Codon-specific effects of tRNA anticodon loop modifications on translational misreading errors in the yeast *Saccharomyces cerevisiae*

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**ABSTRACT**

Protein synthesis requires both high speed and accuracy to ensure a healthy cellular environment. Estimates of errors during protein synthesis in *Saccharomyces cerevisiae* have varied from $10^{-3}$ to $10^{-4}$ errors per codon. Here, we show that errors made by tRNA$_{Glu}$ in yeast can vary 100-fold, from $10^{-8}$ to $10^{-4}$ errors per codon. The most frequent errors require a G$ullet$U mismatch at the second position for the near cognate codon GGA (Gly). We also show, contrary to our previous results, that yeast tRNAs can make errors involving mismatches at the wobble position but with low efficiency. We have also assessed the effect on misreading frequency of post-transcriptional modifications of tRNAs, which are known to regulate cognate codon decoding in yeast. We tested the roles of mcm$^5$s$^2$U$_{34}$ and t$^6$A$_{37}$ and show that their effects depend on details of the codon anticodon interaction including the position of the modification with respect to the base mismatch and the nature of that mismatch. Both mcm$^5$ and s$^2$ modification of wobble uridine strongly stabilizes G$_2$$ullet$U$_{35}$ mismatches when tRNA$_{Glu}$ misreads the GGA Gly codon but has weaker effects on other mismatches. By contrast, t$^6$A$_{37}$ destabilizes U$_1$$ullet$U$_{36}$ mismatches when tRNA$_{Lys}$ misreads UAA or UAG but stabilizes mismatches at the second and wobble positions.

**INTRODUCTION**

Ribosomes decode the information in mRNAs using tRNAs to produce a polypeptide product. The efficiency and fidelity of this process are critical to the health of the cell and systems have evolved both to optimize speed and accuracy (1). A critical step in terms of accuracy and the cause of the most frequent errors is the recruitment of aminoacyl-tRNAs (aa-tRNAs). Recruitment is governed by a suite of interactions between the ribosome and the codon–anticodon complex (2). The occasional ‘misreading’ errors result from the acceptance of an incorrect aa-tRNA resulting in the substitution of one amino acid by another in the protein product. We have shown for *Escherichia coli* that these errors occur *in vivo* at frequencies up to $3.5 \times 10^{-3}$ per decoding event (3) but some errors are no more frequent than $2 \times 10^{-6}$ (4), which is orders of magnitude less frequent than has been supposed (5).

The understanding of how the ribosome discriminates between correct (cognate) and incorrect (near and non-cognate) aa-tRNAs has advanced recently. Aa-tRNAs bind to the A site in a ternary complex with an elongation factor (EF-Tu in bacteria or its cognate EF-1A in eukaryotes) and guanosine triphosphate (GTP). The ribosome controls acceptance in a two-stage process before (selection) or after (proofreading) hydrolysis of the GTP. The two stages are composed of several distinct kinetic steps. Cognate tRNAs are known to accelerate activation of the intrinsic GTPase activity of EF-Tu/EF-1A and accommodation of the aminoacyl-tRNA into the ribosomal P site after GTP is hydrolyzed (6–8). Ogle et al. (9,10) proposed that cognate but not near-cognate ternary complex can efficiently bind the A site and induce a large-scale rearrangement of the ribosome called domain closure that both prevents dissociation of the ternary complex and activates the EF-Tu GTPase. This large-scale rearrangement involves induced fit in which conformational changes in constituents of the A site allows them to contact all three base pairs of the codon–anticodon complex (11) suggesting that the inability of near-cognate complexes to induce these changes explains the preference for cognate complexes. More recently, X-ray crystallography of near-cognate bacterial complexes showed that near-cognate tRNAs can form G$ullet$U mismatches that adopt a geometry indistinguishable from canonical Watson–Crick pairs, interact with the A site equivalently and induce domain closure (12,13). Rozov et al. (14) proposed that closure of the small subunit generates a rigid geometrical mold that constrains some mismatched pairs, but not others (15), to adopt Watson–Crick geometry. A recent ensemble cryoEM study of recruitment of cognate and near-
cognate ternary complexes binding to 70S ribosomes argues strongly for induced fit (16). Ternary complex recruitment was shown to involve three distinct pre-accommodation structures with the final structure of both cognate and near-cognate complexes resembling a previously characterized structure (A/T). In the A/T structure, a cognate ternary complex inserts aa-tRNA into the decoding center such that the paired codon–anticodon complex is fully engaged with three rRNA nucleotides of the decoding site: G530, A1492 and A1493 (17). The interaction of these nucleotides is now known to occur step-wise through intermediate states and is consistent with induced fit. The conformational flexibility of step-wise tRNA recruitment seen in the cryoEM study (16) contradicts a model in which a rigid decoding center forces the mismatched pair into Watson–Crick geometry (the mold model). The fact that the A site interacts equivalently with cognate and certain near-cognate codon–anticodon complexes suggests that some mismatches are indistinguishable from canonical Watson–Crick pairs (molecular mimicry model) (12–15).

Despite advances in understanding the steps leading to aa-tRNA selection in vitro, in vivo analysis of misreading error remains important to understand fully how ribosomes maintain translational accuracy. The higher and lower-frequency errors that we have observed appear to be fundamentally different with the higher frequency events depending on acceptance of tRNAs making a small subset of nucleotide mismatches (4). The nature of these mismatches confirms some predictions based on structural analysis. The most frequent errors predominately involve the same G•U mismatched base pairs shown to mimic cognate Watson–Crick pairs during A site binding (14). Other highly frequent errors require U•U or U•C mismatches, which may also mimic Watson–Crick pairs (14); the frequency of acceptance of aa-tRNAs forming these mismatches contradicts the prediction of Rozov et al. (14) that the lack of hydrogen bonding in U-U pairs would reduce their ability to induce errors. An experiment involving unbiased assays of nearly all possible errors using a mass spectrometry approach produced essentially the same conclusion (18). Two studies measuring misreading of nonsense codons also found similar mismatches at the first two codon positions but identified other mismatches associated with significant frequency of selection including A•C, G•A and A•A wobble mismatches in the third or wobble position (19,20); the structures of these mismatches in the A site are not available.

Our studies of misreading errors by tRNA^{Glu}_{UUC} in E. coli (21) and S. cerevisiae (22) identified some differences in the phenomenology of these errors. Overall, the frequency of misreading errors in S. cerevisiae is less than in E. coli (22,23). In addition, errors involving mismatches at the third, or wobble position of the codon predominate in E. coli but were not detected in yeast suggesting that S. cerevisiae might differ from E. coli fundamentally in its ability to discriminate against this type of error (22). The absolute frequency of misreading errors depends on several variables. One source of variation is the effect of competition by cognate tRNAs for the mutant codons; higher misreading error frequencies result from lower competition by low-abundance cognate tRNAs (3). Post-transcriptional modifications can further modulate misreading errors by stabilizing or destabilizing reading by either the misreading tRNA or its competing cognate. The highest diversity of modifications is within the anticodon loop, particularly positions 34 and 37. These modifications increase the efficiency of cognate decoding (24,25) by increasing codon–anticodon stacking energy (26) and they have been proposed to ‘preorder’ the anticodons into a conformation appropriate for cognate recognition (27). By optimizing decoding rates these modifications are thought to help maintain proteome integrity by reducing co-translational protein misfolding caused by sporadic pausing during elongation (28). Modifications of wobble nucleotide U34 (xm^2U, xm^3U and xm^5U) are thought to restrict decoding to A and G ending codons (29,30). U34 modifications also have important roles in regulating translational errors (31). We have shown that in E. coli the mm^2 modification destabilizes misreading by tRNA^{Glu}_{UUC} but actually stabilizes errors by tRNA^{Lys}_{UUA}. In the former case, the modification appears to increase discrimination against near-cognate decoding by the tRNA but in the latter the modification appears to generally support decoding by stabilizing a functional conformation of the very weakly structured tRNA anticodon. Comparable divergent effects of wobble quenosine (Q) on tRNA^{Asp}_{UUC} and tRNA^{Tyr}_{UUA} can be explained similarly. Modifications on base 37, adjacent to the anticodon, appear to have a different function of increasing codon–anticodon stacking energy (32). The t^6A37 modification decreases frameshifting in yeast (33) while m^2P^3A37 increases misreading errors in bacteria (31,34) and Schizosaccharomyces pombe (35).

Here we validate a reporter-based system to measure misreading errors by tRNA^{Glu}_{UUC} in yeast and use it, and a second reporter of errors by tRNA^{Lys}_{UUA}, to determine the effect of anticodon loop modifications of the two tRNAs on misreading frequencies. We demonstrate that wobble position misreading errors do occur in yeast but with much reduced frequency compared to in bacteria. This difference is not caused by the eukaryotic-specific wobble modifications of these two tRNAs. Rather, the bacterial and yeast systems appear to differ in their intrinsic abilities to reduce these errors. We do find a difference in the effect of anticodon loop modifications in yeast compared to bacteria. In bacteria these modifications affect the decoding activity or stability of tRNA and the lack of modification had similar effects on all misread codons for each tRNA. In yeast, by contrast, the modifications regulate misreading in a codon-specific manner by altering the selection of the tRNA differentially on various misread codons.

MATERIALS AND METHODS

Strains and growth conditions

The E. coli strain used in this study for cloning and plasmid propagation is in DH5α (F− Φ80lacZAM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 phoA supE44 λ− thi-1 gyrA96 relA1) (36). All bacterial strains are cultured at 37°C in Luria-Bertani (LB) media (10 g NaCl, 10 g tryptone and 5 g yeast extract per liter) supplemented with ampicillin (100 μg/ml) or chloramphenicol (25 μg/ml) as required.
The *S. cerevisiae* used in this study is in the BY4742 background (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) (37). Yeast strains were grown either in Yeast peptone dextrose (YPD) media (Difco) or Synthetic Complete media lacking uracil (SC-Ura) (1.7 g Yeast Nitrogen Base w/o amino acids, 5 g ammonium sulphate supplemented with 2% glucose, amino acids and adenine but lacking uracil for selective purposes). Single mutants (elp3Δ, ncs6Δ, sua5Δ) were created by sporulating a corresponding heterozygous diploid strain in the BY4743 background (MATa/α his3Δ1/α his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0) followed by selection of a G418 resistant ascospore on YPD + G418 (200 μg/ml). The double mutants (elp3Δ ncs2Δ, elp3Δ ncs6Δ and elp3Δ sua5Δ) were generated by one-step polymerase chain reaction (PCR)-based gene replacement (38), using the NATMX marker for deletion and positive selection on YPD plates supplemented with nourseothricin (100 μg/ml) (39). Successful deletion was confirmed by PCR. Yeast transformation was carried out as described before (40). To create yeast strains with the hyper accurate and error prone ribosomes, we started with *RPS23Δ* yeast strain in a BY4742 background and subsequently introduce a vector carrying *RPS23A* as either the wild-type or mutant copies (*RPS23A-K62R* and *RPS23A A113V*) (41) Translational errors were induced by addition of a sublethal concentration (200 μg/ml) of the antibiotic paromomycin (42).

**Plasmids**

The construction of the K529 dual luciferase reporter system used in this study, based on the plasmid pDB688 (42) (Supplementary Figure S1), has been described (22). To construct the E537 β-galactosidase reporter plasmids we introduced active site (E537) mutants of β-galactosidase into pANU7 (Supplementary Figure S2), a yeast-based vector (43) that provides *bla* (ampicillin resistance in bacteria) and *URA3* (uracil auxotrophy in yeast) as selection markers. A BamHI–SacI fragment of the pJC27 vector (44) containing the *ura3*Δ marker for deletion and positive selection on YPD plates (Difco) or Synthetic Complete media lacking uracil (SC-Ura) (1.7 g Yeast Nitrogen Base w/o amino acids, 5 g ammonium sulphate supplemented with 2% glucose, amino acids and adenine but lacking uracil for selective purposes). Single mutants (elp3Δ, ncs6Δ, sua5Δ) were created by sporulating a corresponding heterozygous diploid strain in the BY4743 background (MATa/α his3Δ1/α his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0) followed by selection of a G418 resistant ascospore on YPD + G418 (200 μg/ml). The double mutants (elp3Δ ncs2Δ, elp3Δ ncs6Δ and elp3Δ sua5Δ) were generated by one-step polymerase chain reaction (PCR)-based gene replacement (38), using the NATMX marker for deletion and positive selection on YPD plates supplemented with nourseothricin (100 μg/ml) (39). Successful deletion was confirmed by PCR. Yeast transformation was carried out as described before (40). To create yeast strains with the hyper accurate and error prone ribosomes, we started with *RPS23Δ* yeast strain in a BY4742 background and subsequently introduce a vector carrying *RPS23A* as either the wild-type or mutant copies (*RPS23A-K62R* and *RPS23A A113V*) (41) Translational errors were induced by addition of a sublethal concentration (200 μg/ml) of the antibiotic paromomycin (42).

**Preparation of cell extracts and enzyme assays**

β-galactosidase protein assays were performed on yeast strains transformed with reporter plasmids and grown in selective medium to an OD600 of 0.8–1.0. Transformant cells expressing wild-type β-galactosidase were diluted for assay 1000-fold compared to mutants and assayed to quantify β-galactosidase activity using the Promega β-Glo system according to manufacturer’s specification, using 96-well LUMITRAC plates (Greiner Bio One). Activities in Relative Light Units were measured using a in a Modulus II Microplate Multimode Reader (Turner BioSystems) according to manufacturer’s directions. Assays of our dual luciferase reporters were performed using the Dual-Luciferase Reporter Assay System (Promega) essentially as described (3) and quantified similarly. For both assays, three to six replicate biological samples were assayed each in three technical repeats. Statistical significance of results was determined using a two-tailed, homoscedastic Student’s t-test.

**RESULTS**

Misreading errors by tRNA<sub>Glu</sub> occur at the same codons but are less frequent than in bacteria, especially wobble position errors

In previous work we have reported frequencies of misreading errors for several tRNAs in *E. coli* (3,4,31) but only for tRNA<sub>Glu</sub> in *S. cerevisiae* (22). Comparison of the frequency of errors by tRNA<sub>Glu</sub> in *E. coli* and *S. cerevisiae* revealed two significant differences. Errors at individual codons in *S. cerevisiae* were 3- to 5-fold less frequent than in *E. coli* and wobble position errors (on the Asn codons AAU and AAC) appeared to be absent in *S. cerevisiae* whereas they were quite frequent in *E. coli*. The differences suggest that *S. cerevisiae*, and perhaps eukaryotes in general, might have evolved mechanisms to reduce misreading errors, especially with respect to wobble position errors. To test this conclusion, we exploited a set of misreading error reporters based on active site mutants altering glutamic acid 537 (E537) of *E. coli* β-galactosidase, encoded by the lacZ gene (4). Two isoaccepting tRNAs decode Glu codons, tRNA<sub>Glu</sub><sup>Glu</sup> and tRNA<sub>Glu</sub><sup>Lys</sup>. The former is much more abundant (45) so errors in this reporter system probably nearly exclusively reflect errors by that tRNA. As discussed below, mutants that alter wobble U modification of tRNA<sub>Glu</sub><sup>Glu</sup> alter the frequency of all misreading events, which validates this conclusion. To quantify all possible misreading errors by tRNA<sub>Glu</sub><sup>Glu</sup>, we measured the activities of 14 near-cognate and 7 synonymous non-cognate mutants (Figure 1A). As in *E. coli* (4), a majority of the mutants produced very little activity, averaging 2 × 10<sup>-6</sup> times wild-type; these include 10 of the near-cognate and all of the synonymous non-cognate mutants (Figure 1B). The remaining four mutants, the Gly codons GGA/GGG and the Asp codons GAA/GAC, produced 46- to 150-fold more activity. GGA and GGG misreading requiring middle position G<sub>S</sub>•U<sub>35</sub> mismatches (we refer throughout to base mismatches in codon–anticodon order with subscripts to indicate the positions of the nucleotides in the mRNA codon and tRNA) and GAA/GAC requires U<sub>3</sub>•U<sub>33</sub> or C<sub>3</sub>•U<sub>33</sub> third position or wobble error. Comparing the results to *E. coli* shows that these four mutants produced on average 3.5-fold fewer errors in *S. cerevisiae*. Previously we showed that errors by tRNA<sub>Glu</sub><sup>Lys</sup> are also lower in *S. cerevisiae* than in *E. coli* (3).

One of the indications that the activity expressed by a reporter gene results from misreading is that the activity is greater than that of synonymous mutants. This is clear for the GGA and GGG Gly mutants, which have distinctly different activities that are also far greater than that of the synonymous non-cognate mutants (GGU/GGC). The activities of the two wobble position mutants (GAA/GAC) were nearly identical and these codons lack synonymous non-cognates. Thus, the GAA/GAC activity could result not from misreading but from the substitution of the wild-type
We previously failed to demonstrated errors by tRNA_{Lys}^{UUC} in S. cerevisiae involving wobble position mismatches on the Asn codons AAU and AAC and suggested that yeast might lack wobble position errors in general (22). Using the same RPS23A error-modulating mutations we found no decrease in the activity of the AAU and AAC mutants in the presence of RPS23A-A113V but a significant increase in the presence of rps23A-K62R (Supplementary Table S2). These data show that under error-inducing conditions tRNA_{Lys}^{UUC} can misread by wobble misreading although the activity of the AAU/AAC mutants in non-error inducing conditions is that of the mutant protein and any activity due to misreading is obscured by this background.

Wobble uridine modification of tRNA_{Glu}^{UUC} and tRNA_{Lys}^{UUU} regulates misreading in a codon context-dependent manner

Both tRNA_{Lys}^{UUU} and tRNA_{Glu}^{UUC} recognize A or G-ending codons by third position pairing with the modified nucleotide 5-methoxycarbonylmethyl-2-thiouridine (mcm\textsuperscript{s2}U34). This modification has been thought to block misreading by U3\textsuperscript{s2}U34 or C3\textsuperscript{s2}U34 mismatching (47). In S. cerevisiae the mcm\textsuperscript{5} modification is added to the unmodified wobble U34 by the Elongator complex (Elp1-Elp6) (48) and s\textsuperscript{2} by the Ncs6\textsuperscript{s2}Ncs2 complex (49). The hypermodified mcm\textsuperscript{s2}U nucleotide is present on three tRNAs that decode pairs of synonymous codons from the third column of the genetic code (Figure 1A), which includes all codons with A in the middle position. The mcm\textsuperscript{5} and s\textsuperscript{2} are introduced independently (25,48,50). In an elp3\Delta strain the wobble nucleotide of tRNA_{Glu}^{UUC} and tRNA_{Lys}^{Lys} is s\textsuperscript{2}U34 rather than the mcm\textsuperscript{s2}U34 found in the ELp3\textsuperscript{5} wild-type (48,51). In the elp3\Delta strain there is little or no change in any other post-transcriptional modification of tRNA_{Glu}^{UUC} indicating that any elp3\Delta phenotype must result from lack of mcm\textsuperscript{5} (48). In either an ncs2\Delta or ncs6\Delta strain, mcm\textsuperscript{s2}U34 in tRNA_{Lys}^{UUC} is replaced by mcm\textsuperscript{5}U34 (52). A detailed analysis of the effect on tRNA modification of ncs2\Delta or ncs6\Delta has not been performed but lack of the s\textsuperscript{2} modification is known to have no significant effect on either aminocyclation or the concentration of the modified tRNAs (25,52,53). Eliminating both modification systems is lethal in a W303 genetic background but overexpressing tRNA_{Lys}^{Lys} suppresses the lethality, suggesting that the lack of the modification reduces the efficiency of codon recognition (25). In the S288c genetic background lack of both modifications is not lethal (28,51).

The effect on misreading of the mcm\textsuperscript{5} and s\textsuperscript{2} moieties of mcm\textsuperscript{s2}U34 can be determined by comparing the activities of the misreading reporters in a strain with U34 (elp3\Delta ncs6\Delta) to those with mcm\textsuperscript{5}U34 (ncs6\Delta) or s\textsuperscript{2}U34 (elp3\Delta) or the effect of both by comparing with the activities in the wild-type parental strain. We determined the effect of these modifications by quantifying errors by tRNA_{Glu}^{UUC} using the four error-prone mutants of E537 of β-galactosidase, and those by tRNA_{Lys}^{UUU} using mutants of K529 of firefly luciferase. The ratio of enzyme activity in these strains var-

![Figure 1.](image-url)
ied widely according to the codon being misread. For reporters of first and second position misreading, the presence of mcm3 modification significantly increased errors at UAA, UAG and GGA (an average of 1.8-fold), decreased those at GGG (1.4-fold) and had no significant effect on errors at AGG strain (Table 1). The presence of the s2 modification significantly increased errors at all codons except GGG (an average of 2-fold) but the increases were significantly greater for the A-ending than the G-ending codons (UAA and GAA versus UAG and AGG.) The presence of both modifications increased errors at UAA (7.7-fold), UAG (1.6-fold) and GGA (42-fold) and decreased errors at AGG (1.2-fold) and GGG (1.5-fold). A combination the two modifications showed strong positive synergism for errors at UAA and GGA suggesting that the two modifications cooperatively increase the frequency of misreading errors at these two codons. For AGG and GGG, the combination showed weak negative synergism and for UAG no synergism. In general, the greatest individual or combined effects of these modifications were on A-ending codons, UAA and GGA, and the effects on G-ending codons were either significantly less or actually negative.

The effect of U modification on near-cognate decoding is generally similar to their effect on cognate decoding. Introducing s2 at U34 increases the affinity of cognate binding to A but not G-ending codons both in vitro and in vivo (54,55) and recent kinetic analysis shows that s2 slows dissociation of tRNAlys UUU from its cognate codon AAA during both initial selection and proofreading and accelerates acceptance further in two other ways (56). The mcm5 modification also promotes decoding of both A and G-ending codons (57) though the preference for A-ending codons is weaker than for s2 (58); details of the kinetic basis of this effect are not available for mcm5. The synergism we observed is consistent with in vitro data suggesting that the maximum effect of mcm5 on cognate decoding requires s2 (26). The negative synergism on two G-ending codons suggests that at least for near-cognate decoding, the combination of the two modifications interact to limit misreading; the mechanism of this synergism is unclear.

Misreading errors involving wobble position mismatches (U34 or C34) largely were increased by mcm5 and s2 modifications (Table 1). The presence of either or both modification increased all wobble misreading errors by tRNAlys UUU but with no synergism. The effect on wobble errors by tRNAGlu UUC was less consistent. The presence of either mcm5 or s2 had no significant effect on errors involving U34 or C34 matches with the exception of errors at GAU, which were significantly decreased by mcm5 modification. These data are generally inconsistent with the proposal that these wobble U modifications restrict wobble mismatch errors although the negative effect of mcm3 on some errors by tRNAGlu UUC suggests that they can have that effect depending on the codon sequence context.

It had been thought that xm5 modifications block misreading of pyrimidine-ending codons by restricting nucleotide conformation (29) but structural results challenged that proposal for mmm5U in bacteria (27). In vivo analysis in bacteria, however, shows that mmm5 modification does limit recognition of pyrimidine ending codons by tRNAGlu UUC and tRNAlys UUU (31,59). Based on these results, we suspected that the extremely low level of wobble errors in S. cerevisiae might result from mcm5 modification more severely limiting pyrimidine-pyrimidine mismatches. Our data show the opposite, that these errors are extremely low for tRNAs with unmodified wobble U and that the presence of either modification generally increases them. The direct comparison of errors involving a s2U wobble nucleotide pairing with pyrimidines shows that they are much more frequent in E. coli than in S. cerevisiae, which suggests that some other aspect of translation in yeast must limit these errors.

N6-Threonylcarbamoyladenosine modification at position 37 regulates misreading errors in tRNA Lys

We previously demonstrated that nucleotide 37 modifications can modulate translation accuracy in E. coli in the case of 2-methylthio-N6-isopentenyladenosine (ms2A37) in tRNA^Tyr^ (31). We extended this analysis of the role of modifications in this position in yeast but of the two yeast tRNAs studied here only tRNAlys UUU has a modified nucleotide 37, N6-threonylcarbamoyladenosine (t6A) (34,60). The t6A modification is present in all tRNAs that decode codons with a first position A, which corresponds to the codons of the third row of the standard genetic code (Figure 1A). The purpose of this modification appears to be to compensate for the weakness of the A1-U36 pair formed when these tRNAs read their cognate codon (61) by the t6A37 in the anticodon stacking on the first base of the codon (27). The enzyme responsible for modifying tRNAs with t6A in E. coli is essential although the essentiality of the modification itself has not been demonstrated (62). In yeast, however, the modification is not essential, which allows us to test genetically the modification’s role in modulating misreading errors in yeast.

Biosynthesis of t6A is a complex process involving the Sua5 protein and the KEOPS complex (Kae1, Bud32, Gon7, Pcc1 and Cgi121) (34,62). Sua5 is responsible for synthesizing the intermediate threonyl-carbamoyl-AMP (TC-AMP) and the KEOPS complex transfers the threonyl-carbamoyl moiety to tRNAs. To study the effect of t6A modification at position 37 on misreading by tRNAlys UUU we introduced the K529 reporter plasmids into a sua5Δ strain; this analysis was repeated with mutants lacking the Bud32 and Kae1 subunits of the KEOPS complex with similar results (Supplementary Table S3). We compared activities of our firefly luciferase misreading reporters in strains lacking Sua5 (sua5Δ and elp3Δ sua5Δ) and those in which it is present (the wild-type parent and elp3Δ). In each case, the presence of t6A reduced the activity of UAA (4.5-fold) and UAG (9-fold) termination codon mutants (Table 2). Misreading these codons requires a U1-U36 first position mismatch. By contrast, the presence of t6A significantly increased misreading of the other three error-prone codons, AGG (G3-U35 mismatch), AAA and AAC (U34 or C34 mismatches). All of these effects were similar in the presence or absence of mcm5 modification, suggesting that the effect of t6A is independent of the effect of wobble modifications. In the case of the UAA codon, the frequency of
misreading is much greater in the presence of mcm\(^5\) than in its absence, consistent with the stabilizing effect of mcm\(^3\) on \(\text{A}_3\bullet\text{U}_{34}\) pairing. In the absence of \(\text{t}^\text{6A}\), misreading of UAG is increased only 1.4-fold by addition of mcm\(^5\); we attribute this effect to the stabilizing influence of mcm\(^5\) on this ternary complex and elements of the A site (65). The result of our in vivo misreading analysis affords an important commentary on this question because it demonstrates that acceptance of near-cognate tRNAs is largely restricted to those that involve specific nucleotide mismatches including \(\text{G}\text{U}\), \(\text{U}\text{U}\) or \(\text{C}\text{U}\). The \(\text{G}\text{U}\) and \(\text{U}\text{U}\) mismatches interact with the A site nucleotides G530, A1492 and A1493 equivalently with Watson–Crick pairs; \(\text{C}\text{U}\) has not been investigated (65).

A comparison of our results reported here with previous studies of misreading errors in \(E\. coli\) (3,4,31) and \(S\. cerevisiae\) (22) demonstrate that these errors predominate and that other near-cognate errors are either much less frequent or undetectable by our system, with errors no higher than \(2 \times 10^{-6}\) per codon. Clearly, then, there is congruence between those mismatches that can induce cognate-like A site interactions and those that result in substantial misreading errors. It is very attractive to conclude that their ability to interact with the A site similarly to a cognate tRNA explains their propensity to misread and, correspondingly, the infrequency or lack of errors involving other mismatches predicts the inability to interact as stably. Rozov et al. (14,65) show that the distance between the paired \(\text{U}\text{U}\) nucleotides is too great to allow hydrogen bonding and suggested that tRNAs with this mismatch should dissociate more readily than those with a \(\text{G}\text{U}\) Watson–Crick mimic mismatch. Our results show that errors using this mismatch are often as frequent or more frequent than \(\text{G}\text{U}\) mismatch errors, suggesting that lack of \(\text{U}\text{U}\) hydrogen bonding per se does not disqualify near-cognates from inducing errors.

Recently, Blanchet et al. (19) and Roy et al. (20) using nonsense codon readthrough assays demonstrated misreading involving the same \(\text{G}_7\text{U}_{36}, \text{U}_7\text{U}_{36}\) mismatches, but also \(\text{A}_3\text{G}_{36}, \text{G}_3\text{G}_{36}\) and \(\text{C}_3\text{A}_{36}\) mismatches. We have found that errors dependent on purine–purine wobble mismatches were extremely infrequent but could be increased to high levels in error-prone conditions (4). Misreading requiring \(\text{C}_3\text{A}_{36}\) mismatches between tRNA\(^\text{Trp}\) and UGA has long been known (66) but we lack any in vivo reporter for that misreading error. Significantly, Blanchet et al. (19) identified these errors using error-inducing conditions involving a \(\text{PSI}^\text{Trp}\) background deficient in eukaryotic release factor 3 (eRF3); prolonged pausing at the nonsense codon could drastically increase the opportunity for misreading. Roy et al. (20) showed that although errors were elevated in the \(\text{PSI}^\text{Trp}\) background, the distribution of misreading errors was relatively unchanged in normal \(\text{PSI}\) cells. It is significant that these errors are confined to the wobble position where base pair geometry is less constrained. The proposed

**Table 1. Effect on misreading errors of addition to \(\text{U}_{34}\) of mcm\(^5\), s\(^2\) or mcm\(^5\)s\(^2\) modifications**

| Misreading tRNA | Codon misread | Mismatch | \(\text{elp}\Delta\text{nsc6}\Delta\) \(\text{U}_{34}\) | \(\text{nsc6}\Delta\) mcm\(^5\)\(\text{U}_{34}\) | \(\text{elp}\Delta\) \(\text{s}^2\)\(\text{U}_{34}\) | Wild-type mcm\(^5\)\(\text{s}^2\)\(\text{U}_{34}\) |
|-----------------|---------------|----------|-----------------|-------------------|-----------------|-----------------|
| tRNA\(^{\text{Lys}}\)\(\text{U}_{34}\) | UAA | \(\text{U}_1\text{U}_{36}\) | 0.22 \pm 0.01 | 0.45 \pm 0.02** (2.0\(\times\)) | 0.52 \pm 0.09** (2.4\(\times\)) | 1.7 \pm 0.17*** (7.7\(\times\)) |
| tRNA\(^{\text{Lys}}\)\(\text{U}_{34}\) | UAG | \(\text{U}_1\text{U}_{36}\) | 3.0 \pm 0.2 | 5.3 \pm 0.40** (1.8\(\times\)) | 4.4 \pm 0.18** (1.5\(\times\)) | 4.8 \pm 0.35** (1.6\(\times\)) |
| tRNA\(^{\text{Glu}}\)\(\text{U}_{34}\) | AGG | \(\text{G}_2\text{U}_{35}\) | 9.1 \pm 0.2 | 9.2 \pm 0.11 (1.0\(\times\)) | 12 \pm 0.29** (1.3\(\times\)) | 7.9 \pm 0.22** (0.86\(\times\)) |
| tRNA\(^{\text{Glu}}\)\(\text{U}_{34}\) | GGA | \(\text{G}_2\text{U}_{35}\) | 0.05 \pm 0.003 | 0.08 \pm 0.008* (1.6\(\times\)) | 0.14 \pm 0.002*** (2.8\(\times\)) | 2.1 \pm 0.13** (42\(\times\)) |
| tRNA\(^{\text{Glu}}\)\(\text{U}_{34}\) | \(\text{GGG}\) | \(\text{G}_2\text{U}_{35}\) | 0.35 \pm 0.03 | 0.26 \pm 0.02* (0.74\(\times\)) | 0.34 \pm 0.01 (0.97\(\times\)) | 0.23 \pm 0.02*** (0.66\(\times\)) |
| tRNA\(^{\text{Lys}}\)\(\text{U}_{34}\) | AUA | \(\text{Y}_1\text{U}_{34}\) | 0.85 \pm 0.05 | 1.3 \pm 0.11** (1.5\(\times\)) | 1.9 \pm 0.16*** (2.2\(\times\)) | 1.3 \pm 0.08** (1.5\(\times\)) |
| tRNA\(^{\text{Lys}}\)\(\text{U}_{34}\) | AAC | \(\text{Y}_1\text{U}_{34}\) | 0.75 \pm 0.05 | 1.3 \pm 0.11** (1.7\(\times\)) | 1.3 \pm 0.11*** (1.7\(\times\)) | 1.3 \pm 0.10*** (1.7\(\times\)) |
| tRNA\(^{\text{Glu}}\)\(\text{U}_{34}\) | GAU | \(\text{Y}_1\text{U}_{34}\) | 1.8 \pm 0.27 | 0.87 \pm 0.07** (0.48\(\times\)) | 1.9 \pm 0.13 (1.1\(\times\)) | 0.95 \pm 0.06*** (0.53\(\times\)) |
| tRNA\(^{\text{Glu}}\)\(\text{U}_{34}\) | GAC | \(\text{Y}_1\text{U}_{34}\) | 0.65 \pm 0.06 | 0.80 \pm 0.06 (1.2\(\times\)) | 0.76 \pm 0.08 (1.2\(\times\)) | 0.94 \pm 0.06* (1.4\(\times\)) |

Standard errors: *, \(P\)-value < 0.05; **, \(P\)-value < 0.01; ***, \(P\)-value < 0.001.

**DISCUSSION**

Protein synthesis is a kinetically regulated process with tRNA selection in the ribosomal decoding site consisting of many discrete steps. Several of these steps distinguish kinetically between correct (cognate) and incorrect (near or non-cognate) tRNAs with the discrimination resulting from structural dynamics of the ribosome and induced fit (64). Recent X-ray crystallographic results suggest that some near-cognate tRNAs can induce ribosomal structural rearrangements identical to those during cognate tRNA binding including rearrangement of the decoding site to allow non-sequence-specific contacts between the codon–anticodon complex and elements of the A site (65). An important question is whether these interactions occur during initial selection since the solved crystal structures are of complexes post initial selection (64). The result of our in vivo misreading analysis affords an important commentary on this question because it demonstrates that acceptance of near-cognate tRNAs is largely restricted to those that involve specific nucleotide mismatches including \(\text{G}\text{U}, \text{U}\text{U}\) or \(\text{C}\text{U}\). The \(\text{G}\text{U}\) and \(\text{U}\text{U}\) mismatches interact with the A site nucleotides G530, A1492 and A1493 equivalently with Watson–Crick pairs; \(\text{C}\text{U}\) has not been investigated (65).
Table 2. Effect on misreading of addition of the t6A37 modification

| Misreading tRNA | Codon misread | Mismatch | Activity relative to wild-type reporter (× 10⁻⁴) |
|-----------------|---------------|----------|-----------------------------------------------|
|                 |               |          | sua5Δ mcm5s2U34 A37 wild-type tRNA            | elp3Δ elp3Δ sua5Δ elp3Δ |
| UAA             | U1•U36        |          | 7.8 ± 0.37                                   | 1.7 ± 0.17** (0.22×) 0.92 ± 0.02 0.52 ± 0.09 (0.57×) |
| tRNA_Lys_UUU   | UAA           |          | 43 ± 1.6                                    | 4.8 ± 0.35*** (0.11×) 31 ± 1.3 4.4 ± 0.18*** (0.14×) |
| tRNA_Lys_UUU   | UAA           |          | 5.8 ± 0.2                                   | 7.9 ± 0.22*** (1.4×) 6.6 ± 0.03 12 ± 0.29*** (1.8×) |
| tRNA_Lys_UUU   | UAA           |          | 0.75 ± 0.03                                 | 1.3 ± 0.11*** (1.7×) 0.82 ± 0.02 1.9 ± 0.16* (2.3×) |
| tRNA_Lys_UUU   | UAA           |          | 0.72 ± 0.04                                 | 1.3 ± 0.10*** (1.8×) 0.64 ± 0.02 1.3 ± 0.11** (2.0×) |

Watson–Crick mimicry model is limited to the more strictly monitored first and second positions. Wobble position errors of this type, however, could also be explained by the purine–purine pairs adopting Hoogsteen pairing and the CCA pairing through tautomerism (65). The fact that we fail to find these errors at other codon positions suggests that selection of tRNAs making these errors can occur only at the less monitored wobble position presumably because of their steric clashes in the other positions.

The details of the structure of individual tRNAs resulting from post-transcriptional modification is known to modulate translational error frequency (reviewed in 67). Here, we show that modifications modulate misreading in distinct ways in S. cerevisiae and E. coli. Our study in E. coli showed that the presence of anticodon loop modifications either increased or decreased errors by each targeted tRNA largely independent of the codon being misread (31). By contrast, in S. cerevisiae the effect of the anticodon loop modifications differed for a particular tRNA depending on the codon being read. The presence of a particular modification on misreading was frequently opposite on various of its near-cognate codons. The presence of the mcms2U34 modification increased misreading frequency on most codons, but it had no significant effect or actually decreased misreading on several codons. The s2 modification similarly increased misreading on most codons but had little or no effect on several others. Generally, misreading was increased for first and second position mismatches on A-ending codons, especially in the case of the s2 modification, but in several cases the modification actually reduced errors, especially for G-ending codons. The effect of the two modifications, mcms2 and s2, was greatest and highly synergistic for two A-ending codons—for tRNA_Glu Lys misreading GGA and for tRNA_Glu Lys misreading UAA. The strong synergism suggests that the modifications alter near-cognate codon recognition in distinct ways. The effect on third or wobble position mismatches was much more similar for A and G-ending codons and showed no evidence of synergism, which suggests that in this case, where the modified base is mismatched, the two modifications do not play distinct roles in supporting decoding.

A full understanding of how modifications modulate errors will require structural analysis of modified near-cognate tRNAs engaged at the A site. Previous work has demonstrated unexpected cognate A site interactions. The mcm5s2U34 base in bacterial tRNA_Lys stacks on the adjacent U35 and the amino group of the mcm5 group apparently hydrogen bonds with the 2’OH of U35. These interactions stabilize the cognate codon–anticodon helix and influence its conformation (14). Similarly, t6A37 of tRNA_Lys forms a cross-strand stack with codon nucleotide A1 to stabilize the weak A1•U36 base pair (14). The question is whether during near-cognate decoding they might have different or even opposite effects derived from their interacting differently with a mismatched codon–anticodon complex. We know, for example, that mcms2 destabilizes all error-prone near-cognate decoding in E. coli (31), which suggests that it has a different role than increasing stacking energy for these complexes. The stabilization effect of t6A37 on the weak A1•U36 base pair becomes a destabilizing effect on U1•U36 mispairing when tRNA_Lys decodes UA or UAG. Clearly the same stabilizing stacking interaction by t6A37 on A1•U36 is missing for U1•U36, perhaps replaced by an interaction that displaces U1 from pairing with U36. Rozov et al. (15) showed that when tRNA_QUA misreads the His codon CAC the hypermodified base queuosine (Q34) stabilizes a conformation of codon nucleotide of C1 away from pairing with anticodon nucleotide A36, blocking formation of the CCA mismatch. An unusual interaction like this may explain effects of modifications like t6A on near-cognate decoding that are opposite to their effects on cognates.

Recent cryo-electron microscopy results provide a detailed view of the process of tRNA assembly involving stepwise assembly of the final A/T complex in a bacterial ribosome (16). These data identify three steps for recruitment of a cognate or near-cognate EF-Tu ternary complex to the ribosomal A site. In these three steps the tRNA increasingly approaches complete pairing with the mRNA codon. With the third step the codon–anticodon complex fully engages with the ribosomal A site and the ribosome shifts to the ‘closed’ conformation. Important, for the near-cognate tRNA the G3•U35 pair only adopts Watson–Crick geometry in this third complex; in the second step the pair is in a non-Watson–Crick conformation. Also, from the first to the third step the elements that recognize a cognate codon–anticodon complex of increasingly move into position to interact. This result is inconsistent with the ‘mold’ hypothesis of Rozov et al., which proposed that the A site adopts a rigid structure that forces the codon–anticodon complex into Watson–Crick geometry (14). The adjustment of the positions of the interacting nucleotides between the first and third step is consistent with the induced fit model (64). However, the concept proposed by Demeshkina et al. (12) that
acceptance of the near-cognate tRNA does involve Watson–Crick mimicry by the mismatched base pair was confirmed by the new data.

The issue of the reduced occurrence of wobble misreading errors by tRNA\textsubscript{Glu} and tRNA\textsubscript{Lys} in S. cerevisiae relative to E. coli might be explained by differences in the process of tRNA recruitment. The three-step process in bacteria involves a stage before formation of the final cognate A/T complex in which the wobble bases are paired and stabilized by rRNA base C1054 stacking on the anticodon wobble nucleotide and partially stabilized by hydrogen bonding to G530 in a ‘semi-on’ conformation; in the near-cognate complex C1054 stacking is disrupted and G530 is in an ‘off’ conformation (16). The wobble bases pair in the near-cognate case only in the third structure in which the A/T codon–anticodon complex interacts fully with the A site. The weak wobble interaction with the A site in structure 2 no doubt contributes to the instability of near-cognate complexes implied in the relative rarity of structure 3 for the non-cognate complex, which implies a higher degree of EF-Tu ternary complex dissociation consistent with kinetic data. The fact that structure 2 does not monitor the wobble pair for the near-cognate case may explain why complexes with G\textsubscript{29}U\textsubscript{35} and Y\textsubscript{34}U\textsubscript{34} mismatches are accepted at approximately equal frequencies despite the presumably much lower stability of the latter pair. Formation of the wobble pair only in concert with latching of the A site may reduce the destabilizing effect of Y\textsubscript{34} versus R\textsubscript{34}, leading to more frequent wobble misreading in bacteria. The much lower frequency of these errors in S. cerevisiae may result from eukaryotic ribosomes transitioning through an unlocked open complex in which wobble pairing is required and the presence of Y\textsubscript{3}U\textsubscript{34} may destabilize tRNAs with that mismatch. Cryo-electron microscopy of similar pre-A/T structures for yeast ribosomes could resolve this issue.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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