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Ribosome binding of DNA analogs of tRNA requires base modifications and supports the "extended anticodon"

VIVIAN DAO*, RICHARD GUENTHER*, ANDRZEJ MALKIEWICZ†, BARBARA NAWROT†, ELZBIETA SOCHACKA†, ADAM KRASZEWSKI‡, JADWIGA JANKOWSKA‡, KEITH EVERETT§, AND PAUL F. AGRIS*||

Departments of *Biochemistry and §Genetics, North Carolina State University, Raleigh, NC 27695-7622; †Institute of Organic Chemistry, Technical University, Zwirki 36, 90-924 Lodz, Poland; and ‡Institute of Bioorganic Chemistry, Noskowskiego 12/14, 61-704 Poznan, Poland

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ABSTRACT **The efficiency of translation depends on correct tRNA–ribosome interactions. The ability of chemically synthesized yeast tRNA^{Phe} anticodon domains to effectively inhibit the binding of native yeast tRNA^{Phe} to poly(U)-programmed *Escherichia coli* 30S ribosomal subunits was dependent on a Mg^{2+}-stabilized stem and an open anticodon loop, both facilitated by base modifications. Analysis of tRNA sequences has revealed that base modifications which negate canonical hydrogen bonding are found in 95% of those tRNA anticodon loop sequences with the potential to form two Watson–Crick base pairs across the loop. Therefore, we postulated that a stable anticodon stem and an open loop are prerequisites for ribosome binding. To test this hypothesis, DNA analogs of the yeast tRNA^{Phe} anticodon domain were designed to have modification-induced, Mg^{2+}-stabilized stems and open loops. The unmodified DNA analog neither bound to poly(U)-programmed 30S ribosomal subunits nor inhibited the binding of native tRNA^{Phe}. However, specifically modified DNA analogs did bind to ribosomal subunits and effectively inhibited tRNA^{Phe} from binding. Thus, modification-dependent, Mg^{2+}-stabilized anticodon domain structures with open loops have evolved as the preferred anticodon conformations for ribosome binding.**

Base pairing of the tRNA anticodon with the mRNA codon is an integral part of tRNA's functioning as an "adapter" between the genetic information stored in nucleic acids and the amino acid sequences of proteins (1). However, when free in solution, tRNAs bind to their trinucleotide codons only weakly; any related but erroneous triplets are recognized 10^{-1} to 10^{-2} times as efficiently as the correct triplets (2). Therefore, hydrogen bonding of tRNA anticodons to their trinucleotide codon is not sufficiently accurate to account for the genetic code's being read with an error frequency of 5×10^{-3} to 1×10^{-5}, including the proofreading step (3). General properties of RNA structure, such as the overall conformation of the anticodon domain, and particular contributions of the 2′-OH of the ribose, modified nucleosides (4, 5), and ion coordination (6–8) must contribute to the effectiveness of ribosome-mediated codon binding.

The anticodon domain of tRNA appears to be exclusively in contact with the *Escherichia coli* ribosome's 30S subunit (9). An oligomer comprising the tRNA^{Phe} anticodon domain only, tRNA$^{Phe}_{AC}$, binds to the poly(U)-programmed 30S ribosomal subunit with the stoichiometry and binding constant identical to those of deacylated native tRNA^{Phe}. In addition, the anticodon domain and tRNA^{Phe} compete for the same binding site on the ribosome (10). These data indicate that the interaction of tRNA^{Phe} with poly(U)-programmed 30S subunits is primarily a result of contacts in the anticodon domain and not with other parts of the tRNA.

Unmodified yeast tRNA$^{Phe}_{AC}$ binds poly(U)-programmed small ribosomal subunits with an affinity two orders of magnitude lower than the fully modified native tRNA$^{Phe}_{AC}$ (11). Transfer RNAs contain more than 80 differently modified nucleosides, and the nucleosides of eukaryotic tRNAs are as much as 25% modified (4, 5). The introduction of these bases into tRNA is a post-transcriptional event involving specific enzymes. Although the functions of modified nucleosides in tRNA molecules are not yet well understood, several studies show that tRNA structure (12), metal ion binding (6–8), and interaction with cognate aminoacyl-tRNA synthetase are substantially influenced by the presence of modified nucleosides (13–16). By directing the local or global structural changes in tRNA, these base modifications can affect the tRNA's interaction with different macromolecules (5). Structural analogs of the yeast tRNA^{Phe} anticodon domain in which either the entire sequence or just the stem were composed of unmodified deoxyribonucleosides did not bind to poly(U)-programmed 30S ribosomal subunits (11). Therefore, questions remain as to whether modified nucleosides are important in ribosome-mediated codon binding and whether DNA analogs might be altered to bind the ribosome at the same site as tRNA. We have found that a m^{5}C-dependent Mg^{2+}-stabilized yeast tRNA^{Phe} anticodon domain and a m^{1}G-aided open loop conformation are important conformational determinants of a strong yeast tRNA^{Phe}–ribosome interaction. When these structural elements were incorporated into a DNA analogs of the anticodon domain, the analogs effectively inhibited tRNA^{Phe}–ribosome interaction.

MATERIALS AND METHODS

Materials. Brewer's yeast tRNA^{Phe}, poly(A), and poly(U) were purchased from Sigma. Concentrations of native brewer's yeast tRNA^{Phe} were calculated on the basis of 1307 pmol/A_{260} unit. [γ-^{32}P]ATP was purchased from New England Nuclear. Five RNAs with sequences corresponding to the yeast tRNA^{Phe} anticodon stem and loop (Fig. 1 *Upper Left*), but differing in modified nucleosides, were produced by automated chemical synthesis (17). Modified nucleoside phosphoramidites were synthesized from the corresponding modified nucleosides (A.M., B.N., E.S., A.K., and J.J., unpublished work). They were deprotected and purified as previously described (8). The unmodified tRNA$^{Phe}_{AC}$ sequence CCAGACUGAAG^{11}-AUC^{14}UGG is designated tRNA$^{Phe}_{AC}$. The other sequences have been designated according to the presence of a modification: tRNA$^{Phe}_{AC}$-d(m^{5}C^{14}) with d(m^{5}C) substituting for C^{14}, tRNA$^{Phe}_{AC}$-(m^{5}C^{14}) with m^{5}C substituting for C^{14}, tRNA$^{Phe}_{AC}$-(m^{1}G^{11}) with m^{1}G substituting for G^{11}, and the doubly substituted tRNA$^{Phe}_{AC}$-(m^{1}G^{11}, m^{5}C^{14}). In addition, six DNA oligonucleotide analogs (Fig. 1 *Lower Right*) with

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||To whom reprint requests should be addressed at: Department of Biochemistry, Box 7622, North Carolina State University, Raleigh, NC 27695-7622.

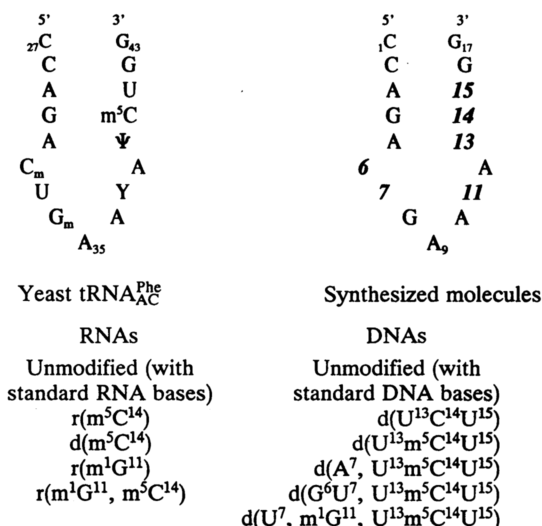

FIG. 1. Comparison of sequences and secondary structures of the native yeast $\mathrm{tRNA}^{\mathrm{Phe}}$ anticodon domain (*Upper Left*) with the chemically synthesized $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domains (*Upper Right*) and DNA analogs. The anticodon stem/loop of yeast $\mathrm{tRNA}^{\mathrm{Phe}}$ has the modified nucleosides 2′-*O*-methyl C (Cm) and G (Gm), 5-methylcytidine (m^{5}C), pseudouridine (Ψ), and the hypermodified tricylic guanosine derivative wyeosine (Y). The five $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ molecules (*Lower Left*) and the six DNA analogs (*Lower Right*) of the yeast $\mathrm{tRNA}^{\mathrm{Phe}}$ anticodon domain were synthesized and purified as described (7, 8).

sequences corresponding to that of the anticodon stem and loop domain of yeast $\mathrm{tRNA}^{\mathrm{Phe}}$ were synthesized and purified as described before (7). The unmodified DNA sequence, d(CCAGACT^{7}GAAG^{11}AT^{13}C^{14}T^{15}GG), is designated unmodified $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$. Five other sequences were synthesized with base modifications and/or base changes; for example, the sequence d(T^{13}C^{14}T^{15}) was changed to d(U^{13}m^{5}C^{14}U^{15}), and the DNA is designated $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$-d(U^{13}m^{5}C^{14}U^{15}). The four remaining sequences have been designated $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$-d(U^{13}C^{14}U^{15}), $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$-d(A^7, U^{13}m^{5}C^{14}U^{15}), $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$-d(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}), and $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$-d(U^7, m^{1}G^{11}, U^{13}m^{5}C^{14}U^{15}). The extinction coefficients of $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ and $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ analogs were calculated as 1.60×10^6 $M^{-1} \cdot cm^{-1}$ (7). Ribosomes, from which 30S subunits were prepared, were isolated from the RNase-deficient *E. coli* strain MRE600 (18, 19). The A_{260}/A_{280} ratio of the 30S ribosomal subunit preparations was between 1.8 and 1.9. Ribosomal subunit concentrations were calculated from the absorbance measurements on the basis of 70 pmol/A_{260} unit (19).

Phosphorylation of the Yeast $\mathrm{tRNA}^{\mathrm{Phe}}$ and $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ and DNA Analogs. The $\mathrm{tRNA}^{\mathrm{Phe}}$ anticodon stem/loop domains and their DNA analogs were 5′-end-labeled with ^{32}P by using [γ-^{32}P]ATP and T4 polynucleotide kinase (20).

Ribosome Binding of $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ and $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$. The interactions of $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ and $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ analogs with poly(U) or poly(A)-programmed 30S ribosomal subunits were assayed by using a nitrocellulose filtration method (10). *E. coli* 30S ribosomal subunits were activated at 37°C for 10 min before the assays. The assay mixtures (50 mM Tris·HCl, pH 7.2/25 mM magnesium acetate/150 mM NH_4Cl; 50 μl) were incubated at 4°C, in icewater, for 30 min. Each assay mixture contained 5 μg of poly(U) or poly(A), 0.56 μM *E. coli* 30S ribosomal subunits, and increasing concentrations of $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ or $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$. Assays without poly(U) or ribosomes or with poly(A) instead of poly(U) were performed as negative controls. Nonspecific binding of $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domains to 30S subunits was assessed in the absence of codon. The nonspecific binding of $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domains was the same as that of native $\mathrm{tRNA}^{\mathrm{Phe}}$. The nonspecific binding of $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ analogs to 30S subunits was higher. The data are presented with these negative controls already subtracted.

There are two tRNA-binding sites on the 30S subunits (21). The binding of $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domains or their DNA analogs, $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$, to poly(U)-programmed 30S ribosomal subunits did not reach saturation in the direct binding study. However, the $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ or $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ molecules were able to effectively inhibit whole $\mathrm{tRNA}^{\mathrm{Phe}}$ from binding 30S subunits in a competition assay.

$\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ and $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ Inhibition of $\mathrm{tRNA}^{\mathrm{Phe}}$ Binding to Poly(U)-Programmed Ribosomes. The binding of 5′-^{32}P-end-labeled native yeast $\mathrm{tRNA}^{\mathrm{Phe}}$ (0.6 μM) to poly(U)-programmed 30S ribosomal subunits was assayed alone and in the presence of various amounts of either unlabeled $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ or unlabeled $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$. The amount of native $\mathrm{tRNA}^{\mathrm{Phe}}$ bound to 30S ribosomal subunits in the presence of increasing amounts of synthesized $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ competitors was compared with the amount bound in the absence of anticodon domains. The ability of each $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ or $\mathrm{tDNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domain to inhibit the binding of native $\mathrm{tRNA}^{\mathrm{Phe}}$ to 30S subunits (percent inhibition) was plotted against the ratio of the concentration of the competitor to that of the native $\mathrm{tRNA}^{\mathrm{Phe}}$. To diminish the possible deviations derived from the different sample syntheses and preparations, unmodified $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ was used as an internal control to normalize the data.

RESULTS AND DISCUSSION

Binding of $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ to the Poly(U)-Programmed 30S Ribosomal Subunit. Five $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domains with sequences corresponding to the yeast $\mathrm{tRNA}^{\mathrm{Phe}}$ anticodon stem and loop, but differing in modified nucleosides, were produced by automated chemical synthesis (Fig. 1). Various $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domains synthesized by us and others (11) and end-labeled with ^{32}P differed in their abilities to bind poly(U)-programmed 30S ribosomal subunits (Fig. 2). For example, approximately 12 pmol of all unmodified $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ in the binding assay was bound to the 30S subunits, whereas only 4 pmol of $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$-d(m^{5}C^{14}) was bound under the same conditions. The $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domains did not bind poly(A)-

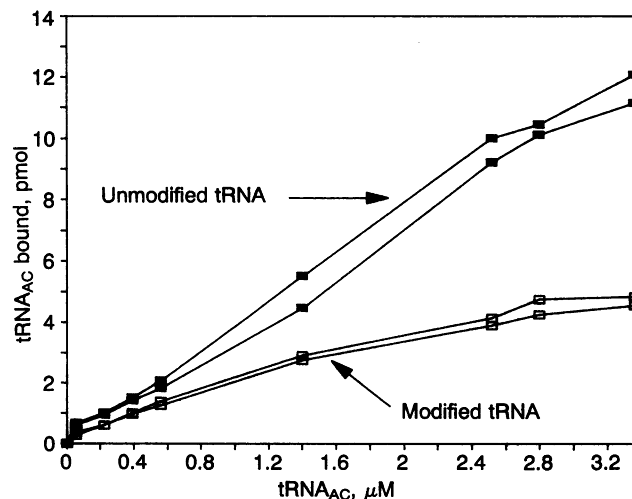

FIG. 2. Ribosome-mediated binding of two $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domains to poly(U)-programmed 30S ribosomal subunits. Each assay mixture contained 5 μg of poly(U) or poly(A). *E. coli* 30S ribosomal subunit concentration was maintained constant at 0.56 μM. The concentrations of the two $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ domains were increased from 0 to 3.5 μM as indicated. Poly(A)-programmed 30S ribosomal subunits were used in the reactions as negative controls. The results shown are for two experiments, unmodified $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$ (■) and $\mathrm{tRNA}^{\mathrm{Phe}}_{\mathrm{AC}}$-d(m^{5}C^{14}) (□). The results of negative controls were subtracted as background in all the experimental results.

programmed 30S ribosomal subunits. Thus, chemically synthesized tRNA$^{Phe}_{AC}$ domains bind directly and specifically to poly(U)-programmed 30S ribosomal subunits, but the quality of that binding is affected by the presence of a modified nucleoside in the stem of the domain. Completely unmodified tRNA$^{Phe}_{AC}$ was bound by 30S subunits with an affinity 2–3 orders lower than that of native tRNA^{Phe}, as had been reported by others (11). The direct binding of tRNA$^{Phe}_{AC}$ domains to programmed ribosomes, although an indication of this interaction, does not determine if the binding is similar in structure and location to that of native tRNA^{Phe}.

tRNA$^{Phe}_{AC}$ Domains Differentially Inhibit tRNA^{Phe} Binding of Poly(U)-Programmed 30S Ribosomal Subunits. To determine if the ribosome binding of tRNA$^{Phe}_{AC}$ domains was similar in location to that of native tRNA, the assay was altered to measure the ability of each unlabeled tRNA$^{Phe}_{AC}$ domain to compete with 5′-^{32}P-labeled native tRNA^{Phe} for programmed 30S ribosomal subunits. In the presence of increasing amounts of the tRNA$^{Phe}_{AC}$ domains, less native tRNA^{Phe} was bound to the 30S subunits (Fig. 3). However, the abilities of the variously modified tRNA$^{Phe}_{AC}$ domains to compete with tRNA^{Phe} for the 30S subunits were quite different. When equal in concentration to tRNA^{Phe}, both tRNA$^{Phe}_{AC}$-(m^1G^{11}, m^5C^{14}) and tRNA$^{Phe}_{AC}$-(m^1G^{11}) were able to inhibit more than 50% of the tRNA^{Phe} from binding the 30S subunit (Fig. 3). Under the same conditions unmodified tRNA$^{Phe}_{AC}$ was less competitive—i.e., capable of inhibiting 45% of tRNA^{Phe} from binding the ribosomal subunits. In contrast, tRNA$^{Phe}_{AC}$ domains with either d(m^5C^{14}) or m^5C^{14} as the only modified nucleoside were poor inhibitors, 15% and 10%, respectively.

tRNA$^{Phe}_{AC}$-d(m^5C^{14}),tRNA$^{Phe}_{AC}$-(m^5C^{14}), and DNA analogs (Fig. 1) have m^5C^{14}-dependent, Mg^{2+}-stabilized stems with five base pairs and a loop closed by two additional base pairs between C^6 and G^{11} and U^7 or T^7 and A^{10} (6–8). Unmodified tRNA$^{Phe}_{AC}$ (Fig. 1) has a weak stem with three or four base pairs and an open 7-membered loop (6–8). However, tRNA$^{Phe}_{AC}$-(m^1G^{11}, m^5C^{14}), the best competitor of tRNA^{Phe} in the ribosome binding assay (Fig. 3), has both a m^5C^{14}-dependent, Mg^{2+}-stabilized hairpin structure and an m^1G^{11}-aided open loop conformation (ref. 6; V.D. and P.F.A., unpublished results). Methylated G, precursor to Y^{37} in tRNA^{Phe} (22, 23), is unable to form a canonical base pair with C^6. Therefore, we hypothesized that tRNA anticodon domains with closed loop structures were inhibited from proper interaction with the codon. A nucleic acid sequence with a stabilized open loop structure usually has a high affinity to a complementary sequence due in part to the stabilized loop conformation, which provides more contacts to a complementary sequence than does a simple linear structure (24–26).

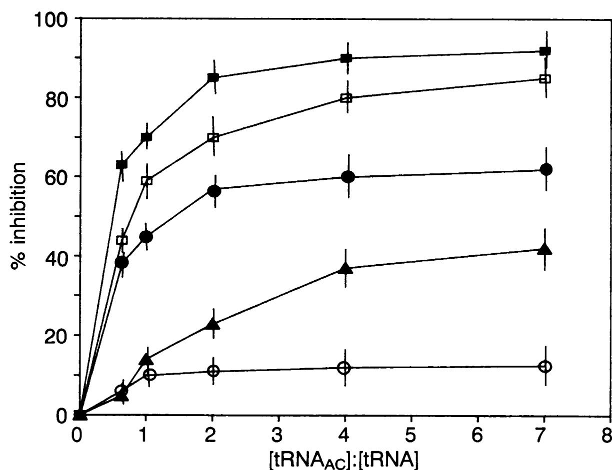

FIG. 3. Ribosome-mediated binding of tRNA^{Phe} to poly(U) in the presence of unmodified and modified tRNA$^{Phe}_{AC}$ molecules. Interactions of 5′-^{32}P-end-labeled tRNA^{Phe} with poly(U)-programmed 30S ribosomal subunits were assayed in the presence of increasing concentrations (0 to 4.2 μM) of the five unlabeled tRNA$^{Phe}_{AC}$ domains: ○, tRNA$^{Phe}_{AC}$-(m^5C^{14}); ▲, tRNA$^{Phe}_{AC}$-d(m^5C^{14}); ●, unmodified tRNA$^{Phe}_{AC}$; ▭, tRNA$^{Phe}_{AC}$-(m^1G^{11}); and ▬, tRNA$^{Phe}_{AC}$-(m^1G^{11}, m^5C^{14}).

Structurally Designed DNA Analogs of the tRNA$^{Phe}_{AC}$ Inhibit Native tRNA^{Phe} from Binding Poly(U)-Programmed 30S Ribosomal Subunits. Our hypothesis was tested with six DNA analogs of tRNA$^{Phe}_{AC}$ (Fig. 1), three of which were designed to have stable open-loop conformations (6, 8). The tDNA$^{Phe}_{AC}$-d(A^7, $U^{13}m^5C^{14}U^{15}$), tDNA$^{Phe}_{AC}$-d(G^6U^7, $U^{13}m^5C^{14}U^{15}$), and tDNA$^{Phe}_{AC}$-d(U^7, m^1G^{11}, $U^{13}m^5C^{14}U^{15}$) were synthesized to form m^5C-dependent, Mg^{2+}-stabilized conformations (6, 8). In addition, A is substituted for T^7 to disrupt the $T^7{\cdot}A^{10}$ base pair in tDNA$^{Phe}_{AC}$-d(A^7, $U^{13}m^5C^{14}U^{15}$) (Fig. 1). In tDNA$^{Phe}_{AC}$-d(G^6U^7, $U^{13}m^5C^{14}U^{15}$), G substitutes for C^6 to disrupt the $C^6{\cdot}G^{11}$ base pair and dU substitutes for T^7. U^7 corresponds to the invariant U^{33} that is important for a sharp "U-turn" in the anticodon loop and for maintenance of both 3′ and 5′ base stacking in the domain (27). Uridine is present at position 33 in 97% of all sequences of prokaryotic, eukaryotic, mitochondrial, and archaebacterial tRNAs (28). A structural rationale for this constancy comes from an examination of the crystal structure of yeast tRNA^{Phe}, which suggests that U^{33} forms two hydrogen bonds to stabilize the sharp turn that occurs in the anticodon loop after position 33 (29). The tDNA$^{Phe}_{AC}$-d(U^7, m^1G^{11}, $U^{13}m^5C^{14}U^{15}$) sequence most closely resembles that of the tRNA anticodon domain in its modification, and the methylation of G^{11} should effectively inhibit the formation of the $C^6{\cdot}G^{11}$ Watson–Crick base pair. Also, dU substitutes for T^7 in this analog. The abilities of the six different tDNA$^{Phe}_{AC}$ domains to compete with tRNA^{Phe} for poly(U)-programmed 30S subunits was dependent on the concentration of the domain and whether or not it had a stabilized, open loop structure (Fig. 4). At molar concentrations equal to the concentration of tRNA^{Phe}, the stabilized open loop structures of tDNA$^{Phe}_{AC}$-(A^7, $U^{13}m^5C^{14}U^{15}$), tDNA$^{Phe}_{AC}$-d(G^6U^7, $U^{13}m^5C^{14}U^{15}$), and tDNA$^{Phe}_{AC}$-d(U^7, m^1G^{11}, $U^{13}m^5C^{14}U^{15}$) were able to inhibit 20–28% of the tRNA^{Phe} from binding poly(U)-programmed 30S subunits. The other three DNA analogs inhibited ribosome binding by 10% or less. With

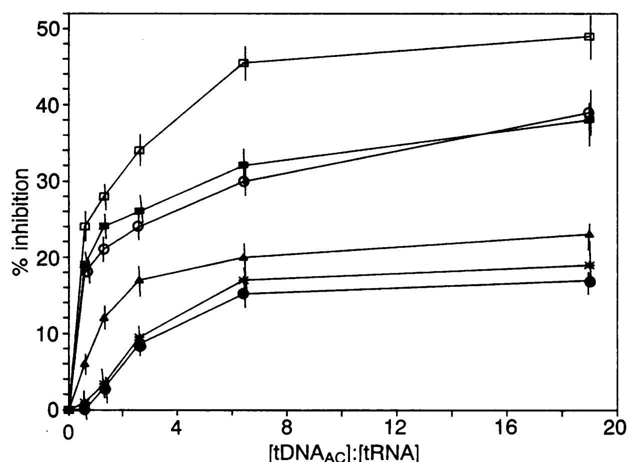

FIG. 4. Ribosome-mediated binding of tRNA^{Phe} to poly(U) in the presence of unmodified and modified tDNA$^{Phe}_{AC}$ molecules. The amounts of tRNA^{Phe} bound to 30S subunits in the presence of the tDNA$^{Phe}_{AC}$ were compared with the amount bound in the absence of competing tDNA$^{Phe}_{AC}$. Reaction mixtures contained increasing concentrations (0–11.4 μM) of the six tDNA$^{Phe}_{AC}$ analogs: ●, tDNA$^{Phe}_{AC}$-d($U^{13}m^5C^{14}U^{15}$); *, tDNA$^{Phe}_{AC}$-d($T^{13}C^{14}T^{15}$); ▲, tDNA$^{Phe}_{AC}$-d($U^{13}C^{14}U^{15}$); ▬, tDNA$^{Phe}_{AC}$-d(A^7, $U^{13}m^5C^{14}U^{15}$); ○, tDNA$^{Phe}_{AC}$-d(U^7, m^1G^{11}, $U^{13}m^5C^{14}U^{15}$); and ▭, tDNA$^{Phe}_{AC}$-d(G^6U^7, $U^{13}m^5C^{14}U^{15}$). The ability of each tDNA$^{Phe}_{AC}$ to inhibit the binding of native tRNA^{Phe} to 30S subunits (percent inhibition) is plotted against the ratio of the tDNA$^{Phe}_{AC}$ concentration to that of tRNA^{Phe}.

a molar concentration 6 times that of the tRNA^{Phe}, tDNA$^{Phe}_{AC}$-d(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}) inhibited 45% of the tRNA^{Phe} from binding the 30S subunits, tDNA$^{Phe}_{AC}$-(A^7, U^{13}m^{5}C^{14}U^{15}) and tDNA$^{Phe}_{AC}$-d(U^7, m^{1}G^{11}, U^{13}m^{5}C^{14}U^{15}) inhibited 30%, and unmodified tDNA^{Phe} and tDNA^{Phe}-d(U^{13}m^{5}C^{14}U^{15}) inhibited subunit binding by only 14–16% (Fig. 4). Therefore, tDNA$^{Phe}_{AC}$-d(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}) was comparable to unmodified tRNA$^{Phe}_{AC}$ and considerably better than tRNA$^{Phe}_{AC}$-(m^{5}C^{14}) and tRNA$^{Phe}_{AC}$-d(m^{5}C^{14}) (Fig. 3), as well as the other DNA analogs, in inhibiting tRNA^{Phe} from binding poly(U)-programmed 30S subunits.

The analog tRNA$^{Phe}_{AC}$-d(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}) was a better inhibitor to native tRNA^{Phe} binding 30S subunits than tDNA$^{Phe}_{AC}$-d(A^7, U^{13}m^{5}C^{14}U^{15}). We expected that tDNA$^{Phe}_{AC}$-d(U^7, m^{1}G^{11}, U^{13}m^{5}C^{14}U^{15}), as the analog most closely resembling tRNA$^{Phe}_{AC}$-(m^{1}G^{11}, m^{5}C^{14}) in sequence, would be better than tDNA$^{Phe}_{AC}$-d(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}) in inhibiting native tRNA^{Phe} from binding the ribosomal subunit, but instead it was comparable to tDNA$^{Phe}_{AC}$-d(A^7, U^{13}m^{5}C^{14}U^{15}). Perhaps the single methylation of G^{11} was not sufficient to block all H-bonding interactions with C^6 in the DNA analog. In tRNA^{Phe}, m^{1}G is the precursor to the tricyclic hypermodified nucleoside Y^{37} and the C at position 32 across the loop is methylated to 2′-*O*-methyl-C. This extensive modification may be required to keep the loop open. Alternatively, the C^6 to G^6 base change in tDNA$^{Phe}_{AC}$-d(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}) may have blocked base pairing across the loop, but it also could have produced a DNA loop conformation more closely related to that of the tRNA.

The importance of the stem sequence modification's contribution to the DNA analogs' abilities to inhibit native tRNA^{Phe} from binding the 30S subunit was investigated by using the analog tDNA$^{Phe}_{AC}$-d(U^{13}C^{14}U^{15}). The tDNA$^{Phe}_{AC}$-d(U^{13}C^{14}U^{15}) analog was a better inhibitor of the tRNA^{Phe}-ribosome binding than tDNA$^{Phe}_{AC}$-d(U^{13}m^{5}C^{14}U^{15}) (Fig. 4), which has a closed loop (6). The tDNA$^{Phe}_{AC}$-d(U^{13}C^{14}U^{15}) was also better than unmodified tDNA$^{Phe}_{AC}$, which has an open loop but is without the modification-dependent, Mg^{2+} stabilization of the structure. However, tDNA$^{Phe}_{AC}$-d(U^{13}C^{14}U^{15}) was a poorer inhibitor than tDNA$^{Phe}_{AC}$-(A^7, U^{13}m^{5}C^{14}U^{15}), tDNA$^{Phe}_{AC}$-d(U^7, m^{1}G^{11}, U^{13}m^{5}C^{14}U^{15}), and tDNA$^{Phe}_{AC}$-d(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}), which have both open loops and Mg^{2+}-stabilized conformations. To ensure that the competitive ability of the DNA analog was independent of the RNA structure of the poly(U), the same experiments were performed with poly[d(T)]-programmed 30S ribosomal subunits. Poly[d(T)] is translated in cell-free systems, but not as effectively as poly(U) (30). Native tRNA^{Phe} was bound to poly[d(T)]-programmed 30S ribosomal subunits only 60% as effectively as to poly(U)-programmed subunits. The relative abilities of two tRNA$^{Phe}_{AC}$ domains and the various DNA analogs to inhibit tRNA^{Phe} from binding poly[d(T)] were unchanged. This suggests that the ability or inability of the various tDNA$^{Phe}_{AC}$ analogs to inhibit tRNA^{Phe} binding to ribosomes is independent of the RNA nature of the coding triplet.

Binding of tDNA$^{Phe}_{AC}$ to the Poly(U)-Programmed 30S Ribosomal Subunit. Unmodified tDNA$^{Phe}_{AC}$ does not bind the poly(U)-programmed 30S ribosomal subunit (11). We have designed DNA analogs with modified nucleosides to have the structural (7) and functional properties important to tRNA$^{Phe}_{AC}$, and these tDNA$^{Phe}_{AC}$ analogs successfully inhibited native tRNA^{Phe} binding to 30S ribosomal subunits (Fig. 4). Fig. 5 shows the results of the direct binding of three tDNA$^{Phe}_{AC}$ analogs, tDNA$^{Phe}_{AC}$-(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}), tDNA$^{Phe}_{AC}$-(m^{1}G^{11}, U^{13}m^{5}C^{14}U^{15}), and tDNA$^{Phe}_{AC}$-(U^{13}m^{5}C^{14}U^{15}), to poly(U)-programmed 30S ribosomal subunits. The tDNA$^{Phe}_{AC}$-(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}), the best inhibitor of all the tDNA analogs studied, also was bound to poly(U)-programmed 30S subunits most effectively. As expected from results with tRNA$^{Phe}_{AC}$-d(m^{5}C^{14}) (Fig. 2), tDNA$^{Phe}_{AC}$-(U^{13}m^{5}C^{14}U^{15}) did not bind to poly(U)-programmed 30S subunits (Fig. 5).

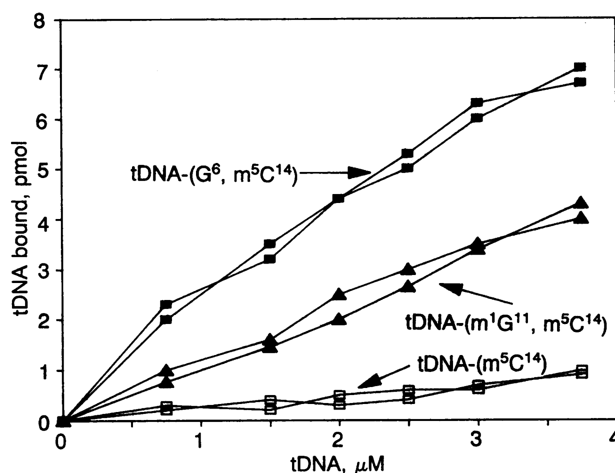

FIG. 5. Ribosome-mediated binding of three tDNA$^{Phe}_{AC}$ domains to poly(U)-programmed 30S subunits. Each assay mixture contained 5 μg of poly(U) or poly(A). *E. coli* 30S ribosomal subunit concentration was maintained constant at 0.56 μM. The concentrations of the three tRNA$^{Phe}_{AC}$ domains were increased from 0 to 3.75 μM, as the points on the figure indicate. Poly(A)-programmed 30S ribosomal subunits were used in the reactions as negative controls. The results of negative controls were subtracted as background in all the experimental results. The results shown are for two experiments: ▭, tDNA$^{Phe}_{AC}$-d(U^{13}m^{5}C^{14}U^{15}); ▲, tDNA$^{Phe}_{AC}$-d(U^7, m^{1}G^{11}, U^{13}m^{5}C^{14}U^{15}); and ■, tDNA$^{Phe}_{AC}$-d(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}).

Nucleoside Modifications Stabilize the Stem and Maintain the Open-Loop Structure of the Anticodon Domain. Although correct anticodon–codon interaction is essential to translational fidelity, the three bases of the anticodon of tRNA are not the only nucleotides of the anticodon domain that are important in the tRNA's interaction with the ribosome and mRNA. The performance of a tRNA in translation is determined by an "extended anticodon" (31). We postulated that m^{5}C^{40} of native yeast tRNA^{Phe} is important in producing a Mg^{2+}-regulated dynamic of the anticodon loop structure in which alternative conformations are recognized for different tRNA functions (6). We also postulated that modified nucleosides of the anticodon loop aid in this dynamic. For instance, the hypermodified base Y at position 37, 3′-adjacent to the anticodon, is a G modified in such a way as to prevent H-bonding to N1 and the C2 amino group. The tricylic Y's inability to base pair facilitates the open-loop conformation (6); its increased hydrophobic character, as compared with G, is important to an improved base stacking of the anticodon (32). Since there are two tRNA-binding sites on 30S ribosomal subunits (21), the affinity of tRNA^{Phe} to the two sites on the ribosome are probably differentially affected by modification and ion binding. To determine if anticodon loop modifications were important to the ribosome binding of other tRNAs, we screened the 507 reported tRNA sequences (European Molecular Biology Laboratory, tRNA sequence data bank, Heidelberg) for their potential to form Watson–Crick base pairs across the anticodon loop. Of the 121 sequences with the potential of forming two canonical base pairs across the anticodon loop, one of which involves an anticodon base, 115, or 95%, were modified in a way as to preclude one base pair (Table 1). All of the remaining six sequences had the potential of forming two A·U base pairs.

The inability of unmodified tDNA$^{Phe}_{AC}$ to bind to poly(U)-programmed 30S ribosomal subunits (11) or to compete with native tRNA^{Phe} for 30S ribosomal subunits is due to the absence of modified nucleoside structural constraints, as well as absence of the 2′-OHs. The tDNA analogs, tDNA$^{Phe}_{AC}$-d(A^7, U^{13}m^{5}C^{14}U^{15}), tDNA$^{Phe}_{AC}$-d(U^7, m^{1}G^{11}, U^{13}m^{5}C^{14}U^{15}), and tDNA$^{Phe}_{AC}$-d(G^{6}U^7, U^{13}m^{5}C^{14}U^{15}), were effective inhibitors of

Table 1. Correlation between base modifications and the potential for Watson–Crick base pairs across tRNA anticodon loops

Sequences	No.	% of total
With potential for two base pairs	121	—
With potential for two base pairs, but at least one prevented by base modification	115	95
With potential for two base pairs, and not prevented by base modifications	6*	5
With potential for one base pair	224	—
With potential for one base pair, but prevented by base modification	42	19
With potential for one base pair, and not prevented by base modification	182†	81

The total number of tRNA sequences screened was 507.
*All six potential base pairs are A·U.
†Of these base pairs, 146 are A·U.

native $tRNA^{Phe}$ binding to poly(U)-programmed ribosomal subunits because they had modified nucleoside-dependent, Mg^{2+}-stabilized stems and open-loop structures. Therefore, structural elements of the extended anticodon, such as the Mg^{2+} stabilization of the stem and the m^1G- (or Y)-aided open loop, and the contribution of individual 2′-OHs to nucleoside conformation, are all determining factors in effective tRNA–ribosome interactions.

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