
Role of the constant uridine in binding of yeast tRNA^{Phe} anticodon arm to 30S ribosomes

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ABSTRACT

Twenty-two anticodon arm analogues were prepared by joining different tetra-, penta-, and hexaribonucleotides to a nine nucleotide fragment of yeast tRNA^{Phe} with T4 RNA ligase. The oligomer with the same sequence as the anticodon arm of tRNA^{Phe} binds poly U programmed 30S ribosomes with affinity similar to intact tRNA^{Phe}. Analogues with an additional nucleotide in the loop bind ribosomes with a weaker affinity whereas analogues with one less nucleotide in the loop do not bind ribosomes at all. Reasonably tight binding of anticodon arms with different nucleotides on the 5' side of the anticodon suggest that positions 32 and 33 in the tRNA^{Phe} sequence are not essential for ribosome binding. However, differences in the binding constants for anticodon arms containing modified uridine residues in the "constant uridine" position suggest that both of the internal "U turn" hydrogen bonds predicted by the X-ray crystal structure are necessary for maximal ribosome binding.

INTRODUCTION

The nucleotide at position 33 on the 5' side of the anticodon is one of the most conserved residues in transfer RNA. Out of more than two hundred tRNA sequences currently available, a uridine (U33) appears at this position in all but a few eukaryotic initiator tRNAs (1). In the X-ray crystal structure of yeast tRNA^{Phe}, U33 helps to form the so called "U-turn" where the stacked conformation on the 3' side of the anticodon is stabilized by internal hydrogen bonds between the N3 hydrogen of the uridine and an oxygen of phosphate 36 and between the 2' hydroxyl of ribose 33 and N7 of adenosine 35 (2). A central role for U33 in protein synthesis has been proposed (3) which recently has received support from the altered conformation of U33 in the *E. coli* initiator tRNA^{Met} X-ray crystal structure (4).

This laboratory recently showed that a semi-synthetic oligoribonucleotide which can form the anticodon stem and loop of yeast tRNA^{Phe} is able to bind *E. coli* 30S ribosomes in the presence of poly U (5). The stoichiometry

and the association constant of this anticodon arm to the 30S ribosome is identical to that of intact yeast tRNA^{Phe}. Since the arm also competes effectively with tRNA^{Phe}, this 15 residue oligonucleotide appears to be a good model compound for the study of the codon-anticodon interaction on the ribosome.

In this paper, we use a similar strategy of semi-synthesis to prepare a series of anticodon arms that vary the nucleotides which correspond to positions 32 and 33 in the tRNA sequence and vary the size of the loop. Since the internal hydrogen bonds in the anticodon loop could possibly be important in the codon-anticodon interaction, modified uridine residues were inserted at position 33 to disrupt this "U turn" structure. The variant anticodon arms then were tested for poly U directed binding to 30S ribosomes.

MATERIALS AND METHODS

Nucleoside 5' diphosphates and dinucleoside monophosphates were purchased from Sigma Chemical Company. Nucleoside 3'(2'),5'-bisphosphates of cytidine, guanosine, uridine, 5-bromouridine (Br⁵U), 5-fluorouridine (f⁵U), pseudouridine (Ψ), 3-methyluridine (m³U), dihydrouridine (D), 3-deazauridine (deaza³U), 2'-O-methyluridine (Um), deoxyuridine (dU), and deoxycytidine (dC) were synthesized from the corresponding nucleosides using pyrophosphoryl chloride as described previously (6).

ApG(pA)_n were synthesized in a 1 ml reaction containing 7 mM ApG, 11 mM ADP, 10 mM MgCl₂, 0.4 M NaCl, 0.1 M Tris-HCl pH 8.3, and 0.1 mg/ml primer dependent *M. luteus* polynucleotide phosphorylase (PNPase). After incubation for 12 hr at 37°C, the reaction was diluted with 1 ml of H₂O, heated to 90°C for 3 min and then incubated at 37°C for 5 hours with 100 μ g bacterial alkaline phosphatase (BAP) to hydrolyze unreacted ADP. The reaction products were separated by descending chromatography on Whatman 3 MM paper using 70:30 (v/v) 1 M ammonium acetate : 95% ethanol. ApGpA (2.1 μ moles, 30%), ApG(pA)₂ (0.9 μ moles, 13%) and ApG(pA)₃ (0.45 μ moles, 7%) were recovered from the paper as described previously (7).

ApGpApU was prepared in a 0.2 ml reaction containing 0.7 mM ApGpA, 21 mM UDP, 10 mM MgCl₂, 0.4 M NaCl, 0.1 M Tris-HCl pH 8.3, 50 μ g/ml pancreatic ribonuclease (RNase A), and 150 μ g/ml PNPase. After incubation for 14 hours at 37°C, the reaction was heated at 90°C and treated with BAP as above. The single product ApGpApU (0.12 μ moles, 80%) was purified by paper chromatography using 40:60 (v/v) 1 M ammonium acetate : 95% ethanol (solvent A).

ApGpApC and ApGpApApU were prepared in a similar fashion.

The twelve different ApGpApCpN were prepared by using T4 RNA ligase to add the appropriate nucleoside 3',5'-bisphosphate to the tetranucleotide (7). Each 100 μ l reaction contained 0.25 mM ApGpApC, 1 mM nucleoside 3'(2'),5'-bisphosphate, 0.5 mM ATP, 20 mM MgCl₂, 50 mM Hepes pH 8.0, 3.3 mM dithiothreitol and 120 μ g/ml T4 RNA ligase (6). The reactions were incubated for 12 hours at 4°C, heated for 2 min at 90°C and further incubated for 2 hours at 37°C with 10 μ g BAP. The products were purified by paper chromatography using solvent A. The yields of pentamers varied from 11 nmoles (44%) for ApGpApCpG to 19 nmoles (76%) for ApGpApCpdeaza³U. ApGpApUpU was prepared in a similar fashion. (Ap)₅C was obtained as described elsewhere (8).

The three different hexamers ApGpApCpUpU, ApGpApCpUpA, and ApGpApCpUpG were prepared in two successive steps. First, 5' phosphorylated dimers were prepared in 75 μ l reactions containing 1.25 mM dimer, 1.5 mM ATP, 20 mM MgCl₂, 50 mM Hepes pH 8.0, 3.3 mM dithiothreitol, and 90 units/ml T4 polynucleotide kinase. After incubation for 2 hours at 37°C, the reactions were heated to 90°C for 2 min. to inactivate the enzyme. To each of these reactions, 85 μ l of 0.6 mM ApGpApC, 1.2 mM ATP and 250 μ g/ml T4 RNA ligase in the same buffer were added and incubated for 12 hours at 4°C. The hexamers were purified by paper chromatography with solvent A. Although products corresponding to multiple additions of each dimer to ApGpApC were obtained, yields of 20 to 35 percent were obtained for ApGpApCpUpU, ApGpApCpUpA and ApGpApCpUpG. An equivalent amount of ApGpApC(pU)₄ was obtained.

The oligonucleotide G_mpApApYpApYpCpUpGp corresponding to positions 34-42 in yeast tRNA^{Phe} was isolated from the intact tRNA^{Phe} by partial hydrolysis at U33 with RNase A (9) followed by total digestion with RNase T₁ and purification on benzoylated DEAE cellulose. 5 mg (0.2 μ moles) of yeast tRNA^{Phe} (Boehringer) was incubated in 15 ml of 0.2 M KCl, 10 mM magnesium acetate, 0.1 M Tris-HCl pH 7.5 and 42 μ g/ml RNase A for 4 hours at 0°C. The reaction was stopped by adding 100 μ l diethylpyrocarbonate and vortexing for 1 min. After phenol extracting the reaction mixture twice, the tRNA half molecules were recovered by ethanol precipitation. The pellet was resuspended in 2 ml of 50 mM Tris-HCl pH 7.5, 0.5 mM EDTA and 250 units/ml of RNase T₁. After incubation for 12 hours at 37°C, the reaction mixture was brought to 1 M NaCl and applied to a 5 ml benzoylated DEAE cellulose column. After washing with 1 M NaCl, 50 mM Tris-HCl pH 7.5 to remove other oligomers, the desired oligonucleotide containing Y base was eluted from the column with 1 M NaCl, 50 mM Tris-HCl pH 7.5, 15% (v/v) ethanol (10). After

desalting on Sephadex G10, 0.14 μ moles (70 percent) of the nonamer was recovered.

Twenty-two different anticodon arm variants with an internal ^{32}P label were synthesized in two steps. The 5' terminus of the 9 nucleotide tRNA fragment was first phosphorylated in a 110 μ l reaction containing 14 μ M oligomer, 14 μ M [γ - ^{32}P] ATP (900 Ci/mmmole), 20 mM MgCl_2 , 50 mM Hepes pH 8.0, 3.3 mM dithiothreitol and 120 units/ml PseT 1 polynucleotide kinase (11). The reaction was incubated for 90 min at 37°C and then heated for 2 min at 90°C. The [$5'$ - ^{32}P] labeled nonamer donor was then joined to each tetramer, pentamer, or hexamer acceptor in 15 μ l reactions composed of 5 μ l of the above kinase reaction and 10 μ l of 60 μ M acceptor, 1 mM ATP, 20 mM MgCl_2 , 50 mM Hepes pH 8.0, 3.3 mM dithiothreitol and 560 μ g/ml T4 RNA ligase. After incubating for 12 hr at 4°C, the reactions were terminated by the addition of 15 μ l of 8 M urea containing 0.67% bromophenol blue and 0.33% xylene cyanole. The internally labeled anticodon arms were purified by polyacrylamide gel electrophoresis (see Fig. 2). The oligomers were eluted from the gel by a crush and soak procedure described in detail previously (12). From 3 to 6 μ Ci of each oligomer was obtained which corresponds to a 5 to 10 percent recovery of the input [γ - ^{32}P] ATP.

The preparation of E. coli 30S ribosomal subunits was carried out by a standard procedure described in detail previously (5). All the data reported in this paper were obtained with a single ribosome preparation. The binding of each ^{32}P labeled oligomer to activated 30S ribosomes was carried out in a series of 20 μ l reactions containing 30 mM magnesium acetate, 150 mM NH_4Cl and 50 mM Tris pH 7.2 (5). Each reaction contained a very low concentration of the oligomer (less than 0.5 nM), a saturating concentration of poly U (60 nM), and one of 18 ribosome concentrations varying from 1 nM to 2 μ M. After incubation at 0°C for 20 min, each tube was diluted to 2 ml with cold 10 mM magnesium acetate, 50 mM NH_4Cl and 10 mM Tris pH 7.2 and filtered onto a nitrocellulose filter. Without further washing, the filter was dried and counted.

RESULTS

(a) Synthesis of Anticodon Arm Variants.

The protocol for the synthesis of the 14 nucleotide anticodon arms (Figure 1) was simplified greatly by the use of a 9 nucleotide fragment of tRNA^{Phe} for the portion of the molecule where no alterations were planned.

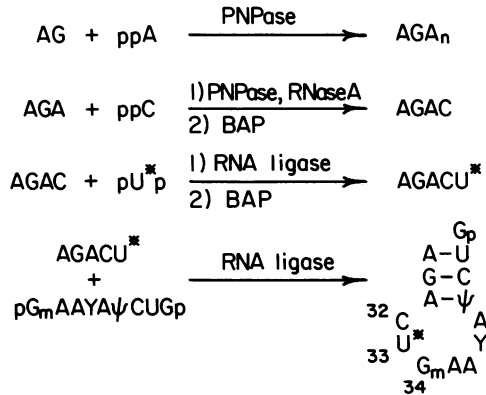


Figure 1. Synthetic scheme for anticodon arms. Enzyme abbreviations are given in text. The numbers refer to the corresponding nucleotides in yeast tRNA^{Phe}.

An anticodon stem of only three base pairs could be used since previous experiments (5) had shown this was all that was necessary for optimal binding of the oligomer to the ribosome. The resulting semi-synthetic oligomer contains all the modified nucleotides present in tRNA^{Phe} except the 2'-O-methyl group at position 32. Since only low concentrations of radiochemically pure oligomer were required for the ribosome binding experiments, the synthesis could be scaled down to conserve oligonucleotides and enzymes.

Figure 2 shows an autoradiogram of a 20% polyacrylamide - 7m urea gel analysis of an aliquot from the polynucleotide kinase reaction in which the tRNA^{Phe} nonamer is [5'-³²p] end-labeled and of aliquots from seven of the RNA ligase reactions where the [5'-³²p] nonamer is joined to different oligomer acceptors. The autoradiogram shows that in each RNA ligase reaction a majority of the radioactivity migrates to a higher position on the gel according to the length of the acceptor added. Although very high concentrations of RNA ligase were used in these reactions to ensure high yields for all the variants, RNA ligase concentrations as low as 100 µg/ml were generally sufficient for quantitative joining. In similar preparative gels where the bands moved somewhat further, the internally labeled oligomers were well resolved from oligomers one residue shorter or longer. The variant anticodon arms were cut from the gel, eluted, ethanol precipitated and re-analyzed by gel electrophoresis to confirm their homogeneity and chainlength.

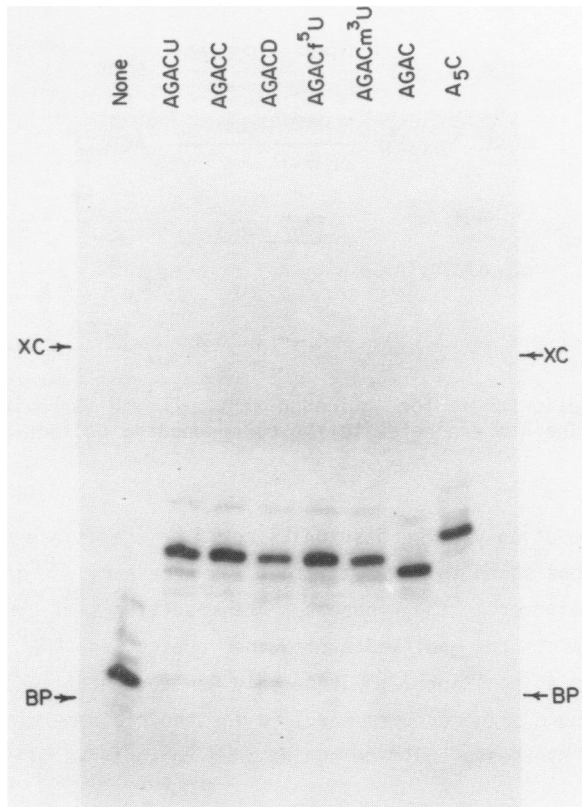


Figure 2. Final step in the synthesis of anticodon arm variants. Aliquots of the ligation reaction between $[5' \text{-}^{32}\text{P}]$ nonamer (left lane) and indicated acceptors are analyzed by electrophoresis and autoradiography in a 20% polyacrylamide gel. Dye markers are xylene cyanol (XC) and bromophenol blue (BP).

Each of the twenty-two purified oligomers also were digested with a mixture of ribonucleases A, T_1 and T_2 , and the products of the reaction analyzed by two dimensional chromatography on a cellulose thin layer plate (13). In every case, as shown for nine examples in Figure 3, the expected unique ^{32}P labeled product was obtained. In most cases the radiolabeled product of the digestion reaction was the $3' \text{-}^{32}\text{P}$ labeled nucleotide in position 33. However, when a ribonuclease resistant nucleotide such as 2'-O-methyluridine was inserted at that position the ^{32}P labeled product was the trinucleoside triphosphate (UmpGmpAp). Since only a single product was

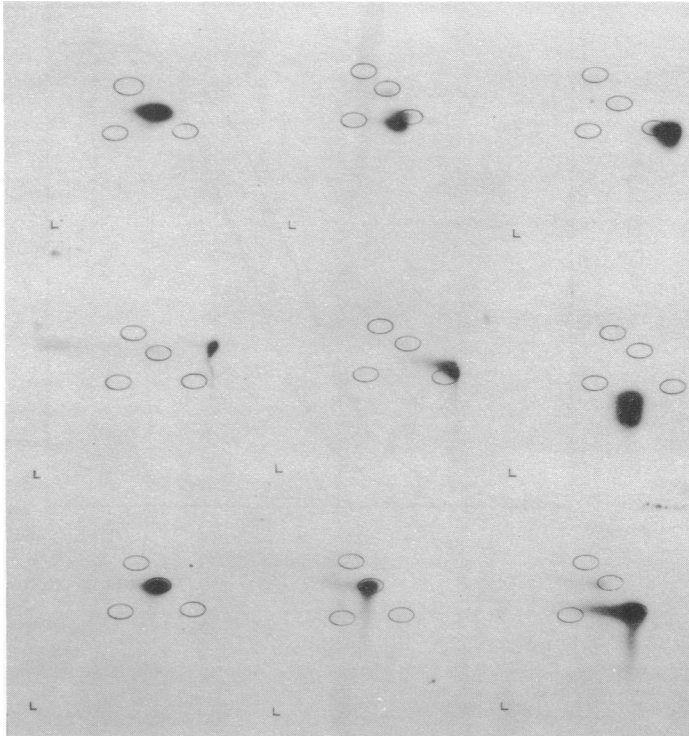


Figure 3. Identification of anticodon arm variants. Autoradiograms of two-dimensional thin layer separations (13) of enzymatic hydrolysates of anticodon arms formed from $[5'-^{32}\text{P}]_3$ nonamer joined with AGACC, AGACD, AGACF⁵U (top row, left to right); AGACm³U, AGACBr⁵U, AGAC ψ (middle row); AGAC, A₅C, and AGACU (bottom row) acceptors are shown.

obtained in each case, these experiments additionally confirm the radiochemical purity of the oligomers.

(b) Ribosome Binding Experiments

In Figure 4 the binding of three representative ^{32}P oligomers is plotted as a function of the ribosome concentration. In the case of the AGACU arm, which most closely resembles the tRNA^{Phe} sequence, binding is detectable at 10 nM ribosomes, has a midpoint at 50 nM and is complete at 500 nM. This binding curve is identical to that found for both $[^{32}\text{P}]$ labeled tRNA^{Phe} and for the model anticodon arm with an additional 5' terminal C and a 2'-O-methyl C at position 34 studied previously (5). Thus, the 14 nucleotide AGACU arm depicted in Figure 1 contains all of the structural elements required for binding of tRNA^{Phe} to 30S ribosomes and

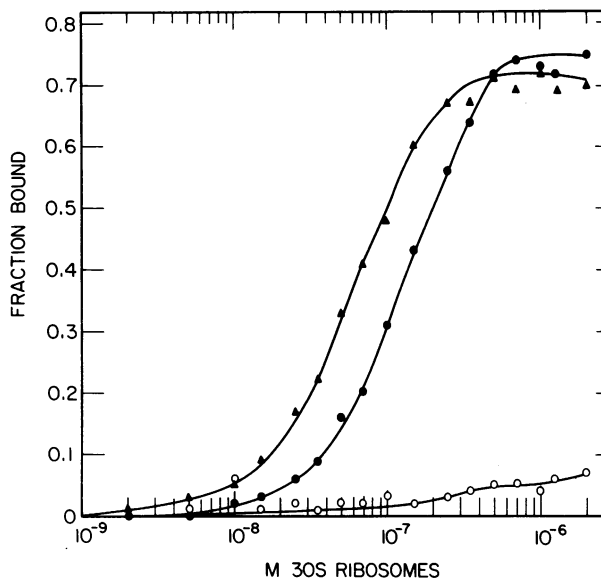


Figure 4. 30S Ribosome binding of AGACU (Δ), AGACC (\bullet), and AGAC (\circ) anticodon arms.

therefore is an appropriate model compound for studying the interaction.

The AGACC arm, which has a cytidine in position 33 instead of a uridine, binds slightly less well than the AGACU arm (Figure 4). The midpoint of the curve is at about 120 nM 30S ribosomes as opposed to 50 nM for the AGACU arm. Although this difference is quite small, it has been reproduced several times with different preparations of oligomers and ribosomes. Thus, the uridine at position 33 has a small but significant effect on the binding of the anticodon arm to ribosomes. If U33 is totally omitted, an anticodon loop with only six residues results and no binding of this AGAC arm to ribosomes is detected (Figure 4).

Similar ribosome binding experiments were carried out for the nineteen other oligomers. In those cases where binding was observed, the shape of the curve was very similar to those in Figure 4 and somewhat sharper than what would be predicted for a simple first order binding reaction between the oligomer and the poly U ribosome complex. However, a thorough analysis of the interaction of the 15 nucleotide anticodon arm with 30S subunit (5) shows that the ribosome concentration at the midpoint of the transition accurately gives the apparent dissociation constant since only one oligomer is bound per 30S subunit.

The data in Table 1 compare the transition midpoints of twelve oligomers which differ by the nucleotide at position 33. All twelve oligomers are able to bind ribosomes reasonably well. Thus, a uridine at position 33 does not appear to be essential for the binding of the oligomer to the ribosome. However, small differences in the binding constants are observed. The three oligomers which contain 5-bromouridine, 5-fluorouridine, and pseudouridine bind with an affinity quite similar to the oligomer containing uridine, while the other eight oligomers bind less well. The oligomer with deoxycytidine in position 33 binds the least well. Although the magnitude of the differences in K_d are only two to five fold, the results are very reproducible. Due to the shape of the binding curve, the difference can correspond to a two fold or more difference in the fraction of loop bound at certain ribosome concentrations.

In Table 2, the binding of nine additional variants is compared with the binding of the natural sequence. Substitution of C32 with either a U or an A leads to only a small decrease in ribosome binding, and thus indicates that the sequence at this position is also not essential for anticodon function. The poor binding of the AGA₃ oligomer, where positions 32 and 33 are both substituted with an A residue, is somewhat unexpected. Since oligomers with a single substitution at either position 32 or 33 bound relatively well, it is unclear why the doubly substituted molecule binds so poorly. One possibility is that the large number of A residues in the loop destabilizes the stem sufficiently to abolish binding. Alternatively, the

Table 1
Binding of Anticodon Arms with Position 33 Variants to 30S Ribosomes

Nucleotide at Position 33	K_d (nM)
U	50
Br ⁵ U	60
f ⁵ U	40
ψ	80
C	120
m ³ U	150
D	160
deaza ³ U	240
Um	160
G	190
dU	230
dC	300

Table 2

Oligonucleotide Acceptor	K_d (nM)
AGACU	52
AGAUU	100
AGAAU	120
AGAAA	>5000
AGAC	>5000
AGAU	>5000
AGACUG	80
AGACUA	200
AGACUU	400
AGACUUUU	>5000
AAAAAC	>5000

AGA₃ oligomer may base pair effectively with poly U and this prevents ribosome binding.

The number of nucleotides in the loop portion of the anticodon arm also has a clear effect on ribosome binding. As noted above, six residue loops without either C32 or U33 do not bind ribosomes. However loops with a single extra nucleotide between positions 33 and 34 can be accommodated by the ribosome. An extra G residue in this position is the least disruptive, while an extra U residue decreases K_d about eight fold. A loop with three extra U residues shows no detectable binding.

DISCUSSION

Radiochemical amounts of twenty-two different anticodon arm analogues 13 to 15 residues long were prepared by enzymatic methods using a nine nucleotide fragment of tRNA^{Phe}. The substrate specificity of T4 RNA ligase permits the incorporation of modified nucleotides and thus allowed the construction of variant anticodon arms which differed from the natural sequence by only a few atoms.

Despite the fact that uridine 33 is a highly conserved nucleotide in tRNA, it does not appear to be essential for binding of the anticodon arm to poly U programmed 30S ribosomes. Variant arms with nucleotides at position 33 as disparate as guanosine or dihydrouridine bind to ribosomes. Since the isolated anticodon arm appears to be a good model for intact tRNA in this assay, these data suggest that the uridine 33 is also not essential for tRNA

binding to ribosomes. It is, of course, quite possible that U33 plays a more important role in a later step in the protein synthesis mechanism. Experiments underway to replace U33 in an intact tRNA will address this point.

Although differences in K_d that are observed with the U33 variants are comparatively small, they correlate quite well with whether the modification disrupts the internal hydrogen bonds proposed for U33 in the X-ray crystal structure. The three variants which bind the best, namely 5-bromouridine, 5-fluorouridine, and pseudouridine, do not interfere with the "U turn" structure, whereas seven of the eight variants which bind less well disrupt one or both of the internal hydrogen bonds. The hydrogen bond involving the hydrogen on N3 of uridine 33 cannot form with the cytidine, 3-methyluridine, 3-deazauridine and guanosine variants and the hydrogen bond involving the 2' hydroxyl of uridine 33 cannot form with the deoxyuridine and 2'-O-methyl uridine variants. Only the dihydrouridine variant is capable of forming both hydrogen bonds and does not bind well. Perhaps the non-planar structure of the dihydrouridine ring results in the lower binding affinity of this variant. It is striking that the oligomer containing deoxycytidine which cannot form either of the two internal hydrogen bonds binds the least well of the twelve oligomers tested. Thus, although the effect is rather small, we have demonstrated a functional consequence of disrupting a tertiary structure by making substitutions at the atomic level.

The size of the loop portion of the anticodon arm clearly influences its ribosome binding capacity. Loops missing one of the pyrimidines on the 5' side of the anticodon show no detectable binding. Presumably the missing residue results in an altered conformation of the anticodon such that it cannot interact properly with the poly U. On the other hand, all three molecules with an extra nucleotide between positions 33 and 34 show detectable ribosome binding. Several tRNAs with eight nucleotides in the anticodon loop have been shown to be active in translation (14-16). However, it is unclear whether four base pairs form between poly U and the enlarged anticodon loops. Although the loop with an extra G residue binds nearly as well as the normal seven-membered loop, the loop with an extra A residue binds nearly as poorly as the loop with an extra non-complementary U residue. This is consistent with the suggestion (17) that a complementary base pair in the fourth position does not lead to more efficient function.

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