Orientation of the tRNA anticodon in the ribosomal P-site: Quantitative footprinting with U₃₃-modified, anticodon stem and loop domains

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ABSTRACT

Binding of transfer RNA (tRNA) to the ribosome involves crucial tRNA–ribosomal RNA (rRNA) interactions. To better understand these interactions, U₃₃-substituted yeast tRNA^{Phe} anticodon stem and loop domains (ASLs) were used as probes of anticodon orientation on the ribosome. Orientation of the anticodon in the ribosomal P-site was assessed with a quantitative chemical footprinting method in which protection constants (K_p) quantify protection afforded to individual 16S rRNA P-site nucleosides by tRNA or synthetic ASLs. Chemical footprints of native yeast tRNA^{Phe}, ASL-U₃₃, as well as ASLs containing 3-methyluridine, cytidine, or deoxyuridine at position 33 (ASL-m³U₃₃, ASL-C₃₃, and ASL-dU₃₃, respectively) were compared. Yeast tRNA^{Phe} and the ASL-U₃₃ protected individual 16S rRNA P-site nucleosides differentially. Ribosomal binding of yeast tRNA^{Phe} enhanced protection of C1400, but the ASL-U₃₃ and U₃₃-substituted ASLs did not. Two residues, G926 and G1338 with K_p s $\approx$ 50–60 nM, were afforded significantly greater protection by both yeast tRNA^{Phe} and the ASL-U₃₃ than other residues, such as A532, A794, C795, and A1339 (K_p s $\approx$ 100–200 nM). In contrast, protections of G926 and G1338 were greatly and differentially reduced in quantitative footprints of U₃₃-substituted ASLs as compared with that of the ASL-U₃₃. ASL-m³U₃₃ and ASL-C₃₃ protected G530, A532, A794, C795, and A1339 as well as the ASL-U₃₃. However, protection of G926 and G1338 (K_p s between 70 and 340 nM) was significantly reduced in comparison to that of the ASL-U₃₃ (43 and 61 nM, respectively). Though protections of all P-site nucleosides by ASL-dU₃₃ were reduced as compared to that of the ASL-U₃₃, a proportionally greater reduction of G926 and G1338 protections was observed (K_p s = 242 and 347 nM, respectively). Thus, G926 and G1338 are important to efficient P-site binding of tRNA. More importantly, when tRNA is bound in the ribosomal P-site, G926 and G1338 of 16S rRNA and the invariant U₃₃ of tRNA are positioned close to each other.

Keywords: anticodon stem/loops; modified nucleosides; quantitative footprinting; ribosome

INTRODUCTION

Transfer and ribosomal RNAs play critical roles in both the decoding (Moazed & Noller, 1986, 1990) and peptidyl-transferase (Barta et al., 1984; Noller et al., 1992; Noller, 1993; Green et al., 1997) activities of the ribosome. Binding of tRNA to the mRNA-programmed ribosome involves tRNA–rRNA, as well as tRNA–mRNA contacts. Some tRNA–rRNA contacts have been identified with chemical footprinting techniques (Moazed & Noller, 1990; von Ahlsen & Noller, 1995). Numerous cross-linking studies have provided insights into tRNA

binding sites on the ribosome (Prince et al., 1982; Doring et al., 1994; Osswald et al., 1995). However, two of the more difficult challenges have been the identification of specific nucleoside interactions of tRNA's anticodon stem and loop with 16S rRNA and the orientation of the tRNA anticodon relative to the 16S nucleosides at the decoding site.

In exploring the specific structural requirements of tRNA for efficient ribosomal binding, we and others have shown that efficient binding of tRNA to the ribosome requires a proper tRNA anticodon U-turn conformation (von Ahlsen et al., 1997; Ashraf et al., 1999). Nucleoside substitutions of the invariant U₃₃, whether in the full-length tRNA or the tRNA anticodon stem and loop domain (ASL), adversely affected ribosomal binding. Here, we report the use of these variously substituted U₃₃ ASLs as probes of tRNA's anticodon interaction with and relative orientation to individual 16S rRNA P-site nucleosides. To determine and compare subtle as well

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as dramatic differences in the way various ASLs interacted with the ribosome, a quantitative footprinting assay was developed. This assay was adapted from the chemical probing of rRNA first reported by Moazed & Noller (1986, 1990). We quantified the subtle differences in chemical protection of individual 16S rRNA P-site nucleosides by native yeast tRNA^{Phe} and a completely unmodified tRNA^{Phe} ASL. Then, orientation of the tRNA anticodon relative to individual 16S rRNA P-site nucleosides was determined using the U₃₃-substituted ASLs and this quantitative footprinting technique.

RESULTS

Experimental design

U₃₃-substituted anticodon stem and loop domains (ASLs), chemically synthesized with the nucleoside sequence of the unmodified yeast tRNA^{Phe}, differed only at position 33, the position of the invariant U₃₃ that is 5'-adjacent to the anticodon (Fig. 1). U₃₃ substitutions were shown to affect structure of the ASL loop and, therefore, anticodon conformation and dynamics, but not the stem thermal stability (Ashraf et al., 1999). Changes in anticodon loop stability and conformation resulted in U₃₃-substituted ASLs binding the P-site of programmed ribosomes to varying degrees (Fig. 1). We used ASLs with quantifiable binding to the ribosome, ASL-U₃₃ and the U₃₃-substituted ASLs with

m³U₃₃, C₃₃, and dU₃₃, as probes of the anticodon's orientation at the ribosomal P-site. In doing so, we assessed the chemical footprinting of each of the four ASLs (as well as yeast tRNA^{Phe}) on the known 16S rRNA P-site nucleosides. However, to adequately differentiate the chemical protection of a particular 16S rRNA nucleoside by one ASL and compare it to another, each protection was quantified.

Quantitative footprinting has been used extensively in studying DNA-protein interactions (Brenowitz et al., 1986; Petri & Brenowitz, 1997). More recently it has been applied to the folding of the group I *Tetrahymena* ribozyme (Banerjee & Turner, 1995; Sclavi et al., 1998), as well as the study of RNA-antibiotic interactions (Wallis et al., 1995). However, an assay to examine quantitatively the chemical protections of ribosomal nucleotides upon tRNA or ASL binding had not been reported. In developing this methodology, criteria already established for valid quantitative footprinting methods (Brenowitz et al., 1986; Petri & Brenowitz, 1997) had to be addressed. Besides meeting these criteria, the use of ASLs rather than native tRNAs required establishing additional conditions. Many heptadecamer ASLs with five-membered base-paired stems and seven-membered loops begin to denature at temperatures lower than 37 °C (Ashraf et al., 1999), the temperature conventionally used for ribosomal footprinting (Moazed & Noller, 1990). Therefore, we determined the temperature as well as the time dependence of kethoxal and dimethyl sulfate (DMS) reactivity of 16S rRNA P-site nucleosides. Before applying the methodology to address the issue of anticodon orientation on the ribosome, we validated our approach by quantifying the subtle differences in footprints between native yeast tRNA^{Phe} and the ASL-U₃₃.

FIGURE 1. Primary sequence and secondary structure of the ASLs, and their relative binding affinities to the poly(U)-programmed 30S ribosomal subunit. ASL-U₃₃ had the same sequence as the unmodified anticodon stem and loop of yeast tRNA^{Phe}. Other ASLs were substituted at position-33 as follows: ASL-m³U₃₃, substituted with 3-methyluridine, ASL-C₃₃ with cytidine, ASL-dU₃₃ with deoxyuridine, ASL-Um₃₃ with 2'-O-methyluridine, ASL-m⁶U₃₃ with 6-methyluridine, and ASL-D₃₃ with dihydrouridine. Dissociation constants (K_d) for tRNA^{Phe} and each ASL were determined using filter binding as described in Materials and Methods.

Temperature dependence of chemical protection of P-site nucleotides by native yeast tRNA^{Phe} and ASL-U₃₃

The protection from chemical probes afforded to 16S rRNA P-site nucleosides (in particular G926, G966, and G693) and G530 by the ASL-U₃₃ and native yeast tRNA^{Phe} was analyzed as a function of temperature (Fig. 2). These experiments determined not only a temperature at which most P-site nucleosides could be probed with ASLs, but also conditions that would be equally suitable for comparisons to native yeast tRNA^{Phe}. The P-site residues G926 and G530 were protected from chemical modification by tRNA^{Phe} at all temperatures tested (4, 15, 25, and 37 °C). However, G966 and G693, though protected from kethoxal reactivity at the higher temperature (37 °C), were not protected by tRNA^{Phe} at the lower temperatures (Fig. 2A). The inability of yeast tRNA^{Phe} to protect G966 and G693 from reacting with kethoxal at lower temperatures probably reflects the dynamic nature of ribosomes, such that at

FIGURE 2. Protection of 16S rRNA nucleosides from kethoxal reactivity at various temperatures by either (A) yeast tRNA^{Phe} or (B) ASL-U₃₃. A, C, G, and U represent the individual nucleoside sequencing lanes and K is the control lane showing the natural stops in reverse transcription of 16S rRNA. —: lanes containing incubation mixtures of 10 pmol 30S ribosomal subunits and 10 μ g poly(U), but without tRNA or ASL-U₃₃. +: lanes containing 10 pmol 30S and 10 μ g poly(U) plus 50 pmol of yeast tRNA^{Phe} (A) or ASL-U₃₃ (B).

low temperatures, these particular rRNA residues are less dynamic and though accessible for chemical reactivity, they are not available for interaction with tRNA.

As with tRNA^{Phe}, protection of P-site nucleosides with the ASL-U₃₃ was also temperature sensitive (Fig. 2B). G926 (data not shown) and G530 were protected at lower temperatures (4, 15, and 25 °C), but not at 37 °C. The inherent instability of the ASL-U₃₃ at 37 °C (Ashraf et al., 1999) probably accounts for the lack of protection of G530 (and G926) at this temperature. However, the ASL did not protect G693 at 37 or at 4 °C, and only weakly at 25 and 15 °C. Probably the inability of the ASL to protect G693 at these temperatures was because of the lack of ribosomal dynamics at lower temperatures and the melting of the ASL loop at higher temperatures. For this reason, only six (A532, A794, C795, G926, G1338, and A1339) of the nine previously identified tRNA P-site footprints (A532, G693, A794, C795, G926, G966, G1338, A1339, and G1401) could be examined at the temperature used for the quantitative footprinting (20 °C). Under conditions where 16S rRNA P-site nucleosides were protected from chemical probes by yeast tRNA^{Phe}, we consistently observed protection of G530, by both yeast tRNA^{Phe} and the ASL-U₃₃ (Fig. 2A and Fig. 2B). Therefore, G530 [which was initially assigned to the ribosomal A-site (Moazed & Noller, 1990)], as well as C1400, which had an enhanced chemical reactivity upon yeast tRNA^{Phe} binding, were included in this study.

Codon dependence of the chemical footprinting and time dependence of the chemical probing were investigated with the ASL-U₃₃. Codon dependence of the footprinting was confirmed by using a heptadecamer ASL in which the poly(U) responsive anticodon of tRNA^{Phe}, G₃₄A₃₅A₃₆, was changed to G₃₄C₃₅A₃₆. As expected, ASL-G₃₄C₃₅A₃₆ did not protect 16S rRNA P-site residues from chemical reactivity (data not shown). A time-dependent evaluation was conducted for both kethoxal and DMS reactions. At the concentrations of kethoxal and DMS chosen for the experiments, the degree of protection of the 16S rRNA nucleosides by the ASL-U₃₃ and by tRNA had reached equilibrium within 20 min at 20 °C (data not shown).

Quantification of yeast tRNA^{Phe}–rRNA interactions at specific 16S rRNA residues

To accurately quantify tRNA–rRNA interactions at individual 16S rRNA P-site nucleosides, poly(U)-programmed 30S ribosomal subunits were titrated with increasing amounts of tRNA^{Phe} or the ASL-U₃₃. The degree of protection from chemical probes afforded by tRNA^{Phe} or the ASL at each of the eight P-site nucleosides was quantified with the aid of a Phosphorimager. For example, titration of poly(U)-programmed 30S ribosomal subunits with increasing amounts of ASL resulted in an increased protection of G1338 from kethoxal reactivity (Fig. 3A). The intensity of the G1338 band in each lane was quantified and was used as a direct measure of “binding” (i.e., the degree to which the 16S rRNA nucleoside was protected from the chemical probe). The protection of G1338 from kethoxal reactivity by the ASL-U₃₃ followed a one-site binding isotherm, and the apparent protection constant (K_p) was determined by both nonlinear regression (Fig. 3B) and Scatchard analysis (Fig. 3C) to be 69 nM and 65 nM, respectively, for this particular experiment, and 61 ± 25 nM after multiple analyses.

Differential protection of individual P-site nucleosides by yeast tRNA^{Phe} and the ASL-U₃₃

The quantitative footprinting of 16S rRNA nucleosides by the ASL-U₃₃ was compared to that of native yeast tRNA^{Phe}. The 16S rRNA nucleosides that were protected from chemical probes by tRNA^{Phe} were also protected by the ASL (Fig. 4A and Fig. 4B). Nonlinear regression was used to derive a quantitative measure for these protections, K_p (Fig. 4 and Table 1). Yeast tRNA^{Phe} differentially protected the various 16S rRNA P-site nucleosides; for example, G530, G926, and G1338 were protected to a higher degree than A532, A794, C795, A1339, and C1400. The ASL-U₃₃ protected the same 16S rRNA residues to approximately the same degrees as yeast tRNA^{Phe}, except for G530. The protection of G530 from kethoxal by the ASL was

FIGURE 3. Quantification of chemical protection (K_p) afforded by ASL-U₃₃ to G1338. Protection of the 16S rRNA nucleotide (G1338) was assessed quantitatively by titrating poly(U)-programmed 30S ribosomal subunits with increasing concentrations of ASL-U₃₃. **A:** Dideoxy-sequencing gel for determining the protection of G1338. A, C, G, and U represent dideoxy sequencing lanes of 16S rRNA, K is the control lane showing the natural reverse transcription stops in 16S rRNA. Lanes 1–10 contain 10 pmol of 30S ribosomal subunits plus 10 μ g poly(U) and increasing amounts of ASL^{Phe} (0, 3, 6, 9, 12, 15, 20, 25, 30, and 50 pmol). The K_p for the protection of G1338 from kethoxal reactivity by the ASL-U₃₃ was determined from a nonlinear regression (**B**) and a Scatchard analysis (**C**). An apparent K_p of 30 nM and a stoichiometry of ASL^{Phe}/30S ribosomal subunit 0.81 were obtained using both analyses.

significantly reduced ($K_p = 96 \pm 60$ nM) as compared to that afforded by tRNA^{Phe} ($K_p = 25 \pm 18$ nM). C1400 reactivity to DMS was enhanced upon binding of yeast tRNA^{Phe}, as previously reported (Moazed & Noller, 1990). Yet, strikingly the binding of the ASL-U₃₃ to 30S ribosomes failed to enhance the reactivity of C1400 (Fig. 4B). Furthermore, C1400 failed to show any enhancement even upon addition of five molar excess of the ASL-U₃₃.

Differential protection of individual P-site nucleotides by various U₃₃-substituted ASLs

The quantitative footprints of ASL-m³U₃₃, ASL-C₃₃, and ASL-dU₃₃, all with reduced but significant ribosomal binding (Fig. 1), were assessed and compared to each other as well as that of ASL-U₃₃. The four ASLs pro-

tected the 16S rRNA P-site nucleosides, A532, A794, C795, G926, G1338, and A1339, and G530 differentially (Fig. 5). Reactivity of C1400 was not enhanced upon binding of any of the ASLs. The degrees of protection conferred by the ASLs at the various P-site nucleosides were not equal. Some ASLs, such as ASL-dU₃₃, did not protect the P-site nucleosides as completely as ASL-U₃₃ or tRNA^{Phe}. Indeed, quantification of the footprinting demonstrated that significant differences existed in the degrees to which the three U₃₃-substituted ASLs differentially protected the P-site nucleosides (Table 2). ASL-m³U₃₃, ASL-C₃₃, and ASL-U₃₃ protected six of the eight 16S rRNA P-site nucleosides similarly. The two exceptions were G926 and G1338. Protection of G926 and G1338 by ASL-m³U₃₃ was reduced to one-half and one-fourth ($K_p = 70 \pm 5$ nM; $K_p = 270 \pm 68$ nM) of that afforded by

FIGURE 4. Yeast tRNA^{Phe} or ASL-U₃₃ protection of 16S rRNA P-site nucleosides from reacting with (A) kethoxal and (B) DMS. Small (30S) ribosomal subunits (10 pmol) and 10 μ g poly(U) were incubated with increasing concentrations of yeast tRNA^{Phe} or ASL-U₃₃ (0, 4, 8, 12, 24, and 50 pmol) as described in Materials and Methods. The 16S rRNA sequencing lanes are denoted with A, C, G, and U.

ASL-U₃₃, respectively. Similarly, protections of G926 and G1338 by ASL-C₃₃ were reduced to one-half and one-sixth ($K_p = 82 \pm 7$ nM; $K_p = 340 \pm 76$ nM) that of the ASL-U₃₃, respectively. ASL-dU₃₃ had a similarly selective effect on the individual K_p s. However, ASL-dU₃₃ protected all of the P-site nucleosides more poorly than the ASL-U₃₃ (Table 2, shaded area).

DISCUSSION

Site-specific substitution of U₃₃ resulted in the production of a set of ASLs with varying degrees of ribosomal binding (Ashraf et al., 1999; Fig. 1). We have assessed the interaction of the various U₃₃-substituted ASLs and yeast tRNA^{Phe} with individual 16S rRNA P-site nucleosides. With the ASLs, we were able to footprint the local space occupied by U₃₃ and the anticodon in the ribosomal P-site. However, to differentiate objectively the footprint of one particular ASL from another, we developed a quantitative footprinting method based on the initial chemical probing approach of Moazed and Noller (1990).

Development of this assay for tRNA's binding to the ribosome included meeting or exceeding the already established criteria for quantitative footprinting (Brenowitz et al., 1986; Petri & Brenowitz, 1997): (1) An equilibrium rather than a kinetic process is being stud-

ied; (2) The experimental procedures (minor base alkylation and sugar substitution) do not dramatically perturb the interaction being studied; and (3) The results are consistent with an independent method (von Ahsen et al., 1997; Ashraf et al., 1999). In addition, the quantitative protection data for the individual 16S rRNA P-site nucleosides were found to correspond to a one-site binding isotherm. Furthermore, in determining the conditions for this method, we found that the 16S rRNA tertiary structure was very sensitive to temperature and that the protection of certain P-site nucleosides (e.g., G530) from chemical probes by ASL cannot be observed above 25 °C (Fig. 2B). This finding and the thermal instability of the ASL compared to that of tRNA required that assays be performed at 20 °C.

Examination of the degree of protection (via protection constants, K_p) afforded the various 16S rRNA P-site nucleosides by yeast tRNA^{Phe} and the ASL-U₃₃ demonstrated that both the tRNA and the ASL interacted with individual P-site nucleosides with different K_p s (Table 1). Quantitative footprinting results for yeast tRNA^{Phe} and ASL-U₃₃ were comparable to the previously published (Moazed & Noller, 1986), visually estimated reactivities of the various 16S rRNA P-site nucleosides (Table 1). However, in addition to protection of the recognized P-site nucleosides, we always observed protection of G530 by both yeast tRNA^{Phe}

TABLE 1. Protection constants^a for the interaction of yeast tRNA^{Phe} or ASL^{Phe} with individual 30S P-site nucleotides.

16S P-site nucleotide	Relative reactivity ^b	yeast tRNA ^{Phe}	ASL-U ₃₃
G530	+++	25 ($\pm$ 18)	96 ($\pm$ 60)
A532	+	200 ($\pm$ 29)	195 ($\pm$ 17)
A794	+	123 ($\pm$ 52)	170 ($\pm$ 44)
C795	$\pm$	176 ($\pm$ 32)	103 ($\pm$ 48)
G926	+++	53 ($\pm$ 23)	43 ($\pm$ 10)
G1338	++	52 ($\pm$ 27)	61 ($\pm$ 25)
A1339	$\pm$	149 ($\pm$ 57)	199 ($\pm$ 71)
C1400 ^c	$\pm$	479 ($\pm$ 18)	Not detected

^aProtection constants are in nM for binding in 20 mM MgCl₂ at 20 °C.

^bVisually estimated reactivity (+++: hyperreactive; $\pm$: marginally reactive) (Moazed & Noller, 1986).

^cBinding of yeast tRNA^{Phe} to programmed-30S ribosomal subunit caused an enhancement of C1400 reactivity, deprotection. However, a protection constant of C1400 upon yeast tRNA^{Phe} binding to 30S ribosomal subunit was calculated for this enhanced reactivity to compare P-site nucleoside reactivities.

FIGURE 5. Chemical footprints derived from protection of 16S rRNA P-site nucleosides by yeast tRNA^{Phe}, ASL-U₃₃, or various U₃₃-substituted ASLs. Poly(U)-programmed 30S ribosomal subunits (10 pmol) were incubated with buffer alone (lane marked -), or 50 pmol of tRNA^{Phe} or ASL as indicated. A, C, G, and U represent the 16S rRNA sequencing lanes. Chemical probing with (A) kethoxal or (B) DMS were carried out as described in Materials and Methods.

and ASL. G530 is considered to be a tRNA A-site footprint. Our observation with G530 is consistent with that reported by Moazed and Noller (1990) who found that P-site bound tRNA weakly protected G529 and G530.

TABLE 2. Protection constants^a for the interaction of variously U₃₃-modified ASLs with individual 30S P-site nucleotides.

16S P-site nucleotide	ASL-U ₃₃	ASL-m ³ U ₃₃	ASL-C ₃₃	ASL-dU ₃₃
G530	96 (± 60)	105 (± 34)	107 (± 43)	293 (± 24)
A532	195 (± 17)	173 (± 66)	106 (± 17)	216 (± 63)
A794	170 (± 44)	135 (± 22)	163 (± 68)	378 (± 38)
C795	103 (± 48)	129 (± 26)	134 (± 25)	349 (± 101)
G926	43 (± 10)	70 (± 5)	82 (± 7)	242 (± 86)
G1338	61 (± 25)	270 (± 68)	340 (± 76)	377 (± 79)
A1339	199 (± 71)	285 (± 111)	226 (± 43)	416 (± 125)
C1400	Not detected ^b	Not detected ^b	Not detected ^b	Not detected ^b

^aProtection constants are in nM for binding in 20 mM MgCl₂ at 20 °C.

^bEnhancement of C1400 accessibility/reactivity to DMS was only observed upon yeast tRNA^{Phe} binding, and not with any of the ASLs tested.

G530 may reside at the boundary of the A- and P-sites and be shared by both the sites. An alternative explanation of the qualitative and quantitative protections of G530 is that the binding of yeast tRNA^{Phe} (or ASL) to the 30S ribosomal subunit results in a conformational change in 16S rRNA such that G530 is footprinted in both the A- and P-sites. We believe further research is warranted before G530 is conclusively assigned to either the A- or P-site, or to a dynamic of the ribosome.

Results from the quantitative footprinting defined two classes of 16S rRNA P-site nucleosides; G530, G926 and G1338 with lower *K_p*s, and A532, A794, C795, and A1339 with significantly higher *K_p*s. The lower *K_p*s for G530, G926, and G1338 as compared to A532, A794, C795, and A1339 may reflect that the two groups of nucleosides reside in spatially different environments or are differentially protected upon tRNA binding. Alternatively, the weakly protected sites may result from "conformational" footprints, for example, an altered conformation of 16S rRNA due to tRNA binding. The 16S rRNA residues that are protected by yeast tRNA^{Phe} (or ASL) to a higher degree may be in close proximity to the anticodon. This hypothesis is supported by data from two laboratories. G926 was identified as one of the 16S rRNA residues that may possibly make inter-molecular contacts between rRNA and tRNA (von Ahsen

& Noller, 1995). Also, nucleoside 32 just 5' adjacent to the invariant U₃₃ of *Escherichia coli* tRNA^{Phe} has been cross-linked with a photoreactive diazirine derivative to G1338 in 16S rRNA (Doring et al., 1994) indicating a close spatial relationship (within 10 Å) between the position 32 of the anticodon loop and G1338.

In addition to showing differential protection of the individual P-site nucleosides in 16S rRNA, quantitative footprinting also highlighted subtle differences between the ribosomal binding of native tRNA^{Phe} and the ASL-U₃₃. The ASL-U₃₃ protected the 16S P-site nucleosides very similarly to yeast tRNA^{Phe}, except for two notable differences, G530 and C1400. Residue G530 was protected to a higher degree by tRNA^{Phe} than by ASL-U₃₃ (tRNA^{Phe}: $K_p = 25 \pm 18$ nM; ASL-U₃₃: $K_p = 96 \pm 60$ nM), whereas C1400, whose chemical reactivity was enhanced upon yeast tRNA^{Phe} binding, did not exhibit any enhancement with the binding of ASL-U₃₃. A fully modified yeast tRNA^{Phe} ASL (with all the nucleoside modifications) binds to 30S ribosomal subunits with the same binding affinity (K_d) as yeast tRNA^{Phe} (Rose et al., 1983) and produces the same footprints (including the C1400 enhancement) on 16S rRNA as yeast tRNA^{Phe} (Moazed & Noller, 1986). The anticodon stem and loop is the only region of the tRNA molecule that is protected from hydroxyl radical probing by the 30S ribosomal subunit (Huttenhofer & Noller, 1992). Therefore, we believe the differences in the K_p s observed for the interaction of the 16S rRNA nucleosides with ASL-U₃₃ versus yeast tRNA^{Phe} (Table 1) are due to the lack of modified nucleosides in the ASL, and not due to its smaller size or sequence length. The enhancement of C1400 reactivity with the binding of yeast tRNA^{Phe} was thought to result from the 2'-O-methyl modification of G₃₄ (Gm₃₄) in that tRNA's anticodon, as this enhancement was not observed upon binding of *E. coli* tRNA^{Phe} that has an unmethylated G at position 34 (Moazed & Noller, 1986). In fact, we have recently observed that Gm₃₄ was the only modification in the yeast tRNA^{Phe} ASL able to produce the C1400 enhancement. This effect was observed only with Gm₃₄-containing ASLs (Ashraf et al., in prep.) and is consistent with the fact that C1400 has been directly cross-linked to the anticodon position 34 of tRNA (Prince et al., 1982). Thus, C1400 lies very close to the tRNA anticodon in the P-site, and probably adjacent to position 34.

Using quantitative footprinting, we have probed 16S rRNA nucleosides with U₃₃-substituted ASLs to gain a better understanding of the orientation of the tRNA anticodon in the ribosomal P-site. This approach depended on the conformational perturbation of U₃₃ and the U-turn rather than the use of bulky cross-linking reagents with the potential to structurally perturb 16S rRNA nucleosides. ASL-m³U₃₃ protected the P-site nucleosides with K_p s similar to ASL-U₃₃ except for G926 and G1338. K_p s for G926 and G1338 were two- and four-fold higher than that observed with ASL-U₃₃, respec-

tively (Table 2). Likewise, the only observed differences in the K_p s between ASL-C₃₃ and ASL-U₃₃ were the significantly higher K_p s for G926 and G1338. Though protections of all P-site nucleosides by ASL-dU₃₃ were reduced as compared to that of the ASL-U₃₃, a proportionally greater reduction of G926 and G1338 protections was observed.

The observation that ASL-m³U₃₃ and ASL-C₃₃ protected 16S rRNA nucleosides to a similar degree, as well as the fact that the K_p s obtained with ASL-C₃₃ and ASL-dU₃₃ were very different (Table 2), were surprising, as they seemed to contradict data from filter-binding experiments (Fig. 1). In the filter-binding assay, ASL-m³U₃₃ bound to programmed 30S ribosomes ($K_d = 220 \pm 20$ nM) almost as well as ASL-U₃₃ ($K_d = 140 \pm 50$ nM), whereas ASL-C₃₃ and ASL-dU₃₃ had dramatically lower affinities (K_d s = $2,190 \pm 30$ nM and $2,930 \pm 140$, respectively) (Fig. 1; Ashraf et al., 1999). Quantification of tRNA's or ASL's protection of individual 16S rRNA nucleosides from chemical probes (K_p) is not immediately comparable to the overall binding affinity (K_d) for tRNA binding to the ribosome. The binding of tRNA at the decoding center of a programmed 30S ribosomal subunit is a complex and poorly understood phenomenon, and involves the dynamic nature of the ribosome (Fig. 2; Woodson & Leontis, 1998). However, quantitative footprinting data obtained from simpler systems such as RNA aptamer-antibiotic interaction (Wallis et al., 1995) can be related to binding constants. In tRNA-ribosome interaction, chemical probing examines individual interactions between specific 16S rRNA nucleosides and the ASLs. Upon tRNA binding, some tRNA footprints on 16S rRNA may be due to conformation-restricted interactions, and may therefore exhibit differential protection from chemical probes. In contrast, the filter-binding assay is reflective of the simultaneous interaction between many parts of the whole 30S ribosome (rRNA and proteins) and the ASL. In fact, S7 and S11 proteins of the small ribosomal subunit have been cross-linked to the anticodon region of P-site bound tRNA (Podkowinski & Gornicki, 1989; Doring et al., 1994). Though, related to K_d , the exact correlation between the individual protection constants and the overall binding constant is yet to be defined. However, protection constants still can be exploited for understanding tRNA's interaction with rRNA, and can be especially helpful in analyzing and comparing the ribosomal binding of a set of ASLs or tRNA.

More significantly, the fact that altering the U-turn conformation of the ASL with three different U₃₃ substitutions selectively affected protection of G926 and G1338 is strongly suggestive that these two 16S rRNA nucleosides are in the vicinity of the P-site tRNA anticodon U-turn. We cannot rule out the possibility that differential protection was an indirect result of the ASLs perturbing the interaction of mRNA (polyU) with 16S rRNA. However, close proximity of G1338 and G926 to

the decoding center of 16S rRNA already has been postulated on the basis of cross-linking data, for example, tRNA derivatized with diazirine at position 32 cross-linked to G1338 (Doring et al., 1994). In addition, G926 cross-linked to the +2 position of the mRNA start codon (diazirine modified U of AUG) only in the presence of tRNA^{met} (Sergiev et al., 1997). Hence, our approach of using locally perturbed ASLs as probes for tRNA binding sites of 16S rRNA provided independent confirmation of tRNA's orientation at the decoding site.

The study presented here outlines a powerful approach to study biomolecular interactions involving nucleic acids. Site-selective incorporation of specifically altered nucleosides to cause local perturbations in nucleic acid structure followed by quantitative footprinting can potentially provide insight into nucleic acid–protein as well as nucleic acid–nucleic acid interactions.

MATERIALS AND METHODS

Materials

Yeast tRNA^{Phe}, nucleosides, deoxynucleosides, and AMV-RT were purchased from Boehringer-Mannheim. Synthetic RNA heptadecamers representing the unmodified anticodon stem and loop of yeast tRNA^{Phe} (ASL-U₃₃) and the various U₃₃-substituted ASLs containing C, dU, and m³U were synthesized by solid phase chemistry by the North Carolina State University Nucleic Acids Facility and were purified by ion-exchange and reverse-phase chromatography (Agris et al., 1995). Primers complementary to *E. coli* 16S rRNA sequences were used for reverse transcription. The complement to 1453–1469 was used to probe G1338, A1339, and C1400; the complement to 861–878 was used to probe A794 and C795; the complement to 950–965 was used to probe G926; the complement to 560–575 was used to probe G530 and A532; the complement to 1016–1032 was used to probe G966 and G926; and the complement to 1016–1032 was used to probe G530 and G693. Poly(U) was purchased from Sigma. Dimethyl sulfate was purchased from Aldrich and kethoxal was from Research Organics. ATP-[γ -³²P] (3,000 Ci/mmol) was purchased from ICN.

Preparation of 30S ribosomal subunits

Small ribosomal subunits (30S) were prepared from *E. coli* according to Ericson et al. (1995). Before conducting the binding of yeast tRNA^{Phe} or the ASLs, 30S ribosomal subunits were activated by incubating in buffer (20 mM Tris, pH 7.5, 200 mM NH₄Cl, 20 mM MgCl₂, 2 mM dithiothreitol [DTT]) at 37 °C for 30 min (Ericson et al., 1995).

Filter binding of yeast tRNA^{Phe} and ASLs to 30S ribosomal subunits

The filter binding assay for determining tRNA and ASL binding affinities to programmed 30S ribosomal subunits was accomplished as described previously (Ashraf et al., 1999). Briefly, binding of yeast tRNA^{Phe} and the various ASLs was

measured by incubating 10 pmol 30S subunit and 10 μ g poly(U) with increasing amounts of yeast tRNA^{Phe} or ASLs (up to 50 pmol) in 40 μ L of 80 mM potassium-cacodylate buffer (pH 7.2, 20 mM MgCl₂, 100 mM NH₄Cl, 3 mM β -mercaptoethanol) for 20 min at 37 °C. A 20-min incubation on ice followed, as previously reported (Ashraf et al., 1999), before passing the incubation mixture through the filter. The codon specificity of the ribosomal binding assay was confirmed by using a synthetic heptadecamer corresponding to the anticodon stem and loop of yeast tRNA^{Phe}, except that the anticodon was changed from 5'-GAA-3' to 5'-GCA-3'.

Chemical modification and primer extension

Chemical probing was accomplished as described by Moazed and Noller (1986). However, chemical modifications with DMS (1 μ L of threefold diluted in 100% ethanol) and kethoxal (2 μ L of 20-fold diluted in 20% ethanol) were conducted at 20 °C for 30 min in 40 μ L of buffer (80 mM sodium cacodylate, pH 7.2, 100 mM NH₄Cl, 20 mM MgCl₂, 3 mM β -mercaptoethanol). In a temperature-dependent study of protection, the amount of kethoxal and the incubation times were varied because of the reduced reactivity of kethoxal at low temperatures. The concentration of kethoxal and incubation time ranged from 1 μ L of 20-fold diluted kethoxal for 25 min at 37 °C to 2 μ L of 20-fold kethoxal for 4 h at 4 °C. Primer extension of chemically modified rRNA was performed by annealing 1 pmol of ³²P-labeled primer to 0.2 μ g of rRNA in 2 μ L of 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 initiated by adding 20 μ L of the mixture containing 50 mM Tris-Cl, pH 8.2, 50 mM KCl, 6 mM MgCl₂, 10 mM DTT, and 0.5 mM of each dATP, dCTP, dGTP, and dTTP plus 1 U of AMV-RT. The reaction was allowed to proceed for 10 min at 42 °C and was then stopped by adding 100 μ L of 10 mM Tris, pH 8.0, and 1 mM EDTA to each tube. After phenol and chloroform extractions, reverse-transcribed cDNA was ethanol precipitated, dried, and resuspended in 10 μ L of $\frac{2}{3}$ -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 μ L each sample) on a 6–8% sequencing gel.

Phosphorimager analysis and data manipulation

For quantifying of the 16S rRNA protections by yeast tRNA^{Phe} or the ASLs, the dried sequencing gels were exposed to a Phosphor-Screen that were scanned on a Molecular Dynamics PhosphorImager. Intensities of bands corresponding to specific residues were measured. Errors due to uneven sample loading were minimized by normalizing the intensities of protected nucleotides against a non-tRNA responsive (natural) reverse transcription stop (usually within 5 nt of the nucleotide to be quantitated). No significant differences were observed when the non-tRNA responsive RT stop used for normalization was located either before or after the 16S nucleotide of interest. The difference in intensity of a specific band (16S rRNA residue) in a lane without yeast tRNA^{Phe} or the ASL (usually the first lane) as compared to the lane with 5-molar excess yeast tRNA^{Phe} or the ASL (usually the last

lane) represented the total tRNA^{Phe} (or ASL) reactive sites. Residual reactivity of the specific 16S rRNA bands at 5-molar excess tRNA^{Phe} or ASL was a measure of inactive sites. Inactive sites were not protected from chemical reactivity at high tRNA^{Phe}/30S or ASL/30S ratios and were used to calculate the percentage of active ribosomes in the preparations (usually 65–85% active). Also, this value (residual, background intensity at 5-molar excess tRNA^{Phe}) was subtracted from each lane to obtain the net intensity of the specific 16S nucleosides. Thus, the 100% bound state was assumed to be achieved at 5-molar excess. Zero percent tRNA^{Phe} (or ASL) bound was determined from the net intensity in the absence of any tRNA^{Phe} or ASL (the first lane). The amount of the bound tRNA^{Phe} (or ASL) was subtracted from the amount of added tRNA^{Phe} (or ASL) to determine the amount of free tRNA^{Phe} (or ASL). Prism software (from Graphpad Inc.) was used for Scatchard and nonlinear regression analysis of the data.

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REFERENCES

- Agris PF, Malkiewicz A, Kraszewski A, Everett K, Nawrot B, Sochacka E, Jankowska J, Guenther R. 1995. Site-selected introduction of modified purine and pyrimidine ribonucleosides into RNA by automated phosphoramidite chemistry. *Biochimie* 77:125–134.
- Ashraf SS, Ansari G, Guenther R, Sochacka E, Malkiewicz A, Agris PF. 1999. The uridine in "U-turn": Contributions to tRNA–ribosomal binding. *RNA* 5:503–511.
- Banerjee AR, Turner DH. 1995. The time dependence of chemical modification reveals slow steps in the folding of a group I ribozyme. *Biochemistry* 34:6504–6512.
- Barta A, Steiner G, Brosius J, Noller HF, Kuechler E. 1984. Identification of a site on 23S ribosomal RNA located at the peptidyl transferase center. *Proc Natl Acad Sci USA* 81:3607–3611.
- Brenowitz M, Seneff DF, Shea MA, Ackers GK. 1986. "Footprint" titrations yield valid thermodynamic isotherms. *Proc Natl Acad Sci USA* 83:8462–8466.
- Doring T, Mitchell P, Osswald M, Bochkariov D, Brimacombe R. 1994. The decoding region of 16S RNA; a cross-linking study of the ribosomal A, P, and E sites using tRNA derivatized at position 32 in the anticodon loop. *EMBO J* 13:2677–2685.
- Ericson G, Minchew P, Wollenzien P. 1995. Structural changes in base-paired region 28 in 16 S rRNA close to the decoding region of the 30 S ribosomal subunit are correlated to changes in tRNA binding. *J Mol Biol* 250:407–419.
- Green R, Samaha RR, Noller HF. 1997. Mutations at nucleotides G2251 and U2585 of 23 S rRNA perturb the peptidyl transferase center of the ribosome. *J Mol Biol* 266:40–50.
- Huttenhofer A, Noller HF. 1992. Hydroxyl radical cleavage of tRNA in the ribosomal P site. *Proc Natl Acad Sci USA* 89:7851–7855.
- Moazed D, Noller HF. 1986. Transfer RNA shields specific nucleotides in 16S ribosomal RNA from attack by chemical probes. *Cell* 47:985–994.
- Moazed D, Noller HF. 1990. Binding of tRNA to the ribosomal A and P sites protects two distinct sets of nucleotides in 16 S rRNA. *J Mol Biol* 211:135–145.
- Noller HF. 1993. tRNA–rRNA interactions and peptidyl transferase. *FASEB J* 7:87–89.
- Noller HF, Hoffarth V, Zimniak L. 1992. Unusual resistance of peptidyl transferase to protein extraction procedures. *Science* 256:1416–1419.
- Osswald M, Doring T, Brimacombe R. 1995. The ribosomal neighborhood of the central fold of tRNA: Cross-links from position 47 of tRNA located at the A, P, or E site. *Nucleic Acids Res* 23:4635–4641.
- Petri V, Brenowitz M. 1997. Quantitative nucleic acids footprinting: Thermodynamic and kinetic approaches. *Curr Opin Biotechnol* 8:36–44.
- Podkowinski J, Gornicki P. 1989. Ribosomal proteins S7 and L1 are located close to the decoding site of *E. coli* ribosome: Affinity labeling studies with modified tRNAs carrying photoreactive probes attached adjacent to the 3'-end of the anticodon. *Nucleic Acids Res* 17:8767–8782.
- Prince JB, Taylor BH, Thurlow DL, Ofengand J, Zimmermann RA. 1982. Covalent cross-linking of tRNA^{Val} to 16S RNA at the ribosomal P site: Identification of cross-linked residues. *Proc Natl Acad Sci USA* 79:5450–5454.
- Rose SJD, Lowary PT, Uhlenbeck OC. 1983. Binding of yeast tRNA^{Phe} anticodon arm to *Escherichia coli* 30 S ribosomes. *J Mol Biol* 167:103–117.
- Sclavi B, Sullivan M, Chance MR, Brenowitz M, Woodson SA. 1998. RNA folding at millisecond intervals by synchrotron hydroxyl radical footprinting. *Science* 279:1940–1943.
- Sergiev PV, Lavrik IN, Wlasoff VA, Dokudovskaya SS, Dontsova OA, Bogdanov AA, Brimacombe R. 1997. The path of mRNA through the bacterial ribosome: A site-directed cross-linking study using new photoreactive derivatives of guanosine and uridine. *RNA* 3:464–475.
- von Ahlen U, Green R, Schroeder R, Noller HF. 1997. Identification of 2'-hydroxyl groups required for interaction of a tRNA anticodon stem-loop region with the ribosome. *RNA* 3:49–56.
- von Ahlen U, Noller HF. 1995. Identification of bases in 16S rRNA essential for tRNA binding at the 30S ribosomal P site. *Science* 267:234–237.
- Wallis MG, von Ahlen U, Schroeder R, Famulok M. 1995. A novel RNA motif for neomycin recognition. *Chem Biol* 2:543–552.
- Woodson SA, Leontis NB. 1998. Structure and dynamics of ribosomal RNA. *Curr Opin Struct Biol* 8:294–300.