# Effects of tRNA modification on translational accuracy depend on intrinsic codon–anticodon strength

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## ABSTRACT

**Cellular health and growth requires protein synthesis to be both efficient to ensure sufficient production, and accurate to avoid producing defective or unstable proteins. The background of misreading error frequency by individual tRNAs is as low as $2 \times 10^{-6}$ per codon but is codon-specific with some error frequencies above $10^{-3}$ per codon. Here we test the effect on error frequency of blocking post-transcriptional modifications of the anticodon loops of four tRNAs in *Escherichia coli*. We find two types of responses to removing modification. Blocking modification of $tRNA_{UUC}^{Glu}$ and $tRNA_{QUC}^{Asp}$ increases errors, suggesting that the modifications act at least in part to maintain accuracy. Blocking even identical modifications of $tRNA_{UUU}^{Lys}$ and $tRNA_{QUA}^{Tyr}$ has the opposite effect of decreasing errors. One explanation could be that the modifications play opposite roles in modulating misreading by the two classes of tRNAs. Given available evidence that modifications help preorder the anticodon to allow it to recognize the codons, however, the simpler explanation is that unmodified 'weak' tRNAs decode too inefficiently to compete against cognate tRNAs that normally decode target codons, which would reduce the frequency of misreading.**

## INTRODUCTION

Transfer RNAs (tRNAs) are adaptors that decode the sequence of codons in an mRNA into a sequence of amino acids in a polypeptide. The specificity of this process depends on two tRNA functions. First, each tRNA is recognized by an aminoacyl-tRNA synthetase that covalently attaches an amino acid to the 3' end of the tRNA (1). Second, the tRNAs use 3-nt tRNA anticodons to pair with one or more 3-nt mRNA codons (2,3). These two functions allow the tRNAs to associate particular amino acids successively with nucleotide codons in the mRNA, accomplishing

the process of translation while optimizing speed and accuracy (4). Correct selection of aminoacyl-tRNAs depend on a suite of interactions between the ribosome and the codon–anticodon complex (5). These interactions insure accuracy of codon–anticodon recognition but the system is not perfect. Occasional 'misreading' errors can occur during tRNA selection when an incorrect aminoacyl-tRNA is used, causing one amino acid to be substituted for another in the protein product. We previously showed that misreading errors at most codons by $tRNA_{UUU}^{Lys}$ in *Escherichia coli* occur with frequencies $\leq 2$ per 10 000 incorporations (6). More recently, we showed that the frequency of errors by $tRNA_{UUC}^{Glu}$ for most codons is about 100-fold lower or about 2 per 1 million events (7), a far lower level of accuracy than has previously been suspected (3). Errors by both tRNAs occur at a small subset of codons at much higher frequencies, up to three orders of magnitude more frequently (6,7).

Decoding accuracy depends on the ribosome discriminating between correct (cognate) and incorrect (near or non-cognate) codon–anticodon complexes (2,3). Near-cognate aminoacyl-tRNAs have been thought to be unable to form the same intimate set of interactions that form between the ribosome and the cognate codon–anticodon complex (5). Aminoacyl-tRNAs enter the A site in a ternary complex with elongation factor Tu (EF-Tu) and guanosine triphosphate (GTP) and acceptance of the tRNA results from cleavage of GTP to guanosine diphosphate (GDP) plus inorganic phosphate by an intrinsic EF-Tu GTPase activated by its interaction with a site on the large ribosomal subunit (8). Under either model of codon–anticodon monitoring, discrimination is thought to result both from accelerating GTPase activation for cognate complexes and increased dissociation of near-cognate complexes before GTP cleavage (9). More recent data, however, suggest that the A site interacts in a similar way with both cognate and near-cognate complexes, forcing mismatched bases to adopt Watson–Crick geometry (10). Some mismatched base pairs appear to be accepted because their conformation mimics that of a Watson–Crick pair and can easily conform to the decoding center (10) while other mismatches result in a

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conformation drastically different from Watson–Crick with codon bases being forced out of the binding site (11). These structures substantiate our argument that the most frequent errors occur when the ribosome cannot determine that a particular mismatch base pair is not one of the canonical Watson–Crick pairs—A•U or G•C—because physical dimensions of the pair are too similar to that of the canonical pairs (7).

Post-transcriptional modifications modulate the function of tRNAs (12). Individual tRNAs overall have an average of eight modifications with a total of 12% of tRNA nucleotides being modified (13). All cells encode substantial tRNA modification systems devoting up to 10% of a genome to modification genes, a proportion greater than that devoted to encoding the tRNAs themselves (14). Many modifications within the structural core of the tRNA are essential to stabilizing the overall structure of the tRNA; loss of these modifications can result in rapid degradation of hypomodified tRNAs (13). Other modifications, especially those targeting the anticodon loop region, affect the function of the tRNA; loss of many of these modifications can reduce protein production or translational accuracy (15). Modifications are most common at the wobble nucleotide (position 34) and the nucleotide immediately 3′ of the anticodon (position 37). Crick (16) in his wobble hypothesis proposed an unusual set of base pairs in the third codon position, allowing, for example, pairing of U34 in the anticodon with A or G. Pairing of U34 with the codon is modulated by modification. Addition of 5-methylene derivatives, usually combined with 2-thiolation, as in 5-methylaminomethyl-2-thiouridine ($mnm^5s^2U$), had been thought to reduce recognition of G, restricting decoding to A (reviewed in 17). More recently, another 5-methylene modification (5-methoxycarbonylmethyl or $mcm^5$) was shown to stabilize pairing to both A and G (18,19), with pairing to G proposed to require protonation of U to support an unconventional G•U pair (20). The tRNAs with wobble 5-methyluridine derivatives occur on tRNAs that decode within a four-codon group ('box') in which codons differ only at the third or wobble position but where the pyrimidine and purine-ending codons specify different amino acids (a 'split box'). These modifications are thought to destabilize pairing between the tRNAs with wobble U34 and the pyrimidine-ending codons (reviewed in 21). In non-split boxes, where all four codons encode the same amino acid, addition of 5-oxyacetic acid to uridine ($cmo^5U34$) actually expands decoding to all four wobble nucleotides (22,23). These position 34 modifications are thought to modulate hydrogen bonding by the modified nucleotide (24), whereas modification of the position 37 nt, which does not base pair with the mRNA, indirectly affect the stability of the adjacent codon–anticodon complex for example by stacking (25). For example, position 37 2-methylthio-$N^6$-isopentenyladenosine ($ms^2i^6A37$) in tRNAs recognizing codons beginning in A is thought to stabilize the weak U36-A1 base pair with the lack of the modification reducing the efficiency of decoding (reviewed in 12).

Most of these studies have concerned correct cognate decoding; less information is available concerning the role of modification on mispaired complexes, which is the main motivation for this study. For this reason we tested the effect of loss of three modifications—at position 34 $mnm^5s^2U$ and the hypermodified base queuosine (26) and at position 37 $ms^2i^6A$—on the frequency of errors. We found that anticodon loop modifications do modulate error frequencies in *E. coli* but that the magnitude and direction of the effect—either increasing or decreasing accuracy—depended on the strength of codon–anticodon pairing; modification of tRNAs that are intrinsically weak tended to increase misreading errors while for tRNAs that form intrinsically stronger pairs with their codons modification tended to decrease misreading errors. The effect of removing specific modifications will be discussed in the context of models for the role played in modulating the stability of cognate and near-cognate codon–anticodon pairing.

## MATERIALS AND METHODS

### Bacterial strains

The *E. coli* strains used in this study were: Xac (*ara∆*[*lac-proAB*] *gyrA*(nalR) *rpoB*(rifR) *argE*[amber]), (Xac *∆tgt770::Kan^R*), (Xac *mnmE::tn983*), (Xac *∆miaA721::Kan^R*). To eliminate any effect of secondary mutations in the genetic background, The *miaA*, *mnmE* and *tgt* mutations were transferred into a common genetic background, Xac (27), by P1 transduction (28) by selecting for the disrupting antibiotic marker using kanamycin resistance (*kan^R*) or resistance to tetracycline and ampicillin (*tn983*). For each modification mutant the phenotype was validated by total hydrolysis of cellular tRNAs and high-performance liquid chromatography (29) which demonstrated the complete loss of the corresponding modification. Bacterial cultures were grown at 37°C in liquid Luria-Bertani (LB) medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per liter of distilled water) supplemented with relevant antibiotics, where required.

### Misreading error reporter systems

Systems of reporter plasmids were developed to assay the frequency of translational errors *in vivo* that are based on the enzymes *Photinus pyralis* (firefly) luciferase (F-luc) (6) and *E. coli* β-galactosidase (7). The reporters genes are carried on a low copy bacterial plasmid, pJC27 (30), and expressed from a regulated Ptac promoter (31). Codons encoding four active site amino acids were targeted for mutagenesis: Lys 529 (K529) of Fluc and either glutamic acid 537 (E537), aspartic acid 201 (D201) or tyrosine 503 (Y503) of β-galactosidase. The construction of the codon substitutions has been described (7). In each case, we constructed a set of 14 near-cognate mutants of each target codon; these were all mutants differing by 1 nt from one of the two synonymous codons encoding the wild-type amino acid. For example, K529, which could be encoded by AAA or AAG, was replaced with six mutants of the first codon position—codons for stop (UAA/UAG), glutamine (CAA/CAG) and glutamic acid (GAA/GAG)—six second position mutants and two wobble position mutants. The error frequency is calculated as the ratio of the activity of the enzyme made from a mutant reporter to that from a gene carrying the wild-type codon. Data on mutant enzyme ac-

tivities in the Xac background reported here have been published previously (6,7).

### Enzyme assays

Plasmids were introduced into bacterial strains using Z-competent cells (Zymo Research) or by the calcium chloride method (32). Transformants were grown in 1 ml of liquid LB media in 96-well culture blocks at 37°C overnight to stationary phase. Cultures were inoculated to a dilution of 1:33 in 1 ml of liquid LB media with chloramphenicol and grown at 37°C to an OD600 of 0.5–0.8. Triplicate assays of β-galactosidase activity on each of three independent transformants were performed using the β-Glo system (Promega) according to manufacturers specifications using Microfluor 96-well microtiter plates (ThermoFisher Scientific) in a Modulus II Microplate Multimode Reader (Promega). The relative light units (RLUs) measured by the luminometer are proportional to the activity of β-galactosidase in the cells. This method was used because of its much greater sensitivity compared to the conventional system using *ortho*-nitrophenyl-β-galactopyranoside. Misreading error frequency was calculated as a ratio of mutant to wild-type RLU. The significance of the difference in gene expression between wild-type and mutant genetic backgrounds were determined using a paired, two-tailed Student's *t*-test.

To assay the expression of dual luciferase reporters cultures of individual clones transformed as above were grown to OD600 of from 0.5 to 0.8 in liquid LB medium with 100 μg/ml ampicillin at 37°C and then pelleted by centrifugation and resuspended in 200 μl 1 mg/ml lysozyme/10 mM Tris–HCl, pH 8.0/1 mM ethylenediaminetetraacetic acid. Cells were incubated on ice for 10 min, frozen on dry ice and thawed on ice (33). Five microliter samples of this extract were assayed for F-luc and *Renilla reniformis* (Sea pansy) luciferase (R–luc) activities using the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using the luminometer function of a Modulus II Microplate Multimode Reader (Promega). Three transformants for each construct were assayed, each in triplicate. The R–luc activity was used as an internal standard and standardized F-luc activity was calculated as the ratio of the F-luc to R-luc activity expressed in RLUs.

## RESULTS

### Misreading error reporter systems based on mutations affecting active site residues

Misreading errors have been quantitated in a variety of ways including amino acid composition analysis (34) and isoelectric point determination (35). A third approach has involved measuring activities of enzymes encoded by genes bearing mutations affecting important active site amino acids (36). We developed four misreading error reporter systems to quantify misreading error frequencies by four tRNAs: $tRNA_{UUU}^{Lys}$ (6), and $tRNA_{UUC}^{Glu}$, $tRNA_{QUC}^{Asp}$ and $tRNA_{QUA}^{Tyr}$ (7). The advantage of these reporter-based systems is that they can provide highly quantitative measures of misreading at all possible codons by single aminoacyl-tRNAs. Because the assays are performed *in vivo* the results are directly

relevant to understanding the mechanism of error correction in living cells and the reporters can be used to quickly and quantitatively test the effect on accuracy of mutants that alter important aspects of protein synthesis. The experiments reported here focus on the effect on misreading of loss of post-transcriptional modification of tRNAs.

The frequency of errors by $tRNA_{UUU}^{Lys}$ is determined using active site lysine-529 (K529) mutants of Fluc. This amino acid plays a central role in catalysis by coordinating the two enzyme substrates in the active site (37). Fluc is a member of a family of adenylate-forming enzymes within which the residues corresponding to K529 are extremely conserved. K529 makes multiple contacts with the substrates, luciferin and adenosine triphosphate, that orients the reactants to promote formation of the luciferin-adenylate product that subsequently reacts with molecular oxygen to generate light. The lack of K529 strongly reduces enzyme activity. Errors by $tRNA_{UUC}^{Glu}$, $tRNA_{QUC}^{Asp}$ and $tRNA_{QUA}^{Tyr}$ are quantified using active site mutants of β-galactosidase affecting glutamate-537 (E537), aspartate-201 (D201) and tyrosine-503 (Y503). E537 is the principle nucleophile during catalysis (38). D201 participates in orienting the substrate in the active site, coordinating and acting as a counter ion for bound monovalent cations to indirectly interact with the substrate (39). Y503 participates in orienting the transition state of the substrate in the active site (40). Mutations in all of these residues strongly reduce activity. Relatively rare misreading events that result in insertion of the wild-type amino acid at the mutant site can restore full enzymatic activity; the frequency of such errors is equal to the ratio of the activity produced from the mutant and wild-type genes (6)

### Methylaminomethyl-modification has opposite effects on misreading error frequency by $tRNA_{UUU}^{Lys}$ and $tRNA_{UUC}^{Glu}$

The two proteins MnmE and MnmG form an $\alpha_2\beta_2$ heterotetrameric complex that catalyzes the first step in adding 5-methylaminomethyl (mnm$^5$) groups to uridine or 2-thiouridine (41). A mutant lacking the MnmE protein was shown to accumulate tRNAs with s$^2$U at the wobble position (41,42). This modification is introduced in *E. coli* to tRNAs that decode NNA/NNG codons of split codon boxes, which includes $tRNA_{UUU}^{Lys}$, $tRNA_{UUC}^{Glu}$, $tRNA_{UUG}^{Gln}$, $tRNA_{UAA}^{Leu}$ and $tRNA_{UCU}^{Arg}$, as well as the GGA/GGG decoding $tRNA_{UUC}^{Gly}$, which decodes codons of a non-split box (43). Using our error reporter systems for Lys and Glu codons, we have measured the effect of *mnmE*Δ on errors by $tRNA_{UUU}^{Lys}$ and $tRNA_{UUC}^{Glu}$.

We confirmed by total hydrolysis and high-performance liquid chromatography (HPLC) of cellular ribonucleotides that the *mnmE::Tn*93 mutant has no detectable mnm$^5$s$^2$U (Supplementary Figure S2). Unfortunately, we were not able to confirm the presence of s$^2$U in this mutant because that species elutes along with the much more abundant guanosine nucleoside in the analytic system used. Elseviers *et al.* (41) showed that an *mnmE* produces tRNAs with s$^2$U34 in at least 80% the abundance of mnm$^5$s$^2$U in wild-type so it is possible that in the *mnmE* strain some of

the tRNAs normally subject to this modification contain unmodified U at the wobble position. Residual $s^2U$ modification of these tRNAs is essential since strains lacking *mnmE* and also lacking the normally non-essential *mnmA* gene, which encodes thiouridylase responsible for catalyzing formation of $s^2U$ (44), are inviable (G. R. Björk, personal communication). The MnmA thiuridylase binds unmodified tRNAs *in vitro* with similar affinity and catalyzes sulfation with equal substrate activity on the two tRNAs (45). Furthermore, the structure of a complex of thiohydrolase bound to a tRNA (46) shows that the enzyme does not interact with the C5 position in binding to the substrate and that the enzyme has a large cavity into which the mnm⁵ modification could be accommodated; the structures implies that the enzyme could catalyze the modification of either U or mnm⁵U equally well. Based on this evidence we conclude that it is likely that the wobble base on the tRNAs is $s^2U$ but we cannot absolutely exclude that one or more of the tRNAs may have wobble U in some or all molecules in the *mnmE* strain. The anticodon loops of tRNA$^{Lys}_{UUU}$ and tRNA$^{Glu}_{UUC}$ each include a second modified nucleotide 37: for tRNA$^{Lys}_{UUU}$ N6-threonylcarbamoyladenosine ($t^6A$) and for tRNA$^{Glu}_{UUC}$ 2-methyladenosine ($m^2A$). We demonstrated that the amounts of these modified bases were unchanged in the *mnmE* mutant (Supplementary Figure S3) so that both modifications are present in the anticodon loop of the two tRNAs lacking mnm⁵s²U34. Therefore, any phenotypic effect of *mnmE* cannot result from lack of these two nearby modified nucleotides. We also measured the steady state level of the two tRNAs by northern blotting using a tRNA-specific oligonucleotide probe and found no difference in steady state level between the wild-type and mutant backgrounds (Supplementary Figure S4).

On theoretical grounds, mnm⁵-modification was proposed to restrict wobble pairing to reduce recognition of U and C-ending codons (21). Surprisingly, Hagervall *et al.* (42) showed tRNA$^{Lys}_{UUU}$ lacking either the mnm⁵ or s² modification caused reduced wobble misreading of AAU/AAC Asn codons, suggesting that the modifications actually stabilized the errors. In order to clarify this conflict, we measured the effect of loss of mnm⁵-modification on the frequency of misreading errors by tRNA$^{Lys}_{UUU}$ and tRNA$^{Glu}_{UUC}$.

Using the Fluc K529 reporter system, we have shown that in wild-type *E. coli* errors by tRNA$^{Lys}_{UUU}$ are restricted to five codons: the termination codons UAA and UAG, the Arg codons AGA and AGG and the Asn codon AAU (see Figure 1). That the increased enzyme activity measured in these codons results from increased misreading errors is shown mainly by two observations. First, that the mutants involving synonymous codons (the termination codon UGA or the Asn codon AAC) have significantly lower activity, and that the activities of the two Arg mutants are significantly different (6). This argues against the observed activities resulting from the intrinsic activity resulting from the mutant amino acid replacing the wild-type amino acid; if this model ('functional replacement') were true, all synonymous mutants should have identical activity. Second, the activity of the putative error-prone mutants was greatly reduced in a hyperaccurate *rpsL* strain (6). So, the higher enzyme activi-
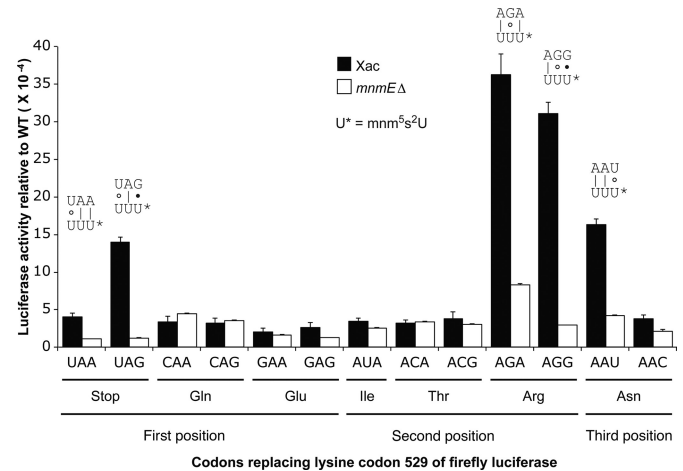


**Figure 1.** Misreading errors by tRNA$^{Lys}_{UUU}$ decrease in the absence of 5-methylaminomethyl-modification of the wobble uridine. The activity of K529 mutants of firefly luciferase expressed in the wild-type (Xac) or *mnmE* background, which blocks mnm⁵-modification, are compared. The activities of seven mutants, six normally error-prone, were significantly reduced in the *mnmE* background suggesting that the modification increases the frequency of misreading errors by the tRNA. For the mutants showing a statistically significant change in activity between the wild-type and *mnmE* backgrounds, the structure is shown of the codon–anticodon complexes required for each of these misreading events (the upper line represents the codon, the lower the anticodon). Vertical lines represent Watson–Crick pairs, filled circles canonical wobble pairs, and open circles pairs requiring a tautomeric shift to form.

ties of the UAG, AGA, AGG and AAU mutants result from increased frequency of their misreading by tRNA$^{Lys}_{UUU}$.

As shown in Figure 1, The Fluc activity of these five error-prone mutants is reduced strongly in the absence of mnm⁵-modification in the *mnmEΔ* strain. The magnitude of this effect varies among the mutants with ~11-fold decreases for the UAG and AGG mutants and ~4-fold decreases for UAA, AGA and AAU; none of the other mutants show significant change. For each of these five codons misreading involves mispairing at one position of either U•U or G•U (when referring to codon–anticodon base pairs throughout the first listed will be an codon base and the second from the anticodon). We have suggested (7) that these errors require formation of a newly discovered base pair that has roughly Watson–Crick geometry (10,11). Pairing between nucleotides capable of adopting this geometry is indicated in Figure 1 and other figures by an open circle to distinguish them from normal Watson–Crick (indicated by a vertical line), classic wobble (filled circle) or apparent clash (X) pairs.

These data are consistent with the idea that mnm⁵-modification, when present, stabilizes mispaired recognition by tRNA$^{Lys}_{UUU}$, consistent with the results of Hagervall *et al.* (42). The effect is observed for errors involving mismatches at all three codon positions—first position (UAA, UAG), second position (AGA, AGG) and third or wobble position (AAU) suggesting that regardless of whether the wobble position is correctly paired, the lack of modification of the anticodon nucleotide reduces the efficiency of decoding by tRNA$^{Lys}_{UUU}$. So, the effect of the lack of mnm⁵-
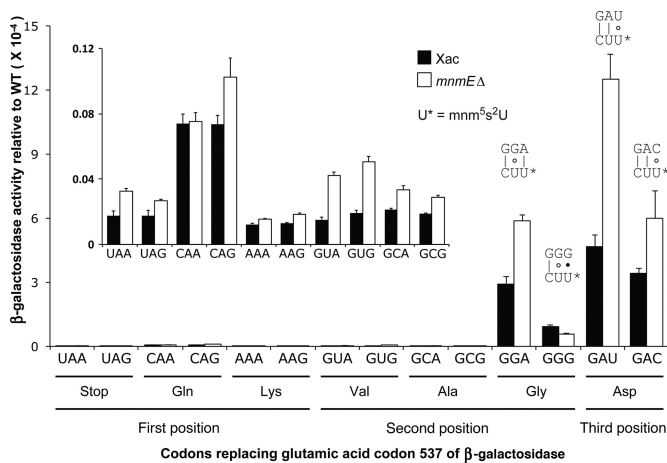
**Figure 2.** Misreading errors by $tRNA_{UUC}^{Glu}$ increase in the absence of 5-methylaminomethyl-modification of the wobble uridine. The activity of E537 mutants of *Escherichia coli* β-galactosidase expressed in the wild-type (Xac) or *mnmE* background are compared as in Figure 1.

modification is not at the level of codon discrimination at the wobble position.

The same wobble modification is found in $tRNA_{UUC}^{Glu}$, allowing a test of the error effect in a different codon context. Using the β-galactosidase E537 mutant, we have shown that significant errors by this tRNA occur only at four codons: the Gly codons GGA and GGG and the Asp codons GAU and GAC (7). The data show that increased error results from misreading involving a second position G•U mismatch at the Gly codons and U•U or C•U wobble mismatches at the Asp codons. All of these mismatches would require tautomeric shifts. As shown in Figure 2 and contrary to the result with $tRNA_{UUU}^{Lys}$, in the absence of mnm⁵-modification the activity of three of the four error-prone mutants increased significantly; only the least error-prone mutant, GGG, showed a significant decrease. For many of the non-error prone mutants there was a statistically significant increase in activity but given that the activities of these mutants in wild-type were extremely low (2 to 7 × $10^{-6}$ of wild-type) these increases are unlikely to be physiologically significant. The magnitude of the change in activity, although mainly in the opposite direction, is somewhat smaller than for the K529 mutants: increases of ~3-fold for GAU and ~2-fold for GGA and GAC and a decrease of ~2-fold for GGG. The opposite effect on GGG could be explained by the existence of a second Gly tRNA with anticodon CCC that successfully competes against the error-inducing hypomodified Glu tRNA only on the GGG codon.

The different effect on errors by $tRNA_{UUU}^{Lys}$ and $tRNA_{UUC}^{Glu}$ is striking. The result suggests that the effect of modification is sensitive to its sequence or structural context and not an autonomous effect of the base itself. The stability of RNA helices including non-Watson/Crick G•U pairs has been shown to be affected by nearest neighbors presumably because of variation in stacking interactions (47). These studies were done in solution so the pairs are expected not to adopt the Watson–Crick geometry imposed

by the decoding center so their relevance to decoding errors is unclear. Our results, however, show that the structural context around the non-canonical base pair has a strong effect on decoding of near-cognate codons.

## Queuosine modification has opposite effects on errors by $tRNA_{QUC}^{Asp}$ and $tRNA_{QUA}^{Tyr}$

To further test the generality of the effect of modification depending on structural context we tested the effect in two tRNAs in which the anticodon wobble nucleotide is modified from G to the hypermodified nucleotide 7-(4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanosine or queuosine (Q). Q-modification is limited to tRNAs that decode NAU and NAC codons (Tyr UAU/UAC, His CAU/CAC, Asn AAU/AAC and Asp GAU/GAC). The *tgt* gene encodes the protein that performs the first step in conversion of wobble G to the hypermodified base Q (48). $tRNA_{QUC}^{Asp}$ undergoes further modification by addition of a glutamic acid residue to Q (GluQ) (49). The exact structure of GluQ and its effect on the activity of the tRNA is unknown; for simplicity we will refer to the base as Q here. We have confirmed by total hydrolysis and HPLC analysis that the *tgt* mutants have no detectable amount of these modified nucleotides and based on the known function of the *tgt*-encoded tRNA-guanine transglycosylase the wobble bases on these tRNAs should be unmodified guanines (Supplementary Figure S5). We were not able to identify GluQ in this analysis, which is labile during purification (48); since GluQ is formed by modification of Q in the tRNA we assume that the wobble nucleotide in this tRNA is G. In addition, we demonstrated that two nucleotide 37 modifications found in $tRNA_{QUA}^{Tyr}$ (2-methylthio-$N^6$-isopentenyladenosine or ms²i⁶A37) is not reduced in the presence of the *tgtΔ* mutation (Supplementary Figure S6) and that the steady state level of the two tRNAs is not reduced by the lack of modification (Supplementary Figure S4).

Two of our misreading reporters, the β-galactosidase mutants of Asp-201 (D201) and Tyr-503 (Y503), provide tests of misreading by Q-modified tRNAs. We saw no evidence of significant misreading errors for mutants of the D201 reporter in a wild-type background but the Gly GGC codon mutant had 6-fold elevated activity (to $1.6 \times 10^{-3}$ times wild-type) in an error-prone *rpsD* mutant background (7), suggesting that we might be able to measure misreading errors at this codon. For $tRNA_{QUA}^{Tyr}$, three codons showed significant misreading: the Cys codon mutants of Y503 UGU and UGC had activities $3.2 \times 10^{-4}$ and $7.3 \times 10^{-4}$ times wild-type and the termination codon mutant UAG had an activity $8 \times 10^{-5}$ times wild-type. All three of these showed significantly reduced activity in the hyperaccurate *rpsL* background (7), again suggesting that we could measure errors at these codons.

As shown in Figure 3, we tested for changes in misreading frequency by unmodified $tRNA_{QUC}^{Asp}$ by measuring the activity of D201 mutants in the *tgtΔ* background. The activity of the Gly GGC mutant is increased 7.4-fold to $1.9 \times 10^{-3}$ times wild-type. There was no significant change for any of
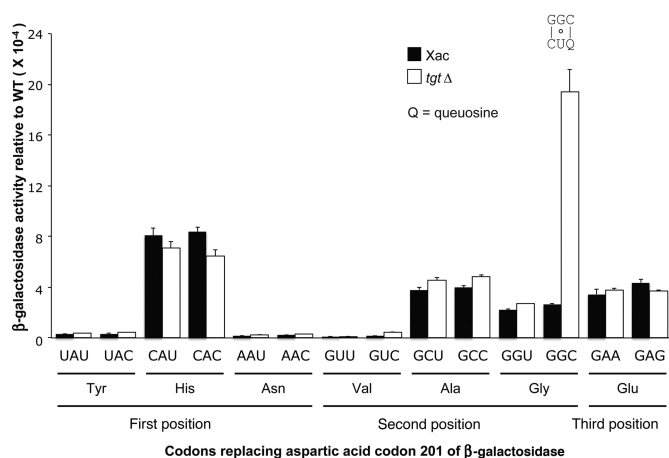
**Figure 3.** Misreading errors by $tRNA_{QUC}^{Asp}$ increase in the absence of the wobble queuosine modification. The activity of D201 mutants of *Escherichia coli* β-galactosidase expressed in the wild-type (Xac) or *tgt*Δ background, which blocks formation of wobble queuosine, are compared as in Figure 1.
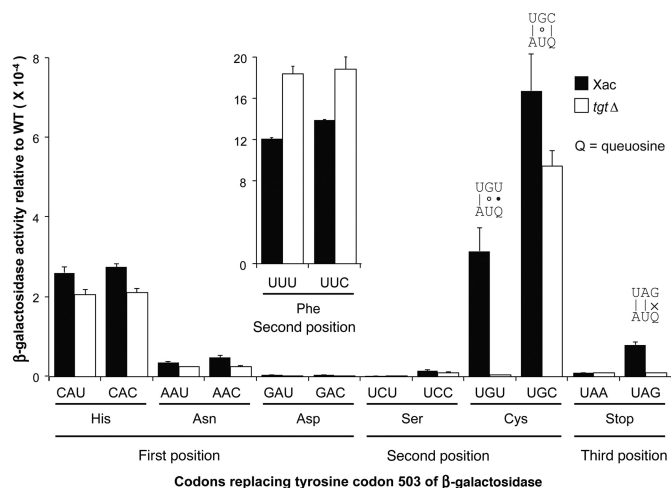


**Figure 4.** Misreading errors by $tRNA_{QUA}^{Tyr}$ decrease in the absence of the wobble queuosine modification. The activity of Y503 mutants of *Escherichia coli* β-galactosidase expressed in the wild-type (Xac) or *tgt*Δ background are compared as in Figure 1.

the other mutants; in particular, there was no increase in activity of the two wobble position mutants, Glu GAA and GAG. These data suggest that the Q-modification destabilizes second-position misreading by $tRNA_{QUC}^{Asp}$ of these three codons since loss of the modification resulted in increased activity of these codon mutants. The lack of an effect on the Glu codon mutants suggests that Q modification is not essential to block wobble misreading by $tRNA_{QUC}^{Asp}$ of these codons; these errors would require a bulky purine-purine mismatched base pair (Q–A or Q–G), which apparently is efficiently rejected even in the absence of Q-modification. Since the activity of all the D201 mutants was not reduced in a hyperaccurate *rpsL* mutant background, suggesting that the wild-type activity does not result from translational misreading, the high activity in these mutants might hide any effect of Q modification. If so, however, the magnitude of such an effect would necessarily be much lower than the effect seen with the Gly GGC mutant. The major effect in terms of misreading of Q modification, then, is to strongly block second-position errors that involve U•G mismatches by $tRNA_{QUC}^{Asp}$ in the context of correct wobble position pairing. The fact that loss of modification causes a large increase in errors on GGC but not GGU is consistent with Q destabilizing G•C pairing much more than G•U.

We tested the effect of Q-modification on decoding by $tRNA_{QUA}^{Tyr}$ using the Y503 mutants of β-galactosidase. Again, we observed significant changes in the activity of three of the four error-prone codon mutants (Figure 4). The mutants introducing the Cys codons UGU and UGC and termination codon UAG mutants each showed significant reductions in activity. The activity of the UGU mutant was reduced 80-fold to $4 \times 10^{-6}$ times wild-type and the UAG mutant was reduced 9.2-fold to $8.9 \times 10^{-6}$ times wild-type. The effect on the Cys UGC mutant was much weaker, reducing activity only 1.4-fold to $5.4 \times 10^{-4}$ per codon. Importantly, the effect in each case is opposite to

the effect on $tRNA_{QUC}^{Asp}$, decreasing activity in the mutant background relative to wild-type rather than increasing activity. The small fold change for the UGC mutant suggests that the Q modification stabilized pairing with C much less than it did wobble pairing with U in UGU or non-canonical pairing with G in UAG. Overall we find that in contrast to Q-modification of $tRNA_{QUC}^{Asp}$, which destabilizes misreading, modification of $tRNA_{QUA}^{Tyr}$ has the opposite effect of stabilizing near-cognate misreading. Thus the data provide a second example of how the effect of modification on misreading is sensitive to the structural context.

## 2-methylthio-$N^6$-isopentenyl modification of A37 of $tRNA_{QUA}^{Tyr}$ increases misreading errors

Both mnm$^5$ and Q-modification occur at the wobble position of the tRNA and the modified nucleotides directly interact with wobble nucleotide of the codon. The wobble nucleotide of the anticodon and the nucleotide at position 37 immediately adjacent to the codon are modified in most tRNAs and are the sites of the widest diversity of modifications in all species (12,14). Our results confirm the conclusion that these modifications are important because they modulate translation efficiency and therefore translational accuracy. The fact that nucleotide 37 is nearly universally modified and its proximity to the anticodon has long suggested that it has a role in translation efficiency. The modified base ms$^2$i6A37 occurs in tRNAs that recognize codons beginning with U and that thus form a weak first position U1•A36 pair. The modification is adjacent to this base pair and is thought to stabilize its formation (50). The lack of ms$^2$i6A modification of $tRNA_{UAA}^{Leu}$ results in decreased misreading of the Phe codon UUU *in vitro* as a result of increased dissociation of the aminoacyl-tRNA during proofreading (51). The relatively few *in vivo* studies of the role of the modification in accuracy have been inconsistent (reviewed in 12) possibly because they were performed
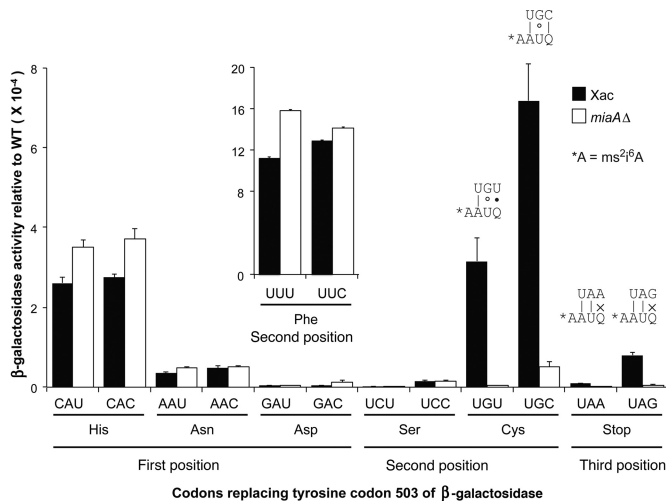
**Figure 5.** Misreading errors by $tRNA_{QUA}^{Tyr}$ decrease in the absence of 2-methylthio-$N^6$-isopentenyl modification of adenosine-37, the nucleotide adjacent to the anticodon. The activity of Y503 mutants of *Escherichia coli* β-galactosidase expressed in the wild-type (Xac) or *miaA*Δ background are compared as in Figure 1.

with different tRNAs and codons or with mutant tRNAs. The *miaA* protein isopentylates its target tRNAs, the first step in addition of 2-methylthio-$N^6$-isopentenyladenosine ($ms^2i^6A37$) at position 37, accumulating A37 in place of $ms^2i^6A37$ (52). We confirmed by total hydrolysis and HPLC that the *miaA* lacks any detectable $ms^2i^6A$ (Supplementary Figure S7). The tRNA has an additional modification introducing wobble Q. We were unable to demonstrate that the tRNA in the *miaA* background has Q in the wobble position. *In vitro* analysis showed that $tRNA_{QUA}^{Tyr}$ with or without $ms^2i^6A37$ were modified with the same kinetics (53). The fact that the four Q-containing tRNAs have three different base modifications at position 37 ($t^6A$, $m^2A$ and $ms^2i^6A$) argues against a function for these modifications in targeting Q modification. Based on these arguments, we assume that in the *tgt* background the tRNA has A37 and Q34. We also show that the lack of modification does not alter the steady state level of the tRNA (Supplementary Figure S4). We have tested the effect of $ms^2i^6$-modification on errors by $tRNA_{QUA}^{Tyr}$ to gain a better insight into how it affects decoding.

We tested the effect of lack of $ms^2i^6A37$ on the activity of the mutants of the codon encoding Tyr 503 (Y503) of β-galactosidase. We showed previously that only four codons showed evidence of translational misreading errors: the Cys codons UGU and UGC and the termination codons UAA and UAG (7). The activity of UGU, UGC and UAG mutants was reduced in the hyperaccurate *rpsL* background and the activity of all four mutants was increased in the error-prone *rpsD* background. All of the other mutants showed no significant changes in either background. We attribute the high activity of the Phe and His mutants to those amino acids partially functionally replacing Tyr presumably because of their structural similarity. As shown in Figure 5, enzyme activity for the four mutants UGU, UGC, UAA and UAG was significantly reduced in the *miaA*Δ back-

ground while the activity of the other eight near-cognate mutants was not significantly changed. The effect on the two Cys mutants was quantitatively different. The activity of the UGU mutant in the *miaA*Δ mutant was much lower ($3.5 \times 10^{-6}$ times wild-type) than for UGC ($5.2 \times 10^{-5}$ times wild-type) and the fold reduction was also greater for UGU (93-fold) than for UGC (14-fold). Although the activity of the termination codon mutants was low the fold reduction was significant (4.7-fold for UAA and 16.7-fold for UAG). These data are consistent with the *in vitro* results of Diaz and Ehrenberg (51). The errors would require $tRNA_{QUA}^{Tyr}$ to form a G•U mispair at the middle position to read Cys codons or a A•Q or G•Q wobble mispair for the termination codons. These data suggest that the presence of the $ms^2i^6$ modification increases the ability to misread and that in three cases the errors appear to depend essentially absolutely on the modification since the level of residual activity is near the background of detection.

We assume that the modification is not introduced with the purpose of so substantially increasing error frequency. It is likely that increased errors are the necessary consequence of enhancing the decoding ability of the tRNA to increase rates of elongation during cognate decoding. We note that for two of the tRNAs tested, $tRNA_{UUU}^{Lys}$ and $tRNA_{QUA}^{Tyr}$, the effect of modification is to increase misreading errors while for the other two, $tRNA_{UUC}^{Glu}$ and $tRNA_{QUC}^{Asp}$, the effect is in general to decrease misreading errors. Importantly, for two modifications, the presence of the modified base increases error frequency in one tRNA but has the opposite effect of reducing error frequency on a second tRNA. This result shows that the effect is not an intrinsic feature of the modification but that it depends on the structural context of the modified tRNA structure.

## DISCUSSION

The effect of post-transcriptional modifications on cognate decoding has been extensively investigated, especially on the question of discrimination between synonymous codons (24). Less is known about the effect of modification on near-cognate decoding and some of that has been controversial. Our purpose in this work was to provide a comprehensive analysis of the role of modification in modulating near-cognate decoding and thus misreading errors by four tRNAs, $tRNA_{UUU}^{Lys}$, $tRNA_{QUA}^{Tyr}$, $tRNA_{UUC}^{Glu}$ and $tRNA_{QUC}^{Asp}$.

The striking result of this work is how varied was the frequency of most errors in the absence of modification. In general, the lack of modification of $tRNA_{UUC}^{Glu}$ and $tRNA_{QUC}^{Asp}$ increased the frequency of misreading errors while the lack of modification of $tRNA_{UUU}^{Lys}$ and $tRNA_{QUA}^{Tyr}$ decreased errors. These effects were apparent even when the two tRNAs carry the same modification. For example, the absence of $mnm^5$-modification increased errors by $tRNA_{UUC}^{Glu}$ but decreased errors by $tRNA_{UUU}^{Lys}$ and the absence of Q increased errors by $tRNA_{QUC}^{Asp}$ but decreased errors by $tRNA_{QUA}^{Tyr}$. These results suggest that modification of $tRNA_{UUC}^{Glu}$ and $tRNA_{QUC}^{Asp}$ increase translational accuracy

while those of $tRNA_{UUU}^{Lys}$ and $tRNA_{QUA}^{Tyr}$ reduce accuracy. These effects could either result from an intrinsic change in behavior of the tRNAs or could result from a some effect specific to the codon being misread, a context effect, which has been documented for cognate decoding by nonsense (54,55) and missense suppressors (56) and was invoked to explain over-representation of certain codon pairs in genomes (57) and the effect of codon pairs on elongation speed (58). tRNA modification can alter the magnitude of the context effect. For example, the lack of $ms^2i^6A37$ modification reduced nonsense suppression and increased the context sensitivity of readthrough (reviewed in 12). In this case, lack of the modification changed the magnitude of the context effect, but not its direction. There is no precedent for a context effect that changes an effect from increasing to decreasing translation efficiency compared with wild-type. For this reason, we suspect that context does not explain the difference between the two classes of misreading events we have found.

The alternative to a context effect is that the direction of the effect depends on a feature of each tRNA's structure. The effect cannot be an intrinsic effect of the modification since a specific modification can have opposite effects on distinct tRNAs. The lack of $mnm^5$ has opposite effects on $tRNA_{UUU}^{Lys}$ and $tRNA_{UUC}^{Glu}$ (compare Figures 1 and 2) as lack of Q has opposite effects on $tRNA_{QUC}^{Asp}$ and $tRNA_{QUA}^{Tyr}$ (compare Figures 4 and 5). The most parsimonious conclusion based on this data is that the distinct changes in accuracy reflect an essential difference between the pairs of tRNAs.

Structural analysis of the *E. coli* $tRNA_{UUU}^{Lys}$ has demonstrated that its anticodon loop is unusually flexible (59). The $mnm^5$-modification studied here *in vivo* performs an important role in stabilizing the U-turn in $tRNA_{UUU}^{Lys}$ between nucleotide U33 and U34 (the wobble nucleotide) that is essential to codon recognition (60). A $tRNA_{UUU}^{Lys}$ lacking $mnm^5$ and $t^6A37$, which flanks the other end of the anticodon, cannot bind *in vitro* to either cognate codon (AAA or AAG) in the ribosomal A site (61). The simplest explanation for the opposite effect of this modification on $tRNA_{UUU}^{Lys}$ and $tRNA_{UUC}^{Glu}$ is that the lack of the modification in $tRNA_{UUU}^{Lys}$, although not $tRNA_{UUC}^{Glu}$, reduces its activity *in vivo* and thus results in reduced misreading. The similar effect with $tRNA_{QUA}^{Tyr}$ suggests that it may also depend on anticodon loop modification, both at positions 34 and 37, to insure its activity. Murphy *et al.* (62) described the effect of anticodon loop modification of *E. coli* $tRNA_{UUU}^{Lys}$ as 'preordering' the anticodon, meaning that it stabilizes a conformation in solution appropriate for recognizing its cognate codons. Similar effects were seen for modifications of $tRNA_{GAA}^{Phe}$ to 1-methylguanosine ($m^1G$), $N^6$-isopentenyladenosine ($i^6A$) and wybutosine (yW) (63) and 2-thiouridine ($s^2U$) was shown to reduce the number of structural conformers of a U•U pair from four for an unmodified U to only one for $s^2U$ (64). In a tRNA like $tRNA_{UUU}^{Lys}$, with its unusual flexibility, this preordering may simply increase the efficiency of the tRNA and, as we have

seen, result in increased translational misreading as a consequence.

In tRNAs like $tRNA_{UUC}^{Glu}$ or $tRNA_{QUC}^{Asp}$ the effect of the modification appears to be different. There is ample evidence that the rate of cognate recognition is increased by the presence of anticodon loop modifications (for example, see 65–67). Our results confirm that the other function of these modifications is to reduce the rate of near-cognate recognition since the frequency of errors increases for these tRNAs when the modification is absent. These two effects appear to be in conflict, increasing cognate coding efficiency while decreasing near-cognate efficiency. We suspect that this difference reflects differences in the details of the cognate and near-cognate codon–anticodon complex. The misreading events that show relatively high frequencies of errors in our analysis appear to result from formation of a single non-canonical pair, either G•U, U•U or C•U (7). Recently, Demeshkina *et al.* (10) presented the structure of a complex in which $tRNA_{QUA}^{Tyr}$ decodes the Cys codon UGC, which we have shown here is a frequent error *in vivo*. They showed that a G•U non-canonical pair in the second codon position conforms closely to Watson–Crick geometry rather than showing the lateral shift of the U away from its position in a Watson–Crick A•U pair, as occurs in the third or wobble position. Rozov *et al.* (11) demonstrated other the non-canonical pairs C•A or A•A do not conform to Watson–Crick geometry and undergo large displacements of the codon nucleotide. Correspondingly, we find no evidence of frequent errors involving C•A or A•A pairs *in vivo* (7). The ability of the G•U pairs to adopt Watson–Crick geometry may explain their increased acceptance by the ribosome. Structures of U•U and C•U near-cognate complexes are not available but the structure formed by $cmo^5U$ with U and C also closely conforms to Watson–Crick geometry (68). U•U (69) and C•U pairs (70) in solved RNA helices have similar conformations. It appears, based on these data, that the more frequent translational misreading errors require that non-canonical base pairs mimic Watson–Crick geometry.

The fact that lack of anticodon loop modification results in increased misreading suggests that in some way the presence of the modifications reduces the efficiency of forming these non-canonical pairs. If the modifications induce preordering then we would propose that for $tRNA_{UUC}^{Glu}$ and $tRNA_{QUC}^{Asp}$ the conformation of the preordered structure interferes with formation of the non-canonical pairs. Alternatively, since the formation of G•U pairs appears to require protonation or formation of alternative tautomers (for a discussion see 11), the modifications may shift the equilibria for these reactions away from the non-canonical form. To understand how modification interferes with near-cognate decoding by these tRNAs structural studies of modified and unmodified tRNAs will be required.

The effect of Q modification on $tRNA_{QUA}^{Tyr}$ may be another example of a modification that modulates decoding but resulting in increased errors. Loss of Q in $tRNA_{QUA}^{Tyr}$ cannot be explained as a general loss of function since substantial misreading for UGC remains in *tgtΔ*. It appears that misreading on UGU and UAG strongly require the

presence of Q whereas misreading on UGC is less dependent. Like $tRNA_{UUC}^{Glu}$ and $tRNA_{QUC}^{Asp}$ this effect could reflect a preordering of the anticodon but in this case supporting near cognate decoding rather than reducing it. A clear understanding of the effect of Q on $tRNA_{QUA}^{Tyr}$ will require solution of the structure of the tRNA bound to A site cognate and near-cognate codons.

Do the effects we have seen depend only on modification dependent on the enzyme encoded by the deleted gene? For example, could the loss of nearby anticodon loop modifications contribute to the observed accuracy phenotype? We demonstrated that the modifications tested do not block other nearby modifications in several cases. In the $mnmE\Delta$ mutant we saw no reduction in $t^6A$ (present at position 37 of $tRNA_{UUU}^{Lys}$) or $m^2A$ (present at 37 for $tRNA_{UUC}^{Glu}$). The $tgt\Delta$ mutation did not block the $ms^2i^6A37$ modification of $tRNA_{QUA}^{Tyr}$ or $m^2A$ modification of $tRNA_{QUC}^{Asp}$. In each case, *in vitro* evidence argues for the lack of effect of the primary targeted modifications on the nearby modifications. *In vitro* evidence shows that the modification enzymes encoded by *tgt* (53) and *miaA* (71) function even on unmodified tRNA transcripts. For all these reasons we believe that it is unlikely that the phenotypes associated with the lack of the modification enzymes result from disruption of other nearby modifications.

In summary, the data we have presented suggest that the effect of anticodon loop modifications depends on their structural context. In some tRNAs, and perhaps most, the modifications appear to fine tune the structure of the anticodon loop to promote efficient cognate decoding while reducing the efficiency of errant, near-cognate decoding. In two cases, however, and perhaps in others, the fundamental instability of the anticodon appears to make the modification essential for either cognate or near-cognate decoding and therefore the presence of the modification has the counterintuitive effect of increasing the frequency of translational misreading errors.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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