

N1-Methylpseudouridine and pseudouridine modifications modulate mRNA decoding during translation

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The ribosome relies on hydrogen bonding interactions between mRNA codons and incoming aminoacyl-tRNAs to ensure rapid and accurate protein production. The inclusion of chemically modified bases into mRNAs has the potential to alter the strength and pattern of hydrogen bonding between mRNAs and aminoacyl-tRNAs to alter protein synthesis. We investigated how the N1-methylpseudouridine (m¹Ψ) modification, commonly incorporated into therapeutic and vaccine mRNA sequences, influences the ability of codons to react with cognate and near-cognate tRNAs and release factors. We find that the presence of a single m¹Ψ does not substantially change the rate constants for amino acid addition by cognate tRNAs or termination by release factors. However, insertion of m¹Ψ can affect the selection of near-cognate tRNAs both *in vitro* and in human cells. Our observations demonstrate that m¹Ψ, and the related naturally occurring pseudouridine (Ψ) modification, exhibit the ability to both increase and decrease the extent of amino acid misincorporation in a codon-position and tRNA dependent manner. To ascertain the chemical logic for our biochemical and cellular observations, we computationally modeled tRNA^{Ile}(GAU) bound to unmodified and m¹Ψ- or Ψ- modified phenylalanine codons (UUU). Our modeling suggests that changes in the energetics of mRNA:tRNA interactions largely correlate with the context specificity of Ile-miscoding events we observe on Ψ and m¹Ψ containing Phe codons. These studies reveal that the sequence context of a given modification within an mRNA plays a large role in determining how (and if) the modification impacts the number and distribution of proteoforms synthesized by the ribosome.

INTRODUCTION

Chemically modified nucleosides are present in all organisms, often playing essential roles in key cellular processes including splicing and translation (1–3). Defects in ribosomal RNA (rRNA) and transfer RNA (tRNA) modifying enzymes are linked to a host of deleterious human health outcomes, illustrating the importance of RNA modifications in protein synthesis (4–7). There are over 150 unique modifications reported in RNAs that range in size and complexity from isomerized or saturated nucleosides (*e.g.* pseudouridine and dihydrouridine) to large chemically diverse functional groups (*e.g.* NAD⁺, N(6)-threonylcarbamoyladenine, glycan and farnesyl) (8–11). RNA modifications have been widely studied for almost three quarters of a century and until recently were thought to be almost exclusively incorporated into non-coding RNA species (ncRNAs). However, the transcriptome wide mapping of 13 RNA modifications revealed that protein coding messenger RNAs (mRNAs) can also contain modifications at thousands of sites (12–26). This discovery has raised the possibility that mRNA modifications might play a previously underappreciated role in post-transcriptionally regulating gene expression.

The majority of enzymes that modify mRNAs also catalyze their incorporation into ncRNAs central to protein synthesis (8). Like their protein post-translational counterparts, mRNA post-transcriptional modifications are generally present at sub-stoichiometric levels, with transcripts existing in a mixed population of modified and unmodified states (15, 26–28). Together these circumstances make ascertaining the impact of mRNA modifications on translation challenging. In cells, any changes to protein output

observed when RNA modifying enzymes are removed are difficult to directly attribute to alterations in a particular mRNA's modification status. Therefore, studies using reconstituted translation systems, where it is possible to uniformly change the modification status of an mRNA without impacting that of ncRNA, have been particularly useful for assessing the consequences of mRNA modifications on translation (29). Initial studies reveal that modifications commonly slow the ribosome, though some only do so only in particular mRNA sequence contexts (29). Additionally, a subset of mRNA modifications, including pseudouridine (Ψ) and inosine (I), also impact the accuracy of mRNA decoding (30–32). These findings suggest that there is a broad range of possible consequences when the ribosome encounters an mRNA modification. Developing a framework for understanding how individual modifications impact translation in differing sequence contexts will be crucial as researchers seek to uncover which of the thousands of chemically modified positions reported in mRNA codons are the most likely to have consequences for protein synthesis in cells.

In addition to being present in naturally occurring RNA molecules, modifications are also heavily incorporated in RNA-based therapeutics and mRNA vaccines (33–36). Indeed, the mRNA transcripts that form the basis of many currently available COVID-19 mRNA vaccines substitute every uridine nucleoside with N1-methylpseudouridine (m¹Ψ) (37). The addition of m¹Ψ limits the cellular innate immune response to dramatically stabilize the mRNA transcript, and ultimately increase the amount of protein synthesized (38–41). Recent studies in a lysate-based translation system suggest that m¹Ψ slows the ribosome in a manner that can

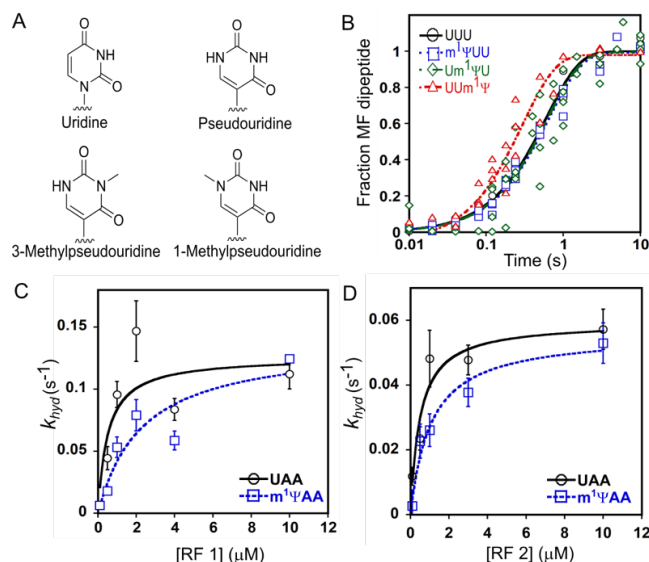


Figure 1. Cognate amino acid addition is modestly increased on UUm¹Ψ, but not m¹ΨUU or UUm¹ΨU, codons. (A) The chemical structures of the nucleobases we investigated. (B) The formation of Met-Phe (MF) dipeptide as a function of time by *E. coli* ribosomes containing ³⁵S-Met-tRNA^{Met} bound to an AUG start codon in the P site, and unmodified (○-UUU) or modified (□-m¹ΨUU, ◇-U^mΨU, △-UUm¹Ψ) codons in the A site. (C) The $K_{1/2}$ curve for RF1. k_{obs} values for RF1-catalyzed ³⁵S-Met release on UAA (○) or m¹ΨAA (□) are displayed as a function of [RF1]. (D) The $K_{1/2}$ curve for RF2. k_{obs} values for RF2-catalyzed ³⁵S-Met release on a UAA (○) or m¹ΨAA (□) are displayed as a function of [RF2].

be alleviated by the addition of membranes (42). However, there is limited information available directly measuring how m¹Ψ influences the rate and accuracy of amino acid addition. This is an important question to ask because m¹Ψ shares much of its structure with pseudouridine (Ψ) (Figure 1A), a modification that has been shown to change translation speed and tRNA selection (30, 43, 44). Even subtle changes in translation rates or fidelity have the potential to impact protein folding or function (45–48). Therefore, establishing if there are situations in which m¹Ψ can alter translation will be critical for the continued development of mRNA-based therapeutics and vaccines in addition to understanding how different types of chemical moieties impact translation.

To ascertain the molecular level consequences of m¹Ψ codon modifications on ribosome decoding, we compared the translation of unmodified and m¹Ψ- modified codons in both a fully reconstituted bacterial *in vitro* translation system and HEK293 cells. These studies reveal that in contrast to Ψ, the presence of a single m¹Ψ does not reduce the rate constant for cognate amino acid addition. However, m¹Ψ does influence the accuracy of amino acid addition. We demonstrate that Ψ and m¹Ψ can both impede and enhance near-cognate tRNA selection depending on the surrounding sequence context and identity of the tRNA. Comparison how Ψ and m¹Ψ modifications affect translation reveal that uridine base isomerization and methylation each contribute to the ability of m¹Ψ to perturb mRNA decoding. Computational modeling of a near cognate tRNA^{Ile,UG} bound to modified and unmodified Phe (UUU) codons in the context of the A site suggests that changes in the energies of

mRNA:tRNA interactions likely account for the context dependent effects of Ψ and m¹Ψ we observe. These findings demonstrate that Ψ and m¹Ψ modulate ribosome decoding and have the potential to modulate the speed and accuracy of protein production from both native and therapeutic mRNA sequences.

RESULTS

m¹Ψ modestly impacts the rate constant for Phe addition and $K_{1/2}$ for peptide release

We used a fully reconstituted *E. coli in vitro* translation system to evaluate the consequences of incorporating m¹Ψ into mRNA codons on translation elongation and termination. In contrast to reporter-based studies in cells and lysates, the *in vitro* system we implemented is not influenced by extra-translational factors that can change observed protein levels (*e.g.* RNases and proteases) and allows us to directly examine individual steps along the translation pathway with high resolution (49). This *E. coli* translation system has long been used to study translation elongation because tRNA binding sites and ribosome peptidyl-transfer center are highly conserved between eukaryotic and bacterial ribosomes (50, 51).

The rate constants for amino acid addition were measured on unmodified (UUU) and m¹Ψ modified (m¹ΨUU, U^mΨU, and UUm¹Ψ) phenylalanine (Phe) codons (Figure 1B). We chose to first evaluate amino acid incorporation rates on a UUU codon because the kinetics of Phe addition on UUU is well established, and UUU codons are present in the Pfizer/BioNTech mRNA COVID-19 vaccine (37). Our translation reactions were initiated by mixing *E. coli* 70S ribosome initiation complexes (ICs; ³⁵S-labeled formylmethionine-tRNA^{Met} [³⁵S-Met] bound to an AUG in the P site and Phe codon in the A site) with an excess of ternary complexes (TCs; Phe-tRNA^{Phe}•EF-Tu•GTP). Reactions were quenched at select time points, and the unreacted ³⁵S-Met and ³⁵S-Met-Phe products are visualized by electrophoretic TLC (eTLC) (SI Figure 1A). These studies reveal that cognate Phe incorporation on m¹Ψ modified codons is largely unchanged, though we observe a slight (2 ± 0.3 -fold) increase in the rate constant for Phe addition when m¹Ψ is in the third position in the codon (Figure 1B, SI Figure 1B, SI Table 1).

All three stop codons begin with uridine (UAA, UAG, UGA) ensuring that modified stop codons will be present in synthetic mRNA-based vaccines and therapeutics. We evaluated the ability of bacterial class I release factors (RF1 and RF2) to hydrolyze peptidyl-tRNA bonds and terminate translation on m¹Ψ modified stop codons. To accomplish this, we reacted termination complexes (*E. coli* 70S ribosomes with ³⁵S-Met bound to an AUG in the P site, and a universal stop codon positioned in the A site (UAA, m¹ΨAA)) with varying concentrations of RF1 and RF2 (0.1–10 μM). The reactions were quenched at a range of time points and ³⁵S-Met hydrolyzed by RFs was detected on an eTLC (SI Figures 2 and 3). At saturating levels of RF1 and RF2 the rate constants for peptide release ($k_{hyd,max}$) on UAA and m¹ΨAA codons are equivalent (~ 0.1 s⁻¹) and comparable previously published termination rates on an unmodified UAA codon (SI Tables 2 and 3) (52, 53). However, this was not the case at sub-saturating conditions, as reflected by the 2- to 4-fold increase in $K_{1/2}$ values obtained for

peptidyl-tRNA hydrolysis by RF1 and RF2 on a m¹ΨAA (Figure 1C-D, SI Tables 2 and 3). The redistribution of the electronegativity around the pyrimidine ring in m¹Ψ can weaken the hydrogen bonding network between the first two nucleotides of the stop codon and release factors, perhaps accounting for this observation (53, 54). Nonetheless, because $k_{hyd,max}$ is unperturbed we do not expect m¹Ψ to impede translation termination in cells unless the concentration of release factors becomes severely limited, or, the termination codon is in a particularly poor sequence context (55). This supposition is supported by numerous observations that reporter peptides and therapeutic RNA sequences generated from fully m¹Ψ-substituted mRNAs yield protein products of the expected length (38, 56).

m¹Ψ influences aminoacyl-tRNA selection by the ribosome in a context dependent manner

Chemical modifications to nucleobases can change the propensity of the ribosome to incorporate alternative amino acids into a growing polypeptide chain (29–32, 57–60). In comparison to uridine, m¹Ψ possesses a repositioned, methylated nitrogen in its pyrimidine ring (Figure 1A). These two changes provide m¹Ψ the opportunity to alter the conformational fit of an mRNA in the ribosome, and increase the variety of mRNA:tRNA base pairing interactions possible. Consistent with this idea, Ψ, which shares a repositioned nitrogen with m¹Ψ, was previously shown to enhance the reaction of some near-cognate tRNAs on UUU codons *in vitro* and in HEK293 cells (30). To begin examining if m¹Ψ similarly influences aminoacyl-tRNA (aa-tRNA) selection, we qualitatively evaluated the impact of m¹Ψ on the propensity of Phe codons to react with near-cognate tRNAs. In these assays, 70S *E. coli* initiation complexes were generated with unmodified (UUU) and modified (m¹ΨUU, Um¹ΨU, and UUm¹Ψ) codons in the A site, and reacted with EF-Tu containing ternary complexes formed using a mixture of total tRNA aminoacylated either by reacting all 20 amino acids with S100 (total aa-tRNA^{aa}), or with a single amino acid and aminoacyl tRNA synthetase (Phe-tRNA^{Phe}, Ser-tRNA^{Ser}, Leu-tRNA^{Leu}, Ile-tRNA^{Ile} and Val-tRNA^{Val}) (49). Relative to UUU, we observed that m¹ΨUU appears to react more robustly with total aa-tRNA^{aa}, and promote the production of higher-levels of miscoded Met-Ile (MI) and Met-Met-Val (MV) peptides (Figure 2A). Um¹ΨU and UUm¹Ψ

codons also exhibit different levels of reactivity with near-cognate tRNAs than an unmodified UUU (SI Figure 4).

Using the information obtained from our qualitative screens, we selected tRNAs for closer, quantitative investigation. We choose to evaluate three near-cognate tRNAs that either appeared to react more, less, or to the same extent on unmodified and m¹Ψ modified codons (tRNA^{Ile}(GAU), tRNA^{Leu}(CAG) and tRNA^{Ser}(UGA)) (Figure 2A). The single-turnover rate constants (k_{obs}) for each amino acid mis-incorporating on unmodified and m¹Ψ substituted Phe (UUU) codons was measured at saturating concentrations of individually purified charged aa-tRNA (Figure 3, SI Figures 5 and 6). An energy regeneration mix was included in our reactions to permit the reformation of ternary complexes (aa-tRNA:EF-Tu:GTP) after an aa-tRNA is rejected by the ribosome (61). We find that tRNA identity and the position of m¹Ψ within a codon influence the rate constants for amino acid substitution (Figure 3, SI Table 4). For example, m¹Ψ substitution at the first position in the Phe codon (m¹ΨUU) does not change the k_{obs} values for Leu or Ser incorporation, but increases the rate constant for Ile addition by 2.2 ± 0.4 -fold (Figures 3D and 4A, SI Table 4). This differs markedly from what we observed on Um¹ΨU-modified codons, which have a much larger effect on tRNA selection. The k_{obs} values are significantly reduced for Ile (10 ± 2 -fold) and Leu (4 ± 1 -fold) addition, while the rate constant for Ser misincorporation is conversely increased by 3.5 ± 0.4 -fold (Figure 3D, SI Table 4). Substitution at the wobble position (UUm¹Ψ) generally had modest impacts on the rate constants for amino acid incorporation; decreasing the k_{obs} for Leu addition (2 ± 0.3 -fold), while not impacting the k_{obs} values for Ile and Ser addition (Figure 3D, SI Table 4). The findings of our kinetic studies are generally consistent with our initial qualitative assays (Figures 2, 3 and SI Figure 5), and together indicate that m¹Ψ codon modifications can both increase and decrease the ability of Phe UUU codons to react with near-cognate tRNAs in the A site.

Uridine isomerization contributes to observed changes in amino acid substitution on m¹Ψ containing codons

To determine how the C5-glycoside uridine isomerization and an N1-methylation individually impact the ability of m¹Ψ to modulate amino acid misincorporation, we measured the rate constants for Leu, Ile and Ser addition on Ψ modified Phe codons (ΨUU, UΨU and UUΨ). Ψ was selected for study because it contains the same isomerized uridine base as m¹Ψ, but lacks the methylation at position N1 (Figure 1A). The impact of Ψ on Ile and Leu insertion was similar to what we observed when m¹Ψ is present in codons (Figure 3, SI Figure 7). For example, the rate constant for Ile is significantly decreased (10 ± 2 -fold) when Ψ is incorporated at the second codon position (UΨU), while Leu is added more slowly when Ψ is at all three positions in the codon (Figure 3, SI Table 5). In contrast to m¹Ψ, Ser incorporation occurs with a 4 ± 0.5 -fold faster rate constant when Ψ is at the first and second positions in the codon, and is not influenced by Ψ substitution at the wobble position (UUΨ). These observations indicate that uridine isomerization largely accounts for changes in how the ribosome decode some tRNAs on for m¹Ψ-substituted mRNAs. However, comparison of the reactivity profiles Ψ and m¹ΨUU reveal the N1 methyl group can suppress the effect of uridine

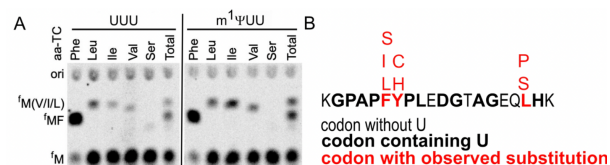


Figure 2. m¹Ψ amino acid selectivity *in vitro* and in HEK293 cells. (A) eTLC displaying dipeptide products from translation reactions performed with 70S initiation complexes (ICs) with an unmodified UUU or m¹ΨUU codon in the A site and total *E. coli* tRNA aminoacylated with a single amino acid (aa-TC). Relative to ICs containing a UUU codon in the A site, higher levels of miscoded MI and MV dipeptide products and lower levels of MS were generated from m¹ΨUU containing ICs. (B) Summary of amino acid substitutions observed by mass spectrometry in a luciferase peptide incorporated on m¹Ψ-containing mRNAs translated in HEK293 cells (SI Table 8).

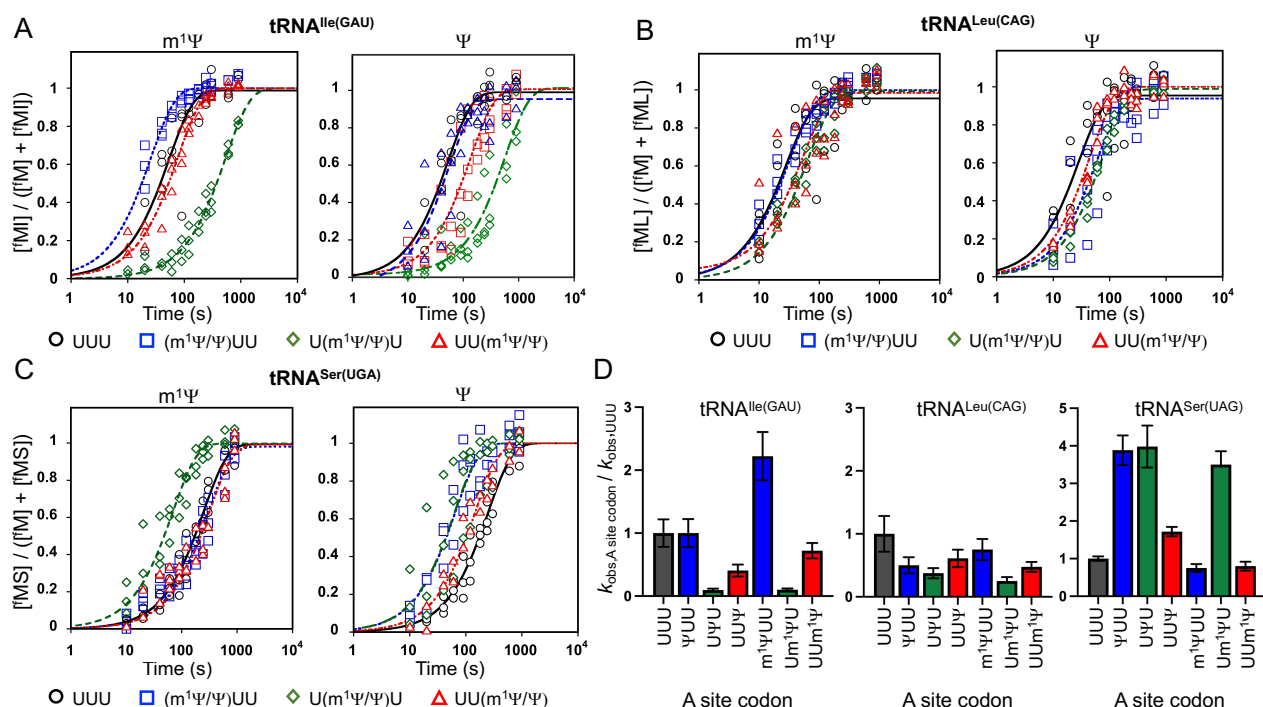


Figure 3. Ψ and $m^1\Psi$ impact the rates of the ribosome reacting with near-cognate tRNAs in a sequence context dependent manner. Plots of dipeptide formation as a function of time (seconds). Miscoding reactions were performed with *E. coli* ICs containing ^{35}S -Met-tRNA^{Met} bound to an AUG start codon in the P site, and unmodified (○-UUU) or modified (□- $\Psi/m^1\Psi UU$, ◇- $U\Psi/m^1\Psi U$, △- $UU\Psi/m^1\Psi$) codons in the A site. Purified ICs were reacted with TCs containing (A) Ile-tRNA^{Ile(GAU)}, (B) Leu-tRNA^{Leu(CAG)}, or (C) Ser-tRNA^{Ser(UGA)}. (D) The rate constants (k_{obs}) for isoleucine, leucine, and serine misincorporation on Ψ - and $m^1\Psi$ -modified codons relative to the rate constants for isoleucine, leucine, and serine misincorporation on a UUU codon.

isomerization on the rate constants for amino acid misincorporation by other near-cognate tRNAs (e.g. ΨUU vs $m^1\Psi UU$ reacting with tRNA^{Ser(UGA)}) (Figure 3D, 4A).

Amino acid substitution in HEK293 cells increases on some $m^1\Psi$ containing codons

Our *in vitro* translation data reveal that $m^1\Psi$ and Ψ affect tRNA selection by *E. coli* ribosomes in different ways depending on their sequence context. We next asked if $m^1\Psi$ has similar effects on amino acid selection in eukaryotic cells. To approach this question, we transfected luciferase encoding mRNAs transcribed *in vitro* with either UTP or $m^1\Psi$ TP into HEK293 cells, where they were translated. The base composition of the unmodified and modified mRNAs was assessed by liquid chromatography-mass spectrometry (LC-MS), and is consistent between the unmodified and modified mRNA species we generated (SI Figure 8). We observed increased levels of luciferase protein expression in the $m^1\Psi$ -substituted mRNAs, consistent with previous reports (SI Figure 9-10) (30). The luciferase proteins generated from both unsubstituted and $m^1\Psi$ -substituted mRNAs were purified and analyzed by mass spectrometry to identify amino acid substitutions.

Our mass spectrometry data analyses focused on a specific luciferase peptide with favorable ionization characteristics (Figure 2B) (30). ~1% of the amino acids in this peptide were substituted. This is a >20-fold increase over the level of amino acid substitution we previously observed for peptides generated from an unmodified version of the same

luciferase reporter (< 0.05% of their amino acids substituted) (30). $m^1\Psi$ -mediated substitutions were detected on variety of codons (e.g. UUU, UAU), though we did not observe amino acid substitutions above background levels for every U-containing codon (e.g. UGG) (SI Table 6). The highest levels of substitution were observed on the two Phe codons (UUU and UUC). Similar to our *in vitro* observations, serine and isoleucine/leucine amino acid substitutions were seen on both Phe codons with a > 6-fold increased frequency of substitution over peptide from unmodified mRNA (Figure 2B, SI Tables S6-8) (30). Isoleucine and leucine have the same mass and therefore cannot be distinguished in this assay. We also noted that the likelihood of substitutions occurring was not uniform across $m^1\Psi$ -containing codons. The levels of miscoding that we detect are consistent with what we would predict from our *in vitro* studies, as is the heterogeneity of amino acid substitution on $m^1\Psi$ -modified codons (Figures 2-3). Furthermore, the lack of uniformity in amino acid substitution was also seen in our previous findings indicating that Ψ also increases the levels of amino acid misincorporation in the same luciferase reporter peptide (30). Our results collectively suggest that the extent of misincorporation on any codon containing a C5-glycoside uridine isomer strongly depends on the sequence context in which the modification is present.

Ψ -derived modifications change the energetics of mRNA:tRNA interactions in the ribosome A site

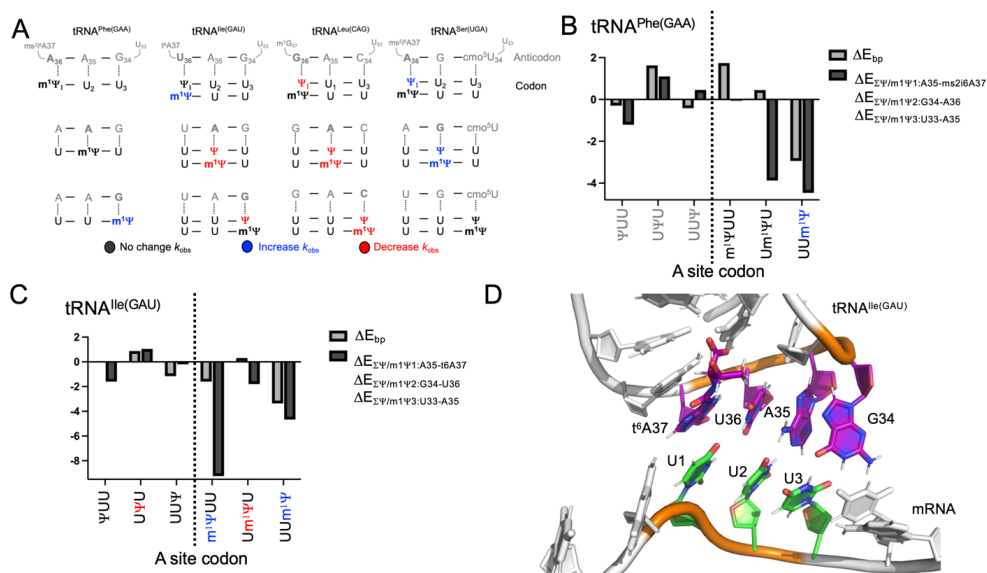
We sought to understand why Ψ and $m^1\Psi$ modifications alter the interactions between mRNAs and tRNAs during translation in a position dependent manner (Figures 1-4A). To approach this question we used molecular modeling (MM) and quantum mechanical calculations to examine unmodified and Ψ -, $m^1\Psi$ - and 3-methylpseudouridine ($m^3\Psi$) modified UUU mRNA codons interacting with a cognate ($tRNA^{Phe(AAG)}$) and near-cognate ($tRNA^{Ile(UAG)}$) tRNAs in a portion of the ribosome A site (Figures 1A, 4). Although we did not investigate the translation of $m^3\Psi$ -containing codons, we included $m^3\Psi$ in our computational studies as a positive control for a modification that should severely perturb mRNA:tRNA interactions; methylation at the uridine N3 position removes the ability of uridine to donate a hydrogen bond and will limit tRNA binding. Our MM studies were conducted using models developed based on previously published crystal structure of the 70S *Thermus thermophilus* ribosome with $tRNA^{Phe}$ bound on a Ψ UUU codon (30). The MM investigations were designed to examine how the location of a modification impacts the pairwise tRNA:mRNA interaction energies. Each modification was modeled in either the first, second, or third codon position and the energetics of $tRNA^{Phe/Ile}$:mRNA interactions on modified codons were compared those on an unmodified UUU codon (ΔE) (Figure 4, SI Figures 11 and 12). Only ΔE values with magnitudes ≥ 1 kcal/mol are considered large enough to potentially change mRNA:tRNA interactions.

We found that the incorporation of pseudouridine-derived modifications affect the energetic landscape for codon interactions with $tRNA^{Phe(GAA)}$. As expected, the ΔE values for base pairing (ΔE_{bp}) between each $tRNA^{Phe(GAA)}$ anti-codon base and all three $m^3\Psi$ modified codons are significantly increased ($\Delta E_{bp} = +8.6$ to 19.7 kcal/mol, SI Figure 11), suggesting the $tRNA^{Phe(GAA)}$ is not likely to interact productively with $m^3\Psi$ -modified codon. Compared to $m^3\Psi$, the effects of Ψ and $m^1\Psi$ that we observed on ΔE were not as uniform across the codon. Ψ UUU and $U\Psi$ U do not alter the energetics

of base pairing ($\Delta E_{bp, \Psi 1:A36} = -0.3$ kcal/mol and $\Delta E_{bp, \Psi 3:G34} = -0.4$ kcal/mol), while substitutions in the second codon position ($U\Psi$ U) are less energetically favorable ($\Delta E_{bp, \Psi 2:A35} = +1.6$ kcal/mol) (Figure 4AB). In contrast to Ψ , $m^1\Psi$ perturbs the ΔE_{bp} of codon:tRNA^{Phe(GAA)} anti-codon interactions when it is included at the first and third, but not second, positions of the codon. However, while $\Delta E_{bp, m^1\Psi 1:A36}$ is increased ($+1.7$ kcal/mol), this change is rendered insignificant by the compensatory alterations in energy between $m^1\Psi$ and the $tRNA^{Phe(GAA)}$ bases (A35 and 2-methylthio-N6-isopentenyladenosine (ms^2i^6A)37) adjacent to the $m^1\Psi 1:A36$ interaction ($\Delta E_{\Sigma m^1\Psi 1:A35-ms^2i^6A 37} = -1.4$ kcal/mol) (Figure 4A-B). $UUm^1\Psi$ has more favorable energetics for interacting with $tRNA^{Phe(GAA)}$ than UUU ($\Delta E_{bp, m^1\Psi 3:G34} = -2.9$ kcal/mol), which are enhanced by $m^1\Psi$ interactions with the two adjacent tRNA nucleotides ($\Delta E_{\Sigma m^1\Psi:U33-A35} = -4.5$ kcal/mol) (Figure 4B). These results are generally consistent with our kinetic studies demonstrating that the rate constant for Phe addition is modestly increased on $UUm^1\Psi$ codons relative to UUU, $m^1\Psi$ UUU and $Um^1\Psi$ U (Figure 1B, SI Table 1).

The substitution of U with Ψ , $m^1\Psi$, and $m^3\Psi$ also impacts how codons interact with near cognate $tRNA^{Ile(GAU)}$ in a manner supported by our *in vitro* translation kinetic findings (Figure 4A, C, D). Just as with $tRNA^{Phe(GAA)}$, the ΔE_{bp} values for individual $m^3\Psi$ -containing codon:tRNA^{Ile(GAU)} anti-codon base pairs were typically less favorable relative to an unmodified UUU codon (up to $+16.2$ kcal/mol; SI Figure 12). Ψ UUU and $U\Psi$ U do not markedly influence $\Delta E_{bp, \Psi 1:U36}$ and $\Delta E_{bp, \Psi 2:A35}$ ($+0.9$ and $+0.05$ kcal/mol, respectively), though the energetics of $tRNA^{Ile(GAU)}$ interactions with these substitutions are altered by ~ 1 kcal/mol when the ΔE values for the flanking tRNA nucleotides are also considered ($\Delta E_{\Sigma \Psi 1:G34-U36} = +1.1$ kcal/mol, $\Delta E_{\Sigma \Psi 2:A35-t^6A37} = -0.8$ kcal/mol) (Figure 4C). This differs from the effects of including Ψ at the third codon position. In the context of a $UU\Psi$ codon, $\Delta E_{bp, \Psi 3:G34}$ is more favorable (-1.2 kcal/mol), but this effect is mitigated by the energetically less favorable interactions

Figure 4. Changes in the energetics of mRNA:tRNA interactions correlate with observed differences in Phe and Ile incorporation on Ψ - and $m^1\Psi$ -containing codons. (A) Summary of data in SI Tables 4 and 5 displaying how a Ψ and $m^1\Psi$ impact the rate constants for the reaction of near-cognate tRNAs. **(B and C)** Summary of MM data. Gray bars reflect the change in energy for interactions between a modified mRNA position ($\Psi/m^1\Psi$) and the base pairing **(B)** $tRNA^{Phe(GAA)}$ or **(C)** $tRNA^{Ile(GAU)}$ residue (ΔE_{bp}) relative to an unmodified mRNA U. Black bars reflect the total change in energy ($\Delta E_{\Sigma \Psi/m^1\Psi:X-Y}$) for interactions



between a modified mRNA position ($\Psi/m^1\Psi$) and three **(B)** $tRNA^{Phe(GAA)}$ or **(C)** $tRNA^{Ile(GAU)}$ residues (the base paired nucleotide, and nucleotides 5' and 3' the bp) relative to an unmodified mRNA U. **(D)** Molecular model of an unmodified sequence coding for a Phe UUU codon and a $tRNA^{Ile(GAU)}$. The hypermodification t^6A37 is also shown the tRNA.

between the wobble Ψ and tRNA^{Ile(GAU)} nearby tRNA residues ($\Delta E_{\Sigma U33-A35} = -0.2$ kcal/mol). Our findings support previous studies demonstrating that tRNA nucleotides adjacent to tRNA anticodon:codon base pairs are important determinants of mRNA decoding (62).

The trend for how m¹ Ψ substitutions alter codon:tRNA^{Ile(GAU)} anticodon base pairing interactions is similar to that of Ψ , however the magnitude of the changes is increased. For example, $\Delta E_{bp,m^1\Psi 1:U36}$ and $\Delta E_{bp,m^1\Psi 3:G34}$ are more negative (-1.6 kcal/mol and -3.4 and, respectively), while $\Delta E_{bp,m^1\Psi 2:A35}$ is largely unchanged (+0.3 kcal/mol) (Figure 4C). As we observed for Ψ , the extent of the m¹ Ψ -mediated changes to the codon:tRNA^{Ile(GAU)} anticodon interactions increases when ΔE is considered in the context of the flanking tRNA nucleotides (e.g. $\Delta E_{\Sigma m^1\Psi 1A35-t6A37} = -9.2$ kcal/mol). Our computational analyses generally support our experimental findings that Ψ -derived modifications differentially affect the interactions between codons and both cognate and near-cognate tRNAs in context dependent manner to alter mRNA:tRNA interactions in the ribosome decoding center (63–68) (Figure 4, SI Figures 11–12).

DISCUSSION

During the selection of aminoacylated-tRNAs the ribosome must compromise between the speed and accuracy of decoding. Chemical modification of the RNAs involved in decoding (e.g. mRNA and tRNA) permit the fine tuning of this balancing act. m¹ Ψ modifications are heavily used in mRNA-based therapeutics and vaccines and we were interested in determining how their inclusion in mRNA transcripts can impact translation elongation (33, 37). Our studies indicate that, depending on where it was located within a codon, m¹ Ψ either has no effect or modestly increases the rate constant (k_{obs}) for cognate amino acid incorporation (Figure 1B). The rate constants ($k_{hyd,max}$) for translation termination are not perturbed when sufficient concentrations of release factors are present (Figures 1C–D). Notably, the effect (if any) of m¹ Ψ on the ribosome reacting with near cognate tRNAs depending largely on the position of the modification within a codon (Figures 2–3). Our computational studies reveal that the context dependent effects of m¹ Ψ we observe might be explained by changes in the energetics of base pairing interactions between m¹ Ψ -substituted UUU codons and their cognate tRNA^{Phe(GAA)} (Figure 4AB), which vary depending on where m¹ Ψ is incorporated in the codon. Perturbations in base pairing energies have the ability to influence central steps in the translation elongation pathway including tRNA selection and accommodation. Although faster elongation rates might help to partially explain greater protein yield from m¹ Ψ containing transcripts in cells (SI Figures 9–10), the preponderance of the increased yields in protein are likely due to m¹ Ψ -induced enhancements in mRNA stability and avoidance of the cell's innate immune system (40, 41, 56, 69).

We found that m¹ Ψ alters not only cognate tRNA interactions with the ribosome, but also those of near-cognate tRNAs. Our kinetic investigations reveal that both m¹ Ψ and Ψ modifications can either enhance or limit amino acid substitution depending on aa-tRNA identity and the position of the modification within the codon (Figures 3 and 4A). These *in vitro* observations are supported by cellular reporter studies indicating that m¹ Ψ can promote miscoding events

when included in full-length transcripts expressed in human cells (Figure 2B, SI Tables 6, 8). The mass spectrometry assay implemented here does not have sufficient sensitivity to identify modified codons that exhibit reduced levels of amino acid substitution, as we might expect on Leu codons based on our kinetics. Our explorations of amino acid substitution in a peptide translated from a m¹ Ψ substituted mRNA are consistent with the increase in miscoding we previously observed on Ψ -containing mRNAs in the same experimental system (30). Comparison of miscoding rates on m¹ Ψ and Ψ -containing codons suggests that the addition of a methyl group, and altered ring electronics resulting from the exchange of the nitrogen, play distinct positional and codon specific roles in the m¹ Ψ -mediated modulation of miscoding (Figure 3). This is further supported by our MM calculations revealing that methylations (m¹ Ψ and m³ Ψ) have larger impact on the energetics of tRNA:mRNA interactions than isomerization at the C5-position alone (Ψ) (Figure 4). These findings are in line with previous work demonstrating that naturally occurring mRNA modifications can differentially affect translation depending on their location within a codon or mRNA sequence (30, 31, 70).

In this work we go beyond observing that the sequence context of a modification matters for codon decoding (Figures 3 and 4) to try and identify *how* pseudouridine derived modifications change the fundamental interactions between mRNAs and tRNAs in a context dependent manner (Figure 4). Comparison of the rate constants for amino acid misincorporation on m¹ Ψ and Ψ containing codons, coupled with MM calculations provide evidence that fundamental changes in the energetics of mRNA:tRNA interactions may largely account for the context dependent outcomes we observe. Indeed, we find that the most energetically favorable interactions between the near cognate tRNA^{Ile} anticodon and Ψ and m¹ Ψ modified UUU Phe codons occur when these modifications are in the first and third position of the codon (Figure 4D–E). This is consistent with our kinetic studies indicating that Ile-tRNA^{Ile} reacts more rapidly with codons containing Ψ and m¹ Ψ in the first and third positions of a codon than in the second position (Figures 3, 4A). Additionally, our data suggest that tRNA ASL hypermodifications may also contribute to the context dependence of near-cognate decoding on modified Ψ and m¹ Ψ codons. We find that the rate constants for near cognate tRNAs possessing hypermodifications (t⁶A37 in tRNA^{Ile(GAU)}, ms²t⁶A37 and cmo⁵U34 in tRNA^{Ser(UGA)}) are more sensitive to the position of mRNA modifications within a codon than the near cognate tRNA^{Leu(CAG)} that contains only a methylation at position 37 (m¹G37) (Figure 4A). In line with this, MM calculations reveal that the hypermodifications adjacent to tRNA^{Phe(GAA)} and tRNA^{Ile(GAU)} anticodons strongly influence the energetics of codon:anticodon interactions with Ψ and m¹ Ψ , and can make tRNA interactions with a near-cognate codon up to 7.6 kcal/mol more energetically favorable. This correlates with our kinetic studies demonstrating that tRNA^{Ile(GAU)} reacts more rapidly on m¹ Ψ UUU, and is further supported by previous studies demonstrating that modified tRNA A37 nucleosides (t⁶A, ms²t⁶A, ct⁶A) improve the stability of the codon:anticodon duplex through enhanced base stacking (30, 41, 56, 57). Taken together, our kinetic and MM results suggest that both tRNA and mRNA

modifications modulate the energetics of codon:anticodon interactions differently in varied sequence contexts to influence the decoding of Ψ and $m^1\Psi$ modified codons (Figure 4D,E and SI Figure 7).

The ability of $m^1\Psi$ and Ψ to change the decoding behavior of the ribosome while only modestly altering amino acid addition and termination has several implications. Given the emerging evidence that Ψ is commonly included into mRNA at increased levels under cellular stress conditions, these findings support the possibility that Ψ -derived modifications can provide the cells with a way to transiently increase the diversity of the proteome under stress to increase fitness (15, 30, 73, 74). Furthermore, this could potentially be advantageous for mRNA vaccines relative to traditional vaccine platforms; greater antigen diversity might provide broader protection against circulating virus populations than single-strain vaccine formulations, similar to the increased efficacy of multivalent vaccines. While potentially beneficial in the context of mRNA vaccines, even small changes translational fidelity need to more carefully considered during the development of other classes of mRNA therapeutics. The complex rules governing the translational outcome of mRNA modifications such as $m^1\Psi$ are only beginning to be elucidated, and may in some cases prove to be critical to designing safe and effective mRNA therapeutics.

ASSOCIATED CONTENT

Supporting Information

Supporting Figures and Supporting Tables are available and associated with this text.

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Author Contributions

J.G. Monroe designed, performed and analyzed *in vitro* translation experiments, and participated in analyzing the MM data generated here. L. Mitchell and B. Roy designed and performed the mass spectrometry analyses of luciferase peptides translated in 293HEK cells. I. Deb and A. Frank designed, performed and the MM experiments. K.S. Koutmou designed and analyzed *in vitro* translation experiments, and participated in analyzing the MM data generated here. All authors contributed to writing the manuscript.

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ABBREVIATIONS

Phenylalanine, Phe, F; Isoleucine, Ile, I; Leucine, Leu, L; Serine, Ser, S; Pseudouridine, Ψ ; N1-methylpseudouridine, $m^1\Psi$; N3-methylpseudouridine, $m^3\Psi$; 2-methylthio-N6-isopentenyladenosine, ms^2i^6A ; N6-threonylcarbamoyladenine, t^6A ; cyclic N6-threonylcarbamoyladenine, ct^6A ; 1-methylguanosine, m^1G ; uridine 5-oxyacetic acid, cmo^5U .

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