{34} also abrogates the need for the extensive array of genes encoding the modified nucleoside pathways of enzymes and substrates required of the simplest to the most complex modifications of U_{34}.[11] However, the superwobble reading of codons compromises translational efficiency.[58]

An unmodified U_{34} seems to be particularly efficient for the organellar $tRNA^{Gly}$ species. The codons for the amino acid glycine, GGA, GGG, GGU or GGC, are found in a 4-fold degenerate codon box, whose first two nucleosides are the same (Fig. 1). They are read by as many as three different tRNA isoacceptors in bacteria with cognate and wobble anticodons. Early in the study of glycine tRNAs, the *Escherichia coli* $tRNA^{Gly}$ isoacceptors were grouped into 3 subspecies based on a chromatographic separation: $tRNA^{Gly1}{}_{CCC}$, $tRNA^{Gly2}{}_{UCC}$, and $tRNA^{Gly3}{}_{GCC}$.[59] In an *E. coli* in which there were multiple copies of suppressors increasing the levels of wild-type $tRNA^{Gly1}{}_{CCC}$, −1 translational frameshifting occurred at the 5′-GGG-3′ codon allowing the near-cognate tRNA to read GGA codons.[60] Surprisingly, experiments with *E. coli* $tRNA^{Gly2}{}_{UCC}$ demonstrated that the unmodified UCC anticodon discriminates among the four glycine codons depending on the nucleoside in position 32, an unmodified U_{32} or C_{32} (Fig. 4A). Thus, the unmodified UCC anticodon reads GGA and wobbles to GGG, but does not recognize GGU and GGC.[61]

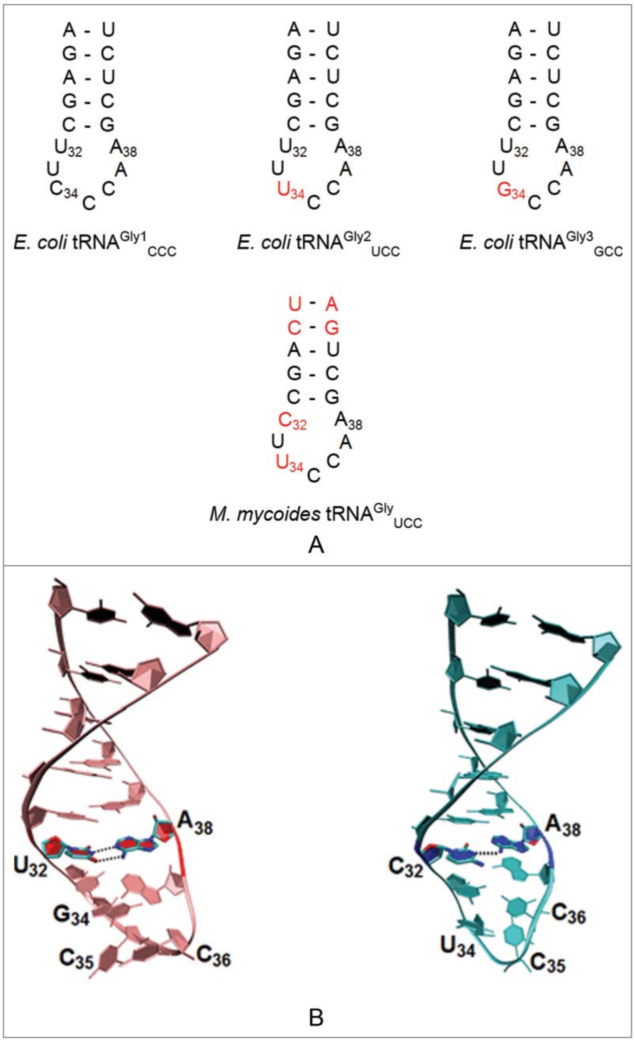


Figure 4. Sequences of unmodified ASL of $tRNA^{Gly}$. A. ASLs of *E. coli* $tRNA^{Gly1}{}_{CCC}$, $tRNA^{Gly2}{}_{UCC}$, and $tRNA^{Gly3}{}_{GCC}$ indicating the changes in nucleoside position 32 and 34 (marked in red) from the $tRNA^{Gly1}$ sequence. All 3 *E. coli* $tRNA^{Gly}$ have the same nucleoside at position 32 (U_{32}). The *Mycoplasma mycoides* $tRNA^{Gly}{}_{UCC}$ differs from the *E. coli* $tRNA^{Gly}$ as a result of a cytidine at position 32 as well as the presence of inverted base pairs in the anticodon stem at positions 27•43 and 28•42. B. Three Dimensional Structure of the $tRNA^{Gly}{}_{GCC}$ and $tRNA^{Gly}{}_{UCC}$ ASLs of *B. subtilis*. The interactions between the U_{32}•A_{38} and C_{32}○A_{38} nucleosides (PDB ID: 2LBJ and 2LBL) are shown. Both ASLs lack the sharp U-turn characteristic of ASLs of other tRNAs. Nucleosides of the anticodon are labeled 34 to 36.

Although the anticodon UCC could discriminate efficiently with a U in position 32, it loses its ability to differentiate when substituted with a C_{32} as was also true for *M. mycoides* glycine tRNA. In wild type *M. mycoides* $tRNA^{Gly}$, the anticodon UCC failed to discriminate between the glycine codons with a position 32 cytidine, but when changed to a U it acted like *E. coli* tRNAs and discriminated between the four glycine codons.[62] In *M. mycoides*, the single $tRNA^{Gly}{}_{UCC}$ that decodes all four glycine codons is devoid of anticodon modifications and has a C_{32}○A_{38} mismatch which leads to decreased fidelity *in vivo* (Fig. 4).[63] The $tRNA^{Gly}{}_{UCC}$ transcript without any modifications reads all 4 codons *in vitro*.[48] Ribosome binding experiments with U_{32}/C_{32} mutants of $tRNA^{Gly}$ showed an increased affinity of the C_{32} mutant to the cognate codon and to codons with third position mismatches in the ribosome's A-site.[64] The rate of dissociation of the U_{32}-containing $tRNA^{Gly}$ from the near-cognate GGC, GGA and GGU codons was much more rapid - 12-fold faster than from the GGG cognate codon - and stabilized the binding of tRNA to codons with third position mismatches.[64] In contrast, the mutated $tRNA^{Gly1}$ with C_{32} dissociated much more slowly from near-cognate codons. Analysis of the UV-thermal denaturation (melting) curves of the anticodon stem and loop domains demonstrated that $tRNA^{Gly}{}_{UCC}$ with a protonated C_{32}○$A^{+}{}_{38}$ non-canonical base pair melted at a temperature 10 °C lower than $tRNA^{Gly}{}_{GCC}$ or np-$tRNA^{Gly}{}_{UCC}$, used exclusively for non-protein cell wall synthesis in *Staphylococcus* species, which exhibited a T_m of 70 °C.[65] Although the sequences of these tRNA differed from one another, none of their solution structures formed the classical U-turn motif seen in other tRNA anticodon loops (Fig. 4B). tRNA molecules fulfill different functional roles by interacting with other cellular molecules, and the structural variations of the glycine ASL of *S. aureus* could contribute to its functional diversity. $tRNA^{Gly}{}_{GCC}$, without any base modification participates in transcriptional regulation and transcription. The $tRNA^{Gly}{}_{UCC}$ which more often contains a U_{34} modification except in some organisms including *M. mycoides*, participates in translation. The np-$tRNA^{Gly}{}_{UCC}$ contains no base modification and participates in cell wall synthesis.[65] The presence of pyrimidine at position 37, along with a reduced affinity for EF-Tu, could limit their involvement in transcription and translation. Modifications of U_{34} could increase or decrease the ability to wobble, thus enhancing discrimination. A more dynamic ASL $tRNA^{Gly}{}_{GCC}$ was observed in the presence of multivalent cations, whereas $tRNA^{Gly}{}_{UCC}$ and np-$tRNA^{Gly}{}_{UCC}$ were more structurally ordered in their presence. A more dynamic loop structure would therefore, better accommodate the different functional roles of an unmodified $tRNA^{Gly}$ in protein translation, in tRNA-dependent gene regulation, and cell wall biosynthesis.[65]

The nucleoside at position 32 of the tRNA's anticodon loop is recognized as important for translation, though the position

32 nucleoside is remote from the anticodon. The intraloop hydrogen bonding between the nucleosides at 32 and 38 strongly influences the affinity of tRNA to the A-site.[64] tRNA species other than those for glycine also have an unmodified wobble position U_{34}, and the nature of the $N_{32}{\circ}N_{38}$ intraloop hydrogen bonding in these tRNAs does appear to regulate expansion/discrimination of codon reading. The $A_{32}{\bullet}U_{38}$ interaction, highly conserved in $tRNA^{Ala}$ and also seen in $tRNA^{Pro}$, decreases the tRNA affinity to the ribosomal A-site compared with other $N_{32}{\circ}N_{38}$ intraloop hydrogen bonding nucleosides.[66] Five *Mycoplasma capricolum* tRNA species with the unmodified anticodon UNN and decoding for five different amino acids have anticodon loop sequences with $C_{32}{\circ}A_{38}$. In addition, $tRNA^{Ala}{}_{UGC}$ has an intraloop interaction of $C_{32}{\circ}C_{38}$. Because these 6 tRNA species have a C_{32} and an unmodified U_{34}, in theory they could efficiently read all four synonymous codons for leucine, valine, proline, alanine, glycine and serine.[67] Although $tRNA^{Thr}{}_{UGU}$ was shown to translate the codons ACU, ACA and ACG, it was inefficient in reading the codon ACC.

Interestingly, the sufD42 mutant of *E. coli* $tRNA^{Gly1}$, encoded by glyU, is a derivative of $tRNA^{Gly}{}_{CCC}$ with an extra C in the anticodon loop and contains no modification in the anticodon loop.[68] This mutant is considered dominant and contains four bases, 5′-CCCC-3′ that make up the anticodon and suppress +1 frameshift mutants with an extra G inserted into a GGN codon (GGGN). The quadruplet translocation theory is used to deduce the pairing of these four cytidines with four bases of the codon in the A site. The tRNA anticodon sequence has been suggested to act as a molecular ruler which determines the codon size during translation, within certain limits,[69] thereby restoring some ribosomes to the wild type frame. Thus, the nature of the $N_{32}{\circ}N_{38}$ base interaction affects the binding of the anticodon to the codon suggesting that the intraloop hydrogen bonding alters the conformation and dynamics of the anticodon stem and loop domain within the ribosomal complex.[62,70]

Very few unmodified, wobble position A_{34} have been found in tRNA sequences: a yeast mitochondrial $tRNA^{Arg}{}_{ACG}$[71] and *Mycoplasma* $tRNA^{Thr}{}_{AGU}$.[50,56,72] In all domains of life, A_{34} of the tRNA transcript is almost always deaminated to form inosine at the wobble position (I_{34}). The modification to I_{34} expands the coding capacity to read the bases U, C and A. In *Mycoplasma* the unmodified A_{34} of $tRNA^{Thr}{}_{AGU}$ efficiently translates the ACC codon,[67] but mutants of *E. coli* $tRNA^{Ser}$ and $tRNA^{Gly}$ with A_{34} could only weakly read UCC and GCC *in vitro*, respectively.[73,74] In *Salmonella typhimurium*, $tRNA^{Pro}{}_{GGG}$ is the only tRNA that reads the CCC codon. With G_{34} replaced by an unmodified A, a mutant with no cognate codon for CCC, grew normally.[75] The mutant $tRNA^{Pro}{}_{AGG}$ efficiently read the CCC codon similarly to its wild-type counterpart with a GGG. It formed a wobble base pair using a protonated A with the third position C in mRNA. Similarly, a mutant $tRNA^{Gly1}$ with a UCC to ACC mutation containing an unmodified A_{34}, lost its ability to discriminate between the third position nucleosides of the glycine codons.[73] The possibility of a purine∘purine base pair being more stable than a pyrimidine∘pyrimidine pair,[76] as well as the two out of three model[77] was suggested to explain the non-discrimination by A in the wobble position.[73] The presence of A in the wobble position could change the conformation of the anticodon by preventing hydrogen bonding between U_{33} and the phosphate of nucleoside 36,[78] which stabilizes the U-turn conformation of the ASL. The rare occurrence of an unmodified A_{34} is supported by the hypothesis that a wobble position A cannot discriminate and ensure translational fidelity of tRNAs reading split box codons. In contrast, the presence of A_{34} would be advantageous in the reading of fully degenerate synonymous codons where there is a lack of discrimination and in cases where only a single tRNA exists for the reading all four codons.[73]

In addition to their primary role of translation, some tRNAs have other functions including regulation of gene expression, bacterial cell wall synthesis, viral replication, antibiotic biosynthesis and suppression of alternative splicing.[64,65] In *Bacillus subtilis* and many other Gram-positive bacteria, tRNA molecules regulate gene expression by the tRNA dependent, ‘T-box’, mechanism of transcription attenuation to maintain a balanced pool of aminoacyl-tRNAs that is essential for cell viability.[79,80] The tRNA ligand for the T-box mechanism regulating the expression of glycyl-tRNA synthetase is $tRNA^{Gly}{}_{UCC}$ with an unmodified U_{34}. A Rho-independent, terminator helix in the 5′UTR of the leader mRNA of the glyQS operon for glycyl-tRNA synthetase prevents the operational binding of an aminoacylated glycyl-$tRNA^{Gly}{}_{UCC}$.[81] Conversely, the anticodon of an uncharged tRNA interacts with a loop, the Specifier Loop, containing the complementary codon and the tRNA's 3′-terminal CCA hydrogen bonds to an anti-terminator helix, re-conformed from the terminator helix. These interactions as well as others between the tRNA and the mRNA stabilize the anti-terminator conformation of the 5′UTR and allow transcription to proceed downstream through the coding sequence. Thus, the unacylated $tRNA^{Gly}{}_{UCC}$ is similar to the much smaller metabolic products that affect the riboswitch mechanisms controlling gene expression; the 5′UTR undergoes a conformational change with the binding of the ligand.[82] The $tRNA^{Gly}{}_{UCC}$ is also predicted to bind to the 5′-GGA-3′ Specifier codon in *Bacillus* and *Staphylococcus* species glycyl T-box riboswitches.[83]

A third glycine tRNA (UCC) without a modified U_{34} has been identified in *Staphylococcus* species as participating in cell wall biosynthesis, but not in protein translation, and was termed non-proteinogenic (np-$tRNA^{Gly}$).[84-87] These np-tRNA have an unmodified U_{34} nucleoside and a cytidine rather than a purine at position 37.[88] In *Thermus thermophiles*, the np-$tRNA^{Gly}$ species are found to have reduced affinity for the elongation factor Tu (EF-Tu) due to base substitutions of A_{51}-U_{63} for G_{51}-C_{63} in the base of the T-stem, thus decreasing their involvement in ribosomal protein synthesis.[65] These weak EF-Tu binders could act as glycine donors in forming essential pentaglycine bridges which stabilize the staphylococcal cell wall.[85,89-91]

The biosynthesis of peptidoglycan in *S. aureus* involves two uridine nucleotide substrates, UDP-MurNAc-pentapeptide and UDP-GlcNAc (N-acetyl glucosamine), which combine to form a lipid intermediate GlcNAc-MurNAC(pentapeptide)-P-P-lipid. The lipid intermediate gets further modified by amidation of the α-carboxylic group of glutamic acid and the addition of a pentaglycine chain to the ε-amino group of lysine. The weak EF-Tu binding glycyl tRNAs serve as intermediates in these reactions to form the pentaglycine bridges, that stabilize the peptidoglycan chains and are essential for cell viability. These short peptide bridges are synthesized in a non-ribosome catalyzed peptidyl transferase reaction, which uses the charged glycyl-tRNA,[1] np-tRNA^{Gly} and a 'pseudo'-tRNA$^{Gly}_{UCC}$ as substrates.[65,85,92] Glycine and serine act as substrates that are successively added to form small peptide bridges which are catalyzed by a family of non-ribosomal peptidyl transferases known as FEM-XAB (Factors Essential for Methicillin Resistance) - mediated cell wall synthesis.[93] The incomplete formation of these interpeptide bridges can lead to increased antibiotic susceptibility or lethality.[85]

The wobble hypothesis and the modulation of inosine wobbling

Though there are instances of unmodified nucleosides at tRNA's wobble position 34 as described, more often than not U_{34} is post-transcriptionally modified and A_{34} is deaminated to inosine. Using specific modifications of U and the modulation of I reading A, U and C, we illustrate here the importance of wobble position 34 modifications to tRNAs' accuracy and efficiency of translation. The modification of adenosine to inosine was first recognized by Francis Crick for enabling tRNA recognition of synonymous codons.[1] Inosine (Fig. 3) results from the deamination of adenosine, a transformation that is facilitated by the adenosine deaminase (ADAR) family of enzymes that act on RNA.[94] Although inosine is a marker of damage or mutation in DNA, the presence of this very same modified nucleoside is considered to be essential in various RNAs.[95] Inosine plays a vital role in the function of tRNA, in particular. As the first recorded nucleoside modification within the sequence of an anticodon,[2] Crick introduced inosine in his 1966 Wobble Hypothesis.[1] While the first two bases of the codon undergo traditional base-pairing without exception,[96] Crick proposed the potential for non-canonical base pairs between the first base of the anticodon ("wobble" position 34) and the third base of the codon, U_{34}○G3, or I_{34}○A3/U3/C3 (Fig. 5).[1] This flexibility of the genetic code is not without limitations; however, in accordance with this hypothesis, a given tRNA isoacceptor may recognize multiple codons, thus explaining the degeneracy of the genetic code.

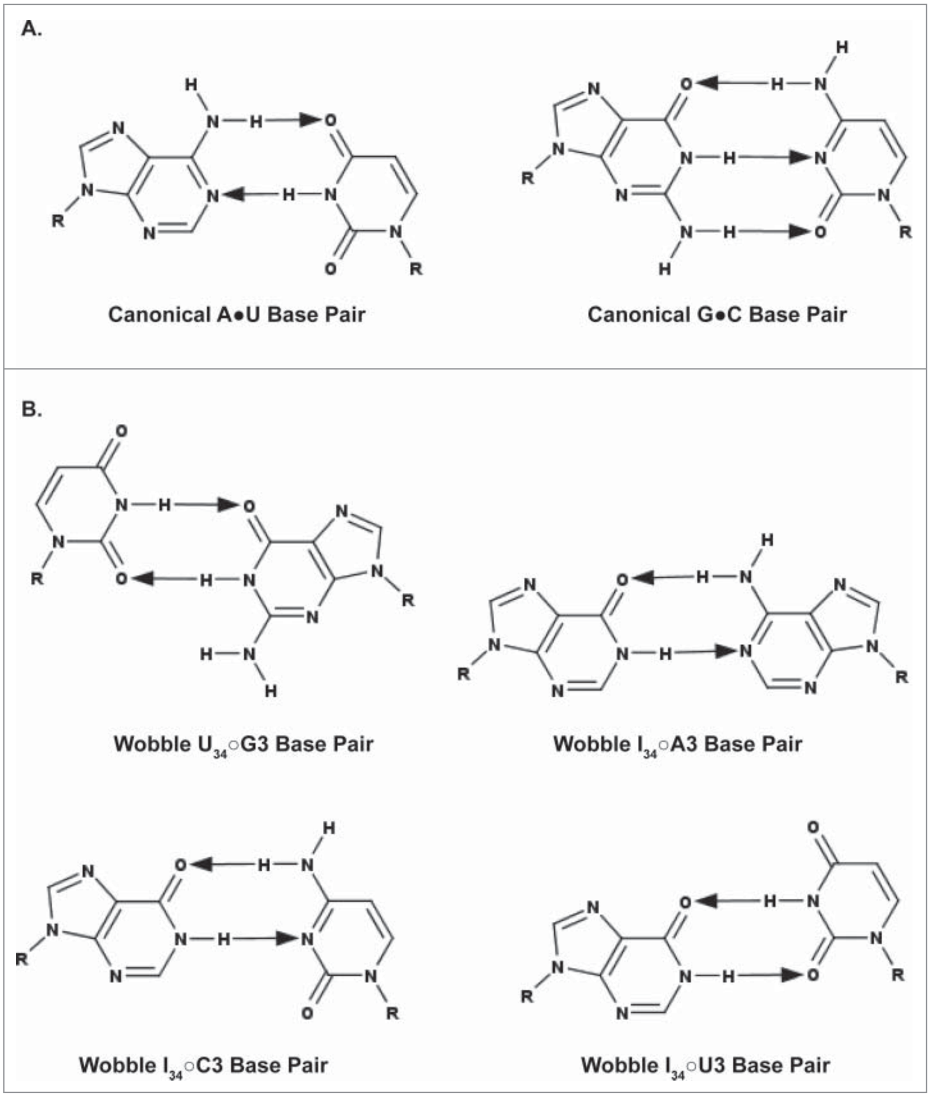


Figure 5. Canonical and wobble base pairing of tRNA to mRNA. A. Canonical A•U and G•C base pairs. B. Wobble U_{34}○G3, I_{34}○A3, I_{34}○C3, and I_{34}○U3 base pairs. G_{34}○U3 pairings are virtually nonexistent; therefore, the pairing is not shown. The arrows point away from the hydrogen bond donor and toward the hydrogen bond acceptor.

The Wobble Hypothesis states that position 34 inosine may base pair with uridine, cytidine, and adenosine. The ability of inosine at the wobble position to promote the reading of multiple codons, in some cases, proves essential to survival. The heterodimeric enzyme consisting of the Tad2p and Tad3p subunits of *Saccharomyces cerevisiae* catalyzes the deamination of adenosine to inosine on tRNA.[97] A strain of *Schizosaccharomyces pombe* containing a mutant *tad3–1*, the homolog of *TAD3*, experienced temperature-dependent arrested growth at Gap 1 and Gap 2 of the cell cycle.[98] The *S. pombe* genome utilizes only 3 $tRNA^{Ala}$ isoacceptors for the 4 alanine codons GCU, GCC, GCG, and GCA: $tRNA^{Ala}{}_{IGC}$, $tRNA^{Ala}{}_{CGC}$, and $tRNA^{Ala}{}_{UGC}$. According to the wobble rules, $tRNA^{Ala}{}_{IGC}$ must be responsible for decoding of the GCC codon. The otherwise unmodified $tRNA^{Ala}{}_{AGC}$ would be able to decode its complementary GCU codon but could not wobble to GCC, inhibiting the translation of gene products vital to the G_1/S and G_2/M transitions of the cell cycle.[98]

A $tRNA^{Arg}{}_{IGC}$ may be modified from A_{34} to I_{34} yet still be unable to decode A3. The *E. coli* $tRNA^{Arg1}{}_{ICG}$and $tRNA^{Arg2}{}_{ICG}$ isoacceptors should bind and effectively decode the CGU and CGC codons, and even the CGA codon. These wobble pairings were confirmed experimentally, as the singly modified anticodon stem and loop of $tRNA^{Arg}{}_{ICG}$, $ASL^{Arg}{}_{ICG}$, is able to bind the CGU, CGC, and CGA codons within the ribosomal A-site.[39] In fact, $tRNA^{Arg1}{}_{ICG}$ and $tRNA^{Arg2}{}_{ICG}$ are the only isoacceptors available to recognize the 3 aforementioned codons. Furthermore, adenosine must be modified to inosine at position 34 of the $tRNA^{Arg1,2}{}_{ICG}$ isoacceptors in order for wobble pairing to occur. Unmodified $ASL^{Arg1,2}{}_{ACG}$ is able to bind its cognate codon, CGU, but unable to bind CGC or CGA as expected, as Crick did not explicitly delineate any wobble capabilities of position-34 adenosine.[1,39]

The $tRNA^{Arg1,2}{}_{ICG}$ decoding of the CGA codon is relatively inefficient compared with translation of the CGU.[99,100] Although the energy barrier for $I{\circ}A$ base-pair formation is greater than those of $I{\circ}C$ and $I{\circ}U$, the increased distance between the N-glycosyl bonds of I_{34} and A3 required to accommodate the purine${\circ}$purine wobble pair can be achieved when the nucleosides adopt an $I_{anti}{\circ}A_{anti}$ conformation.[1,44,101] Additional modifications at position 32 and 37 of the $ASL^{Arg}{}_{ICG}$ may further contribute to the difficulties of $I{\circ}A$ wobble pairing. The *E. coli* $tRNA^{Arg1}{}_{ICG}$ species contains the naturally-occurring 2-thiocytidine at position 32 (s^2C_{32}) and 2-methyladenosine at position 37 (m^2A_{37}). The thrice modified ASL construct could not be synthesized, but the doubly modified $ASL^{Arg}{}_{ICG}$-s^2C_{32} was unable to bind the CGA codon within the ribosomal A-site.[39] Here, the complete wobble capabilities of inosine do not apply due to the restrictive effects of the s^2C_{32} modification with respect to the otherwise feasible $I{\circ}A$ wobble pair.

The $tRNA^{Arg2}{}_{ICG}$ species lacks the s^2C_{32} modification but does contain the m^2A_{37} modification. As evidenced by the aforementioned ribosomal binding study, m^2A_{37} prohibits $I{\circ}A$ pairing.[39] However, reading of the CGA codon defaults to $tRNA^{Arg2}{}_{ICG}$, as the inclusion of the s^2C_{32} modification disqualifies the $tRNA^{Arg1}{}_{ICG}$ from decoding of the CGA codon *in vivo*. Yet the *E. coli* genome still must compensate for the overall poor capacity of the $tRNA^{Arg}{}_{ICG}$ isoacceptors to wobble to the CGA codon by biasing codon usage. The inclusion of CGA codons in mRNA transcripts increases energetic costs and decreases the efficiency of translation. The prevalence of the CGU, CGC, and CGA codons is heavily biased against the CGA codon and in favor of the CGU and CGC codons.[102,103]

The modified wobble hypothesis and decoding at position 34

For many years, Crick's Wobble Hypothesis appeared to sufficiently explain the function of modified nucleosides at tRNA's wobble position without the need for alteration. However, the discovery of numerous new modifications, a large number of which are found exclusively at the wobble position, led to the development of a modified wobble hypothesis.[5] The first base of the anticodon is so often modified to either expand or restrict the binding abilities of the wobble nucleoside, therefore enabling the specific recognition of cognate and synonymous codons. As such, near-cognate codons can be selected against, or the recognition of multiple codons can be made feasible with various chemical moieties introduced onto the wobble base.[5] There are several examples of both expansion as well as restriction of codon recognition which are presented with a focus on the mechanistic details of both expansion and restriction of recognition, and the many factors that must come to play to make either possible.

Expansion of codon recognition through modified nucleosides pre-structuring of the ASL

The high freedom of rotation coupled with the limited chemical variation of the four major nucleosides enables RNAs to adopt multiple conformations with comparable stability yet limited chemistry. Modifications add to the chemistry and can either limit or expand the number of available conformations, thereby influencing the structure toward a more specific architecture or provide dynamics important to translational effectiveness. There are six amino acids represented by 4-fold degenerate codon boxes which include alanine, glycine, proline, serine, threonine, and valine, and are of particular interest in U_{34} modification. As with *S. pombe* and the GCN alanine codon box, *Salmonella enterica* utilizes 3 distinct species of $tRNA^{Pro}$ for recognition of the entire CCN codon box: $tRNA^{Pro}{}_{CGG}$, $tRNA^{Pro}{}_{GGG}$, and $tRNA^{Pro}{}_{UGG}$. Deletion of the genes for $tRNA^{Pro}{}_{CGG}$ and $tRNA^{Pro}{}_{GGG}$ simultaneously is not lethal to *S. enterica*.[104] Only $tRNA^{Pro}{}_{UGG}$ proves necessary for viability of the organism by surpassing its expected decoding capabilities due to the presence of the 5-oxyacetic acid modification (cmo^5) on position-34 uridine in the ASL. The cmo^5U_{34} modification is present in one species of each of the tRNA isoacceptors for alanine, proline, serine, threonine, and valine. Similarly, $tRNA^{Val}{}_{UAC}$-cmo^5 and $tRNA^{Ala}{}_{UGC}$-cmo^5 partially rescue the *E. coli* growth phenotype caused by knockout of the 2 $tRNA^{Val}{}_{GAC}$ isoacceptors and the $tRNA^{Ala}{}_{GGC}$ isoacceptor, respectively, indicating the ability of tRNA containing the cmo^5U_{34} modification to decode an entire codon box, albeit somewhat inefficiently.[105] The presence of the cmo^5U_{34} modification facilitates pre-structuring of the

$\text{ASL}^{\text{Val}}{}_{\text{UAC}}$ that enhances its ability to bind near cognate codons. When binding each of the GUN valine codons, the ribose of $\text{cmo}^5\text{U}_{34}$ of the modified $\text{ASL}^{\text{Val}}{}_{\text{UAC}}$ assumes the C3′-*endo* conformation.[29] Prevalent within A-type helical regions of tRNA, the C3′-*endo* sugar pucker is synonymous with stability and rigidity.[106-108] The existence of a hydrogen bond between the 2′-OH of the almost invariant U_{33} of the ASL and O5 of the cmo^5 modification further constrains $\text{cmo}^5\text{U}_{34}$.[29] This intramolecular hydrogen bond in particular expands the ability of $\text{ASL}^{\text{Val}}{}_{\text{UAC}}$-$\text{cmo}^5\text{U}_{34}$ to decode codons ending in U and C, pre-structuring the ASL such that the entropic cost of a pyrimidine○pyrimidine base pair, initially believed to be unfavorably short, is surpassed.[1,29]

The cmo^5 modification has surprising implications for the U_{34}•G3 wobble pair. Although this pairing was originally predicted without regard for the potential of modified uridines, the uridine at position 34 of the ASL must be modified in order for the U_{34}•G3 pairing to occur.[18,105] Rather than enhancing the ability of U_{34} to wobble to G3, cmo^5 merely enables the interaction. In a second divergence from the original Wobble Hypothesis, the modified $\text{cmo}^5\text{U}_{34}$•G3 does not adopt the predicted wobble geometry with two hydrogen bonds between the two bases. The $\text{cmo}^5\text{U}_{34}$ instead forms three hydrogen bonds with G3 as in the traditional Watson-Crick geometry (Fig. 6).[29] The steric and electronic properties of the cmo^5 modification are thought to promote the enol form of $\text{cmo}^5\text{U}_{34}$, thus allowing for the Watson-Crick geometry of the $\text{cmo}^5\text{U}_{34}$•G3 base pair reminiscent of a C•G base pair.[29,109]

Additionally, *E. coli* $\text{tRNA}^{\text{Val}}{}_{\text{UAC}}$ containing the $\text{cmo}^5\text{U}_{34}$ modification is also methylated at the position-37 adenosine to yield *N*⁶-methyladenosine (m^6A). As seen with $\text{tRNA}^{\text{Arg1}}{}_{\text{ICG}}$-$\text{s}^2\text{C}_{32}$;$\text{m}^2\text{A}_{37}$ and $\text{tRNA}^{\text{Arg2}}{}_{\text{ICG}}$-$\text{m}^2\text{A}_{37}$, it is not uncommon for the ASL of a given tRNA to contain multiple modified nucleosides. Modifications written onto N6 of the universal purine located at position 37 of the ASL are proposed to interfere with intraloop hydrogen bonding and to enhance base-stacking, thereby promoting an open loop structure within the ASL.[18] The open loop structure of the ASL is a prerequisite for anticodon-codon binding within the ribosomal complex.[110] $\text{ASL}^{\text{Val}}{}_{\text{UAC}}$ lacking both the $\text{cmo}^5\text{U}_{34}$ and m^6A modifications does not appreciably bind the GUU, GUC, or GUG codons within the 30S ribosomal subunit.[111]

While G is able to base pair with both C as well as U, the interaction with the latter is thermodynamically less stable. As such, tRNAs coding for aspartate, asparagine, histidine, and tyrosine commonly contain 7-(((4,5-cis-dihydroxy-2-cyclopenten-1-yl)amino)methyl)-7-deazaguanine, queuosine or Q (Fig. 3A), at the wobble position, which enables recognition of codons ending in either C or U without favoring one or the other.[112] This is true even for the more simplistic and somewhat less commonly modified mitochondrial tRNAs, implying an evolutionary necessity for enhanced codon recognition that Q provides.[113] Q is synthesized by bacteria, and eukaryotes acquire Q either as a nutrient or from the intestinal flora as quenine, the free base form of Q, which is then directly inserted into the pertinent tRNAs through substitution of the base quenine.[114] Interestingly, the *E. coli* tRNA^{Tyr} contains pseudouridine at position 35, the second position of the anticodon and adjacent to Q, $\text{ms}^2\text{i}^6\text{A}$ at position 37, and Ψ at position 39. Loss of Ψ at the rarely modified position 35 compromises translational fidelity *in vitro*, implying that some ASLs need even more than three modified nucleosides for accurate function.[115] Like many other large modifications, Q causes a steric effect in the ASL, influencing the structure of the loop. The effect is largely mediated by hydrogen bonding within the ASL, which has been shown to maintain the structure of the ASL *in silico*, thus pre-structuring the ASL for codon recognition in the ribosome. The electrostatic potential of Q compared with G is important. Due to the intramolecular hydrogen bond between O6 and the quaternary amine of the aminomethyl side chain, the electronegativity of O6 in Q is reduced compared with O6 in G. In G, O6 acts as a hydrogen bond acceptor, however, this is not the case for Q. The two amines act as hydrogen bond donors in both species. The side-chain of Q can also engage in hydrogen bonding with the backbone of U_{33}, which in turn enhances the U_{33}○C_{36} interaction, stabilizing the ASL conformation. This same effect is absent or reduced if Q is replaced with G.[112] The pre-structuring is further enhanced by

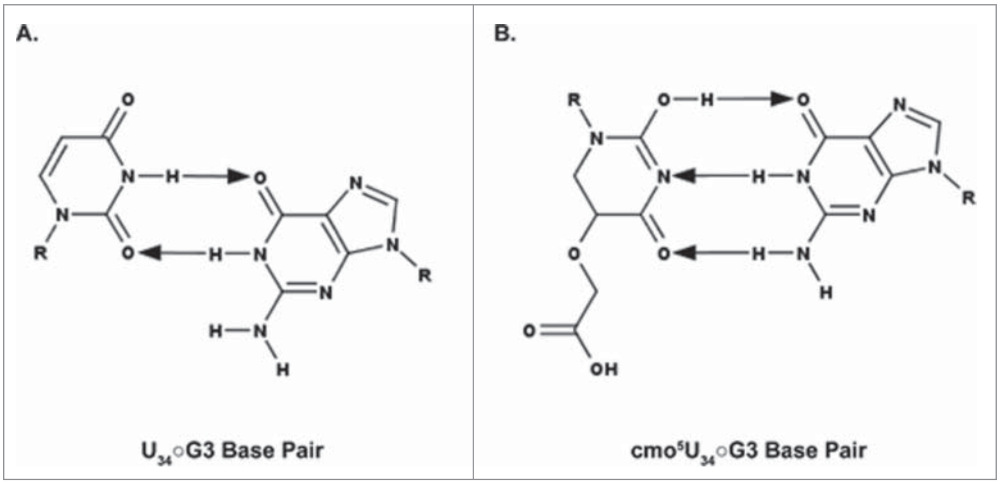


Figure 6. Watson-Crick geometry of the $\text{cmo}^5\text{U}_{34}$○G3 base pair. A. The predicted geometry of the unmodified U_{34}○G3 base pair containing two hydrogen bonds. B. The observed geometry of $\text{cmo}^5\text{U}_{34}$○G3 base pair containing three hydrogen bonds. For the $\text{cmo}^5\text{U}_{34}$○G3 to resemble a C•G base pair, the cmo^5 modification is proposed to facilitate formation of the enol tautomer. The arrows point away from the hydrogen bond donor and toward the hydrogen bond acceptor.

the large modification wyosine, (3,4-dihydro-4,6-dimethyl-3-β-D-ribofuranosyl-9*H*-imidazo[1,2-α]purin-9-one), imG, also derived from G and found at position 37 along with other derivatives often paramount to tRNA structure and function.[112]

The high degree of hydrophobicity of wyosine (Fig. 3A) and its derivatives enhances the nucleoside's ability to form hydrophobic interactions such as base stacking, which enhances codon binding stability. The bulky residue prevents the intraloop hydrogen bond between residues 32 and 37, thus promoting proper pre-structuring of the ASL.[116,117] In mitochondrial tRNAs, imG_{37} or its derivatives are commonly replaced by i^6A_{37} or a derivative thereof, or by a methylated purine, therefore suggesting the evolution of an alternative strategy for establishing the same functional properties by using different chemical moieties in RNA modifications.[113,116]

The nearly universal modification at position 37 that is required for decoding codons beginning with A, *N*6-threonylcarbamoyladenosine, t^6A_{37}, may be further modified to 2-methylthio-*N*6-threonylcarbamoyladenosine, $\text{ms}^2\text{t}^6\text{A}_{37}$.[118] Interestingly, a cyclic derivative of t^6A, ct^6A, has also been shown to exist, functioning in a manner analogous to the more studied t^6A.[118,119] Hydrogen bonding between N1 and N11 of t^6A lead to a pseudocyclic conformation that enhances the rigidity of the conformation.[120] The same hydrogen bonding exists in ct^6A, but a further isomerization occurs as well, where C10 and C13 are linked via an ether, thus forming a oxazolidine.[118-120] The C14 alcohol of ct^6A can hydrogen bond with the N7 of the first codon, an A, increasing the stabilization that enhanced stacking alone provides.[118,120,121]

Restriction of codon recognition and pre-structuring of the ASL

RNAs are highly flexible molecules with each base containing several bonds about which free rotation can take place. The flexibility and the possibility of not only Watson-Crick base pairing but also wobble or Hoogsteen interactions provide suitable conditions for multiple structures to form. Certain modifications restrict intraloop base pairing interactions, thus conforming the architecture to one suitable for ribosomal decoding. Restriction of codon recognition may become necessary in cases where an unmodified nucleoside could base pair with a near-cognate codon, therefore causing misreading of the codon. U, for instance, can base pair with all of the 4 major nucleosides with a strong preference to purines.[18] As such, modification can provide an enhanced specificity. As an example, isoleucine shares a codon box with methionine where AUA, AUC, and AUU code for isoleucine, and AUG codes for methionine, with the exception of mitochondria where AUA also codes for methionine.[6,113,122] Two codons can be read by $\text{tRNA}^{\text{Ile}}{}_{\text{GAU}}$, but the AUA codon requires a different isoacceptor that would also not read AUG as isoleucine. Eukaryotes commonly have a $\text{tRNA}^{\text{Ile}}{}_{\text{IAU}}$ which can read all three codons, but not AUG.[122] The genomes of bacteria and some eukaryotic organelles encode a $\text{tRNA}^{\text{Ile}}{}_{\text{GAU}}$ and $\text{tRNA}^{\text{Ile}}{}_{\text{LAU}}$ where L is lysidine (k^2C).[123] Modification of C to k^2C (Fig. 3B) effectively changes the base pairing capabilities of the nucleoside, causing a switch in preference from G to A for tRNA^{Ile}. The modification is required to prevent the recognition of the near-cognate AUG codon (Fig. 7).[7] Lysidine is a modified cytidine containing a lysine residue in lieu of O2 at the wobble position. The modification lysidine is the recognition determinant for the amino acid specificity in isoleucyl-tRNA synthetase (IleRS) aminoacylation of $\text{tRNA}^{\text{Ile}}{}_{\text{LAU}}$ and not $\text{tRNA}^{\text{Met}}{}_{\text{CAU}}$. An intriguing minor $\text{tRNA}^{\text{Ile}}{}_{\Psi\text{A}\Psi}$ in yeast contains pseudouridine at position 34 instead of lysidine,[121] and many archeal species utilize agmatidine [N-(4-carbamimidamidobutyl)-4-imino-1-(b-D-ribofuranosyl)-1,4-dihydro-2-pyrimidinamine] (Fig. 3B) for decoding of the AUA isoleucine codon. Thus, there have been multiple and convergent evolutionary paths to accomplish specificity in decoding through restriction of codon recognition with the help of modified nucleosides.[122]

Tautomerism: A chemical mechanism by which modified nucleosides restrict codon recognition

Tautomers and rare ionic forms of nucleosides have been shown to exist at low populations *in vitro*;[124] it is commonly postulated that the existence of tautomers of modified nucleosides at the ribosome's decoding site has a strong effect on codon recognition, either preventing or enabling it.[28] The nature of the C5 modification affects the tautomerism of the uridine and 2-thio modified uridine[125] producing either an expanded codon recognition, or one which is restricted. Restriction of codon recognition is a common mechanism for fidelity when a tRNA anticodon can mistakably base pair with a near-cognate codon in a shared codon box. In contrast, expansion of codon recognition by tRNA is often required when an interaction of an unmodified nucleoside would either lack the base pairing ability, thermodynamic stability, or the required structural orientation to achieve accuracy and efficiency of translation.[18]

Both lysidine and agmatidine can exist in several tautomeric forms. The exact tautomeric form through which cognate codon recognition can occur still needs to be elucidated.[122] However, it is a tautomer of lysidine that disallows recognition of the near-cognate AUG codon. The modification contains a secondary amine in place of oxygen at the two position of C, thus switching a hydrogen bond acceptor into a donor. The two tautomers (Fig. 7B) have different base pairing capabilities due to distinct local electron densities. Interestingly, neither tautomer is ideal for base pairing with G, but the non-aromatic tautomer is capable of recognizing A; the primary amine at position 4 on the ring is switched into an imine, thereby enabling it to function as a hydrogen bond acceptor. Similarly, N3 of the base becomes a hydrogen bond donor, thus enabling base pairing with A (Fig. 7B).[126] Agmatidine forms a similar tautomer as lysidine.[122,126] The hydrogen bonding capability of modified nucleosides commonly found at the wobble position of tRNA^{Ile} illustrates how small changes in the chemistry of a single nucleoside can have broad and highly specific effects, in this case altering codon recognition and specificity while simultaneously preserving aaRS recognition.[121]

Tautomers of modified nucleosides expand reading of synonymous codons

U•A base pairs are thermodynamically much weaker than C•G base pairs. In tRNAs such as $\text{tRNA}^{\text{Lys}}{}_{\text{UUU}}$ this problem is

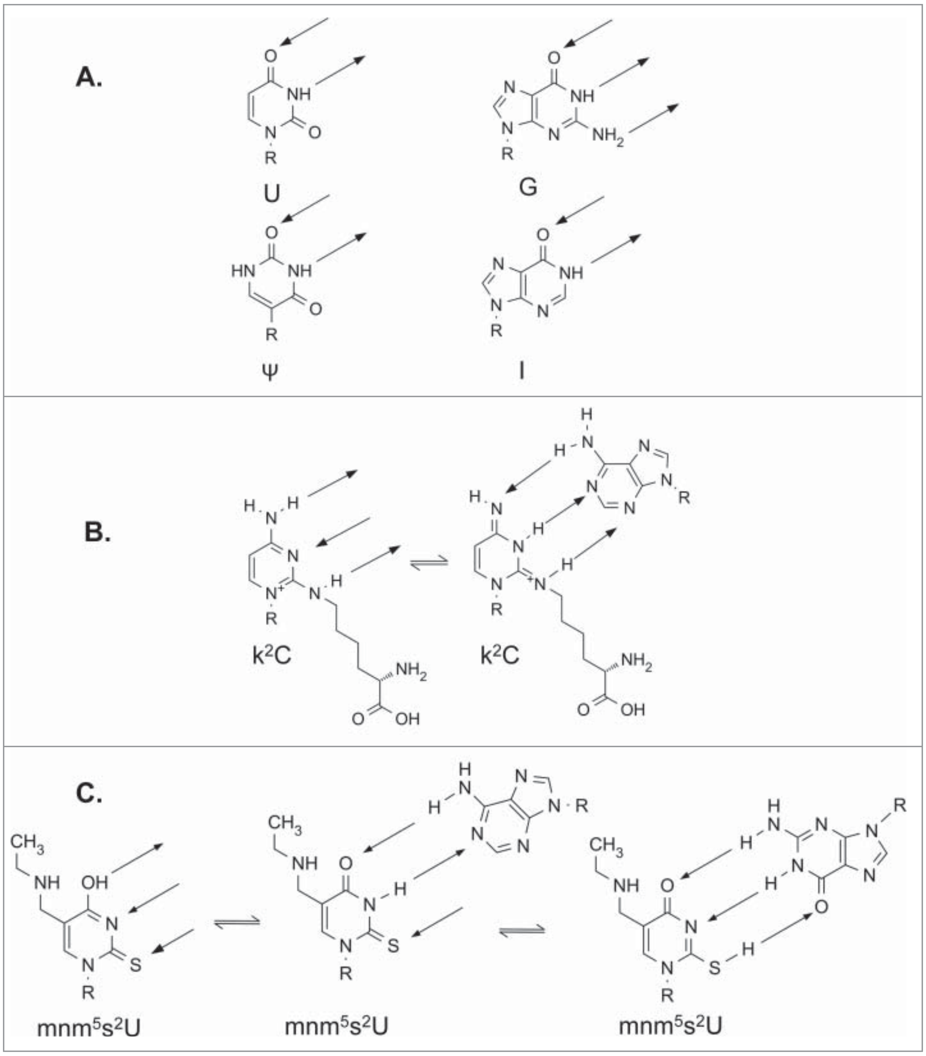


Figure 7. Modifications influence protein and codon recognition of tRNA through altered hydrogen bonding. R represents the ribose sugar. Hydrogen bonding is indicated by arrows. A. Nucleosides commonly found at position 34 of tRNA^{Ile}. All 4 bases are capable of recognition by isoleucyl-tRNA synthetase, IleRS. B. Two possible tautomers of lysidine. Structure on the right can be recognized by IleRS, and could putatively recognize A as shown. C. Possible tautomers of mnm^{5}s^{2}U$_{34}$ of *E. coli* tRNA$^{Lys}{}_{UUU}$. Likely interactions with A and G are shown.

exacerbated, as all anticodon to codon base pairs are U•A pairs. Furthermore, in the absence of modifications, the U-rich ASL cannot effectively form stacking interactions to stabilize its structure nor negate intraloop hydrogen bonding that effectively condenses the size of the ASL.[117] The nearly ubiquitously modified, invariable purine at position 37 can alleviate the thermodynamic penalty of a pyrimidine rich anticodon. In tRNA$^{Lys}{}_{UUU}$ the purine at position 37 is modified to *N*^{6}-threonylcarbamoyladenosine, t^{6}A or a derivative thereof such as 2-methylthio-*N*^{6}-threonylcarbamoyladenosine, ms^{2}t^{6}A$_{37}$ found in mammalian tRNA$^{Lys3}{}_{UUU}$. Additionally, Ψ at position 39 is important for function.[127] The wobble position is extensively modified with an xm^{5}s^{2}U type of modification, illustrating the importance of having three modified nucleosides in the anticodon loop for at least this tRNA.[28,128]

Lysine is coded by AAA and AAG codons. In many organisms, as well as in organelles, a single tRNA$^{Lys}{}_{UUU}$ reads both codons. In mammals, two isoacceptors, tRNA$^{Lys1,2}{}_{CUU}$, decode the AAG codon, and a third, tRNA$^{Lys}{}_{UUU}$, translates both codons. The wobble xm^{5}s^{2}U modification is required for the efficient and specific recognition of both lysine codons, and for translocation on the ribosome.[129] The xm^{5}s^{2}U$_{34}$ modifications, such as bacterial tRNA modification mnm^{5}s^{2}U$_{34}$, can undergo keto-enol tautomerism (Fig. 7C), which allows recognition of both AAA and AAG by expanding the hydrogen bonding capabilities of the nucleoside. Similarly, expanded codon recognition could be achieved via ionization of the nucleoside. In the case of mnm^{5}s^{2}U, the secondary amine in the sidechain could be positively charged and the thio-group negatively charged. This zwitterion would have the same hydrogen bonding qualities as the tautomer (Fig. 7C).[130]

Group (VI) elements at the 2 position of U – the influence of atom size

When U is found at tRNA's position 34 it is nearly always modified. Modifications of U at the wobble position include 2-thiouridine (s^{2}U) and 2-selenouridine (se^{2}U) (Fig. 8)[131] that affect both ribose sugar pucker as well as the glycosidic χ angle resulting in a restrictive reading of codons. Both sulfur as well as selenium have similar chemical properties to the carbonyl oxygen at the

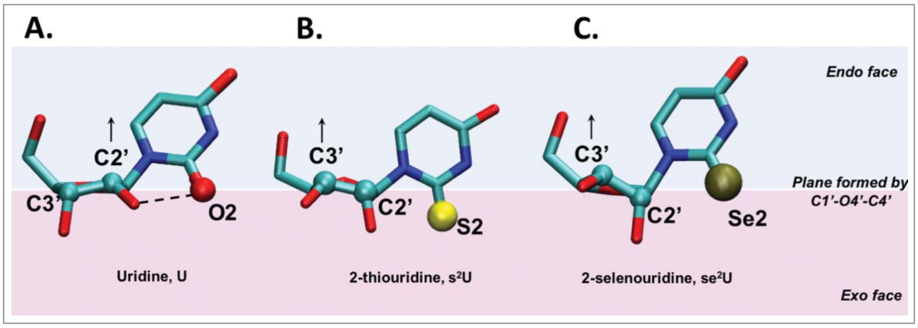


Figure 8. Sulfur and selenium atomic radii affect sugar pucker when modifying the C2 position of uridine. A. Uridine, B. 2-thiouridine and C. 2-selenouridine nucleosides are shown. All 3 nucleosides are depicted in their *anti* conformation. The plane defined by atoms C1′-O4′-C4′ of the sugar separates the *endo* face (the side in which C5′ projects from the plane) from the *exo* face. In the case of uridine, the sugar pucker is C2′ *endo* as indicated by atom C2′ in the *endo* face (above the C1′-O4′-C4′ plane), while in 2-thiouridine and 2-selenouridine, the relative increase in atomic radius of substituent atom X at position C2 and the weakening of the hydrogen bonding interaction between 2′OH and X (where X – O2, S^2 or Se^2) shifts the equilibrium toward a C3′ *endo* conformation.

C2 position of U. However, the larger size of sulfur and selenium elicit different steric properties on their environment, being approximately 40% and 100% larger than oxygen, respectively (Fig. 8). Also the electronegativity of the substituent atoms (sulfur and selenium) is smaller than that of oxygen. As would be expected, the modified U exhibits a dominant *anti* conformation. The larger atomic radii of these modifications also affect the nucleoside's sugar pucker particularly when the glycosidic bond angle χ is in the *anti* conformation. While the ribose of unmodified uridines populate equally the C3′ and C2′ *endo* conformations, the ribose sugar pucker of s^{2}U and se^{2}U exist preferentially in the C3′ *endo* conformation only occasionally switching to the C2′ *endo* pucker. The large size difference in the Van der Waal radius of the group IV elements bonded to C2 of U forces the sugar to adopt the C'3 *endo* conformation; a conformation that has been principally associated with increased stability through enhanced stacking.[28] In the case of the unmodified uridine, the carbonyl oxygen at C2 can form a hydrogen bond with the 2′OH group on the ribose thus stabilizing the C2′ *endo* conformation of the ribose (Fig. 8A). The less electronegative S or Se have a reduced propensity for such hydrogen bond interactions, and combined with the steric effects introduced by the larger size of the atoms, these modifications push the sugar pucker distribution toward C3′*endo* (Fig. 8B and 8C). Thus, the conformation of uridines at wobble position 34 and modified with s^2 and se^2 is *anti*, C3′ *endo*, and the base pairing is preferentially to A, rather than wobbling to G.

Discussion

We and others have extolled the virtues of modifications at wobble position 34 and the invariant purine 3′-adjacent to the anticodon at position 37. Wobble base pairing does not appear to occur at positions other than tRNA position 34 with rare exception such as arthropod mitochondrial translation of the codon AGG as lysine instead of serine (invertebrates) or arginine (standard Universal Genetic Code) in which there is position 35 wobble base pairing, U_{35}○G2.[132] Modifications at tRNA's nucleosides 34 and 37 frame the anticodon and prestructure the ASL for decoding. In doing so, they reduce energy barriers to conformational change required for ribosomal A-site binding, maintain the translational reading frame and either expand or restrict cognate and wobble codon recognition. In *S cerevisiae*, loss of either the xm^5 or s^2 modifications of mcm^{5}s^{2}U$_{34}$ increases observation of +1 translational frameshifts,[133] probably caused by a decrease in the affinity of the tRNA for the ribosomal A-site and an impaired translocation.[9,129] The nearly ubiquitous t^{6}A$_{37}$ modification at position 37 in tRNAs responding to codons beginning with A, ANN, enhances base stacking of U$_{36}$ with A1 of the codon, as well as prevents intraloop base pairing within the ASL.[115] Thereby, t^{6}A$_{37}$ facilitates the U-turn and presentation of the anticodon to the codon on the ribosome. t^{6}A$_{37}$ also facilitates ribosomal codon binding and maintains the translational frame.[26,126] Other modifications at position 37, particularly in tRNAs responding to codons beginning with U, UNN, such as wyosine, contribute similarly to base stacking, maintaining the open loop structure and by doing so maintain the translational reading frame.[26,126] The fact that the ASL can be stabilized by various purine 37 modifications implies convergent evolutionary pathways that relate not only to the identity of the codon, but also to that of the nucleoside at position 34 and perhaps additionally either 32 or 38/39.

Yet, there appears to be a need for some tRNAs to further modify the anticodon loop for acceptance on the ribosome and codon recognition. For instance, the nucleoside N3-methylcytidine (m^{3}C) was first found in tRNA over 50 years ago[134] and appears to be located exclusively at position 32 of the anticodon loop in specific eukaryotic tRNAs.[20] In addition, 2-thiocytidine is found at position 32 of specific prokaryotic tRNAs. The s^{2}C$_{32}$ modification is present in *E. coli* and *Salmonella enterica* tRNA$^{Arg}_{ICG}$ which decodes CGU/C and CGA in the absence of s^{2}C$_{32}$, tRNA$^{Arg}_{CCG}$ that decodes CGG, tRNA^{Arg} with the modified anticodon mnm^{5}UCU that decodes AGA/G, and tRNA$^{Ser}_{GCU}$ decoding AGC/U.[19,39] The presence of s^{2}C$_{32}$ negates I$_{34}$ wobbling to A3.[39] It may affect translational efficiency of rare codons that are intrinsically inefficient in decoding.[19,135] The s^{2}C$_{32}$ modification has also been found in archaeal tRNA.[136] Pseudouridine, Ψ, although present at the anticodon wobble position 34, is another modification found predominantly at positions 32, 38 and 39.[137] Pseudouridine in the anticodon loop apparently increases thermal stability but does not affect ribosome mediated codon binding, nor is its N1 position used for hydrogen bonding.[127]

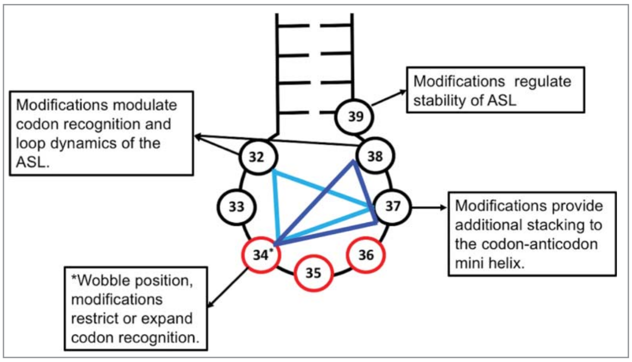


Figure 9. ASL modification triangle. A combination of 3 positions in the ASL (nucleotides 32 through 38) of tRNAs are commonly modified to expand or restrict codon recognition. Position 32, 34 and 37 (light blue) or position 34, 37 and 38 (dark blue) form vertices of the triangle in which modifications work in a co-surgical manner to achieve desired ribosomal binding affinity of the tRNA to different codons. The anticodon is represented in red and the nucleosides in the ASL in black.

Multiple modifications within the ASL may provide a redundancy to the structure and conformational dynamics not otherwise achievable by 40+ individual tRNAs meeting ribosome acceptance and unique codon recognition.[43] Modification of tRNAs anticodon loop positions 32, 38 or 39, along with positions 34 and 37, in effect complete a triangle of modified nucleosides (Fig. 9). It is interesting that the three point presentation of ASL modifications has modified nucleosides spaced almost equally around the anticodon loop at approximately every other position 32, 34, and 37, or 34, 37 and 39. Looking back at $\text{tRNA}^{\text{Gly}}_{\text{NCC}}$, the strong anticodon-codon, C•G interactions require no modifications, whereas $\text{tRNA}^{\text{Lys}}_{\text{UUU}}$, $\text{tRNA}^{\text{Tyr}}_{\text{NUA}}$, and $\text{tRNA}^{\text{Ile}}_{\text{NAU}}$ have at least three functionally and/or structurally essential modified nucleosides in the ASL. Weak interactions with the codon, such as multiple U•A base pairs, may require far more extensive modifications, where chemically diverse modifications affect similar biologic properties, often through structure. The ribosome bound structures of the tRNA ASLs containing post-transcriptional modifications offer molecular insights into their role in modulating structure, stability and interactions of the tRNA. While the uridine at wobble position 34 is the most heavily modified, positions 32, 37 and 38 also show significant levels of modifications. We have gathered the structures of five tRNAs bound to the ribosome at the A-site, and highlighted the interactions of their modifications (Fig. 10). It is interesting to note that U_{34} is primarily modified at C5 adjacent to the base-pairing face and hence the modifications do not interfere with its hydrogen bonding to the codon except in cases where the modifications introduce tautomerism. However, the modifications are either polar (as is true in case of tRNA^{Leu}[138] and tRNA^{Val}[11]) or charged, (tRNA^{Lys}),[44] and therefore participate in hydrogen bonding with neighboring bases or charge-charge interactions with the backbone phosphate group. Modifications on the position 37 purine nucleobase are primarily involved in stacking with the base at position 36 and the base to which it is paired in codon recognition on the ribosome. These modifications are either methylations (tRNA^{Val}) or groups with complex chemistries,

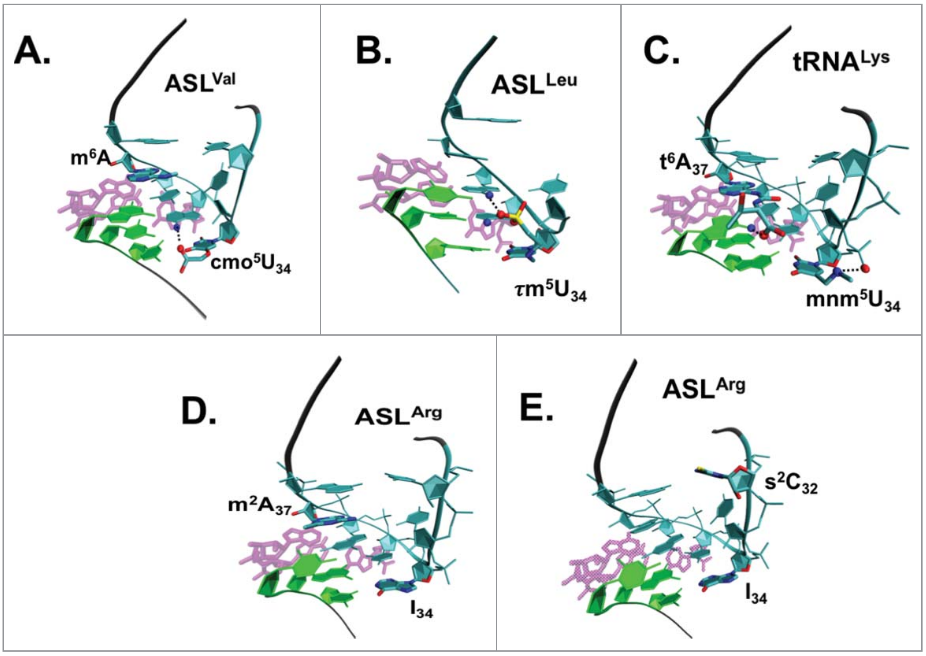


Figure 10. Ribosome-bound structures of the ASLs of 5 tRNA species with modifications. tRNA modified nucleosides at positions 32, 34 and 37 are influential in creating the architecture of the anticodon domain accepted into the ribosome's A-site for cognate and wobble codon binding. The codon on the mRNA is shown in green, the ASL in cyan with the modifications labeled and the rRNA interactions shown in magenta. Possible interactions of the modified groups are represented using a dashed line. A. In $\text{ASL}^{\text{Val}}_{\text{UAC}}$ bound to codon GUU, (PDB ID: 2UUB), the carboxyl oxygen of $\text{cmo}^5\text{U}_{34}$ is within hydrogen bonding distance of the amine group on the neighboring A_{35}. B. In $\text{ASL}^{\text{Leu}}_{\text{UAA}}$ bound to codon UUG (PDB ID: 2VQF), two of the three oxygens bonded to the sulfur atom in the $\tau\text{m}^5\text{U}_{34}$ can form hydrogen bonding interactions with A_{35} and A_{36}. C. In $\text{ASL}^{\text{Lys}}_{\text{UUU}}$ bound to codon AAA (PDB ID: 1XMQ), there are a salt-bridge between the 5-methylaminomethyl group on U_{34} and its phosphate group, an enhanced stacking interaction provided by t^6A_{37}, and a possible hydrogen bond between the threonyl group of t^6A_{37} and A1 on the mRNA. D. $\text{ASL}^{\text{Arg2}}_{\text{ICG}}$ bound to the codon CGC. The ASL has the two modifications I_{34}, m^2A_{37}. E. $\text{ASL}^{\text{Arg1}}_{\text{ICG}}$ bound to the codon CGC. The ASL has the modifications s^2C_{32}, I_{34}. When either of the modifications m^2A_{37} or s^2C_{32} are present, then the tRNA is unable to recognize the rare CGA codon.[39]

like t^6A_{37}, which significantly increases its stacking propensity. Enhanced base stacking of position 37 modified purines provides additional stability to the U-turn structure, and increases the binding strength of the ASL when U_{34} pairs with a near-cognate or non-cognate codon.

The cross-loop interaction between the nucleosides at position 32 and 38 at the beginning of the ASL loop (Fig. 4B) is characterized by a single or bifurcated hydrogen bond.[139] The strength of this interaction appears to be carefully modulated. For instance, pseudouridine at position 32 uses a water mediated base-backbone interaction to stabilize the interaction between nucleosides 32 and 38.[138] While a stronger interaction, like that of a canonical base pair at this position could lead to a loss in flexibility of the loop domain, a weaker interaction could result in loss of stacking in the ASL resulting in a disruption of the functional U-turn conformation of the ASL. Modifications at the 32nd and 38th positions of the ASL may be important as a possible means of modulating the interaction between the two nucleosides and base stacking in the loop.

We hypothesize that the combined chemistries and conformational dynamics of modified nucleosides located at three positions, positions 34, 37 and one other within the ASL loop (Fig. 9) transform the loop architecture and dynamics to that consistent with the constraints that the ribosome places on all tRNAs. It is important that a stable, yet adaptable, U-turn is maintained for presentation of the anticodon resulting in accurate and efficient codon recognition. Multiple modifications mold the anticodon loop architecture of specific tRNAs into thermally stable, malleable triangles of strength recognizable by mRNA-programmed ribosomes (Fig. 9). In order for the architecture of the anticodon's loop to change, an edge of the triangle must collapse, as in an alteration of the 5′-side and U-turn. This is apparent in the three different anticodon loop conformers of $tRNA^{Arg1,2}$ that are recognized by argininyl-tRNA synthetase with a disrupted U-turn, a solution structure that does not exhibit a U-turn and a conformation on the ribosome in the A-site that has the canonical U-turn.[39]

Conjecture about the evolution of site- and chemically specific modifications within tRNA's ASL domain relative to their functions can yield insights into their future, investigator-designed applications. RNA polymerases, with few exceptions, *in vivo* and *in vitro*, do not accept modified nucleoside triphosphates as substrates for transcription, and if they did the modification would be randomly placed throughout the transcript in response to the template. Therefore, it is feasible to hypothesize that the evolution of post-transcriptionally modified nucleosides occurred after the appearance of proteins that could catalyze their limited existence at specific sites to which each modification would contribute chemistry and conformation to that tRNA's function in translation. In acknowledging today's understanding of the manner in which anticodon domain modifications both expand and restrict recognition of cognate and wobble codons, but not near-cognate codons, it is difficult to comprehend life in which new amino acids would be introduced with limited numbers of tRNAs and necessitating 2-fold degenerate codons. For instance, without the restrictions imposed by modifications, one could conceive of asparagine and lysine, aspartic and glutamic acids, and perhaps arginine and serine being mistakenly incorporated. More likely, tRNAs with unmodified anticodon domains much like the very limited number of 22 tRNAs transcribed in mammalian mitochondria decoded 4-fold degenerate codon boxes without error, but perhaps at reduced efficiencies. In contrast, cytoplasmic and mitochondrial translation of codons from 2-fold degenerate codon boxes (Fig. 1) preceded in the absence of modification with significantly reduced translational fidelity and lack of efficiency until restrictive modifications had evolved. If tRNA anticodon domain modifications facilitated the accurate and efficient entry of new amino acids through split codon boxes in the evolution of proteins as we know them, then why couldn't the emergence of still other amino acid incorporations continue by re-appropriating sense codons?[140,141] The use of 4-fold degenerate sense codons to incorporate new amino acids would require the splitting of 4-fold degenerate codons. Perhaps, this could be accomplished by removal of cytoplasmic tRNAs from redundant codon recognition (which does not occur in the mitochondria), restructuring the modification enzyme recognition of specific tRNA species and aminoacyl-tRNA synthetase recognition of tRNA and the non-natural amino acid substrates. The introduction of novel amino acids into proteins portends applications to biomaterial science and medicine. New investigator-designed, protein-based materials and potential therapeutics would become available through biomanufacturing.

In conclusion, The Wobble Hypothesis is being revised continually for as we learn more every year about modified

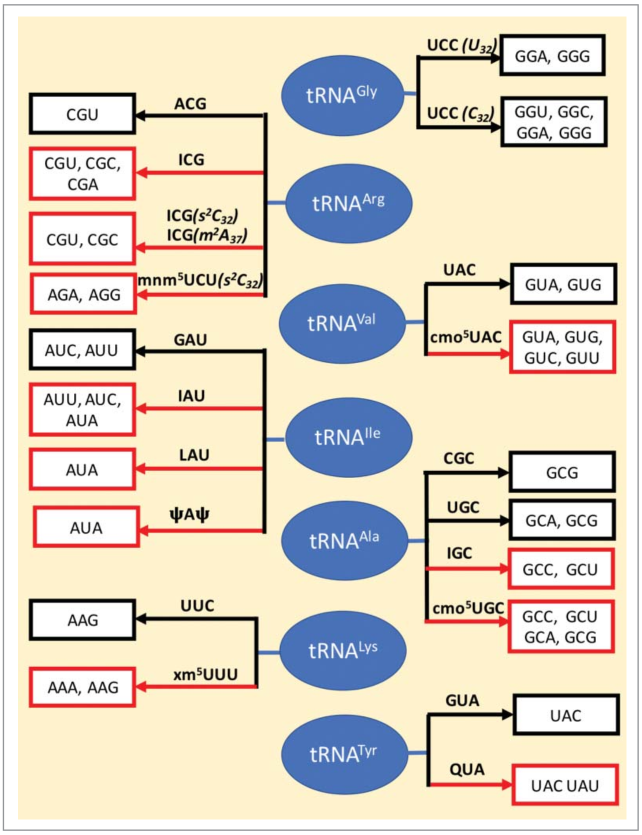


Figure 11. Wobble map. Anticodon domain modified nucleosides play an important role in the wobbling of tRNA's codon recognition, some expanding codon recognition to synonymous codons while others restrict recognition. The different cases of wobbling in the presence and absence of modifications as discussed in this paper, are shown. The black arrow represents no modifications, whereas a red arrow indicates the presence of one or more modifications. The tRNAs are shown in blue, the anticodons are indicated on the arrow and codons decoded by the tRNA bearing the anticodon are enclosed in white boxes.

nucleoside chemistry, structure and function and how it reflects on RNA chemistry, structure and function, we understand the subtleties of translation being able to manipulate and apply them. We have discussed the different instances in which wobble base pairs are used to expand/restrict codon recognition by tRNA's anticodon with modified and unmodified wobble position 34, and the importance of modified nucleoside positions outside of anticodon, 32, 37 and 38 (Fig. 11). Wobble and non-canonical base pairs in the ASL domain of tRNA are integral to the translation of the genetic code. They are modulated either by unique structural and dynamic features introduced in a sequence dependent manner by unmodified bases (e.g., $tRNA^{Gly}$ with C_{32}/U_{32}) or by nucleoside modifications that can expand or restrict decoding capacity of tRNAs by stabilizing or disrupting native interactions at the decoding center of the ribosome. An in-depth understanding of the origins and mechanisms of the variety of ways by which wobbling is achieved will equip us with the tools to tap into this often overlooked potential of using modified nucleoside contributions to translation for therapeutics and other applications.

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

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