The role of modifications in codon discrimination by tRNA(Lys) UUU

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The role of modifications in codon discrimination by $tRNA^{Lys}_{UUU}$

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The natural modification of specific nucleosides in many tRNAs is essential during decoding of mRNA by the ribosome. For example, $tRNA^{Lys}_{UUU}$ requires the modification *N*6-threonylcarbamoyladenosine at position 37 (t^6A37), adjacent and 3′ to the anticodon, to bind AAA in the A site of the ribosomal 30S subunit. Moreover, it can only bind both AAA and AAG lysine codons when doubly modified with t^6A37 and either 5-methylaminomethyluridine or 2-thiouridine at the wobble position (mnm^5U34 or s^2U34). Here we report crystal structures of modified tRNA anticodon stem-loops bound to the 30S ribosomal subunit with lysine codons in the A site. These structures allow the rationalization of how modifications in the anticodon loop enable decoding of both lysine codons AAA and AAG.

tRNA, esterified with its cognate amino acid in ternary complex with elongation factor Tu (EF-Tu) and GTP, enters the ribosome's aminoacyl-tRNA site (A site) and interacts with the codon on mRNA. However, unless their anticodon loops are post-transcriptionally modified, many tRNAs cannot decode their cognate codons.

The decoding center of the 30S ribosomal subunit discriminates between cognate and noncognate codon-anticodon pairing by specific recognition of Watson-Crick base-pairing geometry through interactions with the minor groove of the codon-anticodon duplex[1–3]. Correct geometry is primarily recognized through conformational changes in three universally conserved and essential 16S rRNA bases, A1493, A1492 and G530, such that all three bases make extensive contacts with the first two base pairs via the minor groove of a cognate codon-anticodon duplex. This recognition of the correct base-pairing geometry in the codon-anticodon helix leads to a closure of the 30S subunit around the cognate tRNA, which is postulated to trigger subsequent steps in translation[1,3].

The decoding center is relatively insensitive to the geometry of the wobble base pair: the position of the codon base is restricted, but the anticodon base is only minimally restrained. The O2′ of the codon nucleotide is contacted directly by G530, and by C518 and Pro48 of ribosomal protein S12 via a metal atom[3], fixing the position of the codon base. Concurrently, C1054 interacts with the anticodon nucleotide, placing some restrictions on the orientation and width of the base pair. Nevertheless, there is a distinct lack of direct discrimination of minor groove geometry, allowing certain non-Watson-Crick base-pairing in the wobble position, as observed in the degeneracy of the genetic code[4], with no impact on the recognition of the codon-anticodon duplex[3]. Unlike the close reading of base-pairing geometry at the first two positions, it has been shown that at the wobble position, for anticodon stem-loop $(ASL)^{Phe}_{AAG}$, the ribosome does not add to the discrimination between a C3-G34 and U3•G34 base pair over that present in solution[3]. However, base-pairing at the wobble position is not completely free. The width of the base pair is partially constrained because of packing interactions with C1054, and although not directly monitored by the decoding center, distortions can also affect the thermodynamic stability of the ASL itself. The consequences of these constraints are observable: a U3•G34 wobble base pair is allowed for the unmodified ASL^{Phe}_{AAG} (ref. 3), but a G3•U34 wobble base pair is disallowed for the unmodified ASL^{Lys}_{UUU} (ref. 5).

Modifications of tRNA bases in the anticodon loop are widespread, with only 7 of the 61 sense codons in *Escherichia coli* being read exclusively by a tRNA without modifications at position 34 or 37 (refs. 6,7). Modifications have been shown to affect the speed[8] and accuracy[9] of translation, and are required for proper reading frame maintenance[10] and for translocation from the A to P site[11]. Modifications are so important to translation that organisms devote more genetic information to the modification of tRNA than to tRNA itself[12]. However, the effects of modifications depend on the particular tRNA: some *in vitro* transcripts of tRNA (lacking any modifications) seem to perform on par with the natural tRNAs, whereas others require modification to function at all[13]. This variable requirement for modification indicates that the repertoire of ASL structures that can be generated by the four standard RNA bases (A, G, C and U) is not sufficiently large to properly decode the genome.

All lysine tRNAs have been found with the modification *N*6-threonylcarbamoyladenosine (t^6A) (**Fig. 1**) or the 2-methylthio derivative (ms^2t^6A) at position 37, 3′ to the anticodon[7]. This modification is required, as it restores binding of an otherwise unmodified ASL^{Lys}_{UUU} to a ribosome with the lysine AAA codon in the A site[5]. However, t^6A37 by itself cannot restore binding of ASL^{Lys}_{UUU} to the

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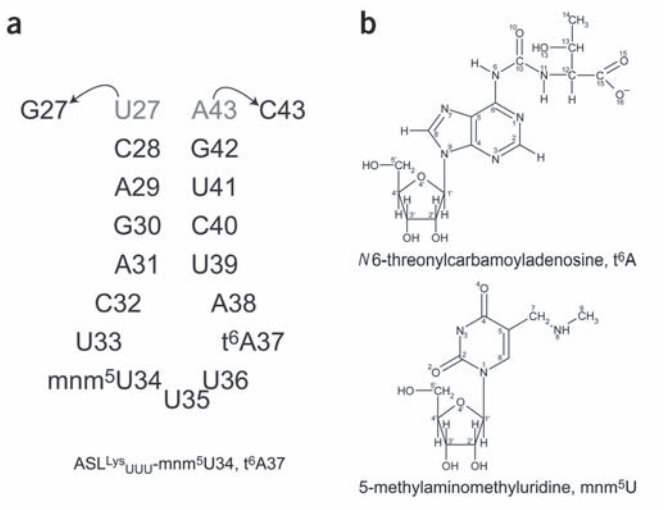


Figure 1 ASL sequence and modified nucleoside structures. (**a**) Nucleotide sequence and secondary structure of the ASL^{Lys} constructs. The U27-A43 base pair in the natural sequence has been replaced by a G27-C43 base pair to increase the stability of the stem. The ASLs synthesized contained either t^6A37 or both t^6A37 and mnm^5U34. (**b**) The structures of the modified nucleosides contained by the ASLs. The text refers to the ureido (N6, C10, O10 and N11) and threonyl (the remainder of the modification) groups of the t^6A.

lysine AAG codon[5] or A- to P-site translocation[11,14]. When present in combination with the modification 5-methylaminomethyluridine at wobble position 34 (mnm^5U34; **Fig. 1**), the now doubly modified ASL^{Lys}_{UUU} binds both lysine codons[9], and can be translocated[11,14]. Here, we present two crystal structures of the 30S subunit (at resolutions of 3.0 and 3.25 Å): the first in complex with a singly modified ASL^{Lys}_{UUU}-t^6A37 and an AAA codon in the A site, and the second in complex with the doubly modified ASL^{Lys}_{UUU}-mnm^5U34, t^6A37 and an AAG codon. This work addresses key questions about the roles of modified tRNA nucleotides in decoding: how does the t^6A37 stabilize codon binding from its position 3′ to the anticodon? How does the mnm^5U34 enable binding of the AAG codon, and why does it not facilitate decoding of the AAU codon?

RESULTS

Structure determination

Crystal structures of the modification-dependent ASL–mRNA complexes were determined after soaking native *Thermus thermophilus* 30S crystals with solutions containing either ASL^{Lys}_{UUU}-t^6A37 and mRNA 5′-AAAAAA-3′ or ASL^{Lys}_{UUU}-mnm^5U34, t^6A37 and mRNA 5′-AAGAAA-3′ (**Fig. 1** and **Table 1**). The antibiotic paromomycin was included because in the context of the crystal lattice it facilitates the conformational change to the closed form of the 30S subunit induced by cognate tRNAs[3], resulting in both improvement in diffraction resolution and better ordered density for the ASL. However, previous studies have shown that although the presence of paromomycin facilitates the transition to the closed form, it does not change the resulting structure for cognate ASLs[2].

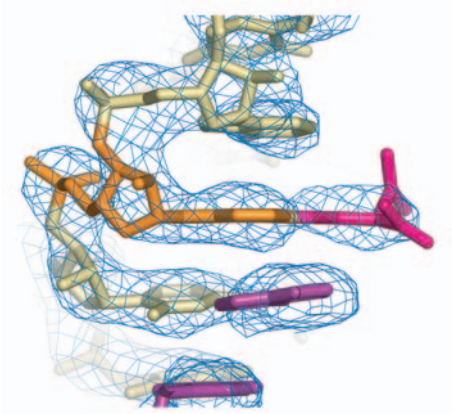
There are no substantial differences in the overall structural features of cognate codon-anticodon recognition by the decoding center between the structures reported here and those reported previously[2,3]. Furthermore, the structures of the 30S subunit are identical within experimental error (data not shown), and alignments of the mRNA and message based on the structurally invariant phosphate atoms of the 16S rRNA are nearly indistinguishable from those based on the mRNA and ASL themselves.

Figure 2 $2F_o - F_c$ density for the t^6A37. The t^6A37 residue is shown in the context of the ASL (ASL, cream; t^6A base, orange; t^6A modification, pink) with A38 directly above t^6A37 and U36 below and to the back. Bases in the mRNA are below the t^6A37 in the foreground colored purple: A1 is above A2. $2F_o - F_c$ density is from the t^6A37-AAA structure, contoured at 1.6 σ.

Structure of t^6A37 and its role in codon-anticodon stability

The t^6A37 modification has two distinct roles: it forms a planar structure that acts as a third heterocycle to stack with nearby bases, and it also makes van der Waals contacts that affect the stacking of the base. The conformation of the t^6A modification (**Fig. 2**) is nearly identical to that reported previously in the crystal structure of the nucleoside alone[15]. The ureido group (**Fig. 1b**) forms a markedly planar structure that is coplanar with the adenine ring, stabilized by a hydrogen bond from N11 of the modification to N1 of the adenine base (**Fig. 3c**). In the crystal structure of the nucleoside alone, substantial delocalization of charge has been observed in the ureido group[15]. The N1-N11 hydrogen bond and delocalization of charge cause the ureido group to act as a third ring in the t^6A base, thus expanding the area of t^6A37 available to stack with adjacent bases. Whereas the ureido group participates in base stacking owing to its planarity, the threonyl moiety seems free to rotate about the N11-C12 bond, as judged by the differences in temperature factors for the base and the ureido and threonyl moieties (67.7, 75.7 and 121.3 $Å^2$, respectively). This rotation of the bulky threonyl moiety prohibits it from being incorporated into the helical stack, restricting the position of t^6A37 (**Figs. 2** and **3**). As a result of the extended ring structure of the ureido group and the restrictions imposed by bulkiness of the threonyl moiety, t^6A37 does not stack with U36 of the anticodon, but instead stacks with A1 of the codon.

The modified t^6A37 base bridges between the 3′ stack of tRNA and the codon-anticodon helix by stacking with A38 of the tRNA and forming a cross-strand stack with the first base of the codon (**Fig. 3a**,**c**). As has been observed for the ASL in solution[16,17], t^6A37 stacks with A38, using the modification in the stacking interactions and forming the 3′ stack (**Fig. 3b**). In the current structures, the modification is also used in stacking to the 5′ side of t^6A37, but instead of forming the expected intrastrand stack with U36, t^6A37 forms an interstrand stack with A1, the base in the first position of the codon (**Fig. 3c**). This stacking arrangement helps to explain the observed increase in the stability of codon-anticodon interactions for the modified ASL versus that of the unmodified case[5,18,19]. Cross-strand stacks usually compensate for the loss of stacking owing to a sheared base or non-Watson-Crick base-pairing[20], but in the case of ASL^{Lys}_{UUU} the t^6A37 is involved in a cross-



strand stack to compensate for the low enthalpy of binding of the (purely A-U) codon-anticodon duplex[21] and the poor stacking properties of the anticodon itself.

Modified bases at position 37 of the tRNA are frequently used to compensate for the low enthalpy of binding of A-U base pairs in the codon-anticodon duplex, but the codon-anticodon duplex involving $tRNA^{Lys}_{UUU}$ is particularly unstable for three reasons: in the same context, A-U base pairs are weaker than G-C pairs owing to less hydrogen bonding[22], the interactions of the 16S rRNA bases with the minor groove of the codon-anticodon base pairs are also weaker for A-U base pairs[23], and stacking within the anticodon loop of $tRNA^{Lys}_{UUU}$ is poor[24,25]. The combined effects of these three factors lead to the inability of unmodified $tRNA^{Lys}_{UUU}$ to bind to its cognate codons. The t^6A37 modification enhances the interaction of the $tRNA^{Lys}_{UUU}$ anticodon with its cognate codons in two ways: by preordering the anticodon loop, thus reducing the entropic cost of tRNA binding, and by bridging base stacking between the codon and the 3′ stack of the tRNA. To preorder the ASL, the modification to t^6A37 prevents the formation of a U33-A37 base pair that would make the required conformation of the anticodon loop unfavorable[16] as well as promoting additional base stacking, for example between t^6A37 and A38. The reduction in entropic cost owing to the modification has been estimated to be 17 cal mol^{-1} K^{-1} (ref. 5). Stacking by bases at position 37 has been investigated for yeast $tRNA^{Phe}$ and showed that stacking interactions of the tricyclic base wybutine make a large energetic contribution to the free energy of the codon-anticodon interaction on the ribosome ($\Delta\Delta G^\circ_{(37^\circ C)} = -1.8$ kcal mol^{-1} versus uracil[19]). Although this is a good estimate of the contribution of t^6A37 to the codon-anticodon interaction, t^6A37 may make an even stronger contribution when U34 is modified owing to extensive preorganization of the anticodon loop by mnm^5U34 (ref. 5,26).

Structure of the mnm^5U34•G wobble base pair

The structure of the doubly modified ASL bound to the AAG codon (**Fig. 4a**) in conjunction with that of the singly modified ASL with an AAA codon allows a detailed comparison of two different codon-anticodon pairings in the third (wobble) position: an A3-U34 Watson-Crick base pair (for AAA-ASL^{Lys}_{UUU}-t^6A37 codon-anticodon) and a G3•mnm^5U34 wobble base pair (for AAG-ASL^{Lys}_{UUU}-mnm^5U34, t^6A37). Although the modification itself is not visible in the density, presumably because it is disordered (temperature factors for the base and modification are 95.6 and 107.8 $Å^3$, respectively), its presence has some readily observable consequences. Superposition of the two structures highlights the differences in the third base pair (**Fig. 4c**): whereas the A3-U34 pair is in the canonical Watson-Crick geometry[22], the G3•mnm^5U34 pair is in an unusual conformation that differs from the expected 'wobble' geometry, and is the result of local stacking interactions, hydrogen bonding and the influence of the mnm^5U34 modification.

Changes in the anticodon to accommodate the G3•mnm^5U34 versus the A3-U34 base pair are limited to the modified nucleotide mnm^5U34. Superposition of the two structures by invariant 16S rRNA phosphates clearly establishes that the position of the codon base in the G3•mnm^5U34 and A3-U34 base pairs does not change (**Fig. 4c**). This is expected, because the third base of the codon is held in place by contacts between the decoding center and the O2′ of the nucleotide. The fixed position of the codon base necessitates certain adjustments within the anticodon to form a base pair at the third position. These include a series of adjustments in the sugar-phosphate backbone, large enough changes to shift the position of the mnm^5U34 phosphate by 1.5 Å, but not to affect the C3′-*endo* puckering of the mnm^5U34 sugar (pseudorotation phase angles of 19.6° and 11.9° for U34 and mnm^5U34, respectively).

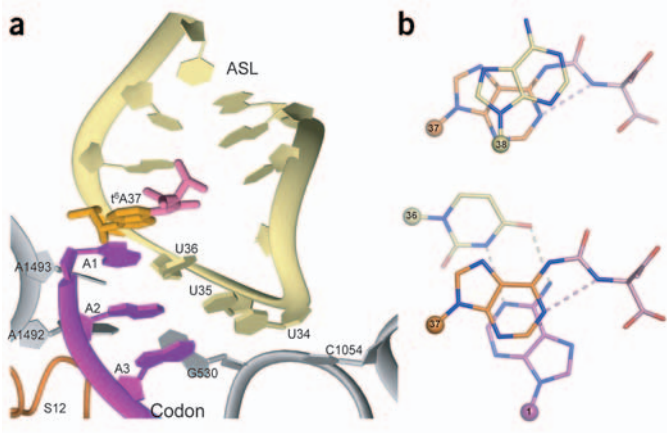


Figure 3 Stacking interactions of t^6A37. (**a**) Overview of the decoding site for ASL^{Lys}_{UUU}-t^6A37-AAA. 16S rRNA is grey, with interacting bases drawn. S12 is orange, codon is purple, and ASL is cream. The base of t^6A37 is orange, and the modification pink. (**b**) A38 and t^6A37 stack strongly, with the t^6A modification taking part in the interactions. This stack is drawn with A38 in the foreground. (**c**) The t^6A37 base forms a cross-strand stack with the first base of the message. The stack is drawn such that t^6A37 is 'above' the U36-A1 base pair. The bases in the ASL are cream and numbered 36 and 38, with the t^6A37 base orange and the modification pink; the mRNA base is purple and labeled 1. C1′ atoms are spheres bearing the labels, and hydrogen bonds are dashes: t^6A37 N1-N11 (pink), 2.9 Å; A1-U36 (cream) O4-N6, 2.8 Å; N3-N1, 2.8 Å.

The G3•mnm^5U34 base pair has a geometry that corresponds neither to the common G•U wobble geometry[4,27] (**Fig. 4d**) nor to the alternatives recently proposed for G-modified•U pairs[28] (although the base pair is tRNA U•mRNA G, the more conventional G•U wobble description has been used). In the most common G•U wobble pair, the U shifts toward the major groove of the codon-anticodon duplex, thus forming two complete hydrogen bonds with G of the codon. In the case of G3•mnm^5U34, however, the mnm^5U34 does not fully make this shift (~1.1-Å difference), rotates slightly about the C1′-N1 bond, and forms a bifurcated hydrogen bond between O2 of mnm^5U34 and N1 and N2 of G3 (ref. 29). One three-center hydrogen bond is not as strong as two two-center hydrogen bonds[30], and to compensate for this the position of the mnm^5U34 O2 carbonyl is positioned directly over the ring of U35, thus maximizing its stacking interactions[22]. In contrast, if it adopted the usual G•U wobble geometry, mnm^5U34 would be destabilized by the loss of base stacking. U•G base pairs at the end of helices are very unstable owing to poor stacking of U with the base 3′ to it[31]. Additionally, local stacking interactions have been observed to strongly influence G•U base pairing in tandem G•U mismatches[32], so an unusual stacking arrangement of a 5′-U•U-3′ base step involved in a G•U base pair is not unexpected. In fact, a structure similar to the G3•mnm^5U34 geometry has been determined by NMR of a tandem G•U mismatch RNA, complete with bifurcated hydrogen bonding (**Fig. 4e**).

Using the current structure it is possible to explain the effects of s^2U (2-thio-), the other modification to U34 present in fully modified $tRNA^{Lys}_{UUU}$. Based on the position of the O2 in U34 and mnm^5U34, an O2-to-S2 change would have two primary effects: (i) the bifurcated hydrogen bonding to N1 and N2 of G3 would become weaker, but have no effect in hydrogen bonding to A3, and (ii) the base stacking with U35 would become stronger for binding to both A and G bases in the codon. Thus the structure provides a rationale for observations that the s^2U modification increases the translation rate of A-ending codons and has a neutral impact on the translation rate of G-ending codons[8].

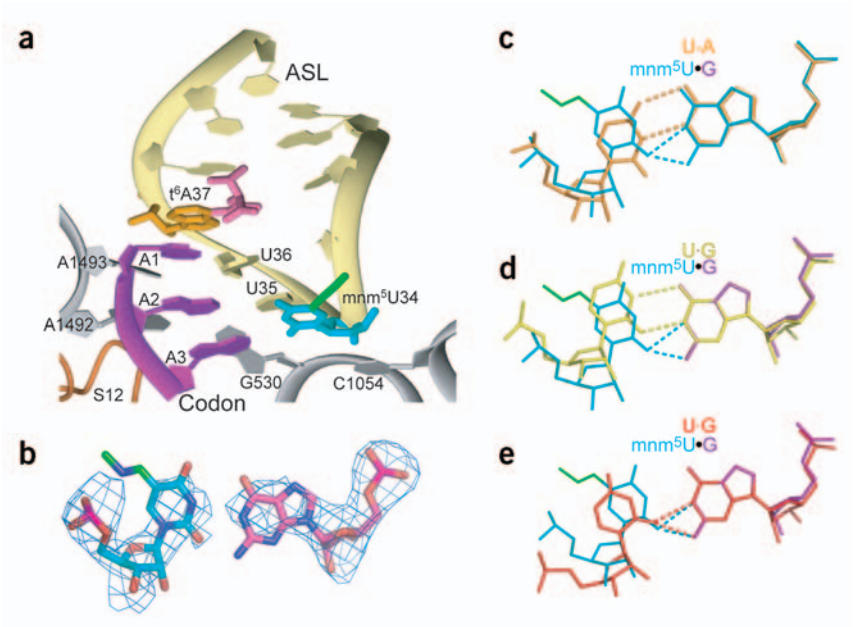


Figure 4 mnm^{5}U•G, U-A and U•G base pairs. (a) Overview of the decoding site for ASL$^{Lys}_{UUU}$-mnm^{5}U34, t^{6}A37-AAG. 16S rRNA is gray, with interacting bases drawn, S12 is orange, codon is purple, and ASL is cream. The base of t^{6}A37 is orange, and the modification is pink; the base of mnm^{5}U34 is cyan, and the modification is green. (**b**) mnm^{5}U34•G3 base pair (carbons for mnm^{5}U34 base are cyan and for modification are green; carbons for G3 are purple) is shown with electron density ($2F_o - F_c$ contoured at 1.5 σ). (**c**) mnm^{5}U34•G3 (cyan and green) and U34-A3 (orange) are superimposed by alignment of nonvariant 16S rRNA phosphate atoms, with anticodon at left and codon at right. The U34-A3 base pair represents the classic Watson-Crick geometry. (**d**) mnm^{5}U34•G3 (cyan and green) and G36•U1 from the X-ray structure of the 30S ribosome bound to noncognate tRNAs[3] (yellow) are superimposed by alignment of the G nucleotide. The G36•U1 base pair is a classic G•U wobble[4], but is flipped to align with the mnm^{5}U34•G3 base pair. (**e**) mnm^{5}U34•G3 (cyan and green) and U5•G4 (magenta) from the NMR structure of the self-complementary oligonucleotide r(GGCGUGCC)$_2$ (ref. 32) are superimposed by alignment of the G nucleotide. Both U•G base pairs use bifurcated hydrogen bonds (dashed lines).

Moreover, s^{2}U occurs only for anticodons with U in the second position[7]; U is the base with the poorest stacking potential, and it alone requires the addition of the s^{2}U on the wobble base to enhance the stacking interactions.

Although the electron density is clear for the phosphate, sugar and base of mnm^{5}U34, no consistent density is present for the modification, leading to the conclusion that it is disordered. Although disordered, the amine moiety of the modification refines to a position within hydrogen bonding distance of a 5′ phosphate oxygen (2.8 Å). However, if there were a strong hydrogen bond between these two atoms, some density would be expected, leading to the conclusion that the primary influence of the mnm^{5}U modification is not through direct hydrogen bonding, but through steric interactions or indirect hydrogen bonding. This is in agreement with NMR data[26] and explains how the mcm^{5}s^{2}U (5-methoxycarbonylmethyl-2-thiouridine) at position 34 of the human tRNA$^{Lys}_{UUU}$ influences codon recognition in the same way as mnm^{5}U even though it has a very different hydrogen bonding potential. The interactions of mnm^{5}U34, whether indirect hydrogen bonding or steric in nature, contribute to the preordering of the anticodon loop[17], and this is the primary mode of action of the mnm^{5}U34 modification.

The present work shows why the normal G•U wobble does not form and provides clues as to why U•U pairs are disfavored. The traditional G•U wobble does not form, owing at least in part to the establishment of 'alternate' hydrogen bonding and base stacking, and possibly in part to interactions of the mnm^{5}U modification. The mnm^{5}U modification is found only in tRNAs that read codons ending in A or G (not U or C) and only in two-fold degenerate codon boxes (arginine, glutamine, lysine and glutamate)[7], leading to the conclusion in the literature that the modification restricts the base-pairing possibilities of the modified mnm^{5}U34 to A and G[27]. Owing to recent structural data[1], it is now clear that there is no a priori physical constraint by the decoding center that would preclude a pyrimidine•pyrimidine base pair (such as U•U) at the wobble position, thus the restriction from U•U base pair formation comes from the tRNA. For U34 to base-pair with a U in the third position of the codon, a shift of the anticodon U toward the major groove similar to that of the normal G•U wobble (**Fig. 4d**) is required.

The present work shows clearly that the normal G•U wobble does not form, owing at least in part to the establishment of 'alternate' hydrogen bonding and base stacking, and possibly partly owing to interactions of the mnm^{5}U modification. This suggests that the reason the U•U base pair is disallowed is that the geometry of the U•U wobble is incompatible with the maintenance of base stacking between U34 and U35. Additionally, if U34 were to move to the position observed for the U•G base pair reported here, there would be no hydrogen bonding between the bases, making the base pair disallowed in this geometry as well. This provides a clear explanation for the lack of U•U base pairs for anticodons with pyrimidines in the second position of the anticodon, as well as a rationale for the prevalence of split codon boxes for such pyrimidine-rich anticodons. The current structures argue against the idea that the mnm^{5}U modification restricts the tRNAs in which it is present from binding to U- and C-ending codons, as it is evident no restriction is required, and no binding is observed in the absence of the modification to begin with[24].

Because work with the UUU codon and the cognate phenylalanine ASL show that the wobble position readily accommodates a U3•G34 without modifications and with the usual wobble geometry[2,3], the question arises why a G3•U34 pair requires both a modification and an unusual geometry. The reasons for this difference are the identity of the base that deforms from Watson-Crick geometry to wobble geometry, and its sequence context. The codon base in the wobble position is fixed by its interactions with the ribosome, so that the formation of wobble pairs requires movement from Watson-Crick geometry by the anticodon base. In the case of tRNA$^{Phe}_{AAG}$, G34 moves, but is able to maintain base stacking with A35. However for tRNA$^{Lys}_{UUU}$, U34 must move (if it were to take up wobble geometry), resulting in unstacking of U34 and U35. In the context of the UUU anticodon, this must be sufficiently unfavorable to prevent decoding unless a modification prevents the unstacking and the assumption of a more favorable, albeit unusual base-pairing geometry.

DISCUSSION

There are several distinct consequences of the modifications examined in this work. The t^{6}A37 and mnm^{5}U34 modifications act in concert to stabilize the ASL$^{Lys}_{UUU}$ through preordering the ASL into a structure

Table 1 Data collection and refinement statistics

	ASL^{Lys}-t^6A_{37}–AAA	ASL^{Lys}-mnm^5U_{34}, t^6A_{37}–AAG
Data collection		
Space group	$P4_12_12$	$P4_12_12$
Cell dimensions		
a, *b*, *c* (Å)	400.7, 400.7, 175.1	400.8, 400.8, 176.1
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	3.0 (3.11–3.0)	3.25(3.37–3.25)
R_{sym}	14.2 (72.2)	14.5 (34.2)
$I / \sigma I$	6.5 (2.1)	7.9 (1.8)
Completeness (%)	97.4 (91.3)	93.1 (91.3)
Redundancy	30.3	17.6
Refinement		
Resolution (Å)	99.0–3.0	99.0–3.25
No. reflections	263,204	199,050
R_{work} / R_{free}	22.19 / 23.60	23.06 / 28.43
No. atoms	52,089	52,071
RNA	32,701	32,683
Protein	19,237	19,237
Ion	109	109
B-factor	77.07	80.38
R.m.s deviations		
Bond lengths (Å)	0.0062	0.0069
Bond angles (°)	1.201	1.218

Values in parentheses are for the highest-resolution shell.

suitable for presentation to the codon in the decoding center. The t^6A37 influences the stability of the codon-anticodon duplex by forming a cross-strand stack with the first position of the codon. By facilitating the formation of an alternate G3•U34 base pair that does not require unstacking of U34, the mnm^5U34 modification enables favorable base-pairing with G at the wobble position.

The stabilization of the ASL^{Lys}_{UUU} by modifications is necessary to offset both the low enthalpy of binding provided by the three A-U base pairs, and the poor stacking in the UUU anticodon[24]. Decoding requires a domain closure of the 30S subunit on binding of cognate tRNA in the A site, the energy for which comes from interactions of the ribosome with the cognate codon–tRNA complex[3]. Modifications specifically increase the affinity of the tRNA for the codon in the context of the decoding center, thereby increasing the free energy available for domain closure of the 30S around the codon–tRNA complex. Thus, the modifications are necessary not only to facilitate binding of the tRNA to the codon, but also to provide energy for the domain closure of the 30S, thus allowing translation to proceed.

The mnm^5U34 modification stabilizes the codon-anticodon duplex, and is required for $tRNA^{Lys}_{UUU}$ to bind the AAG codon in addition to AAA. The mnm^5U34 modification enables base-pairing with G by contributing to the preordering of the anticodon loop[17] and stabilization of an alternative G3•U34 wobble pair through steric interactions or indirect hydrogen bonding. The mnm^5U34 modification has been suggested to enable wobble recognition of G[5] and restrict wobble recognition from U[6,27]. The present work suggests that the mnm^5U34 modification enables base-pairing with G, but does not restrict base-paring to U, as no such restriction is necessary. The formation of a U•U base pair would abrogate any stacking between U34 and a pyrimidine at position 35, incurring an unacceptable free energy penalty[21]. In the cases of U3•U34 base-pairing where position 35 of the anticodon is a purine, the situation is less clear. In *E. coli* the modification uridine 5-oxyacetic acid (cmo^5U34) is used to extend to U3•U34 wobble base-pairing in all but one case, a clear indicator that the normal U3•U34 wobble is not optimal, even for anticodons in which U34 has a purine at position 35 with which to stack. The modification 5-carboxymethylaminomethyl-2′-O-methyluridine ($cmnm^5Um$) is restrictive, preventing the U3•U34 wobbling of $tRNA^{Leu}_{AAU}$ to the UUU codon in *E. coli*, suggesting that the presence of modifications to position 5 of uridines can sterically restrict wobble and that restrictions to base-pairing at the wobble position are necessary in some cases. However, for pyrimidine-rich ASLs such as ASL^{Lys}_{UUU}, in particular when there is a pyrimidine at position 35 of the anticodon, there is clearly no such requirement[5].

The ribosome is a finely tuned machine in which interactions with cognate tRNA induce conformational changes required for decoding, whereas those with noncognate tRNA do not[3,33]. However, the amount of binding energy available for conformational changes can vary considerably even among cognate tRNA-codon pairs. Thus modifications of tRNA seem essential to facilitate binding and decoding in cases where the unmodified tRNA would not have sufficiently favorable interactions to induce the required domain closure in the 30S, or even to bind stably to the ribosome on the timescale required by the living cell. This is an extension of the structural and thermodynamic properties of tRNAs by base modification within the ASL. The current structures show how modifications in the anticodon stem-loop can contribute to the free energy of binding in subtle and yet distinct ways. We expect that other modifications important in decoding will be variations on this theme.

METHODS

Materials and crystallization. *Thermus thermophilus* 30S ribosomal subunits were purified, crystallized and cryoprotected as described (the final solution being 26% (v/v) MPD, 100 mM K-MES, pH 6.5, 200 mM KCl, 75 mM NH_4Cl, 15 mM $MgCl_2$)[34]. The anticodon stem and loop domains of $tRNA^{Lys}_{UUU}$ were designed with a G27-C43 base pair rather than the U27-A43 of the native tRNA. The protected modified nucleoside phosphoramidites were chemically synthesized. ASL^{Lys} with t^6A37 was chemically synthesized by the North Carolina State University's Nucleic Acid Facility with the standard ribonucleoside phosphoramidites (ChemGenes) and with little change in standard synthesis protocols[35]. ASL^{Lys} with t^6A37 and mnm^5U34 was synthesized by RNA-TEC. All of the ASLs were purified by HPLC using described procedures[36] and analyzed by denaturing, 7 M urea PAGE. Nucleoside composition of the modified ASLs was analyzed by HPLC of constituent nucleosides[36] and by mass spectrometry[37]. mRNA oligonucleotides were chemically synthesized and gel-purified (Dharmacon) with the sequences 5′-AAAAAA-3′ and 5′-AAGAAA-3′ (codons underlined). After cryoprotection, the 30S crystals were soaked in cryoprotection buffer containing 80 μM paromomycin, 300 μM ASL and 300 μM of the corresponding mRNA hexanucleotide for at least 48 h as described[2,3]. Crystals were flash-cooled in liquid nitrogen and stored for data collection.

Data collection and refinement. Data were collected at ESRF ID14-4, processed with DENZO and SCALEPACK[38], using Strategy[39] to aid in the efficient collection of data. The resolution difference between the two data sets, which were collected at different times, is more likely a consequence of differences in the X-ray beam than in the crystals. CNS 1.1 (ref. 40) was used for refinement; topologies and parameters of the modifications were taken directly or derived from HIC-Up[41]; O[42] was used for visualization and building; the CCP4 package[43] was used for assorted tasks; and figures were composed using PyMOL (http://www.pymol.org) and MolMol[44]. Alignment of the structures was done using Escet 0.6 (ref. 45), which identifies structurally invariant phosphate atoms in the 16S rRNA, and analysis of RNA bond angles was done using the 3DNA[46].

Coordinates. Atomic coordinates for ASL^{Lys}_{UUU}-t^6A37-AAA (accession code 1XMQ) and ASL^{Lys}_{UUU}-mnm^5U34, t^6A37-AAG (accession code 1XMO) have been deposited in the Protein Data Bank.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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