Role of a tRNA Base Modification and its Precursors in Frameshifting in Eukaryotes

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Little is known about the role of specific base modifications of transfer RNAs. Wyosine bases (Ybs) are tRNA\textsuperscript{Phe}-specific modifications that are distinguished by differentiated, lateral side chains and base methylations appended to the core ring structure of a universally conserved G37, adjacent to the anticodon of Phe tRNAs. Based on previous data, we hypothesized that this modification was needed for -1 frameshifting. Using a reporter system incorporating a SCV-LA yeast virus slippery site for detecting -1 frameshifts \textit{in vivo}, yeast strains were created that enabled chemical-genetic dissection of the role of different functional groups of wyebutosine (yW) that are added in a 3-step post-transcriptional set of reactions. With this system, hypomodification increased Phe-specific frameshifting, with incremental changes in frameshift efficiency following specific intermediates in the progression of yW synthesis. These data, combined with investigations of wild-type and hypomodified tRNA binding to ribosomes, suggest that frameshift efficiency is kinetically and not thermodynamically controlled. The progressive nature of frameshift efficiency with stage of modification is consistent with a stepwise evolution and tuning of frameshift potential. The stepwise tuning of frameshift efficiency could explain why tRNA\textsuperscript{Phe} in some eukaryotes is not fully modified, but rather hypomodified to capture a specific frameshift potential.

Little is known about the biological role of specific base modifications of transfer RNAs. These modifications are found universally with some bacteria investing nearly 1% of their genome into tRNA modification genes (1). Yet most functions of tRNA that can be tested \textit{in vitro} show little dependence on these modifications. Wyosine bases (Ybs)\textsuperscript{1} are eukaryote- and archaea- specific modifications, characterized by a fluorescent, tricyclic \(1H\)-imidazo[1,2-\(\alpha\)]purine core structure. These guanosine modifications occur strictly in phenylalanine-specific tRNA (tRNA\textsuperscript{Phe}) at position 37, on the 3' side of the anticodon. Given the remarkable distribution of wyosine bases in eukaryotic Phe-specific tRNAs, and in the light of our previous genetic studies of wyosine base biosynthesis (2), we set out to investigate its potential functional significance.

Wyosine bases from different organisms are distinguished by differentiated, lateral side chains and base methylation that are appended to the core ring structure (3-8). Wyebutosine (yW), a wyosine-family member of yeast tRNAPhe, is one of the most extensively hypermodified bases (Fig. 1A). In \textit{S. cerevisiae}, yW is posttranscriptionally synthesized from a genetically encoded guanine in a multi-enzyme process whose details are not fully understood (9,10) (Figure 1B). In vitro studies showed that loss of the modification has little effect on the interactions between tRNA\textsuperscript{Phe} and phenylalanyl-tRNA synthetase, or its ability to perform \textit{in vitro} protein synthesis (11,12). The hydrophobic nature of wyebutosine contributes to base stacking interactions with neighboring bases (A\textsuperscript{36} and A\textsuperscript{38}) and restricts conformational flexibility of the anticodon loop (13-17). Removal of yW from tRNA\textsuperscript{Phe} resulted in local changes of the anticodon and was thought to destabilize codon-anticodon interactions in the ribosomal P and A sites (18-21). These observations seem

\textsuperscript{1}http://www.jbc.org/cgi/doi/10.1074/jbc.M703391200 The latest version is at http://www.jbc.org/
relevant to in vitro studies that link yW to the preservation of reading frame at phenylalanine codons and to the influence of yW on -1 ribosomal programmed frameshifting (22,23). Thus, change of the position 37 modification of tRNA\(^{\text{Phe}}\) from wyebutosine-37 to 1-methyl-G37 resulted in a 3-fold increase of -1 programmed ribosomal frameshifting in rabbit reticulocyte lysates.

Many viruses (e.g. retroviruses and totiviruses) rely on programmed -1 frameshifting to allow translation of multiple proteins or protein variants from a single promoter (24-27). For such organisms, the efficiency of frameshifting is critical because it determines the ratio of structural and catalytic proteins and, therefore, controls viral propagation. For example, a small (2-fold) change in -1 frameshifting frequency completely eradicated the SCV-LA virus from S. cerevisiae (28). Given the perspective of this previous work, we set out to explore the potential role of the wyebutosine base modification in -1 frameshifting. Because yW is synthesized through a multi-step pathway, our interest was drawn to the question of how the potential for frameshifting might also be built up, step-by-step. Thus, the significance of yW modification substructures of yeast tRNAPhe to -1 frameshifting, its frequency, and its specificity were investigated by structural ablation, using a series of characterized yeast knock-out strains that are blocked at specific, successive steps of wyebutosine synthesis.

**MATERIALS AND METHODS**

**tRNA Strains.** Wild-type and deletion strains (ΔYPL207w and ΔYML005w) of S. cerevisiae were purchased from Open Biosystems (www.openbiosystems.com). All three strains were of MAT\(a\) leu2Δ0 met15Δ0 ura3Δ0 genotype. Genes ΔYPL207w and ΔYML005w were renamed as TYW1 and TYW2, respectively (9). The YPL207w and ΔYML005w ORFs were substituted with a Kan\(^r\) cassette.

Each deletion strain is blocked at separate, successive steps of wyebutosine biosynthesis (Fig. 1B) and therefore it produces tRNA\(^{\text{Phe}}\) pools that are homogeneously undermodified at position 37. Composition analysis of tRNA\(^{\text{Phe}}\) from S. cerevisiae ΔTYW1 strain by tandem liquid chromatography – mass spectroscopy revealed the presence of the first wyebutosine precursor, 1-methylguanosine (m\(^1\)G, Fig. 1), at position 37 of tRNA\(^{\text{Phe}}\) (2). An analogous study of ΔTYW2 (ΔYML005w) strain demonstrated accumulation of imG14 precursor in tRNA\(^{\text{Phe}}\) (29).

Cell growth of each deletion and wild-type strains was measured in rich YPD medium (2% peptone, 1% yeast extract and 2% glucose). Neither strain showed a detectable defect in rate or yield relative to the parent wild-type strain that might hinder consequent in vivo studies.

-1 PRF reporter constructs. Dual-luciferase reporter construct pJD511 containing ADH1 promoter, lucR, ~200 base sequence from SCV-LA virus, lucF and CYC1 terminator was a generous gift of J. D. Dinman (University of Maryland) (30). The slippery sequence variants (pSCV-I - pSCV-VI) of the SCV-LA derived bicistronic reporter were produced by site directed mutagenesis (Quick change) of pJD511 plasmid. 0-frame control plasmid contained polylinker sequence only (no frame-shifting sequence) and firefly luciferase is in the same reading frame as Renilla luciferase, producing both proteins. Plasmid sequences were confirmed by automated sequencing.

Plasmids were first introduced into S. cerevisiae by chemical transformation using standard protocols. Transformants were isolated on media lacking leucine (0.067% Leu(-) DO supplement (BD biosciences), 0.67% Nitrogenous Base (Sigma), 2% glucose); pSCV restores leucine biosynthesis.

Dual-luciferase assays. Yeast strains (wt, ΔTYW1, ΔTYW2) harboring reporter constructs were grown in Leu(-) dropout media (2 mL) for 7 hours at 30 °C after 1:5 dilution of overnight culture. Cells were harvested by centrifugation, washed with 500 μL of ice-cold 1xPBS (containing protease inhibitor cocktail, Roche), centrifuged again for 10 min at 15700 xg and resuspended in 100 μL of the same buffer. Cells were lysed with 100 μL of glass beads (Sigma, 425-600 μm, prewashed in PBS) in a vortex mixer at 4 °C 5 times 2 min each. Lysates were clarified by centrifugation for 10 min and total protein concentration was determined by Bradford method. Preparation routinely produced 0.1 – 1.0 mg/ml of protein.
Activities of expressed Renilla and Firefly luciferases were determined in a luminometer (MicroLumant LB 96P) with 20 μL of cell lysate using the Dual-Luciferase® Assay System reagents (Promega E1910), following manufacturer’s protocol. Each strain was assayed in 7 replicates. Raw luminescence data was converted to a lucF/lucR ratio for each sample. This ratio was then normalized to the strain-specific 0-frame control and multiplied by 100% to obtain frameshift efficiencies for each recoding signal. By virtue of this manipulation, frameshifting is 100% for control plasmids, though strain variation is not large.

**tRNA preparation and purification.** Bulk tRNA, containing tRNA Phe structural variants, was isolated from yeast strains (wt, ΔTwy1, ΔTwy2). Bulk tRNA purification and fractionation, and purification of aminoacylated Phe-tRNA Phe structural variants were as described in Waas et al (2). Briefly, purified bulk tRNA containing wt or hypomodified tRNA Phe was fractionated by reverse-phase HPLC (Vydac C4 semi-preparative HPLC column, Deerfield, IL). Fractions enriched for tRNA Phe were aminoacylated using yeast phenylalanine-tRNA synthetase. Aminoacylated 3H-Phe-tRNA Phe variants were further purified by reverse-phase HPLC as described (2). Since the retention times of Phe-tRNA Phe and deacylated tRNA Phe are grossly different, the resulting Phe-tRNA Phe pool was free of deacylated tRNA contamination. Using this procedure, we obtained Phe-tRNA Phe structural variants with amino acid acceptances that were higher than 1800 pmol/A260 (2). The uniformity of purified tRNA Phe structural variants was additionally confirmed by MALDI mass spectrometry (2,29).

All three structural variant were aminoacylated simultaneously with the same batch of 3H-Phe and the charged tRNAs were purified under identical conditions. We demonstrated, that no less than 95% of detected 3H-Phe was linked to tRNA. Specific activities of the three 3H-Phe-tRNA Phe variants were uniform and equal to 1.32*10^4 cpm/pmol. The procedure for N-acetylation of Phe-tRNA Phe to prepare P-site substrate NAc-Phe-tRNA Phe was performed similar to previously described (31).

**Ribosome purification.** 80S ribosomes were purified from commercially available *S. cerevisiae* strain (INVSc1, Invitrogen) as described in (32) with the following modification. Yeast cells, resuspended in 30 mL of low salt buffer (100 mM KOAc, 20 mM Hepes-KOH, pH 7.6, 2.5 mM Mg(OAc)2, 1 mg/mL heparin, 1 mini-EDTA free protease inhibitor tablet (Roche), were lysed with 425-600 μm glass beads using Bead-Beater (Biospec) in three pulses 3 min each at 4 °C. Cells were spun for 30 min at 50,000 x g, the supernatant was collected and centrifuged again. KCl was added to the supernatant to the final concentration of 500 mM and centrifuged again for 4 hr at 50,000 x g. Ribosome pellets were resuspended in 6 mL of high salt buffer (500 mM KCl, 100 mM KOAc, 20 mM Hepes-KOH, pH 7.6, 2.5 mM Mg(OAc)2, 1 mg/mL heparin, 2 mM DTT). Puromycin and GTP were added to the final concentration of 1 mM each and the mixture was incubated on ice for 45 min. Ribosome mixture was layered on top of 3 mL of sucrose cushion (500 mM KCl, 100 mM KOAc, 20 mM Hepes-KOH, pH 7.6, 2.5 mM Mg(OAc)2, 2 mM DTT, 1 M sucrose) and centrifuged overnight at 152,000 xg at 4 °C. Pellets were resuspended in ribosome storage buffer (20 mM Hepes-KOH, pH 7.6, 10 mM MgCl2, 1 mM DTT, 250 mM sucrose, 0.1 mM EDTA), aliquoted, frozen in liquid nitrogen and stored in –80 °C. The concentration of 80S ribosomes were calculated based on the assumption that 1 OD260 = 20 pmols (80S extinction coefficient at 260 nM is 5x10^7 cm^-1 M^-1) (32,33).

**Acid-treatment of 3H-AcPhe-tRNA Phe.** In order to remove wyebutosine modification of position 37 of tRNA Phe, wt 3H-Phe-tRNA Phe and 3H-Phe-tRNA Phe (m1G-37) were treated with mild acid as previously described (12). Briefly, aminoacylated tRNA was incubated in 100 mM Ammonium Formate, pH 2.9 for 3 hrs at 37 °C. tRNA was exchanged into 5 mM Ammonium Acetate (pH 4.5) and concentrated using Microcon YM10 filters (Millipore).

**Millipore filter binding assay**

**Saturation experiments with 14C-NAcPhe-tRNA Phe:** 0.8 μM 80S were incubated with 0.8 mg/ml poly(U) RNA (Sigma) in the Binding Buffer (30 mM Heps, pH 7.5, 50 mM NH4Cl, 10 mM MgCl2, 2 mM spermidine, 0.05 mM spermine, 5 mM β-mercaptoethanol) for 20 min at 37 °C followed by 20 min incubation on ice. 2.5 μL
 aliquots were taken into separate tubes, 7.5 µL of an increased concentration ¹⁴C-NAc-Phe-tRNAₚₚ was added (to reach molar ratios of 0.5-10 tRNA to 80S). The incubation was continued for 20 min at 37 °C. Tubes were transferred onto ice, tRNA binding was determined by a filter-binding assay. Each sample was diluted to 100 µL with Binding Buffer and immediately applied onto a presoaked 0.45 µm nitrocellulose filter (Millipore); filters were washed 3 times with 500-750 µL of Binding Buffer containing 0.2 mg/ml total yeast RNA (to decrease non-specific binding of tRNA to filters). Values were corrected for control samples, lacking ribosomes, which were typically 1 - 4 % of applied labeled tRNA. Filters were air dried, placed into a scintillation vial, treated with 100 µL of 0.1 M NaOH to release Phe of tRNA (to reduce quenching) and counted in a scintillation counter using EcoLite scintillant (ICN).

A-site tRNA binding: 80S yeast ribosomes were preincubated with poly(U) mRNA in 140 - 150 µL of Binding Buffer for 20 min at 37 °C. 10 – 20 µL P-site substrate N-AcPhe-tRNAₚₚ in the same buffer was added, giving a final concentration of 60 nM 80S, 0.4 mg/ml poly(U), 75 nM N-AcPhe-tRNAₚₚ. The incubation was continued for 20 min at 37°C, 20 min on ice. For the alternative A-site complex (see Results), 0.1 µM of mRNA with sequence AUG UUU ACG AUU AUU AUU (synthesized by IDT technologies) and 0.1 µM of E. coli tRNAₚₚ as P-site substrate were used (purchased from Chemical Block, Russia). After incubation, 5 µL aliquots of P-site tRNA-80S complex were transferred into separate tubes and mixed with 5 µL of increasing concentration of ³H-Phe-tRNAₚₚ to titrate ribosomal A site. The complexes were incubated additional 20 min at 37 °C, transferred to ice and filtered as described above.

RESULTS

Experimental Strategy- In the yeast S. cerevisiae, the hypermodified wyebutosine is synthesized through post-transcriptional, sequential action of at least five enzymes, designated as TRM5p and TYW1p to TYW4p (Figure 1B) (9). The disruption of TYW1 results in production of m¹G37, a hypomodified tRNAₚₚ lacking both yW sidechains and the core tricyclic ring structure. (It is specifically m¹G37 tRNAₚₚ that has been identified in certain mammalian tumor cell lines (11,34).) After conversion of G37 to m¹G by TRM5p, the next enzyme, TYW1p, converts m¹G to 9H-imidazo[1,2-α]purin-9-one, 3,4-dihydro-6-methyl-3-β-D-ribofuranosyl (4-demethylwyosine, imG14). The actions of TYW2p, 3p, 4p then alter imG14 to yW, through reactions whose details are not fully understood. Thus, a ΔTYW2 strain produces tRNAₚₚ with a tricyclic, archaeal-like (wyosine-family bases at position 37 of tRNAₚₚ (9) are also seen in archaea) form of wyosine that lacks the typical side chains of yW, i.e., 4-demethylwyosine-37 (imG14-37).

Our goal was to work with yeast strains in which one of the genes needed for yW synthesis was knocked-out, so that a specific intermediate, like m¹G37 or imG14-37, would build up. We could then test that tRNA, with a specific hypomodification at position 37, for its activity in programmed ribosomal frameshifting.

For these investigations, isogenic yeast strains were selected that were proven to accumulate the specific hypomodified tRNAₚₚ. Taking advantage of previous work and strain constructions, the common genetic background for the ‘wild-type’ and deletion strains was MAT a leu2Δ0 met15Δ 0 ura3Δ 0 (2). Similar strains encoding the ΔTYW1 and, separately, the ΔTYW2 alleles, were viable and, as demonstrated previously by mass spectrometry, produced tRNAₚₚ with m¹G and imG14, respectively, at position 37 (2,29). A strain carrying a ΔTRM5 allele was also considered, but its viability was marginal. More importantly, the m¹G37 methyltransferase is not pathway-specific. (A ΔTRM5-bearing strain encodes other hypomodified tRNAs (for example, Arg-, Leu-, and Pro – specific tRNAs), in addition to hypomodified tRNAₚₚ (35).) Disruption of the TYW1 and TYW2 gene products, which are pathway-specific, thus provides an important simplification for interpretation of any data generated in vivo. With all these considerations in mind, three different species of tRNAₚₚ (wild-type, m¹G, and imG14) were used for investigations of frameshifting in vivo.

Experimental Rationale for Use of Yeast Double Stranded RNA Virus as Part of Reporter System- Our reporter constructs were developed to
mimic viral frameshifting in which “-1” (or -1 PRF) is most common. In viruses, programmed -1 ribosomal frameshifting redirects translating ribosomes into a new reading frame, thus bypassing an in-frame stop codon and continuing synthesis of an extended protein. As a consequence, frameshifting allows for expression of two polypeptide chains, from a single mRNA, at a ratio specified by viral gene’s primary sequence as well as by the host’s predisposition for frameshifting.

We noted that many laboratory strains of *S. cerevisiae* carry a double stranded RNA virus known as SCV-LA. The viral genome codes for a capsid polypeptide (*cap*) and cap-RNA polymerase fusion (*cap-pol*) which is produced as a result of -1 PRF (36-38). The signal for frameshifting is provided by an ~200 base sequence between *cap* and *pol* genes. Contained within this sequence are three elements that are each required for -1 PRF - a heptameric slippery site, a linker region, and a secondary structure element (36,39). In SCV-LA virus, similarly to other viruses (including retroviruses) efficiency of –1 PRF is crucial because it determines the ratio of structural and enzymatic proteins. Growing evidence suggest that modulation of frameshifting efficiency can reduce viral infectivity and have a dramatic effect on virus propagation and viability (25,40,41).

For our experiments, the functional -1 PRF unit from SCV-LA virus was transplanted into a bicistronic LucF/LucR reporter system (see below) (Figure 2). In yeast that harbors SCV-LA, a specific UUA-reading tRNA_{Leu} slips one nucleotide from UUA to insert leucine at a UUU (codes for Phe) codon. This construction allowed the inherent frameshifting frequency to be determined in detail (30). When placed in the wild-type or ΔTYW1 and ΔTYW2 genetic backgrounds, the reporter system enabled us to analyze in vivo relationship between the discrete (but substantial) modification state of tRNA_{Phe} and -1 programmed ribosomal frameshifting efficiency in a codon-specific manner. The dependence of frameshifting on the slippery sequence context could also be studied by incorporating several variants of the SCV-LA heptameric frameshift element into our test system. For these purposes, we changed the “pre-slippage” UUA codon to either UUU or UUC (codons for Phe) so as to specifically position tRNA_{Phe} in the ribosomal A site (Figure 2).

**Combining Yeast RNA Virus Construct with Dual Luciferase Reporter System** - In order to have a direct visual readout, we used a dual luciferase reporter system developed by Harger and Dimman (30,42). The construct specifies synthesis of two reporters that are driven by an ADH1 promoter. The two reporters are Renilla luciferase (encoded by lucR) and Firefly luciferase (encoded by lucF). As shown in Figure 2, viral coding sequences for the *cap* and *pol* proteins were replaced, respectively, with the coding sequences for lucR and lucF. Thus, the SCV-LA sequence that promotes frameshifting was placed between the coding sequences for the two luciferases. This arrangement promoted constitutive expression of lucR, while the production of lucF depended on frameshifting at the SCV-LA intervening sequence. Therefore, a -1 frameshift produced a protein harboring both Firefly and Renilla luciferases connected by a small peptide linker.

A construction encoding a 0-frame control was also assembled. A control plasmid was used to measure the translation level of an in-frame firefly luciferase coding sequence relative to that of the Renilla luciferase. The luminescence from lucR and lucF was measured sequentially in the same experiment with two different molecular substrates – with a quench step after the first measurement. (Thus, in these experiments, luminescence from lucR served as a convenient internal control.) The frequency of programmed ribosomal frameshifting was then determined by dividing the lucF/lucR luminescence ratio, from cells carrying the frameshift reporter vector, by the same ratio, from the cells carrying the 0-frame control plasmid.

The major advantage of this system is the internally controlled nature of the constructs. Normalization allowed for comparison of frameshifting rates in different cell types (strains) and minimized the potential for artifacts due to inherent variability of protein synthesis rate and differences in mRNA abundance/stability. Because, LucF acts as an internal control, there was no need to depend on error-prone protein quantification or standardization procedures for individual samples (30).
Modification Status of G37 of tRNA\textsuperscript{Phe} Correlates with -1 PRF at Phenylalanine-Specific A-site Codons- Six different variations of the heptameric slippery site were created (Figure 2). These were designated as pSCV-I to VI. We reasoned that, because wyebutosine is a tRNA\textsuperscript{Phe}-specific modification, frameshifting would respond specifically to codons (UUU and UUC) for phenylalanine. To demonstrate this point, we used constructs pSCV-I and pSCV-II containing G GGU\textsubscript{UUU} and G GGU\textsubscript{UUU} heptameric sequences, respectively. Because UUU codes for phenylalanine, frameshifting at the slippery sequence of pSCV-II was expected to respond to yW hypomodification. In contrast, the pSCV-I A-site sequence codes for leucine and should not be sensitive to the modification status of tRNA\textsuperscript{Phe} or deletion of the corresponding biosynthetic gene. With all three strains of \textit{S. cerevisiae} - wild-type, \(\Delta\)TYW1 and \(\Delta\)TYW2 - -1 frameshifting was observed and its frequency was substantially increased by hypo-modification at position 37. In particular, -1 frameshifting was at a frequency of 35\% (compared to the 0-frame control) for the hypomodified m\textsuperscript{1}G37-containing tRNA\textsuperscript{Phe} (Figure 3). This level of frameshifting was almost twice that for wild-type tRNA\textsuperscript{Phe}. For the next intermediate, the more modified imG14-37 tRNA\textsuperscript{Phe}, the frameshift frequency was between that of wild-type and m\textsuperscript{1}G37 tRNA\textsuperscript{Phe}. However, with the UUA codon of pSCV-I replacing the UUU codon of pSCV-II, little frameshifting was observed and no sensitivity to the status of tRNA\textsuperscript{Phe} modification could be seen. These results support the idea that the modification status of tRNA\textsuperscript{Phe} affects -1 frameshifting at the slippery sequence and does so in a way that is specific to the A-site codon for Phe. The data also demonstrate the incremental nature of the effects of guanosine-37 modification states.

Interplay Between G37 Modification State and Slippery Sequence of mRNA- The codon-anticodon interaction is a critical event for translation and for ribosomal recoding by frameshifting. Previous work showed that -1 PRF correlates with the theoretical strength between A- and P-site interactions of tRNAs with the mRNAs to which they are bound (43). In order to consider the role of G37 modifications in establishing these interactions, we designed experiments where strains harboring tRNA\textsuperscript{Phe} G37 variants were challenged with reporter constructs coding for permutations of the slippery sequence (pSCV-II-pSCV-VI). Because tRNA\textsuperscript{Phe} has only a single isoacceptor with a GAA anticodon, only UUU and UUC A-site codons need be considered. We made two P-site sequence variants (GGU (wild-type) and GAU) in pairwise combinations with each of the UUU and UUC codons. Thus, for example, differences in -1 PRF frequencies were compared using pSCV-II \textit{versus} -III (or -IV \textit{versus} -V) constructs that contain alterations of the A-site mRNA sequence. In addition, we made a sixth construct – pSCV-VI – that had a GUU codon at the P site and a UUU codon at the A site. This construct gave us a third P-site variation to test in conjunction with the UUU A-site. The frequency of -1 PRF was markedly reduced by changing U to C at the terminal position of the slippery sequence (Figure 4A). This reduction was seen with both the GGU and the GAU P-site triplet that immediately precedes the Phe codon. This result is consistent with the previous observation of Brierley \textit{et al} (43) with the IBV virus sequence in a rabbit reticulocyte lysate cell-free translation system, where frameshifting was more likely in cases where the codon-anticodon interaction in the “pre-slip” ribosomal A site is weaker. The simplest explanation for this observation is that the specific interaction between the A-site Phe codon of the mRNA and the tRNA\textsuperscript{Phe} GAA-anticodon sequence is operationally weaker with UUU than with UUC.

Interestingly, the frameshifting efficiencies remained largely unaffected when the P-site codon was changed from GGU to GAU (compare SCV-II \textit{versus} SCV-IV, and SCV-III \textit{versus} SCV-V), indicating that requirement for specific nucleotides in the P-site sequence are less stringent (Figure 4B). Similarly, Brierley \textit{et al} concluded that only minimal mRNA-tRNA pairing in the P site might be required for efficient frameshifting (43).

Surprisingly, for the slippery sequence G GUU UUU (SCV-VI contains a third P-site variation codon), sensitivity to hypomodification is lost, though -1 PRF frequency is robust. At present we cannot fully explain this observation. However, Brierley \textit{et al} (43) have demonstrated reduced sensitivity of frameshifting events to the absence/presence of downstream secondary
elements in the context of extended uridine stretches at the slippery sequence (where position 7 is also uridine). Similarly, Carlson et al (23) observed no influence by yW37 on -1 PRF at UUU UUU/C sequences. It is plausible that, by substituting U at position 3 for pSCV-VI (versus G for pSCV-II, or A for pSCV-IV), and thereby increasing (U)n content in the slippery sequence, sensitivity to modification at position 37 is attenuated.

Most importantly, the putative strength of the codon-anticodon interaction did not change the relative order of the efficiency of frameshifting that correlates with the state of modification. That is, the greater the state of modification, the lower the frequency of frameshifting. This relationship can be seen more clearly by assigning an arbitrary value of “1” to the frameshift efficiency seen with wild-type tRNA^Phe, and replotting the data (Figure 4B). In this format, it is easy to see that deletion of the wyebutosine side chain, as occurs with the ΔTYW2 genetic background, increased the -1 PRF frequency by 150 – 200% relative to the isogenic wild-type yeast strain. Further ablation of the structure (from a tri-cyclic core of 4-demethylwyosine to a purine of m^1G, ΔTYW1) produced an even more significant increase (>200% relative to the wild-type yeast strain). Thus, hypomodification increased the frequency of -1 ribosomal frameshifting. These incremental changes in frameshift efficiency, therefore, correlate inversely with the increasing structural complexity of G37 modifications of A-site bound tRNA^Phe that are associated with specific intermediates in the progress of the wyebutosine biosynthetic pathway.

Binding of tRNA^Phe Variants to Eukaryotic Ribosomes- The correlation in vivo between -1 frameshifting and the apparent strength of tRNA interactions at the ribosomal A-site was further investigated in vitro. This correlation could be due to an inherent stronger equilibrium binding of the hypomodified tRNAs to the A site. From an experimental standpoint, equilibrium studies were most accessible and were pursued. For this purpose, we performed equilibrium binding studies of Phe-tRNA^Phe to yeast 80S ribosomes, using two different mRNA systems.

Limited data are available on the functional role of yW-37 modification in tRNA binding to the ribosomal A site. Thus, Katunin and coworkers demonstrated that the presence of wyebutosine base increased the strength of binding of aminoacylated tRNA^Phe to the A site of E. coli 70S ribosomes 10-fold (18,20). In their work, binding of a fully modified yeast tRNA^Phe was compared to the binding of acid-treated tRNA^Phe, specifically depurinated at the position 37. Such treatment removes wyebutosine from wild-type tRNA without breaking the RNA chain, thereby producing tRNA^Phe depurinated at position 37 (12). Because frameshifting is a subtle, conformationally sensitive event, we worried that removal of a wyebutosine base could have a significant effect on the conformation of anticodon loop that, in turn, would result in destabilization of codon-anticodon interactions and tRNA binding to the ribosomes. This concern not withstanding, we first decided to determine whether, acid-directed ablation of yW-37 had the same effect in an in vivo yeast as in the E. coli system.

We carried out acid-treatment of wild-type tRNA^Phe (yW-37), and purified yeast ribosomes that accepted up to 1.5 molecules of N-AcPhe-tRNA^Phe (per 80S particle in the presence of poly(U) mRNA). Because N-AcPhe-tRNA^Phe is able to bind to both P and A sites, we concluded that 75% of our preparation of yeast ribosomes was active for tRNA binding. Then, to determine the affinity of Phe-tRNA^Phe for the A site, conditions had to be found where the P site was fully occupied, while the A site was vacant. For E. coli ribosomes, the P site is occupied first and the half-life of N-AcPhe-tRNA^Phe dissociation is ~2 hours (provided there is no tRNA exchange between P- and A-rubosomal sites during the experimental procedure) (44). (Interference from the third, E-site, binding locus is unlikely because that site is specific for deacylated tRNA and, in any case, binding to that site is obscured by the filter binding technique that we used (45-47).) Thus, by use of ribosomes whose P-site is preoccupied with a peptidyl-tRNA analog, the titration of the A-site with aminoacyl- tRNA^Phe reduces to a single-site model and allows for a determination of the K_d for the A-site complex.

Yeast 80S ribosomes were first incubated with a 1.25-fold excess of N-AcPhe-tRNA^Phe in the presence of poly(U). This procedure quantitatively filled the P site. Increasing concentrations of aminoacylated of ^3H- Phe-
tRNA\textsuperscript{Phe} were then added to the P-site-filled ribosomes. Ribosomal complexes were subjected to nitrocellulose filtration. Similar to \textit{E. coli} ribosomes, the A-site binding of wild-type Phe-tRNA\textsuperscript{Phe} to yeast 80S particles decreased at least 5-fold after acid-dependent depurination of yW-37. (As a control, the binding affinity of acid-treated tRNA\textsuperscript{Phe} (m\textsuperscript{1}G-37) remained unchanged (within the error of experiment) as expected, because m\textsuperscript{1}G-containing tRNA is not susceptible to acid depurination). Because acid-ablation actually removes the entire base and not just a pendant group, and because we demonstrated that frameshifting at Phe-codons is sensitive to modification changes far more subtle than acid-ablation of yW-37 from the tRNA anticodon loop, we decided to proceed with studies of binding affinities of hypomodified Phe-tRNA\textsuperscript{Phe} (m\textsuperscript{1}G and imG14) to the A site of the yeast 80S particle.

Using the approach described above, we identified a \(K_d\) for yeast wild-type Phe-tRNA\textsuperscript{Phe} (yW-37) binding to the A site as 154 ± 50 nM (Table 2). (This value is similar to the reported dissociation constants of 20 -100 nM for yeast Phe-tRNA\textsuperscript{Phe} binding to the A site of \textit{E. coli} 70S ribosomes (18,44).) Interestingly, binding of structural analogs of Phe-tRNA\textsuperscript{Phe} containing the hypomodified nucleotide in position 37, tRNA-m\textsuperscript{1}G or tRNA-imG14, demonstrated somewhat stronger binding to the ribosomal A site, with dissociation constants of 49 ± 27 nM and 46 ± 21 nM, respectively. This difference in binding of wild-type and hypo-modified tRNA, while small (3-fold), was reproducible and had a p-value < 0.006.

In order to avoid possible ambiguity coming from the degenerate nature of the poly (U)-programmed system and, in addition, the possibility of a P-site binding contribution to the A-site \(K_d\) value, similar binding studies were performed with 80S ribosomes programmed with an mRNA coding for tRNA\textsubscript{\textsuperscript{i}Met} and tRNA\textsuperscript{Phe} at the first two codons (AUG UUU). In this system, the ribosomal P site was occupied by deacylated \textit{E. coli} tRNA\textsubscript{\textsuperscript{i}Met}, while the A site was titrated by structural variants of Phe-tRNA\textsuperscript{Phe}, as described above. Essentially the same results were obtained for this system. A-site binding of fully modified Phe-tRNA\textsuperscript{Phe} (\(K_d = 96 \pm 9\) nM) was 2.5-3 - fold weaker than that of the hypo-modified tRNA\textsuperscript{Phe} isoacceptors (\(K_d = 41 \pm 6\) or 30±9 nM for imG-14 or m\textsuperscript{1}G, respectively) (Table 2).

Thus, acid ablation of the entire base produced a result opposite to that observed with the more subtle, natural changes produced by genetics.

**DISCUSSION**

The major motivation for this work was to find at least one rationale for the strong selective pressure that has designated phenylalanine-specific tRNAs in eukaryotes for wyosine-family base modification on the 3’-side of the anticodon. Generally, base modifications at any position in a tRNA do not produce large effects, at least with respect to the available assays related to proteins synthesis. For choosing to do \textit{in vivo} studies of frameshifting in \textit{S. cerevisiae}, we were particularly struck by the realization that only the smallest change in frameshift efficiency (e.g., 2-fold, \textit{vide supra}) could result in the difference between viability and non-viability in specific circumstances. We therefore surmised that, if the effects of wyosine base modifications on frameshifting were small but at least measurable, then the small effects could in themselves be sufficient to account for the widespread distribution of wyosine modifications in eukaryotes and archaeae. The data presented in Figures 3 and 4 establish clearly that yW-related modifications at position 37 alter \(-1\) frameshifting \textit{in vivo} by a magnitude that is well outside experimental error. Thus, the effects of yW-family modifications on \(-1\) frameshifting may in themselves offer the explanation for the widespread distribution of these modifications in eukaryotes and archaeae.

Our data also show that \(-1\) frameshifting itself is dependent on the degree of modification of each intermediate state in the multi-step biosynthetic pathway. We were able to study 3 progressive states of modification—m\textsuperscript{1}G, imG14, and yW (wild-type). Frameshifting was greatest with the least modified m\textsuperscript{1}G intermediate, and then dropped progressively as additional elaborations to the core base were added. Because the entire multi-step pathway most likely developed in a progressive, step-by-step way, our results suggest that the differences in frameshift efficiency of each substructure could itself provide the selective
force for the pathway to evolve progressively. In addition, the appearance, in some organisms, of mature tRNA Phe’s that are hypomodified with m1G or imG14 (4-6,48,49), for example, could be a result of a need for a finely tuned tRNA Phe-dependent frameshift potential in these organisms.

The general mechanism of –1 PRF is thought to reflect ribosome pausing by cis-acting elements of the mRNA that induce a –1 shift. Based on in vitro studies, frameshifting at a particular site has been proposed to be determined, at least in part, by the strength of interaction of the tRNA anticodon with the A-site codon, before tRNA slippage (43). According to this hypothesis, if the codon-anticodon interaction in the ribosomal A site before tRNA slippage is relatively weak, then slippage is more likely to occur. Indeed, we observed in vivo a decrease in frameshifting efficiency upon switching the A-site codon triplet from UUU to the stronger pairing UUC (Figure 4A). But we cannot determine whether, from the in vivo analysis, the effects of a codon difference on frameshift efficiency are under kinetic (transition state) or thermodynamic control. The in vivo data in general do not address whether the effects of the modification state on frameshift efficiency are from kinetic or thermodynamic parameters. This question is of some interest because a potentially related phenomena, translational fidelity, is generally thought to depend primarily on kinetic rather than thermodynamic discrimination (50).

To understand better the thermodynamic side, we used poly(U) as the mRNA for studying in vitro the A-site binding of three different position 37-modified tRNA Phe s. In these studies, we found a consistently stronger binding of m1G and imG14-modified substrucures, compared to the wild-type, fully modified structure (Table 2). Also, the m1G and imG14-modified substrucures are more efficient in frameshifting. If thermodynamics determined the efficiency of frameshifting, then we would expect tighter A-site binding to yield less frameshifting (because the bound tRNA would have more trouble “slipping” ahead). This lack of correlation of binding in vitro with frameshifting in vivo is consistent with the idea that the results in vivo are determined more by kinetic considerations. Indeed, while mG37-tRNA Phe is more active than imG14-37 tRNA Phe in frameshifting (Figures 3 and 4), no significant difference could be seen between the two tRNAs in A-site mRNA binding in vitro (Table 2). Thus, if frameshifting is viewed as a specific example of a perturbation of translational fidelity, then our data can be harmonized with the idea that fidelity is kinetically determined.

While our experiments show clearly that wosine base modifications can modulate frameshifting, we do not know whether frameshifting per se is the driving force in evolution for the strong preservation of these modifications, or whether frameshifting is secondary to a more fundamental role for these modifications. Because of the strong role of frameshifting in determining the viability of specific infectious viruses in eukaryotes, the selective pressure is obvious in these circumstances (virus-infected cells). Less clear is the role for frameshifting and position 37 modifications of tRNA Phe in non-virus infected cells. However, it is becoming increasingly apparent that PRF is not limited to virus-infected cells, but is more widespread and is likely employed by many organisms. Thus, frameshifting is used for expression of the E. coli (and certain other bacteria) dnaX gene, encoding two subunits of DNA polymerase III, i.e., τ and γ (51). Frameshifting occurs with 50 % efficiency at an A AAA AAG slippery site, which encodes for A- and P-site tRNA Lys containing the hypermodified nucleotide t6A at position 37. Recently, computational analyses revealed that functional –1 PRF signals are widespread in the S. cerevisiae genome. Confirming the analyses, selected tested putative -1 PRF signals promoted efficient frameshifting in vivo (52). Interestingly, the majority of –1 PRF events would direct translating ribosomes to premature stop codons suggesting that, in yeast PRF could be used to post-transcriptionally regulate gene expression through nonsense-mediated decay (52).

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FOOTNOTES

1The abbreviations used are: Ybs, wyosine bases; yW, wyebutosine; m1G, N1-methylguanosine; imG14, 4-demethylwyosine; wt, wild-type; PRF, programmed ribosomal frameshifting.

FIGURE LEGENDS

Figure 1. (A) Secondary structure of tRNA_{Phe} from S. cerevisiae with modified nucleotides (Wyebutosine yW at position 37 is boxed); (B) Biosynthetic pathway of yW in S. cerevisiae tRNA_{Phe} (9).

Figure 2. Schematic of –1 PRF reporter construct. Dual luciferase reporters were designed to mimic viral frameshifting (bottom panel). The frameshifting signal encoded between Luc_R and Luc_F is provided by a ~200 nts sequence from SCV-LA virus and contains a heptameric slippery sequence, a linker, and a secondary structure element. The slippery sequence codons are shown in 0-frame (“Pre-slippage”) and in –1 frame (“Post-slippage”) with corresponding A-site codons underlined (top panel).
**Figure 3.** Frameshifting efficiencies in cells bearing the pSCV-I and pSCV-II constructs (%). WT and deletion yeast strains producing tRNA\(^{Phe}\) with Wyebutosine (light gray), imG14 (dark gray) or 1-methyl-G (black) modifications at position 37 and harboring indicated reporter plasmids (slippery sequences are indicated for each reporter) were prepared as described in Materials and Methods. Frameshift efficiencies were determined by dividing the ratio of the firefly to Renilla luciferase activities from each sample to the ratio obtained from the ‘0-frame’ control strain (both luciferases are in-frame and constitutively expressed).

**Figure 4.** Frameshifting efficiencies of pSCV-II – pSCV-IV constructs. (A) ‘Pre-slip’ A-site-dependent effects on frameshift efficiencies. (B) Replot of data in (A) as normalized frameshifting efficiencies, which show large relative increase in efficiency with ablation of yW side chains: -1 PRF efficiency of each sample was normalized to −1 PRF efficiency of the wild type yeast strain.
### Table 1. Isogenic *S. cerevisiae* strains producing tRNA^Phe^ with specific structural variants of G37.
The common genetic background of the strains was *MAT* α *leu2Δ0 met15Δ0 ura3Δ0*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype (tRNA^Phe^ – 37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td><em>matα</em></td>
<td>Wyebutosine (yW)</td>
</tr>
<tr>
<td>ΔTYW1 (ΔYPL207w)</td>
<td><em>matα, TYW1ΔKan^R</em></td>
<td>N1'-methylguanosine (m1G)</td>
</tr>
<tr>
<td>ΔTYW2 (ΔYML005w)</td>
<td><em>matα, TYW2ΔKan^R</em></td>
<td>4-demethylwyosine (imG14)</td>
</tr>
</tbody>
</table>

### Table 2. Dissociation constants K_d (nM) of binding of ^3^H-Phe-tRNA^Phe^ variants to the A site of yeast 80S ribosomes at pH 7.5, 10 mM MgCl_2_. Binding was measured using Millipore filter method as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Structural variants of Phe-tRNA^Phe^</th>
<th>yW-37 (wt)</th>
<th>m1G-37</th>
<th>imG14-37</th>
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</thead>
<tbody>
<tr>
<td>poly(U) programmed 80S</td>
<td>154 ± 50</td>
<td>49 ± 27</td>
<td>46 ± 21</td>
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<tr>
<td></td>
<td>p = 0.0057</td>
<td>p = 0.0055</td>
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<tr>
<td>AUG UUU~ programmed 80S</td>
<td>96 ± 9</td>
<td>41 ± 6</td>
<td>30 ± 9</td>
</tr>
<tr>
<td>acid-treated tRNAs (poly(U) programmed 80S)</td>
<td>780 ± 19</td>
<td>72 ± 3</td>
<td>N/D</td>
</tr>
</tbody>
</table>
Figure 1

A

B

G  \rightarrow  m^1G  \rightarrow  imG-14  \rightarrow  yW-86  \rightarrow  yW

TRM5  \rightarrow  TYW1p  \rightarrow  TYW2p  \rightarrow  TYW3p-TYW4p

G  m^1G  imG-14  yW-86  yW
Figure 2.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Slippery site variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-slippage</td>
</tr>
<tr>
<td>pSCV-I</td>
<td>G GGU UUA</td>
</tr>
<tr>
<td>pSCV-II</td>
<td>G GGU UUU</td>
</tr>
<tr>
<td>pSCV-III</td>
<td>G GGU UUC</td>
</tr>
<tr>
<td>pSCV-IV</td>
<td>G GAU UUU</td>
</tr>
<tr>
<td>pSCV-V</td>
<td>G GAU UUC</td>
</tr>
<tr>
<td>pSCV-VI</td>
<td>G GUU UUU</td>
</tr>
</tbody>
</table>

Slippery Site

5’ G GGU UUA

Secondary structure element

CAP
SCV-LA
POL

Luc_R
SCV-LA
Luc_F
Figure 3.
Figure 4.