tRNA-Guanine Transglycosylase from *Escherichia coli*: Gross tRNA Structural Requirements for Recognition†

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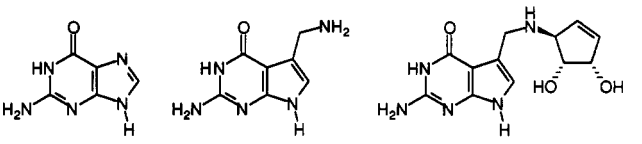
ABSTRACT: tRNA-guanine transglycosylase (TGT) is the enzyme responsible for the post-transcriptional modification of specific tRNAs (those for Asn, Asp, His, and Tyr) with the hypermodified base, queuine. In *Escherichia coli* this enzyme catalyzes the exchange of guanine-34 in the anticodon with $preQ_1$, which is subsequently further modified to queuine. There is evidence that such hypermodified tRNA molecules may play a role in the control of cell proliferation and differentiation. In order to perform detailed, in vitro mechanistic studies and to probe the tRNA–enzyme interaction, we have generated unmodified *E. coli* $tRNA^{Tyr}$ and truncated analogues using an in vitro RNA synthesis system suggested by Milligan and Uhlenbeck [Milligan, J. F., & Uhlenbeck, O. C. (1989) *Methods Enzymol. 180*, 51-62]. From this system we have generated three tRNA analogues totally devoid of any post-transcriptional modifications. In order to compare the unmodified tRNA with the true physiological substrate for TGT, that is, tRNA that contains all modified bases except queuine, we have isolated *E. coli* $tRNA^{Tyr}$ from an overexpressing clone in a TGT-deficient strain of *E. coli*. We report here that unmodified, full-length $tRNA^{Tyr}$ serves as a substrate for TGT with kinetic parameters that are, within experimental error, the same as those for in vivo isolated $tRNA^{Tyr}$. This indicates that other post-transcriptional modifications have negligible effects upon TGT recognition of tRNA. A 17-base oligoribonucleotide, corresponding to the anticodon loop and stem, is also a substrate for TGT with only a 20-fold loss in V_{max}/K_M, versus the full-length tRNA. These results indicate that 80% of the tRNA structure is dispensible, suggesting that the recognition elements for the TGT reaction are localized in the anticodon loop and/or stem of the cognate tRNAs.

Accurate recognition and discrimination of tRNAs by aminoacyl-tRNA synthetases is requisite for faithful translation of the genetic code. Recently, significant progress has been made to elucidate this recognition process for some tRNAs, most notably the alanyl-tRNA synthetase (Schimmel, 1989). In a number of studies, tRNAs and tRNA analogues have been synthesized in vitro using the T7 RNA polymerase to transcribe DNA templates (Sampson & Uhlenbeck, 1988; Samuelsson et al., 1988; Francklyn & Schimmel, 1989). In these cases it has been shown that the in vitro-generated tRNA, containing no modified bases, has similar, if not identical, kinetic parameters to fully modified tRNA. Singhal (1983) has reported that queuine-containing $tRNA^{Asp}$ is only a slightly better substrate (2-fold higher V_{max}/K_M) for the aspartyl-tRNA synthetase in vitro.

Studies of the tRNA methyltransferase that is responsible for the ribothymidine in the TΨC loop of tRNA have been reported by Gu and Santi (1991). They found that this modifying enzyme is able to recognize a truncated form of tRNA that corresponds to the T arm alone. The ribothymidine in the TΨC loop of tRNA is found in virtually all tRNAs. We have embarked upon studies of tRNA-guanine transglycosylase (TGT, E.C. 2.4.2.29), the enzyme responsible for the incorporation of the modified base queuine (Q, 7-[[(4,5-*cis*-dihydroxycyclopenten-2-yl)amino]methyl]-7-deazaguanine, Figure 1), which is found only in the anticodon wobble position (QUN) of the tRNAs for Asn, Asp, His, and Tyr.



GUANINE (G) preQUEUINE$_1$ (preQ$_1$) QUEUINE (Q)

FIGURE 1: Structures of guanine, $preQ_1$, and queuine.

Queuine has been found in the cognate tRNAs of virtually all organisms studied with the exception of yeast. Significant alterations of the extent of queuine in the cognate tRNAs from various biological sources have been found. A number of studies have shown that, in eukaryotes, queuine is involved in differentiation, proliferation, and perhaps cellular signaling [summarized in Kersten and Kersten (1990)]. It has been proposed that queuine may be involved in the metabolic control of respiration and lactate metabolism in prokaryotes (Kersten & Kersten, 1990).

tRNA-guanine transglycosylase has been isolated from a number of different sources and exhibits differences in gross structural features such as molecular weight and subunit composition (Singhal, 1983). There appear to be two functionally different classes of the enzyme. The first class of TGT, represented by the eukaryotic enzymes, exchange queuine into tRNA. The second class, represented by the enzyme from *Escherichia coli*, does not recognize queuine itself but exchanges a queuine precursor ($preQ_1$, Figure 1), which lacks the cyclopentene diol moiety, for the genetically encoded guanine-34.

We have recently reported the isolation and characterization of *E. coli* TGT from an overexpressing clone (Garcia et al., 1993). We report here the generation of unmodified $tRNA^{Tyr}$

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Table I: Oligonucleotides Used in ptRNA2 Construction and in Truncated tRNA Transcriptions

oligo	sequence (5′ to 3′)	T_H[a] (°C)
TRNA1 (85-mer)	GTG GTG GGG GAA GGA TTC GAA CCT TCG AAG TCG ATG ACG GCA GAT TTA CAG TCT GCT CCC TTT GGC CGC TCG GGA ACC CCA CCT A	n/a
TRNA2 (21-mer)	TGG TGG TGG GGG AGG GAT TCG	67
TRNA3 (21-mer)	GAA TTC GGT GGG GTT CCC GAG	65
TRNA4 (30-mer)	GAA AGC TTA TGA AAT TTG AAC TGG	85
G2A (15-mer)	TGG GGG AAG GAT TCG	45
TZSEQ (17-mer)	ACG CCA GGG TTT TCC CA	51
IVSEQ (18-mer)	AAA CGA CGG CCA GTG CCA	55
dT7P (17-mer)	TAA TAC GAC TCA CTA TA	41
dECY-A1 (34-mer)	GCA GAT TTA CAG TCT GCT ATA GTG AGT CGT ATT A	n/a
dECY-D1 (52-mer)	GCA GAT TTA CAG TCT GCT CCC TTT GGC CGC TCG GGT ATA GTG AGT CGT ATT A	n/a
dECY-D2[b] (53-mer)	GCA GAT TTA CAG TCT GCT CCC TTT GGC CGC TCG GGC TAT AGT GAG TCG TAT TA	n/a

[a] $T_H \equiv 2(A + T) + 4(G + C) - 3$; n/a, not applicable. [b] The added cytosine is underlined.

and truncated $tRNA^{Tyr}$ analogues via in vitro transcription of DNA templates and the isolation of queuine-deficient $tRNA^{Tyr}$ from an overexpressing clone in vivo. These tRNAs and tRNA analogues are substrates for TGT. The results of the present study indicate that other post-transcriptional modifications are not necessary for TGT recognition of tRNA and that 80% of the tRNA structure can be removed with only a 20-fold diminution in V_{max}/K_M.

MATERIALS AND METHODS

Reagents. Buffers and inorganic pyrophosphatase were purchased from Sigma. Nucleoside triphosphates, M13K07 helper phage, and the plasmid pTZ18U were from Pharmacia. The GeneAmp kit was from Perkin-Elmer. The Sequenase Version 2.0 kit was from United States Biochemicals. *Bst*NI restriction endonuclease was from Stratagene. [8-^{14}C]Guanine was from Moravek Biochemicals. Centiprep-10 concentrators (no. 4304) were from Amicon. TGT was isolated from an overexpressing clone as described previously (Garcia et al., 1993). T7 RNA polymerase was isolated from *E. coli* BL21/pAR1219 following the procedure of Grodberg and Dunn (1988). Oligodeoxynucleotides (Table I) were synthesized at the University of Michigan Biomedical Research Resources Core Facility.

Template DNA (ptRNA2) Construction. The PCR[1] primers TRNA2 and TRNA3 were designed to amplify the $tRNA^{Tyr}$ gene from the oligo TRNA1. The forward primer TRNA2 incorporated an *Eco*RI site in the beginning of the tRNA gene, to enable cloning into the *Eco*RI site of pTZ18U. The reverse primer TRNA3 was designed to leave a blunt end after the last base of the tRNA coding sequence. The last three bases of the tRNA gene are CCA, and when this end of the amplified gene ligates into the *Sma*I site (CCC′GGG) of pTZ18U, a new *Bst*NI restriction site (CC′AGG) is formed. Amplification of the $tRNA^{Tyr}$ gene was done following the vendor's protocol. The annealing temperature used was 35 °C. After 30 cycles, the amplified $tRNA^{Tyr}$ gene was extracted with phenol and chloroform. The DNA was ethanol precipitated, and the pellet was resuspended in 20 μL of sterile water.

Subcloning of the PCR product into pTZ18U was performed as outlined in Figure 2. Approximately 680 μg of pTZ18U was digested using 8 units of *Sma*I in a 170-μL reaction mixture for 1 h at 30 °C. After phenol and chloroform extractions, the digested DNA was ethanol precipitated, and the pellet was resuspended in 85 μL of sterile water. Ten microliters of the *Sma*I-restricted pTZ18U and 20 μL of PCR product from above were digested with 5 units of *Eco*RI in a single 40-μL reaction mixture for 1 h at 37 °C. The DNA was extracted with phenol/chloroform, as before, and ethanol precipitated. The pellet was then resuspended in 50 μL of sterile water. The 50-μL mixture of *Eco*RI-digested vector and tRNA gene DNA was ligated using 5 units of T4 DNA ligase in a 60-μL reaction mixture at 16 °C overnight. The following day, 50 μL of the ligation mixture was incubated with 200 μL of competent *E. coli* TG2 at 0 °C for 40 min. Blue/white selection was used to detect transformants containing inserts into the multiple cloning site of pTZ18U. The transformed cells were heat shocked at 45 °C for 2 min by adding the cells to 3 mL of H-top (45 °C) containing 134 μg/mL IPTG and 267 μg/mL X-Gal. This mixture was then spread onto an L-Amp plate and incubated at 37 °C overnight. Twelve white colonies were picked from the developed plate, and template DNA was generated using the M13K07 protocol from Pharmacia.

The 12 templates were screened for correct inserts via dideoxy sequencing using TZSEQ (Table I) as a sequencing primer following the Sequenase (USB) protocol. Seven out of twelve of the templates contained inserts, and two of the seven had inserts that were determined to be the complete *E. coli* $tRNA^{Tyr}$ gene; however, a point mutation (A to G) had been introduced into the gene. This construct was designated ptRNA1. Kunkel-style site-directed mutagenesis was performed on ptRNA1 to change the PCR-induced guanine point mutation back to the correct adenine base (Ausubel et al., 1987). The uracil-containing ptRNA1 (U-ptRNA1) was generated using published protocols (Ausubel et al., 1987). Two units of PNK was used to phosphorylate 420 pmol of the G2A oligo, in a 20-μL reaction mixture. A 10-μL annealing reaction mixture was set up, containing 5 pmol of U-ptRNA1 and 100 pmol of phosphorylated G2A oligo in 10 μL of 1× annealing buffer (20 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, and 50 mM NaCl). Annealing was carried out by heating the samples for 3 min at 70 °C followed by 30 min at 37 °C and then allowing them to cool slowly to room temperature. The annealed template and primer were subjected to primer extension and ligation to complete the mutant strand. The annealed primer/template mixture was adjusted to synthesis conditions by adding 1 mg of T4 gene 32 protein, 1 unit of T4 DNA polymerase, 5 units of T4 DNA ligase, and enough 10× synthesis buffer to bring the final buffer conditions to 23 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 35 mM NaCl, 1.5 mM DTT, 0.5 mM ATP, and 0.5 mM dNTPs. The synthesis/ligation mixture was incubated sequentially at 0 °C for 5 min, 25 °C for 5 min, and 37 °C for 90 min. A 20-μL aliquot of the completed mutagenesis mixture was used to transform 200 μL of competent *E. coli* TG2. Twelve colonies picked from the developed plate were used to generate single-stranded DNA for sequencing purposes. Template DNA preparation

[1] Abbreviations: PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; IPTG, isopropyl β-thiogalactoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol, HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanelsulfonic acid; PNK, polynucleotide kinase; dNTP, deoxynucleotide triphosphate; NTP, ribonucleotide triphosphate; BSA, bovine serum albumin. Abbreviations for oligonucleotides are defined in Table I. Abbreviations for RNA transcripts are defined in Figure 5, where EC stands for *E. coli* and Y stands for tyrosine.

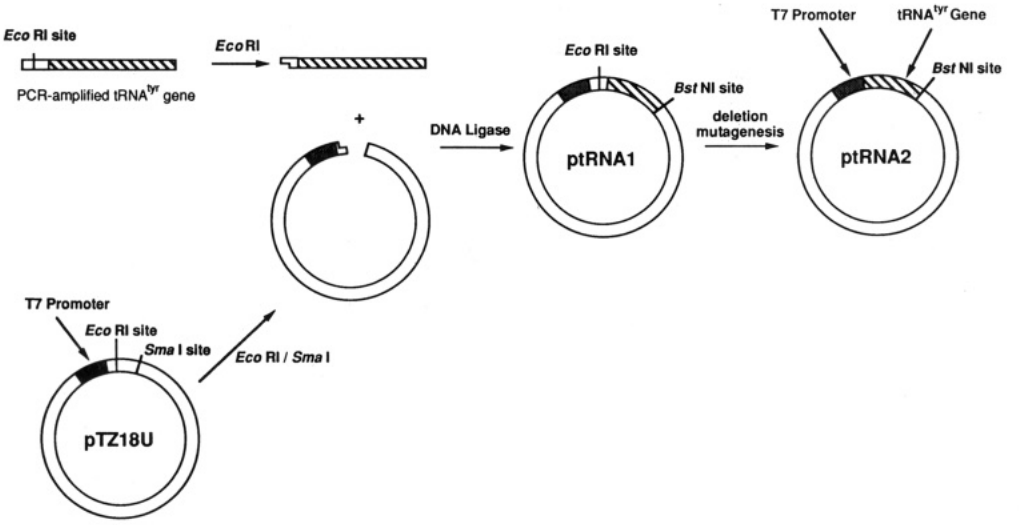

FIGURE 2: ptRNA2 construction. There are five other *Bst*NI sites in this vector. Restriction by *Bst*NI generates six fragments; however, only one fragment contains the T7 promoter sequence and was transcribed.

was performed exactly as before. Of the twelve templates, two samples were determined to have the correct G to A revertant mutation by DNA sequence analysis. The sample labeled C6 was used for additional mutagenesis to remove the eight bases that include the *Eco*RI cloning site.

Uridine-containing template DNA from the above C6 mutant strain (U-ptRNA$_{C6}$) was prepared as described above. Two hundred picomoles of the mutant oligo TRNA4 were phosphorylated in a 30-μL reaction mixture containing 4.5 units of T4 PNK, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, and 8.6 mM Mg·ATP complex. The incubation was for 45 min at 37 °C, followed by heat inactivation at 65 °C for 10 min. A 10-μL annealing reaction mixture was set up containing 6 pmol of phosphorylated TRNA4 and 0.35 pmol of U-ptRNA$_{C6}$ under the same buffer conditions mentioned in the G2A mutagenesis section. Annealing of the primer to the template DNA was performed by heating the sample to 93 °C for 5 min, followed by an incubation at 75 °C for 20 min and then cooling to room temperature slowly. Primer extension and ligation to complete the mutant strand was performed under the same buffer conditions mentioned in the G2A mutagenesis section. After the final incubation at 37 °C, the mutagenesis mixture was diluted using 90 μL of TE8. A 40-μL aliquot of the diluted mixture was used to transform 200 μL of competent *E. coli* TG2. Colonies from the developed plate were screened for the presence of the mutation via dideoxy DNA sequencing. Ten out of the twelve colonies screened were determined to have the eight-base deletion. A 1-L plasmid preparation was performed on the strain which exhibited the clearest sequence during screening, and the resulting plasmid was designated ptRNA2.

Template DNA Preparation. The plasmid ptRNA2 upon restriction digestion provides the template for full-length tRNA transcription (Figure 3). A 1-mL restriction digestion is performed using 2 μM plasmid and 40 units of *Bst*NI restriction endonuclease in Stratagene Buffer no. 7 (150 mM NaCl, 10 mM Tris-HCl, pH 7.7, 10 mM $MgCl_2$, 1 mM DTT, and 10 μg/mL BSA). The mixture is incubated for 2 h at 60 °C. The digestion mixture is then extracted with equal volumes of phenol and chloroform and ethanol precipitated at –20 °C overnight. The template strands for ECY-D1, ECY-D2, and ECY-A1 (see Table I and Figure 5) transcriptions consist of the gene to be transcribed abutting a 17-base sequence corresponding to the Φ10 T7 RNA polymerase promoter. These strands (5 nmol) are mixed with the 17-base sequence (dT7P, Table I) corresponding to the complement of the T7 promoter (6 nmol) in 0.1–0.5 mL of 10 mM Tris, pH 8. The strands are annealed by heating at 90 °C for 10 min and allowed to cool to room temperature (approximately 30 min). This process generates a synthetic DNA template that is double-stranded in the T7 promoter region and single-stranded over the length of the template sequence.

Transcription Reaction. A typical transcription (Figure 3) is carried out using the templates generated above (2 μM plasmid template and 5 μM synthetic DNA template) in a 1-mL transcription mixture composed of the transcription buffer, 40 mM Tris-HCl (pH 8 at 37 °C), 20 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 4 mM each NTP (ATP, CTP, GTP, and UTP), 2500 units of T7 RNA polymerase, 5 units of RNase inhibitor, and 2 units of inorganic pyrophosphatase. The mixture is allowed to incubate at 37 °C for approximately 6 h. The mixture is extracted with equal volumes of phenol and chloroform and ethanol precipitated at –20 °C overnight. (In order to generate ^{3}H-labeled transcripts, 50 μM [2,8-^{3}H]ATP can be added to the transcription reaction.)

Transcript Purification. The precipitate is resuspended in 10 mM Tris, pH 8, and 1 mM EDTA (TE8), and 7 M urea and is heated to 80 °C for 10 min and cooled rapidly on ice. The transcript is then applied to a MonoQ column (HR 10/10, Pharmacia) and eluted with a linear gradient from 0 to 1 M NaCl in TE8 with 7 M urea at 4 °C. Fractions containing RNA product are pooled and desalted using Centiprep-10 concentrators and exchanged into 10 mM Tris, pH 8. The RNA fractions are then ethanol precipitated overnight at –20 °C. The transcript is stored as the dried precipitate at –20 °C. When used for kinetics experiments, the transcript is resuspended in 10 mM Tris, pH 8, and 10 mM $MgCl_2$ to 100 μM RNA, heated at 80 °C for 3 min, and cooled rapidly on ice.

The concentrations of the transcripts were determined spectrophotometrically using extinction coefficients at 260 nm calculated from the base composition of each transcript (Table II).

In Vivo tRNA^{Tyr} Overexpression Vector (ptRNA3) Construction (Figure 4). Uridine-containing single-stranded DNA from the C6 mutant strain (U-ptRNA$_{C6}$) was used as a template for a PCR reaction designed to amplify the tRNA^{Tyr} gene. The oligos TZSEQ and TRNA4 were used in a PCR reaction identical to that described above. The 50-μL PCR reaction mixture was phenol extracted, ethanol precipitated,

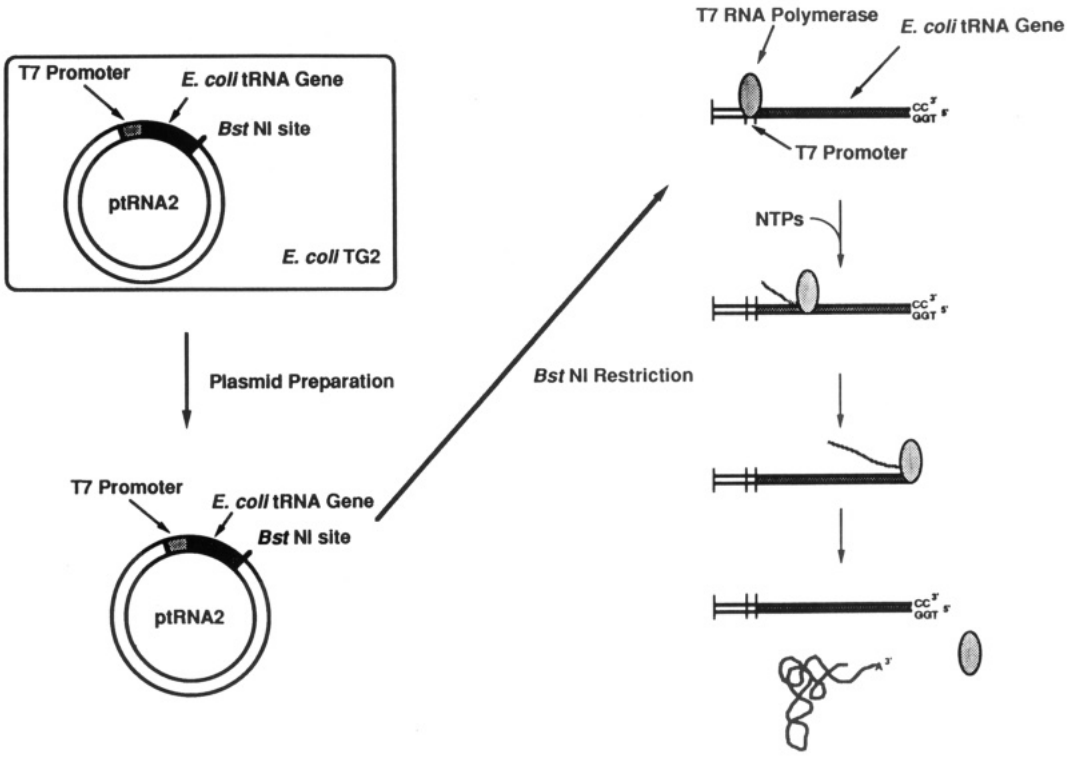

FIGURE 3: In vitro transcription of full-length *E. coli* $tRNA^{Tyr}$. Plasmid template for full-length tRNA transcription was generated and transcribed as described in Materials and Methods. Synthetic oligodeoxynucleotides served as templates for truncated tRNA transcriptions as described in Materials and Methods.

Table II: Extinction Coefficients for $tRNA^{Tyr}$ and Analogues[a]

tRNA	base composition	A_{260} (OD/mM cm)
ECY2	$A_{19}G_{23}C_{27}U_{16}$	914
ECY-D2	$A_{10}G_{12}C_{10}U_4$	406
ECY-A1	$A_5G_4C_4U_4$	192

[a] The individual extinction coefficients used were A = 15.2, G = 12.0, C = 7.0, and U = 10.0 OD/mM cm.

and resuspended in 40 μL of sterile water. Ten microliters of the resuspended amplification fragment was digested with 100 units of *Bam*HI at 37 °C for 1 h. The restricted fragment was ethanol precipitated, and the restriction site was "blunt ended" using T4 DNA polymerase as described in section 3.5.11 of Ausubel et al. (1987). The blunt-ended fragment was then digested with 30 units of *Eco*RI in the vendor's buffer at 37 °C for 2 h. The prepared insert was then ethanol precipitated and resuspended in 15 μL of sterile water.

The plasmid pTB9,[2] a derivative of pGFIB1 (Masson & Miller, 1986) containing the gene for the *Bacillus stearothermophilus* $tRNA^{Tyr}$ in an *Eco*RI–*Pst*I fragment, was prepared in a similar fashion. Approximately 200 μg of pTB9 were digested with 50 units of *Pst*I in the vendor's buffer at 37 °C for 6 h. The linearized vector was ethanol precipitated and blunt ended using T4 DNA polymerase as above. The blunt-ended vector was digested with 20 units of *Eco*RI in the vendor's buffer at 37 °C for 2 h. The prepared vector was ethanol precipitated and resuspended in 15 μL of sterile water.

Ten microliters of prepared insert DNA and 1 μL of prepared vector DNA were ligated using 20 units of T4 DNA ligase in the vendor's buffer overnight at 16 °C. The ligation mixture was transformed into competent *E. coli* TG2. Template DNA was prepared from 12 of the colonies using the M13K07 protocol. Dideoxy sequencing using IVSEQ (Table I) as a sequencing primer revealed that clone 7 contained the insert. This clone was named ptRNA3.

Plasmid DNA from ptRNA3/TG2 was used to transform competent *E. coli* K12 (Δ*tgt*), a K12 strain of *E. coli* in which the *tgt* gene has been deleted.[3]

In Vivo $tRNA^{Tyr}$ Isolation and Purification. A 2-L culture of ptRNA3/*E. coli* K12 (Δ*tgt*) in 2× TY media with ampicillin was incubated at 37 °C overnight. Cells were harvested via centrifugation at 4500*g* for 15 min and resuspended in 200 mL of lysis buffer (10 mM Bis-Tris, pH 5.5, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 1% SDS). Total nucleic acids were isolated by extracting the cell pellet with phenol/chloroform followed by ethanol precipitation. The nucleic acid pellet was resuspended in 95 mL of DNAase I buffer (100 mM sodium acetate, pH 5.0, and 5 mM $MgSO_4$) and was incubated with 2100 units of DNAase I at 37 °C for 1 h. The solution was extracted with phenol/chloroform, and an equal volume (≈100 mL) of 8 M LiCl was added to the aqueous layer, which was then incubated at −20 °C for 2 h. The LiCl precipitate was removed by centrifugation at 18000*g* for 15 min. The supernatant was dialyzed against 10 mM Tris, pH 8, and 10 mM $MgCl_2$ overnight at 4 °C and then ethanol precipitated. The $tRNA^{Tyr}$ was then purified by anion-exchange chromatography (Pharmacia MonoQ HR 10/10) with a gradient from 0 to 1 M NaCl in TE8 buffer with 7 M urea. Fractions containing the $tRNA^{Tyr}$ were desalted into 10 mM Tris, pH 8 (Pharmacia fast desalting column), and pooled. The concentration of ECY1 was determined spectrophotometrically using the extinction coefficient calculated for ECY2 (Table II).

Kinetic Assays. The K_M determination for guanine was carried out in a reaction mixture containing 100 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 5 mM DTT, 10 μM ECY2, 300 nM (monomer) TGT, and from 0.25 to 20 μM [8-^{14}C]guanine.

[2] T. Borgford and A. R. Fersht, unpublished.

[3] H. Kersten, unpublished.

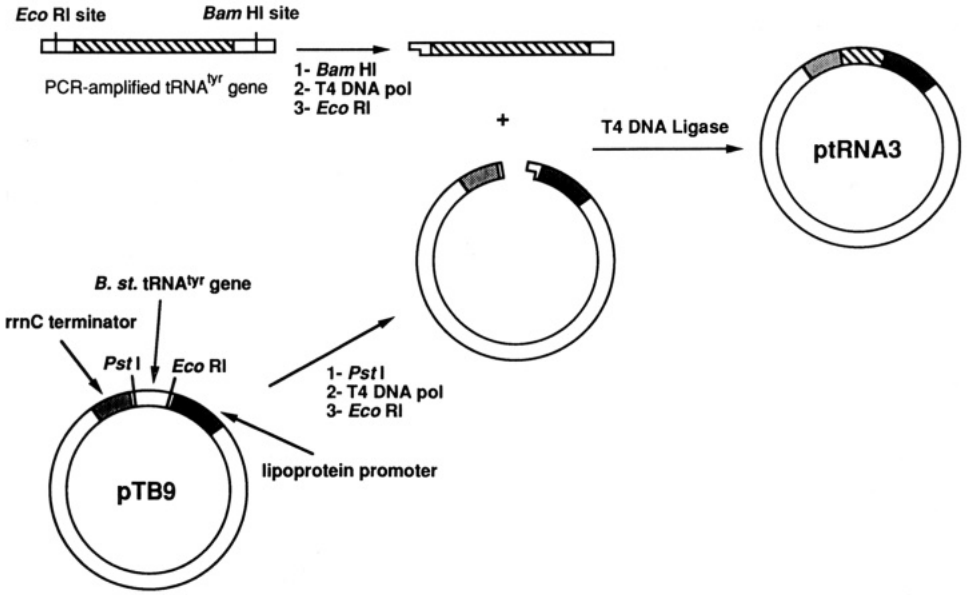

FIGURE 4: ptRNA3 construction The plasmid pTB9 is a derivative of pGFIBI where the gene for the *B. stearothermophilus* $tRNA^{Tyr}$ has been inserted between the *Eco*RI and *Pst*I sites. In this scheme, the *B. stearothermophilus* $tRNA^{Tyr}$ gene has been replaced by the *E. coli* $tRNA^{Tyr}$ gene.

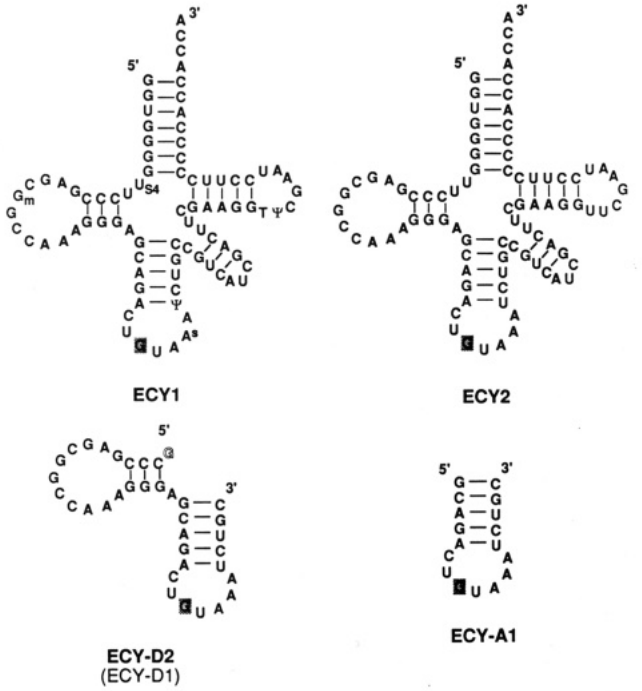

FIGURE 5: *E. coli* $tRNA^{Tyr}$ and in vitro-transcribed analogues. Guanosine-34 is denoted by the shaded box. The guanosine that was added to the 5′ end of ECY-D2 is shown in outline font. The modified bases in ECY1 are as follows: U_{S4}, 4-thiouridine; G_m, 2′-*O*-methylguanosine; A^s, N^6-isopentenyl-2-methylthioadenosine; Ψ, pseudouridine; T, ribothymidine.

The tRNA and truncated tRNA K_M determinations were carried out in the same buffer system in the presence of 10 μM[8-^{14}C]guanine. The concentrations of ECY1, ECY2, ECY-A1, and ECY-D2 (Figure 5) were varied from 0.1 to 75 μM. The reaction mixtures (400 μL) were incubated at 37 °C, and aliquots (75 μL) were taken at various time points. Upon removal from the reaction mixture the aliquots from ECY-A1 were ethanol precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of ethanol. The aliquots from ECY1, ECY2, and ECY-D2 were precipitated by adding 2 mL of 5% trichloroacetic acid (TCA) solution. The precipitates were then collected on glass-fiber filters (Whatman GF/C). The filters were then rinsed, dried, and quantitated via liquid scintillation counting. Initial velocities were determined by linear regression of DPM versus time plots. Michaelis–Menten parameters were determined from the average of three replicate determinations of initial velocity data (converted to M s^{-1}) using the program GraFit (Leatherbarrow, 1990).

RESULTS

In Vitro Synthesis System. The template strands for ECY-D1, ECY-D2, and ECY-A1 (see Figure 5 and Table I) transcriptions consist of the gene to be transcribed abutting a 17-base sequence corresponding to the Φ10 T7 RNA polymerase promoter. These strands are annealed to the 17-base sequence (dT7P, Table I) corresponding to the complement of the T7 promoter. This process generates a synthetic DNA template that is double-stranded in the T7 promoter region and single-stranded over the length of the template sequence. The template for ECY2 transcription is obtained by digestion of the plasmid ptRNA2 with *Bst*NI. A number of fragments are generated, but only the fragment of interest contains the T7 promoter. It is not necessary to isolate this fragment as the other fragments will not interfere in the transcription. Run-off transcription of this double-stranded template generates the full-length, unmodified *E. coli* $tRNA^{Tyr}$, ECY2.

In our initial transcription reactions, we found that after approximately 30 min a precipitate formed. We assumed that the precipitate was magnesium pyrophosphate. In subsequent transcription reactions we included 2 units of inorganic pyrophosphatase. We found that the pyrophosphatase prevented the formation of the precipitate and dramatically increased our yield of transcript. Following the procedures above, we routinely obtain 2.5 mg of transcript per 10 mL of transcription reaction mixture from *Bst*NI-digested plasmid and 2 mg of transcript per 5 mL of transcription reaction mixture from synthetic DNA templates. The entire process, from DNA template to purified transcript, typically takes 2–3 days.

In Vivo $tRNA^{Tyr}$ Isolation and Purification. ECY1 tRNA was prepared from a 2-L culture of ptRNA3/*E. coli* K12 (Δ*tgt*). Approximately 4 mg of purified $tRNA^{Tyr}$ was

Table III: Kinetic Parameters for TGT Substrates[a]

	K_M (10^{-6} M)	V_{max} (10^{-9} M s^{-1})	V_{max}/K_M (10^{-4} s^{-1})	V_{max}/K_M ratio[b]
guanine	0.98(0.01)	1.48(0.01)	1.50(0.1)	n/a
ECY1	2.4(0.8)	1.9(0.2)	7.9(2.8)	1:1
ECY2	1.8(0.3)	1.7(0.1)	9.4(1.6)	≈1:1
ECY-D2	8.2(1.7)	2.3(0.2)	2.8(0.6)	1:3
ECY-A1	9.6(0.7)	0.38(0.08)	0.40(0.09)	1:20

[a] Standard errors are shown in parentheses; n/a, not applicable. [b] The V_{max}/K_M ratio was calculated relative to that for ECY1, the full-length, in vivo-generated tRNA.

obtained. The concentration of ECY1 determined spectrophotometrically using the extinction coefficient calculated for ECY2 is in good agreement with a concentration calculated from an endpoint analysis of guanine acceptance of ECY1 (data not shown). However, tyrosine acceptance assay of ECY1 using the tyrosyl-tRNA synthetase from *B. stearothermophilus* indicates that approximately 45% of the ECY1 is capable of being aminoacylated with tyrosine.

Kinetics. In order to perform kinetic studies on the synthetic tRNA analogues at saturating guanine, the K_M for guanine had to be determined. Incubations performed in the presence of 10 μM ECY2 determined the K_M for guanine to be 0.98 μM (Table III). All subsequent tRNA assays were carried out at 10 μM guanine.

The inclusion of [2,8-^{3}H]ATP in the ECY2 transcription reaction generates labeled ECY2 (data not shown). This labeled tRNA was used to confirm that the TCA precipitation would result in essentially 100% recovery of tRNA. It was found during the initial kinetic experiments with unlabeled ECY-A1 that this anticodon arm analogue did not precipitate completely in the TCA procedure (data not shown). We determined that an ethanol precipitation protocol was necessary to give basically 100% yield of the ECY-A1 analogue (data not shown). A slightly higher background of radioactivity (200 versus 60 dpm in the TCA protocol) was observed when the kinetics of ECY-A1 were determined using the ethanol precipitation protocol. (The range of radioactivity determined in these experiments was 700–7000 dpm.) The higher background results in slightly higher intercepts in plots of dpm versus time for the ECY-A1 reactions, but does not influence the slopes which were used to determine the initial velocities.

Plots of initial velocity versus tRNA (or analogue) concentration (Figure 6) demonstrate that Michaelis–Menten kinetics are followed for these analogues. Nonlinear regression analysis (Leatherbarrow, 1990) was used to fit the initial velocity data; the calculated fits are displayed as the solid curves in Figure 6. The kinetic parameters (K_M, V_{max}, and V_{max}/K_M) for guanine, ECY1, ECY2, ECY-D2, and ECY-A1 from the above fits are shown in Table III along with the standard errors of the fits.

DISCUSSION

The system we used for the in vitro generation of tRNA analogues was suggested by the work of Milligan. This system utilizes T7 RNA polymerase to transcribe synthetic DNA template strands. T7 RNA polymerase is utilized since it is a single polypeptide, is available in an overexpressing clone, is highly processive, and recognizes a specific promoter sequence not normally found in the *E. coli* genome. The promoter region is a 17-base-pair, double-stranded sequence which is required for recognition and initiation by T7 RNA polymerase. The template portion of DNA need not be double-stranded; thus the noncoding (promoter-only strand, dT7P; Table I) can be synthesized and purified on a large scale, while the template strand (promoter–template strand, dECY-A1, dECY-D1, dECY-D2; Table I) can be synthesized and purified as needed according to the complementary sequence desired in the RNA transcript. The promoter region of T7 RNA polymerase actually extends approximately two bases into the gene to be transcribed. Transcription is most efficient if these two bases are guanines. Since the dihydrouridine stem of $tRNA^{Tyr}$ begins with cytidine, the transcription of the template dECY-D1 was not efficient enough to produce useful quantities of RNA product. To overcome this, we added one cytosine residue (underlined in Table I) to the 3′ end of the template sequence (to yield the requisite guanine in the transcript) and created dECY-D2, which was transcribed in sufficient quantity to study. The other tRNA analogues we were interested in fortunately began with guanosine and therefore were efficiently transcribed.

The pGFIB1 vector (Masson & Miller, 1986) utilizes the *E. coli* lipoprotein promoter to direct the in vivo transcription of an inserted tRNA gene. The rrnC terminator sequence is used to terminate the transcription after the tRNA gene. The resulting transcript should then be processed by the cellular machinery to the mature tRNA form. The cell line used for in vivo expression of $tRNA^{Tyr}$ is one in which the *tgt* gene had been genetically deleted.[3] The $tRNA^{Tyr}$ isolated from this strain should contain all of the modified bases except queuine. In fact, previous experiments had shown that crude tRNA prepared from this cell line did indeed serve as a substrate for the guanine exchange reaction catalyzed by TGT (data not shown). There are two possible explanations for the observation that only 45% of ECY1 can be aminoacylated with tyrosine. First, we may have purified a mixture of tRNAs for Asn, Asp, His, and Tyr, which are all substrates for TGT but of which only 45% is $tRNA^{Tyr}$. A second, and more likely, explanation is that during the isolation and purification of ECY1, an exonuclease activity has digested some of the 3′ terminus of the tRNA such that only 45% of the ECY1 has the intact 3′ CCA terminus required for aminoacylation. The observation that ECY1 migrates identically to ECY2 on native gel electrophoresis (data not shown) is most consistent with the second explanation.

In order to perform the kinetic studies with our tRNA analogues, we determined the kinetic parameters for guanine in the presence of 10 μM ECY2. It turned out that this concentration of ECY2 is 5 times its K_M and therefore the guanine experiments were performed under nearly saturating conditions of ECY2. These experiments determined the K_M for guanine to be 0.98 μM. The K_M for guanine with the *E. coli* TGT has been reported by Okada et al. (1979) to be 0.053 μM. The experiments of Okada et al. (1979) were performed in the presence of 1 A_{260} unit of unfractionated yeast tRNA. This corresponds to approximately 15 μM yeast tRNA, only a fraction of which is substrate tRNA which, given the K_M of approximately 2 μM that we have determined for $tRNA^{Tyr}$, is probably at much less than saturating concentration. While it remains to be determined, the vast majority of nonsubstrate tRNA present in unfractionated yeast tRNA may interfere with the TGT reaction. Depending upon the kinetic mechanism of the reaction, inhibition by noncognate tRNA could raise or lower the apparent K_M for guanine. Either or both of these observations may account for the discrepancy between the guanine K_M that we have determined, with highly purified enzyme and homogeneous substrates, and that reported by Okada et al. (1979).

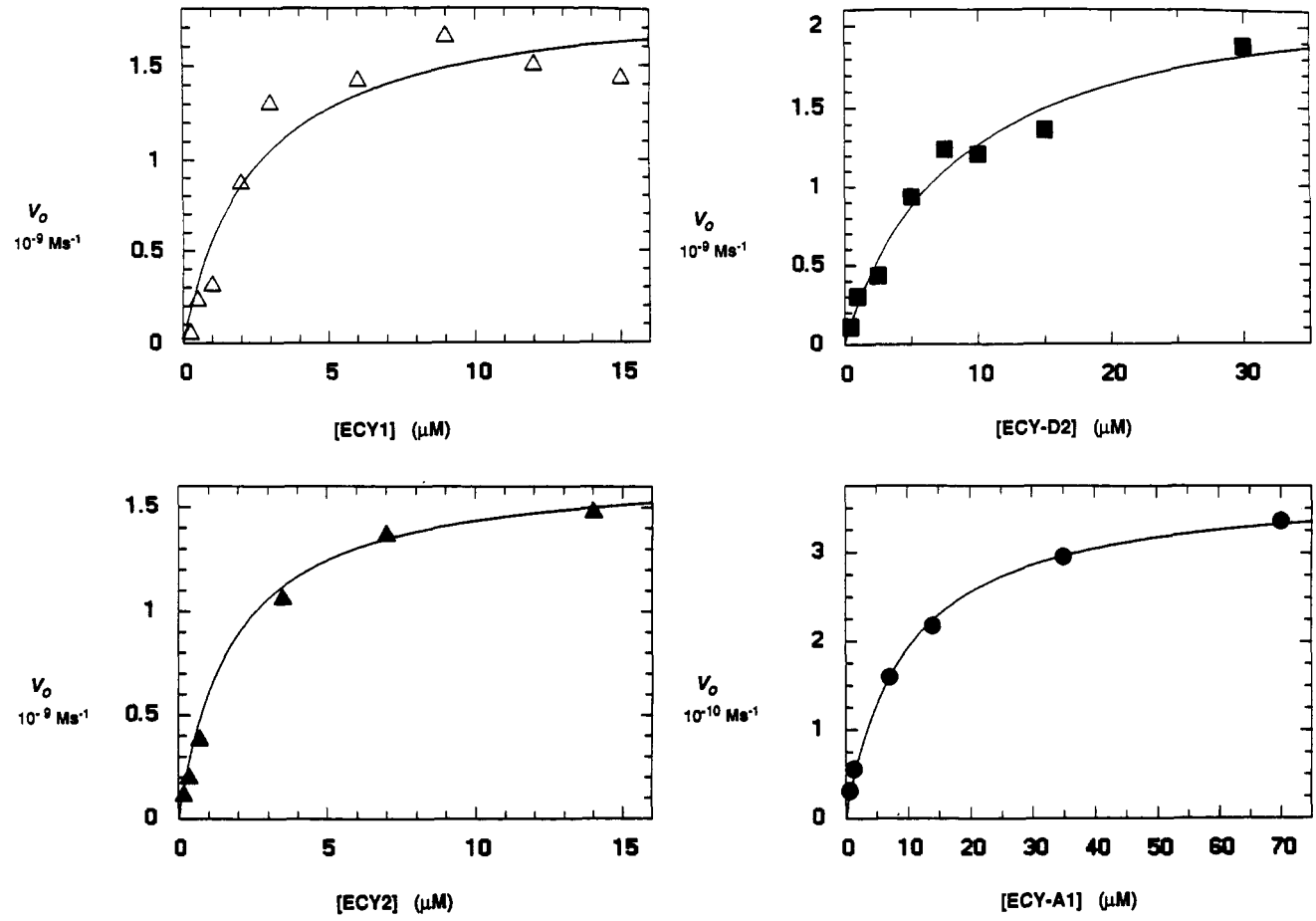

FIGURE 6: Michaelis–Menten plots of *E. coli* $tRNA^{Tyr}$ and in vitro-transcribed analogues Data were fit using the program GraFit. Note that the axes have been adjusted so that the data will fit in a similar area. Solid curves are calculated fits of the data.

Queuine insertion into tRNA is one of the last events in pre-$tRNA^{Tyr}$ processing (Nishikura & De Robertis, 1981). One of the first questions that arises is, does TGT require other post-transcriptional modifications to be present in order to recognize and modify tRNA? The tRNA analogues that we have generated by in vitro transcription lack the modified bases added to *E. coli* $tRNA^{Tyr}$, in vivo, prior to its modification by TGT. ECY2, the unmodified, full-length $tRNA^{Tyr}$, was found to have kinetic parameters in the TGT reaction that are identical, within experimental error, to those determined for ECY1, the Q-deficient but otherwise modified $tRNA^{Tyr}$ isolated from *tgt*-deficient *E. coli*. This demonstrates that post-transcriptional modifications are not required to be present in $tRNA^{Tyr}$ for efficient recognition and catalysis by TGT.

In Table III we have reported V_{max} values and not k_{cat} values because we do not know the actual concentration of TGT active sites. We can, however, estimate a lower limit for k_{cat} by assuming that all of the TGT is active and contains one active site per monomer. This then gives us a $k_{cat} \geq 6 \times 10^{-3}$ s^{-1} for ECY1. This turnover rate appears to be rather slow. However, if one makes a reasonable estimate of the amount of Q-tRNA (≈25% of the 160 000 tRNA molecules per *E. coli* cell; Lewin, 1990) in the cell and assumes a tRNA turnover time of 20 min, an amount of TGT corresponding to 0.3% of the total cell protein would be sufficient to incorporate queuine into the tRNA. Additionally, it should be noted that these kinetic parameters have been determined using radiolabeled guanine as the heterocycle substrate. The true physiological substrate is $preQ_1$ (Figure 1). The k_{cat} for $preQ_1$ incorporation may be significantly higher than that for the guanine exchange reaction, consequently reducing the amount of TGT required in the cell.

Francklyn and Schimmel (1989) reported that a 24-base oligoribonucleotide (microhelix^{Ala}) corresponding to the aminoacyl acceptor stem of $tRNA^{Ala}$ has a k_{cat}/K_M that is 57-fold lower, and a K_M that is 18-fold higher, than that for the full-length $tRNA^{Ala}$. The authors concluded that the deletions in tRNA structure had their predominant effect on binding and not upon catalysis. Gu and Santi (1991) reported that an 11-base oligoribonucleotide corresponding to the TΨC arm of $tRNA^{Val}$ has a k_{cat}/K_M that is 300-fold lower, and a K_M that is only 5-fold higher, than that for the full-length $tRNA^{Val}$. In this case, it appears that the predominant effect of the loss of tRNA structure is upon catalysis. Is there something intrinsic to the aminoacyl-tRNA synthetase reaction that is different in its interaction with tRNA than that for post-transcriptional modification enzymes? Our data suggest that this is not the case.

We find that a 17-base oligoribonucleotide corresponding to the anticodon arm of $tRNA^{Tyr}$ (ECY-A1 in Figure 5) has a V_{max}/K_M that is 20-fold lower, and a K_M that is 4-fold higher, than that for the full-length $tRNA^{Tyr}$. These data are similar to the results for the alanyl-tRNA synthetase and suggest that protein–tRNA interactions may not be easily classified by the type of reaction catalyzed.

This trend in tRNA activity with TGT is supported by our results for the 36-base oligoribonucleotide corresponding to the dihydrouridine and anticodon arms of $tRNA^{Tyr}$ (ECY-D2 in Figure 5). In this case, V_{max} is essentially unchanged and K_M is 3-fold higher than that for the full-length $tRNA^{Tyr}$, a value intermediate between those found for the anticodon

arm alone and the full-length tRNA. These data suggest that positive recognition elements in the TGT reaction are localized in the anticodon arm of tRNA. These in vitro experiments do not address the possibility that there may be negative recognition elements that allow TGT to reject nonsubstrate tRNAs localized elsewhere in the tRNA structure.

The possiblity that TGT recognizes an oligoribonucleotide containing the UGU sequence found in the anticodon loop of these tRNAs (Asp, Asn, His, and Tyr) cannot be ruled out by these experiments. However, the fact that there is no energy coupling for this enzymic reaction suggests that binding energy is used to catalyze this base exchange. If so, it is reasonable to assume that a significant amount of tRNA structure may be required to provide this binding energy. Our results indicate that the anticodon arm alone is sufficient for this process to occur. It remains to be determined if smaller oligoribonucleotides can also provide enough structure for binding and catalysis.

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