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Identification of 2-hydroxyl groups required for interaction of a tRNA anticodon stem-loop region with the ribosome

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ABSTRACT

Synthetic RNA stem loops corresponding to positions 28–42 in the anticodon region of $tRNA^{Phe}$ bind efficiently in an mRNA-dependent manner to ribosomes, whereas those made from DNA do not. In order to identify the positions where ribose is required, the anticodon stem-loop region of $tRNA^{Phe}$ (*Escherichia coli*) was synthesized chemically using a mixture of 2-hydroxyl- and 2-deoxynucleotide phosphoramidites. Oligonucleotides whose ribose composition allowed binding were retained selectively on nitrocellulose filters via binding to 30S ribosomal subunits. The binding-competent oligonucleotides were submitted to partial alkaline hydrolysis to identify the positions that were enriched for ribose. Quantification revealed a strong preference for a 2-hydroxyl group at position U33. This was shown directly by the 50-fold lower binding affinity of a stem loop containing a single deoxyribose at position U33. Similarly, defective binding of the corresponding U33-2-O-methyl-substituted stem-loop RNA suggests that absence of the 2-hydroxyl group, rather than an altered sugar pucker, is responsible. Stem-loop oligoribonucleotides from different tRNAs with U33-deoxy substitutions showed similar, although quantitatively different effects, suggesting that intramolecular rather than tRNA-ribosome interactions are affected. Because the 2-hydroxyl group of U33 was shown to be a major determinant of the U-turn of the anticodon loop in the crystal structure of $tRNA^{Phe}$ in yeast, our finding might indicate that the U-turn conformation in the anticodon loop is required and/or maintained when the tRNA is bound to the ribosomal P site.

Keywords: RNA structure; RNA synthesis; translation; U-turn

INTRODUCTION

Binding of transfer-RNA to the ribosome is determined in part by codon–anticodon interactions that are responsible for the specificity of selection of aminoacyl-tRNA and maintenance of the correct translational reading frame. However, tRNAs also interact with the ribosome itself via mechanisms that are more general and independent of codon–anticodon interactions. For example, tRNAs can be bound to the ribosomal P site either in an mRNA-dependent or mRNA-independent manner, depending on the specific ionic conditions, giving an identical footprint on 16S rRNA in both cases (Moazed & Noller, 1986, 1990). Furthermore, the tRNA anticodon stem loop of $tRNA^{Phe}$ contains all of the determinants for binding to the 30S subunit P site (Rose et al., 1983) and confers the same chemical protection pattern in 16S rRNA as full-length tRNA (Moazed & Noller, 1986). In fact, advantage was taken from this finding in a modification-interference approach to identify bases in the 16S rRNA that are involved in tRNA P-site binding (von Ahsen & Noller, 1995).

There is little absolute sequence conservation between the anticodon stem-loop regions of different tRNAs, with the exception of a conserved uridine at position 33 (Steinberg et al., 1993). Base substitutions at this position, however, still allow binding to the ribosome (Uhlenbeck et al., 1982). Therefore, it is likely that molecular interactions between the anticodon stem loop and the ribosome involve more general features of tRNA, such as the ribose–phosphate backbone. Accordingly, it has been shown that, whereas a chemically synthesized RNA oligonucleotide analogue of the anticodon stem-loop region of $tRNA^{Phe}$ binds efficiently to the P site of 30S ribosomal subunits, the corresponding DNA oligonucleotide does not (Koval'chuke et al., 1991; Dao et al., 1994). One possible explanation is that the

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ribosome simply requires an A-helical conformation, 3′-endo sugar pucker, or other ribose-specific conformational properties. Another possibility is that there is a requirement for a 2′-hydroxyl group at one or more specific positions.

In this study, we generated synthetic tRNA anticodon arm pools containing varying ratios of ribo- and deoxynucleotides, and identified critical ribose positions by virtue of the ability of the different chimeric RNA–DNA stem loops to bind to ribosomes. The importance of a 2′-hydroxyl group at these positions was tested directly by assaying the binding of single 2′-deoxy-substituted RNA stem loops. We show that the requirement for ribose is quite specific; deoxyribose is tolerated at all positions of the anticodon stem loop, except U33. We further show that substitution of 2′-O-methyl ribose is tolerated at positions G29, U32, C40, and C41, but not at positions G30 and U33. We infer that there is a specific requirement for a 2′-hydroxyl group at position U33.

RESULTS

Experimental strategy

In order to identify 2′-hydroxyl groups in the anticodon arm that are required for P-site binding in the 30S subunit, we created pools of synthetic anticodon stem-loop 15-mers containing randomly incorporated mixtures of ribo- and deoxynucleotides (Fig. 1). Oligonucleotides whose 2′-hydroxyl composition permitted binding to the ribosome were retained selectively by filter binding. Nucleotide positions enriched in ribose in the binding-competent subpopulation were identified by partial alkaline hydrolysis. The importance of specific 2′-hydroxyl groups was tested by assaying singly substituted 2′-deoxy- or 2′-O-methyl-oligoribonucleotides for binding to ribosomes.

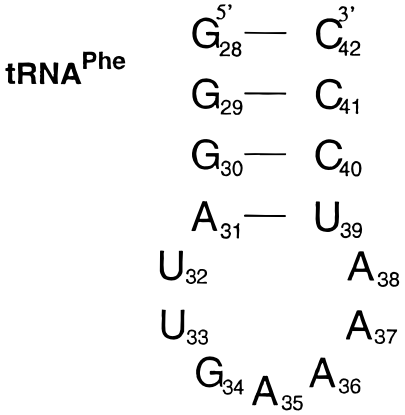

FIGURE 1. tRNA^{Phe} (*E. coli*) anticodon arm used in these studies. In pools where varying amounts of ribose and 2′-deoxyribose were incorporated, the 3′-terminal nucleotide (C42) was maintained as ribose.

Synthesis and characterization of tRNA anticodon stem-loop pools

Oligonucleotides comprising the anticodon stem-loop sequence from tRNA^{Phe} (*Escherichia coli*, corresponding to positions 28–42, Fig. 1) were synthesized as described in the Materials and methods. RNA phosphoramidites generally exhibit a lower coupling efficiency than DNA phosphoramidites. Because detailed data were not available for the relative efficiencies of incorporation of the two kinds of monomers, they were determined empirically for three different ribo:deoxy ratios. Oligonucleotide pools were synthesized using phosphoramidite mixtures containing 0, 2, 10, and 50% deoxyribose, respectively (called AcRNA, Acd02, Acd10, and Acd50, see Table 1). The actual 2′-deoxynucleotide content of each synthetic oligonucleotide pool was determined by completely digesting 3′-[^{32}P]-pCp-end-labeled oligonucleotides with RNAse T_2 and resolving the products by gel electrophoresis. Because 2′-deoxynucleotides are not susceptible to cleavage by RNAse T_2, the deoxyribose content of the various pools can be calculated by comparing the ratios of dimer to trimer, trimer to tetramer, and so on (Table 1). For example, pool Acd02, which was synthesized using 2% DNA phosphoramidites, was found to have a deoxyribose content of 8–10% in the actual oligonucleotide pool. From these data, we infer that the coupling efficiency of DNA phosphoramidites is about four times higher than that of RNA phosphoramidites.

Next, we compared the binding efficiencies of the three oligonucleotide pools to 30S ribosomal subunits, relative to the all-RNA anticodon arm. Binding of 3′-[^{32}P]-end-labeled oligonucleotides to 30S subunits was conducted under standard conditions in the presence of poly(U) and assayed by filter binding. In the absence of poly(U) mRNA, essentially no binding was detected for any of the oligonucleotides (data not shown). As expected, the ability to bind to 30S subunits decreases with increasing deoxynucleotide substitution (Fig. 2); about 10% DNA content (compare Table 1, pool Acd02) can be tolerated without signifi-

TABLE 1. Oligonucleotides with respective deoxyribose content synthesized in this work.

Name	RNA:DNA[a]	DNA content[b]
AcRNA	All RNA	0% (control)
Acd02	50:1	8–10%
Acd10	10:1	30–35%
Acd50	1:1	n.d.
Acd10/2[c]	n.d.	40–45%

[a]Ratios of ribo- and deoxyribose phosphoramidites during synthesis.

[b]As determined by RNAse T_2 digest.

[c]Generated from pool Acd10 after partial alkaline hydrolysis; n.d., not determined.

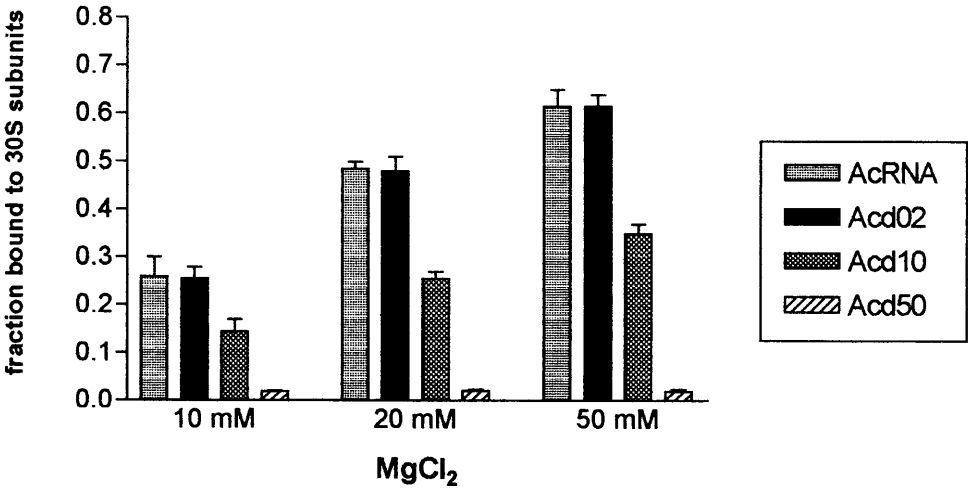

FIGURE 2. Binding efficiency of 3′ end-labeled anticodon arm pools to 30S subunits in the presence of poly(U) under varying magnesium ion concentrations as indicated in the figure. Shown is the fraction of the input tRNA (0.2 pmol) bound to 30S subunits (5 pmol). Error bars indicate variations in different experiments.

cantly affecting binding efficiency. However, the DNA content in pool Acd50 is too high, because almost no binding is detected. Although increasing the magnesium ion concentration generally results in higher anticodon arm binding, it does not compensate for the overall deleterious effect of the 2′-deoxynucleotide substitutions. Thus, in order to generate a pool with a more intermediate level of binding (relative to Acd10 and Acd50), the RNA content of pool Acd10 was decreased by partial alkaline hydrolysis and subsequent gel-isolation of full-length oligonucleotides. Following this treatment, the deoxyribose content measured by 3′-[^{32}P] end-labeling and total RNAse T_2 digestion was 40–45% and this pool, called Acd10/2 (Table 1), was used for selection experiments.

Selection for 30S subunit P-site binding

Pool Acd10/2, [^{32}P]-pCp-labeled at the 3′ end, was bound to 30S subunits in the presence of mRNA and the samples were subjected to the nitrocellulose filter binding assay. Only those oligonucleotides whose ribonucleotide composition permitted binding to 30S subunits were retained on the filter. The binding-competent tRNA analogues were subjected to partial alkaline hydrolysis and analyzed by gel electrophoresis. The requirement for a 2′-hydroxyl group at a specific position is reflected in enhanced susceptibility to alkaline cleavage at that position, relative to the starting pool. A typical autoradiograph is shown in Figure 3A. The band patterns of gels from several independent experiments were quantified by phosphorimaging. The ratios of the mean values of individual positions from the selected oligonucleotides relative to the unselected oligonucleotide pool are shown in Figure 3B (compare the Materials and methods). Position U33, and to a lesser extent, C40 and C41, showed increased susceptibility to alkaline hydrolysis. This same selectivity was observed with 30S subunits isolated from two different *E. coli* strains (MRE-600 and D10) and under several different magnesium ion concentrations between 10 and 50 mM $MgCl_2$ (data not shown). In a control experiment using an all-RNA anticodon arm, no position-specific enhancement of alkaline cleavage was observed (data not shown).

Singly 2-deoxy- or 2-methoxy-substituted oligonucleotides

To test directly the requirement for 2′-hydroxyl groups at specific positions, we synthesized oligoribonucleotides containing single 2′-deoxynucleotide substitutions and assayed their ability to bind to 30S subunits, relative to the all-RNA stem loop. In most cases, substitution with 2′-deoxyribose caused little or no decrease in binding (Table 2). However, introduction of a 2′-deoxynucleotide at position U33 caused a severe decrease in binding to 30S subunits. Deoxy substitution at C40 resulted in slightly (ca. twofold) reduced binding at 10 mM Mg^{2+}, but this effect was suppressed at increased Mg^{2+} concentrations (Table 2). These results provide direct confirmation for the ribose preferences identified in the ribosome selection experiments.

The requirement for ribose at a specific position could reflect the need for a 2′-hydroxyl group as a potential hydrogen bond donor or acceptor, or for the 3′-endo sugar pucker usually observed for ribo-, but not deoxyribonucleotides (Saenger, 1984). To distinguish between these possibilities, we synthesized oligonucleotides containing a single 2′-O-methyl ribose, which preferentially adopts the 3′-endo conformation, but is

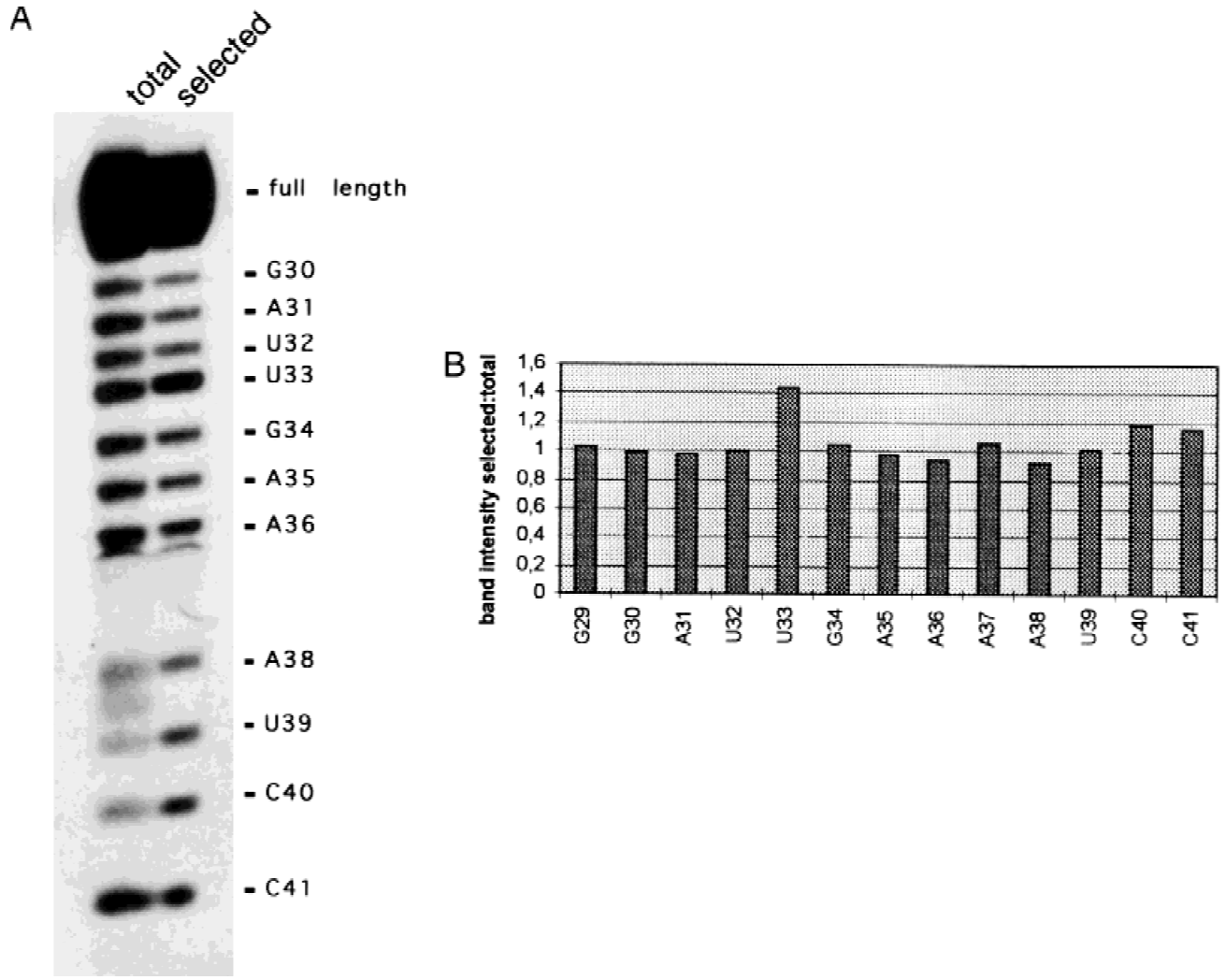

FIGURE 3. A: Autoradiograph of total and selected (binding-competent) pools after partial alkaline hydrolysis and separation on 20% polyacrylamide gels. Positions of the nucleotides are indicated on the right. Selection was performed at 10 mM $MgCl_2$ and buffer conditions as indicated in the Materials and methods. About 0.2 pmol 3′-[^{32}P] end-labeled tRNA anticodon pool (Acd10/2) was bound to 5 pmol 30S subunits in the presence of 10 μg poly(U) in a 50-μL volume. After nitrocellulose filter binding, the population retained on the filter was reeluted and treated as described in the Materials and methods. **B:** Ratios of band intensities of individual nucleotides of selected over total pools after partial alkaline hydrolysis (compare Fig. 3A). Mean values from at least three independent experiments for every position are shown. A value of 1.0 indicates no enrichment of 2′-hydroxyl groups. Variations from mean values were at all positions lower than 0.1.

unable to act as hydrogen bond donor (see, for example, Herschlag et al., 1993, and references therein). Similar to the effects of single deoxynucleotide substitution, a 2′-methoxy substitution at position U33 caused severe loss of binding affinity. In contrast, the 2′-O-MeC40 and 2′-O-MeC41 constructs showed only small decreases (Table 2). Interestingly, introduction of a 2′-methoxy group at position G30 (an unselected position) caused a significant (ca. sevenfold) decrease in binding efficiency, whereas a 2′-deoxy substitution at this position had no effect. Increasing the magnesium ion concentration to 20 mM compensated only slightly for the deleterious effects of 2′-O-MeG30 observed at 10 mM $MgCl_2$.

Binding affinities of the anticodon stem-loop analogues

Dissociation constants (K_d values) were estimated for three of the singly substituted anticodon arms (dU33, 2′-O-MeU33, and 2′-O-MeG30) as well as for the all-RNA analogue (AcRNA). Figure 4 shows the results of filter binding experiments as a function of 30S subunit concentration for the various anticodon analogues. For the all-RNA anticodon arm (AcRNA), we estimate a K_d of 130 nM ($\pm$35 nM) at 25 mM $MgCl_2$ and 0 °C with maximum molar binding ratio of 0.6. This agrees well with that observed for binding of full-length tRNA containing the natural modifications to 30S subunits (Gnirke & Nierhaus, 1986), as well as with that of the

TABLE 2. Binding efficiencies of anticodon arms containing single-2′-modified riboses at the indicated positions in an oligoribonucleotide in the presence of poly(U) relative to the all-RNA anticodon arm (*: 44%, **: 65% binding of input tRNA) under standard conditions.[a]

	Binding efficiencies (rel.)	
Anticodon arm	10 mM $MgCl_2$	20 mM $MgCl_2$
AcRNA + poly(U)	(1.0)*	(1.0)**
AcRNA -poly(U)	0.004	0.002
AcdG29	1.11	1.08
AcdG30	1.09	0.94
AcdU32	0.75	0.75
AcdU33	0.052	0.10
AcdC40	0.38	0.74
Ac2′-O-MeG29	0.93	1.08
Ac2′-O-MeG30	0.15	0.33
Ac2′-O-MeU32	0.65	0.69
Ac2′-O-MeU33	0.038	0.06
Ac2′-O-MeC40	0.50	0.58
Ac2′-O-MeC41	0.50	0.61

[a]Variations in binding efficiencies between different experiments was always smaller than 10% of the values shown.

anticodon arm of $tRNA^{Phe}$ from yeast containing the natural modifications (Uhlenbeck et al., 1982; Rose et al., 1983; Nekhai et al., 1994). As expected, the affinity of the singly substituted anticodon analogues is strongly diminished; indeed, we were unable to saturate binding and can therefore only estimate the K_d values of these analogues by extrapolation, assuming a saturation of 0.6, as for the all-RNA analogue (Table 3). K_d values for the deoxy-U33 and 2′-O-methyl-U33 oligonucleotides are up approximately 50-fold and 100-fold, respectively, compared with the all-ribo version.

TABLE 3. Binding of 5′-[^{32}P]-labeled oligonucleotides to 30S subunits was performed at 25 mM $MgCl_2$ using 0.2 pmol anticodon arm, 2–10 μg poly(U), and 30S concentrations as indicated in the graph in Figure 4.[a]

Anticodon arm	K_d
AcRNA	1.3 ($\pm$0.35) $\times$ 10^{-7} M^{-1}
AcdU33	6.1 ($\pm$0.75) $\times$ 10^{-6} M^{-1}
Ac2′-oMeU33	1.6 ($\pm$2.2) $\times$ 10^{-5} M^{-1}
Ac2′-oMeG30	7.1 ($\pm$1.25) $\times$ 10^{-7} M^{-1}

[a]For estimation of K_d values of the singly substituted anticodon arms, a binding stoichiometry of 0.6 was assumed as for the all-RNA analogue.

Anticodon arm analogues of other tRNAs

To test whether there is a general requirement for a 2′-hydroxyl group at position U33, oligoribonucleotides corresponding to the anticodon region of additional tRNAs were synthesized and assayed for binding to 30S subunits (Table 4). Two of those tested, threonine and asparagine, exhibited significant mRNA-dependent binding (Fig. 5). Anticodon arm analogues of $tRNA^{Asn}$ and $tRNA^{Thr}$ containing a single 2′-deoxy substitution at position U33 were then synthesized and their binding to 30S subunits tested. Binding of $tRNA^{Thr}$-dU33 was decreased strongly compared with that of the all-RNA $tRNA^{Thr}$ anticodon arm. In contrast, the $tRNA^{Asn}$-dU33 anticodon analogue showed only a modest loss of binding affinity compared to its all-RNA analogue. Finally, the singly substituted 2′-deoxy-U33 variant of the yeast $tRNA^{Phe}$ anticodon analogue showed one-third the binding efficiency of the corresponding all-RNA version (Table 5). Thus, the effects of deoxy substitution at position 33 are tRNA-specific, and are most pronounced for *E. coli* $tRNA^{Phe}$ among the anticodon arms tested in this study.

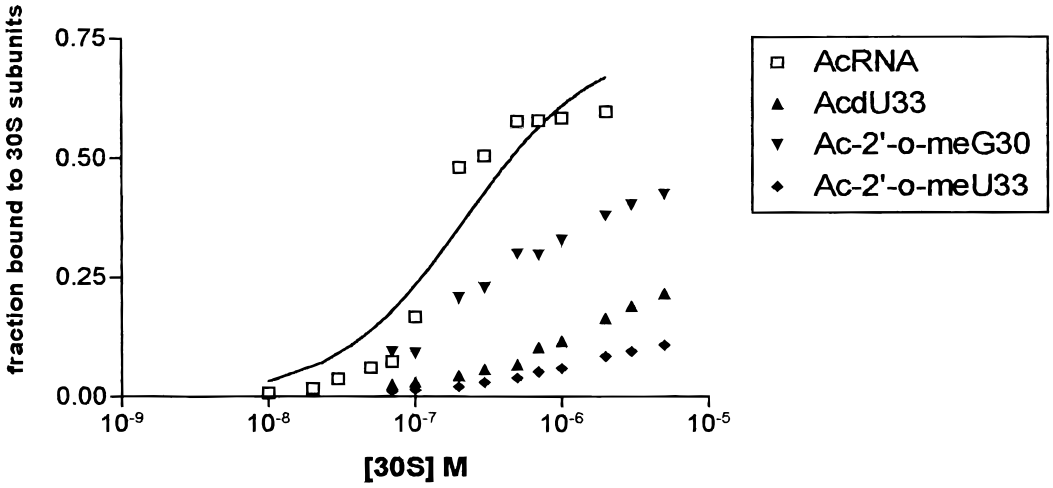

FIGURE 4. Graph showing K_d determinations of anticodon arms. The continuous line shows the theoretical first-order binding curve for anticodon arm AcRNA with an assumed initial K_d value of 33 nM and an assumed initial maximum binding of 0.6.

TABLE 4. Sequences of anticodon arm analogues used in this work.

tRNA	Sequence	mRNA
Phe (*E. coli*)	5′ GGG AUU GAA AAU CCC 3′	poly(U)
Phe (yeast)	5′ C CAG ACU GAA GAU CUG G 3′	poly(U)
Asn	5′ CGG ACU GUU AAU CCG 3′	poly(A,C)
Gln	5′ CGG UUU UUG AUA CCG 3′	poly(A,C)
Pro	5′ CUG GUU UGG GAC CAG 3′	poly(A,C)
Thr	5′ ACC CUU GGU AAG GGU 3′	poly(A,C)
Lys	5′ UUG ACU UUU AAU CAA 3′	poly(A)
LysGC[a]	5′ G UUG ACU UUU AAU CAA C 3′	poly(A)

[a]An additional GC pair for stabilization of the stem was introduced. Anticodons are underlined.

TABLE 5. Binding efficiencies of anticodon arms from various tRNAs to 30S subunits, determined in the presence of the appropriate mRNA (compare Table 4) at 20 mM $MgCl_2$ under standard conditions.

Anticodon arm	Binding efficiency
$tRNA^{Phe}$ (*E. coli*)	51.0% ± 5
$tRNA^{Phe}$-dU33 (*E. coli*)	2.1% ± 1.1
$tRNA^{Phe}$ (yeast)	53.0% ± 5
$tRNA^{Phe}$-dU33 (yeast)	18.0% ± 4
$tRNA^{Asn}$	16.4% ± 3
$tRNA^{Asn}$-dU33	10.3% ± 2
$tRNA^{Thr}$	11.7% ± 4
$tRNA^{Thr}$-dU33	1.8% ± 0.9

DISCUSSION

Our studies demonstrate the specific requirement for a 2′-hydroxyl group at position 33 of *E. coli* $tRNA^{Phe}$ for mRNA-dependent binding of the anticodon arm to 30S ribosomal subunits. Substitution of ribose 33 by 2′-deoxyribose or 2′-O-methylribose causes an increase in K_d of about 50- and 100-fold, respectively (Table 3), suggesting that the absence of this critical 2′-OH group results in disruption of one or more weak molecular interactions. Our finding that this effect is tRNA-specific provides evidence that disruption of intramolecular interactions within tRNA, rather than between tRNA and the ribosome, is responsible for the observed decrease in binding affinity.

In view of the inability of the all-deoxy anticodon arm to bind to 30S subunits, it is worthwhile asking, "What is the minimal number of riboses required for binding?" A stem containing all 2′-deoxy groups likely assumes the structure of a B-form helix, possibly disturbing the interaction of the stem with the ribosome. It has been shown, however, that the introduction of a single 2′-hydroxyl group in an otherwise completely deoxy-stem converts this stem into A-form helical structure (Ban et al., 1994). In an initial attempt to synthesize the maximally deoxy-substituted oligonucleotide that supports binding to 30S subunits, we synthesized a DNA anticodon arm analogue containing only two riboses at position 33 (in the loop) and 41 (in the stem). This oligonucleotide bound weakly but significantly to 30S subunits (4% at 10 mM $MgCl_2$, 7% at 20 mM $MgCl_2$), whereas binding of the all-deoxy analogue was undetectable under our experimental conditions. These results confirm our identification of at least one, and possibly two, critical positions in the anticodon arm where 2′-hydroxyls are required for binding of $tRNA^{Phe}$ to the P site of the 30S ribosomal subunit.

In a previous study using an oligonucleotide analogue of yeast $tRNA^{Phe}$, Uhlenbeck et al. (1982) showed that substitution of deoxyribose at position 33 caused an approximately fivefold decrease in binding affinity for the 30S subunit. They interpreted this finding as

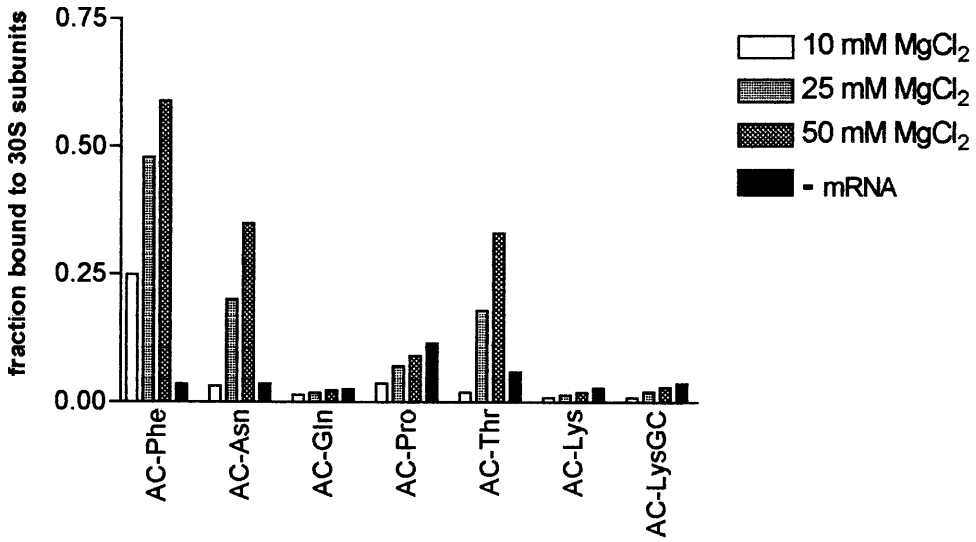

FIGURE 5. Binding efficiencies of anticodon arms from various tRNAs to 30S subunits as fraction of input tRNA under standard conditions and magnesium ion concentrations as indicated in the legend. All samples contained mRNA (compare Table 4) except where indicated; the magnesium ion concentration in the samples without mRNA was 50 mM.

evidence for a hydrogen bond between the 2′-hydroxyl of ribose 33 and N7 of adenine 35 that was predicted from the crystal structure of yeast $tRNA^{Phe}$ (Quigley & Rich, 1976).

Further support for this interpretation comes from our results using anticodon arms from different tRNAs (Table 5). Deoxy substitution at position 33 of the $tRNA^{Thr}$ analogue causes a strong decrease in binding, similar to that seen for the $tRNA^{Phe}$ analogues. Therefore, we conclude that our results are consistent with the notion that, during P-site binding, the anticodon loop adopts a similar U-turn conformation, as described for the crystal structure. Deoxy substitution at position 33 has only a minor effect on binding of the $tRNA^{Asn}$ analogue. This discrepancy can be explained by the fact that phenylalanine- and threonine-specific tRNA anticodons both have a purine at position 35, whereas asparagine-tRNA has a pyrimidine at this position, and so lacks an N7 hydrogen bond acceptor for the 2′-OH of ribose 33. Presumably, tRNAs that contain pyrimidines at position 35 use some alternative strategy to constrain the conformation of their anticodon loops. Quigley and Rich (1976) suggested hydrogen bonding to O4 and N4 of uracil or cytosine, respectively. In case of the N4 amino group of cytosines, the base would act as hydrogen bond donor, and the 2′-hydroxyl of U33 would then accept rather than donate its hydrogen. Another possibility may be the use of posttranscriptional modifications. For example, Dao et al. (1994) have reported that specifically modified DNA analogues of the yeast $tRNA^{Phe}$ anticodon stem loop are able to bind to *E. coli* 30S subunits. Synthetic anticodon arm analogues of $tRNA^{Phe}$ or $tRNA_f^{Met}$ lacking base modifications have been shown previously to mimic tRNA binding to ribosomes (e.g., Hartz et al., 1990; Koval'chuke et al., 1991). The inability of some of the synthesized anticodon arm analogues to bind to 30S subunits (Gln, Pro, Lys; compare Fig. 5) indicates that, in these cases, additional factors are required for binding. It was suggested that base modification of the N1 position of G37 in the anticodon loop of yeast $tRNA^{Phe}$ by methylation prevents base pairing to U32. Anticodon arms containing this modification exhibited an enhanced affinity for 30S subunits compared to an unmodified anticodon arm and an "open loop" conformation was postulated to facilitate binding (Dao et al., 1994). Although these modifications seem to be of less importance for $tRNA^{Phe}$ or $tRNA_f^{Met}$, they might be required for other anticodon arm analogues.

The approach presented here should be useful for addressing similar questions concerning the structure and function of other interesting RNAs. In addition, the data obtained regarding the relative coupling efficiency of RNA and DNA phosphoramidites may be of use in the synthesis of other chimeric oligonucleotides. A similar method has been described for the random introduction of 2′-methoxyl groups (Green et al., 1995); however, no detailed data regarding the coupling efficiency were presented.

MATERIALS AND METHODS

Oligonucleotide synthesis and quantification of RNA:DNA ratios

All oligonucleotides were synthesized on a Millipore Expedite Nucleic Acid Synthesis System. CPG-columns (500 Å, 1 μmol) and phosphoramidites were purchased from Millipore, except for 2′-methoxyribonucleotide and 2′-deoxyuridine phosphoramidites, which were purchased from Glen Research Corporation. For mixed oligonucleotide synthesis, various amounts of equimolar DNA and RNA phosphoramidite solutions were mixed and used for synthesis on a 1-μmol scale. All pools had a 2′-hydroxyl nucleotide (originating from the CPG support, cytidine) at the 3′ end, allowing for more uniform 3′ labeling. Oligonucleotides containing single-2′-modifications were synthesized automatically until the position of modification. At this point, the preceding DMT group was removed and the column removed from the machine. The phosphoramidite (2′-methoxy or 2′-deoxyuridine; 100 μmol) was dissolved in 1.2 mL acetonitrile; 0.6 mL of the phosphoramidite solution was mixed together with 0.6 mL activator solution and applied to the CPG support using a 1-mL syringe at each opening. After 30–45 min of coupling, with occasional mixing, the column was washed with acetonitrile and the synthesis was completed on the machine. Base deprotection was done with 1.5 mL NH_4OH/EtOH (3:1) at 55 °C overnight. The supernatant was dried down, dissolved in ethanol, and redried. Then, to remove the 2′ silyl protecting groups, 660 μL of 1 M tetrabutylammonium fluoride in tetrahydrofuran was added (40-fold molar excess), vortexed, and incubated for 24 h at room temperature. Next, an equal volume of water was added and the oligonucleotides were precipitated with EtOH/NaOAc, extracted with neutralized phenol, reprecipitated, and purified on denaturing polyacrylamide gels. For quantification of RNA:DNA ratios in the three synthesized pools, the oligonucleotides were 3′-end-labeled (with [^{32}P]pCp, specific activity 3,000 Ci/mmol) using standard procedures (England et al., 1980) and digested with RNAse T_2 in 40 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 1 mM DTT for 30 min at 45 °C in 15 μL volume; the products were resolved on 21% denaturing polyacrylamide gels. Quantification of individual bands was performed with a Molecular Dynamics PhosphorImager.

Binding of synthetic anticodon arm analogues to 30S ribosomal subunits

Subunits were isolated from *E. coli* strains MRE600 and D10-can20-12E using standard procedures (Rheinberger et al., 1988). mRNAs (poly(U), poly(A,C), poly(A)) were purchased from Sigma. In all cases of tRNA binding to 30S subunits, the tRNA anticodon arms were activated in binding buffer (see below) for 1 min at 65 °C and then for 5 min at 37 °C before addition to the 30S-mRNA complex. Standard binding conditions included at least a 10-fold molar excess 30S subunits (usually 10 pmol in 50 μL) over tRNA (0.2–0.4 pmol), a saturating amount of mRNA (10 μg in a 50 μL reaction volume)

in buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NH_4Cl, 1 mM DTT, 2 mM spermine, 0.05 mM spermidine, and 20 mM $MgCl_2$ if not indicated otherwise. After binding of the 3′-labeled RNA/DNA pools to 30S subunits for 10 min at 37 °C and 20 min on ice, samples were passed through nitrocellulose filters (0.45 μm) and washed three times with ice-cold buffer. The bound RNA/DNA mix was eluted from the filter following a procedure similar to that described by Tuerk et al. (1990). Briefly, the RNA/DNA was eluted with hot (65 °C) sodium citrate/EDTA/urea buffer and subsequently extracted with sodium citrate-buffered phenol. After precipitation, the samples were extracted twice more with phenol to remove remaining ribosomal proteins and again precipitated with EtOH/NaOAc. For partial alkaline hydrolysis, the selected and unselected (total) population were incubated for 10 min at 90 °C in 50 mM $NaHCO_3$, pH 9, the total population containing an amount of carrier rRNA to match the total RNA concentration in the selected population. The treated samples were resolved on a 20% denaturing polyacrylamide gel, in parallel with a T_1-digested pool as marker. For quantification, the gels were scanned on a phosphorimager. Bands of interest were normalized to five surrounding bands to compensate for possible differences in hydrolysis rates in the total and selected pools; the 5′- and 3′-terminal nucleotides were not quantified.

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