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The Role of RNA Modifications in Translational Fidelity

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Abstract

The posttranscriptional modifications of tRNA's anticodon stem and loop (ASL) domain represent a third level, a third code, to the accuracy and efficiency of translating mRNA codons into the correct amino acid sequence of proteins. Modifications of tRNA's ASL domain are enzymatically synthesized and site specifically located at the anticodon wobble position-34 and 3'-adjacent to the anticodon at position-37. Degeneracy of the 64 Universal Genetic Codes and the limitation in the number of tRNA species require some tRNAs to decode more than one codon. The specific modification chemistries and their impact on the tRNA's ASL structure and dynamics enable one tRNA to decode cognate and "wobble codons" or to expand recognition to synonymous codons, all the while maintaining the translational reading frame. Some modified nucleosides' chemistries prestructure tRNA to read the two codons of a specific amino acid that shares a twofold degenerate codon box, and other chemistries allow a different tRNA to respond to all four codons of a fourfold degenerate codon box. Thus, tRNA ASL modifications are critical and mutations in genes for the modification enzymes and tRNA, the consequences of which is a lack of modification, lead to mistranslation and human disease. By optimizing tRNA anticodon chemistries, structure, and dynamics in all organisms, modifications ensure translational fidelity of mRNA transcripts.

Keywords

Modifications prestructure tRNA; Wobble position tRNA modifications; Modifications 3'-adjacent to the anticodon; Modification chemistry and structure; Human disease and tRNA modifications; Codon degeneracy and decoding

1 Introduction

1.1 Universal Genetic Code

The Universal Genetic Code of 64 triplet codons encoded in DNA and transcribed into each messenger RNA (mRNA) is degenerate (Fig. 1). As a general rule, 61 codons represent the 20 amino acids during translation on the ribosome. Three codons are read by protein factors as translation stops. Transfer RNAs (tRNAs) decode the mRNA codons while bringing the cognate amino acid to the ribosome for incorporation into the growing peptide chain. The ribosome is one of the most complex of enzymes. With the bacterial and eukaryote decoding or aminoacyl site (A-site) and the peptidyl transferase center (PTC) consisting almost completely of RNA [1,2], the ribosome is an RNA-based enzyme with large RNA substrates.

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Even the mitochondrial ribosome, which is more highly proportioned of proteins, has an RNA-rich decoding and PTC [3]. In humans, the ribosome's substrates include some 25,000 mRNAs for proteins of less than 100 amino acids in length to more than 20,000 amino acids [4]; GTP; a number of protein factors; and some 40 transfer RNAs (tRNA) each aminoacylated with one specific amino acid. The ribosome's enzymatic activity in translating mRNA codons into the amino acid sequences is a "processive" mechanism of action. The ribosome sequentially and covalently links the growing peptide chain to the amino terminus of the incoming amino acid that is bound to its specific tRNA through an ester bond. tRNAs are aminoacylated at their universal 3'-terminal adenosine by amino acid-specific, aminoacyl-tRNA synthetases. The aminoacylated tRNA is brought to the ribosome by a protein, elongation factor, that recognizes that the tRNA is aminoacylated, but is not engaged in codon recognition and specificity.

Codon recognition by aminoacylated tRNA, acceptance and accommodation of the tRNA by the ribosome, peptide bond formation, translocation of the peptidyl-tRNA from the decoding or aminoacyl site (A-site) to the peptidyl-site (P-site), and the movement of unacylated tRNA from the P-site to the exit (E-site) are processive. Without leaving the mRNA template, the ribosome progresses from codon to codon from the mRNA's translational start sequence and first codon AUG for methionine to the 3'- termination codons UAG, UAA, and UGA, and release sequences. The three nucleosides of each aminoacylated tRNA's anticodon recognize a three-nucleotide mRNA codon and bind to the codon in a sequence- and frame-specific manner to deliver the 20 common amino acids for protein synthesis. The aminoacylated tRNA is accepted at the A-site in response to the complementary mRNA codon, and a proofreading mechanism consisting of some nine hydrogen bonds formed between the ribosomal RNA (rRNA), tRNA, and codon ensures a high fidelity in translation. Accurate interaction of the anticodon with codon is very much dependent on the initial Watson-Crick (A●U; G●C) complementarity of the hydrogen bonding of the first two base pairs. Other enzymes having processive mechanisms of action include the DNA- and RNA polymerases. While protein synthesis is accurate to 1 in 10,000 or 20,000 amino acids at a rate of 10–20 peptide bonds formed per second [5,6], DNA- and RNA polymerases have errors of less than 1 in 10^9 at 50–100s of base pairs per second including proofreading steps [7,8] and 1 in 10^5 at a rate of 8–85 bases per second [9,10], respectively, in vivo.

tRNA has to be considered one of the most unique of enzyme substrates. The ribosome structure and its processive catalytic mechanism [2] require the tRNA substrates to have a uniformity in chemistry and structure. Nevertheless, some 40 tRNA molecules, 22 within the human mitochondria, possess enough unique chemical diversity and structural malleability to be recognized by 20 aminoacyl-tRNA synthetases and to decode 61 mRNA codons on the ribosome. How do tRNAs achieve uniformity of chemistry and structure and yet a necessary distinctiveness for protein synthesis? Although seemingly contradictory, tRNAs use posttranscriptional modification chemistries to achieve both a stable structure mandated by the ribosome and the originality required for protein recognition determinants and codon reading.

1.2 Posttranscriptional RNA Modification

All RNAs are involved in activities that contribute directly or indirectly to the regulation, accuracy, and efficiency of gene expression that is fulfilled in translating the mRNA codons into the amino acid sequences of proteins. As in translating one language into another, the accuracy of that translation is dependent not only on a literal word-to-word equivalency but also on context, frame, and punctuation. The enzymatically synthesized, site specifically positioned, posttranscriptional modifications of RNA (Fig. 2) could be considered the punctuation marks in the translation of the genetic code. It is difficult to find among RNA species (mRNA; tRNA; ribosomal, rRNA; small nuclear, snRNA; micro, miRNA; long noncoding, lncRNA) one that is not posttranscriptionally modified [11].

The Universal Genetic Code for the 20 amino acids, the first code, and the operational RNA code for the recognition of tRNA by its cognate aminoacyl-tRNA synthetase, the second code [12], did not take into account the posttranscriptional modifications of RNA, for little was known about them at the time, except that they existed. The ubiquitously occurring, conserved modified nucleosides constitute a yet to be fully appreciated third code in regulating translation, its accuracy, and efficiency [13]. Even with more than half a century of research, more than 100 different posttranscriptional modifications of RNAs remain mostly an undetermined regulating factor to translation [14,15]. The posttranscriptional modification of RNA, designated as the epitranscriptome [16], now usually refers exclusively to mRNAs. mRNAs have a half-dozen different types of modifications identified to date [17]. These represent some of the most common of all modifications, the 2'-O methylations of the four major nucleosides adenosine (Am), guanosine (Gm), cytosine (Cm), and uridine (Um); the isomer of uridine with a carbon-carbon glycosidic bond pseudouridine (Ψ); and the base methylations N^6 -methyladenosine (m^6A), 1-methyladenosine (m^1A), 7-methylguanosine (m^7G), 5-methylcytosine (m^5C), and 5-hydroxymethylcytosine (hm^5C) [18]. Though these are simple chemical alterations, their influence in regulating translation appears to be truly significant [19-21]. We are only just beginning to understand the relevance of mRNA modifications to decoding [22].

In recent years, the coding regions of mRNA have been found punctuated with posttranscriptional modifications [17]. Early in the study of mRNA biochemistry it was recognized that mRNA had modified nucleosides in noncoding regions, such as the 5'-CAP [23]. Some of the mRNA modifications are transient in that methyltransferases “write” a methylation and demethylases “erase” the methyl group while other proteins, as “readers,” recognize the “mark” and bind the RNA, affecting its structure and function [24]. Some have been proven to affect translation and gene expression [25-28]. The biochemical mechanism by which translation is affected is at present speculative. However, nucleobase modifications of nucleosides within the mRNA coding region will alter recognition of codons by tRNA. Any modification of the Watson-Crick hydrogen bonding face of a nucleoside will change tRNA's anticodon base pairing to the codon. Thus, the methyl of an m^6A in an mRNA coding region negates canonical Watson-Crick tRNA base pairing, possibly changing the accuracy and rate of decoding or affecting premature termination, and even the eventual acceptance of the mRNA for translation. The erase mechanism can then functionalize that codon for tRNA reading.

1.3 RNA Modification and the Accuracy and Efficiency of Translation

Accuracy of decoding mRNA at the ribosome's aminoacyl- or A-site depends on tRNA recognition of a complementary mRNA codon. tRNA as the decoder of mRNA is also posttranscriptionally modified for the accuracy and efficiency of reading mRNA codons [11,29-33]. More than 90 different modifications found in tRNAs are site specifically and enzymatically synthesized (Fig. 3). Fully one-quarter of a eukaryotic, cytoplasmic tRNA's nucleosides can be found in a modified form [34,35]. The locations of modifications within tRNA's conserved cloverleaf secondary and "L"-shaped tertiary structures have been known for decades because those sites of modification have been found to be conserved for specific types of modified nucleosides. Modifications outside of the anticodon region are usually of the most common type found in all RNAs, such as 2'-O-methyl, Ψ, m⁷G, and m¹A, but also include modifications that have become synonymous with tRNA structural domains, the dihydrouridine, D, stem and loop and the ribothymidine, T, stem and loop. For the most part, their individual contributions to directing the folding of tRNA into its functioning form have only of late become clearly apparent in physicochemical terms.

tRNAs are also modified in their anticodons and surrounding sequences. In fact, modifications in the anticodon stem and loop domain of tRNAs (ASL) (Figs. 2 and 3) are the most diverse and the most complex chemically and structurally of all modifications found in RNAs [36]. The ASL domain of tRNAs is critical to accurate and efficient translation, and thus, the modifications found in this domain are equally important. ASL modifications contribute directly to the correct and operationally effective decoding of mRNA codons. After the Universal Genetic Code and the operational recognition of tRNA, they constitute a third regulatory level of translational control no less, and probably more important, than those modified nucleosides found in the coding regions of mRNAs.

The 40 some tRNA species outnumber the 20 amino acids, except for instance in the human mitochondria where tRNAs and their genes number 22. Thus, there are fewer tRNA species than there are codons by a considerable amount in the mitochondrion but also in the cytoplasm of any organism. tRNA recognition of a codon through Watson–Crick canonical base pairing is irrespective of the amino acid attached to the tRNA, depending almost completely on the tRNA's anticodon sequence [37,38]. Naturally occurring mutations that give rise to premature stop codons only to be suppressed by tRNAs prove this point. Suppressor tRNAs in responding to premature stop codons in mRNAs are a naturally occurring example of the introduction of an amino acid in which the tRNA anticodon complements a stop codon, not an amino acid codon [39,40]. tRNA complementation of a stop codon has allowed for natural expansion of the genetic code [41] as well as the introduction of nonstandard amino acids via stop codon recognition by tRNA [42].

The efficiency and accuracy of translation in reading mRNA codons is shaped by the base modifications of tRNA's anticodon, the anticodon loop, and the stem [29]. With the codes being degenerate and with fewer tRNA species than the 61 amino acid codes, some tRNAs need to respond to multiple codons. In reading multiple codons effectively, tRNAs recognize exact Watson–Crick complements of their anticodon, G●C, C●G, A●U, and U●A at the first two base pairs. Degeneracy of the codes and limitation in the number of tRNA species predicates recognition of a "wobble" codon at the third base pair [43]. Wobble codon

reading occurs when the nucleoside of the codon is recognized by a nucleoside of the tRNA anticodon other than the complementary base. Such a “wobble” base recognition has been realized for 50 years ever since Crick proposed the wobble hypothesis [43] for the third position of the codes, for instance U●G and G●U, and I●A/U/C [30]. For accurate insertion of the correct amino acid, tRNA discrimination of the third codon base is critically important to “mixed codon boxes” that have more than one amino acid represented. In contrast, some tRNA anticodon nucleoside modifications expand the ability of tRNA species to read as many as all four codons in a fourfold degenerate codon box [44-46]. In this review, we present examples of tRNA anticodon and loop modifications that make possible unique solutions for individual tRNA recognition of a single codon, two synonymous codons, all four codons of a fully degenerate codon box, and the six codons of a single amino acid.

2 Human Mitochondrial tRNA^{Met} Decodes the 1:3 Degenerate Codon Box

2.1 Human Mitochondrial and Cytoplasmic tRNA^{Met}

The mammalian mitochondrial DNA encodes all of the 22 functional tRNAs, one for each of the 18 amino acids and two each for serine and leucine [47] that are required for the synthesis of 13 critical proteins necessary for ATP synthesis and energy production [48,49]. In contrast to cytoplasmic protein synthesis, human mitochondrial tRNA for the amino acid methionine (mt tRNA^{Met}_{CAU} where CAU is the anticodon) is unique in that a single gene codes for the tRNA used for initiation of the translation initiation start codon and for elongation. The universal AUG codon and what is nominally the cytoplasmic isoleucine codon, AUA, are both read as methionine by this one mitochondrial tRNA^{Met}_{CAU}. In the cytoplasm of all cells of all living organisms, two classes of methionine tRNAs decode the only AUG methionine codon: (i) initiator tRNA^{Met}_{CAU} used for initiation of protein synthesis and (ii) elongator tRNA^{Met}_{CAU} that functions to insert methionine into the growing peptide chain. In the cytoplasm, AUA codes for isoleucine and is not used as a methionine codon. However, the AUA codon is present as 20% of the initiator codon in mitochondrial mRNAs and as 80% of internal elongator methionine codons [50,51]. The prevalence of the AUA codon in the mammalian mitochondrial genome indicates that a unique modification exists that allows the recognition of the AUA codon by the single tRNA^{Met}_{CAU} (mt tRNA^{Met}_{CAU}). Although the Crick wobble hypothesis allows for one tRNA to read more than one codon [43], it does not predict the unfavorable base pairing of CAU to AUA due to the COA mismatch. This is overcome by the presence of a unique posttranscriptional modification of tRNA^{Met}_{CAU} at wobble position-34 transforming cytidine to 5-formylcytidine (f⁵C₃₄). This modification is critical for the single mt tRNA^{Met} to recognize both the nonuniversal AUA and the universal AUG codons [52,53]. N-terminal formylation of methionine is important for proper initiation of translation by bacterial and eukaryotic organelle tRNA^{Met}_{CAU}[54,55]. In mitochondria, it is catalyzed by the mitochondrial methionyl-tRNA^{Met} transformylase (MTFMT) [56,57]. The formylated fMet-tRNA^{Met} is used as an initiator tRNA, while the nonformylated methionyl-tRNA^{Met} incorporates methionine into the elongating peptide chain.

Although the basic cloverleaf structure of mammalian mitochondrial tRNAs resembles the canonical tRNAs, they have weaker tertiary interactions in their three dimensional structure due to the lack of several conserved nucleotides that facilitate and contribute to the L-shaped tertiary structure. These features in the human mt tRNA^{Met}_{CAU} include (i) a smaller D-loop that lacks G₁₈ and G₁₉ residues which enables interactions with the T-loop in the tertiary structure; (ii) a Mg²⁺-binding site critical to folding of the full-length tRNA; (iii) a short variable loop lacking G₄₇; (iv) two adjacent pyrimidine:pyrimidine base pairs, UOU and UOΨ in the T-stem; (v) the presence of only six nucleotides instead of seven in the T-stem; and (vi) the absence of TΨC in the T-loop [58,59]. The loss of these conserved residues indicates that the tertiary interactions occurring in the shortened D- and T-loops of hmtRNA^{Met} are weaker and different from canonical cytoplasmic tRNAs [58] with the highly conserved (Type 0) cloverleaf structure (Fig. 4).

2.2 Posttranscriptional Modification of Mitochondrial tRNA^{Met}

Posttranscriptionally modified cytidines have been shown to control switching between the Universal Genetic Code and a deviant code in the isoleucine/methionine codon box. The unique posttranscriptional modification of the wobble position-34 C to f⁵C₃₄ allows mt tRNA^{Met}_{CAU} to engage in unfavorable base pairing of the CAU anticodon to the AUA codon [52,53]. This facilitates the single human mt tRNA^{Met}_{CAU} to be brought to the P-site of the ribosome for translation initiation as well as to interact with mitochondrial elongation factor Tu (EF-Tu_{mt}) and bind to the aminoacyl A-site of the ribosome during peptide elongation. The single modification of f⁵C₃₄ alters the binding kinetics enabling the ASL to bind the Ile codons AUU and AUC, as well as AUA, in the P-site and to initiate translation in mitochondria [59]. An in vitro reconstituted mitochondrial translation system demonstrated that the modified f⁵C₃₄ nucleoside of tRNA^{Met}_{CAU} was critical for the recognition of the nonuniversal AUA codon as methionine [62]. The f⁵C modification is not only found in the mt tRNA^{Met}_{CAU} of bovine and rodents but is thought to be universal in all mammalian mt tRNA^{Met}_{CAU}[53,63,64]. In addition, it is also present in the mt tRNA^{Met}_{CAU} of several other species including nematodes, squids, frogs, chicken, and fruit flies [65-67].

The first reported synthesis and codon binding analysis of the human mt tRNA^{Met} ASL (hmtASL^{Met}-f⁵C₃₄) compared to the unmodified ASL showed that the f⁵C₃₄ modification enhanced the motional dynamics of the loop, destabilizing the ASL [68]. The modification decreased the thermodynamic stability of the RNA, observed as a diminished melting temperature. It also reduced base stacking interactions as seen in the decreased ellipticity of the circular dichroism (CD) spectrum at 270nm. NMR and CD studies made apparent that the modification directed the conformational dynamics of the anticodon bases, facilitating binding to the P- and A-sites. A five-base-paired stem consisting of three G●C/C●G base pairs proximal to the anticodon loop also contributes to the thermodynamic stability of RNA stacking in tRNA^{Met} species, increasing the affinity of initiator tRNA^{Met} to binding to the P-site [59,68,69]. The wobble position f⁵C₃₄ was important for the binding of the hmtASL^{Met}_{CAU} to the AUA codon at the A-site in canonical Watson–Crick geometry, and the rare imino-oxo tautomer of cytidine stabilized the f⁵C₃₄ base pair with two hydrogen bonds [70] (Fig. 5). This is in contrast to the restrictive modification of 4-acetylcytidine (ac⁴C₃₄) that prevents the cytoplasmic tRNA^{Met}_{CAU} from reading the AUA codon [71], and

of lysidine (k^2C) [72] and agmatidine (agm^2C) [73,74] from preventing $tRNA^{Ile}_{CAU}$ from reading AUG in bacteria and archaea.

A recent report demonstrated the synthesis of the 5-formyl group of f^5C through consecutive reactions involving methylation, hydroxylation, and oxidation [75]. The previously uncharacterized methyltransferase, NOL1/NOP2/Sun domain-containing protein 3 (NSUN-3), catalyzed the methylation of C_{34} of mt $tRNA^{Met}$ in the presence of S -adenosylmethionine (AdoMet) to form 5-methylcytidine (m^5C) in the initial step of f^5C generation in human mitochondria. Human embryonic kidney cells lacking NSUN-3 showed reduced mitochondrial protein synthesis and oxygen consumption, causing deficient mitochondrial activity [75]. Mass spectrometric analysis indicated that f^5C_{34} was absent in the NSUN3-KO cells and was replaced by m^5C in these cells.

2.3 Other Human Mitochondrial $tRNA^{Met}$ Properties

Base pairing between f^5C_{34} and A3 of the AUA codon was observed through crystallographic studies of the f^5C_{34} -containing ASL bound to the 30s subunit of *Thermus thermophilus* [70]. The authors concluded that the most likely base pairing occurs due to an exceptional shift in the tautomeric equilibrium of f^5C_{34} from the more common amino-oxo form to the less commonly formed imino-oxo tautomer that enables interaction with A₃ (Fig. 5) [70]. In addition, this study included a structural investigation of the substitution of cytidine for “invariant” uridine at position-33 (C_{33}) as it occurs in mammalian initiator $tRNA^{Met}$. The invariant U_{33} is responsible for the sharp U-turn in the backbone between U_{33} and the nucleoside in the wobble position, allowing anticodon to codon binding. One characteristic of the U-turn is the hydrogen bonding between U_{33} and the phosphate of nucleoside 36 (CAU). A weak interaction was seen between C_{33} and U_{36} , stabilizing the sharp turn of the backbone and base pairing of f^5C_{34} . These studies using an induced-fit model not only resulted in the A-site binding conformation but also suggested a favorable canonical U-turn allowing interaction between C_{33} and U_{36} , permitting it to fit into the P-site in a slightly different conformation [70].

The $hmtASL^{Met-f^5C}$ lacks the universal t^6A_{37} (N^6 -threonylcarbamoyladenosine) modification present in most cytoplasmic methionyl tRNAs and found in almost all tRNAs responding to codons beginning with A (ANN codons) [76]. The A_{37} modification in elongator tRNAs promotes stacking of the bases that stabilize the first anticodon–codon base pairing. The absence of t^6A has been implicated in an increase in +1 and –1 frameshift in cytoplasmic translation, as well as an increase in non-AUG start codons [76]. Since unmodified, $hmtASL^{Met-f^5C}$ forms a stable $U_{36} \bullet A_1$ base pair [70], it is possible that the lack of this modification enables efficient binding to the AUA nonstart codon along with the f^5C_{34} modification that enhances the motional dynamics of the ASL [68]. The unusual intraloop base pairing between C_{32} and the unmodified A_{37} [70] can also promote a more elongator-like conformation; this would expand the codon recognition of f^5C_{34} , allowing a single tRNA to perform both as an initiator and an elongator. The human mt $tRNA^{Met}_{CAU}$ is similar to the cytoplasmic elongator $tRNA^{Met}_{CAU}$ in *Escherichia coli*, whereby they both have a wobble position-34 modification. In *E. coli*, it is N^4 -acetylcytidine (ac^4C), whereas in humans this modification is 2'-*O*-methylcytidine (Cm) [59,77].

2.4 Human Mitochondrial tRNA Disease

Mutations of mitochondrial DNA have been associated with numerous mitochondrial diseases caused by pathogenic mutations in mitochondrial tRNA. Mutations have been found in every mitochondrial gene and are associated with several maternally inherited human genetic diseases. More than 250 mt-tRNA mutations [78] represent a large proportion of all reported mutations leading to disease. Mitochondrial dysfunction caused by point mutations in mitochondrial tRNA is associated with most maternally inherited human genetic diseases. Other mutations affecting tRNA modifications are nuclear-encoded point mutations in the genes encoding mitochondrial tRNA modification. They have been linked to mitochondrial dysfunction and contribute to molecular pathogenesis [79,80]. Three diseases caused by mutations in the gene for human mt tRNA^{Met} arise from a single point mutation: (i) the T4409C mutation leads to a change in the conserved U₈ to C in the corner of the acceptor and D-stems, causing mitochondrial myopathy that results in dystrophic muscles and intolerance to exercise [81]; (ii) the A4435G mutation resulting in an A₃₇ to G₃₇ change in the anticodon loop acts as a modulator of Leber's hereditary optic neuropathy (LHON), a maternally inherited disorder leading to rapid bilateral loss of central vision [82]; and (iii) the G4450A mutation causes the loss of the third G●C base pair in the T-stem, leading to splenic lymphoma, mainly in lymphocytes resulting in abnormal mitochondria, causing severe disruption of the respiratory chain (Fig. 4A; mt tRNA^{Met}) [83].

The single point mutation of T4409C, resulting in a U₈-to-C₈ replacement, in the gene for hmtRNA^{Met}_{CAU} causes mitochondrial myopathy [58,81]. Structural probing and molecular reconstitution experiments revealed that the C₈ substitution leads to a drastic disruption of the tRNA structure, and the loss of one or more critical Mg²⁺ binding sites on the tRNA [58]. The U₈-to-C₈ substitution reduced aminoacylation and resulted in the lack of a formylated mt methionyl-tRNA^{Met}_{CAU}. Thus, correct initiation and the degree of initiation were reduced, which led to the absence of a stable ternary complex formation with eEF1 to participate in chain elongation [58].

The maternally transmitted LHON disease impaired vision of an Asian family of three generations. They had the rare A4435G mutation which is associated with the ND4 G11778A mutation in families of Chinese origin [82]. The A4435G mutation is located in the 3'-end adjacent to the anticodon (A₃₇) of mt tRNA^{Met}_{CAU}. A₃₇ in tRNA^{Met} species is highly conserved from bacteria to human mitochondria, and contributes to high fidelity of codon recognition and structural stabilization of tRNAs. Molecular and genetic analysis of mt DNA identified this novel A₃₇ mutation as contributing to a significant loss of steady-state levels of tRNA^{Met}_{CAU}. The presence of the A4435G mutation increased the expression of the mitochondrial dysfunction associated with the G11778A mutation [82]. In addition, the A4435G mutant has been shown to have reduced m⁵C₃₄ activity, the first step in f⁵C₃₄ biosynthesis, indicating a possible lack of NSUN3, and hypomodified f⁵C₃₄ in these patients [75].

2.5 Summary

Human mitochondria have a unique mechanism to utilize the single hmtRNA^{Met}_{CAU} between initiation of protein synthesis and elongation of the protein chain. The modification

5-formylcytidine at wobble position-34 permits this single tRNA^{Met} to recognize and decode the universal AUG codon as well as the AUA codon as methionine. Codon binding and structural and thermodynamic studies have indicated stable base pairing between the noncanonical CAU and AUA bases at the wobble position, facilitated by the presence of the unique f⁵C₃₄ modification found in the single tRNA. The rare imino-oxo tautomer of the modified cytidine stabilizes this base pairing and expands the use of the AUA codon in human mitochondria. Substitution of C₃₃ for U₃₃ in hmtASL^{Met}_{CAU} supported a C-turn conformation similar to the U-turn conformation seen with the invariant uridine-33. Importantly, the f⁵C₃₄ modification contributes a third element of translational control by enabling wobble base pairing to isoleucine codons.

3 tRNA Decoding of the Twofold, 2:2, Degenerate Codon Box

3.1 tRNA^{Lys} Twofold Degenerate Codons AAA and AAG

Asparagine and lysine share a twofold degenerate codon box in the Universal Genetic Code (Fig. 1) [29]. Their codons differ only in the last base being a pyrimidine, U or C for asparagine, and an A or G for lysine. Aspartic and glutamic acids, and histidine and glutamine share twofold degenerate codon boxes and similarly have codons differing only in the third base, pyrimidine or purine. Though phenylalanine, tyrosine, and cysteine are encoded by two codons each, phenylalanine shares a twofold degenerate codon box with leucine, which is one of the three amino acids encoded by six codons. Tyrosine's two codons differ from two translational stop codons only in the third base, and cysteine's two codons differ only in the third base from a stop codon and that for tryptophan. Thus, the accurate and efficient reading of mRNA codons by the tRNAs for these amino acids requires discrimination at the third base of the codon. The ability of tRNA's first or wobble anticodon base at position-34 becomes all that more important in distinguishing between codons. The number of tRNAs used for the recognition of these codons varies from amino acid to amino acid and from organism to organism [29,84]. Here, we discuss a twofold codon degeneracy using the AAA and AAG lysine codons as examples. The importance of correctly modified tRNA^{Lys}_{UUU} is not limited to accurate translation, because it is also important in maintaining the translational reading frame and translocation on the ribosome. Hypomodified or incorrectly modified mt tRNA^{Lys}_{UUU} has been linked to mitochondrial diseases such as MELAS and MERRF in humans [85], and cytoplasmic tRNA^{Lys}_{UUU} is the primer for reverse transcriptase of all lentiviruses including HIV. Mutations in modification of human tRNA^{Lys}_{UUU} have been associated with a possible mistake in proinsulin translation and are a risk factor in Type 2 Diabetes (T2D) [86,87]. The low enthalpy of the three U●A base pairs in the interaction of tRNA^{Lys}_{UUU} with its cognate codon must be compensated with stabilizing modifications [88]. The modifications present in the ASL are essential for not only codon recognition but additional functions as well [88-90].

E. coli, unlike eukaryotic cells, contains only one tRNA for lysine and it has the anticodon UUU. tRNA^{Lys}_{UUU} recognizes both AAA and AAG codons [88,90]. Archaea, like prokaryotes, for the most part contain but a single tRNA^{Lys}_{UUU} which recognizes both AAA and AAG codons [91]. The number of eukaryotic tRNA^{Lys}_{UUU} species varies among organisms. Mammals such as humans and rabbits contain three species of tRNA^{Lys} that

differ in their anticodons, as well as in nucleoside sequence and modifications. Both tRNA^{Lys1}_{CUU} and tRNA^{Lys2}_{CUU} contain the CUU anticodon which recognizes AAG. tRNA^{Lys3}_{UUU} contains the UUU anticodon capable of recognizing both AAA and AAG with a preference for the former (Fig. 6). In developing tissue, tRNA^{Lys2}_{CUU} may appear in a hypomodified form, sometimes referred to as tRNA^{Lys4}_{CUU}, whereas tRNA^{Lys5}_{CUU} and tRNA^{Lys6}_{CUU} refer to the various stages in between [92].

The smaller genomes of organelles encode fewer tRNAs than the nuclear genome in eukaryotes, approximately 22 in mitochondria and 30 in chloroplasts [94]. Indeed, much like in *E. coli*, mitochondria utilize a single mt tRNA^{Lys}_{UUU} for the recognition of both AAA and AAG codons [95,96]. Additionally, it has been shown that it is feasible to design *E. coli* strains with reduced codon degeneracy, where degenerate codons are replaced with allowed codons [97]. There are some exceptions to the Universal Genetic Code, however. In some species, such as echinoderms and platyhelminthes, the lysine AAA codon in the mitochondria is translated as asparagine, and in starfish mitochondria, mt tRNA^{Lys}_{CUU} is the sole lysine tRNA [98]. One of the most common coding errors for lysine tRNA is mistranslation as asparagine [92,99]. Similar to mammalian mitochondria, chloroplasts utilize a single tRNA^{Lys}_{UUU} for translation [100]. Therefore, tRNA^{Lys}_{UUU} must be able to read both AAA and AAG codons in all organisms where it is utilized. This is made possible by extensive modifications of the ASL (ASL^{Lys}_{UUU}) at positions-34 and -37, in particular [29,88,101].

3.2 Wobble Position Uridine-34 Modifications

In humans and other mammals tRNA^{Lys1}_{CUU} and tRNA^{Lys2}_{CUU}, position-34 contains an unmodified cytosine; these tRNA^{Lys}_{CUU} species read only the AAG codon. tRNA^{Lys3}_{UUU} as well as the bacterial and organelle tRNA^{Lys}_{UUU} contains the UUU anticodon that is capable of decoding both AAA and AAG codons [92,93]. In order for codons ending in a purine to be efficiently read, cognate tRNAs often contain a modified uridine nucleoside at the wobble position, where 5-methyl-2-thiouridine derivatives (xm⁵s²U) are commonly found (Fig. 6) [99,102]. In *E. coli*, the one tRNA^{Lys}_{UUU} contains 5-methylaminomethyl-2-thiouridine at position-34 (mnm⁵s²U₃₄). Similarly, human tRNA^{Lys3}_{UUU} contains 5-methoxycarbonylmethyl-2-thiouridine at position-34 (mcm⁵s²U₃₄; Fig. 6) [90], and mammalian mt tRNA^{Lys}_{UUU} has 5-taurinomethyl-2-thiouridine (τm⁵s²U₃₄; Fig. 6) in the wobble position [96,103,104]. In several bacteria as well as in a murine leukemia cell line, the sulfur in carbon position-2 of uridine-34 can be replaced by selenium in an ATP-dependent process, resulting in a mixture of thiolated and selenated tRNAs for the amino acids glutamate, glutamine, and lysine. The s²U is a required precursor for the reaction to occur, but the mnm⁵ group is not [92].

3.2.1 Functional Significance of tRNA U₃₄ Modifications—The functional significance of U₃₄ modifications is substantial. Many of these modifications are 5-position derivatives of 5-methyl-2-thiouridine, or xm⁵s²U (Fig. 6). The xm⁵s² modifications restrict the conformational flexibility of the anticodon, thus providing translational fidelity [45,96,105]. A complete knockout of U₃₄ modifications is lethal in *Saccharomyces cerevisiae* [106]. Hypomodified tRNA^{Lys}_{UUU} cannot efficiently induce the expected

conformational changes in the 30S ribosomal subunit during translation as it cannot form favorable enough interactions with its cognate codon, causing the translation to stall [88,107]. The xm⁵U modification in bacterial and mt tRNA^{Lys}_{UUU} (mnm⁵U, mcm⁵U, and τm⁵U) at the wobble position stabilizes the invariant U-turn that occurs between U₃₃ and U₃₄. This in turn stabilizes the anticodon–codon duplex [88,108,109]. In prokaryotes, this is achieved through a hydrogen bond between 2′-OH of U₃₃ and the amino group of mnm⁵s²U₃₄[109,110]. The xm⁵U modification is essential for a sure and stable recognition of the AAG wobble codon in addition to the AAA codon. For instance, the mcm⁵s²U₃₄ shifts from the keto to enol tautomeric form in binding to G3, achieving a stable wobble base pair in the Watson–Crick geometry [111]. Due to its contribution to enhanced structural rigidity and preordering of the anticodon loop, mnm⁵s²U₃₄ enables wobble base pairing with G, and thus allows recognition of both codons [88,112].

The mnm⁵U modification in the absence of the s² group does not appear to restrict a U₃₄•pyrimidine interaction. However, the poor base stacking of the UUU anticodon makes such restriction unnecessary as a U₃₄•U3 interaction would abrogate any base stacking between U₃₄ and U₃₅, leading to a free energy penalty that would make codon recognition unfeasible. Thus, the role of the xm⁵U modification is to increase translational fidelity by enhancing purine recognition, but not to restrict pyrimidine recognition; this is perhaps predictable from thermodynamic stabilities [88,113]. NMR studies have shown that a modified wobble base is important for the stacking between anticodon positions-34 and -35, since in the absence of modifications noncanonical and closed conformations are highly populated for the UUU anticodon. However, in human tRNA^{Lys3}_{UUU} the mcm⁵U modification does not seem to enhance base stacking between U₃₄ and U₃₅ in a significant manner [110]. Therefore, the thio modification is required to compensate for the poor stacking of uridine [110]. The 2-thio group allows the ribose of U₃₄ to adopt C3′-endo puckering augmenting the U-turn and thus facilitates binding with purines and restricting recognition of NNY codons [105,110,114-117]. The thio group stabilizes the U-A base pair, but it is mildly destabilizing for U-G base pairs [29,116].

Hypomodified tRNAs, such as the mammalian tRNA^{Lys3}_{UUU} lacking the fully modified mcm⁵s²U at the wobble position, cannot efficiently decode their cognate codons, resulting in codon-specific ribosomal pausing, which in turn effects negative consequences on protein homeostasis [107]. Hypomodified tRNA^{Lys}_{UUU} is defective in translocation from the A- to P-sites of the ribosome [89]. Interestingly though, the hypomodified bacterial tRNA^{Lys}_{UUU} is less frequently misread as asparagine than fully modified tRNA^{Lys}_{UUU}[99,108]. The increased misreading may simply be due to increased efficiency induced by the modifications. The fact that misreading increases for the mnm⁵U-modified tRNA^{Lys}_{UUU} compared to unmodified tRNA, but has the opposite effect for tRNA^{Glu}_{UUC}, implies that the effect of the modification depends on the structural context as well as the chemical nature of the modification [108]. Thus, hypomodification can lead to mistranslation of alternate codons in twofold degenerate codon boxes of the Universal Genetic Code, or to changes in the translational reading frame leading to aberrant protein synthesis.

3.2.2 Modified Nucleoside-Dependent Synonymous Codon Bias at Uridine-34 and Stress—A mechanism of modified nucleoside-dependent synonymous codon bias

regulates gene expression through wobble base pairing at position-34, and is most affected by the uridine-34 modifications. tRNA reading of synonymous codons, those degenerate codons for which the identical amino acid is represented, is “silent” in that the protein is not altered (Fig. 1). Yet, the reading of synonymous codons can lead to different functional outcomes [118-120]. Because tRNA modifications alter tRNA's codon usage or bias, translation can be affected by changes in modifications that support synonymous codon bias in the mRNAs of specific proteins. Various modified aminoacyl-tRNAs are not necessarily prevalent in equal concentration to codons present, nor is the hypomodified state of the wobble-34 modification most favorable for anticodon–codon binding [119]. Also, optimal reading of codons by fully modified tRNA resulting at what appears to be the most advantageous translation rates is not always the most suitable for a particular cell condition. Thus, particular codons are read by hypomodified tRNA with the result that specific proteins harboring these synonymous codons are expressed. This regulation of translation can occur in response to factors external to cells, such as signal transduction, extracellular RNA, environmental factors and contaminants, illegal and legal drug use. The wobble position-34 uridine modifications have been of particular interest in that their codon biases are altered in response to cellular stress, such as oxidative stress [121-123]. These changes in U₃₄ modification are associated with the translation of specific stress response proteins, the mRNAs of which have a particular codon bias, including the recoding of stop codons [124,125]. In this way, tRNA modifications are “reprogrammed” to support a cell's efficient translation of specialized proteins. Selenated or thiolated uridine-34 may help sense damage caused by irradiation [92]. A lack of the U₃₄ thiolation has also been shown to increase sensitivity to oxidative stress in *S. cerevisiae* [126], whereas incomplete U₃₄ modifications did not affect a drug-induced stress response in *S. cerevisiae* compared to controls [107].

3.3 Modifications of Adenosine-37, 3'-Adjacent to the Anticodon

Modifications at position-37 of tRNA are commonly found in a variety of tRNAs, with the type of modification depending on the base found at position-36 [127]. While not essential in all bacteria, the *N*⁶-threonylcarbamoyladenosine (t⁶A) modification at position-37 is found in tRNAs reading ANN codons in all domains of life [88,110,128-130]. It is required for efficient aminoacylation of several tRNAs, tRNA binding to the A-site of the ribosome and codon recognition in ANN codons, efficient translocation, reading frame maintenance, and preventing leaky scanning of initiation codons, and proper recognition of stop codons [88-90,131,132]. The lysine tRNAs of prokaryotes, mammalian mitochondria as well as mammalian tRNA^{Lys3} contain the t⁶A modification [88,110,128]. Adenosine at position-37 can also contain the 2-methylthio-*N*⁶-threonylcarbamoyladenosine (ms²t⁶A) or the cyclic-*N*⁶-threonylcarbamoyladenosine (ct⁶A) [131,133,134].

The t⁶A modification improves the stability of the codon–anticodon duplex through cross-strand stacking with the first base of the codon [88,110]. Furthermore, neither t⁶A nor ms²t⁶A modification results in an increase in stability of free tRNA in molecular dynamics simulations, which would be expected for a modification responsible for stabilizing the codon–anticodon interaction in the ribosome [135]. In fact, the unmodified tRNA^{Lys3}_{UUU} ASL elicits a higher melting temperature and increased stability because of intraloop hydrogen bonding compared to the open loop, t⁶A₃₇-modified ASL^{Lys3}_{UUU}[90].

Pseudouridine (Ψ) at position-39 enhances the stability of both unmodified and t^6A_{37} -modified $ASL^{Lys^3}_{UUU}$, yet the unmodified ASL with or without Ψ_{39} is incapable of binding poly-A programmed ribosomes [90,132].

A model of $tRNA^{Lys}_{UUU}$ with the circular t^6 modification, ct^6A_{37} , at the ribosomal A-site [133] compared to the structure of $tRNA^{Lys^3}_{UUU}$ containing the t^6A modification at the ribosomal A-site [88] predicts the effects of the ct^6A modification on codon recognition and ribosomal function. The ct^6A_{37} modification has been shown to enhance the decoding efficiency much like the native t^6A_{37} modification [133]. In both cases, the modification allows the nucleoside to stack with the first adenosine (A1) of the codon and across the anticodon–codon base pair, thus stabilizing the interaction (Fig. 7). A hydrogen bond between N1 and N11 is found in both cases [88,133], enhancing the base stacking ability of t^6A_{37} with A1 as this causes the formation of a third ring that expands the area of the base [88,90]. The C14-OH group in ct^6A_{37} can form a hydrogen bond with N7 of A1, further stabilizing the interaction [133]. The oxazolone ring is also thought to be planar with respect to the adenine [133]. The threonyl group of t^6A_{37} has rotational freedom about the N11-C12 bond [88]. This, due to steric hindrance, prohibits the incorporation of t^6A into the helix, which in turn together with its tricyclic structure allows the stacking interaction with the first adenine of the codon [88].

3.3.1 Functional Significance of tRNA A_{37} Modifications—In a number of organisms, there is but one $tRNA^{Lys}_{UUU}$ that reads both AAA and AAG codons [88,90,91,94]. In humans and other mammals, $tRNA^{Lys^3}_{UUU}$ decodes AAA and AAG, and is expressed at a ratio of 1:2 with $tRNA^{Lys^{1,2}}_{CUU}$ that reads only AAG [94]. To enhance the affinity of what is an energetically weak UUU-AAA anticodon–codon interaction, the tRNA is heavily modified with U_{34} and A_{37} contributing to binding affinity, translocation ability, and maintenance of the translational reading frame [88,109]. Modifications are necessary in order for $tRNA^{Lys}_{UUU}$ to read the AAG codon [88,89,109]. The t^6A_{37} modification restores cognate codon binding to unmodified $tRNA^{Lys}_{UUU}$, but does not enable translocation of the ASL^{Lys}_{UUU} from the A-site to the P-site of the ribosome [89]. The modification mnm^5U_{34} also restores codon binding but not translocation. Interestingly, the modification s^2U_{34} enhanced translocation, but not to the degree of t^6A_{37} and mnm^5U_{34} together.

Together the modifications enabled cognate and wobble codon binding and resulted in a 25-fold increase in translocation in comparison to the unmodified ASL^{Lys}_{UUU} [89]. Besides its role in translocation, the modification t^6A_{37} is an essential, strong positive specificity determinant of many but not all prokaryote isoleucyl-tRNA synthetases [129]. The modification may also be a recognition determinant of eukaryote and viral proteins. The viral nucleocapsid protein of HIV, NCp7, recognizes t^6A_{37} [136]. Peptides, composed of 15 amino acids and selected to mimic NCp7, not only recognize the modification but are also equally capable of denaturing the modified $ASL^{Lys^3}_{UUU}$ as does the protein [136,137]. Modification of already modified N^6 -adenosine-37 (t^6A_{37} , i^6A_{37}) with the 2-methylthio moiety (ms^2) is found in specific tRNA species in almost all organisms [14]. Mutations in the gene for the enzyme, *Cdkal1*, responsible for ms^2 -group of $ms^2t^6A_{37}$ in human $tRNA^{Lys^3}_{UUU}$ have been shown to be a reliable risk assessment gene for T2D [86,87]. The homozygous recessive mutation of *cdk11* has a 1.50 risk of T2D, comparable to the *brca 1*

and 2 risks of breast cancer. With an incompletely modified tRNA^{Lys3}_{UUU}, responsible for inserting Lys88 positioned adjacent to Arg89 at which a crucial protease cleavage separates the insulin A-chain from the C-peptide, the Lys88 codon may not be read, resulting in a proinsulin processing issue, or aberrant protein.

3.4 Summary

The twofold degeneracy within the Universal Genetic Code requires a distinction in decoding between NNA/G and NNU/C. Nature's solution to the twofold degeneracy is to ensure that tRNA anticodons read the appropriate, amino acid-specific codons and not those of the amino acid or stop codons sharing the first and second codon bases. Here again, modifications contribute a third code to controlling translation of mRNAs. In the decoding of twofold degenerate codons, modifications of tRNA's wobble position-34 add precision to the reading of the third codon base. Keto-enol tautomerism enables strong wobble codon reading in a canonical base pair geometry. Modification of the invariant purine at position-37 prestructures and restricts dynamics of the ASL domain to maintain the translational reading frame. The purine-37 modification is all that more important when the first anticodon-codon base pair is a U●A or A●U. The inherent weakness of these base pairs is counterbalanced by the van der Waals forces of the 3'-adjacent modification, creating a hydrophobic platform that is evident for such tRNAs as tRNA^{Lys}_{UUU}.

4 tRNA Decoding of Codon Fourfold Degeneracy

4.1 Single tRNA Reads All Four Codons of the Prokaryote Fourfold Degenerate Codon Box

The amino acids alanine, leucine, proline, serine, threonine, and valine each claim, at least, an entire set of four codons, a completely degenerate codon box within the Universal Genetic Code (Fig. 1). Within each set of fourfold degenerate codons, the first two nucleosides of the codons are conserved, while the divergence occurs at the third nucleoside. For each of these amino acids within prokaryotic organisms, there is a single corresponding tRNA that can recognize all four of these codons. In prokaryotes, these tRNAs contain the 5-oxyacetic acid modification at the position-34 uridine (cmo⁵U₃₄) (Fig. 8) [138]. Although pyrimidine:pyrimidine base pairs were originally thought to be disallowed under the original wobble hypothesis, tRNAs containing cmo⁵U₃₄ can recognize codons ending in U and even C in addition to codons ending with the canonical A or the traditional wobble G [30,139-142]. The interaction between the cmo⁵U₃₄ modification and the phosphate backbone has been found to promote the C2'-endo conformation of the ribose ring, which subsequently allows for the UOU base pair that would otherwise be too short (Fig. 9) [142].

There are multiple tRNAs for the aforementioned fourfold degenerate amino acid codons, but the tRNA species containing the cmo⁵U₃₄ modification may be sufficient for viability of prokaryotic organisms. Three tRNAs are responsible for decoding four proline codons in *Salmonella enterica*: tRNA^{Pro}_{CGG}, tRNA^{Pro}_{GGG}, and tRNA^{Pro}_{UGG-cmo⁵U₃₄}. When the genes for tRNA^{Pro}_{CGG} and tRNA^{Pro}_{GGG} are deleted, the organism is still viable, indicating the ability of tRNA^{Pro}_{UGG-cmo⁵U₃₄} to read all four proline codons [143]. Furthermore, knockout of *cmoA* and *cmoB*, two genes implicated in the synthetic pathway of the

$\text{cmo}^5\text{U}_{34}$ modification, in a strain of *S. enterica* containing only $\text{tRNA}^{\text{Pro}}_{\text{UGG-cmo}^5\text{U}_{34}}$ resulted in reduced decoding efficiency of the four proline codons by the hypomodified tRNA; the $\text{cmo}^5\text{U}_{34}$ modification, as opposed to the 5-hydroxyuridine 34 (ho^5U_{34}) and 5-methoxyuridine 34 (mo^5U_{34}) modifications, must be vital for recognition of $\text{tRNA}^{\text{Pro}}_{\text{UGG-cmo}^5\text{U}_{34}}$ by near-cognate codons within the *Salmonella* system [143].

In *E. coli*, the $\text{cmo}^5\text{U}_{34}$ -modified $\text{tRNA}^{\text{Ala}}_{\text{UGC}}$ can bind to the GCC codon within the ribosomal A-site, but this binding was found to be seven times less stable than binding to the GCA codon. The rate of GTP hydrolysis during ribosomal translocation with the GCC codon differed from that of the cognate codon only by a factor of 2.5 [144]. In fact, the $\text{cmo}^5\text{U}_{34}$ modification may be necessary in order to prevent the ribosome from stalling in the presence of an anticodon–codon pair in which a UOU or COU base pair occurs at the wobble position. Translocation from the A-site to the P-site of the ribosome did not occur when unmodified *E. coli* $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ was bound to the GUU valine codon [89].

4.2 Eukaryote tRNAs That Wobble in Reading Fourfold Degenerate Codons

While the $\text{cmo}^5\text{U}_{34}$ modification is exclusive to prokaryotic tRNA, eukaryotic tRNA species that decode for the amino acids with fourfold degenerate codons, among others, contain similar modifications at the 5-position carbon of uridine-34 (Fig. 1) [145]. These eukaryotic modifications lack the methoxy group connectivity of $\text{cmo}^5\text{U}_{34}$ yet include a carboxylic acid derivative. Perhaps this resemblance is enough to enhance recognition at the wobble position in more complex organisms, expanding the wobble capacity of eukaryotic tRNAs as well. In *S. cerevisiae*, the 5-carbamoylmethyluridine-34 modification ($\text{ncm}^5\text{U}_{34}$) present in $\text{tRNA}^{\text{Val}}_{\text{UAC}}$, $\text{tRNA}^{\text{Thr}}_{\text{UGU}}$, and $\text{tRNA}^{\text{Ser}}_{\text{UGA}}$ enhances the efficiency of wobble pairing to the GUG, ACG, and UCG codons, respectively [112]. In fact, fully 25% of the *S. cerevisiae* tRNA wobble position-34 uridines are modified to be 5-carbamoylmethyluridine, $\text{ncm}^5\text{U}_{34}$ or $\text{mcm}^5\text{U}_{34}$ for translational accuracy and efficiency. Synthesis of these modifications requires the Elongator complex associated with elongating RNA polymerase II transcription (see chapter “Structures and activities of the Elongator complex and its co-factors” by Kolaj-Robin and Séraphin). The Elongator complex is composed of six elongator protein subunits and appears conserved among eukaryotes. In addition, three protein subunits of the yeast *Kluyveromyces lactis* killer toxin (zymocin) insensitivity genes are involved in this modification [146]. tRNAs with the modifications mcm^5s^2 , $\text{tRNA}^{\text{Glu}}_{\text{UUC}}$, $\text{tRNA}^{\text{Lys}^3}_{\text{UUU}}$, and $\text{tRNA}^{\text{Gln}}_{\text{UUG}}$ are cleaved by zymocin toxin (γ -toxin) at the 3'-side of the modified nucleoside. Resistance to γ -toxin is observed in the modification-deficient mutants [146]. Lack of Elongator activity results in hypomodified tRNA which, in turn, causes defects [147]. ELPC-1 and ELPC-3, the *Caenorhabditis elegans* homologues to the *S. cerevisiae* Elongator complex subunits Elp1 and Elp3p, are essential to the synthesis of the $\text{ncm}^5\text{U}_{34}$ and $\text{mcm}^5\text{U}_{34}$ modifications within nematode tRNAs [148]. The xm^5 - and s^2 modifications are primary to maintaining the translational reading frame and thus accuracy of protein synthesis. As ELPC-1 and ELPC-3 are expressed in ASE neurons, *elpc-1* and *elpc-3* mutants demonstrate defective neuronal function, as exhibited through the inability of the mutants to adapt the expected experience-dependent behavioral changes in response to having previously been exposed to 100mM sodium chloride in nutrient-deficient conditions [148].

4.3 Roles of Both Wobble Positions-34 and -37 Modifications in Reading Fourfold Degenerate Codons

In addition to the $\text{cmo}^5\text{U}_{34}$ modification, the *E. coli* isoaccepting $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ contains another modification on the adenosine adjacent to the anticodon. The N^6 -methyladenosine modification (m^6A_{37}) (Fig. 8), which naturally occurs at position-37 of the $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ species with $\text{cmo}^5\text{U}_{34}$, plays an important role in structurally ordering the ASL for binding to the codon. Modifications at this conserved purine have been proposed to negate intraloop hydrogen bonding, thereby allowing the ASL to attain an open loop structure for binding to the codon, and to simultaneously enhance base stacking (Fig. 8). Base stacking effectively orders the ASL structure [30]. Determination of thermodynamic stability by UV melting of the ASL of *E. coli* tRNA^{Phe} containing the N^6 -isopentenyladenosine (i^6A_{37}) modification at nucleoside-37 supported the hypothesis that an intraloop hydrogen bond was disrupted [149]. Similar results were determined for the ASL of human $\text{tRNA}^{\text{Lys}}_{\text{UUU}}$ containing the N^6 -threonylcarbamoyladenine (t^6A_{37}) modification at position-37 [90].

Differences in thermal stabilities were observed for the fully modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ with $\text{cmo}^5\text{U}_{34}$ and m^6A_{37} in comparison to that of the unmodified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ [45]. While the melting temperatures, T_m , did not differ substantially, the hyperchromicity of the modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ was more than twice that of the unmodified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ [45]. Coupled with the entropic contributions of the modifications, this increased hyperchromicity indicated the increased base stacking, resulting in a more ordered ASL that arose from the presence of the $\text{cmo}^5\text{U}_{34}$ and m^6A_{37} modifications [45]. The increased ellipticity of the fully modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ hairpin in comparison to that of the unmodified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ hairpin, obtained by CD spectroscopy, also confirms the increased base stacking within the ASL of the fully modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ [45].

The fully modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ with $\text{cmo}^5\text{U}_{34}$ and m^6A_{37} decodes the GUA, GUG, GUU, and GUC codons [140]. Within the A-site of the 30S ribosomal subunit, the binding affinities of both the fully modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ and the unmodified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ to each of the four codons were measured [45]. There was a little difference between the dissociation constants for fully modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ and the unmodified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ when binding the cognate GUA codon. Whereas the fully modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ bound to the GUG and GUU codons ($K_d=1.96$ and $1.93\mu\text{M}$, respectively), the unmodified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ did not have measurable dissociation constants for these three valine codons [45]. The K_d for $\text{tRNA}^{\text{Val}}_{\text{UAC}}\text{-cmo}^5\text{U}_{34};\text{m}^6\text{A}_{37}$ binding to the GUC codon remains undetermined [45]. Crystal structures have been elucidated for the binding of the fully modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$ on the small subunit of the ribosome in response to mRNA containing each of the valine codons, including the GUC codon [46]. The previously unexpected UOC wobble pairing in the presence of the $\text{cmo}^5\text{U}_{34}$ modification must occur, despite being a weak interaction.

The $\text{cmo}^5\text{U}_{34}$ and m^6A_{37} modifications play important roles in structuring the ASL domain of the fully modified $\text{tRNA}^{\text{Val}}_{\text{UAC}}$. The comparatively low root-mean square deviation values obtained from NOESY spectra suggest that the fully modified $\text{ASL}^{\text{Val}}_{\text{UAC}}$ is more ordered than unmodified $\text{ASL}^{\text{Val}}_{\text{UAC}}$ due to the steric hindrance encountered by the N^6 -methyl of the m^6A_{37} modification and to the geometry of the $\text{cmo}^5\text{U}_{34}$ modification brought about by hydrogen bonding between the base and the 5-oxy group [45]. Additionally, both

the fully modified and modified ASLs contained some, but not all, defining characteristics of a U-turn at U₃₃, a type of π -turn that confers stability onto the ASL through base-phosphate stacking and hydrogen bonding [45]. With respect to this feature, the ASL^{Val}_{UAC} with cmo⁵U₃₄ and m⁶A₃₇ contains the shorter hydrogen bond between U₃₃ O2' and A₃₅ N7 [45].

4.4 Summary

The existence of a single species of tRNA that can decode each codon of a fourfold degenerate set is undoubtedly advantageous. Referring back to *S. enterica*, the presence of the modified tRNA^{Pro}_{UGG} with cmo⁵U₃₄ in the absence of tRNA^{Pro}_{CGG} and tRNA^{Pro}_{GCG} allows for survival of the organism, albeit at a reduced growth rate [143]. In some cases, the cmo⁵U₃₄ modification may prove absolutely essential, even for reading of cognate codons. For instance, the unmodified tRNA^{Ala}_{UGC} cannot recognize the cognate GCA codon or the near-cognate GCG in accordance with traditional base pairing and wobble pairing, respectively, in the ribosomal P-site [150]. In decoding of the wobble codons ending in G, cmo⁵U₃₄ is crucial [44]. The cmo⁵U₃₄ is apparently in the enol form and facilitates formation of a Watson–Crick geometry when binding G3. An intramolecular hydrogen bonding stabilizes the conformation of the anticodon for codon binding [46].

5 Three Amino Acids Are Encoded by Six Synonymous Codons Each

5.1 Sixfold Degeneracy and Reading of the Arginine Codons

Within the Universal Genetic Code, three amino acids are coded by six different codons: arginine, leucine, and serine. These six synonymous codons always comprise one whole and an additional half of a twofold degenerate codon box, such that ambiguity exists not only in the identity of the base at the third codon position, but in that of the bases at the first and second positions as well (Fig. 1). This poses a chemical challenge for their set of tRNA isoacceptors, which must together recognize and discriminate among all six degenerate codons while sharing the specific structure and chemistry required for their common recognition by a sole cognate aminoacyl-tRNA synthetase and by the ribosomal A-site. In the case of the *E. coli* arginine tRNA isoacceptors, a compelling case may be made that a suite of posttranscriptional nucleoside modifications, combined with the chemistry imparted by the identity of the anticodon nucleosides themselves, provides the necessary identity determinants for these whole-and-split codon box tRNA isoacceptors to achieve their function.

In *E. coli*, the six codons for arginine are decoded by five tRNA isoacceptors [77,151,152]. Usage of these six codons varies widely, from >2% of all codons for the most common CGU and CGC to <0.2% for the very rare AGA and AGG [51,153-155]. Of the four less common codons, three (CGG, AGA, and AGG) are decoded by individual isoacceptors tRNA^{Arg3}, tRNA^{Arg4}, and tRNA^{Arg5}, respectively, while the fourth, CGA, is decoded by the same isoacceptors (tRNA^{Arg1} and tRNA^{Arg2}) that also decode the two most common codons CGU and CGC [77]. The primary sequences, and therefore chemical identities, of the *E. coli* arginine tRNA isoacceptors differ between members of the family; this is particularly true in the region of the anticodon, which must vary in its sequence among isoacceptors to account for the variation in the first and third nucleosides in the six synonymous arginine codons

(Fig. 10). Despite these chemical differences, sufficient chemical similarity must exist to permit the single *E. coli* arginyl-tRNA synthetase (ArgRS) to charge all five isoacceptors [156]. Furthermore, three of the isoacceptors contain bases in the first position of the anticodon that theoretically permit wobble recognition of near-cognate codons, an expansion tRNA decoding capacity that appears to be either regulated or disallowed in vivo [77]. These conflicting chemical requirements—the recognition of a variety of mRNA codons, sufficient similarity to enable charging by ArgRS, and functional regulation of wobble decoding—are facilitated by a suite of isoacceptor-specific posttranscriptional nucleoside modifications.

5.2 Modification-Dependent tRNA Reading of the Arginine Codons

A number of chemical signatures and naturally occurring posttranscriptional modifications are in fact common to all or nearly all of the *E. coli* tRNA arginine isoacceptors. A highly conserved adenosine occupies position-38 at the 3'-terminus of the tRNA's ASL domain in all five isoacceptors, and thus, a mismatch base pair between A₃₈ and C₃₂/s²C₃₂ can be formed [77,138]. These mismatches typically feature a stable hydrogen bond between the O2 of C₃₂ and the N6 of A₃₈ and may, together with the invariant uridine at position-33, be important for permitting the ASL to adopt the “U-turn” structural motif for correct tRNA recognition in the ribosomal A-site [110,138,157,158].

A conserved cytidine at position-35, the second position of the anticodon, is also common to all five *E. coli* tRNA isoacceptors for arginine [138]. C₃₅, positioned to form a Watson–Crick base pair with the invariant guanine that occupies position two of all six arginine codons, is known to provide at least a portion of the common chemical signature that serves as a strong identity determinant for aminoacylation; it is recognized through backbone interactions with ArgRS His22 and His23 and a stacking interaction with Trp569 [159]. Structurally, because C₃₅ must be exposed to the solvent in order to make specific contacts with the ArgRS backbone and/or side chains, but must also accommodate ribosomal binding and mRNA decoding, the ASL domain of arginine tRNA isoacceptors must be significantly deformable. A significant conformational change must occur between binding to the aminoacyl-tRNA synthetase and adopting the canonical U-turn. This deformability is, in fact, visible in a comparison between the ribosome-bound and synthetase-bound structures of the tRNA, which differ considerably in the ASL region [159,160]. Elements of their respective modification schemes are also common among the arginine tRNA isoacceptors. Each has a modified purine at position-37, either a 2-methyladenosine (m²A; tRNA^{Arg1} and tRNA^{Arg2}), an N⁶-threonylcarbamoyladenine (t⁶A₃₇; tRNA^{Arg3} and tRNA^{Arg4}), or a 1-methylguanosine (m¹G; tRNA^{Arg5}) [77,138].

The two *E. coli* isoacceptors responsible for decoding not only the common CGU and CGC codons but also the seldom-used CGA sequence are tRNA^{Arg1}_{ICG} and tRNA^{Arg2}_{ICG}. These isoacceptors, which have identical primary sequences and differ only in that tRNA^{Arg1}_{ICG} lacks the adenosine nucleotide at position-20 (A₂₀) in its dihydrouridine loop and contains the rare modification 2-thiocytidine at position-32 (s²C₃₂), are sometimes referred to jointly as tRNA^{Arg1,2}_{ICG} [161,162]. Interestingly, both A₂₀ and s²C₃₂ are present in the other three arginine isoacceptors, tRNA^{Arg3,4,5} [77,161,162]. Both tRNA^{Arg1}_{ICG} and tRNA^{Arg2}_{ICG} also contain posttranscriptional nucleoside modifications at position-34 (inosine, I₃₄) and

position-37 (2-methyladenosine, m^2A_{37}), which have also been shown to play significant roles in allowing $tRNA^{Arg1,2}$ to achieve its functionality [77,138]. In $tRNA^{Arg1}$, the absence of A_{20} from the molecule's dihydrouridine loop has been shown to negatively affect aminoacylation efficiency [163]. Mutant $tRNA^{Arg2}$ transcripts in which the expected A_{20} is either substituted or deleted result in a 370-fold decrease in aminoacylation activity by ArgRS, suggesting that $tRNA^{Arg1}_{ICG}$ is constitutively aminoacylated and available to participate in translation at a lower rate of efficiency than $tRNA^{Arg2}_{ICG}$ [164].

5.3 The Modification 2-Thiocytidine Modulates Inosine Reading of A, U, and C

The unusual 2-thiocytidine modification also plays a role in differentiating between the properties of the $tRNA^{Arg1}$ and $tRNA^{Arg2}$ isoacceptors. s^2C_{32} is a relatively uncommon modification, present only in a subset of bacterial and archaeal species; in *E. coli*, it can be found in four of the five arginine tRNA isoacceptors ($tRNA^{Arg1,3,4,5}$) and $tRNA^{Ser}_{GCU}$ at position-32 in the ASL [14,77]. Functionally, the presence of s^2C_{32} was first demonstrated to promote reading frame maintenance and to reduce the rate of tRNA selection into the ribosomal A-site and was suggested to play a role in improving translational accuracy by reducing the speed of translation, particularly during suboptimal growth conditions [165]. A series of ribosomal A-site binding assays using differentially modified $tRNA^{Arg1,2}$ ASLs later definitively demonstrated that the $ASL^{Arg1,2}_{ICG}$ containing either a single s^2C_{32} or m^2A_{37} modification cannot efficiently wobble decode CGA, while the unmodified $ASL^{Arg1,2}_{ICG}$ recognizes, per the expectations of the wobble hypothesis, CGC, CGU, and CGA [43,166]. This experiment definitively established the role of s^2C_{32} in $tRNA^{Arg1,2}$ as restricting the ability of the $tRNA^{Arg1}_{ICG}$ isoacceptor to decode the rarest of its three wobble codons [166].

The functional difference imparted by the s^2C_{32} modification in $tRNA^{Arg1}_{ICG}$ cannot be explained by structural differences in solution; NMR structural analysis of the differentially modified $tRNA^{Arg1,2}_{ICG}$ ASL constructs employed in the ribosomal binding assay showed that neither the presence of s^2C_{32} nor of m^2A_{37} altered the solution structure of the $ASL^{Arg1,2}_{ICG}$ significantly from the highly stable 5'-U₃₃NCG₃₆-3' motif observed for the unmodified ASL and found as a tetraloop [166-168]. When a subset of the $ASL^{Arg1,2}_{ICG}$ constructs were crystallized bound to their cognate mRNA in the context of the 30S bacterial ribosomal subunit, they adopted the characteristic U-turn structural motif but failed to indicate any significant source of structural difference among them (W. Cantara, personal communication). However, biophysical characterization suggested that s^2C_{32} contributions to stability and thermodynamics properties, reducing the melting temperature of the $ASL^{Arg1,2}_{ICG}$ hairpin, increasing the free energy of folding, and decreasing base stacking interactions compared to the unmodified ASL [166]. A computational analysis of the possibly dynamic effect of s^2C_{32} on the properties of $tRNA^{Arg1,2}_{ICG}$ in its mRNA-bound context on the ribosome led to the suggestion that the presence of a purine:purine I₃₄-A₃ basepair in the A-site wobble position of the unmodified $tRNA^{Arg1,2}_{ICG}$ distorts the U-turn from its canonical structural motif and precipitates the formation of a network of water molecules that stabilize the distorted U-turn. Subsequent substitution of the larger, less electronegative sulfur atom for oxygen in the position-32 cytosine disrupts this hydrogen bonding network, such that the unconventional distorted U-turn cannot be simultaneously

maintained within the geometric constraints of a large purine:purine I₃₄-A₃ base pair (S. Ranganathan and S. Vangaveti, personal communication).

The posttranscriptional modification of some tRNA^{Arg1,2}_{ICG} transcripts with s²C₃₂ provides the cell with a regulatory strategy to address the allotment of cellular resources to the translation of the rare codon CGA. Of the two isoacceptors tRNA^{Arg1} and tRNA^{Arg2}, only tRNA^{Arg2}, bearing an unmodified C₃₂, is capable of decoding CGA [166]. Under typical circumstances, when CGA-containing mRNA transcripts are rare, the tRNA^{Arg1,2} population may be regulated at the level of posttranscriptional modification to favor s²C₃₂-modified tRNA^{Arg1} and the decoding of CGC and CGU only; under stress responses or in other circumstances where a more robust response to CGA is required, the modification of tRNA^{Arg1,2} transcripts can be reduced, favoring tRNA^{Arg2}.

Although the tRNA^{Arg1,2}_{ICG} isoacceptors have the ability, depending upon modification profile, to decode three codons, the remaining three isoacceptors recognize only one codon each [169,170]. Two of these, tRNA^{Arg3} and tRNA^{Arg5}, contain the anticodons CCU and CCG, respectively, and, consistent with the expectations of the wobble hypothesis, do not form non-Watson–Crick wobble base pairs with any nucleosides other than guanosine at the third position of their cognate codons AGG and CGG [43,77]. Both isoacceptors contain a modified purine at position-37 and a 2-thiocytidine modification at position-32 [138]. While the function of s²C₃₂ in these arginine tRNA isoacceptors is not known and cannot be accounted for by a need for the restriction of wobble decoding, the position-37 purine modifications t⁶A₃₇ and m¹G₃₇ have been, respectively, shown to order the ASL domain in an open conformation for the formation of the U-turn motif and to prevent translational frameshifting while enhancing codon– anticodon affinity [90,111,171,172].

5.4 tRNA^{Arg} Species Decoding a Twofold Degenerate Codon Box

tRNA^{Arg4}_{UCU}, however, provides another example in which nucleoside identity and modification scheme combine to achieve specific functional results. A wide variety of bacterial and archaeal species contain verified tRNA^{Arg} species with UCU anticodons for the decoding of the codon AGA; candidate sequences for an equivalent tRNA species in several eukaryotic genomes, while not yet confirmed experimentally, have also been identified [77,173]. The *E. coli* tRNA^{Arg4}_{UCU}, known to be solely responsible for the decoding of the codon AGA in that species, contains four endogenous posttranslational modifications: 2-thiocytidine at position-32 (s²C₃₂); 5-methylaminomethyluridine at wobble position-34 (mnm⁵U₃₄); t⁶A₃₇; and Ψ₄₀[77,174]. tRNA^{Arg4}_{UCU} shares with the bacterial tRNA^{Lys}_{UUU}, which decodes both AAA and AAG, a nearly identical ASL sequence and both the mnm⁵U₃₄ and t⁶A₃₇ modifications [77]. Although the well-studied human tRNA^{Lys}_{UUU} does not share s²C₃₂ or Ψ₄₀, its biophysical properties are otherwise so similar to those of *E. coli* tRNA^{Arg4}_{UCU} that it was expected that functional properties of two tRNA species would likewise resemble each other [111]. However, it was found that in tRNA^{Arg4}_{UCU}, the mnm⁵U₃₄ is required for this isoacceptor to recognize the rare codon AGA, but does not allow wobble pairing to AGG. In contrast, mnm⁵U₃₄ in *E. coli* tRNA^{Lys}_{UUU} demonstrably confers the ability to read both AAA and AAG codons [132,175]. The only differences in the loop sequences of *E. coli* tRNA^{Lys}_{UUU} and

tRNA^{Arg4}_{UCU} lie in the identity of the second anticodon nucleoside (C₃₅ for tRNA^{Arg4}; A₃₅ for tRNA^{Lys}) and the s²C₃₂ modification in tRNA^{Arg4}_{UCU}[138]; like tRNA^{Arg1,2}_{ICG}, it was initially thought that the presence of s²C₃₂ would serve an inhibitory role in AGG recognition [166]. However, A-site codon-specific ribosome binding assays performed on three synthesized tRNA^{Arg4}_{UCU} ASL constructs (unmodified ASL^{Arg4}_{UCU}, singly modified ASL^{Arg4}_{UCU}-s²C₃₂, and triply modified ASL^{Arg4}_{UCU}-mnm⁵U₃₄;t⁶A₃₇;Ψ₄₀) showed that only the triply modified ASL bound AGA with a physiological dissociation constant (188±21nM), while no construct, with or without modifications, bound AGG (W. Cantara, personal communication).

While the presence of s²C₃₂ surprisingly appears not to restrict the wobble interaction of tRNA^{Arg4} with the AGG codon as it does in tRNA^{Arg1,2}, biophysical analysis shows that its modification suite contributes to a reduction in base stacking and overall molecular order, similar to effects observed in the fully modified ASL of ASL^{Lys}_{UUU}. Preliminary NMR analysis further indicated that modifications modulate the p*K*_a of A₃₈ to stabilize the noncanonical C₃₂○A₃₈ base pair and enhance cognate decoding by enhancing the base stacking interactions between G₃₁ and s²C₃₂. In this instance, although modifications clearly affect the thermodynamic properties and overall function of tRNA^{Arg4}_{UCU}, the chemical determinant for the ability of tRNA^{Arg4}_{UCU} to discriminate between A- and G-ending codons lies in the presence of modifications together with the sequence identity of the nucleotide, C₃₅, occupying the second position of the anticodon (W. Cantara, personal communication).

The *E. coli* tRNA arginine isoacceptors provide an excellent example of a system in which the chemical identity of the nucleotides comprising the RNA, together with their posttranscriptional modifications, operates in tandem to achieve several functional goals for the family of isoacceptors: discrimination between six chemically distinct arginine codon from whole- and split-codon boxes and differing from each other at their first and third nucleotide positions; universal recognition for amino acid charging by a single arginyl aminoacyl-tRNA synthetase; and regulation of wobble decoding to influence the ability of the cell to decode the rare codons CGA, AGA, and AGG. For example, the modification state of tRNA^{Arg1,2}_{ICG} regulates whether the isoacceptor decodes all three codons to which it theoretically wobble pairs or only to the two common codons (CGU and CGC) [166]; this regulatory role for modifications counteracts the need for multiple isoacceptors to decode the common and rare codons and be recognizable to the aminoacyl-tRNA synthetase, while also allowing the translation of CGA-containing genes to be regulated by a single modification enzyme under appropriate cellular conditions. It is also clear from the tRNA^{Arg} family of isoacceptors that modifications perform different roles depending upon their chemical environment; the rare s²C₃₂ modification, for example, has a clear inhibitory effect on wobble decoding of tRNA^{Arg1,2} but permits and enhances cognate codon binding for tRNA^{Arg4}_{UCU}[166]. Such dual functionality may also exist for mnm⁵U₃₄ in tRNA^{Arg4}_{UCU} and tRNA^{Lys}_{UUU} to explain their respective abilities to inhibit or allow wobble decoding.

5.5 Summary

Sixfold degenerate codons across two codon boxes differ not only in the nucleoside occupying their wobble positions but also in the nucleoside present in their first (and, for serine, second) positions. The tRNA isoacceptors decoding these degenerate codons must simultaneously recognize and discriminate between these divergent codons while remaining universally recognizable to a single aminoacyl-tRNA synthetase and able to bind the ribosomal A-site. In *E. coli*, posttranscriptional modifications assist the five arginine tRNA isoacceptors in accomplishing distinguishing their cognate and wobble codons. In the tRNA^{Arg1,2}_{ICG} isoacceptors, the variable presence of a 2-thiocytidine at position-32 inhibits the ability of the tRNA to wobble decode the rare arginine codon CGA due to steric and hydrogen bonding effects resulting from the replacement of an oxygen with a larger and less electronegative sulfur atom. tRNA^{Arg4}_{UCU}, by contrast, is also restricted in its wobble recognition of the near-cognate AGG codon, but depends for this effect not on its s²C₃₂ modification but on the chemical identity of the codon nucleosides together with the presence of four endogenous posttranscriptional modifications. The *E. coli* arginine tRNA isoacceptors provide an excellent example of the functional specificity conferred by posttranscriptional modifications acting in unique local chemical environments, as well as pointing out how RNA modification-based strategies of cellular regulation might influence the cell's response to codon bias and the translation of rare codons.

6 Posttranscriptional Modification of tRNA Is Required for Accurate and Efficient Decoding

Posttranscriptional modifications evolved and were conserved at tRNA nucleoside positions-34 and -37 to both expand and restrict the capability of isoaccepting tRNAs to read cognate and wobble codons. Though the effects are opposite in function, in both cases the modifications ensure translational fidelity of mRNA transcripts. These site-specific, context-dependent roles for tRNA modifications emphasize their ability to fine-tune tRNA molecular function through chemistry and conformational dynamics in control of gene expression [29,31,45]. This occurs even to the extent that a single modification may perform different functions depending on the sequence and chemical context of the ASL [108].

In their use of modification chemistries, tRNA molecules achieve the simultaneous goals of structural uniformity and ASL sequence variety through the use of a conformational dynamics and chemistries. tRNA structural uniformity is prescribed by the constraints of the ribosomal translational machinery [2,176,177]. Every tRNA species must have ASL domain structures that conform to the spatial limitations and chemistries of the A- and P-sites of the ribosome. To be productively accepted into the decoding site, the backbone chemistries of tRNA's ASL must be structured to engage in stable hydrogen bonding to the rRNAs, bacterial 16 and 23S RNAs, and eukaryote 18 and 28S RNAs. Yet, within the variety of their individual sequence chemistries, each of approximately 40 tRNAs needs to read one or more of the 61 amino acid codons. The individual chemistries, structures, and conformational dynamics required for accurate and efficient decoding of mRNA are provided to the tRNA in the form of prestructuring the ASL domain with modified nucleosides [111].

Instances in which a codon box is split between multiple amino acids (Fig. 1) exemplify how modifications prestructure a tRNA to distinguish the correct codon(s) from the incorrect codon(s). As described, mt tRNA^{Met} modifications allow this one tRNA to read AUG and AUA codons as the initial and internal methionine codes. The tRNA^{Val}_{UAC} cmo⁵U₃₄ and m⁶A₃₇ modifications are crucial for accurate codon recognition of fourfold degenerate codons. Prokaryote and mammalian tRNA^{Lys}_{UUU} are fully functional in accurate codon recognition, translocation, and maintenance of the reading frame only when fully modified. When the first and second codon bases are shared as they are for lysine and asparagine codons, the introduction of a modification at wobble position-34, such as the 2-thiouridine-34, enables the tRNA^{Lys}_{UUU} to bind the AAA and AAG lysine codons while hindering recognition of the asparagine AAC and AAU codons [150]. This level of translational regulation through the contribution of modified nucleosides leads to the accurate binding of the anticodon to the cognate and wobble codons within the ribosomal A-site and peptide bond formation. There is still opportunity for the anticodon-codon mismatch within the P-site to trigger early termination of translation [6].

Modified nucleosides are required of tRNA^{Arg} species for accurate distinction of sixfold degenerate codons and modulation of wobble position inosine-34 recognition of A, U, and C. The sixfold degenerate codons for leucine claim the entire CUN codon box and share the UUN (where N is A, C, G, or U) mixed codon box with phenylalanine. MiaA, a tRNA isopentenyltransferase implicated in the biosynthesis of the N⁶-isopentenyl-2-methylthioadenosine-37 (ms²i⁶A₃₇) modification found in tRNA^{Leu}_{UAA} and tRNA^{Leu}_{CAA} [178], is required for the accurate translation of the leucine-containing *ipoS* and *iraP* gene products in *E. coli*, whose mRNA transcripts code for leucine using the complementary UUA and UUG codons [179].

Nature rarely discards chemistries that prove successful for one purpose only to be advanced and duplicated for another purpose. In doing so with RNA-modified nucleosides, the value has been conserved. Through site-specific placement and structure/activity relationships, modifications evolved to optimize translational accuracy and efficiency. The ASL was optimized with modifications that prestructures conformation, lowers energy barriers, and negates the need to refashion the chemistry, structure, and dynamics for codon recognition and ribosome binding. The first reportings of modified nucleosides in RNA in general, and in tRNA (s-RNA) in particular, occurred over 50 years ago [180]. The modifications of tRNA's ASL domain are the most studied and understood. Yet, scientists worldwide continue to gain insight into how this encoded third level of translational control within tRNA and other RNAs provide accurate, efficient, and responsive decoding of the Universal Genetic Codes.

Acknowledgment

Our review emphasizes and cites some of the most recent findings regarding the chemistry, structure, and functions of posttranscriptional RNA modifications, focusing on tRNA's anticodon domain. As in any such digest of work, we apologize for inadvertently missing the contributions of some, particularly since interest in RNA modifications has exponentially increased and publications are in a large variety of journals. The summation presented here was only possible through half a century of collaborations that we have had with undergraduate and graduate students, postdoctoral fellows, and colleagues during a long and continuing career supported by grants from NIH, NSF, and

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		Second											
		U		C		A		G					
First	U	xi^6A_{37}	UUU	Phe	Gm_{34}	UCU	Ser	xm^5U_{34}	UAU	Tyr	UGU	Cys	U
		m^1G_{37}	UUC	Phe	Gm_{34}	UCC	Ser	xm^5U_{34}	UAC	Tyr	UGC	Cys	C
			UUA	Leu	xm^5U_{34}	UCA	Ser	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	UAA	Stop	UGA	Stop	A
			UUG	Leu	xm^5U_{34}	UCG	Ser	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	UAG	Stop	UGG	Trp	G
	C	m^2A_{37}	CUU	Leu		CCU	Pro	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	CAU	His	CGU	Arg	U
		m^1G_{37}	CUC	Leu		CCC	Pro	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	CAC	His	CGC	Arg	C
			CUA	Leu		CCA	Pro	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	CAA	Gln	CGA	Arg	A
			CUG	Leu		CCG	Pro	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	CAG	Gln	CGG	Arg	G
	A	t^6A_{37}	AUU	Ile		ACU	Thr	$\text{xm}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	AAU	Asn	AGU	Ser	U
		m^6A_{37}	AUC	Ile		ACC	Thr	$\text{xm}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	AAC	Asn	AGC	Ser	C
			AUA	Ile	f^6C_{34}	ACA	Thr	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	AAA	Lys	AGA	Arg	A
			AUG	Met		ACG	Thr	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	AAG	Lys	AGG	Arg	G
	G	m^6A_{37}	GUU	Val	$\text{xm}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	GCU	Ala	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	GAU	Asp	GGU	Gly	U
		m^1G_{37}	GUC	Val	$\text{xm}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	GCC	Ala	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	GAC	Asp	GGC	Gly	C
		m^2A_{37}	GUA	Val	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	GCA	Ala	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	GAA	Glu	GGA	Gly	A
			GUG	Val	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	GCG	Ala	$\text{xo}^5\text{U}_{34}; \text{xm}^5\text{U}_{34}$	GAG	Glu	GGG	Gly	G

FIG. 1.

The Universal Genetic Code. Twofold degenerate codons are highlighted in *tan*; threefold (Ile) in *gray*, fourfold in *yellow*, and sixfold in *blue*. The single codons of Met and Trp are highlighted in *green* and *orange*, respectively, and the three stop codons are highlighted in *red*. The figure is annotated with the abbreviations for those modified nucleosides found in the anticodon domain of tRNAs responding to the codons and discussed in this review. The chemical structures and full names of the modifications are found in Fig. 3.

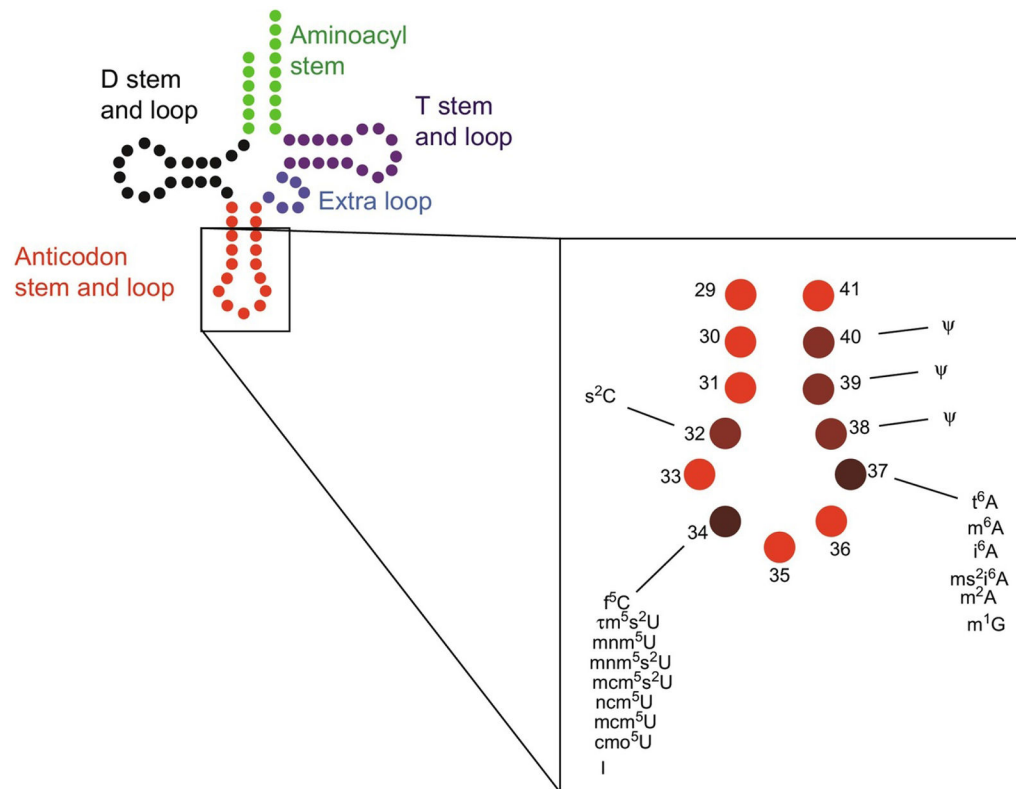


FIG. 2. tRNA and its anticodon stem and loop (ASL) domain. *Left:* General cloverleaf structure of a 76-nucleotide tRNA with the aminoacyl-accepting stem in *green*, dihydrouridine (D) stem and loop in *black*, ASL in *red*, extra loop in *blue*, and ribothymidine (T) stem and loop in *plum*. *Right:* An enlargement of the ASL domain, with the abbreviations of the important modifications discussed in this review listed. A *darker color hue in plum* represents more important modification sites.

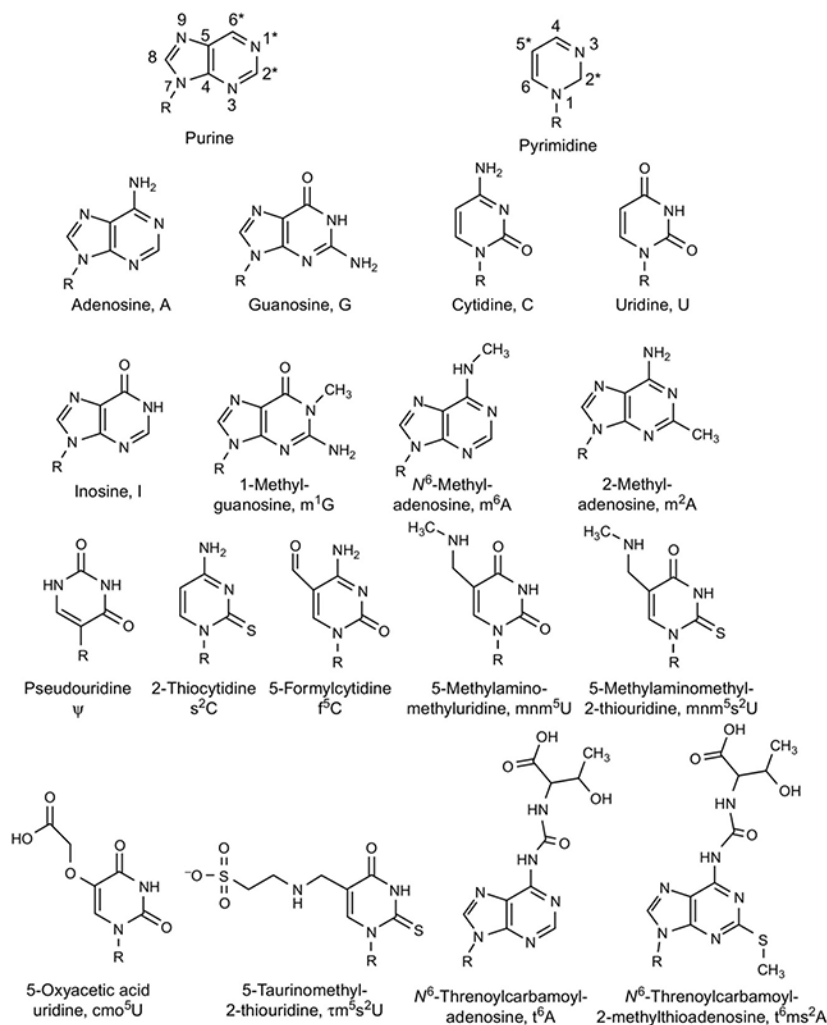


FIG. 3. Chemical structures of the major and modified nucleosides of tRNA. *Top row:* General structures of purine and pyrimidine nucleosides with numbering of the atoms. R represents ribose and the asterisk (*) marks the most important and/or frequent sites of modification. *Second row:* Conventional RNA nucleosides and their letter abbreviations. *Third through fifth rows:* The modified nucleosides discussed in this review with their shorthand notions [14,15].

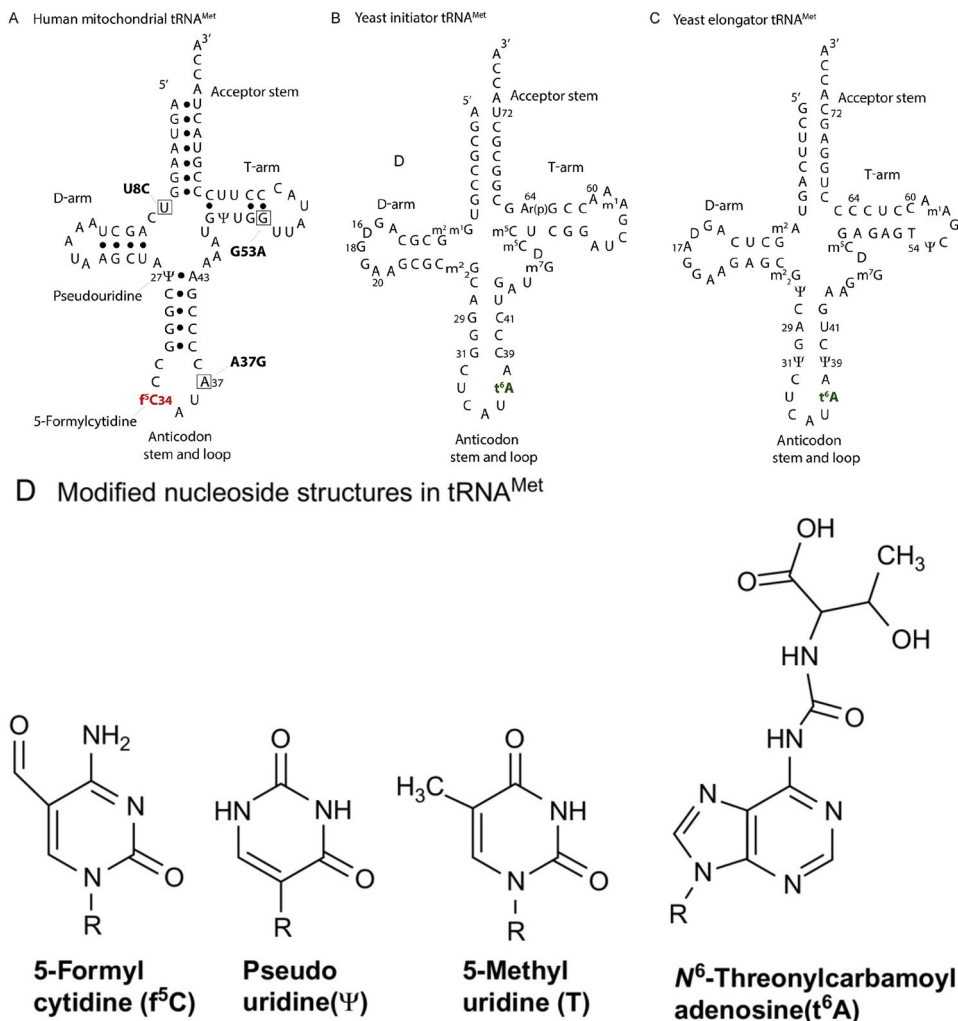
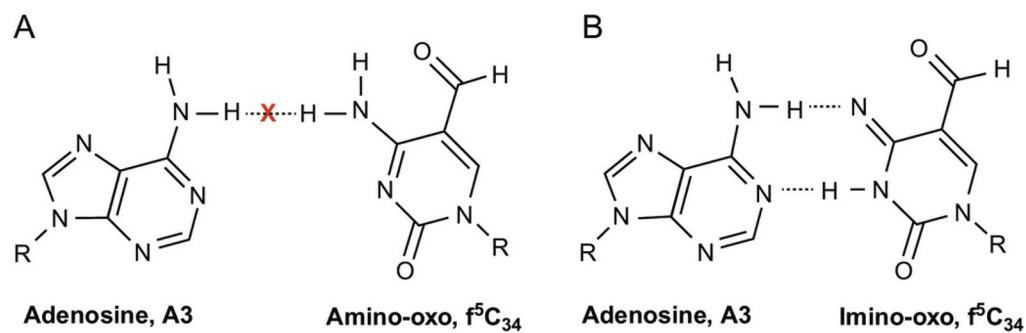


FIG. 4. Nucleoside sequence and cloverleaf structures of tRNA^{Met}. (A) Human mitochondrial tRNA^{Met} displaying the condensed variable loop and the shortened D- and T-arms. Nucleotides affected by disease-causing point mutations are *boxed*. (B) *Saccharomyces cerevisiae* initiator tRNA^{Met}. Ar(p) at position-64 refers to the 2'-O-ribose phosphate modification, which is an identity element of initiator tRNA^{Met} in yeast [60]. (C) *S. cerevisiae* elongator tRNA^{Met}; 5-methyluridine at position-54, indicated by T, is an important determinant of elongator tRNA^{Met}. (D) Chemical structures of the modifications f⁵C₃₄, Ψ at various noted positions, and t⁶A₃₇ found in the three methionyl tRNA [61].

**FIG. 5.**

The tautomeric forms of 5-formylcytidine, f⁵C₃₄. Tautomerism of f⁵C₃₄ allows for the stereochemistry of the Watson–Crick base pairing between tRNA's f⁵C₃₄ and A3 of the AUA codon to approximate that of a canonical U●A pair. (A) Steric repulsion between the common amino-oxo form of f⁵C₃₄ N4H and A₃N⁶H marked by an X. (B) Favorable interactions between the imino-oxo form of f⁵C₃₄ and A₃[70]. *Dotted lines* indicate hydrogen bonding between nucleoside residues [70].

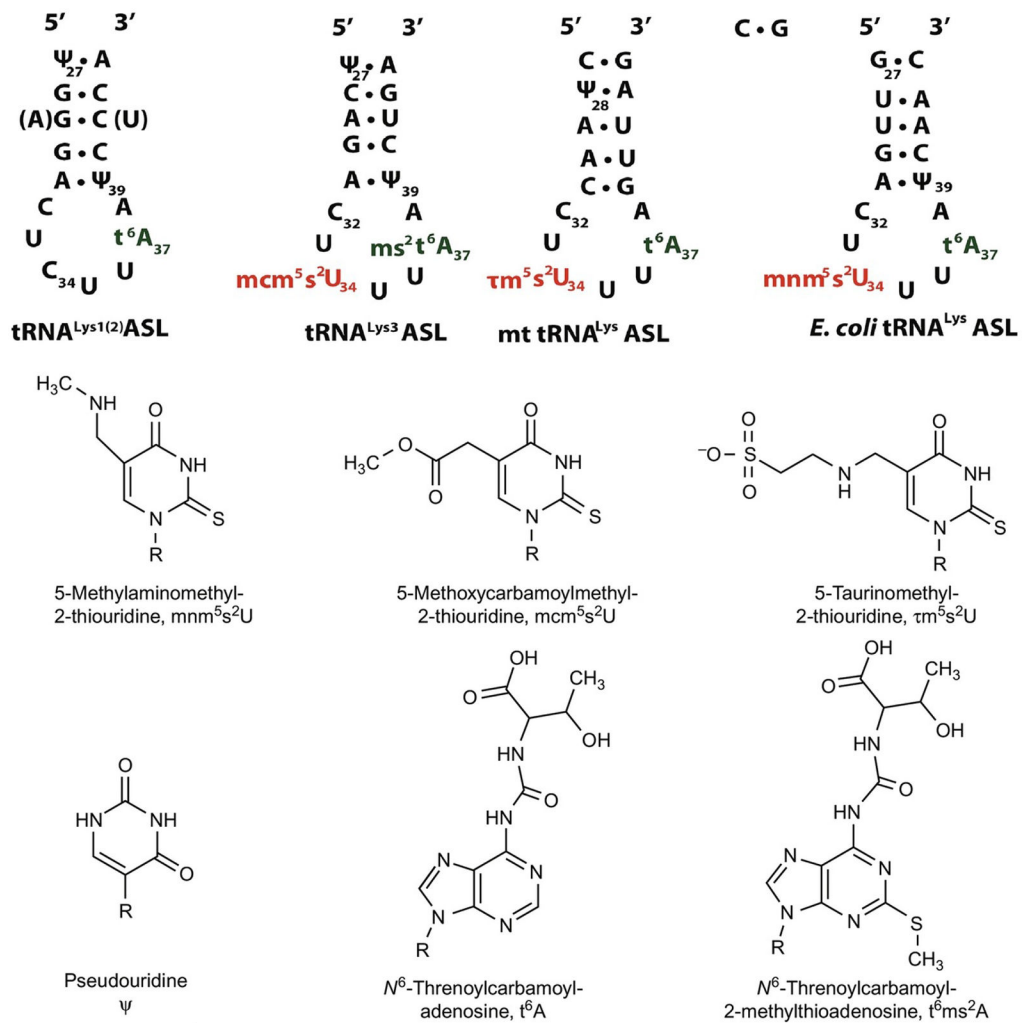


FIG. 6. tRNA^{Lys} ASL sequences and the chemical structures of their modified. *Top:* Sequences and secondary structures of the anticodon stem and loop (ASL) domains of human tRNA^{Lys1,2}_{CUU}, tRNA^{Lys3}, human mitochondrial tRNA^{Lys}_{UUU}, and *E. coli* tRNA^{Lys}_{UUU}. Nucleosides in parenthesis indicate differences between tRNA^{Lys1} and tRNA^{Lys2} sequences. *Bottom:* Chemical structures of the modified nucleosides in the ASLs above and their shorthand notations [92,93].

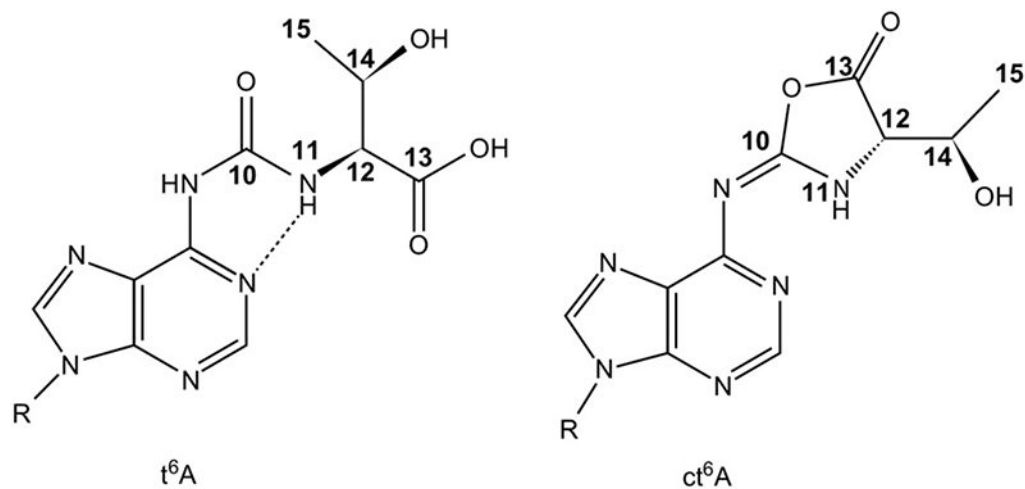
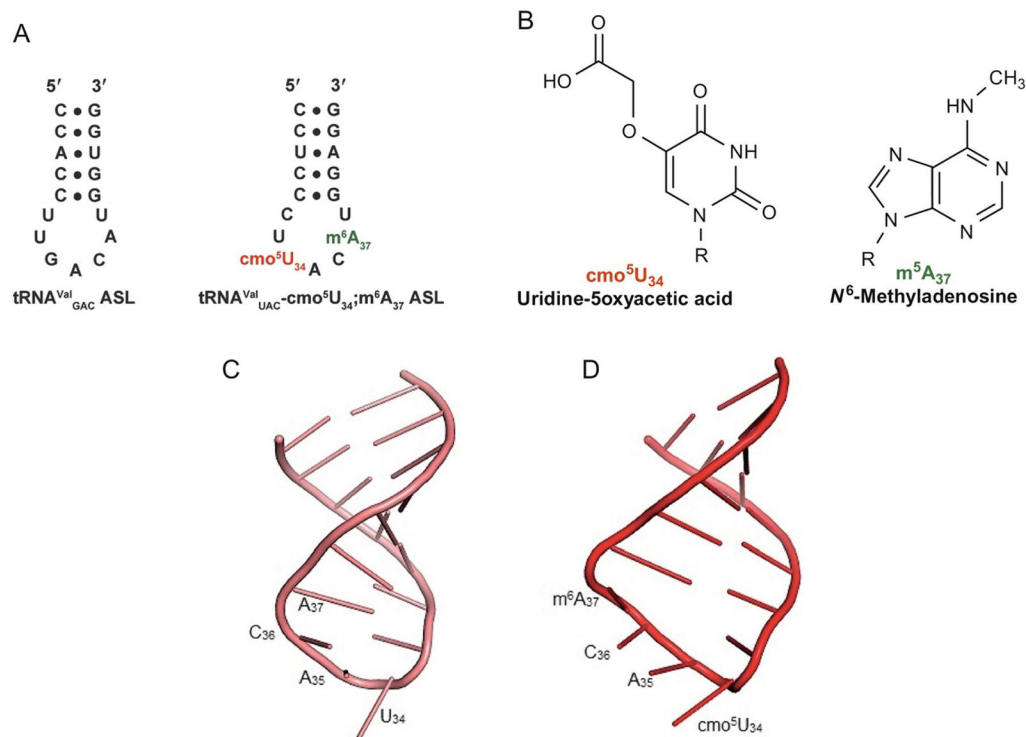


FIG. 7. N^6 -Threonylcarbamoyladenine and its cyclic derivative, t^6A and ct^6A . *Left:* Chemical structure of t^6A with the atoms of the modification numbered. The hydrogen bonding that produces a pseudocyclic conformation of the modified nucleoside is shown as a *dashed line*. *Right:* The cyclic form of t^6A , ct^6A , with the corresponding atoms numbered [88,133].

**FIG. 8.**

The primary sequences and nucleoside modifications of the *E. coli* tRNA^{Val} isoacceptors and the structures of ASL^{Val}_{UAC} and ASL^{Val}_{UAC}-cmo⁵U₃₄;m⁶A₃₇ compared. (A) The anticodon domain primary sequences of the three tRNA^{Val} isoacceptors from *E. coli* and their modifications: uridine-5-oxyacetic acid at position-34, *red* and N⁶-methyladenosine at position-37, *green*. The two tRNA^{Val} species containing the GAC anticodon have identical ASLs with variations in sequence in other regions of the tRNA molecule. (B) The chemical structures, names, and abbreviations of the modifications that appear in the *E. coli* tRNA^{Val}_{UAC}-cmo⁵U₃₄;m⁶A₃₇. (C) Structure of unmodified ASL^{Val}_{UAC}, *pink*. (D) Fully modified ASL^{Val}_{UAC} containing the modifications cmo⁵U₃₄ and m⁶A₃₇, ASL^{Val}_{UAC}-cmo⁵U₃₄;m⁶A₃₇, *red*, showing the base stacking of cmo⁵U₃₄ and an open loop structure. The structures of the unmodified and modified ASL^{Val}_{UAC} were determined by NMR [45] (Protein Data Bank 2JR4 for unmodified ASL^{Val3}_{UAC} and 2JRG for ASL^{Val3}_{UAC}-cmo⁵U₃₄;m⁶A₃₇).

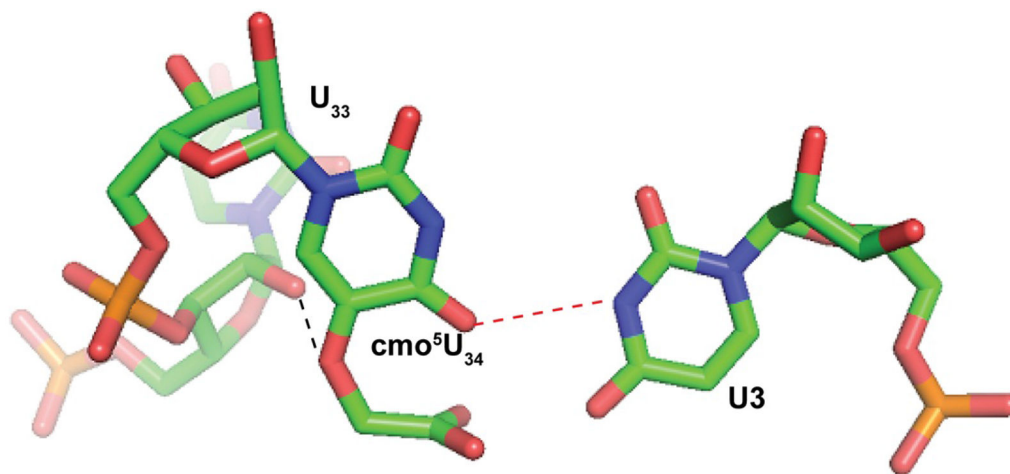
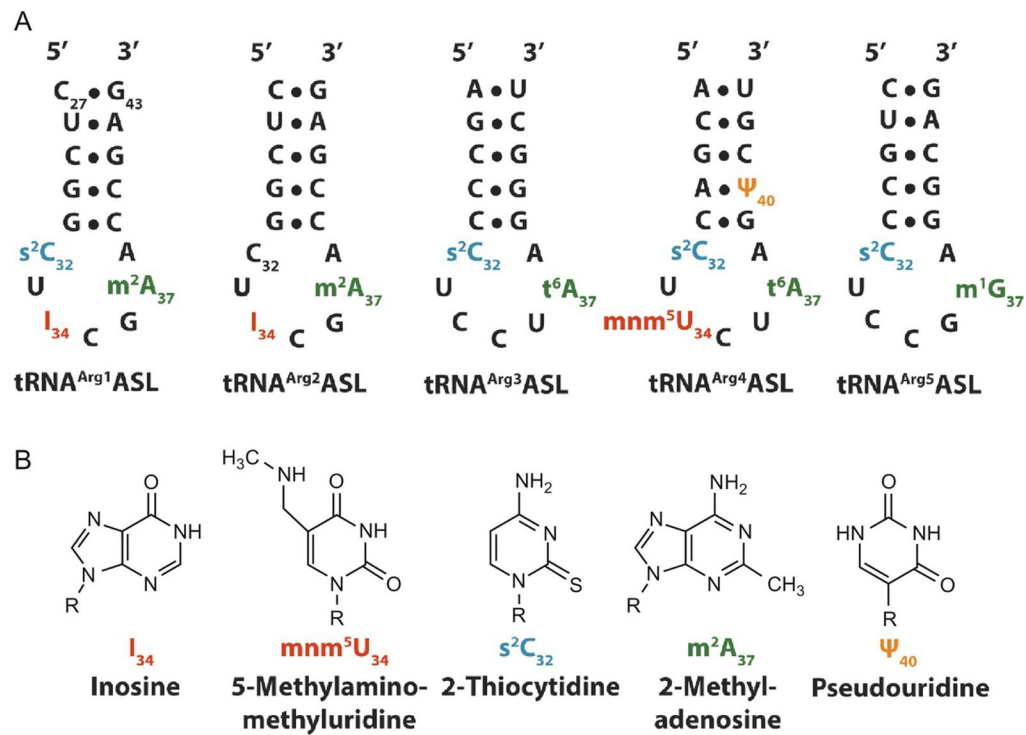


FIG. 9. $\text{cmo}^5\text{U}_{34}\text{OU}_3$ wobble pair. Base pairing between $\text{cmo}^5\text{U}_{34}$ of the $\text{ASL}^{\text{Val}}_{\text{UAC}}$ and U_3 of the GUU codon [46] (Protein Data Bank 2UUB). A hydrogen bond occurs between the 2'-OH of the invariant U_{33} (*background*) of the $\text{ASL}^{\text{Val}}_{\text{UAC}}$ and O_5 of $\text{cmo}^5\text{U}_{34}$ (*black dashed line*), structuring $\text{cmo}^5\text{U}_{34}$ for base pairing with U_3 . The hydrogen bond that allows for the base pair between O_2 of $\text{cmo}^5\text{U}_{34}$ and N_2 of U_3 (*red dashed line*).

**FIG. 10.**

The primary sequences and nucleoside modifications of the *E. coli* tRNA^{Arg} isoacceptors. (A) The anticodon domain primary sequences of the five tRNA^{Arg} isoacceptors from *E. coli* and their modifications: 2-thiocytidine at position-32 (*blue*); inosine and 5-methylaminomethyluridine at position-34 (*red*); 2-methyladenosine, *N*⁶-threonylcarbamoyladenine, and 1-methylguanosine at position-37 (*green*); and pseudouridine at position-40 (*orange*). (B) The chemical structures, names, and abbreviations of the modifications that appear in the five *E. coli* tRNA^{Arg} isoacceptors.