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## SURVEY AND SUMMARY

# Decoding the genome: a modified view

**Paul F. Agris***

Department of Molecular and Structural Biochemistry, 128 Polk Hall, Campus Box 7622, North Carolina State University, Raleigh, NC 27695-7622, USA

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## ABSTRACT

**Transfer RNA's role in decoding the genome is critical to the accuracy and efficiency of protein synthesis. Though modified nucleosides were identified in RNA 50 years ago, only recently has their importance to tRNA's ability to decode cognate and wobble codons become apparent. RNA modifications are ubiquitous. To date, some 100 different posttranslational modifications have been identified. Modifications of tRNA are the most extensively investigated; however, many other RNAs have modified nucleosides. The modifications that occur at the first, or wobble position, of tRNA's anticodon and those 3′-adjacent to the anticodon are of particular interest. The tRNAs most affected by individual and combinations of modifications respond to codons in mixed codon boxes where distinction of the third codon base is important for discriminating between the correct cognate or wobble codons and the incorrect near-cognate codons (e.g. AAA/G for lysine versus AAU/C asparagine). In contrast, other modifications expand wobble codon recognition, such as U·U base pairing, for tRNAs that respond to multiple codons of a 4-fold degenerate codon box (e.g. GUU/A/C/G for valine). Whether restricting codon recognition, expanding wobble, enabling translocation, or maintaining the messenger RNA, reading frame modifications appear to reduce anticodon loop dynamics to that accepted by the ribosome. Therefore, we suggest that anticodon stem and loop domain nucleoside modifications allow a limited number of tRNAs to accurately and efficiently decode the 61 amino acid codons by selectively restricting some anticodon–codon interactions and expanding others.**

## INTRODUCTION

Very often, our understanding of biology is derived from a testable hypothesis and the experimental results that follow. So it was in understanding how genetic information encoded in DNA was transformed by the cell into a unique sequence of a protein's amino acids. With the first mention of the adaptor molecule hypothesis came a search for and the testing of sRNA's (tRNA's) ability to translate genetic information (1). A set of coding triplets or three letter codes composed of the nucleosides adenosine (A), guanosine (G), cytidine (C) and uridine (U) was envisioned to account for the 20 amino acids (2). The three letter code was supported by experiments *in vivo* including some of the earliest applications of translational frameshifting (3). *In vitro*, specific coding triplets were associated with individual amino acids through ribosome-mediated aminoacyl-tRNA binding to either homopolymers, enzymatically synthesized heterotriplets (4,5) or chemically synthesized polynucleotides (6–9). The deciphering of the triplets resulted in what is now the historical presentation of the Genetic Code (Fig. 1). The first two letters of the code, A, G, C or U, create 16 possible combinations, each of which is displayed in a separate 'codon box'. Each codon box is composed of four, three letter codes, 64 in all. Sixty-one codons are recognized by tRNAs for the incorporation of amino acids, and three codons signal the termination of protein synthesis. Eight of the codon boxes each code for only a single amino acid and therefore, are 4-fold degenerate (Fig. 1). The remaining 12 amino acids have codons in 2-fold degenerate codon boxes (e.g. asparagine and lysine, or tyrosine and stop), are 3-fold degenerate (isoleucine), or have only one codon (e.g. methionine and tryptophan).

The evident degeneracy of the genetic code required that amino acid specific tRNAs respond to multiple coding triplets that differed only in the third letter and gave rise to the 'Wobble Hypothesis' (10) and a considerable number of questions, not the least of which has to do with the importance of posttranscriptional modification to the decoding process. This review focuses on the functional contributions of tRNA's modifications to the decoding of genomic information. In particular, decoding that requires modification of tRNA's anticodon stem and loop domain (ASL) for accurate and effective protein elongation will be emphasized. With this information, the codes (Fig. 1) are presented in a mechanistic light for their being productively recognized by tRNA for protein synthesis.

The establishment of the Genetic Code (11), the first sequencing of a tRNA, yeast $tRNA^{Ala}$ (12,13), the presence of a modified nucleoside, inosine ($I_{34}$), at what was thought to be the first position of the anticodon, position 34 (14) (Fig. 1), and Francis Crick's Wobble Hypothesis (10) and its confirmation (15) focused attention on the detailed principles of

*Tel: +1 919 515 6188; Fax: +1 919 515 2047; Email: Paul_Agris@ncsu.edu

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

**Figure 1.** The Genetic Code. The 61 codons for the 20 amino acids and three codons for the translational stop signals are shown in the historical coding chart. Codon boxes with white backgrounds contain four codes for one amino acid and are therefore 4-fold degenerate. Codon boxes with shaded backgrounds contain codons for more than one amino acid, or an amino acid plus stop codons and therefore are 2-fold and 3-fold degenerate codons.

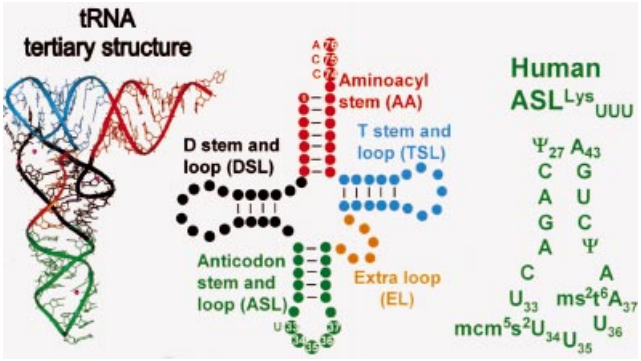


**Figure 2.** The structure and domains of tRNA. The three-dimensional structure of tRNA is represented on the left by the crystallographic structure of yeast $tRNA^{Phe}$ (32). The cloverleaf secondary structure in the center is color coded to identify the structural domains of the crystal structure: amino acid accepting stem, or aminoacyl-stem (AA) is in red; dihydrouridine stem and loop domain (DSL) in black; anticodon stem and loop domain (ASL) in green; extra loop (EL) in gold; and the ribothymidine, or T$\Psi$C, stem and loop (TSL) in light blue. The positions of the invariant U33 and the amino acid accepting 3′-terminus (C74, C75 and A76) are shown. The sequence and secondary structure of the ASL from human $tRNA^{Lys3}$ is shown on the right. The nucleosides are numbered according to the accepted protocol for tRNAs, with the anticodon nucleosides as 34, 35 and 36. The modified nucleosides in $ASL^{Lys3}$ are: pseudouridine, $\Psi_{27,39}$; 5-methoxycarbonylmethyl-2-thiouridine, $mcm^5s^2U_{34}$; and 2-methylthio-N6-threonylcarbamoyladenosine, $ms^2t^6A_{37}$.

anticodon–codon interaction. As early as the 1966 Cold Spring Harbor Symposium on Quantitative Biology, modifications within tRNA's anticodon stem and loop domain (ASL) were thought to play pivotal roles in codon recognition and binding, and/or in aminoacyl-tRNA synthetase recognition of cognate tRNA (16). However, $I_{34}$ was far from the first modified nucleoside identified in RNA. Probably, the ubiquitous pseudouridine, $\Psi$, has that distinction, being discovered in 1951 (17) and identified in 1959 (18,19). Over the next two decades, some 35 modified nucleosides were identified in RNAs (20). With improvements in the site-selected introduction of modified nucleosides through automated chemical synthesis (21), the Wobble Hypothesis could be tailored to include uridine modifications (22). Advances in analytical instrumentation and methodology (23) greatly accelerated the discovery and identification of modified nucleosides. In particular, tRNAs from hyperthermophile organisms were found to provide a rich source of new compounds and structures (24). Today the list of modified nucleosides identified in RNA has grown to about 100, and is still growing by several nucleotides a year.

In general, the chemical properties that modifications contribute to nucleosides are similar to those of the amino acid side chains, hydrophilic (polar and charged), or hydrophobic (aromatic or aliphatic) (25). Some of the modifications are as simple as methylations, whereas others involve multiple step additions of aromatic rings, amino acid derivatives and sugars. Modified nucleoside identification, RNA of origin, biosyntheses, organism differences and possible functions are catalogued in databases and a number of books (26–31). Some modified nucleosides that are so highly conserved in type and location in tRNA (dihydrouridine or D, ribothymidine or T, and pseudouridine or $\Psi$) have become part of the nomenclature associated with tRNA structure (Fig. 2). These and other modifications that are highly common in type and location in RNA sequences appear to be important to RNA folding (33,34). The availability of many whole genome sequences, and the assignment of many unidentified open reading frames or genetic markers to tRNA modifying enzymes (35) has given new impetus to the investigation of the enzymes and mechanisms of RNA modification (36). However, connecting modified nucleoside chemistry and structure to the decoding process has been challenging (25,31,37).

The term decoding, once limited to the actual event in translation, has been appropriated to genomics to identify the products of coding genes, and to systems biology for the monitoring of pathways and timing in responses to stimuli. Recoding has been used to refer to the natural changes in a specific mRNA such that genetic readout is a programmed translational mRNA frameshift, or a redefinition of codons (38,39), as well as the man-made manipulations to insert non-natural amino acids (40–43). Translational bypassing has been used to describe a ribosome's skipping over a number of mRNA nucleosides and resulting in the joining of two non-contiguous, open reading frames (44). Non-standard decoding continues to refer to an organism's or organelle's use of a code for a different amino acid than that traditionally ascribed to it, or for using a stop code for an amino acid insertion (45–50), other than suppression (51). In general, decoding in the cytoplasm is not altered from that established some 40 years ago. However, a number of yeasts of the genus *Candida* have a reassigned cytoplasmic decoding of the leucine codon CUG to serine (Fig. 1). The codon is read by a novel $tRNA^{Ser}$ in which the invariant $U_{33}$ (Fig. 2) is replaced by a $G_{33}$ (52,53). This $tRNA^{Ser}$ has the modified nucleoside N1-methylguanosine ($m^1G_{37}$), as do the leucine isoacceptors, and accepts leucine *in vitro* and *in vivo* indicating the only known ambiguous use of the codon CUG. tRNAs are edited by many eukaryotes and sometimes serve the purpose of expanding the ability to read codons. The first example of thiolation of the invariant $U_{33}$ was demonstrated in an edited mitochondrial and cytosolic

$tRNA^{Trp}$ (54). An understanding of why the universal 64 codes exist for the 20 amino acids and three stop codons has evolved for decades and many exceptions have proved the rule. In contrast, the evolution of thought on modified nucleoside function in decoding is still developing.

## SURVEY AND DISCUSSION

### The decoding process

The RNA and protein components of the ribosome are active participants in protein synthesis. The RNA of the large subunit catalyzes the peptidyl-transferase reaction (55). Decoding of mRNA on the small subunit is a multistep process involving the small subunit RNA and one or two ribosomal proteins. In protein elongation, decoding of mRNA codons begins with a ternary complex of aminoacyl-tRNA, elongation factor and GTP entering the ribosome's aminoacyl-, or A-site, to bind either cognate or wobble codons. A productive placement of tRNA in the A-site requires that the anticodon–codon interaction chronologically precedes formation of any significant interactions of the ternary complex with the ribosome. Results from cryo-EM of the ribosome place the entering aminoacyl-tRNA initially at a location different from its final position in the A-site where it is in preparation for the peptidyl-transferase reaction (56). The establishment of a correct anticodon–codon base pairing heralds the hydrolysis of GTP and release of elongation factor. Thus, it is the A-site at which the correct anticodon–codon interaction must be assessed and verified, and incorrect associations discarded. A 20-year-old hypothesis (57) envisioned that the decoding site ribosomal components recognized the architectural correctness of the anticodon–codon interaction. In fact, fidelity of codon recognition by tRNA's anticodon involves small ribosomal RNA dynamics (58,59). tRNA decoding of the cognate codon results in the small ribosomal subunit transitioning between open and closed forms (60). Crystal structures of the small 30S ribosome subunit characterize a dynamic in which correct codon recognition by tRNA results in a closure of small subunit domains around the decoding site, whereas in the large 50S subunit of the ribosome, elements involved in intersubunit contacts or in substrate binding are flexible, but overall there is a greater order to the crystal structure (61). Structures of *Escherichia coli* wild-type and hyper-accurate ribosomes at resolutions of 10 and 9 Å, respectively, indicated that mRNA decoding is coupled primarily to movement within the small subunit body (62). The functional contributions of tRNA's anticodon domain modifications to small subunit recognition of a stereochemically correct anticodon–codon interaction could be crucial to the accuracy and stability of the interactions, and the rate of protein synthesis.

*Decoding accuracy.* Ribosome mediated decoding is far more accurate than that expected from simple anticodon base pairing with codon (63). In addition, Watson–Crick and wobble codon binding by tRNA does not account energetically, structurally or mechanistically for the A-site selection of tRNAs by the ribosome. Thus, the ribosome contributes significantly to the selection of the correct cognate and wobble codon interactions and to the affinity and dissociation of codon with the correct anticodon (64,65). Several incorrect aminoacyl-tRNA ternary complexes may be tried and dissociated at the A-site until the correct one is found and a closed 30S subunit conformation is stabilized concluding A-site tRNA selection (60). Mechanistically, the anticodon domain of A-site tRNA interacts with ribosomal components, as well as with codon. Correct cognate and wobble codon–anticodon binding enables universally conserved, A-site, 16S rRNA nucleosides G530, A1492 and A1493 to stabilize anticodon–codon base pairing by forming hydrogen bonds with the tRNA anticodon domain backbone and with the mRNA backbone (66). tRNA's 2′-hydroxyl groups at anticodon positions 35 and 36 interact with the 16S rRNA nucleosides A1492 and A1493, respectively. A1492 and A1493 must flip out from the rRNA's helix 44 to be able to enter the minor groove of a correctly formed anticodon–codon mini-helix where hydrogen bonding to and stabilization of the first and second base pairs takes place. The glycosidic bond of G530 is *syn*, but rotates to *anti* to bond to and stabilize the anticodon–codon interaction at the third base pair. With near-cognate codon, anticodon–codon interaction deviates sufficiently from Watson–Crick geometry that 16S rRNA nucleosides folding into the anticodon–codon minor groove do not stabilize the interaction. Thus, the transition to a small subunit closed form is unfavorable for near-cognate, anticodon–codon pairs.

The 2′-hydroxyl groups of tRNA's universally conserved $U_{33}$, and of anticodon nucleosides 35 and 36 are important for tRNA's translocation from the ribosome's aminoacyl-site to the peptidyl-site, A- to P-site (67). Methylation of the 2′OH of wobble position 34 occurs in a number of tRNAs, for instance $Gm_{34}$ in yeast phenylalanine tRNA (25,68). However, modifications of the 2′OH of anticodon positions 35 and 36 have not been found in native tRNAs. Though 2′-O-methylation of a C at position 34 enhanced recognition of the cognate codon ending in G, chemically introduced 2′O-methyls at positions 35 and 36 significantly decreased the tRNA's decoding ability (69). Therefore, at the very minimum, the 2′OH of anticodon nucleosides 35 and 36 are part of a universally conserved mechanism of ribosomal translocation. In addition, lysines in particular of small subunit protein S12, and possibly S13, appear to provide counter charges to the anionic tRNA in the A-site (66,70). Thus, the ribosomes stabilizing components are able to discern structurally correct anticodon interactions with cognate and wobble codons, and discriminate against stereochemically incorrect interactions with similar, near-cognate and dissimilar, non-cognate codons.

*Rates and free energy considerations.* At the A-site of the ribosome, the tRNA with the correct anticodon is competing for codon binding with other tRNAs. Yet, the different aminoacyl-tRNA ternary complexes and their interactions with the ribosome are presumably identical. *Escherichia coli* has 41 tRNAs and some will have anticodon sequences that are similar to the correct sequence. The ribosome's ability to select the correct tRNA is exacerbated by having to distinguish a correct three base pair cognate or wobble interaction from a near-cognate interaction differing in only one base pair. This hydrogen bonding is truly a very small part of what must be multiple contacts between the ternary complex and the ribosome (71). Could the all important distinction between

correct and incorrect anticodon–codon pairing be based solely on the small energy differences in cognate/wobble base pairing versus near-cognate base pairing, and in the architectural correctness of the pairing? The high accuracy and speed exhibited in the process of anticodon–codon pairing could be achieved in a two-step process in which accuracy occurs at the decoding recognition step followed rapidly by an A-site accommodation of the tRNA (71). The free energy difference between cognate and near-cognate base pairing is small leading one to predict an error rate of 1 in a 100 amino acids. Yet, *in vivo* the ribosome incorporates the wrong amino acid approximately once for every 10 000 peptide bonds formed under normal growth conditions (72). Independent of ribosomes, the binding of tRNA to a coding triplet or the binding of one tRNA to another with a complementary anticodon exhibit dissociation constants and dissociation rate constants that are 10- to 100-fold higher in solution than on the ribosome. The kinetics of tRNA binding to the ribosome have been thoroughly examined (73). In addition, there is a 70- to 100-fold difference in rate constants for accommodating cognate versus near-cognate codon–tRNA interactions on the ribosome (74). Thus, the selectivity and lower dissociation constants of anticodon–codon interactions on the ribosome may be achieved kinetically and mechanistically by participation of ribosomal components in the stabilization of the cognate and wobble codon–anticodon interactions (66,75). The decoding process includes discrimination between correct and incorrect tRNA anticodons on the basis of the stabilities of anticodon–codon interaction. Rejection rates are due to differences in stabilities between a correct and incorrect anticodon–codon pairing (74). Correct and therefore, stable pairing is followed by a conformational rearrangement of the small subunit that invokes the enzyme analogy of an induced-fit model (74). Could the contributions of tRNA's anticodon domain modifications generate the differential stability?

## Modified nucleoside contributions to decoding

*Wobble, degeneracy and modified nucleoside-dependent codon binding.* The degeneracy of the codes for the 20 amino acids results in some amino acids being coded for by as many as six codons (leucine, serine), whereas others as few as one (methionine, tryptophan). To account for this, the original Wobble Hypothesis envisioned a decoding mechanism that included a broad spectrum of wobble for position 34 of tRNA. tRNA's anticodon position 34 (Fig. 2) would be responsible for wobble to the third base of the codon. Uridine at tRNA's position 34 would recognize A and wobble to G, whereas the modified nucleoside inosine would recognize C and wobble to A or U (10). In 1991, this was altered to include the directed wobble of the many modified nucleosides found at position 34, especially the 2-thionyl modified uridines, $s^2U$ (22). Ten years later, wobble position decoding rules were amended with regard to the codon binding by various derivatives of 5-substituted uridines (5-methyl-2-thiouridine, $xm^5s^2U_{34}$ and methoxy-5-uridine, $xmo^5U_{34}$) (76). Thus, uridine would bind A, and wobble to all four bases, $xm^5s^2U$ would bind A and wobble to G, and $xmo^5U$ would bind A and wobble to both G and U.

With only four exceptions, the wobble position of tRNAs contains U, C, G or I, the latter derived from adenosine. I and not A appears at position 34 because I binds C and has a wobble capacity to A and U exceeding that of A, and A in the wobble position of the P-site tRNA could destabilize the A-site anticodon–codon duplex (77). The four tRNAs found with an unmodified wobble position 34 adenosine include two mitochondrial arginine tRNAs (78,79), a *Mycoplasma capricolum* threonine tRNA (80), and a mutant of *Salmonella typhimurium* in which the wobble nucleoside $G_{34}$ had been replaced by an unmodified A in $tRNA^{Pro}_{GGG}$ (81). The binding of the two mitochondrial $tRNA^{Arg}_{ACG}$, $tRNA^{Pro}_{AGG}$ and $tRNA^{Thr}_{AGU}$ to codons CCC, CGC and ACC, respectively, would presumably require a wobble position $A^{+}_{34}$·C base pair.

A theoretical analysis of the effect of tRNA modification on wobble base pair formation found that a productive wobble base pairing would require compensation for loss of hydrogen bonds or polar atom–ion bonds (77). Thus, modifications of $U_{34}$ would restrict decoding at the wobble position to purines (with the exception of 5-oxy derivatives of uridine, $xo^5U$, that would decode A, G and U). The 2-thio modification of uridine, $s^2U_{34}$, would be expected to weaken the wobble base pairing with G because one of the two hydrogen bonds of the guanosine $NH_2$ group would be deformed. The model predicted that modifications of the first anticodon residue in the P-site tRNA would affect the stability of the A-site duplex (82). The possibility that the $xo^5$- and $xmethyl^5s^2$-modified uridines would change the decoding rules was first envisioned over 30 years ago (83). NMR analyses of modified nucleosides has found that the 2-thio group restricts uridine dynamics to the *anti*, 3′-endo, gauche$^+$ conformation and thereby promotes binding to adenosine (84,85) (Table 1). The $s^2U$ also influences 3′-adjacent nucleosides to take a similar conformation (99,100). In contrast, $xo^5U$ takes the C2′-endo form, as well as the C3′-endo form, possibly enabling wobble to guanosine and uridine, as well as binding to adenosine as the third letter of the codon (85). However, a number of mechanistic questions remain. For those amino acids with many codons (4-fold degenerate, Fig. 1), but far fewer isoaccepting tRNA species, do modifications expand wobble and contribute to a discernibly correct anticodon–codon architecture on the ribosome? For those amino acids with one or two codons (mixed codon boxes, Fig. 1), do ribosomes distinguish productive cognate and wobble anticodon–codon interactions from incorrect near-cognate interactions with the aid of tRNA modifications? A surprising number of modification-deficient tRNAs and ASLs fail at ribosome mediated codon binding, translocation and mRNA reading frame maintenance.

*Decoding of mixed codon boxes with modification-deficient tRNA.* We (92–94) and others (96) have found that a considerable number of unmodified tRNA transcripts and ASLs (Fig. 2) will not bind codon in the A- or P-sites of the ribosome's small subunit (30S) (Table 1). ASLs composed of five base-paired stems and seven nucleoside loops bind cognate codons on the 30S subunit with an affinity similar to that of the entire tRNA (101). However, if a particular unmodified ASL binds its cognate or wobble codon poorly, the full transcript of the tRNA, lacking modifications, is also found to bind poorly (92). Lack of modifications also enhances translational frameshifting *in vivo* (102) (Table 1). Of immediate interest are the many anticodon–codon interactions that decode 2-fold degenerate codons in mixed codon boxes

**Table 1.** tRNA decoding and reading frame maintenance of codons in mixed codon boxes[a]

| tRNA/ASL-anticodon | Relative codon binding[b] | | Modification(s) present | Restored codon binding[b] | | Translocation[c] | | Frameshifting[d] |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Unmodified | Modified | |
| Lys-UUU | AAA | AAG | | AAA | AAG | AAA | AAA | $s^2U_{34}$ or $mnm^5U_{34}$: |
| | – | – | $s^2U_{34}$ | ++++ | +++ | – | ++ | AAG or AAA |
| | | | $mnm^5U_{34}$ | +++ | – | | – | |
| | | | $t^6A_{37}$ | +++ | – | | – | |
| | | | $\Psi_{39}$ | + | – | | – | $U_{39}$, AAA: None |
| | | | $mnm^5U_{34}$ and $t^6A_{37}$ | ++++ | ++ | | ++++ | |
| Lys-CUU | AAA | AAG | $t^6A_{37}$ | AAA | AAG | | | |
| | – | ++++ | | – | ++++ | | | |
| Arg-UCU | AGA | AGG | $Mcm^5U_{34}$ | AGA | AGG | | | |
| | – | ND | | ++ | – | | | |
| Glu-UUC | GAA | GAG | | GAA | GAG | | | |
| | | ND | $s^2U_{34}$ | +++ | + | | | |
| | | | $mnm^5U_{34}$ | ++ | +++ | | | |
| | | | $s^2U_{34}$ | ++++ | +/– | | | |
| | | | $mnm^5s^2U_{34}$ | +++ | ++ | | | |
| Gln-UUG | CAA | CAG | | CAA | CAG | | | $mnm^5U_{34}$: |
| | – | ND | | ND | ND | | | CAA |
| Ser-GCU | AGC | AGU | | | | | | |
| | + | ++ | | | | | | |
| Cys-GCA | UGC | UGU | | | | | | |
| | – | ND | | | | | | |
| Trp-CCA | UGG | | | UGG | | | | |
| | – | | | ND | | | | |
| Phe-GAA | UUU | UUC | $Cm_{32}$; $Gm_{34}$; $yW_{37}$ | UUU | UUC | | $Gm_{34}$:UUU | $A_{37}$ or $i^6A_{37}$: None |
| | +++ | ND | $ms^2i(o)^6A_{37}$; $\Psi_{39}$; $m^5C_{40}$ | ++++ | +++ | | ++++ | $U_{39}$: None |
| Tyr-GUA | UAU | UAC | $Q_{34}$; $ms^2io^6A_{37}$; $\Psi_{39}$ | UAU | UAC | | | $G_{34}$: UAU |
| | ND | ND | | ND | ND | | | $A_{37}$ or $i^6A_{37}$: UAU/C |
| Ile-GAU | AUC | AUU | $t^6A_{37}$ | AUC | AUU | | | |
| | ++ | ND | | ++++ | ND | | | |

ND, not determined.

[a]Aminoacyl- (A-) site and peptidyl- (P-) site ribosome binding by anticodon stem and loop domains (ASL) not previously reported were accomplished according to published methods (92–94). Translocation of ASLs from the A- to the P-sites was accomplished with published methods (67). Published results are from 86–98.

[b]Relative codon binding relates binding affinity to that of fully modified tRNA or ASL: ++++ equals tRNA or fully modified ASL ($K_d$ ~ 100 nM); –, no binding ($K_d$ > 2000 nM).

[c]Translocation for $ASL^{Lys}$ normalized to $ASL^{Lys}_{UUU}$ with $mnm^5U_{34}$ and $t^6A_{37}$; for $ASL^{Phe}_{GAA}$ normalized to $ASL^{Phe}_{GAA}$ with $Gm_{34}$; for $ASL^{Val}$ normalized to $ASL^{Val}$ with $cmo^5U_{34}$.

[d]Frameshifting by hypomodified tRNAs having the noted modifications and on the specified codons in mutant cells relative to fully modified tRNAs in wild-type cells.

(Fig. 1) and that are also affected by a lack of modifications. For instance, codons for glutamine, lysine, glutamic acid and arginine occur in mixed codon boxes requiring their respective tRNAs to distinguish cognate and wobble codons from near-cognate codons of histidine, asparagine, aspartic acid and serine, respectively (Fig. 1). In addition, these tRNAs have pyrimidine-rich anticodon loops composed of a pyrimidine at position 32 and the invariant $U_{33}$. Lysine and glutamic acid tRNAs have all-pyrimidine anticodons and glutamine's anticodon has a G at position 36. The anticodons have either a modified $U_{34}$, or a $C_{34}$ (Fig. 2). Unmodified ASLs of glutamine, lysine, glutamic acid, arginine (UCU anticodon) and cysteine did not bind their cognate codons (Table 1). Obviously ASLs, such as $ASL^{Lys}_{UUU}$, that do not bind codon in the A-site will not translocate. In contrast, the unmodified $ASL^{Phe}_{GAA}$ with a purine-rich anticodon loop binds UUU in the A-site quite effectively, and translocates (Table 1). tRNAs for glutamine, lysine and glutamic acid all have a derivative of $s^2U$ at wobble position 34. The anticodon of *E.coli* $tRNA^{Glu}_{UUC}$ is complementary to that of $tRNA^{Phe}_{GAA}$. The effect of the naturally occurring $s^2U_{34}$ on complementary anticodon–anticodon association of $tRNA^{Glu}_{UUC}$ with $tRNA^{Phe}_{GAA}$ demonstrated that replacement of the $tRNA^{Glu}_{UUC}$ thio group ($s^2U_{34}$) with a keto group ($U_{34}$) destabilized complex formation and its maintenance (88). The $mnm^5$- modification facilitated $tRNA^{Glu}_{UUC}$ recognition of wobble codon GAG while reducing recognition of cognate codon GAA, whereas $s^2$- increased $tRNA^{Glu}_{UUC}$ recognition of GAA (95). $tRNA^{Ser}_{GCU}$ responds to codons (AGU/C) in a mixed codon box also containing the arginine codons (AGA/G). $ASL^{Ser}_{GCU}$ bound cognate codon poorly (Table 1). Unmodified $ASL^{Arg}_{UCU}$ would not bind the cognate codon AGA (Table 1). In the case of isoleucine, there are three codons. Unmodified $ASL^{Ile}_{GAU}$ bound its cognate codon AUC, but binding to its wobble codon AUU has yet to be assessed (Table 1). The single tryptophan codon also occurs in a mixed codon box. The unmodified $ASL^{Trp}$ bound poorly to its codon. The $tRNA^{Trp}$ anticodon loop is also pyrimidine-rich and usually has a $C_{32}$ followed by $U_{33}C_{34}C_{35}A_{36}$ (68). Lack of, or inefficient codon binding by unmodified tRNAs with pyrimidine-rich anticodon loops was predicted by the 'Modified Wobble Hypothesis'. The hypothesis describes

these types of tRNAs as requiring modification to correctly conform the entire anticodon loop structure for proper codon recognition and binding (22). In fact, pyrimidine-rich ASLs have precluded structure determination of the unmodified loop due to conformational heterogeneity, whereas the addition of a single modification was enough to stabilize the loop (92,103).

Some tRNAs that respond to codons in mixed codon boxes do not have pyrimidine-rich anticodon loops. Wobble position 34 of tRNAs for aspartic acid, asparagine, histidine and tyrosine, all with codons in mixed codon boxes (Fig. 1), are conventionally found to have G modified to various deazaguanosines, derivatives of queuosine $Q_{34}$ (25), that are synthesized *de novo* by bacteria, but provided to vertebrates by their digestive tract organisms. The presence of $Q_{34}$ clearly affected the *in vivo* choice of codon by two histidine isoacceptors from *Drosophila* (104). $tRNA^{His}_{GUG}$ without the modification preferred CAC to the codon CAU. In contrast, $tRNA^{His}_{QUG}$ had little preference for the codon CAU. An *E.coli* strain lacking queuosine in its tRNAs was readily out-grown by an *E.coli* strain with queuosine (105). Similar to the empirical results with $tRNA^{His}_{GUG}$, a very stable association was formed in models of the unmodified (guanosine-containing) $tRNA^{Asp}_{GUC}$ binding to GAC, but was much less stable in complex with a GAU (106). Also similar to the experimental results with $tRNA^{His}_{QUG}$, the modeled $tRNA^{Asp}_{QUC}$ with $Q_{34}$ exhibited no bias for either codons GAC or GAU and had a lower binding energy to the GAU codon than that of the guanosine-containing tRNA (106). Thus, it appears from the experimental (104,105) and modeling (106) results that the presence of $Q_{34}$ is restrictive in that it seems to be involved in codon choice. Therefore, when unmodified, many tRNAs that respond to codons in mixed codon boxes and have pyrimidine-rich anticodon loops will not bind cognate codons, and some without pyrimidine-rich loops will have their codon selection affected.

*Modifications restore decoding accuracy and maintain the reading frame.* Correct cognate and wobble codon binding is restored with the incorporation of modifications at wobble position 34 and/or position 37, 3′-adjacent to the anticodon (Fig. 2). The one methionine codon shares a codon box with the three isoleucine codons (Fig. 1). A clear example of a modification that restricts codon recognition and binding involves codon discrimination by $tRNA^{Ile}_{CAU}$. AUG is decoded for methionine in the initiation of protein synthesis in the ribosomal P-site and during protein elongation in the A-site. The gene for a minor isoleucine tRNA that responds to the codon AUA, was found to have a C in the wobble position and thus, a methionine anticodon CAU (107). Modification of the 2 position of $C_{34}$ with lysine, creating the modification lysidine, $k^2C_{34}$, altered both the aminoacylation and codon recognition from that of methionine to isoleucine. All of the other sequenced bacterial, animal and plant cytoplasmic $tRNA^{Ile}$ species have N6-threonylcarbamoyladenosine ($t^6A_{37}$) and either $G_{34}$ or $I_{34}$. $tRNA^{Ile}$ isolated from *E.coli* cells grown on a suboptimal concentration of threonine was found to contain an average of 50% less $t^6A_{37}$ than tRNA isolated from cells grown under optimal conditions. Ribosome binding of the codon AUC indicated that $t^6A$ was required for $tRNA^{Ile}$ to have an accurate anticodon–codon interaction (87). Therefore, purine 37 modifications adjacent to the anticodon, as well as wobble position 34 modifications within the anticodon, are important for correct cognate and wobble codon recognition.

The incorporation of the $s^2U_{34}$ or $mnm^5U_{34}$ modifications (92,93) or $t^6A_{37}$ (94) into otherwise unmodified ASLs for lysine substantially restored cognate codon AAA binding at the ribosome's A- and P-sites (Table 1). The $s^2U_{34}$ modification, but not the $mnm^5U_{34}$ or $t^6A_{37}$, restored wobble codon binding to AAG, and some A- to P-site translocation for $ASL^{Lys}_{UUU}$ (Table 1). A combination of $mnm^5U_{34}$ and $t^6A_{37}$ restored wobble codon binding and translocation (94; Phelps,S.S., Malkiewicz,A., Agris,P.F. and Joseph,S., submitted). A difference in the decoding preference by the mammalian isoaccepting species $tRNA^{Lys}_{UUU}$ for AAA and $tRNA^{Lys1,2}_{CUU}$ for AAG and a tendency for only the former species to wobble was observed as early as 1981 (89). Fully modified mammalian $tRNA^{Lys}_{CUU}$ with $t^6A_{37}$ decoded AAG faster than the hypomodified tRNA, but poorly decoded AAA, which is coded better by the hypomodified tRNA (90). We found that $t^6A_{37}$ did not alter the high affinity binding of human $ASL^{Lys}_{CUU}$ to AAG, and did not produce binding to AAA (Table 1). Therefore, $tRNA^{Lys}_{CUU}$ apparently does not wobble to AAA, and the presence of $t^6A_{37}$ appears to ensure this. The modification $t^6A$ is also found in *E.coli* $tRNA^{Arg}_{UCU}$ which decodes AGA/G in the mixed codon box with serine. The modification had a small, but significant stabilization on polynucleotide-directed binding of $tRNA^{Arg}_{UCU}$ on the ribosome, ribosome-free trinucleotide binding to codon, and complementary anticodon–anticodon binding (86). Thus, modifications at either wobble position-34 or purine-37 can restore and/or influence specific codon recognition.

In evaluating $tRNA^{Phe}$ function in translation *in vitro*, individual rate constants for the elongation process showed that modifications increased the accuracy of translation by decreasing the rate of dipeptide synthesis and by increasing the rate of rejection with non-cognate codons (101). The modification $ms^2i^6A_{37}$ stabilized anticodon–codon interaction (Table 1), thus preventing misreading of the genetic code (108). Though experiments with suppressors lacking the 2-methylthio- ($ms^2$) group of 2-methylthio-(*cis*-hydroxy)-isopentenyladenosine ($ms^2io^6A_{37}$), also known as 2-methylthio *cis*-ribozeatin (109), indicated that $ms^2$- is important to the decoding efficiency of tRNA, the major contribution apparently comes from the $io^6$- group alone (110). The anticodon stem modification $m^5C_{40}$ of yeast $tRNA^{Phe}$ actually negated ribosome binding of the ASL, yet significantly increased its thermal stability (111,112). Addition of 1-methylguanosine, $m^1G_{37}$, to the $m^5C_{40}$-modified ASL increased affinity for codon 10-fold, but also dramatically decreased thermal stability. Thus, modifications in the anticodon loop at wobble position 34 and position 37 appear to restructure the loop for correct decoding, and may do so by sacrificing overall thermal stability.

*In decoding 2-fold degenerate codons, tRNA modifications aid in reading frame maintenance.* Not surprisingly, some of the same unmodified tRNAs or ASLs that are ineffective at cognate or wobble codon binding *in vitro* are also prone to translational frameshifting *in vivo* when site-specifically unmodified or hypomodified. Messenger RNA reading frame shifting errors occur during translation. Although some

frameshifting is enabled by certain viral RNA and mRNA sequences, frameshift errors in the normal course of translation with fully modified tRNAs are less than $5 \times 10^{-5}$ per codon (113). The ability of anticodon modifications to diminish the frameshifting effects of 5′- and 3′-neighboring codons, i.e. codon context frameshifting, has been explored extensively. It has been proposed that the affinity of the P-site tRNA for its codon is key to P-site frameshifting. The lower the anticodon affinity for codon, the more likely frameshifting will occur (114,115). In comparison to codon binding *in vitro*, frameshifting analyses are accomplished *in vivo*. Relative to wild-type $tRNA^{Lys}_{UUU}$ with 5-methylaminomethyl-2-thiouridine ($mnm^5s^2U_{34}$), hypomodified $tRNA^{Lys}_{UUU}$ with either $s^2U_{34}$ or $mnm^5U_{34}$ exhibited significant +1 frameshifting in response to the wobble codon AAG, and less in response to the cognate codon AAA (Table 1) (102,116). Modifications at other nucleosides of mutant $tRNA^{Lys}$ would be expected to be equivalent to that of wild-type, in particular those of the anticodon domain, $t^6A_{37}$ and $\Psi_{39}$. Excessive frameshifting was observed for $tRNA^{Gln}_{UUG}$ that has the same wobble position uridine modification as $tRNA^{Lys}_{UUU}$, but a considerably different modified $A_{37}$, 2-methyladenosine ($m^2A_{37}$). When the $s^2U_{34}$ modification was absent and CAA was being decoded, $tRNA^{Gln}_{UUG}$ frameshifted more often than fully modified $tRNA^{Gln}_{UUG}$ in wild-type cells (Table 1). Presumably, excessive frameshifting would be observed for $tRNA^{Glu}_{UUC}$ of the same mutant because that tRNA also has the $mnm^5s^2U_{34}$ modifications. In contrast to +1 frameshifting, the same two modification deficiencies had considerably little effect on –1 frameshifting (117).

As early as 1969, it was observed that in *E.coli*, one of three tyrosine tRNA isoacceptors lacking a modification 3′-adjacent to the anticodon at position 37 did not support protein synthesis and did not bind the appropriately programmed ribosome (118). The modification $ms^2io^6A_{37}$ appears in eubacterial tRNAs for phenylalanine and tyrosine. Cells deficient in modifications of $A_{37}$ exhibited increased +1 frameshifts for $tRNA^{Phe}$s and $tRNA^{Tyr}$s deficient in either $ms^2$- or $ms^2io^6$- (102). Although Ψ at position 39 has been shown to stabilize anticodon domain structure (119,120) with little effect on an otherwise unmodified $ASL^{Lys}_{UUU}$ to bind cognate codon (92,119), Ψ at positions 38 or 39 enhances suppressor efficiency for read through of stop codons and promotes +1 frameshifting in at least one tRNA (121), but its deficiency did not enhance either +1 or –1 frameshifting with tRNAs for asparagine, lysine, phenylalanine and leucine (116). Two other tRNAs that respond to codons in mixed codon boxes, tyrosine and histidine, differ in their frameshifting when unmodified at wobble position 34 though they have the same hypermodification, $Q_{34}$ (Table 1). In comparison to the fully modified $tRNA^{Tyr}_{QUA}$ in wild-type cells, $tRNA^{Tyr}_{GUA}$ frameshifted in response to the codon UAU in $Q_{34}$ deficient cells (102,116). However, $tRNA^{His}_{GUG}$ exhibited little to no frameshifting (Table 1). Thus, anticodon loop modifications, particularly those of purine-37 are important for reading frame maintenance by tRNAs responding to codons in mixed codon boxes.

*Limited decoding of 4-fold degenerate codons by modification-deficient tRNA.* The decoding of codons from completely degenerate codon boxes could be accomplished by four isoaccepting tRNA species responding to their corresponding cognate codons. Since A has only been found in four tRNAs at wobble position 34 (78–81), and in all others A is modified to I, minimally two isoaccepting tRNAs could respond to four codons. However, only 12% of tRNA genes for tRNA species that respond to 4-fold degenerate codon boxes are encoded with wobble position adenosines (68). The percentage of wobble position adenosines is dramatically higher (37%) in eukaryotic tRNAs. Significantly, 47% of all tRNAs (31.2% in eukaryotic tRNAs) responding to completely degenerate codons are encoded with a wobble position U. The remaining tRNAs are encoded with wobble positions Cs and Gs in a proportion close to the expected random appearance of 25% (22 and 19%, respectively). Thus, one could conclude that wobble position Us, that are almost always modified (68), are responsible for the majority of wobble codon recognitions by these tRNAs (22,122). An unmodified anticodon stem and loop of $tRNA^{Pro}_{UGG}$ binds to its cognate codon (CCA) with high affinity (Table 2). However, $ASL^{Pro}_{UGG}$ would not bind to its wobble codon (CCG) (Table 2). $ASL^{Val}_{UAC}$ bound its cognate codon (GUA) and translocated from the A- to P- sites, but bound all three wobble codons (GUG, GUC and GUU) very poorly. Unmodified $ASL^{Ala}_{UGC}$ bound neither cognate (GCA) nor wobble (GCG) codons (Table 2). An $ASL^{Ser}_{UGA}$ was found to bind cognate codon (UCA) moderately well, but did not bind the wobble codon (UCG) (Table 2). An unmodified form of *E.coli* $tRNA^{Ser1}_{UGA}$, which normally has the $cmo^5U_{34}$ modification and recognizes the UCU/A/G codons, recognized the UCA codon (125). However, the UCU codon was recognized with low efficiency, and the UCC and UCG codons were not recognized at all. $tRNA^{Arg}$ species with the anticodons CCG and ACG have the modification 2-thiocytidine-32 ($s^2C_{32}$), but differ in wobble position and position 37 modifications. $tRNA^{Arg}_{CCG}$ is not modified at position 34 (68) and has an $m^1G_{37}$, whereas $tRNA^{Arg}_{ACG}$ is modified to $I_{34}$ and has an $m^2A_{37}$. We found that the unmodified $ASL^{Arg}$ constructs with either anticodon, CCG and ACG, would not bind their cognate codons (Table 2). Thus, some unmodified ASLs responding to 4-fold degenerate codons will bind cognate codon and translocate from the A- to P-site, but will not bind wobble codon, whereas other unmodified ASLs even bind cognate codon poorly and do not translocate.

*tRNA modifications maintain the reading frame in decoding 4-fold degenerate codons.* The same tRNAs when unmodified or hypomodified at specific positions by mutation of modification enzymes were more likely to frameshift *in vivo* than their fully modified, wild-type counterparts (Table 2). In the absence of the $m^1G_{37}$-tRNA methyltransferase activity, $tRNA^{Pro}$, $tRNA^{Leu}$ and $tRNA^{Arg}$ frameshifted considerably more often than the corresponding fully modified tRNAs in wild-type cells (102,126). The lack of $m^1G_{37}$ in the $m^1G_{37}$-tRNA methyltransferase mutant may actually slow decoding by tRNAs normally containing the modification (127). $tRNA^{Leu}$ minus $m^1G_{37}$ frameshifted in response to three of its four codons (Table 2). With mutation of a pseudouridine-tRNA synthase for the very common modifications $\Psi_{38}$ and $\Psi_{39}$, $tRNA^{Leu}$, with $m^1G_{37}$ but lacking the Ψ, frameshifted in response to the same three codons. $tRNA^{Pro}$ minus $m^1G_{37}$ also frameshifted more than the wild-type tRNA in response to all

**Table 2.** tRNA decoding and reading frame maintenance for 4-fold degenerate codons[a]

| tRNA/ASL and anticodon | Relative codon binding[b] | | Modification(s) present | Restored codon binding[b] | Translocation[c] | | Frameshifting[d] |
|---|---|---|---|---|---|---|---|
| | | | | | Unmodified | Modified | |
| Arg CCG | CGG<br>– | CGA<br>ND | $s^2C_{32}$; $m^1G_{37}$ | CGG/A<br>ND | | | $G_{37}$: CGG |
| Arg ACG | CGU<br>– | CGC<br>ND | | | | | |
| Pro UGG | CCA<br>++++ | CCG<br>– | $Um_{32}$; $cmo^5U_{34}$; $m^1G_{37}$; $\Psi_{40}$ | CCA/G/U/C<br>ND | | | $G_{37}$: CCA/G and CCU/C |
| Ser UGA | UCA<br>+++ | UCU<br>+<br>UCC/G<br>– | | | | | |
| Val UAC | GUA<br>+++<br>GUU<br>– | GUG<br>+<br>GUC<br>ND | $cmo^5U_{34}$ | GUA<br>+++<br>GUG ++<br>GUU ++ | GUG/U<br>– | $cmo^5U_{34}$:<br>GUA/G/U<br>++++ | |
| Ala UGC | GCA<br>– | GCG<br>– | | | | | |
| Leu | CUU/C<br>ND | CUA/G<br>ND | $m^1G_{37}$; $\Psi_{38-39}$ | CUU/C/A/G<br>ND | | | $G_{37}$: CUU/A/G (CUC in P-site)<br>$U_{38-39}$: CUU, CUA/G |

ND, not determined.

[a]Aminoacyl- (A-) site and peptidyl- (P-) site ribosome binding by anticodon stem and loop domains (ASL) not previously reported were accomplished according to published methods (92–94). Translocation of ASLs from the A- to the P-sites was accomplished with published methods (67). Published results are from references 94,96,123–125.

[b]Relative codon binding relates binding affinity to that of fully modified tRNA or ASL: ++++ equals tRNA or fully modified ASL ($K_d$ ~ 100 nM); –, no binding ($K_d$ > 2000 nM).

[c]Translocation for $ASL^{Val}$ normalized to $ASL^{Va}{}_{UAC}$ with $cmo^5U_{34}$.

[d]Frameshifting by hypomodified tRNA having the noted modifications and on the specified codons in mutant cells relative to fully modified tRNA in wild-type cells.

four of its codons, but lacking $\Psi_{40}$ in the middle of its anticodon stem (Fig. 2) had little effect on codon reading (128). Ψ residues, depending on their locations in tRNA, rRNA and even snRNA, may contribute differently to various RNA functions (129). Thus, for tRNA responding to 4-fold degenerate codon boxed, $m^1G_{37}$, and $\Psi_{38}$ and $\Psi_{39}$ are important to the maintenance of the translational reading frame.

*Modifications expand tRNAs decoding of 4-fold degenerate codes.* Four-fold degenerate coding boxes encompass half of the 64 codons and represent only eight of the 20 amino acids (Fig. 1). Using a MS2 RNA programmed protein synthesizing system *in vitro*, $tRNA^{Val}{}_{UAC}$ with the wobble position modification $cmo^5U_{34}$ read the codon GUU quite efficiently and $tRNA^{Val}{}_{IAC}$ (with $I_{34}$) was just as effective in reading the codon GUG (123). We found that the modification $cmo^5U_{34}$ restored wobble codon binding of $ASL^{Val}{}_{UAC}$ (Table 2). Of particular interest, $cmo^5U_{34}$ restored binding to GUU. A $cmo^5U_{34}$·U base pairing occurred in a translocation assay requiring A-site codon binding prior to translocation from the A- to P-sites (Phelps,S.S., Malkiewicz,A., Agris,P.F. and Joseph,S., submitted). Thus, the $cmo^5U_{34}$ modification enables $tRNA^{Val}{}_{UAC}$ to read three of the four valine codes and translocate (Phelps,S.S., Malkiewicz,A., Agris,P.F. and Joseph,S., submitted). A variant of the $tRNA^{Val}$ modification, 5-methoxyuridine, found in $tRNA^{Ser}{}_{UGA}$ enhanced the wobble reading of UCU and UCG codons (124). Therefore, the family of $xo^5U_{34}$ modifications expands the wobble recognition of tRNAs responding to 4-fold degenerate codons.

## Mitochondrial and chloroplast decoding by tRNAs

The organellar tRNAs are discussed separately because there are far fewer tRNAs encoded than the nuclear encoded, cytoplasmic tRNAs, there appears to be more extensive wobble codon recognition in the mitochondrion, and modifications are both negative and positive determinants for importation of cytoplasmic tRNAs into organelles. A few of the modifications that appear in the anticodon stem and loop domains of eubacteria and eukaryotic cytoplasmic tRNAs also appear in mitochondrial and chloroplast tRNAs. Modifications of nuclear encoded tRNAs appear to both restrict decoding occurring in mixed codon boxes, and enhance wobble for 4-fold degenerate codons. Even though some mitochondria import nuclear encoded tRNAs, the total number of tRNAs operating in the organelle is less than in the cytoplasm. Some nuclear encoded tRNAs appear to require specific modifications for importation (130–132), or a particular modification, such as $s^2U_{34}$, would serve as a negative determinant for the tRNA's importation (133,134). The chloroplast and mitochondrion of green plants translate the codes on the ribosome with a mechanism similar to that of cytoplasmic protein synthesis. However, chloroplasts use only 31 anticodons in translating the codes. Ten CNN anticodons have been eliminated. Green plant mitochondria augment their tRNA population by importing nuclear encoded tRNAs from the cytoplasm.

Mitochondria from other organisms have evolved with recoded codons, such as AUA for methionine, AG(U/C) for serine and stop, AAA for asparagine, CU(A/C/G/U) for threonine, UGA for tryptophan, and may use as few as 22 anticodons for translation. One modification, $\Psi_{35}$, at the center of the echinoderm mitochondrial $tRNA^{Asn}$ anticodon appears to serve to decode the unusual asparagine codon AAA, resulting in the alteration of the genetic code in echinoderm mitochondria (135).

Therefore, in order for all codons to be effective and efficiently decoded, mitochondrial translation relies on modification enhanced wobble and recoding of codons. Whether the reduced number of tRNAs results from natural pressure for a small mitochondrial genome, or from some other stressors, altered amino acid acceptance by some tRNAs, loss of or changes in release factors, anticodon modifications, or disappearance of codons from coding sequences, is debatable (136,137). The nucleotide sequences of all 29 *Mycoplasma capricolum* tRNA species indicate that this organism's tRNAs have similarities to that of mitochondrial tRNAs (80). There is a single tRNA for each of 4-fold degenerate codon boxes of alanine, glycine, leucine, proline, serine and threonine and these tRNAs have unmodified uridines in wobble position 34. The two threonine tRNA species with anticodons UGU and AGU are unmodified at position 34. Of the metazoan mitochondrial tRNAs sequenced and responding to codons in mixed codon boxes, all, but one, lack modification of the position 34 nucleoside. In contrast, the modification $t^6A_{37}$ appears to be important for mitochondrial $tRNA^{Lys}_{CUU}$ to respond to its cognate codon AAG in a mixed codon box (135). We had found that cytoplasmic $tRNA^{Lys}_{CUU}$, whether or not modified with $t^6A_{37}$, bound cognate codon AAG and not its wobble codon AAA (94). Mitochondrial modification deficiencies have been associated with human disease. Mutant mitochondrial $tRNA^{Lys}_{UUU}$ from the pathogenic disease myoclonic epilepsy with ragged red fibers (MERRF), a mitochondrial encephalopathy, lack the wobble position 2-thiouridine derivative. In contrast to wild-type human mitochondrial $tRNA^{Lys}$, the mutant tRNA did not bind to mRNA programmed ribosomes, but could be aminoacylated (138). Thus, the defect in anticodon modification negatively affects anticodon–codon pairing that is crucial to mitochondrial translation. Obviously anticodon domain modifications selectively contribute chemistry and/or structure to decoding and frameshift maintenance in cytoplasmic and organellar protein synthesis.

*Anticodon domain contributions to structure and dynamics of decoding*. Do modifications enable decoding for many tRNAs by creating the canonical anticodon structure of yeast $tRNA^{Phe}$? Or do modifications enable decoding by destabilizing the anticodon loop to be later conformed by the ribosome in an induced-fit model to codon reading? Uridine modifications at position 34 seem to fall into two categories with regard to codon binding: $s^2U_{34}$ and its derivatives tend to bind codons ending in A better than those ending in G, though $ASL^{Lys}$ with $s^2U_{34}$ bound its wobble codon quite well (Table 1). In comparison, $xm^5U_{34}$ anticodons tend to bind G ending codons better than A ending codons (Table 1). It would be simple if only the restricted *anti* C3′-endo, gauche$^+$ conformation of $s^2U_{34}$ versus the less restricted sugar pucker of $xnm^5U$ were responsible for the difference in A and G binding. However, the 3′-adjacent nucleoside-35, and the presence or absence of a hypermodified nucleoside-37 probably influences the wobble base pairing (Table 1). In order to account for the $xnm^5U_{34}$ preferred binding to codons ending in G, a recent survey of the selective binding by anticodons with $xnm^5U_{34}$ suggests a non-conventional U·G pairing (122). Through protonation of the secondary amine of $xnm^5U_{34}$, it is proposed that the 2-thionyl becomes single bonded and negatively charged and the N-1 proton is lost. The G N1 imino proton is donated to either the $S^-$ or the N-1 of the uridine. The unique geometry of the suggested base pairing is intriguing even though the model may need to change in order to accommodate contributions of purine 37 modifications to anticodon architecture. The U·G base pair geometry should become apparent in a high resolution crystal structure determination of the appropriately modified ASL bound to a G-ending codon on the 30S ribosomal subunit. Recently, the crystal structure of $ASL^{Lys}_{UUU}$ with $t^6A_{37}$ bound to AAA on the 30S subunit was solved to 3 Å (Murphy,F., Agris,P.F., Malkiewicz,A. and Ramakrishnan,V., unpublished). The $t^6A_{37}$ is required for binding AAA and acted as a platform for the first codon–anticodon base pair (Cover Figure of ths issue). Binding of the wobble codon AAG could by achieved with an $xm^5U_{34}$ modification plus $t^6A_{37}$ (Table 1) and could reveal the exact base $xm^5U_{34}$·G pairing geometry. Thus, modified nucleoside conformation or geometry is partly the answer to enabling or enhancing codon binding, but not the full answer.

The importance of some anticodon conformational dynamics in codon binding on the ribosome has been suggested (139). Cryo-EM structure, kinetics and mutant tRNA suppressor data together appear to support the possibility of a deformed or waggling aminoacyl-tRNA transitional structure required for effective proofreading by the ribosome during decoding at the A site (140,141). Because of its fundamental importance to decoding, the structure and conformational dynamics of the anticodon have been the subject of numerous studies with sometimes contradictory results (142). Three distinct conformations of the yeast $tRNA^{Phe}$ anticodon domain were suggested by time and polarization resolved fluorescence measurements (143) and an NMR study found that anticodon domain modifications were particularly restricted in motion compared to other modifications (144) perhaps indicating slow exchange among a very small number of conformations. Completely unmodified yeast $ASL^{Phe}$ (111), a DNA analogue of the yeast $ASL^{Phe}$ (145) and *E.coli* $ASL^{Phe}$ (146) molecules exhibited two intra-loop base pairs extending the stem of the unmodified constructs. Whereas incorporation of the individual modifications $m^5C_{40}$ and $\Psi_{39}$ in the stem increase thermal stability of the yeast $tRNA^{Phe}$, incorporation of $m^1G_{37}$ in the yeast $ASL^{Phe}$, $i^6A_{37}$ in the *E.coli* $ASL^{Phe}$, and $t^6A_{37}$ in human $ASL^{Lys}$ decrease overall thermal stability (103,112). We had demonstrated that incorporation of $m^1G_{37}$ contributed to codon recognition on the ribosome (112) for both the yeast $ASL^{Phe}$ and its DNA analogue (147). Ribose 2′-O methylations within the loop of the heptadecamer anticodon stem and loop of eucaryotic $tRNA^{Phe}$, $Cm_{32}$ and $Gm_{34}$, also affect reading frame maintenance (148). All of the naturally occurring purine modifications at residue 37, including $m^1G$, negate the possibility of intra-loop hydrogen bonding by interfering with the Watson–Crick base pairing 'face' of the

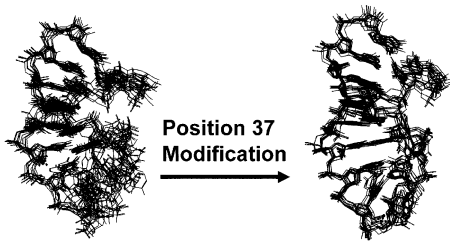


**Figure 3.** Purine 37 modifications order the anticodon loop. The structure and dynamics of triply (left) and quadruply (right) modified ASLs for yeast $tRNA^{Phe}$ have been compared (142). The only difference in sequence between the two constructs is the incorporation of $m^1G_{37}$ versus $G_{37}$. Each structure is represented by a family of the 10 lowest energy structures derived from NMR and restrained molecular dynamics.

nucleoside (25). In fact, 95% of all tRNA sequences have the potential for two anticodon intra-loop base pairs that are modified at position 37 (147). More recently we have found that the anticodon conformation of yeast $ASL^{Phe}$ is realized in solution by modifications altering the conformational space sampled by the anticodon loop (142). For instance, human $tRNA^{Lys3}_{UUU}$ and possibly other $tRNA^{Lys}_{UUU}$ require modifications at position 37, as well as at wobble position 34, to achieve the canonical U-turn structure (149,150) and function (93,94). The incorporation of $m^1G_{37}$, the precursor to the hypermodification wyosine, into an already triply modified ($Cm_{32}$, $Gm_{34}$, $m^5C_{40}$) anticodon stem and loop domain of yeast $tRNA^{Phe}$ directed the conformation dynamics of anticodon loop toward the structure found in the crystal (Fig. 3) (142). The $i^6A_{37}$ in *E.coli* $tRNA^{Phe}$ may enhance ribosome binding by negating intra-loop hydrogen bonding in the anticodon domain (146), as does modification of $G_{37}$ in yeast $tRNA^{Phe}$ (147), thus enabling anticodon–codon helix formation. The predicted most stable conformation of $i^6A_{37}$ and its derivatives including $ms^2i^6A_{37}$ has the isopentenyl substituent away from the adenosine imidazole ring and coplanar with the adenine ring (151). The same is true of $t^6A_{37}$ (103). In a study of the effects of modified adenosines, particularly the $i^6$-derivatives, on duplex stability, most of the modifications destabilized the duplexes (152). The nature of the modification and the buffer composition significantly affected the thermodynamic stability of the RNA duplexes. However, the decreased thermal stability of duplex formation is exactly what is required to achieve an open anticodon loop that will be ordered for codon recognition and binding (103,142,147). Therefore, some conformational freedom of the anticodon loop with some order and boundaries to motion conveyed by modification, particularly at position 37, is also important for codon recognition.

In contrast to most other modifications, Ψ and the 2′O-methyl modified nucleosides are by far the most highly conserved in location and are the most ubiquitous modifications. Both appear in the ASL domains of tRNAs. Pseudouridine is most often found in the stem and adjacent to the anticodon loop at position 39, but also at 31, or adjacent to the stem at position 38. It is also found in the anticodon at position 35. The 2′-O-methylated nucleosides have been found at position 32 adjacent to the loop and in the anticodon. The thermal stabilization of structure contributed by $Ψ_{38,39}$ immediately adjacent to, or in the anticodon stem does not appear to be through direct hydrogen bonding afforded by the additional imino proton at N1 (119,120). Hydrogen bonding through water has not been excluded. Though $Ψ_{39}$ contributes thermal stability, it is incapable of restoring codon binding to $ASL^{Lys}_{UUU}$ which in the absence of other modifications will not bind cognate or wobble codons in the ribosomal A- or P-sites (92–94). By Ψ coordinating structural water via its free N1-H, and/or enhancing base stacking, it has a significant negative effect on RNA dynamics. This effect on structure and dynamics will impact RNA function and may be the reason that Ψ is so prevalent. Thus, it appears that anticodon domain modifications balance structure with dynamics in a direction that creates the correct anticodon conformation and mobility for ribosome binding.

## CONCLUSIONS

Organisms have evolved different patterns of bias in codon usage of the degenerate Genetic Code (153). In general, tRNAs with the appropriate anticodons are expressed in proportion to the codons appearing in mRNAs. However, any particular tRNA species in a cell may be composed of molecules that are not equivalent in modification. Usually a small proportion of tRNAs are undermodified at particular positions. Because modification enzyme activities seem to be unable to keep pace with tRNA transcription and processing in rapidly dividing cells, the proportion of site-specifically undermodified tRNA molecules would be expected to be higher in those cells. Indeed, tRNAs undermodified at various positions, but particularly wobble position 34 and position 37, have been reported (27,28) to occur in rapidly dividing cells and in cloned overexpression of tRNAs (154–160). As discussed, undermodified species of some tRNAs may not bind codon, whereas others may. In some instances, a cell's rarely used codons could be read by undermodified tRNA species, whereas the modified species would be more codon selective. Disparities have been reported for codon binding results *in vitro* in comparison to codon selection *in vivo* (161). *In vitro* codon binding experiments may indicate a requirement for a modification for cognate or wobble decoding, whereas *in vivo* a modification enzyme mutation indicates a tendency to frameshift or read near-cognate codons more or less effectively. Surprisingly, the hypomodified *E.coli* $tRNA^{Lys}_{UUU}$ was reported to misread asparagine codons *in vivo* less than the fully modified tRNA (91). This result not only is at odds with the *in vitro* data, but needs to be clarified with respect to the frameshifting experiments *in vivo* in which selected hypomodification increased frameshifting (102,116). The $s^2U_{34}$ modification has been found to be quite restrictive in its recognition of complementary A versus G and that $mnm^5U_{34}$ is less so (124). The presence of the $t^6A_{37}$ modification in combination with the hypomodified $U_{34}$ may have contributed to the undermodified tRNA's restrictive decoding behavior *in vivo*.

We have found that anticodon loops modified at positions 34, $s^2U_{34}$, and 37, $t^6A_{37}$, carry a redundancy in their ability to effectively read cognate and wobble codons *in vitro* (Table 1). The disparity between results *in vitro* and *in vivo* may very well be due to the redundancy built into the system. There are many redundant systems in biology, and modification is not without them. For instance, some years ago we had found that

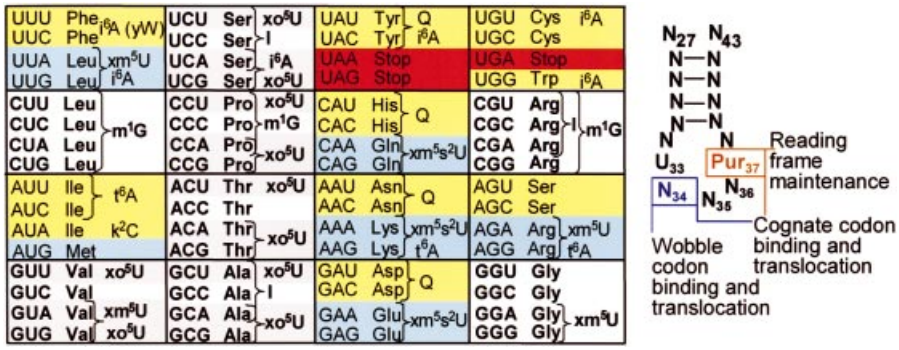


**Figure 4.** The Genetic Code and its decoding by tRNA. The traditional representation of the codons (left) is accompanied by tRNA's anticodon stem and loop domain modifications known to influence decoding, translocation and/or frameshifting (Tables 1 and 2). Cognate and wobble codons that are possibly read by a wobble position, modified uridine-34 responding to one of the 4-fold degenerate codon boxes have backgrounds of white and gray. Codons representing a single amino acid in each of the mixed codon boxes are shaded in yellow and light blue. The stop codons are shaded in red. Modifications are abbreviated to represent all derivatives of the modified nucleoside, such as derivatives of $xm^5s^2U$ and $i^6A$. The wobble position 34, and purine-37 modifications (ASL right) have been discussed. Briefly, wobble position uridine modifications that include 2-thiolation ($xm^5s^2U$) can be required for codon binding and are restrictive to binding A and wobbling to G. The 2-thiolation promotes translocation. Modifications of the 5-carbon of uridine-34, in the absence of 2-thiolation, bind the cognate codon ending in A and promote binding to the wobble codon ending in G ($xm^5U$). Oxy-modifications expand wobble beyond G to U ($xo^5U$). $I_{34}$ binds C and wobbles to A and U. $Q_{34}$ promotes binding to the cognate codon ending in C over that ending in U. The position 37 modification $t^6A$ and its derivative $ms^2t^6A$ promote binding to cognate codon and with position 34 uridine modifications ensure wobble codon binding and translocation. A number of position 37 modifications ($i(o)^6A$, $m^1G$) maintain the translational reading frame.

antisuppressor mutations reduced the efficiency of nonsense suppressors in *Schizosaccharomyces pombe* (162). Mutation of the sin4 or sin3 genes led to loss of 5-(methoxycarbonylmethyl)-2-thiouridine ($mcm^5s^2U_{34}$) from the first anticodon position of tRNAs. The major sulfur-carrying nucleoside in wild-type *S.pombe* tRNA is $mcm^5s^2U_{34}$. It was reduced but not devoid in the mutant strains. Two other thiolated nucleosides were also present, $s^2U$ and a nucleoside of unknown structure, and neither was affected by the antisuppressor mutations. Independent from their effect on suppressors, the two mutations sin3 and sin4 reduced the growth rate of cells, and sin3 also increased cell length. *In vivo* decoding of the serine codon UCG by the UCA reading serine tRNA was not promoted by the two antisuppressor mutations (163). Where differences between results *in vitro* and *in vivo* have been found, investigations of double and triple mutants that negate all modifications at 34 and 37 may resolve the discrepancies between experiments *in vitro* and *in vivo* and yield a greater understanding of redundancy. *In vitro* data taken together would suggest that such multiple mutations could be lethal. Interpretation of results from codon binding by modification deficient tRNAs, thus necessitates consideration of the effects of remaining modifications.

Modifications and the enzymes responsible for their syntheses demonstrate biological redundancy and duality of function. For some tRNAs, anticodon stem and loop modifications are important determinants for aminoacylation (164–167), whereas others, such as methylations of the anticodon loop, enhance protein recognition but are not required (101,168,169). Some of the most ubiquitous modification enzymes such as those for Ψ (36) and 2′-O-methylation have multiple substrates, tRNA and rRNA, tRNA and snRNA. But this plurality of substrates is not readily predictable (170). When $\Psi_{55}$ is missing from tRNA of the human pathogen *Shigella flexneri*, there is a decreased expression of several genes associated with virulence (171).

Other modifying enzymes appear to have functions other than modification of RNAs (172). When *E.coli*'s *trmA* gene for the enzyme $m^5U_{54}$-tRNA methyltransferase that synthesizes the highly conserved $T_{54}$ of tRNA's TΨC loop was mutated (173), wild-type cells had a slight advantage in culture but no dramatic physiological result was witnessed (174). However, insertions in the gene produced nonviable cells and the conclusion that the enzyme had multiple functions (175). Similar results of mutant, and missing *trm2* genes for the same enzyme were observed in *Saccharomyces cerevisiae* (176). The *truB* gene product synthesis of $\Psi_{55}$ may not be critical, but contributes to *E.coli*'s tolerance of thermal stress (177). This may be associated with $\Psi_{55}$'s positive effect on the interaction of tRNA's D-loop with its T-loop (33). The *E.coli trmE* gene product involved in synthesis of $mnm^5s^2U_{34}$ also is a GTPase that can be essential under certain conditions (178).

The data on anticodon modified nucleosides, in particular uridines at tRNA's wobble position 34, prompted the composition of a 'Modified Wobble Hypothesis', in which specific base modifications had evolved to select particular codons (22). At the extreme, this hypothesis is exemplified by a wobble position 34 modification restricting an isoleucine tRNA species to reading the isoleucine codon (AUA) and not reading the methionine codon AUG (107). In general, modifications of $U_{34}$ for tRNAs responding to codons in mixed codon boxes restrict codon reading, $s^2U_{34}$, for instance (Fig. 4). We proposed that this is accomplished by 2-thio modified pyrimidines having well defined *anti*, C3′-endo, gauch$^+$ conformations that may also influence 3′-neighboring nucleoside conformations (22). Modifications of tRNAs responding to 4-fold degenerate codes expand wobble (Fig. 4). The modification $cmo^5U_{34}$ enhances wobble by having a less well defined conformation and a tendency to be in the C2′-endo conformer rather than C3′-endo (85). Position 37 purine modifications, such as $m^1G_{37}$, $t^6A_{37}$ and

$(ms^2)i(o)^6A_{37}$, maintain the mRNA reading frame and counter frameshifting, as do some modifications at wobble position 34, and $\Psi_{38,39}$ (Fig. 4) (102,116,121,179–181). The purine 37 modifications open the anticodon loop by negating intra-loop base pairing and order loop nucleosides, thereby potentiating a significant effect on the anticodon and other loop modifications.

## OUTLOOK

With the completion of over 50 genomic sequences, tRNA gene sets are now available for study and comparison. It is no surprise that the number of tRNA gene copies correlate with the expression level of those tRNAs, a specific codon bias by the organism and its amino acid usage, and that particular tRNA gene sequences are conserved, particularly that of initiator methionine (122,182–184). However, it is too early to extract detailed information about modification-dependent decoding from direct comparisons of genomic information. One can conclude that the application of the general wobble rules will be altered when the wobble position 34 modifications of eukaryotic tRNAs are considered in comparison to those of prokaryotes (183). The rapidly increasing genomic information pool is attractive as a base for development of testable hypotheses (122,183). However, empirical evidence, such as that for purine 37 modifications having significant roles in decoding, translocation and reading frame maintenance in both bacterial and eukaryotic tRNAs, precludes developing codon recognition rules based solely on wobble position 34 nucleosides and their possible modifications. With an ever increasing understanding of the recognition determinants for the many enzymes involved in modification of anticodon domain nucleosides, we will be able to accurately predict the modified nucleosides that will be found in the products of these tRNA genes. Though many unmodified tRNAs and ASLs have been found devoid of cognate codon or wobble codon binding *in vitro*, site-specific modification-dependent restoration of cognate and wobble codon binding and translocation of most tRNA species awaits analyses. The effects of selectively incorporating modifications, individually and in combinations need to be assessed *in vitro* and *in vivo*. Anticodon domain modifications are just as likely to affect translation by contributing to the on and off rates of anticodon–codon interaction, as they are to affect the stereochemistry of a correct interaction on the ribosome. The tRNA modifications found in thermophilic and cryophilic organisms and their contributions to anticodon domain conformational dynamics may provide clues to codon selection and translational rates at temperature extremes. Finally, application of technical advances in oligonucleotide chemical synthesis, spectroscopy and crystallography will accelerate our understanding of the physiochemical contributions of tRNA's modified nucleosides to decoding the genome.

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