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The uridine in "U-turn": Contributions to tRNA-ribosomal binding

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ABSTRACT

"U-turns" represent an important class of structural motifs in the RNA world, wherein a uridine is involved in an abrupt change in the direction of the polynucleotide backbone. In the crystal structure of yeast $tRNA^{Phe}$, the invariant uridine at position 33 (U_{33}), adjacent to the anticodon, stabilizes the exemplar U-turn with three non-Watson–Crick interactions: hydrogen bonding of the 2′-OH to N^7 of A_{35} and the N^3-H to A_{36}-phosphate, and stacking between C_{32} and A_{35}-phosphate. The functional importance of each noncanonical interaction was determined by assaying the ribosomal binding affinities of $tRNA^{Phe}$ anticodon stem and loop domains (ASLs) with substitutions at U_{33}. An unsubstituted ASL bound 30S ribosomal subunits with an affinity (K_d = 140 ± 50 nM) comparable to that of native yeast $tRNA^{Phe}$ (K_d = 100 ± 20 nM). However, the binding affinities of ASLs with dU-33 (no 2′-OH) and C-33 (no N^3-H) were significantly reduced (2,930 ± 140 nM and 2,190 ± 300 nM, respectively). Surprisingly, the ASL with N^3-methyluridine-33 (no N^3-H) bound ribosomes with a high affinity (K_d = 220 ± 20 nM). In contrast, ASLs constructed with position 33 uridine analogs in nonstacking, nonnative, and constrained conformations, dihydrouridine (C2′-endo), 6-methyluridine (*syn*) and 2′*O*-methyluridine (C3′-endo) had almost undetectable binding. The inability of ASLs with 6-methyluridine-33 and 2′*O*-methyluridine-33 to bind ribosomes was not attributable to any thermal instability of the RNAs. These results demonstrate that proton donations by the N^3-H and 2′OH groups of U_{33} are not absolutely required for ribosomal binding. Rather, the results suggest that the overall uridine conformation, including a dynamic (C3′-endo > C2′-endo) sugar pucker, *anti* conformation, and ability of uracil to stack between C_{32} and A_{35}-phosphate, are the contributing factors to a functional U-turn.

Keywords: anticodon conformation; anticodon stem/loop analogs; ASL; nucleoside modifications; ribosome; translation; $tRNA^{Phe}$ U-turn

INTRODUCTION

Transfer RNAs have the general and well-recognized shape defined by the first X-ray-derived crystal structure of an entire RNA, that of yeast $tRNA^{Phe}$ (Kim et al., 1973). Certain nucleotides within the sequence of yeast $tRNA^{Phe}$ and other tRNAs are invariant and associated with structural motifs that are common to other RNAs as well. Uridine at position 33 is one of the most conserved residues in tRNAs; 97% of the 2,716 tRNA genes sequenced have a uridine at position 33. All eucaryotic tRNAs have the invariant uridine at position 33, U_{33}, except for initiator tRNAs from higher eucaryotes and some tRNAs from the genus *Candida* (Sprinzl et al., 1998). Based on the crystal structures of yeast $tRNA^{Phe}$ and $tRNA^{Asp}$, U_{33} in the anticodon domain and pseudouridine, ψ, at position 55 in the TψC loop are intrinsically involved in sharp turns of the phosphodiester backbone (Quigley & Rich, 1976). These turns, designated uridine- or U-turns, were initially observed only in $tRNA^{Phe}$ and $tRNA^{Asp}$, but examples of U-turn motifs have been recently reported in numerous other RNA molecules (Pley et al., 1994; Doudna, 1995; Huang et al., 1996; Stallings & Moore, 1997). Thus, the U-turn may be an important and abundant structural motif in the RNA world (Jucker & Pardi, 1995).

In yeast $tRNA^{Phe}$, the exemplar anticodon U-turn is responsible for an abrupt change in the direction (~180°) of the RNA's phosphodiester backbone just prior to the three anticodon nucleotides. The stability of the turn has been attributed to three noncanonical interactions

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involving U_{33}. Two of the interactions are hydrogen bonds from the 2′-OH of U_{33} to the N^7 of A_{35} and from the N^3-H of U_{33} to the phosphate of A_{36} (Fig. 1). The third interaction is the continuation of base stacking that begins on the 5′ side of the anticodon stem and terminates in the loop with U_{33} stacked on the phosphate of A_{35} (Fig. 1; Quigley & Rich, 1976). Although considerable effort has been invested in elucidating the role of the ubiquitous U_{33} in the anticodon U-turn (Uhlenbeck et al., 1982; Bare et al., 1983; Wittenberg & Uhlenbeck, 1985; Dix et al., 1986; Schnitzer & von Ahsen, 1997; von Ahsen et al., 1997), examination of each of the three proposed stabilizing factors has not yielded a clear understanding of the intramolecular interactions required for a functional U-turn. Recently, von Ahsen et al. (1997) reported that the 2′-OH of U_{33} was essential for the interaction of the *Escherichia coli* $tRNA^{Phe}$ anticodon stem and loop (ASL) with the ribosome. However, these results contradicted a previous study of yeast $tRNA^{Phe}$ in which only a marginal decrease in ribosomal binding was observed when U_{33} was changed to 2′-*O*-methyluridine (Uhlenbeck et al., 1982). To determine the properties of U_{33} that are important to the anticodon U-turn structure/function relationship during mRNA decoding, we have designed and synthesized a set of ASLs with specific nucleotide substitutions at U_{33} and have analyzed their relative ribosomal binding affinities.

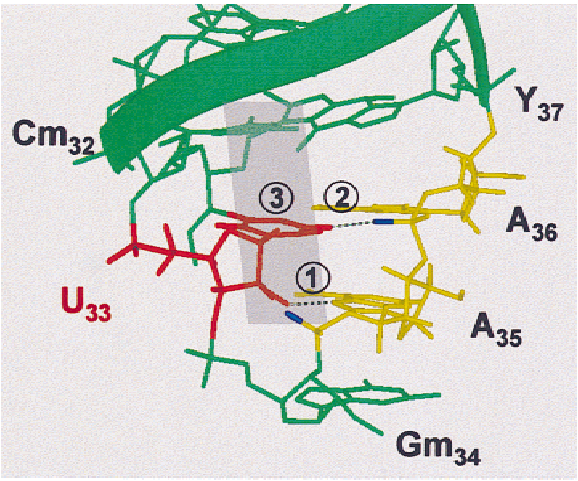

FIGURE 1. The three-dimensional X-ray crystallographic structure of the anticodon loop of yeast $tRNA^{Phe}$ showing the anticodon U-turn (Quigley & Rich, 1976). The two noncanonical hydrogen bonds involving the invariant uridine at position 33 (U_{33}) are highlighted and numbered. ①: H-bonding from 2′-OH (U_{33}) to N^7 of A_{35}; ②: H-bonding from N^3-H (U_{33}) to A_{36}-phosphate. The stacking interactions of Cm_{32}, U_{33}, and phosphate-35 are within the shaded area of the figure that is labeled as ③. Bases of the anticodon triplet (Gm_{34}, A_{35}, and A_{36}) and the hypermodified tricyclic nucleoside Y_{37} are also labeled. C_m: 2′*O*-methylcytidine, G_m: 2′*O*-methylguanosine, Y: wyebutosine. Oxygen atoms colored dark blue are the pro-Rp-phosphate oxygen atoms of A_{35} and A_{36} that are important for efficient ribosomal binding (Schnitzer & von Ahsen, 1997).

RESULTS

Design of "U_{33}-variant" ASLs and experimental strategy

Six ASLs were synthesized with various substitutions at the position of U_{33} (Fig. 2). Each substitution was designed to test the functional importance of one or more of the interactions contributed by U_{33} to the U-turn structure (Table 1). Two of the ASLs were designed such that nucleoside-33 lacked the ability to donate a proton from the 2′-OH: 2′-*O*-methyluridine (ASL-Um_{33}) and 2′-deoxyuridine (ASL-dU_{33}). Others were designed to negate proton donation from the N^3-H position: cytidine (ASL-C_{33}), N^3-methyluridine (ASL-m^3U_{33}), and 6-methyluridine (ASL-m^6U_{33}). The latter precludes proton donation from N^3 to the phosphate of A_{36} because the N-glycosidic bond of m^6U_{33} takes the *syn* conformation both in the mononucleoside (Felczak et al., 1996) and within the ASL. The *syn*, C3′-endo conformation of m^6U_{33} in the ASL was determined by NMR spectroscopy (R. Cain and P.F. Agris, pers. comm.). Two ASLs were designed to force the dynamic U_{33} sugar pucker (~50% C3′ endo in solution) to either the C3′-endo conformation by methylation (ASL-Um_{33}; Kawai et al., 1992) or the C2′-endo conformation by elimination (ASL-dU_{33}; Basti et al., 1996). An additional ASL containing dihydrouridine (ASL-D_{33}) was designed to have the C2′-endo sugar pucker, but unlike ASL-dU_{33}, would retain the ability to donate a proton from the 2′-OH. Dihydrouridine is known to be highly constrained to the C2′-endo conformation as a mononucleoside (Sundaralingam et al., 1971) and within tRNAs and oligomers (Dalluge et al., 1996, 1997; Stuart et al., 1996). Particular nucleoside substitutions, such as m^6U_{33} and D_{33}, were also designed to affect the stacking interactions that characterize position 33. The *syn* conformation of m^6U_{33} and the nonplanar, nonaromatic character of D_{33} would negatively affect stacking interactions. Thus, nucleosides substituted for U_{33} were chosen for their abilities to disrupt local noncanonical H-bonds in the loop or affect nucleoside conformation and stacking interactions. These substitutions could, however, have affected structure beyond that of the anticodon loop and would, in turn, affect the assessment of ribosome binding.

Thermal denaturation of synthetic ASLs

To confirm that the substitutions of the various nucleosides for U_{33} in the ASLs had little or no effect on overall RNA structure, we analyzed the thermal stability of each of the seven ASLs. Thermal denaturations of the ASLs monitored by UV spectroscopy (Fig. 3) revealed that none of the substitutions had any significant effect on the melting temperature (T_m) of the molecules (Table 2), with one exception. Incorporation

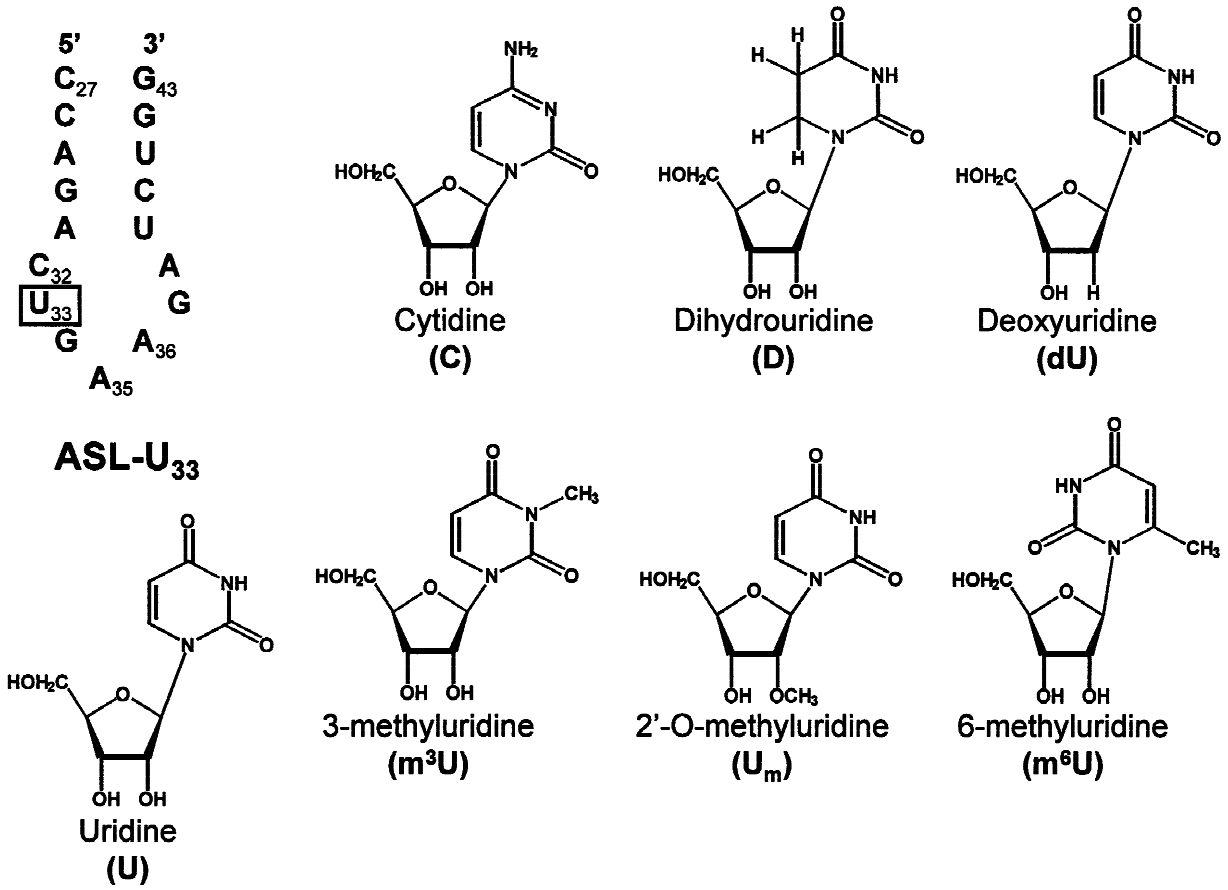

FIGURE 2. Nucleotide sequence and secondary structure of yeast $tRNA^{Phe}$ anticodon stem-loop domain (ASL) and the modified uridines used in this study. The uridine at position 33 (U_{33}, boxed) of the ASL was substituted with C, Um, dU, m^3U, m^6U, or D to produce various position 33-substituted ASLs.

of the nonplanar D_{33} appeared to have destabilized the ASL stem, as well as the loop (Fig. 3). Dihydrouridine dramatically affects local conformation within RNA by transference of the C2′-endo conformation to 3′-adjacent nucleosides (Dalluge et al., 1996, 1997; Stuart et al., 1996). Other substitutions of U_{33} did not alter the T_m of the ASLs, which varied little (62–64 ± 0.6 °C). However, some differences in stability were detected at temperatures (20–40 °C) that preceded the major transition, in particular for ASL-m^6U_{33} (Fig. 3). Analysis of

TABLE 1. Anticodon stem-loop domains (ASLs) used in this study and their expected structural properties.

ASL	Nucleoside at position 33	Availability of 2′-OH	Availability of N^3-H	Availability of base stacking	Expected sugar pucker	Expected base pucker	Base conformation
ASL-U_{33}	Uridine	+	+	+	C3′ > C2′ endo[a]	planar	*anti*
ASL-m^3U_{33}	3-methyl-uridine	+	−	+	C3′ > C2′ endo[a]	planar	*anti*
ASL-C_{33}	Cytidine	+	−	+	C3′ > C2′ endo[a]	planar	*anti*
ASL-dU_{33}	Deoxyuridine	−	+	+	C2′ endo[b]	planar	*anti*
ASL-Um_{33}	2′*O*-methyl-uridine	−	+	+	C3′ endo[c]	planar	*anti*
ASL-m^6U_{33}	6-methyl-uridine	+	−	−	C3′ > C2′ endo[d]	planar	*syn*
ASL-D_{33}	Dihydro-uridine	+	+	−	C2′ endo[e]	nonplanar, puckered	*anti*

[a]The sugar pucker of three of the nucleosides (U, C, and m^3U) at position 33 are expected to be dynamic, but predominantly 3′ endo (C3′ > C2′ endo). Sugar puckers of [b]deoxyuridine (dU) in the anticodon loop (Basti et al., 1996) and [e]Dihydrouridine (D) (Sundaralingam et al., 1971) have the C2′ endo conformation. [c]Methylation of the 2′-OH of uridines is known to strongly constrain sugar pucker to the C3′ endo conformation (Kawai et al., 1992). [d]Sugar pucker of m^6U in ASL-m^6U_{33} was determined to be >50% C3′ endo (data not shown).

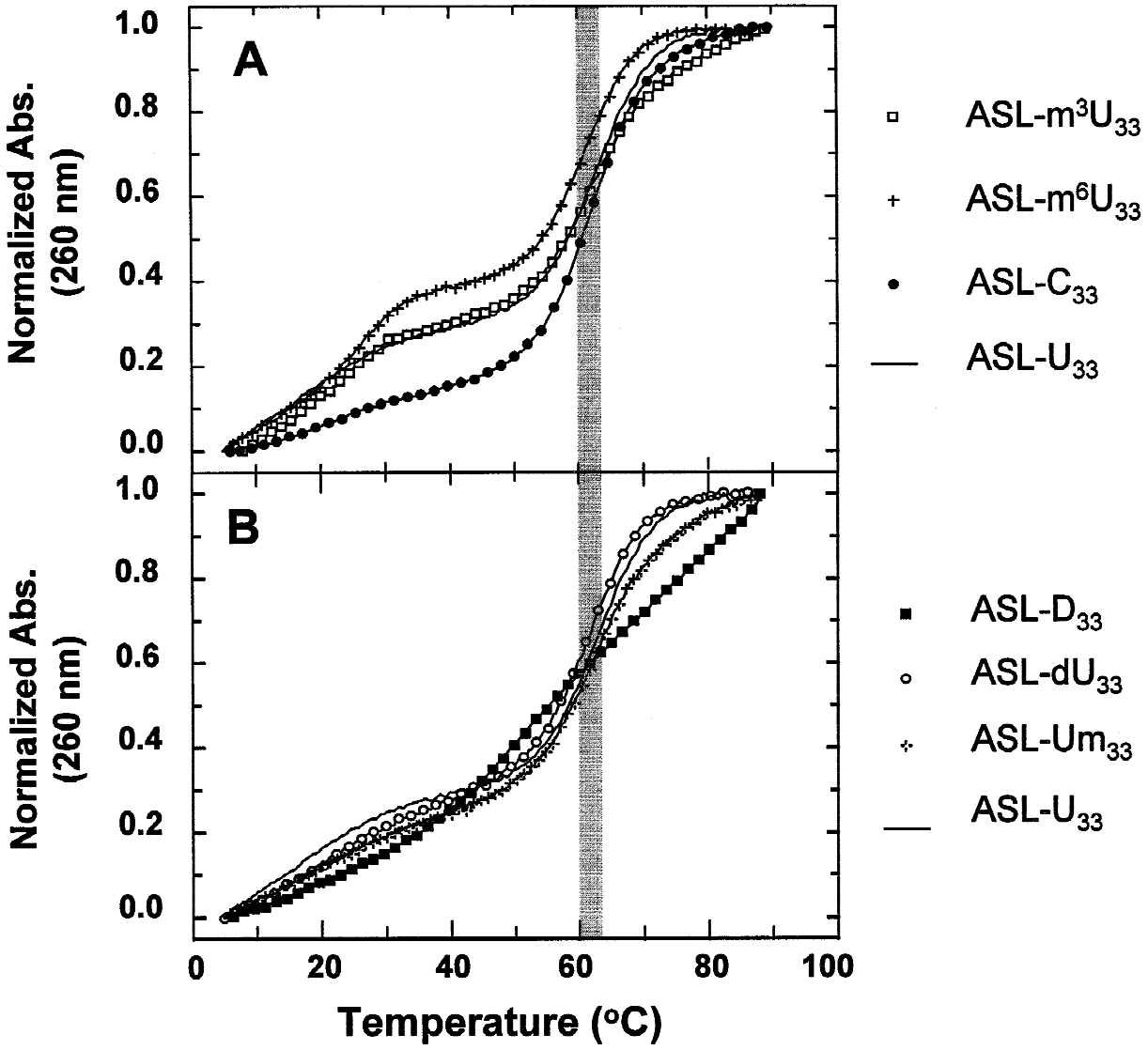

FIGURE 3. UV-monitored thermal denaturations of the various position 33-substituted ASLs in comparison to ASL-U_{33}. **A**: ASLs with U_{33} substitutions (m^3U, m^6U, and C) that disrupt N^3-H bonding in comparison to that of unsubstituted ASL-U_{33}. **B**: ASLs with U_{33} substitutions (dU and Um) that prevent H-bonding from 2′-OH, as well as the ASL containing a nonplanar base (ASL-D_{33}), in comparison to that of the unsubstituted ASL-U_{33}. The perpendicular gray shading indicates the average T_m of the ASLs (62.6 ± 0.6 °C). UV-monitored denaturation profiles of the ASLs were obtained as described in Materials and Methods.

the pretransition as well as the major transition over a tenfold range of RNA concentrations indicated the T_m for pretransitions and that for all the major transitions were temperature independent. Thus, the pretransitions were not due to hairpin–duplex equilibrium, and within the concentration range used, a unimolecular denaturation was being observed. We believe that the pretransition region of the thermal denaturation profile is indicative of the denaturation of loop interactions in the ASL. Thus, except for D_{33}, incorporation of modified uridines only caused changes in the structure and/or dynamics of the ASL loop. Attempts to detect any thermodynamic differences in the major transition (stem denaturation) by curve-fitting the profile to a two-state model did not yield any significant differences among six of the seven ASLs (Table 2).

Binding of ASLs to 30S ribosomal subunits

The ability of the variously U_{33}-substituted ASLs to bind to the poly(U)-programmed 30S ribosomal subunit was assessed and compared to that of the unsubstituted ASL-U_{33} (Fig. 4). Dissociation constants (K_d) for the ASLs binding to 30S ribosomal subunits were calculated from binding isotherms using nonlinear regression (Table 3). The K_d for the yeast $tRNA^{Phe}$ ASL-U_{33} (140 ± 50 nM) was in good agreement with the previously reported K_d for the *E. coli* $tRNA^{Phe}$ ASL binding to

TABLE 2. Thermal denaturation analysis of variously substituted and ASL-U_{33}.

ASL	T_m (°C)[a]	ΔH_{VH} (Kcal/mol)[a]	ΔS_{VH} (Cal/mol∗K)[a]	ΔG_{VH} (Kcal/mol)[a]
ASL-U_{33}	63.5 ± 0.7	−56.0 ± 1.9	−166.4 ± 5.9	−4.40 ± 0.1
ASL-m^3U_{33}	62.6 ± 0.5	−57.3 ± 2.2	−170.7 ± 6.5	−4.4 ± 0.2
ASL-C_{33}	62.7 ± 0.4	−56.0 ± 0.5	−166.8 ± 1.6	−4.3 ± 0.04
ASL-dU_{33}	63.1 ± 0.4	−58.2 ± 2.6	−172.9 ± 7.4	−4.6 ± 0.3
ASL-Um_{33}	61.9 ± 0.6	−62.6 ± 0.4	−187.0 ± 1.1	−4.7 ± 0.1
ASL-m^6U_{33}	61.6 ± 0.6	−59.3 ± 2.2	−177.2 ± 6.8	−4.4 ± 0.2
ASL-D_{33}[b]	no data	no data	no data	no data

[a]Melting point (T_m) and other thermodynamic parameters (ΔH, ΔS, and ΔG) were extracted from the major thermal transition (~50–65 °C) (Fig. 3).
[b]Denaturation of ASL-D_{33} did not yield data from which T_m or other parameters could be extracted (Fig. 3).

30S subunits (130 ± 40 nM; von Ahsen et al., 1997). Binding of the unsubstituted ASL-U_{33} to the ribosome was also comparable to that of the native yeast $tRNA^{Phe}$ (K_d = 100 ± 20 nM). In comparison to the unsubstituted ASL-U_{33}, ASLs lacking the ability to donate protons for H-bonding from either the N^3-H (ASL-C_{33}) or from the 2′-OH (ASL-dU_{33} and ASL-Um_{33}) had substantially reduced K_ds, 2,190 ± 300 nM, 2,930 ± 140 nM, and 13,700 ± 4,600 nM, respectively. ASL-m^6U_{33}, in which the *syn* conformation negated proton donation from the N^3 to the A_{35}-phosphate, also bound very poorly to 30S subunits (Fig. 4). In fact, we could not calculate an accurate K_d (>300 × 10^{-7} M) for the binding of the ASL-m^6U_{33}. Surprisingly, ASL-m^3U_{33}, which lacked the N^3 proton for H-bonding, bound to 30S ribosomal subunits with a K_d (220 ± 20 nM) that was comparable to that of ASL-U_{33} (Table 3). Ribosomal binding could not be detected for the ASL-D_{33}, in which D_{33} had protons available for H-bonding at both the 2′-OH and N^3, but had a nonplanar base and a highly constrained C2′-endo sugar pucker.

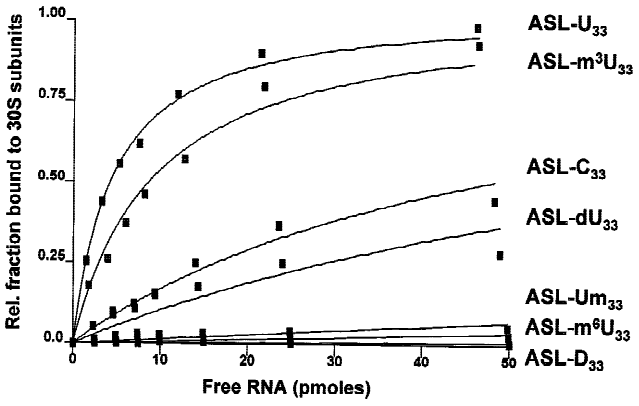

FIGURE 4. Binding of variously substituted ASLs to programmed 30S ribosomal subunits in comparison to ASL-U_{33}. Ribosomal binding assays were conducted as described in Materials and Methods by using ^{32}P-labeled ASLs and poly(U)-programmed 30S ribosomal subunits. The fraction of the ASL bound to ribosomes was normalized to the fraction of ASL-U_{33} bound to 30S subunits according to previously published methods (von Ahsen et al., 1997).

DISCUSSION

Stability of the anticodon U-turn within the X-ray crystallographic structure of yeast $tRNA^{Phe}$ is dependent on U_{33} contributing two noncanonical hydrogen bonds and stacking interactions (Quigley & Rich, 1976; Fig. 1). To test the functional importance of these H-bonds and stacking interactions, six ASLs were synthesized with substitutions at U_{33} designed to disrupt the individual contributions of the nucleoside to the U-turn. This approach of site selective incorporation of natural and nonnatural modifications into synthetic ASLs has proven very useful for addressing specific structure/function-related questions (Uhlenbeck et al., 1982; Bare et al., 1983; Wittenberg & Uhlenbeck, 1985; Dix et al., 1986; Chen et al., 1993; Dao et al., 1994; von Ahsen et al., 1997). In fact, we have recently shown that a specific, single-atom substitution ($U_{34} \rightarrow s^2U_{34}$) in the anticodon stem-loop of $tRNA^{Lys}$ is sufficient to restore ribosomal binding of an otherwise biologically inactive ASL (Ashraf et al., 1999).

In this report, the degree to which each of the six ASLs bound the ribosome in comparison to that of the unsubstituted ASL-U_{33} was used as a measure of the functional importance of U_{33} H-bonding and stacking interactions. However, in this approach we and others (Uhlenbeck et al., 1982; Bare et al., 1983; Wittenberg & Uhlenbeck, 1985; Dix et al., 1986; Schnitzer & von Ahsen, 1997; von Ahsen et al., 1997) have assumed that the various substitutions at U_{33} negate specific uridine

TABLE 3. Binding affinities (K_d) of variously substituted ASLs and ASL-U_{33} to poly(U)-programmed 30S ribosomal subunits.

ASL	K_d (× 10^{-7} M)
ASL-U_{33}	1.4 (± 0.5)
ASL-m^3U_{33}	2.2 (± 0.2)
ASL-C_{33}	21.9 (± 3.0)
ASL-dU_{33}	29.3 (± 13.6)
ASL-Um_{33}	137.0 (± 46.0)
ASL-m^6U_{33}	>300
ASL-D_{33}	Could not be detected

contributions to the U-turn. In fact, it is possible that some or all of the substitutions also sterically distort the anticodon loop conformation, which would lead to reduced ribosomal binding. Yet, Uhlenbeck and colleagues (1982) have reported only marginal effects on ribosomal binding by fully modified ASLs with the same substitutions at position 33 as ours. Thus, these substitutions probably do not grossly disturb the modified anticodon loop structure. Even in the absence of natural modifications, thermal denaturations of the six ASLs with U_{33} substitutions strongly indicated that all the ASLs, with the exception of ASL-D_{33}, were as stable as the ASL containing U_{33} (Table 2; Fig. 2). In addition, in comparing the degree to which the ASLs bound the ribosome, we and others also assumed that the ASLs are all binding at the same site on the 30S subunits. To confirm this, we assessed the abilities of the ASLs to protect two 16S rRNA P-site nucleotides, G926 and G1338, from chemical modification (Fig. 5; Moazed & Noller, 1990). ASLs with m^3U_{33}, C_{33}, and dU_{33} protected nucleosides G926 and G1338 from kethoxal reactivity, as did ASL-U_{33} and the complete yeast $tRNA^{Phe}$ molecule. However, the ASLs that exhibited little (ASL-Um_{33}) or no (ASL-m^6U_{33} and ASL-D_{33}) binding to the ribosome demonstrated little or no protection of the two 16S P-site nucleotides (Fig. 5). Hence, ASLs that bound the ribosome in our filter binding assays were in fact binding at the same tRNA P-site in 30S ribosomal subunits.

One of the three interactions of U_{33} that stabilizes the anticodon U-turn in the yeast $tRNA^{Phe}$ structure is the hydrogen bonding of its 2′-OH to the N^7 of A_{35}. This interaction could be important to tRNAs that have a purine at anticodon position 35, that is, roughly half of all tRNAs. When hydrogen bonding of the 2′-OH at position 33 was disrupted by substituting dU_{33} and Um_{33} for U_{33}, the ASLs' abilities to bind the ribosome were reduced to 5% and 1% of that of the unmodified ASL, respectively. These results are in agreement with that of von Ahsen et al. (1997), and we concur with their conclusion that the 2′-OH of U_{33} is important for ribosomal binding. Yet ASL-C_{33}, ASL-m^6U_{33}, and ASL-D_{33}, all of which had the 2′-OH groups available, also showed significantly reduced or hardly detectable ribosomal binding. Perhaps the important contribution of the 2′-OH of U_{33} to the functional U-turn is some property other than the ability to hydrogen bond. Deoxynucleosides (as in ASL-dU_{33}) are highly constrained to the C2′-endo conformation (Basti et al., 1996) and 2′-*O*-methyl nucleosides (as in ASL-Um_{33}) are highly constrained to the C3′-endo conformation (Kawai et al., 1992). Unaltered ribose, particularly those of uridines, have a dynamic sugar pucker that favors the C3′-endo conformation (Smith et al., 1992). Therefore, we propose

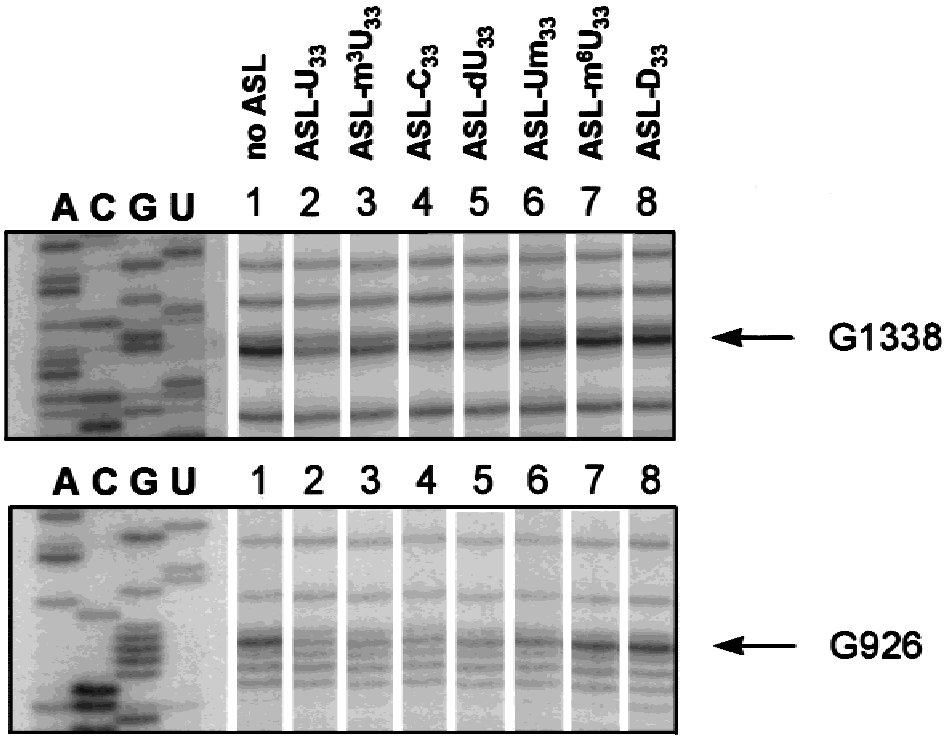

FIGURE 5. Protection of 16S P-site nucleotides (G1228 and G926) from kethoxal reactivity by variously substituted ASLs and ASL-U_{33}. Poly(U)-programmed 30S ribosomal subunits (10 pmol) were incubated with buffer alone (lane 1) or 50 pmol of one of the various ASLs (lanes 2–8). Lane 2: ASL-U_{33}; lane 3: ASL-m^3U_{33}; lane 4: ASL-C_{33}; lane 5: ASL-dU_{33}; lane 6: ASL-Um_{33}; lane 7: ASL-m^6U_{33}; and lane 8: ASL-D_{33}.

that the conformational dynamics of the U_{33} sugar pucker provided by the 2′-OH is functionally more important than its ability to hydrogen bond to the N^7 of position 35 purines. The need for a conformationally dynamic sugar pucker of U_{33} would be important to all tRNAs and not just those with a position 35 purine and suggests that nucleoside conformation and/or dynamics is critical for a functional U-turn.

The hydrogen bond from U_{33} N^3-H to the phosphate of nucleoside-36 also stabilizes the yeast $tRNA^{Phe}$ anticodon U-turn. This H-bond could be important to the ribosome binding of all tRNAs and important for the structure of all anticodon U-turns. ASLs containing C_{33} and m^6U_{33}, both of which preclude N^3-H bonding to phosphate-36, bound the ribosome with affinities that were 5% and $<0.5\%$ of that of the ASL-U_{33}, respectively. These results are in accord with a recent study in which substitution of pro-Rp-phosphate-36 with Rp-thiophosphate (which disrupts N^3-H bonding to phosphate-36) reduced ribosomal binding to about 6% of that of controls (Schnitzer & von Ahsen, 1997). Thus, the interaction N^3-H of U_{33} with phosphate-36 is important for a functional U-turn.

However, our experiments yielded one result that seemingly contradicted this conclusion. ASL-m^3U_{33} lacked the N^3-H but was bound by the ribosome almost as well as ASL-U_{33} (Fig. 4; Table 3). Methylated pyrimidines stack better than their unmodified counterparts (Sowers et al., 1987; Wang & Kool, 1995). N^3-methyluridine, though unable to form a hydrogen bond from N^3, would engage in stacking interactions to a higher degree than the unmodified uridine. The third contribution of U_{33} to the stability of the U-turn in the crystal structure of yeast $tRNA^{Phe}$ is its stacking interactions with C_{32} and the phosphate of nucleoside-35 (Fig. 1). In the previously cited study of individual thiophosphate substitutions (Schnitzer & von Ahsen, 1997), Rp-thiophosphate substitution of pro-Rp-phosphate-35 resulted in a reduction of ribosomal binding even more significant than substitution of phosphate-36. Although unexplained at the time, we believe that the decrease in ribosomal binding was probably due to the fact that substitution of the phosphate-35 oxygen with the less electronegative Rp-thiophosphate sulfur reduced the effectiveness of the ion-induced dipole interaction involving uracil-33 on phosphate-35 (Quigley & Rich, 1976). ASLs with Um_{33} and dU_{33} had the ability to hydrogen bond through N^3-H, yet they bound the ribosome poorly. Their strongly constrained C3′-endo and C2′-endo sugar puckers, respectively, could have changed the orientation of the N-glycosidic bond, thereby disrupting the stacking interactions between C_{32}, U_{33}, and phosphate-35. In contrast, the enhanced stacking of m^3U_{33} between C_{32} and phosphate-35 compensated for the lack of H-bonding from N^3 to the phosphate-36, thus stabilizing the ASL-m^3U_{33} and maintaining the conformation of the U-turn. The lack of ribosomal binding by both ASL-m^6U_{33} and ASL-D_{33} further demonstrated the importance of stacking on phosphate-35. Although both ASLs had the 2′-OH groups, the *syn* conformation of the m^6U_{33} and the puckered rather than the planar character of D_{33} prevented the bases from stacking between C_{32} and phosphate-35, as well as forming the N^3-H bond to phosphate-36. Thus, stacking of U_{33} between C_{32} and phosphate-35 may be a very important contribution of U_{33} to the functional U-turn.

Earlier studies by Uhlenbeck and coworkers using yeast $tRNA^{Phe}$ anticodon domains containing naturally occurring nucleoside modifications revealed that the invariant uridine at position 33 is not important for ribosomal binding (Uhlenbeck et al., 1982; Bare et al., 1983; Dix et al., 1986). Substitution of the uridine at position 33 to any nucleoside, including D, Um, or dU, was tolerated with only marginal effects on ribosomal binding. For example, substitution of U_{33} to Um_{33} in the ASL containing the naturally modified nucleosides reduced the K_d for poly(U)-programmed 30S ribosomal subunits to 30% of controls (Uhlenbeck et al., 1982). The disparity between results reported here and by von Ahsen et al. (1997) and that reported by Uhlenbeck et al. is most likely due to the presence of the natural modifications in the ASL/tRNA used by Uhlenbeck et al. We believe that in ASLs (or tRNAs) containing naturally occurring nucleoside modifications, substitutions at position 33 are tolerated because the nucleoside modifications help maintain the overall conformation of the anticodon U-turn. In fact, the ASL used by Uhlenbeck et al. had pseudouridine at position 39, wyebutosine at position 37, and 2′-*O*-methylguanosine at position 34, all of which contribute to the conformational rigidity of the anticodon loop (Davanloo et al., 1979; Davis & Poulter, 1991; Kawai et al., 1991; Kowalak et al., 1994). Further support for the role of modified nucleosides in conformational restraint of yeast $tRNA^{Phe}$ anticodon stem and loop comes from NMR studies of native yeast $tRNA^{Phe}$ as well as unmodified ASLs. Under conditions in which spectra of $tRNA^{Phe}$ (with naturally modified nucleosides) exhibited the U-turn signature phosphate (Gorenstein & Goldfield, 1982), ^{31}P-NMR analysis of the ASL failed to show the expected phosphorus signal (data not shown). The failure to observe the U-turn phosphate in the unmodified ASL is probably indicative of a dynamic unmodified anticodon loop with an equilibrium between various conformations. In fact, we have previously reported NMR data demonstrating that the anticodon loop of the unmodified ASL is dynamic (Chen et al., 1993), whereas the methyl groups in the anticodon loop of the fully modified native $tRNA^{Phe}$ are considerably more restricted than methyl groups in other regions of the molecule (Schmidt et al., 1987). Our results and that of von Ahsen et al. (1997) demonstrate that, unlike ASLs containing modified nucleosides, the functional conforma-

tion of the anticodon U-turn of unmodified ASLs is extremely sensitive to changes in U_{33} conformation. In fact, we have demonstrated that two site-selectively placed, simple methylations at positions 37 and 40 are sufficient to transform a nonbinding DNA analog of $tRNA^{Phe}$ ASL with dU at position 33 into a functional ribosome binder (Dao et al., 1994).

The data reported here confirm and elucidate results from previous studies demonstrating a requirement for the 2′-OH group (von Ahsen et al., 1997) as well as the N^3-H and the importance of the pro-Rp-phosphate oxygen at position 35 (Schnitzer & von Ahsen, 1997). In addition to explaining these previous observations, we clearly show (with an array of ASLs variously substituted at U_{33}) that the U_{33} nucleoside conformation, important for effective stacking of the uridine between C_{32} and phosphate-35, is a critical determinant for a functional U-turn in the anticodon. Proper U_{33} nucleoside conformation/dynamics may not only be critical for a functional anticodon U-turn, but also for U-turn motifs in RNA structures in general.

MATERIALS AND METHODS

Synthesis of ASLs and preparation of 30S ribosomal subunits

Unsubstituted ASLs and ASLs containing C, Um, dU, m^3U, m^6U, or D at position 33 were synthesized using standard solid phase chemistry for RNA synthesis and then purified by ion-exchange HPLC (Ogilvie et al., 1988; Agris et al., 1995). Synthesis of the m^6U phosphoramidite and structural analysis of the m^6U containing ASL are described elsewhere (E. Sochacka, G. Czerwinska, R. Cain, R. Guenther, G. Ansari, C. Yarian, P.F. Agris & A. Malkiewicz, in prep.). Denaturing polyacrylamide gel electrophoresis and HPLC analyses confirmed the purity of ASLs to be greater than 90%. Modification of the ASLs was confirmed by determining nucleoside composition (Gehrke et al., 1982). Small ribosomal subunits (30S) were prepared and activated as previously described (Ericson et al., 1995).

Thermal denaturation of the ASLs

Thermal denaturations of the $tRNA^{Phe}$ ASLs (in 10 mM sodium phosphate buffer, pH 7.2, 100 mM NaCl, and 0.1 mM EDTA) were monitored with UV using a Cary 3E spectrophotometer. Melting point temperature and other thermodynamic parameters were extracted from the major thermal transition occurring between 50 and 65 °C. The data was analyzed using Origin software and assuming a two-state model of denaturation. Curve-fitting for determining ΔS_{VH}, ΔH_{VH}, ΔG_{VH}, and T_m was restricted to the major transition because of the low temperature transition (~20–40 °C) in some ASLs. However, a two-state model of denaturation was justified because neither transition exhibited changes in T_m with concentration and were independent of each other, as judged by analysis of first derivative plots of the denaturation profiles (data not shown).

Binding of tRNAs and ASLs to 30S ribosomal subunits

To determine the binding affinities (K_d) of ribosomes for tRNA and various ASLs, 30S subunits (10 pmol) and poly(U) (10 μg) were incubated for 20 min at 37 °C with increasing amounts of ^{32}P-labeled ASL (up to 50 pmol) 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). The reaction was incubated for another 20 min on ice, passed through nitrocellulose filters (0.45 μm) and the filter-bound RNA washed twice with ice-cold CMN buffer (100 μL). The filters were then air-dried and counted. Disassociation constants and their standard deviations were determined using nonlinear regression analysis on four replicates of each experiment. To estimate the K_d values of ASLs substituted at U_{33}, a stoichiometry of 0.5–0.7 (as obtained for ASL-U_{33}) was assumed (von Ahsen et al., 1997).

Chemical probing of 16S P-site nucleotides protected by ASLs

Chemical probing for those 16S P-site nucleotides that were protected by the ASLs was accomplished as described (Moazed & Noller, 1990). Modifications with kethoxal (2 μL of 20-fold diluted in 20% ethanol) were conducted at 20 °C for 30 min in 40 μL reaction buffer (80 mM sodium cacodylate, pH 7.2, 100 mM NH_4Cl, 20 mM $MgCl_2$, 3 mM β-mercaptoethanol). Primer extension of chemically modified rRNA was performed by annealing 1 pmol of ^{32}P-labeled primer to 0.2 μg of rRNA in 2 μL of annealing buffer (40 mM Pipes, 400 mM NaCl, 1 mM EDTA, 80% formamide) for 10 min at 45 °C, followed by 10 min incubation on ice. Reverse transcription was started by adding 20 μL of the primer extension mixture (50 mM Tris-Cl, pH 8.2, 50 mM KCl, 6 mM $MgCl_2$, 10 mM DTT, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, and 1 U of AMV-RT). The reaction was allowed to proceed for 10 min at 42 °C and was then stopped by adding 100 μL of 10 mM Tris, pH 8.0, 1 mM EDTA to each tube. After phenol and chloroform extractions, reverse transcribed cDNA was ethanol precipitated, dried, and resuspended in 10 μL of 2/3 diluted formamide containing Bromophenol Blue and Xylene Cyanol dyes. The samples were heat denatured (2 min at 100 °C) before being subjected to gel electrophoresis (3 μL each sample) on an 8% sequencing gel.

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