Modified Nucleoside Dependent Watson−Crick and Wobble Codon Binding by $tRNA^{Lys}_{UUU}$ Species†

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ABSTRACT: Nucleoside modifications are important to the structure of all tRNAs and are critical to the function of some tRNA species. The transcript of human $tRNA^{Lys3}_{UUU}$ with a UUU anticodon, and the corresponding anticodon stem and loop domain (ASL^{Lys3}_{UUU}), are unable to bind to poly-A programmed ribosomes. To determine if specific anticodon domain modified nucleosides of $tRNA^{Lys}$ species would restore ribosomal binding and also affect thermal stability, we chemically synthesized ASL^{Lys} heptadecamers and site-specifically incorporated the anticodon domain modified nucleosides pseudouridine (Ψ_{39}), 5-methylaminomethyluridine (mnm^5U_{34}) and *N*6-threonylcarbamoyl-adenosine (t^6A_{37}). Incorporation of t^6A_{37} and mnm^5U_{34} contributed structure to the anticodon loop, apparent by increases in ΔS, and significantly enhanced the ability of ASL^{Lys3}_{UUU} to bind poly-A programmed ribosomes. Neither ASL^{Lys3}_{UUU}-t^6A_{37} nor ASL^{Lys3}_{UUU}-mnm^5U_{34} bound AAG programmed ribosomes. Only the presence of both t^6A_{37} and mnm^5U_{34} enabled ASL^{Lys3}_{UUU} to bind AAG programmed ribosomes, as well as increased its affinity for poly-A programmed ribosomes to the level of native *Escherichia coli* $tRNA^{Lys}$. The completely unmodified anticodon stem and loop of human $tRNA^{Lys1,2}_{CUU}$ with a wobble position-34 C bound AAG, but did not wobble to AAA, even when the ASL was modified with t^6A_{37}. The data suggest that $tRNA^{Lys}_{UUU}$ species require anticodon domain modifications in the loop to impart an ordered structure to the anticodon for ribosomal binding to AAA and require a combination of modified nucleosides to bind AAG.

Transfer RNAs contain at least 75 different posttranscriptional modifications, affecting chemistry, structure and function (*1*). The anticodon stem and loop domains of tRNAs are highly modified with the greatest variety of modifications occurring at wobble position 34 of the anticodon and at position 37, 3′-adjacent to the anticodon (*2*). Anticodons and the loops in which they reside have significant sequence differences, yet they are all bound by programmed ribosomes. Therefore, anticodon domain structures are similar in conformation or are sufficiently dynamic to conform to the requirements of the ribosomal binding sites while maintaining accurate, in-frame, reading of codons. We postulated that the ribosomal binding of some tRNA species would require the physicochemical contributions of modifications to achieve the proper anticodon loop architecture. Though modification of position 37, 3′-adjacent to the anticodon, in the yeast $tRNA^{Phe}$ structure was important for maintaining an open anticodon loop conformation (*3*), modifications contribute only a slight improvement to this tRNA's aminoacylation and ribosome binding activities (*4*−*6*). In contrast, unmodified tRNAs for glutamic acid, glutamine, lysine, and proline and their respective heptadecamer anticodon stem and loop domains (ASLs)[1] are almost nonfunctional, exhibiting an inability to bind appropriately programmed ribosomes (*7*, *8*).

The anticodon stem and loop domain of $tRNA^{Lys}_{UUU}$ contains nucleoside modifications at positions 34, 37, and 39 (Figure 1) which are common to many tRNA species from procaryotes, eucaryotes, and archaea (*2*). Uridine modifications at position 34 are usually derivatives of 5-methyluridine (xm^5U_{34}), with or without an additional 2-thiolation, s^2U (*2*, *9*). Uridine 5-position modifications have been implicated in aminoacyl-tRNA synthetase recognition and aminoacylation of $tRNA^{Glu}$ (*10*, *11*) and $tRNA^{Lys}$ (*12*) and appear to be crucial for $tRNA^{Lys}$, $tRNA^{Gln}$, and $tRNA^{Glu}$ to bind to appropriately programmed ribosomes (*8*). The uridine at position 34 in human $tRNA^{Lys3}$ is modified to the methoxycarbonyl-derivative of $xm^5s^2U_{34}$, $mcm^5s^2U_{34}$, whereas that in *Escherichia coli* $tRNA^{Lys}$ is the methylamino-derivative, $mnm^5s^2U_{34}$. The $xm^5s^2U_{34}$ type modifications are found in tRNAs reading A or G in the third position of the codon in mixed codon family boxes that code for two amino acids. In organisms that have only one tRNA encoded for this amino acid, $xm^5s^2U_{34}$ must enable the tRNA to wobble to G but not recognize C or U. Thus, correct reading of the third

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[1] Abbreviations: ASL, anticodon stem and loop domain; Ψ, pseudouridine; mcm^5s^2U, 5-methoxycarbonylmethyl-2-thiouridine; ms^2t^6A, 2-methylthio-*N*6-threonylcarbamoyladenosine; mnm^5U, 5-methylaminomethyluridine; t^6A, *N*6-threonylcarbamoyladenosine; xm^5U, variously modified 5-methyluridine.

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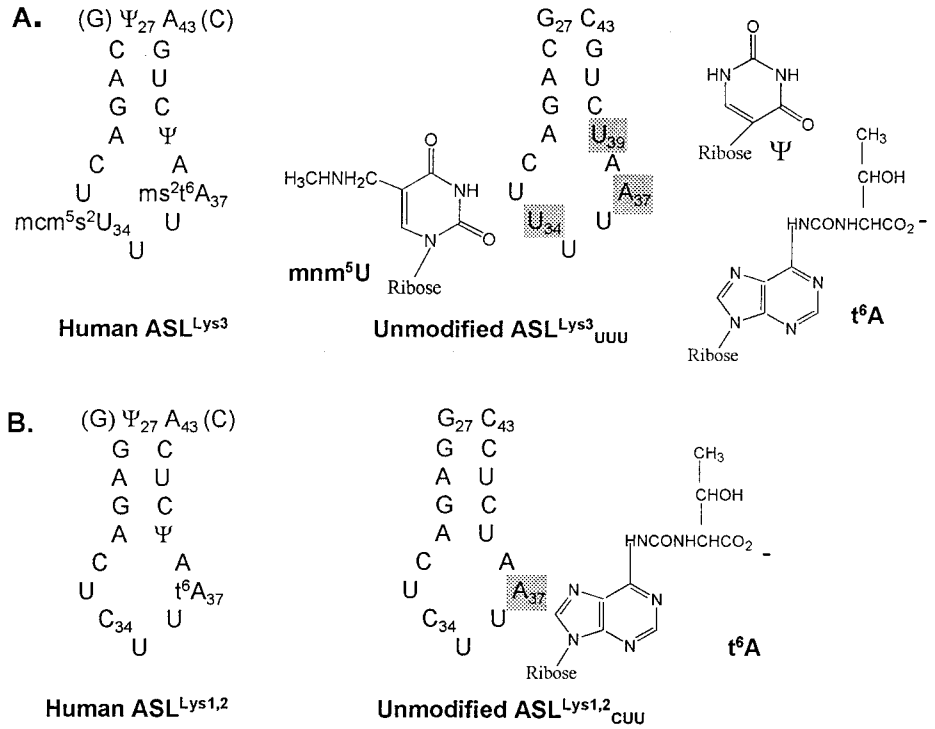

FIGURE 1: ASL^{Lys} constructs. (A) Nucleotide sequence and secondary structures of the anticodon stem and loop domain of human tRNA^{Lys3}. The naturally occurring sequence on the left includes the modifications, Ψ, pseudouridine; mcm^{5}s^{2}U, 5-methoxycarbonylmethyl-2-thiouridine; and ms^{2}t^{6}A, 2-methylthio-N6-threonylcarbamoyladenosine. The unmodified ASL$^{Lys3}_{UUU}$ sequence on the right was synthesized with and without the modified nucleosides Ψ_{39}, mnm^{5}U$_{34}$, and t^{6}A$_{37}$. (B) Nucleotide sequence and secondary structures of anticodon stem and loop of human tRNA$^{Lys1,2}_{CUU}$. The naturally occurring sequence is on the left and the corresponding, unmodified and chemically synthesized, sequence of ASL$^{Lys1,2}_{CUU}$ is on the right. The U$_{27}$-G$_{43}$ terminal base pairs were changed to G$_{27}$−C$_{43}$ for synthesis purposes. Structures of the modified nucleosides, shown to the side of the unmodified ASLs, are those which were site-specifically substituted at the positions identified by shading.

position of the codon by xm^{5}s^{2}U$_{34}$-modified tRNAs is crucial to the incorporation of the proper amino acid.

In all species of lysine tRNA in all organisms, a hypermodified adenosine is present at position 37 (Figure 1) as *N*6-threonylcarbamoyladenosine, t^{6}A$_{37}$, or a derivative of t^{6}A$_{37}$ (*2*). The nucleoside t^{6}A$_{37}$ is a chemically interesting modification involving a carbamoyl-linked threonine to adenosine's N6-amino group. Human tRNA^{Lys3}, the tRNA recruited by HIV-1 for priming reverse transcription, has position 37 further modified to the 2-methylthio-derivative, ms^{2}t^{6}A$_{37}$. However, the biological function of this hypermodified nucleoside is not clear (*9*).

Pseudouridine, Ψ, appears at positions 27 and 39 in the anticodon domain stem; the latter is a conserved modification for all tRNAs with an encoded U$_{39}$, 40% of all tRNAs (*13*). The tRNA^{Lys} position 39 modification is always a Ψ_{39} base paired to A$_{31}$ at the bottom of the anticodon stem (Figure 1) (*2*). Ψ_{39} increases the stability of the anticodon stem and loop domains of both tRNA^{Lys} and tRNA^{Phe} in comparison to their respective unmodified RNAs (*13*−*15*) and may contribute to extending the tRNA^{Lys} anticodon stem into the loop by promoting formation of an adjacent, pH dependent, C$_{32}$·A$^+_{38}$ base pair (*15*).

Previously, we reported that the naturally occurring 2-thiolation (s^{2}U) of U$_{34}$ significantly restored the poly-A programmed ribosomal binding ability of an otherwise unmodified and nonfunctional anticodon stem and loop of human tRNA^{Lys3} (ASL^{Lys3}), but did not affect the RNA's melting temperature (*8*). Upon determining that the single-atom modification of U$_{34}$ (thiolation) restored poly-A programmed ribosomal binding (*8*), we undertook an investigation of the possible structural and functional contributions of the other ASL^{Lys} modifications. We have assessed the abilities of three anticodon stem and loop modifications, mnm^{5}U$_{34}$, t^{6}A$_{37}$ and Ψ_{39}, to restore programmed ribosomal binding and affect the thermodynamics of the otherwise nonfunctional, unmodified human ASL$^{Lys3}_{UUU}$. The ASLs were produced by automated RNA chemical synthesis with site-selective incorporation of modified nucleosides, including the first report of oligoribonucleotide chemical synthesis with the introduction of a hypermodified nucleoside, t^{6}A. We report here that the modifications, mnm^{5}U$_{34}$ and t^{6}A$_{37}$, are critical to tRNA$^{Lys}_{UUU}$ species for effective binding to AAA-programmed ribosomes and are absolutely required for wobbling to AAG codons.

EXPERIMENTAL PROCEDURES

Materials: ASLs, tRNAs, mRNAs and 30S Ribosomal Subunits. Heptadecamer anticodon stem and loop domains, ASLs, were synthesized with base sequences corresponding to that of the human tRNA^{Lys} species, ASL$^{Lys1,2}_{CUU}$, and ASL$^{Lys3}_{UUU}$ (Figure 1). The nucleotide sequence of the anticodon stem and loop of human tRNA^{Lys1} and human tRNA^{Lys2} differ by only one base pair in the stem. ASL^{Lys} constructs were synthesized with a G$_{27}$·C$_{43}$ terminal base pair instead of the naturally occurring U$_{27}$-A$_{43}$ base pair (Figure 1) for better yield and increased thermostability necessary for structure determination by NMR (see accompanying paper). Because the methyl ester of 5-methoxycarbonylmethyluridine (mcm^{5}U$_{34}$) in human tRNA^{Lys3} is not stable during

solid-phase chemical synthesis (A.M. and E.S., personal communication), 5-methylaminomethyluridine (mnm^5U_{34}), a modification found in procaryotic tRNAs, was incorporated. ASLs and 30mer mRNAs were synthesized using ribonucleoside phosphoramidite chemistries and methods (*16*, *17*). ASL^{Lys3}_{UUU} containing the Ψ_{39} modified nucleoside was purchased (Dharmacon). RNAs were purified by ion-exchange high-performance liquid chromatography (*17*). Successful incorporation of the modified nucleosides was verified by quantitative nucleoside composition analysis (*18*). *E. coli* $tRNA^{Lys}$ and yeast $tRNA^{Phe}$ were purchased (Sigma Chemical Co.). Ribosomal 30S subunits were prepared from *E. coli* MRE600 and activated according to Ericson et al. (*19*).

Methods: (i) Determination of Thermodynamic Parameters. ASL samples were dissolved in buffer (10 mM sodium phosphate, pH 7.2, 100 mM NaCl, 0.1 mM EDTA) to approximately 2.0 μM. Thermal denaturations of ASL constructs were monitored by UV absorbance at 260 nm as previously described (*13*). Thermodynamic parameters were calculated with a van't Hoff analysis of the data (*20*) using Origin software (Microcal). Previous studies using the unmodified ASL^{Lys3}_{UUU} with the $U_{27}{\cdot}A_{43}$ terminal base pair in the presence of 100 mM NaCl reported the melting temperature (T_m) to be 48 ± 1 °C (*8*) and 50.6 ± 0.1 °C (*15*). Substitution of the $U_{27}{\cdot}A_{43}$ terminal base pair with a $G_{27}{\cdot}C_{43}$ added stability to the molecule. Under the same conditions, completely unmodified ASL^{Lys3}_{UUU} with $G_{27}{\cdot}C_{43}$ had a T_m of 57.5 °C, an increase in T_m of about 8 °C. However, since all of the ASL^{Lys} constructs used in this study had a $G_{27}{\cdot}C_{43}$ terminal base pair, changes in thermostability resulting from incorporation of modified nucleosides could be compared to that of the unmodified ASL^{Lys3}. NMR determinations of diffusion coefficients and structure at 1.2 mM (following paper in this issue) indicated that the RNA heptadecamers were hairpins. Therefore, the incorporation of modified nucleosides did not affect the global hairpin conformation of the heptadecamers.

(ii) Filter Binding Assay. E. coli $tRNA^{Lys}$ and ASLs were bound to 30S ribosomal subunits as previously described (*7*, *8*, *13*). Yeast $tRNA^{Phe}$ was bound to appropriately programmed 30S ribosomal subunits as a positive control. Briefly, 10 pmol of 30S ribosomal subunits and 10 μg of poly-A or 100 pmols of $(AAG)_{10}$ or $(UUC)_{10}$ were incubated at 37 °C for 20 min with increasing amounts of 3′-(^{32}P) endlabeled tRNA or ASL (0−50) in 40 μL of CMN buffer (80 mM potassium cacodylate, pH 7.2, 20 mM $MgCl_2$, 100 mM NH_4Cl, and 3 mM β-mercaptoethanol), incubated on ice for 20 min, passed through nitrocellulose filters (0.45 μm) and washed twice with 100 μL ice-cold CMN buffer. The filters were air-dried and counted in a scintillation counter. K_ds and their standard deviations were determined using a nonlinear regression analysis.

(iii) Chemical Probing and Primer Extension. Small ribosomal subunits (30S) were activated by incubating at 37 °C for 30 min. The 30S subunits were programmed with poly-A and incubated with 50 pmoles of *E. coli* $tRNA^{Lys}$, unmodified ASL^{Lys3}_{UUU} or variously modified ASL^{Lys3}_{UUU}. Chemical probing of 16S rRNA P-site nucleosides with kethoxal and dimethyl-sulfate followed by primer extension was accomplished as described by Moazed and Noller (*21*) except that chemical modifications were conducted at 20 °C for 30 min in 40 μL of CMN buffer (*8*, *13*).

Table 1: Thermodynamic Parameters[a] of ASL^{Lys} Constructs

ASL construct	T_m[b] (°C)	ΔG[c] (kcal/mol)	ΔH[d] (kcal/mol)	ΔS[e] (cal/mol K)
ASL^{Lys3}_{UUU}-unmodified	57.5	−3.4	−55.4	−168
ASL^{Lys3}_{UUU}-t^6A_{37}	54.7	−2.7	−49.5	−151
ASL^{Lys3}_{UUU}-mnm^5U_{34}	59.6	−3.1	−45.8	−138
ASL^{Lys3}_{UUU}-Ψ_{39}	64.2	−4.3	−52.6	−156
ASL^{Lys3}_{UUU}-mnm^5U_{34};t^6A_{37}	57.7	−2.8	−45.1	−136
$ASL^{Lys1,2}_{CUU}$ unmodified	60.8	−3.5	−49.3	−148
$ASL^{Lys1,2}_{CUU}$-t^6A_{37}	56.9	−2.6	−44.1	−133

[a] Thermodynamic parameters calculated by a van't Hoff analysis of UV monitored thermal denaturations in 10 mM phosphate buffer, 100 mM NaCl and 0.1 mM EDTA. RNA concentrations were approximately 0.2 μM. [b] T_m error, ± 0.9 °C. [c] ΔG values were calculated at 37 °C; error, ±0.4. [d] ΔH error, ±3. [e] ΔS error, ±7.

RESULTS

Thermodynamic Contributions of Modified Nucleosides to ASL^{Lys}. The introduction of modified nucleosides into ASL^{Lys} constructs affected thermodynamic parameters of the RNA. Substitution of Ψ_{39} for U_{39} increased the melting temperature (T_m) of the ASL^{Lys3}_{UUU} by 7 °C corresponding to a 1 kcal/mol decrease in free energy (Table 1) as had been shown previously (*14*, *15*). Incorporation of mnm^5U_{34} into an otherwise unmodified ASL^{Lys3}_{UUU} construct also stabilized the RNA. The increase in T_m (2.1 °C) was accompanied by a significant increase in ΔS of 30 cal/mol K in comparison to that of the unmodified ASL^{Lys3}_{UUU} (Table 1). In contrast, incorporation of t^6A_{37} into the ASL^{Lys3}_{UUU} and $ASL^{Lys1,2}_{CUU}$ resulted in considerable decreases in T_m (2.8 and 3.9 °C, respectively) and correspondingly higher free energies, but also contributed to increases in ΔS of 17 and 15 cal/mol K, respectively. When both mnm^5U_{34} and t^6A_{37} were introduced into ASL^{Lys3}_{UUU}, the resulting melting temperature (T_m = 57.7 °C) was between that of ASL^{Lys3}_{UUU}-t^6A_{37} (T_m = 54.7 °C) and that of ASL^{Lys3}_{UUU}-mnm^5U_{34} (T_m = 59.6 °C), perhaps indicating an ASL conformation and/or dynamics different from either of the modifications alone. Interestingly, a comparison of entropy values for substitutions at the first position of the anticodon U_{34} revealed that the presence of nucleosides having either an exocyclic amine or amino, i.e., mnm^5U_{34} in ASL^{Lys3}_{UUU} with or without t^6A_{37} and C_{34} in ASL^{Lys3}_{CUU}, was correlated with a significantly higher ΔS (20−30 cal/mol K) than that of the unmodified ASL^{Lys3}_{UUU} (Table 1).

Ribosomal Binding Affinities of Unmodified and Variously Modified ASL^{Lys} Constructs. The incorporation of loop modifications significantly restored poly-A programmed ribosomal binding activity to the unmodified and nonbinding ASL^{Lys3}_{UUU}. The ability of the ASL^{Lys3}_{UUU} and $ASL^{Lys1,2}_{CUU}$ constructs to bind to appropriately programmed 30S ribosomal subunits was assessed with a filter binding assay. Appropriately programmed 30S ribosomal subunits were titrated with increasing amounts of native $tRNA^{Lys}$ or ASL^{Lys} (Figure 2). Introduction of the individual loop modifications t^6A_{37} and mnm^5U_{34} restored poly-A programmed ribosomal binding to ASL^{Lys3}_{UUU} (ASL^{Lys3}_{UUU}-t^6A_{37}, K_d = 290 nM and ASL^{Lys3}_{UUU}-mnm^5U_{34}, K_d = 260 nM) compared to that of the completely unmodified ASL^{Lys3}_{UUU} (K_d = 2000 nM)

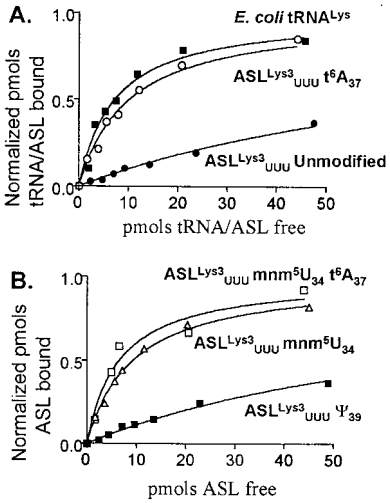

FIGURE 2: Binding of tRNA^{Lys} and variously modified ASL$^{Lys3}_{UUU}$ constructs to poly-A programmed 30S ribosomal subunits. (A) Increasing amounts of *E. coli* tRNA^{Lys} (■), ASL$^{Lys3}_{UUU}$-t^{6}A$_{37}$ (○), and unmodified ASL$^{Lys3}_{UUU}$ (●) were incubated with 10 pmol of poly-A programmed 30S ribosomal subunits as described in Experimental Procedures. The amounts of tRNA or ASL bound were normalized to maximum binding by tRNA^{Lys}. (B) Comparison of ribosomal binding affinities of variously modified ASL$^{Lys3}_{UUU}$ constructs: doubly modified ASL$^{Lys3}_{UUU}$-mnm^{5}U$_{34}$;t^{6}A$_{37}$ (□); ASL$^{Lys3}_{UUU}$-mnm^{5}U$_{34}$ (△); and ASL$^{Lys3}_{UUU}$-Ψ$_{39}$ (■). Data presented here are representative of binding curves obtained in assays conducted at least in triplicate.

Table 2: Binding Affinities of tRNA^{Lys} and ASL^{Lys} Constructs to AAA and AAG Programmed Ribosomes[a]

	message K_d (nM)	
tRNA/ASL	AAA	AAG
E. coli tRNA^{Lys}	170 ± 20	
ASL$^{Lys3}_{UUU}$ unmodified	2000 ± 600	nb[b]
ASL$^{Lys3}_{UUU}$-t^{6}A$_{37}$	290 ± 80	nb
ASL$^{Lys3}_{UUU}$-mnm^{5}U$_{34}$	260 ± 40	nb
ASL$^{Lys3}_{UUU}$-Ψ$_{39}$	1600 ± 700	nb
ASL$^{Lys3}_{UUU}$-mnm^{5}U$_{34}$-t^{6}A$_{37}$	180 ± 20	600 ± 200
ASL$^{Lys1,2}_{CUU}$ unmodified	nb	240 ± 50
ASL$^{Lys1,2}_{CUU}$-t^{6}A$_{37}$	nb	248 ± 34

[a] The 30S ribosomal subunits were programmed with either poly-A or an $(AAG)_{10}$ message and binding of tRNA or ASLs was assessed in the filter binding assay described in Experimental Procedures. K_d values were determined by nonlinear curve-fit of the data. Error is expressed as standard deviation from at least three replicates. [b] nb = negligible binding with a $K_d \gg 2000$ nM.

(Table 2). The combination of the two modified nucleosides further increased the ribosome binding activity of ASL$^{Lys3}_{UUU}$ (K_d = 180 nM) to that of native *E. coli* tRNA^{Lys} (K_d = 170 nM). However, incorporation of the modified nucleoside at the base of the anticodon stem, Ψ$_{39}$, had no effect on the ability of the otherwise unmodified ASL$^{Lys3}_{UUU}$ to bind to poly-A programmed ribosomal subunits (K_d = 1600 nM).

Interestingly, neither of the individually modified ASL$^{Lys3}_{UUU}$ constructs (ASL$^{Lys3}_{UUU}$-mnm^{5}U$_{34}$ or t^{6}A$_{37}$) that had restored binding to poly-A programmed ribosomal subunits were able to restore binding to 30S ribosomal subunits programmed with message containing the lysine codon $(AAG)_{10}$ (Table 2). However, when the two loop modifications were incorporated in combination, the binding of ASL$^{Lys3}_{UUU}$-mnm^{5}U$_{34}$;t^{6}A$_{37}$ to $(AAG)_{10}$-programmed ribosomes was significantly enhanced (K_d = 600 nM).

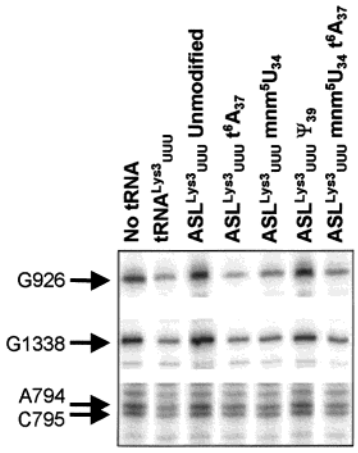

FIGURE 3: Chemical protection of 16S rRNA nucleosides by tRNA^{Lys} and variously modified ASL$^{Lys3}_{UUU}$ constructs. The P-site nucleosides of 16S rRNA are protected when tRNA or an ASL construct is bound (*22*). Small (30S) ribosomal subunits were incubated without tRNA or ASL as a control, and with *E. coli* tRNA^{Lys}, the unmodified ASL$^{Lys3}_{UUU}$, the singly modified ASL$^{Lys3}_{UUU}$-t^{6}A$_{37}$, ASL$^{Lys3}_{UUU}$-mnm^{5}U$_{34}$, ASL$^{Lys3}_{UUU}$-Ψ$_{39}$, or doubly modified ASL$^{Lys3}_{UUU}$-mnm^{5}U$_{34}$;t^{6}A$_{37}$ as labeled in the figure. After chemical treatment by kethoxal or dimethyl sulfate, 16S rRNA was reverse transcribed with radiolabeled primers as described in Experimental Procedures. Primers were chosen to assess protection of the 16S rRNA nucleosides G926, G1338, A794, C795 from chemical modification.

In contrast to the unmodified and singly modified ASL$^{Lys3}_{UUU}$ constructs, the unmodified ASL$^{Lys1,2}_{CUU}$, corresponding to human tRNA^{Lys1,2} with a CUU anticodon was able to efficiently bind to $(AAG)_{10}$ programmed 30S ribosomal subunits (K_d = 240 nM). However, introduction of t^{6}A$_{37}$ into ASL$^{Lys1,2}_{CUU}$ did not improve binding to AAG programmed ribosomes. Neither the unmodified ASL$^{Lys1,2}_{CUU}$ nor the t^{6}A$_{37}$-modified ASL$^{Lys1,2}_{CUU}$ were bound by poly-A programmed ribosomal subunits (Table 2).

Protection of 16S rRNA Nucleosides from Chemical Modification. To determine if the binding of the variously modified ASLs to the ribosomal P-site was similar to that of native tRNA, we compared their abilities to protect certain 16S rRNA nucleosides from chemical modification when bound to 30S ribosomal subunit. Specific 16S rRNA nucleosides in 30S ribosomal subunits are susceptible to chemical modification by kethoxal and dimethyl sulfate. When bound to the P-site of 30S ribosomal subunits, tRNAs or ASLs protect particular 16S rRNA nucleosides from chemical modification (*21*). Using a primer extension reaction with a radiolabeled primer complementary to sequences in 16S rRNA, we assessed the protection of the commonly probed P-site 16S rRNA nucleosides, G926, G1338, A794 and C795 (*22*). With the 30S subunit programmed with poly-A, the unmodified ASL$^{Lys3}_{UUU}$ did not protect these nucleosides, whereas *E. coli* tRNA^{Lys} did (Figure 3). The introduction of t^{6}A$_{37}$ and mnm^{5}U$_{34}$ either individually or in combination afforded protection of the 16S rRNA nucleosides. As expected, the incorporation of the Ψ$_{39}$ into the otherwise unmodified ASL$^{Lys3}_{UUU}$ did not protect 16S rRNA nucleosides from chemical modification.

DISCUSSION

The individual incorporations of mnm^{5}U$_{34}$ and t^{6}A$_{37}$ restored ribosome binding activity to the otherwise unmodi-

fied and nonbinding anticodon stem and loop of human $tRNA^{Lys3}_{UUU}$. ASL^{Lys3}_{UUU} modified with either mnm^5U_{34} or with t^6A_{37} bound to poly-A programmed ribosomes, but did not bind to ribosomes programmed with the alternate lysine codon, AAG (Table 2). Previously, we had reported that s^2U_{34} also restored binding to poly-A programmed ribosomes (*8*). These results suggest that a redundancy of function may have evolved for anticodon loop modifications in $tRNA^{Lys}_{UUU}$ such that the individual modifications impart effective AAA-programmed ribosomal binding to species of lysine tRNA with UUU anticodons. Introduction of both modifications, mnm^5U_{34} and t^6A_{37} in combination, resulted in an affinity for poly-A programmed ribosomes comparable to that of native $tRNA^{Lys}_{UUU}$.

We had postulated that pyrimidine-rich anticodon loops would lack effective stacking interactions and would require modification-induced structure for effective ribosome binding (*23*). Five of the seven anticodon loop nucleosides of lysine tRNAs are pyrimidines. Incorporations of mnm^5U_{34} and t^6A_{37}, individually and together, not only restored ribosomal binding, but also affected the ASL's thermodynamic stability. The introduction of these two quite different modifications at different positions in the anticodon loop had similar effects on ΔS, even though they had opposite effects on T_m (Table 2). The presence of the hypermodified t^6A_{37} increased ΔS of ASL^{Lys3}_{UUU} by 17 cal/mol K and that of $ASL^{Lys1,2}_{CUU}$ by 15 cal/mol K in comparison to their respective unmodified ASLs. Substitution of U_{34} in ASL^{Lys3}_{UUU} with the amine derivative mnm^5U_{34} considerably increased ΔS by 30 cal/mol K. $ASL^{Lys1,2}_{CUU}$, with a position 34 amine in the form of C_{34}, also exhibited a considerable increase in ΔS (20 cal/mol K) compared to ASL^{Lys3}_{UUU}. $ASL^{Lys1,2}_{CUU}$ did not require modification for it to be bound by AAG-programmed ribosomes. Other than substitution of a C_{34} for a U_{34}, the only difference between $ASL^{Lys1,2}_{CUU}$ and ASL^{Lys3}_{UUU} is the opposite orientation of the C·G base pair at positions 28−42 (Figure 1). Thus, the increase in ΔS with introduction of mnm^5U_{34} and t^6A_{37}, and perhaps substitution of U_{34} with C_{34} as well, is consistent with our hypothesis that chemistries must be contributed to pyrimidine-rich anticodon domains to produce an ordered anticodon domain, structured for ribosome binding.

Position 37, 3′-adjacent to the anticodon proper, appears to be crucial in some tRNAs for shaping the anticodon loop into a functional architecture. The t^6A_{37} modification commonly appears in tRNAs that read codons beginning with adenosine (*9*) and is found in all $tRNA^{Lys}$ species sequenced to date (*2*). Interaction of the third position of the anticodon, nucleoside 36, with the first position of the codon is critical to correct codon recognition. Results from previous studies suggest that the t^6A_{37} modifications in *E. coli* $tRNA^{Ile}$ (*24*) and in yeast $tRNA^{Arg3}$ (*25*) are necessary for proper interaction of the anticodon with its codon. Incorporation of t^6A_{37} into the unmodified ASL^{Lys3}_{UUU}, unmodified $ASL^{Lys1,2}_{CUU}$, and the modified ASL^{Lys3}_{UUU}-mnm^5U_{34} lowered the T_m of the RNAs (Table 1) yet significantly restored the ability of the unmodified ASL^{Lys3}_{UUU} to bind to AAA-programmed 30S ribosomal subunits (Table 2). The structure determination of ASL^{Lys3}_{UUU}-t^6A_{37} (following paper in this issue) showed that the threonyl-modification negates A_{37} base pairing to U_{33} and hinders formation of a $C_{32}{\cdot}A^+_{38}$ base pair that would extend the stem and reduce the anticodon loop size. Fifteen percent of all tRNA sequences have the potential to form two canonical base pairs across the anticodon loop but 95% of these are modified to negate at least one of the base pairs (*3*). In contrast, incorporation of Ψ_{39} into ASL^{Lys3}_{UUU} contributed significant thermostability to the ASL^{Lys3}_{UUU} (Table 1), presumably promoting $C_{32}{\cdot}A^+_{38}$ base pair formation and extending the base-paired stem (*15*) yet did not restore AAA or AAG programmed ribosomal binding (Table 2). Thus, the physicochemical contributions of t^6A_{37} to the ribosome binding of $tRNA^{Lys3}$ includes disrupting intraloop base pairs, $U_{33}{\cdot}A_{37}$ and $C_{32}{\cdot}A^+_{38}$, as well as contributing an ordered structure to the anticodon domain.

Only when mnm^5U_{34} and t^6A_{37} were incorporated in combination was ASL^{Lys3}_{UUU} able to bind to AAG-programmed ribosomes (K_d = 600 nM), although certainly not to the extent that $ASL^{Lys1,2}_{CUU}$ bound AAG (K_d = 240 nM). The lower affinity of the doubly modified ASL^{Lys3}_{UUU} for AAG may be for lack of the 2-thiolation of the wobble position uridine. The ability of the doubly modified ASL^{Lys3}_{UUU} to wobble for reading AAG is particularly critical for organisms such as *E. coli* that encode only the one lysine tRNA, $tRNA^{Lys}_{SUU}$ modified with $xm^5s^2U_{34}$ and t^6A_{37}. Lysine codon preference in *E. coli* is consistent with it having only $tRNA^{Lys}_{SUU}$; almost 75% of all lysine codons are AAA. The opposite is true in humans where AAA represents only 40% of lysine codons, while AAG represents 60%. Thus, humans have retained the redundant functions of the multiple anticodon loop domain modifications in $tRNA^{Lys3}_{UUU}$ for reading AAA but employ two additional $tRNA^{Lys}$ species, $tRNA^{Lys1,2}_{CUU}$, that do not require modification to read the preferred AAG codon.

The diverse chemistries in nature that modify position 34 nucleosides are likely used to enable the anticodon to wobble during translation (*1*, *23*). The xm^5U_{34} modifications probably contribute to translation of codons terminating in A or G and limit mistranslation of codons terminating in U or C (*26*). The ribosomal binding and thermal stability studies of the doubly modified ASL^{Lys3}_{UUU} presented here, together with structural studies of a model pentanucleotide (*27*) and the ASL^{Lys3}_{UUU}-t^6A_{37} (following paper in this issue), support our hypothesis that $tRNA^{Lys}_{UUU}$ has an unconventional anticodon architecture (*27*). The $mnm^5s^2U_{34}$ and t^6A_{37} modifications in the anticodon loop of $tRNA^{Lys}_{UUU}$ species interact to contribute to a distinctive anticodon conformation with only U_{35} and U_{36} available for reading the codon. This unconventional anticodon structure would explain the $tRNA^{Lys}_{UUU}$ ability to wobble between AAA and AAG, misread Asn codons, facilitate −1 frameshifts, and prematurely terminate translation more often than other tRNAs (*27*).

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