Recognition of the T-Arm of tRNA by tRNA (m^5U54)-Methyltransferase Is Not Sequence Specific†

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ABSTRACT: tRNA (m^5U54)-methyltransferase (RUMT) catalyzes the methylation of U54 of tRNAs. In contrast to enzymes which recognize a particular tRNA, RUMT recognizes features common to all tRNAs. We have shown that these features reside in the T-arm of tRNA and constructed a minimal consensus sequence for RUMT recognition and catalysis (Gu *et al.*, 1991b). Here, we have mutated each conserved T-loop residue and conserved T-stem base pair to bases or base pairs which are not observed in *Escherichia coli* tRNA. The substrate specificity of RUMT for 30 *in vitro* synthesized T-arm mutants of $tRNA^{Phe}$ and 37 mutants of the 17-mer analog of the T-arm derived from $tRNA_1^{Val}$ was investigated. A 2−5 base pair stem was essential for recognition of the T-arm by RUMT, but the base composition of the stem was unimportant. The 7-base size of the T-loop maintained by the stem was essential for RUMT recognition. For tRNA, most base substitutions in the 7-base loop did not eliminate RUMT activity, except for any mutation of the methyl acceptor U54 and the C56G mutation. The effect of base and base pair mutations on k_{cat} or the rate of methylation by RUMT was more striking than the effect on the K_d for binding to RUMT. In comparison with mutations in the T-loop of intact tRNA, base mutation in the T-loop of the 17-mer T-arm had a more deleterious effect on binding and methylation. Surprisingly, recognition of tRNA by RUMT appears to reside in the three-dimensional structure of the seven-member T-loop rather than in its primary structure.

tRNA (m^5U54)-methyltransferase (RUMT; EC 2.1.1.35),[1] the trmA gene product, catalyzes the transfer of the methyl group of AdoMet to U54 of most tRNAs. Regardless of its near ubiquitous presence, the role of m^5U at position 54 of tRNA and the physiological role of RUMT remain uncertain (Bjork *et al.*, 1975; Davenloo *et al.*, 1979; Kersten *et al.*, 1981).

As currently understood, the catalytic mechanism of RUMT is similar to that of the well-characterized enzyme thymidylate synthase and to the DNA (m^5C)-methyltransferases (Gu *et al.*, 1992; Santi *et al.*, 1984, 1987; Wu *et al.*, 1987). The reaction involves initial formation of a covalent Michael adduct between a Cys thiol (Cys 234 in *Escherichia coli* RUMT) and the 6-position of the target U54 of tRNA or analogs to activate the 5-position of the pyrimidine for subsequent one-carbon transfer from AdoMet (Kealey *et al.*, 1991). Reversible binary covalent adducts are formed between tRNA and RUMT which are sufficiently stable to be isolated by SDS−PAGE (Gu *et al.*, 1992).

The interaction of RUMT with tRNA presents an interesting problem in protein−RNA recognition. Since RUMT methylates U54 of nearly all tRNAs, RUMT must recognize structural elements which are common to all tRNAs. This is in contrast to tRNA-specific enzymes, such as the aminoacyl-tRNA synthetases, which must distinguish cognate tRNAs among some 40−60 tRNAs. An important question is what are the common elements of tRNA recognized by RUMT? Recently, we showed that the 17-nucleotide stem−loop structure of the T-arm of tRNA is an excellent substrate for RUMT (Gu *et al.*, 1991b). The minimal RNA structure for substrate activity contained the 7 bases of the loop and a 2-base pair stem of the T-arm (Gu *et al.*, 1991b). This observation suggested that the minimal tRNA specificity determinants for RUMT are contained within the T-arm and provided a small RNA substrate for study of catalysis, recognition, and structure. By comparing the T-arm sequences of known tRNAs from *E. coli*, we obtained a consensus sequence for T-arm methylation by RUMT (Figure 1A). Since this consensus sequence may contain constraints imposed by other functions of tRNA, it is reasonable to assume that the "true RNA consensus sequence" for RUMT could be considerably more relaxed. A knowledge of the true consensus sequence for RUMT binding/catalysis could lead to the identification of other RNAs which interact with the enzyme and are important, and as yet, unknown substrates *in vivo*. This approach led to the discovery that unmodified *E. coli* 16S rRNA could be methylated at U788 by RUMT (Gu *et al.*, 1994).

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[1] Abbreviations: RUMT, *Escherichia coli* tRNA (m^5U54)-methyltransferase; $tRNA_1^{Val}$, unmodified *E. coli* $tRNA_1^{Val}$ prepared by *in vitro* transcription; $tRNA^{Phe}$, unmodified yeast $tRNA^{Phe}$ prepared by *in vitro* transcription; T-arm, the 17-mer oligoribonucleotide corresponding to nucleotides 49−65 of *E. coli* $tRNA_1^{Val}$ (GGCGGUUCGAUCCCGUC); 11-mer T-arm, the 11-mer oligoribonucleotide corresponding to nucleotides 52−62 of *E. coli* $tRNA_1^{Val}$ (GGUUCGAUCCC); 9-mer T-arm, the 9-mer oligoribonucleotide corresponding to nucleotides 53−61 of *E. coli* $tRNA_1^{Val}$ (GUUCGAUCC); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; AdoMet, *S*-adenosylmethionine; Py, pyrimidine; Pu, purine; m^5U, 5-methyluridine; DTT, dithiothreitol. Mutant T-arms or RNAs are designated by the wild-type base, followed by the position (tRNA numbering), followed by the mutation.

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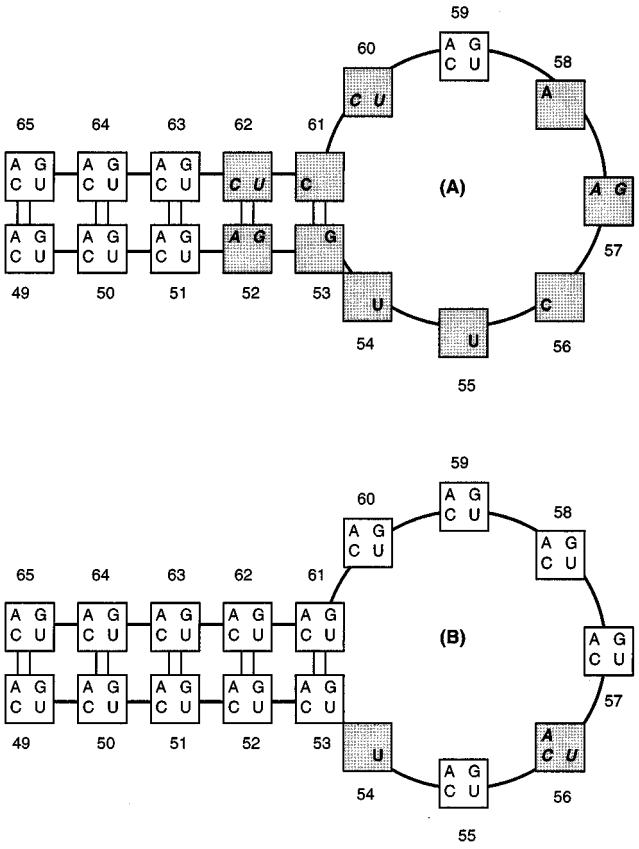


FIGURE 1: (A) Consensus sequence of the T-arm of tRNA derived from the structures of all *E. coli* tRNAs known to be methylated at U54 (Sprinzl *et al.*, 1987). Bold indicates complete conservation of the base, bold italics indicates conservation of Py or Pu, and no shading indicates nonconserved positions. (B) Consensus sequence derived from tRNA sequences as in (A), plus the data of Tables 1 and 2 for tRNA and 17-mer T-arm mutants.

In the present work, we have extensively mutagenized the T-arm of tRNA, both as a short sequence of the T-arm (from $tRNA_1^{Val}$) and in intact $tRNA^{Phe}$, and investigated the binding and catalytic properties of the mutants. Our results extend the knowledge of the structural elements of RNAs necessary for binding and methylation by RUMT.

MATERIALS AND METHODS

The plasmids p67YF0, p67YF1, p67YF13, p67YF30, p67YF31, p67YF34, p67YF35, p67YF40, p67YF46, and p67YF82 used for preparation of yeast $tRNA^{Phe}$ mutants were gifts from O. C. Uhlenbeck (Department of Chemistry and Biochemistry, University of Colorado). The plasmid pVal119−21 used for preparation of unmodified *E. coli* $tRNA_1^{Val}$ was a gift from J. Horowitz (Department of Biochemistry and Biophysics, Iowa State University). The plasmids p67YF101−131 containing variant tRNA mutant genes were constructed by cassette mutagenesis of the yeast $tRNA^{Phe}$ gene in plasmid p67YF0 (Sampson *et al.*, 1988). The appropriate oligonucleotide duplex cassettes were inserted into the *Bgl*II and *Eco*RI sites of p67YF0 to give the plasmids containing tRNA mutant genes. T4 RNA ligase was from New England Biolabs. T7 RNA polymerase was isolated from *E. coli* BL21 harboring the plasmid pAR1219 (J. J. Dunn, Brookhaven National Laboratory, Upton, NY) and purified as described (Grodberg *et al.*, 1988), except that S-Sepharose (Pharmacia) was used instead of trisacyl-SP. Oligonucleotides were prepared at the UCSF Biomolecular Resource Center and purified as described (Ivanetich *et al.*, 1991). [5′-^{32}P]pCp (3000 Ci/mmol) was from Amersham. Qiagen tip-20 was from Qiagen Inc. Nitrocellulose filter membranes (2.4 cm) were from Schleicher & Schuell. RUMT was purified as previously described (Gu *et al.*, 1990, 1991a) and was homogeneous as assessed by SDS−PAGE. All molecular biology procedures not described were according to Sambrook *et al.* (1989).

T-Arm and tRNA Synthesis. T7 RNA polymerase-catalyzed *in vitro* synthesis of the 17-mer T-arm mutants and the 11-mer T-arm was performed using appropriate templates and primer, and the T-arm was purified on 7 M urea−20% PAGE (Gu *et al.*, 1991b; Milligan *et al.*, 1987). The 9-mer T-arm was chemically synthesized at the UCSF Biomolecular Resource Center on an Applied Biosystems Inc. 380B DNA synthesizer and was purified by HPLC (Kealey *et al.*, 1995). The concentrations of the T-arm mutants were calculated from the sum of extinction coefficients of the component nucleotides with correction for a hyperchromic effect of 1.21 (Gu *et al.*, 1992).

T7 RNA polymerase-catalyzed *in vitro* synthesis of tRNA mutants was performed with appropriate *Bst*NI linearized plasmids, and the *in vitro* transcribed tRNA was purified with Qiagen tip-20 as described (Gu *et al.*, 1992; Sampson *et al.*, 1988). [3′-^{32}P]RNAs were prepared using T4 RNA ligase and [5′-^{32}P]pCp (England *et al.*, 1980) and were purified on 7 M urea−15% PAGE. The concentrations of the tRNAs were calculated assuming 1600 pmol per 1 A_{260} (Stanley *et al.*, 1978).

Nitrocellulose Binding Assay. Reaction mixtures containing 1 nM [^{32}P]T-arm mutant or [^{32}P]tRNA mutant [$(1-2) \times 10^4$ cpm] and varying concentrations of RUMT (5−5000 nM) in binding buffer (50 mM TES-HCl, pH 6.6, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM DTT, and 50 mM NaCl) were incubated at 15 °C for 30 min (T-arm) or 60 min (tRNA) and assayed by nitrocellulose filtration (Gu *et al.*, 1992; Santi *et al.*, 1974). With saturating RUMT, 50−65% of the T-arm or 50−70% of the tRNA was trapped, which we assume represents the filtration efficiency for both noncovalent and covalent complexes for these species. The apparent dissociation constants ($K_{d,app}$) for the T-arm and tRNA mutants were obtained by nonlinear least-squares fit of the binding data to the equation:

$$RNA_{bound}/RNA_{total}/(1 + K_{d,app}/E_{total})$$

Gel Shift Assay. Gel shift assays were performed as previously described (Gu *et al.*, 1992; Santi *et al.*, 1987). Reaction mixtures (40 μL) contained 1 μM RUMT and 0.2 μM [^{32}P]T-arm mutant or [^{32}P]tRNA-arm mutant [$(1-2) \times 10^4$ cpm] in binding buffer. After incubation at 15 °C for 10 min (T-arm mutants) or 30 min (tRNA mutants), 20 μL of 2× loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 1.5 M 2-mercaptoethanol, 20% glycerol, and 0.005% Bromophenol blue) was added, and the mixture was incubated at 90 °C for 3 min. Aliquots (20 μL) were removed for 12% SDS−PAGE. The ^{32}P-containing bands were excised, extracted, and counted in 5 mL of Aquasol II. Covalent complexes were quantitated as the fraction of total RNA (Gu *et al.*, 1992).

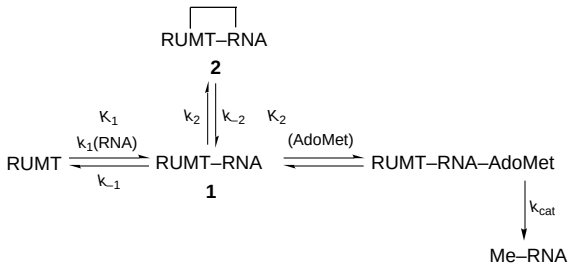
Methylation Assay. Typically, reaction mixtures (60−80 μL) containing 10 μM T-arm or 0.01−10 μM tRNA, 50 μM [^{3}H-Me]AdoMet (1−2 Ci/mmol), and 0.1−0.5 μM RUMT

in methylation assay buffer (50 mM Tricine, pH 8.4, 5 mM DTT, 2 mM $MgCl_2$, 1 mM EDTA, 40 mM NH_4Cl, and 20 mM spermidine) (Santi *et al.*, 1987) were incubated at 15 °C. Aliquots (18 μL) were removed at 1, 2, 10, and 30 min for DEAE-paper disk assay (Gu *et al.*, 1991b). The assay efficiency was about 60% for [^{3}H]methyl incorporation into the T-arm or tRNA (X. Gu, unpublished data). Kinetic parameters were assessed by nonlinear least-squares fit of the data to Michaelis−Menten equation, and in some cases the data were displayed as double-reciprocal plots.

RESULTS

Mechanism of RUMT. A minimal mechanism for the interaction of RUMT with tRNA or T-arm substrates is depicted in Scheme 1 (Gu *et al.*, 1992). There is reversible formation of one or several noncovalent RUMT−RNA binary complexes (**1**) characterized by apparent rate constants k_1 and k_{-1} and the apparent dissociation constant K_1. In the absence of AdoMet, the noncovalent complex (**1**) is converted to covalent Michael adducts (**2**) characterized by apparent rate constants k_2 and k_{-2} and the apparent dissociation constant K_2. In the presence of bound AdoMet, the noncovalent complex (**1**) forms a ternary complex which is converted to products (k_{cat}).

Scheme 1



With tRNA as the substrate, the first step includes both the binding of exposed surface residues of tRNA to form an initial reversible complex and the subsequent disruption of the tertiary structure of tRNA necessary to permit interaction of the T-arm with the enzyme. At this stage, U54 of the T-arm must be exposed so that AdoMet and the catalytic thiol of the enzyme have appropriate access to the 5- and 6-positions of the heterocycle, respectively. Finally, catalysis is a multistep event which involves methylation and disruption of covalent enzyme−substrate bonds to give the products (Gu *et al.*, 1992; Santi *et al.*, 1987).

The minimal essential features for recognition and methylation of tRNA by RUMT are contained within the 17-base T-arm (Gu *et al.*, 1991b). RUMT binds to and methylates the 17-mer T-arm of *E. coli* $tRNA_1^{Val}$ almost as well as the entire 76-base $tRNA_1^{Val}$.

Binding and Kinetic Measurements. We have studied the binding and substrate properties of 30 mutants of the T-arm of yeast $tRNA^{Phe}$ and 37 mutants of a 17-mer T-arm analog of *E. coli* $tRNA_1^{Val}$ (Tables 1 and 2). The parameters measured include (i) apparent dissociation constants for binary RUMT−RNA complexes, (ii) the ability of mutants to form covalent binary complexes with RUMT, and (iii) substrate properties for methylation.

For binding constants, [^{32}P]RNA was titrated with varying concentrations of RUMT, and protein−[^{32}P]RNA complexes were isolated on nitrocellulose filter membranes (Gu *et al.*, 1992). Figure 2 shows titration curves for the wild type and three mutants at position 55 of the T-arm. The $K_{d,app}$ values obtained represent composite binding constants for both noncovalently and covalently bound forms, as shown in the equation:

$$K_{d,app} = \frac{K_1 K_2}{1 + K_2}$$

Binary covalent complexes between RUMT and the 17-mer or tRNA T-arm mutants were assayed using a gel-shift assay (Gu *et al.*, 1992) which monitors the retardation of covalent RUMT−[^{32}P]RNA complexes on SDS−PAGE (Figure 3, Table 2). In general, covalent complexes were formed with mutants of the 17-mer T-arm analog that were substrates for methylation but not with those that were not substrates (Table 2).

For tRNAs, steady-state kinetic parameters for methylation were determined by varying the concentration of tRNA in the presence of 50 μM AdoMet and limiting concentrations of RUMT. For most T-arm analogs, it was impractical to obtain sufficient amounts of RNA to obtain complete kinetic data. Thus, single rates were determined over 2 min using 50 μM AdoMet ($3 \times K_m$ for the wild-type T-arm) (X. Gu, unpublished data) and 10 μM RNA ($2 \times K_m$ for the wild-type T-arm) (see Figure 4). These initial velocities were assumed to reflect V_{max} values (Table 2).

T-Stem Mutations. The first three base pairs of the T-stem of tRNAs are not conserved (Figure 1A) and therefore cannot be primary determinants in recognition or catalysis. Indeed, the 11-mer stem−loop analog of the T-arm, which does not contain the first three base pairs of the T-stem, binds to RUMT as well as the 17-mer T-arm and is a substrate, but with a 20-fold decrease in k_{cat} (Table 1).

All *E. coli* tRNAs contain Pu52·Py62 and G53·C61 base pairs in the stem positions adjacent to the loop (Figure 1A). The U52·A62 base pair found in yeast $tRNA^{Phe}$ (Table 1, B0) and the C52/G62 (B1) mutant are good substrates, showing that Py52·Pu62 is also acceptable. Likewise, the G52·C62 base pair of the 17-mer T-arm could be substituted by any canonical base pair with retention of good substrate properties (A1−A3). The G52A T-arm (A32), which probably forms an A52·C62 base pair to maintain the stem (Puglisi *et al.*, 1990), also showed little loss in binding but a 17-fold decrease in methylation activity. An analog which could not form a 52·62 base pair, i.e., U52C/A62C tRNA (B2), neither bound to nor was a substrate for RUMT. Thus, the only constraint for positions 52 and 62 is that the bases at those positions must be able to form a 52·62 base pair.

The conserved G53·C61 base pair of tRNA or the 17-mer T-arm analog could be replaced by any canonical base pair (B3, B5, B7, A4−A6) and retain good binding and substrate properties. Unusual base pairs such as U53·G61 in tRNA (B8) and A53·C61 in the 17-mer (A31) were also acceptable. In contrast, mutants which cannot form a 53·61 base pair, such as G53A/C61A (B4) and G53C/C61A (B6) tRNA, and the G53C T-arm (A33), were inactive in both the binding and methylation assays. The substrate properties of several other T-arm analogs are worthy of note. The 9-mer T-arm (A37) which contains the loop sequence but cannot form a base-paired stem (Turner *et al.*, 1988) neither bound to nor was a substrate for RUMT. Substitution of the T-stem of the T-arm by A_5-U_5 (A35), which is not

Table 1: Substrate Activity of Mutants of Yeast $tRNA^{Phe}$

mutant	plasmids	mutation	$K_{d,app}$, μM[a]	K_m, μM[b]	k_{cat}, s^{-1} [b]	k_{cat}/K_m, 10^4 $M \cdot s^{-1}$
B0	p67YF0	wild type[c]	0.045	0.82	0.091	11
B1	p67YF122	U52C/A62G	0.11	0.40	0.001	0.25
B2	p67YF121	U52C/A62C	–	–[d]	–[d]	–[d]
B3	p67YF125	G53A/C61U	0.058	0.40	0.027	6.8
B4	p67YF123	G53A/C61A	–	–[d]	–[d]	–[d]
B5	p67YF127	G53C/C61G	0.072	0.38	0.004	1.0
B6	P67YF126	G53C/C61A	–	–[d]	–[d]	–[d]
B7	p67YF128	G53U/C61A	0.075	0.72	0.015	2.1
B8	p67YF129	G53U/C61G	0.069	0.45	0.002	0.44
B9	p67YF130	dU59[e]	–	–[d]	–[d]	–[d]
B10	p67YF131	U60.1[f]	–	–[d]	–[d]	–[d]
B11	p67YF40	U54A	0.70	–[d]	–[d]	–[d]
B12	p67YF30	U54C	0.42	–[d]	–[d]	–[d]
B13	p67YF101	U54G	0.79	–[d]	–[d]	–[d]
B14		U54T[g]	1.1	–[d]	–[d]	–[d]
B15	p67YF46	U55A	0.018	0.19	0.083	43
B16	p67YF13	U55C	0.17	0.41	0.041	10
B17	p67YF102	U55G	0.091	0.82	0.001	0.1
B18	p67YF103	C56A	0.10	0.54	0.007	1.3
B19	p67YF1	C56G	0.14	–[d]	–[d]	–[d]
B20	p67YF104	C56U	0.081	1.1	0.008	0.7
B21	p67YF105	G57C	0.081	0.78	0.003	0.4
B22	p67YF82	G57U	0.028	0.28	0.060	21
B23	p67YF106	A58C	0.056	0.48	0.006	1.3
B24	p67YF31	A58G	0.12	0.80	0.024	3.0
B25	p67YF107	A58U	0.10	0.87	0.003	0.3
B26	p67YF34	U59C	0.031	0.80	0.092	12
B27	p67YF108	C60A	0.071	0.56	0.010	1.8
B28	p67YF109	C60G	0.078	0.90	0.015	1.7
B29	p67YF35	C60U	0.035	0.66	0.045	6.8
B30	p67YF110	U55A/C56A/G57A/U59A/C60A	0.077	0.63	0.004	0.6
		unmodified *E. coli* $tRNA_1^{Val}$	0.054	2.8	0.048	1.7
		yeast $tRNA^{Phe}$ [h]	1.6	–	–	–
		17-mer T-arm	0.14	5.0	0.033	0.66
		11-mer T-arm	0.11	3.7	0.0015	0.04

[a] Reactions were performed at pH 6.6 and 15 °C. A dash indicates that saturated binding of tRNA (plateau about 50−70%) was not observed and the fraction bound at 5 μM RUMT was less than 30%. [b] Reactions were performed at pH 8.4 and 15 °C and at 50 mM [^{3}H-Me]AdoMet. The K_m for AdoMet is 13 μM (unpublished data). A dash indicates undetectable activity. [c] The wild-type sequence is U52,G53,U54,U55,C56,-G57,A58,U59,C60,C61,A62. [d] Reaction mixtures (20 μL) contained 0.5 μM RUMT, 4 μM RNA, and 5 μM [^{3}H-Me]AdoMet (10 Ci/mmol) in methylation buffer at 15 °C for 30 min. The limit of detection for [^{3}H]methyl incorporation was less than 2-fold above background. A dash indicates that methylation was undetectable. [e] U59 was deleted. [f] U60.1 was inserted between C60 and C61. [g] From methylation of unmodified tRNA by RUMT (Gu *et al.*, 1992). [h] Native *in vivo* modified tRNA.

found in natural tRNAs but permits stem formation, produces a mutant which binds to and is methylated by RUMT. However, the analogous A_5-C_5 T-arm analog (A36), which cannot form a base-paired stem, did not bind to or serve as a substrate for RUMT.

From the above results, it was concluded that a stem−loop structure is required for RUMT activity; although no particular bases in the stem are essential, the base pairs are. A main function of the stem probably involves positioning and presenting the loop in a configuration acceptable to RUMT.

T-Loop Mutations. All tRNA substrates for RUMT possess a 7-base T-loop in which 4 bases are completely conserved (U54, U55, C56, A58), 2 are semiconserved as Pu or Py (Pu57, Py60), and 1 (N59) is nonconserved (Figure 1A). In the present work, we modified the size and composition of the T-loop.

First, we examined the effects of decreasing and increasing the size of the T-loop. Deletion of the nonconserved nucleotide 59 (B9, A28) to give a 6-base loop, insertion of U between positions 60 and 61 (B10, A29) to give an 8-base loop, and the G53A/C61A (B4), G53C/C61A (B6), or G53C (A33) mutations to give a 9-base loop resulted in analogs which neither bound to nor served as substrates for RUMT. From these results, the 7-base size of the T-loop appears to be necessary for binding and catalysis.

Next, we studied mutations in the loop of the T-arm of tRNA and the 17-mer T-arm analog in which each base was substituted by every other (B11−B29, A7−A27). U54 is the site of methylation, and its substitution by any other base resulted in decreases in binding efficiency and the complete loss of substrate activity (B11−B13, A7−A9). The loss of activity was expected since substrate activity is dictated by the unique chemistry of Ura methylation (Carreras *et al.*, 1995; Ivanetich *et al.*, 1992). It is noteworthy that the T54 tRNA product of methylation (B14) showed the largest deleterious effect on binding compared to other base mutations at position 54, which is in accord with the hypothesis that a reduction in affinity of the product contributes to its release from the enzyme (Gu *et al.*, 1992).

The conserved U55 is posttranscriptionally modified to Ψ in most tRNAs. Since U55 participates in H-bonding to G18 of the D-loop to help maintain the tertiary structure of tRNA (Kim *et al.*, 1979), it would not have been surprising to find that this base was important for methylation. However, U55A (B15) and U55C tRNA (B16) were excel-

Table 2: Substrate Activity of Wild-Type and Mutant T-Arms[a]

mutants of 17-mer T-arm		$K_{d,app}$,[b]	covalent complex,[c]	rate of methylation,[d]
name	mutation	μM	pmol/10 min	pmol/min
wild type	none	0.14	0.86	3.0
A1	G52A/C62U	0.11	1.27	3.3
A2	G52C/C62G	0.10	0.54	1.1
A3	G52U/C62A	0.09	0.84	1.5
A4	G53A/C61U	0.11	0.38	1.0
A5	G53C/C61G	−	0.02	0.27
A6	G53U/C61A	0.11	0.34	0.95
A7	U54A	−	−	−
A8	U54C	−	−	−
A9	U54G	−	−	−
A10	U55A	0.15	0.36	2.9
A11	U55C	−	−	−
A12	U55G	−	−	−
A13	C56A	0.40	0.24	0.51
A14	C56G	−	−	−
A15	C56U	−	−	−
A16	G57A	0.10	0.72	0.95
A17	G57C	−	−	−
A18	G57U	0.44	0.02	0.23
A19	A58C	−	−	−
A20	A58G	−	0.20	0.10
A21	A58U	−	−	−
A22	U59A	0.13	0.14	0.38
A23	U59C	0.11	0.24	1.8
A24	U59G	0.11	0.36	1.0
A25	C60A	0.45	0.02	0.20
A26	C60G	−	−	−
A27	C60U	0.18	0.18	0.74
A28	U59 deleted	−	−	−
A29	U60.5 inserted	−	−	−
A30	11-mer (G52−C62)	0.11	0.18	0.14
A31	G53A	0.22	ND	0.16
A32	G52A	0.18	ND	0.17
A33	G53C	−	ND	−
A34	U55A/U59C/U64C	0.13	ND	2.1
A35	G48/A49−53/U61−65[e]	0.63	0.02	0.10
A36	G48/A49−53/C61−65[e]	−	ND	−
A37	9-mer (G53-C61)	−	ND	−
$tRNA^{Phe}$		0.045	0.86	11.6
$tRNA_1^{Val}$		0.054	ND	4.8

[a] All experiments were performed two or more times. Results agreed whthin 20%. −, not detectable; ND, not determined. [b] Measured by nitrocellulose binding assay. Reactions were performed at pH 6.6 and 15 °C. A dash indicates that saturated binding of RNA (which usually plateaus at about 60%) was not seen and that the $K_{d,app}$ could not be calculated. For these mutants, the percentage of RNA bound at 5 μM RUMT ranged from 17% to 31%. It is not known if this represents specific or nonspecific binding. [c] Measured by SDS−PAGE. Reactions were performed at pH 6.6 and 15 °C. [d] The initial velocity of methylation at 10 μM RNA was determined at pH 8.4 and 15 °C. [e] G was added to the 5′ end of the T-arm to increase the yield of transcripts (Milligan *et al.*, 1987).

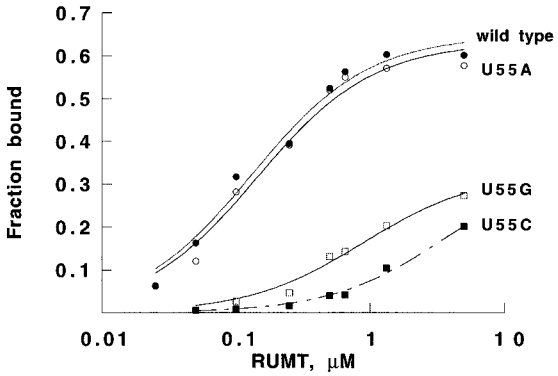


FIGURE 2: Titration of T-arm mutants with RUMT. The reaction mixtures (20 μL) containing 1 nM [^{32}P]T-arm mutant [(1−2) × 10^4 cpm] and variable concentrations of RUMT (20−5000 nM) in binding buffer were incubated at 15 °C for 60 min. Aliquots (18 μL) were removed for the nitrocellulose binding assay. The curve is a nonlinear least-squares fit of the data to the equation: $RNA_{bound}/RNA_{total} = 1/(1 + K_{d,app}/E_{total})$.

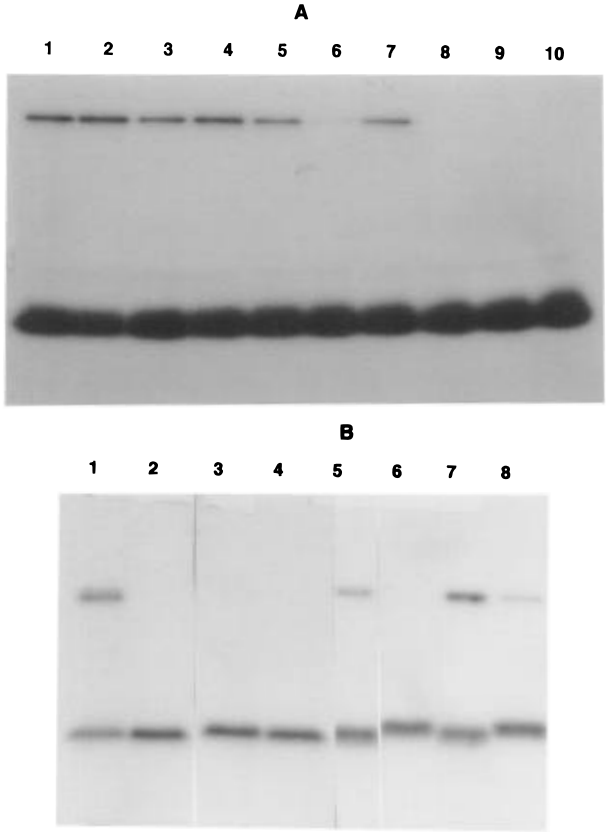


FIGURE 3: (A) SDS−PAGE of covalent complexes between RUMT and 17-mer T-arm mutants. Lanes: 1, wild-type T-arm; 2, G52A/C62U T-arm; 3, G52C/C62G T-arm; 4, G52U/C62A T-arm; 5, G53A/C61U T-arm; 6, G53C/C61G T-arm; 7, G53U/C61A T-arm; 8, U54A T-arm; 9, U54C T-arm; 10, U54G T-arm. (B) SDS−PAGE of complexes between RUMT and tRNA mutants. Lanes: 1, wild-type tRNA; 2, U54A tRNA; 3, U54C tRNA; 4, U54G tRNA; 5, C56A tRNA; 6, C56G tRNA; 7, C56U tRNA; 8, U54A/U55A/C56A/U59A/C60A tRNA.

lent substrates for RUMT. Only U55G tRNA (B17) showed a detrimental effect on catalysis, with a 90-fold reduction in k_{cat}. A $tRNA^{Gly}$ from *Staphylococcus epidermis* has G at position 55 and is also poorly or not methylated at U54 *in vivo* (Robert, 1974). The T-arm analogs U55A (A10) and U55G (A12) showed similar effects to the tRNA mutants, but the U55C mutant (A11) was inactive.

C56 is conserved in *E. coli* tRNAs and interacts with the D-loop G19 through H-bonding interactions (Kim *et al.*, 1979). However, C56 is not essential for RUMT activity since bacteriophage RB69 codes for a tRNA with A at position 56 but is methylated at U54 *in vivo* (Moen *et al.*, 1978). Indeed, C56A tRNA (B18) and C56U tRNA (B20) had $K_{d,app}$ and K_m values approximately equal to those of wild-type tRNA and showed approximately 12-fold decreases in k_{cat}. The 17-mer C56A T-arm (A13) retained activity, but the C56U T-arm (A15) was inactive. The C56G tRNA (B19) and T-arm (A14) represented the only mutation of C56 that was completely inactive in both tRNA and the T-arm.

There is a Pu at 57 in all tRNAs, and as expected, the G57A T-arm (A16) was a good substrate. Pu57Py mutant tRNAs were also substrates for RUMT. The G57U mutation in tRNA (B22) had little effect on kinetic parameters, but the C mutation at position 57 (B21) showed a 30-fold

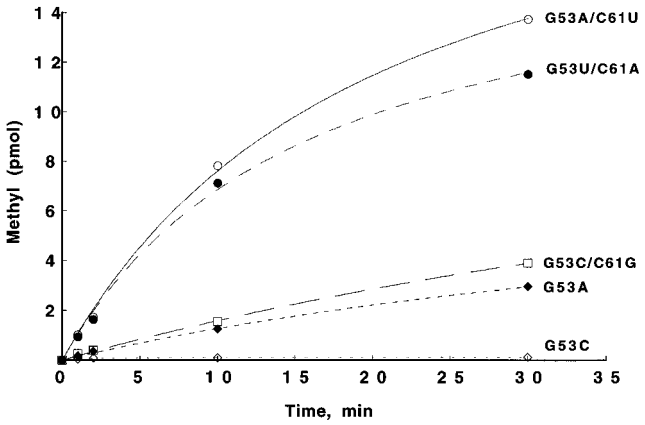


FIGURE 4: Methylation of mutants of the 17-mer T-arm. Reaction mixtures (80 μL) contained 10 μM [^{3}H-Me]AdoMet (2 Ci/mmol), and 0.1 μM RUMT in methylation buffer and were incubated at 15 °C. Aliquots (19 μL) were removed at 0, 1, 2, 10, and 30 min for DEAE-paper disk assay. Curves were generated with the smooth curve option of Kaleidagraph on a Macintosh computer.

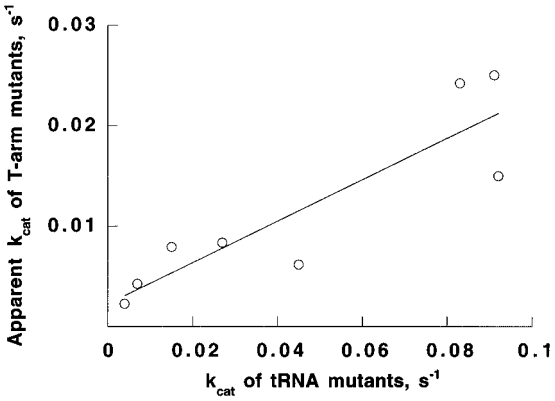


FIGURE 5: Plot of the apparent k_{cat} values for methylation for 17-mer T-arm mutants versus the k_{cat} values for the equivalent tRNA mutants. Data are shown only for mutants where both the T-arm and tRNA mutant exhibited k_{cat} or apparent k_{cat} values greater than 0.002 s^{-1}. Apparent k_{cat} values for methylation for 17-mer T-arm mutants were calculated from the initial velocity per second per 2 pmol of RUMT.

decrease in k_{cat}. The T-arm was more sensitive to mutation at position 57 than was tRNA, with the G57U T-arm (A18) exhibiting a more than 10-fold decrease in the rate of methylation and G57C (A17) showing no activity.

All tRNAs contain A at position 58 which forms a reverse Hoogsteen base pair with U54 that stacks over the G53·C61 base pair to stabilize the conformation of the T-arm (Romby *et al.*, 1987). A58G tRNA (B24) was almost as good a substrate as the parent tRNA^{Phe}, but the k_{cat} values of mutants which contain Py58 (B23, B25) were decreased 15−30-fold. The T-arm A58G mutant (A20) showed a 30-fold decrease in the rate of methylation compared to the wild-type T-arm, while the A58C and A58U T-arms (A19, A21) did not bind to RUMT or show activity.

Nucleotide 59 in tRNA is the only nonconserved base of the T-loop, and T-arm mutants with any base at position 59 bound well to and served as good substrates for RUMT. Only the U59C mutant of tRNA^{Phe} (B26) was examined here, and it showed substrate properties identical to those of unmodified tRNA^{Phe}. T-arm U59A, U59C, and U59G mutants (A22-A24) exhibited $K_{d,app}$ values comparable to the wild-type T-arm but 2−8-fold decreases in the rate of methylation.

The conserved Py60 in tRNA forms a hydrogen bond with C61 (Romby *et al.*, 1987). The tRNA mutants with any base at position 60 were substrates, although Py (B29) to Pu substitutions (B27, B28) resulted in a 3−5-fold decrease in k_{cat}. In the T-arm (A25−A27), only Py60G was ineffective as a substrate.

The above findings indicate that the specificity of RUMT for bases or base pairs of the T-arm of tRNA was much less stringent than anticipated from the "natural" consensus sequence found in *E. coli* tRNAs (Figure 1A). We next prepared an unnatural tRNA where all the loop bases except U54 contained A (B30). This analog has three of the six conserved or semiconserved residues of the T-loop of tRNA replaced by A and a poly(A) sequence not found in any natural tRNA; in effect, it is a composite of the U55A, C56A, and C60A mutations. Assuming the mutations were non-interactive (Wells, 1990), from the additivity of free energies of kinetic parameters of the single mutants we calculated a k_{cat}/K_m of 8.5×10^3 M^{-1} s^{-1}. This mutant was indeed a substrate, and the experimental k_{cat}/K_m of 6×10^3 M^{-1} s^{-1} was in excellent accord with the calculated value.

Overall, the intact tRNA mutants had lower $K_{d,app}$ values than did the comparable T-arm 17-mer mutants. For 13 tRNA mutants with measurable $K_{d,app}$ values, the comparable mutation in the T-arm 17-mer resulted in increases in $K_{d,app}$ so that the parameter was not measurable (>5 μM). For the remaining 9 mutants plus wild type, there was no direct correlation between $K_{d,app}$ values for the T-arm mutant versus the comparable tRNA mutant, but in all cases, the $K_{d,app}$ was lower for the tRNA mutant. There was a reasonable correlation ($R = 0.80$) between the k_{cat} values of the tRNA mutants and the apparent k_{cat} values for the comparable T-arm mutants, with a slope of 0.20 indicative of the lower values for the T-arm mutants (Figure 5). Seven T-arm mutants had no measurable apparent k_{cat} values compared to k_{cat} values of 0.001−0.04 s^{-1} for the comparable tRNA mutants.

DISCUSSION

The methylation of tRNA by RUMT involves (i) the initial binding of RUMT to tRNA, (ii) a conformational change of tRNA to expose the modification site in the T-arm, and (iii) the catalytic events involved in methylation. The minimal features for recognition of tRNA and catalysis by RUMT are contained within the 17-base T-arm (Gu *et al.*, 1991b). Indeed, RUMT binds to and methylates the 17-mer T-arm of *E. coli* tRNA$_1^{Val}$ almost as well as tRNA$_1^{Val}$. Thus, the T-arm serves as a model for recognition of tRNA and catalysis of methylation by RUMT which bypasses the initial recognition and conformational changes required of tRNA.

The T-arm stem−loop represents one of the most highly conserved regions of tRNA and has a natural consensus structure (Figure 1A) possessing the following features. First, the bases of the 3 base pairs of the 5 base pair stem that are furthest from the loop are not conserved. Second, all *E. coli* tRNAs contain Pu52·Py62 and G53·C61 base pairs in the stem positions adjacent to the loop. Finally, the 7-base T-loop has 4 bases which are completely conserved (U54, U55, C56, A58), 2 which are semiconserved as Pu or Py (Pu57, Py60), and 1 (N59) which is nonconserved. At the outset of these studies, it was reasonable to believe that recognition and catalysis by RUMT was governed by the conserved elements of the tRNA T-arm.

In the present work, we have extensively mutated the T-arm within tRNA and the 17-mer T-arm oligonucleotide

and determined the binding and catalytic properties of these mutants with *E. coli* RUMT. Certain generalities emerged from these studies which are worthy of note. First, the tRNA and corresponding T-arm mutants showed similar structure−activity relationships (Figure 5), verifying that the elements required for specificity and catalysis are indeed contained within the T-arm. Second, tRNA mutants showed tighter binding and had higher k_{cat} values than the corresponding 17-mer ribonucleotides (Tables 1 and 2 and Figure 5). The greater efficacy of the tRNAs is attributed to contributions of tertiary structure elements of tRNA to the initial interaction with RUMT or to detrimental effects of the T-arm because of its higher structural disorder in solution. Finally, where examined, 17-mer T-arm mutants which were substrates for methylation also formed binary, covalent RUMT−RNA complexes. Such complexes were not observed with non-substrates, and the ability to form such binary complexes seems coupled to catalytic competency.

From the results of the current study, the following conclusions were reached regarding requirements of the T-arm of tRNA for RUMT substrate activity (Figure 1B): (i) There is no particular requirement for base composition in the T-stem, provided that H-bonding base pairs are maintained across the stem. The stem seems to serve a function in positioning the loop for appropriate presentation to the enzyme. (ii) The 7-base loop size is essential for binding and catalysis. (iii) In the entire T-loop of intact tRNA, U54 is the only base of the natural consensus sequence which is essential for RUMT substrate activity. With the exception of C56, any single base of the loop could be substituted by any other base without a significant decrease in K_m and without complete loss in activity. Besides U54 mutants, C56G tRNA (B19) was the only mutant which was completely inactive.

The primary conclusion of this study is that the consensus T-arm structure for RUMT substrates is not based on the primary sequence of the T-loop; rather, it involves a *consensus conformation* in which the stem−loop structure presents U54 and tertiary structural elements of the 7-base loop required for specificity. The latter could include appropriately positioned planar base moieties, sugar functional groups, phosphate diesters, and other elements common to all RNA sequences.

The absence of base specificity in the recognition of the T-loop by RUMT prompts us to address several important questions.

Why Has the T-Loop Base Sequence of tRNA Been Conserved? Since the sequence of the T-arm has not been conserved for RUMT activity, it must be conserved for one or several other functions of the T-arm of tRNA. For example, the T-arm is involved in Ψ55 synthesis, 1-MeA synthesis in eukaryotes, binding to elongator factor EF-Tu (Antonson *et al.*, 1986), and binding to ribosomes (Helk *et al.*, 1985). Bases of the T-loop which do not appear to be essential for RUMT activity may be essential for other critical functions, and one or more such functions would require a consensus sequence which reflects composite requirements. For example, although U55 is not required for RUMT, it is for Ψ synthesis, and U55 is presumably conserved to allow this modification.

Why Do Not Other RNAs Which Have the RUMT Consensus Become Methylated? Although the T-loop sequence specificity is broad, certain base substitutions are more detrimental than others to RUMT substrate activity; since the effects of such base substitutions on RUMT activity appear to be additive, sequences with several such detrimental bases could reduce methylation to the extent where it is undetectable. Moreover, the bases of loop structures of RNAs are expected to interact with other bases or components within the tertiary structure of larger macromolecules; in their native state, T-loop sequences in other RNAs might be inaccessible to RUMT and thus unavailable for methylation. Indeed, we have previously shown that 16S rRNA is methylated by RUMT *in vitro* at U788 of a loop with the T-arm consensus (Gu *et al.*, 1994) but is not methylated *in vivo*. Finally, it is possible that some methylated U residues have escaped detection since most known RNA sequences have been predicted from the corresponding DNAs. Indeed, methylation of RNAs other than tRNAs may explain the enigma of why RUMT is necessary for cell viability but tRNA methylation is not (Persson *et al.*, 1992).

What Governs the Specificity of RUMT for tRNA? Since the T-loop is buried in tRNA, the first level of recognition of tRNA by RUMT must involve interactions of exposed residues of tRNA other than the T-loop and conformational changes of tRNA which expose the T-loop to the enzyme. We propose that this initial recognition of the tRNA structure is what distinguishes T-arm sequences within tRNA that are methylated from those in other RNAs which are not methylated. Such interactions would involve elements common to all tRNAs, such as planar bases, and ribose and phosphate groups which are appropriately presented by the higher order structure of tRNA. Further, since the T-arm itself dictates aspects of the tertiary structure of tRNA, this first level of recognition may also be attributed in part to T-arm structure.

In summary, the minimal features required for specific recognition of tRNAs by RUMT are contained within the T-arm. However, specificity is not determined by base sequence of the T-arm but rather by other elements of RNA presented to the enzyme in an appropriate stem−loop structure. Specific protein−RNA interactions that are governed by RNA tertiary structure may be a common mechanism in biological systems which involve recognition of multiple RNAs by a single protein. If so, the RUMT−tRNA interaction will serve as a useful paradigm for further studies and understanding of the structural features involved in protein−RNA recognition.

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