Improvement of reading frame maintenance is a common function for several tRNA modifications

Jaunius Urbonavičius, Qiang Qian,
Jérôme M.B.Durand, Tord G.Hagervall and
Glenn R.Björk¹

Department of Microbiology, Umeå University, S-90 187 Umeå,
Sweden

¹Corresponding author
e-mail: glenn.bjork@micro.umu.se

Transfer RNAs from all organisms contain many modified nucleosides. Their vastly different chemical structures, their presence in different tRNAs, their occurrence in different locations in tRNA and their influence on different reactions in which tRNA participates suggest that each modified nucleoside may have its own specific function. However, since the frequency of frameshifting in several different mutants [*mnmA*, *mnmE*, *tgt*, *truA* (*hisT*), *trmD*, *miaA*, *miaB* and *miaE*] defective in tRNA modification was higher compared with the corresponding wild-type controls, these modifications have a common function: they all improve reading frame maintenance. Frameshifting occurs by peptidyl-tRNA slippage, which is influenced by the hypomodified tRNA in two ways: (i) a hypomodified tRNA in the ternary complex may decrease the rate by which the complex is recruited to the A-site and thereby increasing peptidyl-tRNA slippage; or (ii) a hypomodified peptidyl-tRNA may be more prone to slip than its fully modified counterpart. We propose that the improvement of reading frame maintenance has been and is the major selective factor for the emergence of new modified nucleosides.

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Introduction

The capacity of the translation apparatus has evolved to read long messages and thereby to make sophisticated proteins required for life as we see it today. Although the translation apparatus has the ability to decode faithfully, errors occur with frequencies of 10^{-3} – 10^{-4} per codon (Kurland *et al.*, 1996). Most missense errors are not harmful to proteins, since many amino acids can be substituted without affecting the stability or the activity of the protein. In sharp contrast to the missense errors, almost all of the frameshift errors are detrimental to the synthesis of a functional protein, since following such a shift in frame the amino acid sequence becomes completely different and eventually the ribosome usually encounters a stop codon. This results in a truncated, usually unstable or inactive, peptide. Clearly, during evolution, features of the translation apparatus that are pivotal for reading frame maintenance have evolved. To understand the mechanism

by which the ribosome traverses the mRNA in a faithful manner, one has to unravel the features of the translation apparatus that are important for maintaining the correct reading frame.

There are several examples of how frameshift errors occur by peptidyl-tRNA slippage induced by a pause occurring in the A-site (reviewed in Farabaugh, 1997; Farabaugh and Björk, 1999; see also Figure 1). The length of the pause in the A-site, the fitness of the tRNA in the P-site and the mRNA sequence determine the frequency of slippage by the peptidyl-tRNA and thus the frequency of frameshift. Changes in the tRNA structure, such as that induced by a deficiency of a modified nucleoside, may therefore affect reading frame maintenance. Modified nucleosides are derivatives of the four major nucleosides U, C, A and G, and at present 81 different modified nucleosides have been characterized (Rozenski *et al.*, 1999). Although they are present in tRNA in all organisms and in many different positions of the tRNA, many of them are present in the anticodon region and especially in position 34 (the wobble position) and 37 (3' and adjacent to the anticodon). These two positions are not only frequently modified, but also contain a plethora of modified nucleosides. The abundance and presence of modified nucleosides in tRNA from all organisms suggest that they have a pivotal role in the function of tRNA.

Base modification contributes in several ways to the efficiency and accuracy of translation (reviewed in Björk, 1995, 1996; Curran, 1998), e.g. by improving the efficiency of the tRNA, influencing the fidelity of translation, improving the recognition by elongation factors or aminoacyl-tRNA synthetases, influencing codon choice, improving the efficiency of translation, decreasing the codon context sensitivity and preventing frameshifting. The structures of the modified nucleosides are quite different, e.g. *mnm*⁵*s*²U34 is a thiolated pyrimidine with a side chain at position 5, whereas *ms*²*io*⁶A37 is a methylthiolated purine with an extended aliphatic side chain on the amino group at position six of the adenine base. Moreover, they are located in different positions (e.g. position 34 versus position 37) and in different tRNAs (e.g. *mnm*⁵*s*²U34 in tRNA^{Lys}_{*mnm**s**2*UUU} versus *ms*²*io*⁶A37 in tRNA^{Phe}_{*GAA*}). Their vastly different chemical structures, their presence in different tRNAs, their occurrence in different locations in the tRNA and their influence on different reactions in which tRNA participates suggest that each modified nucleoside may have a specific function. Thus, no common function of modified nucleosides has so far been suggested. This question is addressed here and our results show that several structurally different modified nucleosides present in different tRNAs and at different positions of the tRNA have a common function: they all improve reading frame maintenance. The improvement occurs in two principally different ways: by promoting the

Fig. 1. A dual-error model for frameshifting. (A) Hypomodified cognate tRNA is defective in the aa-tRNA selection step, thereby allowing a wild-type near-cognate tRNA to be accepted instead at the A-site. After a normal three nucleotide translocation, the near-cognate tRNA slips into either -1 or $+1$ frame, depending on the sequence of the mRNA. (B) The hypomodified cognate tRNA is very slow in entering the A-site, inducing a pause and thereby allowing the wild-type cognate tRNA in the P-site to frameshift. (C) As in (A), but the hypomodified cognate tRNA is accepted in the A-site, and after a normal three nucleotide translocation to the P-site, the hypomodification induces slippage into the -1 or $+1$ frame. For clarity, only one tRNA is depicted as residing on the ribosome, although two tRNAs are always present in the A- and P-sites or P- and E-sites.

recruitment of the ternary complex to the A-site codon and thereby shortening the pause in the A-site, or by preventing slippage of the peptidyl-tRNA. The results are consistent with our model of how frameshifting occurs (Figure 1). They also emphasize that a small structural change in the tRNA, e.g. by the introduction of certain modified nucleosides, is an important feature of reading frame maintenance. Since some of these modified nucleosides are only found in tRNAs from one phylogenetic domain, others in two domains and some in all three domains (Motorin and Grosjean, 1998), our results also suggest that improvement of reading frame maintenance in conjunction with the appearance of new modified nucleosides started early and has been an ongoing process during evolution.

Results

The model and experimental systems

Figure 1 shows our model of how tRNA modification may influence reading frame maintenance. Figure 1A shows the

case when the hypomodification decreases the ability of the cognate tRNA to enter the A-site so much that instead a near-cognate wild-type tRNA enters the A-site. After a normal three nucleotide translocation, the near-cognate peptidyl-tRNA is not optimally fitted in the P-site and the frequency of slippage will increase, resulting in frameshifting (A-site effect by the hypomodified tRNA). Alternatively (Figure 1B), the hypomodified cognate tRNA enters the A-site slowly, causing a pause that allows the cognate wild-type peptidyl-tRNA to slip, resulting in frameshifting in the P-site (A-site effect by the hypomodified tRNA). However, the hypomodified cognate tRNA may not be seriously defective in the A-site selection step, and after a normal translocation it will reside as a hypomodified cognate peptidyl-tRNA (Figure 1C). The hypomodification makes the anticodon-codon interaction less optimal, similar to the interaction by a near-cognate tRNA, and this will result in an increased frequency of frameshifting (P-site effect by the hypomodified tRNA). Of course, hypomodification may cause frameshifting by mediating both A- and P-site effects. This model has features similar to previously presented models to explain frameshifting caused by peptidyl-tRNA slippage (Horsfield *et al.*, 1995; Farabaugh, 1997; Farabaugh and Björk, 1999). We have used two assay systems to investigate the influence of various modified nucleosides on frameshifting according to either an A-site or a P-site effect induced by the hypomodified tRNA.

To measure an A-site effect by the hypomodified tRNA, we have used plasmids devised by Curran and Yarus (1989) or by Weiss *et al.* (1987) (Figure 2A). In both these systems, the *lacZ* gene is placed downstream of a short frameshifting window in such a way that the β -galactosidase activity is a direct measurement of the frequency with which the ribosome shifts frame within this window (Figure 2A). In these constructs, either CUU, GUU, CCC or UUU codons were placed adjacent to and 5' of the test codon (NNN in Figure 2A). These P-site codons are read by tRNA^{Leu}_{GAG}(CUU), tRNA^{Val}_{GAC} or tRNA^{Val}_{cmo5UAC}(GUU), tRNA^{Pro}_{cmo5UGG}(CCC) and tRNA^{Phe}_{GAA}(UUU), respectively. We will refer to these various constructs by indicating the P-site codon or by referring to which tRNA is frameshifting in the P-site. The frequency of frameshifting is dependent on the competition between the P-site peptidyl-tRNA to slip $+1$ base and the rate of aminoacyl (aa)-tRNA selection at the A-site. A slow entry by the aa-tRNA·EF-Tu·GTP ternary complex to the A-site results in an elevated frequency of frameshifting and thus a higher β -galactosidase activity.

To measure the P-site effect by the undermodified tRNA (Figure 2B), a stop codon was placed just downstream of the P-site codon (Curran, 1993). It is known that the release factor acts in the A-site (Tate *et al.*, 1996), and since the *lacZ* gene is placed in the $+1$ frame downstream of this site, the β -galactosidase activity will be a measurement of the P-site frameshifting event (Qian and Björk, 1997). In several combinations (*mnmE*/CAA, *mnmE*/AAA and AAG, *miaA*/UUU, *miaA*/UAC and UAU, and *trmD3*/CGG), the frequency of frameshifting decreased on overexpression of RF1 (data not shown), consistent with the view that the assay monitors a P-site effect by the hypomodified tRNA.

Fig. 2. The assay systems for measuring +1 frameshifting. (A) Measurement of A-site effect by the hypomodified tRNA. The *lacZ* gene is placed downstream of a short frameshifting window in such a way that β -galactosidase (b-gal) activity is a direct measurement of the frequency with which the ribosome shifts frame within the short frameshifting window. This frequency of frameshifting is dependent on the competition between the peptidyl-tRNA slipping +1 base and aa-tRNA selection at the A-site. The different test codons [NNN in (A)] are placed just downstream of the frameshifting peptidyl-tRNA. (B) Measurement of P-site effect by the hypomodified tRNA. In this case, a stop codon (most often UAG) is placed just downstream of the P-site codon and the *lacZ* gene is placed in the +1 frame downstream of the A-site stop codon. The P-site frameshifting competes with the release factor activity in the A-site and the β -galactosidase activity is a direct measurement of the efficiency of frameshifting.

These assay systems, which are present on plasmids, were introduced into *Salmonella typhimurium* or *Escherichia coli* mutants defective in the synthesis of different modified nucleosides. The frameshifting frequencies were compared with those in the wild type. All strains used are congenic except for the allelic state of the modification gene. Although the frequency of +1 frameshifting is low in many cases, some of them are ~10% and in one case as high as 50%.

A-site effects by the hypomodified tRNA

Q34. The hypermodified base 7-[(4.5-*cis*-dihydroxy-2-cyclopenten-1-yl)-amino]methyl]-7-deazaguanosine, or queuosine (Q), is found exclusively in position 34 in the tRNAs specific for Tyr, His, Asn and Asp. In a *tgt* mutant, these tRNAs have an unmodified G34 instead of Q34. We introduced plasmids containing, as test codons, either the two His codons CAU/C, the two Tyr codons UAU/C or the Asn codon AAU into congenic strains, which only differ in the allelic state of the *tgt* gene.

Figure 3A shows that lack of Q in tRNA^{His}_{CAU} had no influence on frameshifting mediated by the

tRNA^{Pro}_{cmo5UGG} interacting with the proline CCC codon in the P-site when the His codons CAU or CAC were placed in the A-site. However, a significant 30% increase was noted on the tRNA^{Phe}_{GAA}-mediated frameshifting on UUU in the P-site with the His codon CAU as test codon in the A-site (Figure 3B). Lack of Q in tRNA^{Tyr}_{UAU} increased P-site slippage of either tRNA^{Pro}_{cmo5UGG} (2-fold) at CCC or tRNA^{Phe}_{GAA} (by 60%) at UUU for the Tyr test codon UAU in the A-site (Figure 3A and B). However, no effect was noticed with the synonymous Tyr test codon UAC (Figure 3A). Using the Asn test codon AAU, no effect by Q34 on frameshifting was observed.

We conclude that when UAU (Tyr) or CAU (His), but not UAC (Tyr), CAC (His) or AAU (Asn) codons were in the A-site, Q34 deficiency induced P-site frameshifting due to slow entry of the hypomodified tRNA into the A-site. Apparently, the Q modification is more important for efficient interaction with codons ending in U than with codons ending in C. Both wild-type and Q-deficient His- and Tyr-tRNAs were recruited more efficiently to C-ending codons than to U-ending codons, suggesting, as expected, that Q34 and G34 interact more efficiently with C than with U in the mRNA.

mmn⁵s²U34. The modified nucleoside 5-methylamino-methyl-2-thiouridine (mmn⁵s²U34) is present in the wobble position in tRNAs specific for Gln, Lys and Glu. These tRNAs read codons ending with a purine in 'split' codon boxes (more than one amino acid is encoded by codons in one codon box). We tested the influence of mmn⁵s²U34 modification on peptidyl-tRNA^{Pro}_{cmo5UGG}-mediated frameshifting by using plasmids containing Lys or Gln codons as A-site test codons. The *mmnE* mutant lacks the mmn⁵ modification and thus contains s²U34 in its tRNA. The *mmnA* mutant lacks the s² modification and thus contains mmn⁵U34 in its tRNA. Therefore, use of either *mmnE* or *mmnA* mutant allowed us to address specifically the contribution of the mmn⁵ or the s² group to reading frame maintenance.

Frameshifting increased 2.7-fold compared with that observed in the wild type when the mmn⁵ modification was absent when testing the Lys codon AAA (Figure 3C). A 5.5-fold increase in frameshifting was observed when the synonymous codon AAG was tested. When the s² group was missing, a similar increase in frameshifting was observed when both Lys codons were tested (2.6-fold for AAA and 5.4-fold for AAG). Thus, lack of the s² or the mmn⁵ group influenced the decoding of the AAA Lys codon in the A-site similarly, and both groups were especially important to decode the AAG codon.

We have previously shown that at CCC-NNN sequences it is the cmo⁵U34-containing near-cognate tRNA^{Pro}_{cmo5UGG} interacting with the CCC codon in the P-site that is frameshifting (Qian *et al.*, 1998). The synthesis of the cmo⁵ modification is abolished by an *aroD* mutation (Björk, 1980) and so is frameshifting (Qian *et al.*, 1998). In the above experiments, the CCC codon was placed upstream of the AAA or AAG test codons. Since the presence of the *aroD* mutation abolished the *mmnA/E*-mediated frameshifting (Figure 3C), it was caused by the cmo⁵U34-containing tRNA^{Pro}_{cmo5UGG} interacting with the CCC codon at the P-site. Amino acid sequencing of the N-terminus of the purified frameshifting products (β -

Fig. 3. Influence of the Q34 or $\text{mnm}^5\text{s}^2\text{U34}$ on A-site effect by hypomodified tRNA. (A) Influence of Q34 on A-site selection at two His and two Tyr codons. Although the standard error for the His codon CAU suggests a difference between wild type and mutant, the *t*-test analysis did not (Table I). (B) Influence of Q34 on A-site selection at CAU (His), UAU (Tyr) and AAU (Asn) codons. (C) Influence of $\text{mnm}^5\text{s}^2\text{U34}$ on A-site selection at two Lys codons. Values obtained using the *mnmA* mutant were normalized to those using the *mnmE* mutant. (D) Influence of $\text{mnm}^5\text{s}^2\text{U34}$ on two Gln codons. In (A), (C) and (D), the Pro codon CCC is present at the P-site, and in (B) the Phe codon UUU is present at the P-site. Values obtained using the *mnmA* mutant were normalized to those using the *mnmE* mutant. The modification status of tRNAs reading A-site codons is indicated above the bars. The frequency of frameshifting is expressed as the β -galactosidase activity normalized to the β -lactamase activity encoded by the *bla* gene present on the same plasmid as that encoding the *lacZ* gene.

galactosidase) supported the view that frameshifting occurred by a pro-tRNA (data not shown). We conclude that the frameshifting occurred at the P-site CCC codon as a result of a slow recruitment of $\text{mnm}^5\text{s}^2\text{U34}$ -deficient tRNA to the A-site, consistent with the frameshifting model (Figure 1). However, when the CCC codon was followed by the AAG codon, an ~50% decrease in frameshifting occurred in the *aroD* derivatives of the *mnmA/E* mutants (Figure 3C), suggesting that 50% of the frameshifting was caused by a tRNA other than $\text{tRNA}^{\text{Pro}}_{\text{cmo5UGG}}$.

Figure 3D shows that lack of the mnm^5 group of the A-site $\text{tRNA}^{\text{Gln}}_{\text{mnm5s2UUG}}$ (i.e. $\text{s}^2\text{U34}$ is present) did not induce slippage of the P-site $\text{tRNA}^{\text{Pro}}_{\text{cmo5UGG}}$ when testing Gln codons CAA or CAG in the A-site. However, when the s^2 group was absent (i.e. $\text{mnm}^5\text{U34}$ is present), frameshifting was increased 1.6-fold due to a slow entry of $\text{tRNA}^{\text{Gln}}_{\text{mnm5s2UUG}}$ to the A-site CAA codon. Frameshifting was abolished in the presence of the *aroD* mutation (Figure 3D), demonstrating that frameshifting was caused by the $\text{tRNA}^{\text{Pro}}_{\text{cmo5UGG}}$ interacting with the CCC codon in the P-site. No effect was observed for CAG as the test codon. This may be expected, since CAG is also decoded by the iso-accepting $\text{tRNA}^{\text{Gln}}_{\text{CUG}}$, which has C34 as wobble nucleoside. This tRNA should decode CAG efficiently, but not CAA, and should therefore not compete with $\text{tRNA}^{\text{Gln}}_{\text{mnm5s2UUG}}$ in decoding the CAA codon.

In summary, whereas lack of the mnm^5 modification strongly decreased the recruitment of the lys-tRNA, especially to the G-ending codon, we did not observe any effect by the mnm^5 group on the recruitment of $\text{tRNA}^{\text{Gln}}_{\text{mnm5s2UUG}}$ to either of the two Gln codons CAA and

CAG. Lack of the s^2 group reduced the recruitment to the A-ending codon for both Gln- and Lys-tRNAs, whereas it reduced the recruitment for only $\text{tRNA}^{\text{Lys}}_{\text{mnm5s2UUU}}$ to the G-ending codon.

P-site effect by the hypomodified tRNA

Q34. To measure the P-site effect by Q-deficient $\text{tRNA}^{\text{Tyr}}_{\text{QUA}}$, the Tyr codons UAU/C were placed in the P-site followed by a stop codon (Figure 2B). Lack of Q34 in $\text{tRNA}^{\text{Tyr}}_{\text{QUA}}$ residing in the P-site and decoding the UAU or UAC codons did not increase frameshifting (Figure 4A). Thus, no P-site effect by Q34 deficiency was observed.

$\text{mnm}^5\text{s}^2\text{U34}$. To test the influence on peptidyl-tRNA slippage by $\text{mnm}^5\text{s}^2\text{U34}$, we introduced plasmids containing the P-site codons AAA (Lys), AAG (Lys), CAA (Gln) or CAG (Gln) followed by a UAG stop codon into mutants defective in the synthesis of $\text{mnm}^5\text{s}^2\text{U34}$. When AAA or the AAG codon was placed in the P-site, frameshifting on both codons increased ~3-fold for mnm^5 -deficient $\text{tRNA}^{\text{Lys}}_{\text{mnm5s2UUU}}$ and by ~50% when the same tRNA lacked the s^2 group (Figure 4B). Frameshifting increased similarly (2.4- to 2.6-fold) when $\text{tRNA}^{\text{Gln}}_{\text{mnm5s2UUG}}$ lacked either the mnm^5 or the s^2 group and decoded the CAA codon (Figure 4C). When the same tRNA decoded the synonymous codon CAG, mnm^5 deficiency increased frameshifting 1.5-fold, whereas s^2 deficiency had no effect.

$\text{ms}^2\text{i}^6\text{A37}$. The modified nucleoside 2-(methylthio- N^6 -isopentenyl)adenosine $\text{ms}^2\text{i}^6\text{A37}$ is present in all *E. coli* tRNAs reading codons starting with U, except $\text{tRNA}^{\text{Ser}}_{\text{GGA}}$ (Grosjean *et al.*, 1985). In *S. typhimurium*, the

Fig. 4. Influence of Q34 or mnm^5s^2U34 tRNA modifications on P-site effect by hypomodified tRNA. (A) Influence of Q34 on slippage at two Tyr codons. (B) Influence of mnm^5s^2U34 on slippage at two Lys codons. (C) Influence of mnm^5s^2U34 on slippage at two Gln codons. The stop codon UAG was present at the A-site in all cases. The modification status of tRNA reading P-site codon is indicated above the bars. The frequency of frameshifting is expressed as a percentage of the β -galactosidase activity expressed from the test plasmid compared with that activity of a pseudowild-type β -galactosidase encoded by the control plasmid.

hydroxylated derivative ms^2io^6A37 is present in the corresponding tRNAs. Three genes involved in the synthesis of ms^2io^6A37 have been identified: *miaA*, *miaB* and *miaE* (Björk, 1996). The *miaA* mutant contains A37 instead of ms^2io^6A37 in the tRNA, whereas the *miaB* mutant lacks the ms^2 group and contains io^6A37 . The *miaE* mutant contains ms^2io^6A37 instead of ms^2io^6A37 in its tRNA.

We tested the influence of ms^2io^6A37 on frameshifting at the P-site Tyr codons UAC or UAU. A 9-fold increase in frameshifting was observed in both the *miaA* and *miaB* mutants when $tRNA_{QUA}^{Tyr}$ decoded the UAU codon, whereas a 6- to 7-fold increase was observed in both mutants when the same tRNA decoded UAC (Figure 5A). Thus, the effects were similar for both Tyr codons whether $tRNA_{QUA}^{Tyr}$ lacked both the ms^2 and the io^6 modifications (as in the *miaA* mutant) or whether this tRNA lacked only the ms^2 group (as in the *miaB* mutant). We conclude that the ms^2 group was the main contributor to reading frame maintenance for P-site $tRNA_{QUA}^{Tyr}$ interacting with the UAU or the UAC codons.

To test the influence of ms^2io^6A37 on P-site slippage by $tRNA_{GAA}^{Phe}$ at codon UUU, a stop codon was placed adjacent to and 3' of it. Compared with wild type, frameshifting increased similarly (3.4- to 3.9-fold) in both

Fig. 5. Influence on P-site effect of ms^2io^6A37 -deficient tRNA.

(A) Influence of ms^2io^6A37 on slippage at two Tyr codons. Stop codon UAG is present at the A-site. (B) As in (A), but the codon UUU is present at the P-site. (C) Influence of ms^2io^6A37 on slippage at Phe codon UUU. The His codon CAU is present at the A-site. The modification status of tRNA reading P-site codon is indicated above the bars.

the *miaA* and the *miaB* mutants (Figure 5B). Frameshifting occurs at a rather high frequency at the UUU-CAU site (Fu and Parker, 1994). If the frameshifting occurs in the P-site, the frequency at this site should increase upon starvation of His, since the CAU codon is decoded by the His-tRNA. This indeed explains (Qian, 1997) why we consider this system suitable for measuring P-site slippage by peptidyl- $tRNA_{GAA}^{Phe}$ on UUU followed by a sense codon (His) instead of a stop codon. Using this system, frameshifting at the UUU codon by the undermodified P-site $tRNA_{GAA}^{Phe}$ increased similarly (2.6-fold) in both the *miaA* and the *miaB* mutants (Figure 5C). Thus, modification deficiency of P-site $tRNA_{GAA}^{Phe}$ decoding UUU induced a similar increase in frameshifting (2.6- to 3.9-fold) irrespective of whether a stop codon or a sense codon followed the P-site UUU codon. Lack of the hydroxyl group, as in the *miaE* mutant, increased frameshifting by 20% (data not shown). We conclude that when either $tRNA_{GAA}^{Phe}$ or $tRNA_{GUA}^{Tyr}$ resides in the P-site, the ms^2 group is the main contributor to reading frame maintenance, whereas the io^6 group or the OH group has only a minor role in this respect.

m¹G37. The *m¹G37* is present in all organisms in $tRNA_{Leu}^{Leu}$ species that read CUN codons (N denotes any of the four major nucleosides), in $tRNA_{Pro}^{Pro}$ reading CCN codons and in $tRNA_{Arg}^{Arg}$ that reads the Arg CGG codon (Björk, 1986). When we placed the CUU, CUA or CUG leucine codons in

Fig. 6. Influence on P-site effect by m¹G37-deficient tRNA. (A) Influence of m¹G37 on slippage at Leu codons CUU, CUA, CUG and CUC. The value for the CUU codon is 100-fold higher than shown in the figure. (B) Influence of m¹G37 on slippage at the Arg codon CGG. (C) Influence of m¹G37 on slippage at the Pro codon CCG. In (A) and (B), the stop codon UAG is present in the A-site, and in (C) the stop codon UGA or UAA is present in the A-site. The modification status of tRNA reading P-site codon is indicated above the bars.

the P-site, no frameshifting was observed in a strain deficient in m¹G37 (Figure 6A). A 30% increase in frameshifting was noted for the CUC codon (Figure 6A). However, when the Arg codon CGG, which is decoded by the m¹G37-containing tRNA^{Arg}_{CCG}, was in the P-site, frameshifting increased 2.9-fold (Figure 6B). Although there was no increase in frameshifting when the proline codon CCG was placed in the P-site (Figure 6C), a P-site effect was previously shown for the proline codons CCU and CCC followed by a stop codon, but also when these codons were followed by a sense codon (Hagervall *et al.*, 1993b). We conclude that the m¹G37 deficiency results in a P-site effect for one Leu codon, for the only Arg codon read by an m¹G37-containing tRNA, and for two of the three proline codons tested.

ψ 38, 39 and 40. The formation of the modified nucleoside Ψ in the anticodon stem and/or loop is catalyzed by the *truA* (*hisT*) gene product. The modification is present in 20 different tRNA species. A *truA* mutant therefore contains unmodified U instead of Ψ in these positions in tRNA. We measured P-site frameshifting by tRNA^{His} at the CAU

codon, tRNA^{Leu} at the CUU, CUC and CUG codons, and tRNA^{Pro} at the CCC codon in wild-type and *truA* (*hisT*) mutant cells. No significant difference in frameshifting between wild type and the *truA* (*hisT*) mutant was observed (Table I). However, compared with the wild type, a 50% increase in frameshifting was observed for Ψ 38-deficient tRNA^{Leu} on the P-site CUA codon (Table I).

Discussion

We show here that several modified nucleosides present in the anticodon region of tRNAs improve reading frame maintenance. The present results and literature data relevant for the discussion are summarized in Tables I and II. Since the literature data were not interpreted previously in relation to our present model for frameshifting (Figure 1), we will combine these data with our present results in the discussion. Generally, if an A-site effect was observed for the hypomodified tRNA, there was also an effect induced by the same hypomodified tRNA in the P-site (e.g. Lys-tRNA and mnm⁵s²U34; Tyr-tRNA and ms²io⁶A37; Pro-tRNA and m¹G37; Arg-tRNA and m¹G37). In two cases (ms²io⁶A37-deficient Phe-tRNA and mnm⁵s²U34-deficient Gln-tRNA), no A-site effect was induced by hypomodification, although a strong P-site effect was observed. Leu-tRNA deficient in m¹G37 or Ψ 38–40 induced no P-site effect, although a weak A-site effect was observed. Thus, several modified nucleosides improve reading frame maintenance by promoting efficient A-site selection, preventing peptidyl-tRNA slippage, or both. This function of the modified nucleoside was tRNA specific and dependent on the codon read. We conclude that although the structure and the location of the tested modified nucleosides are different (compare the structures of mnm⁵s²U34 and m¹G37) and that they reside in different tRNA species, a common function for several of them is to improve reading frame maintenance. This does not rule out that, in addition, each modified nucleoside may also have its specific function in some other reaction in which tRNAs participate (e.g. in the aminoacylation reaction).

To measure an A-site effect by the hypomodified tRNA, we have used two plasmid systems. One system is based on the programmed frameshifting site in the structural gene encoding release factor 2 (RF2). It is well established that this system measures the aa-tRNA selection rate (Curran and Yarus, 1988, 1989; Gao *et al.*, 1995). This is also consistent with the fact that the *miaA* mutation does not influence the Phe-tRNA selection rate *in vivo* (Table II) or *in vitro* (Diaz and Ehrenberg, 1991). In the other plasmid system, which was used to monitor the A-site effect for the combinations of the *tgt* mutation and codons CAU/C and AAU and the *mnmE/A* mutations with the Lys and Gln codons, a P-site CCC codon was followed by the test codons. Using this system, we have previously shown that the frameshifting event occurred in the P-site by the near-cognate pro-tRNA^{Pro}_{cmo5UGG} (Qian and Björk, 1997; Qian *et al.*, 1998), and this was also the case for several of the combinations in Figure 3C and D. However, at test codon AAG, ~50% of the frameshifting in the *mnmE/A* mutants was caused by a tRNA other than tRNA^{Pro}_{cmo5UGG}. Since a P-site effect was observed for the hypomodified forms of tRNA^{Lys}_{mnm5s2UUU}, it is most likely that this tRNA

Table I. Influence of tRNA modifications Q34, mnm⁵s²U34 and Ψ38–40 on reading frame maintenance

Test codon	Amino acid encoded	Nucleoside mutant/wild type	A-site effect ^a (frameshifting, fold wild type)	P-site effect ^a (frameshifting, fold wild type)
CAU CAC	His His	G/Q34 G/Q34	no (1.5)/yes (1.3) no (1.4)	ND ND
UAU UAC	Tyr Tyr	G/Q34 G/Q34	yes (2.0)/(1.6) no (1.4)	no (1.1) no (1.0)
AAU	Asn	G/Q34	no (0.9)	ND
AAA AAA AAG AAG	Lys Lys Lys Lys	s ² U/mnm ⁵ s ² U34 mnm ⁵ U/mnm ⁵ s ² U34 s ² U/mnm ⁵ s ² U34 mnm ⁵ U/mnm ⁵ s ² U34	yes (2.7) yes (2.6) yes (5.5) yes (5.4)	yes (3.0) yes (1.5) yes (3.4) yes (1.6)
CAA CAA CAG CAG	Gln Gln Gln Gln	s ² U/mnm ⁵ s ² U34 mnm ⁵ U/mnm ⁵ s ² U34 s ² U/mnm ⁵ s ² U34 mnm ⁵ U/mnm ⁵ s ² U34	no (1.1) yes (1.6) no (0.9) no (0.8)	yes (2.6) yes (2.4) yes (1.5) no (1.0)
CUU CUC CUA CUG	Leu Leu Leu Leu	U/Ψ38 U/Ψ38 U/Ψ38–39 U/Ψ38–40	yes (1.8) ^b no (1.5) ^b yes (2.8) ^b yes (3.2) ^b	no (1.0) no (1.0) yes (1.5) no (1.0)
CCC	Pro	U/Ψ40	no (0.8) ^b	no (1.0)
CAU	His	U/Ψ38–39	ND	no (1.0)

ND, not determined.

^a‘Yes’ denotes a significant difference and ‘no’ denotes no significant difference in frameshifting in mutant and wild type as judged by a *t*-test of the means with two tails.**Table II.** Influence of tRNA modifications ms²io⁶A37 and m¹G37 on reading frame maintenance

Test codon	Amino acid encoded	Nucleoside mutant/wild type	A-site effect ^a (frameshifting, fold wild type)	P-site effect ^a (frameshifting, fold wild type)
UAU UAU UAC UAC	Tyr Tyr Tyr Tyr	A/ms ² io ⁶ A37 i ⁶ A/ms ² io ⁶ A37 A/ms ² io ⁶ A37 i ⁶ A/ms ² io ⁶ A37	yes (4.4) ^b yes (1.4) ^b yes (4.9) ^b yes (1.6) ^b	yes (9.0) yes (8.8) yes (6.0) yes (7.1)
UUU UUU	Phe Phe	A/ms ² io ⁶ A37 i ⁶ A/ms ² io ⁶ A37	no (1.1) ^b no (0.8) ^b	yes (2.6/3.4) yes (2.6/3.9)
CUU CUC CUA CUG	Leu Leu Leu Leu	G/m ¹ G37 G/m ¹ G37 G/m ¹ G37 G/m ¹ G37	yes (1.5) ^b no (1.0) ^b yes (1.4) ^b yes (1.7) ^b	no (1.1) yes (1.3) no (1.2) no (1.2)
CCU CCC CCA CCG	Pro Pro Pro Pro	G/m ¹ G37 G/m ¹ G37 G/m ¹ G37 G/m ¹ G37	yes (4.3) ^b yes (2.2) ^b yes (3.3) ^b yes (8.5) ^b	yes (3.5) ^c yes (5.2) ^c ND no (1.3/1.2)
CGG	Arg	G/m ¹ G37	yes (4.3) ^b	yes (2.9)

ND, not determined.

^aSee Table I.^bAdapted from Li *et al.* (1997).^cAdapted from Hagervall *et al.* (1993b).

is responsible for the 50% frameshifting not related to tRNA^{Pro}_{cmo}SUGG. These results are consistent with our model, but suggest that in these cases the assay monitored a mixture of A- and P-site effects by the hypomodified tRNA. However, these considerations do not influence our

major conclusion that the hypomodified tRNA causes a frameshifting event. In the P-site assay, the hypomodified tRNA may cause an A-site effect preceding the translocation into the P-site. Thus, instead of measuring an event according to mechanism C, it may also monitor an

event according to mechanism A (Figure 1). Since no A-site effect was observed for the combinations *mmnE*/CAA and CAG, *miaA*/UUU, *miaB*/UUU, and *trmD*/CUC, we find such an explanation unlikely. The Tyr codon box contains no other sense codons than those for Tyr. Therefore, the A-site effect observed for Tyr codons cannot be caused by a near-cognate tRNA.

The *mmn*⁵ and *s*² groups ensure a restrictive reading of G (Yokoyama *et al.*, 1985). We observed a strong A-site effect of tRNA^{Lys}_{mmn5s2UUU} by *mmn*⁵ or *s*² deficiency; the largest effect was for the G-ending codon (Table I). Similarly, a shift in the opposite direction (−1) at the sequence UUU-AAAA, in which AAA is decoded by tRNA^{Lys}_{mmn5s2UUU}, was stimulated by the absence of the *mmn*⁵ modification (Brierley *et al.*, 1997). For tRNA^{Gln}_{mmn5s2UUG}, no A-site effect was observed for codons CAG or CAA when the *mmn*⁵ group was lacking, whereas *s*² deficiency caused an A-site effect for tRNA^{Gln}_{mmn5s2UUG} in decoding CAA. In all combinations, there was a similar P-site effect, i.e. lack of either the *s*² or the *mmn*⁵ group induced frameshifting to a similar degree (2- to 3-fold) when the hypomodified tRNA^{Lys}_{mmn5s2UUU} or tRNA^{Gln}_{mmn5s2UUG} resided in the P-site. The fact that a P-site slippage was dependent on *mmn*⁵*s*²U34 shows that although the CAG codon is also decoded by the iso-acceptor tRNA^{Gln}_{CUG}, occasionally the *mmn*⁵*s*²U34 containing tRNA^{Gln}_{mmn5s2UUG} outcompetes the tRNA^{Gln}_{CUG} in the A-site selection and will thereafter be translocated to the P-site. Thus, the A-site effect by *mmn*⁵*s*²U34 depended on the tRNA and the codon, and mostly affected tRNA^{Lys}_{mmn5s2UUU}. However, lack of either modification increased slippage of both tested tRNAs in the P-site. A P-site effect induced by hypomodified tRNA (Figure 4B) requires a shift by the hypomodified lysine tRNA from either AAA to the near-cognate codon AAU or from AAG to the non-cognate codon AGU. In the first case, the +1 shift results in a mismatch at the third position of the codon [hypomodified U34-U(III); N34 denotes the wobble nucleoside in the tRNA and N(III) denotes the third nucleoside in the codon], whereas the second case resulted in a U35-G(II) second position wobble. Surprisingly, the degree of frameshifting was similar (Figure 4B). In the case of Glu-tRNA, the +1 frameshift resulted in a shift from the cognate codon CAA to the non-cognate codon AAU or from CAG to the non-cognate codon AGU. Such frameshifts result in a mismatch in the first position of the codon [G36-A(I)] in both cases, and in the latter case also in an additional mismatch, the U35-G(II) second position wobble. The frequency of frameshifting was also lower in the latter case (Figure 4C). Thus, the observed frameshifts resulted in an anticodon–codon interaction in the +1 frame, involving one or more mismatches. This indicates that the anticodon–codon interaction in the P-site may not always follow the same rules as that observed in the A-site, as also suggested by Lim (1997). Apparently, the peptidyl-tRNA can form a wide variety of non-canonical base pairs in any of the positions of the P-site duplex, even simultaneously in all three positions. We suggest that one role of modified nucleosides is to fit the tRNA optimally in the P-site pocket, thereby counteracting an intrinsic tendency of the peptidyl-tRNA to slip along the mRNA, and thereby changing the frame.

The presence of the *ms*²*io*⁶ modification stabilizes the anticodon–anticodon interaction, as determined by the stability of tRNA–tRNA dimers with complementary anticodons. Such experiments also revealed that the *ms*² group was the major contributor to this stabilizing effect (Houssier and Grosjean, 1985). Still, an *ms*²-dependent A-site effect was only observed on the two Tyr codons, whereas no A-site effect was observed for Phe (UUU) (Table II), similar to what was observed previously for Trp (UGG), Cys (UGU and UGC) and Ser (UCG) (Li *et al.*, 1997). However, when both the *ms*² and the *io*⁶ groups were lacking (as in the *miaA* mutant), an A-site effect was observed for Tyr-, Trp-, Cys- and Ser-tRNAs, but not for tRNA^{Phe}_{GAA} (Table II; Li *et al.*, 1997). Thus, the *io*⁶ modification is the major contributor in preventing tRNA-dependent A-site pausing for all tRNAs having *ms*²*io*⁶A37, except tRNA^{Phe}_{GAA}. The influence of the *ms*² group is tRNA dependent, since it only affects tRNA^{Tyr}_{QUA}. The P-site slippage observed for the hypomodified tRNA^{Phe}_{GAA} requires a shift from the cognate UUU to the synonymous cognate codon UUC (Figure 5C) or to the identical cognate codon UUU (Figure 5B). Lack of only the *ms*² group or the *ms*²*io*⁶ group similarly increased the frequency of frameshifting on both codons. Thus, for hypomodified tRNA^{Phe}_{GAA} we observed a P-site effect, in contrast to the absence of any A-site effect. Since the P-site effect was similar when one or both modifications was absent, we conclude that the effect was mainly caused by the lack of the *ms*² group. Interestingly, the phe-tRNA in the P-site is also prone to slip in the −1 direction upon starvation of the next aa-tRNA (Barak *et al.*, 1996). This −1 frameshifting is stimulated in a *relA* background, and it has been speculated that in such a genetic background accumulation of hypomodified tRNAs occurs (Gallant *et al.*, 2000). Thus, also in this case, the stimulation of −1 frameshifting by the Phe-tRNA in the P-site may be caused by a hypomodified Phe-tRNA. The P-site slippage observed for the hypomodified tRNA^{Tyr}_{QUA} requires a shift from the cognate UAU to the non-cognate codon AUU (Ile), or from the cognate codon UAC to the non-cognate codon ACU (Thr). Thus, similar to what we observed for tRNA^{Lys}_{mmn5s2UUU} lacking *mmn*⁵*s*²U34 (see above), mismatches seem to occur in the P-site. This reinforces our suggestion that peptidyl-tRNA apparently makes non-canonical base pairs, as was also predicted previously (Lim, 1997). The overall frequency of frameshifting was much lower for the UAC to ACU shift (Figure 5), although the increase, relative to the frequency observed in the wild type, was similar (Table II). Similarly to the P-site effect for tRNA^{Phe}_{GAA}, the frequency of frameshifting for tRNA^{Tyr}_{QUA} was similar or even somewhat higher when only the *ms*² group was lacking. Thus, the major effect for tRNA^{Tyr}_{QUA}, as was the case for tRNA^{Phe}_{GAA}, was also caused by the *ms*² group (Table II). In both these cases, the results are consistent with results presented by Houssier and Grosjean (1985), who showed that the major stabilizing effect was caused by the presence of the *ms*² group. In the A-site assay, the tRNA is part of the ternary complex in which the EF-Tu is bound to the aminoacyl stem, which alters the anticodon conformation (Wikman *et al.*, 1982). EF-Tu may influence the anticodon conformation in such a way that the modification at position 37 is positioned in space such that it cannot interfere with the stability of the

Table III. Bacterial strains used

Strain	Relevant genotype or phenotype	Source/reference
<i>S.typhimurium</i>		
LT2	<i>wt</i>	J.Roth (UT, USA)
GT 5880	<i>tgt2522::Tn10dCm</i>	this work
GT 522	<i>miaA⁺</i>	Ericson and Björk (1986)
GT 523	<i>miaA1</i>	Ericson and Björk (1986)
GT 2351	<i>miaB⁺, zbf-99::Tn10</i>	Esberg <i>et al.</i> (1999)
GT5444	<i>miaB27, zbf-99::Tn10</i>	Esberg <i>et al.</i> (1999)
GT2176	<i>miaB2508::Tn10dCm, phs</i>	Esberg <i>et al.</i> (1999)
GT1513	<i>trmD⁺, hisO1242, hisD3749</i>	Hagervall <i>et al.</i> (1993)
GT1514	<i>trmD3, hisO1242, hisD3749</i>	Hagervall <i>et al.</i> (1993)
GT2529	<i>hisT⁺, zej-635::Tn5</i>	Li <i>et al.</i> (1997)
GT2530	<i>hisT1504, zej-635::Tn5</i>	Li <i>et al.</i> (1997)
GT3034	<i>miaE2507::MudJ</i>	Persson and Björk (1993)
<i>E.coli</i>		
TH98	<i>ara, Δ(pro-lac), argE(am), Val^R, ilv-135::Tn10, nalA, rif, mnmE⁺</i>	this work
TH99	<i>ara, Δ(pro-lac), argE(am), Val^R, ilv-135::Tn10, nalA, rif, mnmE</i>	this work
TH193	<i>ara, Δ(pro-lac), nalA, argE (am), rif, thi, mnmA</i>	this work
TH194	<i>ara, Δ(pro-lac), nalA, argE (am), rif, thi, mnmA⁺</i>	this work
GRB1758	<i>ara, Δ(pro-lac), nalA, argE (am), rif, Val^R, ilv-135::Tn10, mnmE⁺, aroD::Tn5</i>	this work
GRB1760	<i>ara, Δ(pro-lac), nalA, argE (am), rif, Val^R, ilv-135::Tn10, mnmE, aroD::Tn5</i>	this work
GRB2162	<i>ara, Δ(pro-lac), nalA, argE (am), rif, thi, mnmA⁺, aroD45::Tn10</i>	this work
GRB2163	<i>ara, Δ(pro-lac), nalA, argE (am), rif, thi, mnmA, aroD45::Tn10</i>	this work

anticodon–codon interaction. This may be the reason why we observed no tRNAs specific for Phe, Trp, Cys, Ser or only a minor tRNA^{Tyr}_{QUA} effect by ms² deficiency in the A-site assay, whereas in the P-site assay the largest effect was observed when the ms² group was lacking. Clearly, the ms²io⁶ modification strongly influences reading frame maintenance by affecting both the recruitment to the A-site and slippage in the P-site.

We have previously shown that lack of m¹G37 induces frameshifting at runs of C, and we suggested that this frameshifting was according to a quadruplet translocation model (Björk *et al.*, 1989; Hagervall *et al.*, 1993b). However, previous results (Hagervall *et al.*, 1993a), which are not consistent with the quadruplet translocation model, are consistent with the present model (Figure 1). Since we also now know that the frameshifting event occurs at the P-site (Qian and Björk, 1997), we favor the present model to explain how m¹G37 deficiency causes frameshifting.

The results obtained in this study and previous results (Björk *et al.*, 1989; Hagervall *et al.*, 1993a,b; Li *et al.*, 1997; Schwartz and Curran, 1997) are all consistent with the present model of how +1 frameshift occurs (Figure 1). The data also demonstrate how small changes in the structure of the tRNA, such as modification deficiency, influence reading frame maintenance. The model is also consistent with the suggestion that modifications in the wobble position make the tRNA more ‘cognate’ and thereby reduce decoding by the frameshifting-prone near-cognate tRNA (Sundararajan *et al.*, 1999). One would think that the function of modified nucleosides, as suggested by the model, might primarily be associated with those modified nucleosides present in the anticodon loop and stem. However, we have previously shown that alterations far from the anticodon, e.g. in the aminoacyl stem, also induce frameshifting (Qian and Björk, 1997). Therefore, one can expect that deficiency of modifications

located in other regions of the tRNA may also induce frameshifting. Although the interaction between the anticodon and codon in the P-site allows many non-canonical interactions (Lim, 1997), the nucleotide sequence of the +1 frame partly present in the A-site must be important. Therefore, at certain sequences no frameshifting may be noticed, although at another sequence a frameshift may occur if the modified nucleoside is not present. Moreover, the way in which the pause is manifested will also influence the ability to detect a frameshift event experimentally. Therefore, it may be difficult to show, in some cases, the influence on reading frame maintenance of certain modified nucleosides. Reading frame maintenance is an important feature of the translation apparatus. Its optimization may have occurred by small changes of the tRNA structure, of which the formation of modified nucleosides may have been a simple and a diversified way to accomplish such fine-tuning of the tRNA. Consistent with this suggestion is the fact that one tested modified nucleoside (m¹G37) was most likely present in the tRNA of the last common ancestor (Björk *et al.*, 2001), indicating that the mechanism for reading frame maintenance evolved early, and before the emergence of the three phylogenetic domains. Some others of the tested modified nucleosides (e.g. ms²io⁶A37, mnm⁵s²U34) are present only in one domain (bacteria), and one tested modified nucleoside (Q34) is present in two domains (eukaryotes and bacteria). Therefore, evolution of the mechanism for reading frame maintenance through the formation of modified nucleosides started early and is an ongoing process. Since aberrations in reading frame maintenance have such dramatic consequences for a sophisticated translation apparatus like that present today, they must be kept to a minimum. We show in this paper that important contributors to reading frame maintenance are the modified

nucleosides in tRNA. We therefore suggest that the improvement of reading frame maintenance has been, and is, the major driving force for the emergence of new modified nucleosides.

Materials and methods

Strains, plasmids and growth conditions

The strains used are listed in Table III. For the pTHF series of plasmids (Hagervall *et al.*, 1993b), frameshifting windows were created by the insertion of different synthetic oligonucleotides into the *Hind*III and *Apa*I sites in the 5' end of the *lacZ* gene of the vector p90.91, as described by Weiss *et al.* (1987). The frameshifting window for these plasmids is 5'-ATG-AAA-AGC-TTA-AAC-XXX-NNN-AGC-TAA-CGG-CCC-, where the underlined stop codon in the +1 and 0- frame is indicated. Using this system, the XXX codon (Figure 2) was always CCC. For pJC27 derivatives, frameshifting windows were created by insertion of different synthetic oligonucleotides in the 5' end of the *lacZ* gene using *Hind*III and *Bam*HI sites (Curran and Yarus, 1986). To obtain pJC27tet derivatives, *Hind*III-BssII fragments were exchanged with frameshifting windows containing *Hind*III-BssII fragments from pJC27 derivatives. In these plasmids, the sequence around the frameshifting site is 5'-ATG-ATT-ACG-CCA-AGC-TTC-CTT-AGG-GGG-TAT-XXX-NNN-CTA-CGG-GAT-3'. Underlined is the Shine-Dalgarno sequence required for frameshifting to occur at the XXX-N site (Figure 2). The XXX codon was CUU or GUG when monitoring the A-site effect. To monitor the P-site effect, the XXX-NNN sequence was exchanged to the NNN-UAG sequence. The underlined UAC codon in the +1 frame is the beginning of the fused *lacZ* gene.

Salmonella typhimurium strains harbouring various plasmids were grown at 37°C in the undefined rich medium NB+AV+ADE [Difco nutrient broth (0.8%); Difco Laboratories, Detroit, MI) supplemented with the aromatic amino acids, aromatic vitamins and adenine (Davis *et al.*, 1980). Strains of *E.coli* were grown at 37°C in Luria-Bertani medium (Bertani, 1951).

Determination of β -galactosidase and β -lactamase activity

β -galactosidase and β -lactamase activities, and determination of frameshifting frequency, were as described previously (Hagervall *et al.*, 1993b; Li *et al.*, 1997; Qian and Björk, 1997). The frequency of frameshifting is expressed as β -galactosidase activity relative to that of a pseudowild-type enzyme (% pseudowt) or normalized to the β -lactamase activity encoded from the *bla* gene present on the plasmid (gal/lac). Each measurement is based on three replicates and the statistical variations as indicated in the figures are standard errors. A *t*-test of the means with two tails was used to evaluate whether the frameshifting in the mutant was statistically different from that in the wild type ($p < 0.05$ in Tables I and II).

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