

COMMUNICATION

Modified Nucleotides in tRNA^{Lys} and tRNA^{Val} are Important for Translocation**Steven S. Phelps¹, Andrzej Malkiewicz², Paul F. Agris³ and Simpson Joseph^{1*}**

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Ribosomes translate genetic information encoded by messenger RNAs (mRNAs) into proteins. Accurate decoding by the ribosome depends on the proper interaction between the mRNA codon and the anticodon of transfer RNA (tRNA). tRNAs from all kingdoms of life are enzymatically modified at distinct sites, particularly in and near the anticodon. Yet, the role of these naturally occurring tRNA modifications in translation is not fully understood. Here we show that modified nucleosides at the first, or wobble, position of the anticodon and 3'-adjacent to the anticodon are important for translocation of tRNA from the ribosome's aminoacyl site (A site) to the peptidyl site (P site). Thus, naturally occurring modifications in tRNA contribute functional groups and conformational dynamics that are critical for accurate decoding of mRNA and for translocation to the P site during protein synthesis.

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In all organisms, tRNAs contain numerous post-transcriptional modifications.¹ Modifications are important for maturation and intracellular sorting of tRNAs between the cytosol and the mitochondria.² Some synthetase enzymes, which aminoacylate tRNAs, are dependent on sequence specific interactions with modified nucleotides in the tRNA anticodon.^{3–5} In fact, serious diseases are associated with a missing modification at the wobble position⁶ of the tRNA^{Lys} and tRNA^{Val} anticodon.^{7,8} The role of modified tRNA nucleotides in translation has proven to be elusive. Uhlenbeck and co-workers showed that an unmodified *Escherichia coli* tRNA^{Phe} transcript is active in the early steps of translation.⁹ Recently, evidence has developed which indicates that tRNA modifications enhance decoding,¹⁰ restore ribosomal binding,¹¹ and influence reading frame maintenance.^{12,13} However, very little is known about the role of tRNA modifications in individual steps of the translational cycle.

Translocation is one of the complex steps in the peptide elongation process. After the peptidyl

transferase reaction, deacylated tRNA is bound to the P site and peptidyl tRNA is bound to the A site. Elongation factors (elongation factor G in *E. coli*) bind to the ribosome and hydrolyze GTP, which triggers translocation.¹⁴ During translocation, the ribosome moves along the mRNA a distance of one codon (three nucleotides), and the tRNA bearing the elongating peptide is shifted from A site to P site. The end result of this process is to free the A site for the next aminoacyl-tRNA, and eject the deacylated tRNA from the E site. Large-scale conformational rearrangements occur during translocation that accommodate movement of the tRNA–mRNA complex. Crystal structures¹⁵ and biochemical data¹⁶ indicate that universally conserved ribosomal RNA (rRNA) nucleotides monitor the interaction between the codon and the anticodon at the A site before translocation. Therefore, it is likely that the ribosome is capable of restricting translocation until the proper codon–anticodon interaction has taken place at the A site.

Inspection of the genetic code reveals that accurate decoding of mRNA requires the first base of the tRNA anticodon, the wobble base⁶ at position 34, to read the third codon base, the most degenerate of coding positions. *E. coli* tRNAs having a uridine at position 34 commonly have the modification 5-methylaminomethyluridine

Abbreviations used: ASL, anticodon stem-loop; mnm, methylaminomethyluridine.

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($\text{mnm}^5\text{U}_{34}$), or its 2-thio (s^2) derivative, and another modification at position 37 for decoding twofold degenerate codon boxes (Figure 1(a)). For example, tRNA^{Lys} requires these modifications at positions 34 and 37 for effective binding to cognate AAA and wobble AAG codons at the A and P sites and for discriminating between these codons and those of tRNA^{Asn} , AAU/C.¹¹ These modifications increase stability¹⁷ and impart the proper anticodon stem-loop (ASL) structure for correct codon binding.^{18,19} Here, we show that modifications at

positions 34 and 37 affect translocation of tRNA from the ribosomal A site to the P site.

Modifications of tRNA^{Lys} restore ribosomal translocation of cognate and wobble codons

Previous studies have demonstrated that an anticodon stem-loop (ASL) analog of tRNA^{Phe} will bind and translocate from the ribosomal A site to the P site.^{16,20,21} Therefore, to precisely study the role of modification in translocation, we synthesized ASL analogs of *E. coli* tRNA^{Lys} containing one or more base modifications. An unmodified anticodon stem and loop domain of tRNA^{Lys} (ASL^{Lys}) does not bind cognate AAA codon well (<10%), therefore, translocation from the ribosomal A site was not observed in the translocation assay^{16,22} (Figure 2(a) and (g)). The single, conserved mnm^5 modification at uridine 34 ($\text{mnm}^5\text{U}_{34}$) enhanced cognate codon binding by twofold, but failed to restore translocation from the A site to P site (Figure 2(c)). Incorporation of another conserved modification, the threonylcarbamoyl moiety at N6 of adenine 37 (t^6A_{37}), also restored cognate codon binding by threefold, but again did not restore ribosomal translocation (Figure 2(d)). The presence of both $\text{mnm}^5\text{U}_{34}$ and t^6A_{37} modifications increased binding by four- to fivefold, and substantially re-established ribosomal translocation of ASL^{Lys} (25-fold enhancement) (Figure 2(e) and (f)). The s^2U_{34} modification of ASL^{Lys} increased A site binding affinity to the same extent as the other single mnm^5 or t^6 modifications; however, the single s^2U_{34} modification restored ribosomal translocation significantly (Figure 2(b)). Translocation of the s^2U_{34} ASL^{Lys}, though approximately tenfold more effective than that of the unmodified ASL^{Lys}, was still considerably less than that of the doubly modified, $\text{mnm}^5\text{U}_{34}$ and t^6A_{37} ASL^{Lys} (Figure 2(e)). It is likely that both mnm^5 and s^2 modifications observed *in vivo* at position 34, as well as t^6A_{37} , are required to achieve the anticodon stem-loop structure and dynamics that result in accurate decoding of the wobble base-pair¹¹ and translocation.

The "U-turn" structural motif of the ASL is important for proper ribosomal binding^{23–25} and translocation.¹⁶ The U-turn of the tRNA^{Phe} anticodon is stabilized by a hydrogen bond between the 2'-OH of the invariant uridine 33 and the N7 atom of purine 35. However, many tRNAs with a position 35 pyrimidine, such as tRNA^{Lys} , are unable to form this important hydrogen bond. Crystal²⁶ and solution structures of ASL^{Lys}, with single¹⁸ (t^6A_{37}) and double modifications¹⁹ ($\text{mnm}^5\text{s}^2\text{U}_{34}$ and t^6A_{37}), reveal that with increasing modification, the anticodon stem-loop approaches the U-turn conformation as in the crystal structure of yeast tRNA^{Phe} . Solution structures of ASL^{Lys} showed that each modification plays a distinct structural role. The t^6A_{37} modification prevents formation of a $\text{U}_{33}:\text{A}_{37}$ base-pair, yet induces order to the loop structure by enhancing the appropriate 3'

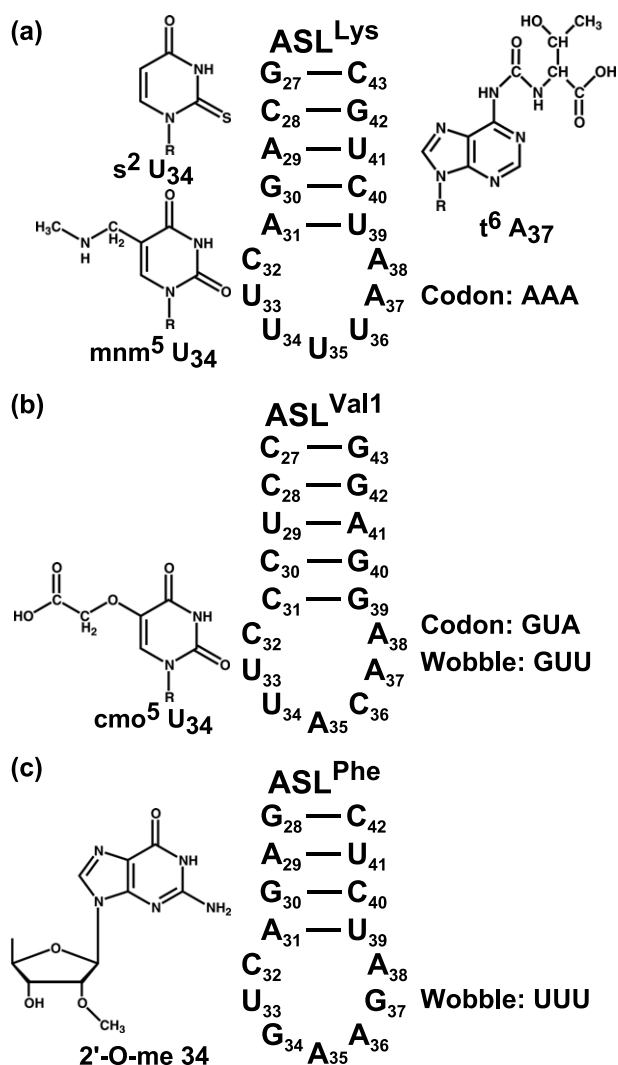


Figure 1. Secondary structure of the anticodon stem-loop (ASL) of *E. coli* tRNA^{Lys} , *E. coli* $\text{tRNA}^{\text{Val1}}$, and yeast tRNA^{Phe} showing several nucleotide modifications observed in the corresponding wild-type tRNAs. (a) Secondary structure of ASL^{Lys}, and the 2-thiouridine (s^2), or methylaminomethyluridine (mnm^5) modification at position 34 as well as the N⁶-threonylcarbamoyladenosine (t^6) modification at position 37. (b) Secondary structure of ASL^{Val1}, and the uridine 5-oxyacetic acid (cmo^5) modification at position 34. (c) Secondary structure of ASL^{Phe}, and the 2'-O-methyl modification at positions 32 and 34. The corresponding mRNA codons are indicated on the right.

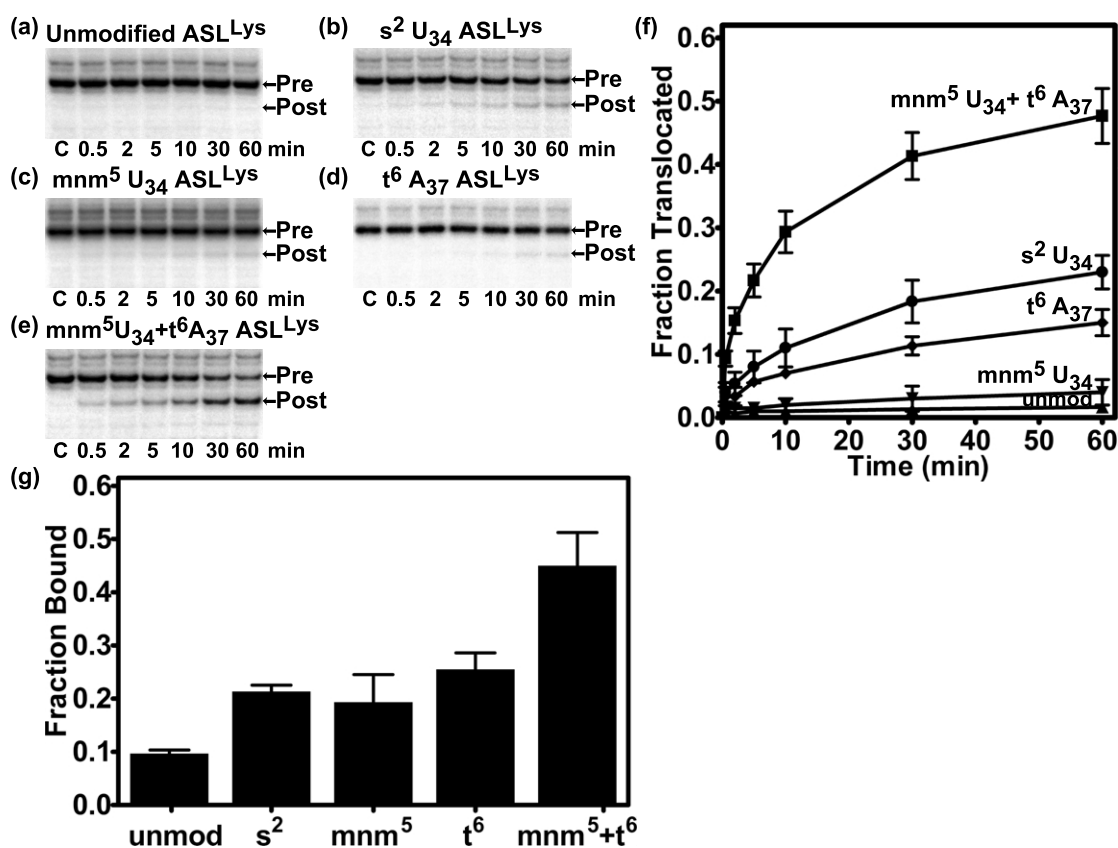


Figure 2. Ribosomal A site binding and time course of translocation of ASL^{Lys}. (a)–(e) Toeprinting assay to monitor translocation of unmodified and modified ASL^{Lys} as indicated. Arrows indicate toeprints before (Pre) and after (Post) translocation. Toeprinting assays^{20,40} were used to determine the extent of translocation, under previously established conditions.¹⁶ Briefly, a radiolabeled DNA primer ([³²P]AL2) was annealed to the mRNA downstream from the initiation site and used to form the ribosomal pre-translocation complex at 37 °C (80 mM potassium cacodylate (pH 7.2), 20 mM MgCl₂, 150 mM NH₄Cl, 1 mM β-mercaptoethanol, 10 Mm 70 S, 20 pM gene 32 mRNA, 20 pM tRNA^{Met}, and 150 pM ASL in 25 μl final volume). Translocation was triggered by incubating with fivefold excess EF-G at 25 °C. Reverse transcriptase (RT) was added to separate aliquots at time points during translocation to extend the AL2 primer upstream until RT is blocked by the ribosome. Translocation would result in an extension product that is three nucleotides (one codon) shorter. The pre and post- extension products are separated by denaturing polyacrylamide gel electrophoresis, visualized by autoradiography, and quantified by phosphorimaging. (f) Extent of translocation of unmodified and modified ASL^{Lys}. Translocation of the mnm⁵U₃₄ + t⁶A₃₇ ASL^{Lys} (squares); s²U₃₄ ASL^{Lys} (circles); t⁶A₃₇ ASL^{Lys} (diamonds); mnm⁵U₃₄ ASL^{Lys} (inverted triangles); unmodified ASL^{Lys} (triangles) with cognate AAA codon. The results are the average of at least three independent experiments. (g) A site binding of unmodified and modified ASL^{Lys}. Fraction of 70 S ribosomes having the A site bound unmodified (unmod) or modified ASL^{Lys} was evaluated using the same conditions as those used in the translocation assay. Ribosomal pre-translocation complexes having radiolabeled ASL bound to the A site were filtered through nitrocellulose and quantified using previously established methods.¹⁶

stack.¹⁸ The mnm⁵ modification enhances conformational rigidity of uridine-5'-phosphate²⁷ and in combination with s², as seen in naturally occurring mnm⁵s²U₃₄, would restrain the nucleotide to the *anti*, C3'-*endo* conformation.²⁸ The restriction of uridine 34 to the *anti* conformation and the ribose to the C3'-*endo* conformation,²⁹ are both thought to be critical for ribosome-mediated discrimination between the wobble U:G base-pair and the unorthodox U:U pair.²⁸ Thus, the anticodon solution structure and subsequent conformational rearrangements that are known to occur upon the A site binding of yeast tRNA^{Phe} may be possible only with the fully modified ASL^{Lys} and it is likely that these rearrangements are crucial for translocation.^{30–32}

Modifications of tRNA^{Val} are required for ribosomal translocation of wobble, but not cognate codons

In contrast to *E. coli* tRNA^{Lys}, the ability of *E. coli* tRNA^{Val11} to achieve the canonical U-turn may explain why an unmodified ASL^{Val11} (as with other unmodified ASLs having a purine at position 35) will bind its cognate codon (GUA) in the A site,¹¹ and translocate (Figure 3(a) and (g)). Efficient decoding of the fourfold degenerate codon for valine requires that a tRNA^{Val} with a wobble position uridine be capable of U:U, as well as U:A and U:G base-pairings. However, the results show that translocation of unmodified ASL^{Val11} on its wobble GUU codon is severely diminished

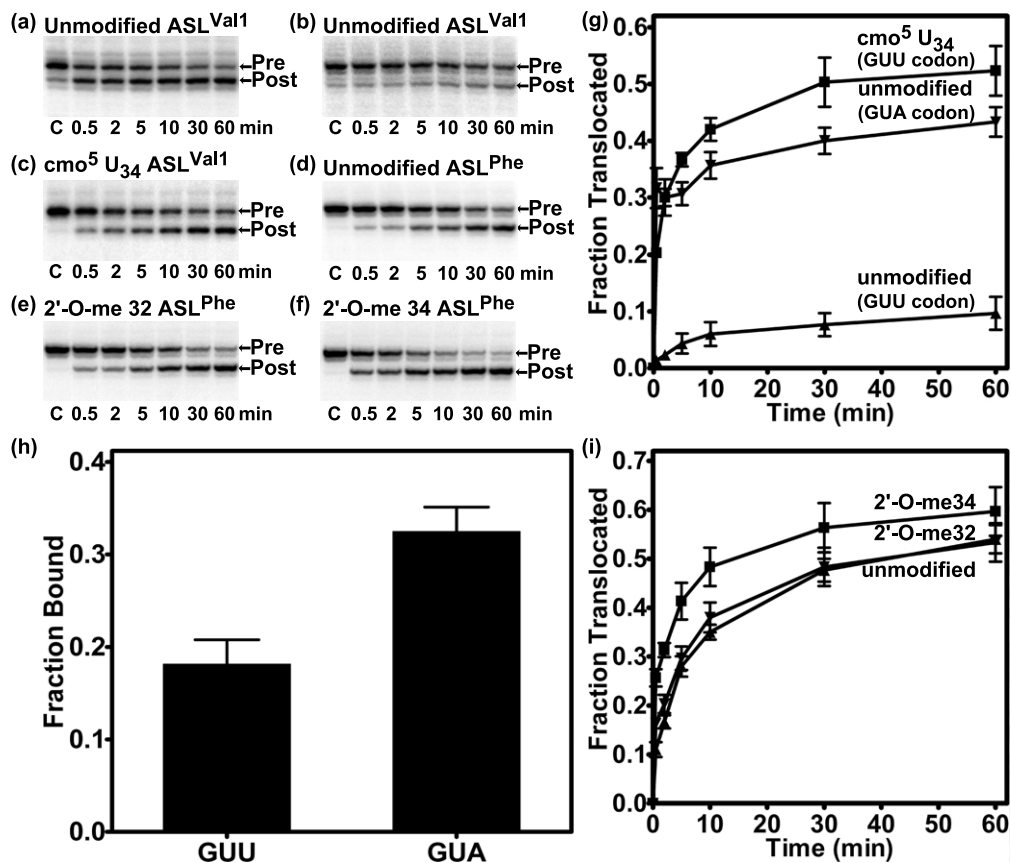


Figure 3. Ribosomal A site binding of ASL^{Val1}, and time course of translocation of ASL^{Val1} and ASL^{Phe}. Toeprinting assay to monitor translocation of (a) unmodified ASL^{Val1} with cognate GUA codon; (b) unmodified ASL^{Val1} with wobble GUU codon; (c) cmo⁵U₃₄ ASL^{Val1} with wobble GUU codon; (d) unmodified ASL^{Phe}; (e) 2'-O-me 32 ASL^{Phe}; (f) 2'-O-me 34 ASL^{Phe}. (g) Extent of translocation of unmodified and modified ASL^{Val1}. Translocation of cmo⁵U₃₄ ASL^{Val1} with GUU codon (squares); unmodified ASL^{Val1} with GUU codon (triangles); unmodified ASL^{Val1} with GUA codon (inverted triangles). (h) A-site binding of ASL^{Val1}. Fraction of 70 S ribosome with unmodified ASL^{Val1} bound to wobble (GUU) and cognate (GUA) codons in the A site. (i) Extent of translocation of unmodified and modified ASL^{Phe}. Translocation with UUU codon of 2'-O-me 34 ASL^{Phe} (squares); 2'-O-me 32 ASL^{Phe} (inverted triangles); unmodified ASL^{Phe} (triangles). Results are the average of at least three independent experiments.

(Figure 3(b)). This is noteworthy considering that binding to the wobble codon GUU in the A site is reduced by only 1.3-fold when compared to binding the cognate GUA codon (Figure 3(h)). In this case a potential U:U mismatch is introduced, and binding is allowed, but translocation does not occur. Remarkably, the single cmo⁵U₃₄ modification (Figure 1(b)) enabled ASL^{Val1} translocation from A to P site (Figure 3(c)). Translocation of the unmodified ASL^{Val1} occurred without the N⁶-methyladenosine modification normally observed in *E. coli* tRNA^{Val1} at position 37, which indicates that the position 37 modification is not essential for translocation on its cognate codon.

The cmo⁵U₃₄ modification alters the C2'-endo/C3'-endo isomerization of uridine 34 towards the C2'-endo conformation through interaction between the -OCH₂ moiety and the 5'-phosphate backbone (33P34)²⁸. The cmo⁵U₃₄-modified tRNA^{Val} decodes two wobble codons (GUU and GUG) in addition to the cognate codon. Therefore, organisms require only two tRNA^{Val} isoacceptors (UAC and GAC as seen in wild-type *E. coli* tRNAs) to decode a four-

fold degenerate codon box, which enhances translational efficiency.

Modifications of tRNA^{Phe} are not required for ribosomal translocation of wobble, or cognate codons

The tRNA^{Phe} anticodon is GAA, which binds the cognate codon UUC and the wobble codon UUU, but does not bind to the leucine codons UUA and UUG that share the same codon box. In the case of tRNA^{Phe}, no modifications are vital for decoding and translocation.^{3,20,33} Consistent with this result, the unmodified ASL^{Phe} translocates efficiently using a UUU wobble codon (Figure 3(d) and (i)). However, yeast tRNA^{Phe} has been shown to have a 2'-O-methyl modification of the ribose at position 32 as well as position 34 (Figure 1(c)). The 2'-O-methyl modification supports the C3'-endo ribose ring conformation,^{34,35} thereby facilitating the wobble interaction with U at the last position of the codon. A single 2'-O-methyl modification at either position 32 or 34 in ASL^{Phe} shows a modest

enhancement of translocation (Figure 3(e) and (f)). The modification at position 34 has the greater effect on translocation. This is consistent with a previous study, which showed that a 2'-O-methyl modification at position 34 of tRNA^{Ser} enhanced cell-free translation³⁶. Potentially, a tRNA with a purine nucleotide having a C3'-endo conformation at anticodon position 34 and a purine at position 35, will have a U-turn conformation that is optimal for ribosomal decoding and translocation. Components necessary for folding and stabilization that are available in full length tRNAs are also likely to play a role in the stability of the ASL. Thermal denaturation studies have demonstrated that the stem of the ASL is stable,³⁷ therefore it is likely that these modifications are necessary for local stabilization in the anticodon loop.

Genomes do not contain all of the genes for tRNAs that are theoretically necessary for Watson-Crick base-pairing of anticodons with the codons for the 20 common amino acids. Therefore, a limited repertoire of tRNAs has evolved to decode a maximal number of codons accurately. Some tRNA anticodon domain modifications may have evolved to facilitate the decoding of fourfold degenerate codons by only two tRNAs (tRNA^{Val}, for example). In addition, to decode genomic information into the protein amino acid sequence accurately, modifications may have evolved for tRNAs to distinguish between purine and pyrimidine nucleotides for twofold degenerate codons, such as that for lysine and asparagine, and phenylalanine and leucine. Modifications at the tRNA wobble position 34 and position 37 appear to have established the selective decoding properties of some tRNAs and the enhanced wobble of others by contributing unique structure and conformational dynamics to the anticodons.^{35,38} Furthermore, our study shows that these modifications are also important for translocation, which is consistent with the idea that decoding and translocation may be functionally linked.¹⁶ Considering that as much as 1% of the bacterial genome is devoted to tRNA modification,²³ it is easy to infer that the enhancement of translational efficiency³⁹ is critical to cell survival.

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