In Vitro Methylation of *Escherichia coli* 16S rRNA by tRNA (m^5U54)-methyltransferase†

Xiangrong Gu,‡ James Ofengand,§ and Daniel V. Santi*,‡

Departments of Biochemistry and Biophysics and Pharmaceutical Chemistry, University of California, San Francisco, California 94143, and Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110

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ABSTRACT: 16S rRNA, isolated from *Escherichia coli* or synthesized *in vitro*, is methylated by tRNA (m^5U54)-methyltransferase (RUMT) and *S*-adenosyl-L-methionine to give ribothymidine (m^5U). By methylation studies of 16S rRNA fragments, nearest-neighbor analysis, and nuclease protection experiments, the site of methylation was identified as U788. We have previously shown that the substrate consensus sequence for the T-arm of tRNA consists of a 2–5 base-pair stem and a 7-base loop, with certain constraints on base substitutions within the loop, and in the first two bases which close the loop [Gu, X., & Santi, D. V. (1991) *Biochemistry 30*, 2999–3002]. U788 of 16S rRNA is within a 9-base loop of a predicted stem–loop structure of 16S rRNA. If Ado substitution is allowed at the third and seventh positions of the loop and the first and ninth bases of the loop form an A–C base pair, the resulting stem–loop falls within the RUMT consensus sequence of the T-arm of tRNA. Individual mutants of the tRNA T-arm at these positions confirm that the substitutions are allowable, and expand the previous consensus sequence. Further, prevention of 7-base loop formation by requiring C–C base-pair formation at the loop closure abolishes substrate activity. RUMT forms a complex with Syn 16S rRNA which can be isolated on nitrocellulose filters or by SDS–PAGE electrophoresis. The enzyme also catalyzes exchange of tritium of [^{3}H]Ura-16S rRNA for protons of water. By analogy with studies with tRNA [Gu, X., & Santi, D. V. (1991) *Biochemistry 31*, 10295–10302], the mechanism of methylation is proposed to involve formation of a covalent, albeit reversible, Michael adduct with the target U788 of 16S rRNA.

Escherichia coli 16S rRNA contains 10 different methylated nucleotides over 13 known sites of methylation (Noller, 1984; Hsuchen & Dubin, 1980), but the list does not include m^5U. The enzymes which catalyze methylation of base and sugar moieties in 16S rRNA have not been well characterized, although the m^5C967 and m^2G966 methylases have been partially purified (Negre et al., 1989; Weitzmann et al., 1991). The functions of the modified bases in 16S rRNA are also largely unknown, although a deletion mutant at C967 and a substitution mutant at C1407 were found to be lethal (Thomas et al., 1988, Jemiolo et al., 1991).

tRNA (m^5U54)-methyltransferase (RUMT, EC 2.1.1.35), the *trm*A gene product, catalyzes the transfer of the methyl group of AdoMet to U54 of most tRNAs. Although m^5U at position 54 of tRNA is not essential for growth and survival, the *trm*A gene product appears to have a second function which is vital (Persson et al., 1992). Recently, we have shown that only the stem–loop structure corresponding to the T-arm[1] of tRNA is necessary for methylation by RUMT. This permitted the proposal of a consensus sequence for methylation (Figure 1) and suggested that the enzyme might recognize and methylate other RNAs (Gu & Santi, 1991).

In the absence of AdoMet, RUMT forms binary covalent complexes with tRNA or a 17 nucleotide fragment corresponding to the T-arm of tRNA, and we suggested that RUMT might form transient covalent adducts with other RNAs which contain the T-arm consensus sequence (Gu & Santi, 1991; Gu & Santi, 1992). Further, in *E. coli* over 40% of RUMT is associated with a heterogeneous population of RNA, including a RNA fragment corresponding the 3′ end of 16S rRNA (Ny et al., 1988; Gustafsson & Bjork, 1993). In the present work, we examined the interaction of RUMT with Syn 16S rRNA *in vitro*. Although m^5U is not normally found in 16S rRNA, *E. coli* 16S rRNA was methylated at U788 by RUMT.

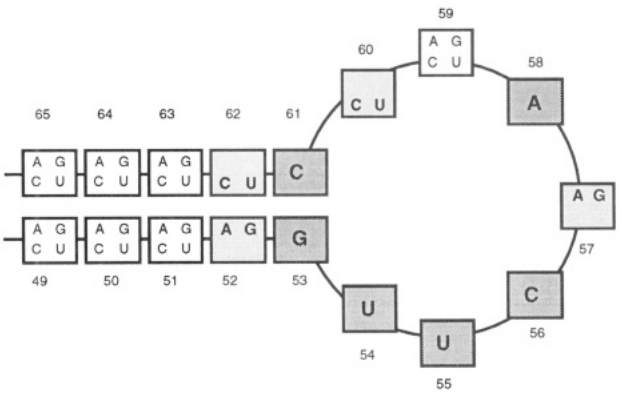

FIGURE 1: Consensus sequence of T-arm of tRNA derived from the structures of *E. coli* tRNAs (Sprinzl et al., 1987). Dark shading indicates complete conservation of base, light shading indicates conservation of Py or Pu, and no shading indicates nonconserved positions.

MATERIALS AND METHODS

The plasmid p67YF0 used for preparation of yeast $tRNA^{Phe}$ and FUra-$tRNA^{Phe}$ and U54C $tRNA^{Phe}$ were gifts from O. C. Uhlenbeck (Department of Chemistry and Biochemistry, University of Colorado, Boulder). Plasmid pWK1, containing

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* To whom correspondence should be addressed.
‡ University of California.
§ Roche Research Center.
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the *E. coli* 16S rRNA gene, has been described (Krzyzosiak et al., 1987). T7 RNA polymerase was isolated from *E. coli* BL21 harboring the plasmid pAR1219 (J. J. Dann, Brookhaven National Laboratory, Upton, NY) and purified as described (Grodberg & Dunn, 1988), except that S-Sepharose (Pharmacia) was used instead of Trisacryl SP. w+ 16S rRNA was isolated from *E. coli* 70S ribosomes (Krzyzosiak et al., 1987). Oligonucleotides were prepared at the UCSF Biomolecular Resource Center and were as follows: ON-T7Fa, CAGGGTATCTAATCCTGCTATAGTGAGTC-GATTTA, containing the T7 promoter and template of fragment Fa (complementary to residues 783–799 of 16S rRNA); ON-Fb, CCCATGGTGTGACGGGCGGT (complementary to residues 1398–1417 of 16S rRNA). [5′-α-^{32}P]ATP (3000 Ci/mmol), [5′-α-^{32}P]CTP (3000 Ci/mmol), [5′-α-^{32}P]GTP (3000 Ci/mmol), [5′-α-^{32}P]UTP (3000 Ci/mmol), [5-^{3}H]UTP (9.2 Ci/mmol), [^{3}H-Me]AdoMet (85 Ci/mmol) were purchased from Amersham. Nucleoside standards and calf thymus DNA were purchased from Sigma. RNase T1, Nuclease P1, and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim. RNase T2 was from BRL. Formamide (nucleic acid hybridization grade) was from EM Science. Qiagen tip-20 was from Qiagen Inc. RUMT was expressed from pJKtrma(pET15b) in BL21 (DE3) cells and purified as described (J.T. Kealey and D.V. Santi, manuscript in preparation), employing a tRNA affinity column as the final step of purification. All molecular biology procedures not explicitly outlined were according to Sambrook et al. 1989.

Templates for 16S rRNA Fragments. pWK1 contains the 16S rRNA sequence immediately preceded by the T7 polymerase promoter which has a 5′ *Kpn*I cloning site (Krzyzosiak et al., 1987). The 3′ truncated 16S rRNAs (D1–D8) were prepared by run-off *in vitro* transcription of pWK1 digested with *Tth*III (D1, 16S rRNA nts 1 to 331), *Sac*II (D2, nts 1 to 526), *Mlu*I (D3, nts 1 to 869), *Rsr*II (D4, nts 1 to 1136), *Nru*I (D5, nts 1 to 1266), *Bsm*I (D6, nts 1 to 1365), *Nco*I (D7, nts 1 to 1415), and *Dra*III (D8, nts 1 to 1469).

For internal fragments of 16S rRNA, plasmid pWK1 was digested with *Kpn*I and a second restriction enzyme within the 16S rRNA sequence; this excised the T7 polymerase promoter endogenous to pKW1 (by use of *Kpn*I) along with adjacent 5′ RNA sequences of various lengths. Synthetic T7 polymerase promoters containing a 5′ *Kpn*I and a 3′ site corresponding to the other restriction enzyme used for excision were ligated into each digested plasmid. In this manner we generated templates pWK1.1 from *Kpn*I-*Sac*II excision/replacement of pWK1, pWK1.2 from *Kpn*I-*Bgl*II excision/replacement, pWK1.3 from *Kpn*I-*Apa*I excision/replacement. Fragments F1 and F2 were prepared by run-off transcription of pWK1.1 digested with *Hin*dIII and *Bgl*II, respectively; fragments F3, F4, F5 and F6 were prepared by run-off transcription of pWK1.2 digested with *Dde*I, *Hinc*II, *Mlu*I, and *Apa*I, respectively; fragment F6 was prepared by run-off transcription of pWK1.3 digested with *Xba*I.

RNA Synthesis. In vitro tRNA synthesis was performed using the appropriate DNA template, nucleotide triphosphates and T7 RNA polymerase (Sampson & Uhlenbeck, 1988; Gu & Santi, 1992). Syn 16S rRNA, 3′ truncated 16S rRNA, and internal fragments of 16S rRNA were synthesized by using appropriate, linearized templates and run-off *in vitro* transcription (Cunningham et al., 1990). [5-^{3}H]Ura-16S rRNA or [^{32}P]16S rRNA was prepared and purified as described above, except 0.1 mM of [5-^{3}H]UTP (2 Ci/mmol) or 0.1 mM [5′-α-^{32}P]CTP (1 Ci/mmol) instead of 4 mM UTP or CTP was used in transcription reactions. RNA was purified on Qiagen-tip 20 columns (larger than 200 nts) or by denaturing (7 M urea) 10% PAGE (less than 200 nts) (Gu & Santi, 1992). The size and homogeneity of rRNA preparations were verified by 1.5% agarose gel electrophoresis. The oligoribonucleotides (fragment Fa, T-arm, and T-arm mutants) were synthesized and purified as described (Milligan et al., 1987; Gu & Santi, 1991).

Concentrations of 16S rRNA fragments larger than 500 nts were calculated assuming that one A_{260} unit (OD) of 16S rRNA is equal to 67 pmols[2] (Denman et al., 1989). For 16S rRNA fragments less than 500 nucleotides, a 1 mg/mL RNA solution was assumed to have an A_{260} of 24 (Zimmermann, 1979). The concentrations of 17mer oligoribonucleotides were calculated from the sum of extinction coefficients of component nucleotides with corrections for a hyperchromic effect of 1.21 (Gu & Santi, 1992).

Enzyme Assays. For methylation assays, reaction mixtures (20 μL) contained 1 μM RNA, 100 μM [^{3}H-Me]AdoMet (0.5–1.0 Ci/mmol), and 10 μM RUMT in methylation buffer (100 mM Tris-HCl, pH 7.5, 40 mM NH_4Cl, 2 mM $Mg(OAc)_2$, 6 mM DTT). The assay was performed at 37 °C, and methylated RNAs were determined by a DEAE–paper disk method (Gu & Santi, 1991) or, when specified, by nucleoside analysis. Nitrocellulose binding assays (Gu & Santi, 1992), SDS–PAGE gel shift assay (Gu & Santi, 1992) and [5-^{3}H]Ura tritium release assays (Santi & Hardy, 1987) were performed in binding buffer (50 mM Tes, pH 6.6, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM DTT, and 40 mM NaCl) at 15 °C as described.

Nucleoside Analysis. [^{3}H-Me]Methylated RNA (20–40 pmol) was extracted with phenol and twice precipitated with ethanol. For F4 or Fa, 10 μL of single-stranded calf thymus DNA (1 μg/μL) was added as a carrier prior to ethanol precipitation. The methylated RNA was digested with Nuclease P1, followed by alkaline phosphatase as described (Negre et al., 1989). For HPLC analysis, each reaction mixture (30 μL) was supplemented with 10 μL of a nucleoside mixture containing 2 mM of each of the following: Ado, Cyd, Guo, Urd, m^{5}Cyd, m^{3}Urd, m^{5}Urd. Reverse-phase HPLC was performed on a Ranin Rabbit HP system, equipped with a Ranin Dynamax-300A column (83-203-C5; 4 × 25 mm) using the following conditions: buffer A, 0.25 M NH_4OAc, pH 6.0; buffer B, 40/60, (v/v), acetonitrile/water (Buck et al., 1983); gradient, 0 min, 0% buffer B; 10 min, 5% buffer B; 25 min, 25% buffer B; 30 min, 50% buffer B; 33 min, 100% buffer B; 35 min, 0% buffer B; 45 min, 0% buffer B; flow rate, 1 mL/min. The column effluent was monitored at 260 nm, and 1-mL fractions were collected and counted in 5 mL of

[1] Abbreviations: Syn 16S rRNA, unmodified *Escherichia coli* 16S rRNA prepared by *in vitro* transcription; w+ 16S rRNA, wild-type *E. coli* 16S rRNA; Syn tRNA^{Phe}, unmodified yeast tRNA^{Phe} prepared by *in vitro* transcription; U54C tRNA^{Phe}, *in vitro* transcribed yeast tRNA^{Phe} containing C substitution at U54 position; FUra-tRNA^{Phe}, *in vitro* transcribed yeast tRNA^{Phe} containing substitution of 5-fluorouracil for uracil; T-arm, the 17mer oligoribonucleotide corresponding to nucleotides 49–65 of *E. coli* tRNA$_1^{Val}$ (GGCGGUUCGAUCCCGUC); mutant RNAs are designated by the wild-type base, followed by the position, followed by the mutation (e.g. U54C tRNA^{Phe} *in vitro* transcribed yeast tRNA^{Phe} containing C at the 54 position); AdoMet, *S*-adenosyl-L-methionine; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 2d-TLC, two-dimensional thin-layer chromatography; nts, nucleotides.

[2] Concentrations of RNA fragments (>500 nts) were calculated as follows: [RNA fragment] = (OD_{260})(67 pmol/OD_{260})(1542 nts/nts of fragment).

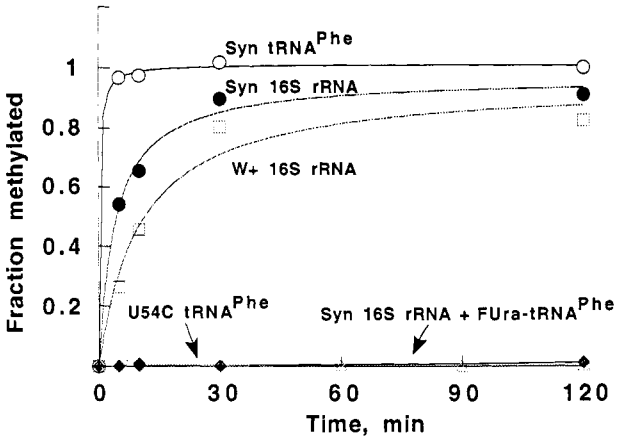


FIGURE 2: Methylation of natural and *in vitro* synthesized 16S rRNA by RUMT. Reaction mixtures (100 μL) contained 1 μM RNA (Syn 16S rRNA, Syn tRNA^{Phe}, or U54C tRNA^{Phe}), 100 μM [^{3}H-Me]-AdoMet (1 Ci/mmol), and 10 μM RUMT in methylation buffer and were incubated at 37 °C. Aliquots (18 μL) were withdrawn when specified for DEAE–paper disk assay. Also shown is inhibition of methylation of Syn 16S rRNA by 5-min pretreatment of RUMT with 1.5 nmol FUra-tRNA^{Phe} before addition of substrates; aliquots (18 μL) were removed and [^{5}H]m^{5}U was measured by HPLC nucleoside analysis (see Materials and Methods).

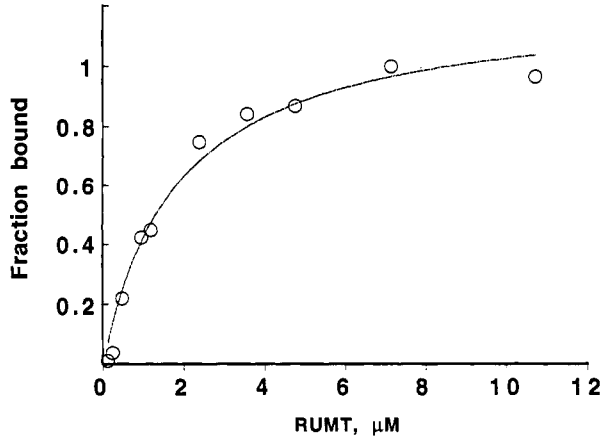


FIGURE 3: Titration of Syn 16S rRNA with RUMT. Reaction mixtures (20 μL) containing 2.5 nM [^{32}P]16S rRNA (5 × 10^5 cpm/pmol) and varying concentrations of RUMT (0.1–10.5 μM) in binding buffer were incubated at 15 °C for 60 min. Aliquots (18 μL) were removed for the nitrocellulose binding assay. Points are experimental and were corrected for filtration efficiency as described in results. The curve is a nonlinear least-squares fit of the data to equation: $RNA_{bound}/RNA_{total} = 1/(1 + K_d/E_{total})$.

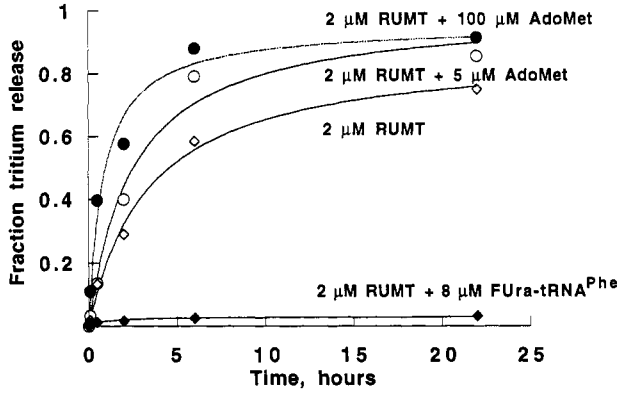


FIGURE 4: The tritium release of [5-^{3}H]Ura-16S rRNA into water catalyzed by RUMT. Reaction mixtures (100 μL) contained 0.1 μM of [5-^{3}H]Ura-16S rRNA (1.4 × 10^6 dpm/pmol 16S rRNA, 4570 dpm/pmol Ura) and 2 μM RUMT with 5 μM AdoMet, 100 μM AdoMet, or 8 μM FUra-tRNA^{Phe}, or without both AdoMet and FUra-tRNA^{Phe}, and incubated at 15 °C. Aliquots (18 μL) were removed when specified for the charcoal adsorption assay.

Aquasol II. Retention volumes of modified nucleosides were as follows: m^{5}C, 12.0 min; m^{5}U, 14.5 min; m^{3}U, 17.2 min.

Nearest-Neighbor Analysis. Fragments F4 and Fa, each prepared separately with one of four [5′-^{32}P]NTPs (20 pmol), were methylated with 500 μM AdoMet and 10 μM RUMT in a 20-μL reaction mixture containing methylation buffer. Following methylation, the samples were diluted with 30 μL of water, extracted with an equal volume of phenol, and precipitated with 3 volumes of cold 95% ethanol. After the precipitant was washed with 80% ethanol and dried, the pellets were dissolved with RNase T2 digestion buffer and digested to completion with RNase T2 (Silberklang et al., 1979). The [3′-^{32}P]NMPs produced were subjected to 2d-TLC on cellulose plates, using the following mobile phases: (1) isobutyric acid–concentrated NH_4OH–H_2O (66/1/33, v/v/v), (2) 0.1 M sodium phosphate (pH 6.8)–ammonium sulfate–*n*-propanol (100/60/2, v/w/v) (Silberklang et al., 1979), and the nucleotides were detected by autoradiography.

RESULTS

Methylation of 16S rRNA. RUMT catalyzes the methylation of *in vivo* or *in vitro* synthesized 16S rRNA by [^{3}H-Me]AdoMet (Figure 2). With the use of our most highly purified preparation of RUMT to catalyze methylation, the methylated base was identified as [^{3}H-Me]m^{5}U (0.8 pmol m^{5}U/pmol RNA), by both HPLC analysis of nucleosides and 2d-TLC analysis of nucleotides (not shown).[3] Treatment of RUMT with the specific inhibitor FUra-tRNA^{Phe} completely prevented methylation of Syn 16S rRNA.

Nitrocellulose Binding Assay. A nitrocellulose filter binding assay was employed to measure the binding of Syn 16S rRNA and RUMT (Gu & Santi, 1992). Figure 3 shows the titration of 2.5 nM [^{32}P]16S rRNA with varying concentrations of RUMT. At saturating enzyme, 80% of the 16S rRNA was bound to RUMT, which represents the filtration efficiency of the assay (Santi et al., 1974). The apparent dissociation constant of the binary RUMT-Syn 16S rRNA complex was 1.8 μM (Figure 3). The binding of Syn 16S rRNA to RUMT was completely inhibited by 5 μM Syn tRNA^{Phe}; as a negative control, the binding was not affected by the 16S rRNA fragment F3 (nts 705–759), which is not methylated by RUMT (data not shown). When a preformed complex of RUMT and Syn 16S rRNA was electrophoresed on 5% SDS–PAGE, the RNA band (stained with ethidium bromide) which slightly penetrates the gel also contained the protein (stained with Coomassie Blue R250), which alone migrates through the gel (data not shown). As previously described (Gu & Santi, 1992), the mobility shift of RUMT in SDS-PAGE is indicative of a covalent RNA–protein complex.

Tritium Release from [5-^{3}H]Ura-16S rRNA. Upon methylation of [5-^{3}H]Ura-16S rRNA by RUMT and AdoMet, there is a release of 0.92 mol of tritium into solvent per mole of 16S rRNA (Figure 4). In the absence of AdoMet, RUMT catalyzes the exchange of tritium between [5-^{3}H]Ura-16S rRNA (0.75 mol tritium/mol RNA) and protons of water. The observation of a tritium exchange reaction is consistent with reversible Michael addition of a nucleophile of RUMT to the 6-carbon of the target pyrimidine of the RNA (Ivanetich & Santi, 1992). Recently, a Michael adduct between Cys 324 of RUMT and FUra-tRNA^{Phe} has been isolated and identified (Kealey & Santi, 1991) and provides direct evidence

[3] With some preparations of RUMT, we observed methylation of C967, which we attribute to contamination with the known m^{5}C967-methylase.

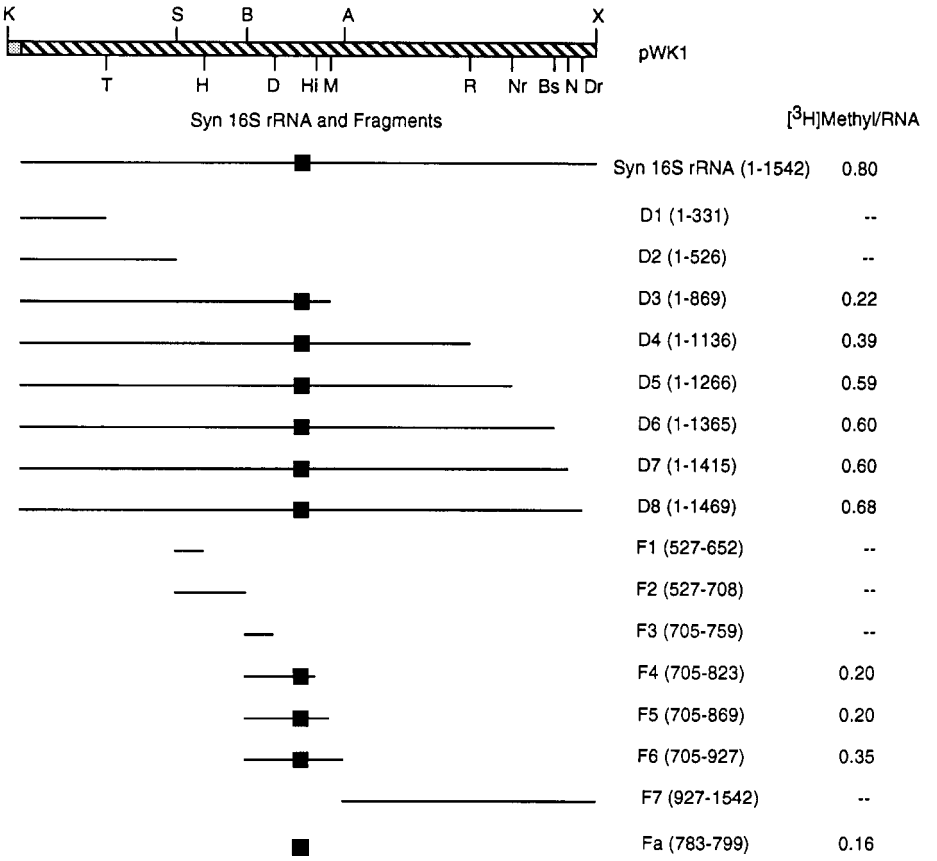


FIGURE 5: Methylation of truncated 16S rRNAs. The 16S rRNAs truncated at 3′ ends (D1–D8) were prepared by run-off *in vitro* transcription using *Tth*III I-, *Sac*II-, *Mlu*I-, *Rsr*II-, *Nru*I-, *Bsm*I-, *Nco*I-, and *Dra*III-digested pWK 1 as templates. Fragments of 16S rRNA (F1–F7) were prepared by *in vitro* transcription and using *Hind*III, *Bgl*II-digested pWK1.1, *Dde*I, *HinC*II, *Mlu*I, *Apa*I-digested pWK1.2, and *Xba*I-digested pWK1.3 as templates. Methylation assays (100 μL) contained 1 μM RNA, 100 μM [^{3}H-Me]AdoMet (1 Ci/mmol), and 10 μM RUMT in methylation buffer. Aliquots (18 μL) were removed after 30, 60, and 120 min incubation at 37 °C for DEAE–paper disk assay; data show the picomoles of methyl group per picomoles of RNA after 120-min incubation. Symbols are as follows: K, *Kpn*I; T, *Tth*III I; S, *Sac*II; H, *Hind*III; B, *Bgl*II; D, *Dde*I; Hi, *Hinc*II; M, *Mlu*I; A, *Apa*I; R, *Rsr*II; Nr, *Nru*I; Bs, *Bsm*I; N, *Nco*I; Dr, *Dra*III; X, *Xba*I; gray shading, T7 promoter; striping, coding sequence of 16S rRNA; ■, nts 783–799 of 16S rRNA; - -, undetectable.

for the intermediate in the RUMT-catalyzed 5-H exchange [5-^{3}H]Ura-tRNA^{Phe} (Santi & Hardy, 1987; Gu & Santi, 1992). As for the inhibition of binding of Syn 16S rRNA to RUMT by FUra-tRNA^{Phe}, FUra-tRNA^{Phe} completely inhibits the [5-^{3}H]Ura-16S rRNA tritium exchange reaction (Figure 4).

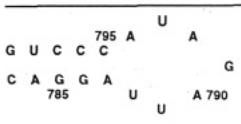
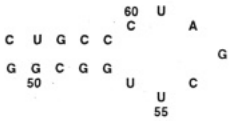
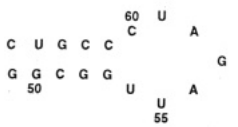
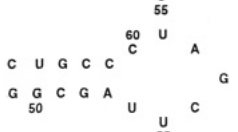
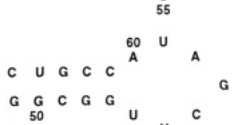
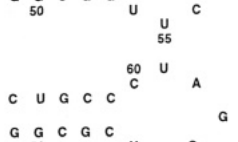
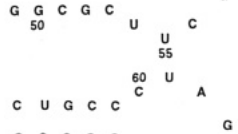
RNA Fragments as Substrates for RUMT. As an initial approach toward identification the site of methylation within the 1542 nt 16S rRNA, eight rRNA fragments (D1–D8) truncated at the 3′ end were prepared by endonuclease digestion of pKW1 and run-off *in vitro* transcription; each fragment was tested for RUMT-catalyzed methylation. m^{5}U was not found in transcripts corresponding to the first 526 bases of 16S rRNA (D1, D2), but was observed in the 869 base transcript (D3) and all larger transcripts (D4–D8) (Figure 5). It was concluded that m^{5}U was located in the 526–869 base region of 16S rRNA.

Next, fragments of 16S rRNA were prepared by subcloning different restriction fragments of pWK1 behind the T7 RNA polymerase promoter to give pWK1.1 (16S rRNA nts 527–1542), pWK1.2 (nts 705–1542), and pWK1.3 (nts 927–1542) (Figure 5). Each of these plasmids was then digested with restriction enzymes, yielding DNA fragments containing the T7 polymerase promoter, followed by a fragment of 16S rRNA sequence. After *in vitro* transcription, the following 16S rRNA fragments were produced: F1 (527–652), F2 (527–708), F3 (705–759), F4 (705–823), F5 (705–869), F6 (705–927), and F7 (927–1542). Each of these fragments was tested in an *in vitro* methylation assay with RUMT and [^{3}H-Me]AdoMet, and each rRNA was analyzed for the extent of methylation and identity of methylated nucleoside. m^{5}U was present in fragments F4, F5, and F6, but not in the F1, F2, F3, and F7 (Figure 5). It was therefore concluded that m^{5}U was contained within fragment 759–823 of 16S rRNA. Finally, we prepared the 17mer RNA fragment Fa, corresponding to residues 783–799 of 16S rRNA, and tested it for methylation by RUMT. As shown in Table 1, this fragment was methylated by RUMT, yielding m^{5}U.

Nearest-Neighbor Analysis and Nuclease Protection. Fragments F4 (nts 705–823) and Fa (nts 783–799), prepared with [5′-α-^{32}P]UTP, were methylated with [^{3}H-Me]AdoMet and RUMT and completely digested with RNase T1, and the resulting T1 products were analyzed by 7M urea–PAGE; the only fragment containing tritium was a pentanucleotide (data not shown). Since there is only one pentanucleotide expected in the complete RNase T1 digests of F4 or Fa (AUUAG, nts 787–791), it was concluded that the site of methylation was either U788 or U789.

Samples of 16S rRNA fragment F4 (nts 705–823), each labeled with one of the four [5′-α-^{32}P]NTPs, were prepared by *in vitro* transcription, purified by 7 M urea–PAGE and methylated by RUMT and AdoMet. The products were subjected to nearest-neighbor analyses using RNase T2 digestion and 2d-TLC analysis of nucleotides (Silberklang et al., 1979; Milligan et al., 1987). [3′-^{32}P]m^{5}UMP was observed only from the fragment labeled with [5′-^{32}P]UTP (Figure 6).

Table 1: Relative Rates of Methylation[a]

structure	RNA	rate (pmol/min)
	Fa, 17mer of 16S rRNA (16S rRNA numbering)	0.10
	wild-type T-arm (tRNA numbering)	3.2
	C56A T-arm	0.51
	G53A T-arm	0.16
	C60A T-arm	0.20
	G53C T-arm	*b*
	U54C T-arm	*b*
	Syn 16S rRNA	0.26
	Syn tRNA^{Phe}	11

[a] Reaction mixtures (60 μL) contained 10 μM RNA, 50 μM [^{3}H-Me]AdoMet (2 Ci/mmol), 0.1 μM RUMT, 50 mM Tricine, pH 8.4, 5 mM DTT, 2 mM $MgCl_2$, 1 mM EDTA, 40 mM NH_4Cl, and 20 mM spermidine. Assays were performed at 15 °C. Aliquots (18 μL) were withdrawn at 1, 2, and 30 min for DEAE–paper disk assay (Gu & Santi, 1991). [b] Undetectable.

Quantitative analysis of 3′ [^{32}P]NMPs in a reaction which contained 0.2 mol of [^{3}H-Me]m^{5}U per mole of F4 yielded the following ratios of AMP/CMP/GMP/UMP/m^{5}UMP: observed, 5/5.5/6.7/0.88/0.16; calculated, 5/5/7/0.8/0.2. Since the 3′-^{32}P of the [3′-^{32}P]m^{5}UMP product was donated by the 5′-α-^{32}P of [5′-α-^{32}P]UTP used for 16S rRNA synthesis, this showed that the methylation occurred at the 5′ base of a UU sequence. Since U788U789 is the only such sequence in fragment F4, we concluded that U788 was the site of methylation.

The above experiments identified the site of methylation in fragment F4, but it was considered possible that the F4 methylation site differed from that of wild-type 16S rRNA. To address this, *in vitro* trnascribed 16S rRNA was methylated by RUMT and [^{3}H-Me]AdoMet and then hybridized to DNA fragments ON-T7Fa (complementary to residues 783–799 of 16S rRNA) or ON-Fb (complementary to residues 1398–1417 of 16S rRNA, as a control). DEAE–cellulose columns (0.1 mL) were employed to separate complete digestion products from protected hybrids. In control experiments, 0.2 M NaCl eluted more than 90% of the ^{3}H from complete RNase T1 digests of [^{3}H-Me]16S rRNA, but did not elute [5′-^{32}P]-ON-T7Fa or [5′-^{32}P]ON-Fb; hybrid-protected RNase T1 digested-fragments were eluted with 1 M NaCl. RNase T1

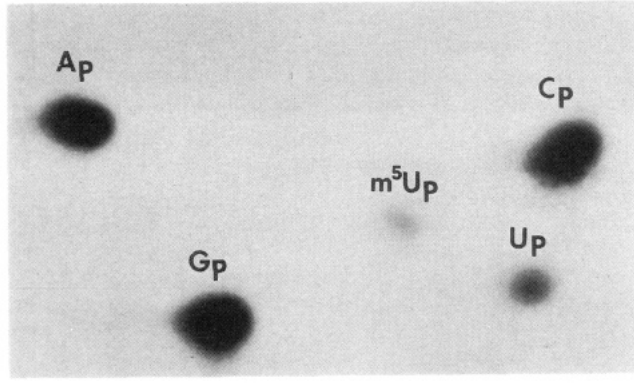


FIGURE 6: Nearest-neighbor analysis of ^{32}P-F4. Individual F4 fragments containing [5′-α-^{32}P]AMP, [5′-α-^{32}P]CMP, [5′-α-^{32}P]-GMP, and [5′-α-^{32}P]UMP were prepared by *in vitro* transcription using *Hinc*II-digested pWK1.2 as template and each of the four [5′-α-^{32}P]NTPs. The methylation reactions (20 μL) contained 1 μM [5′-α-^{33}P]NMP-F4 [(1–5) × 10^4 cpm], 500 μM AdoMet, and 10 μM RUMT in methylation buffer and were incubated at 37 °C for 60 min. The reactions were diluted to 50 μL with H_2O, extracted with phenol and precipitated with ethanol (5 μg of carrier tRNA was added). The four methylated [5′-^{32}P]NMP-F4 fragments were digested with RNase T2 (2 units) in 50 mM NaAc, pH 4.5, 2 mM EDTA, at 37 °C for 5 h, and analyzed by 2d-TLC on cellulose plates. Shown is the 3′-^{32}P-nucleotide analysis of RNase T2 digests of [5′-α-^{32}P]UMP-F4.

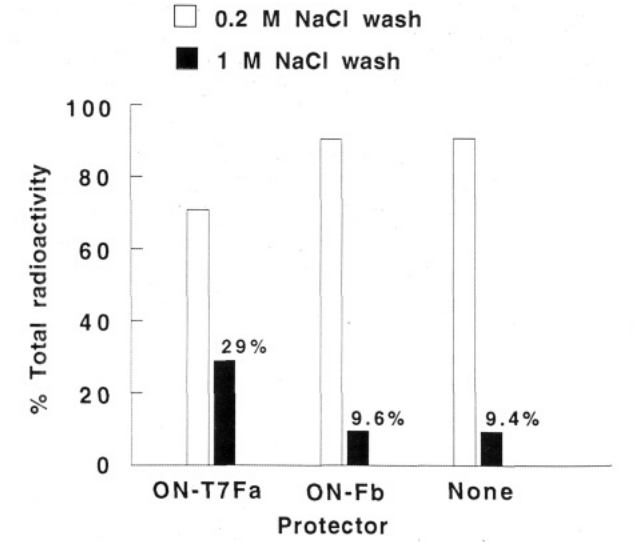


FIGURE 7: Determination of the site of methylation by hybridization–protection. Hybridization mixtures (50 μL) containing 1 pmol of [^{3}H-Me]16S rRNA [(5–6) × 10^3 dpm] and 50 pmol of oligonucleotides (ON-T7Fa or ON-Fb) in hybridization buffer (40 mM Mes, pH 6.4, 0.4 M NaCl, 1 mM EDTA and 80% (vol/vol) formamide) were incubated at 90 °C for 10 min and slowly cooled to room temperature. To this solution were added 450 μL of 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, and 50 units of RNase T1, and digestion was carried out for 30 min at 30 °C. The digests were applied to small DEAE–cellulose columns (100-μL bed volume) previously equilibrated with 50 mM Tris-HCl, pH 7.5. The columns were washed with 5 × 1 mL of 50 mM Tris-HCl and eluted with 5 × 1 mL of 0.2 M NaCl–50 mM Tris-HCl, pH 7.5, followed by 5 × 1 mL of 1 M NaCl–50 mM Tris-HCl, pH 7.5. The radioactivity eluted with 0.2 M NaCl–50 mM Tris-HCl, pH 7.5 (a) and the radioactivity eluted with 1 M NaCl–50 mM Tris-HCl, pH 7.5 (b) were used to calculate the ratio of protected to total methylation sites [b/(a + b)].

(20–50 U) was used to digest 1 pmol of [^{3}H-Me]16S rRNA previously hybridized to fragments ON-T7Fa and ON-Fb. As shown in Figure 7, about 20% m^{5}U was protected by fragment On-T7Fa, but ON-Fb afforded no protection.

Relative Rates of Methylation. The rate of methylation of 16S rRNA is quite slow, and determinations of kinetic

constants were not feasible because of the inability to reach saturating concentrations of 16S rRNA. Under nonsaturating initial velocity conditions at 15 °C, comparative measurements showed that Syn 16S rRNA is methylated at least 40-fold slower than Syn $tRNA^{Phe}$. Relative rates of methylation for the 17mer T-arm of tRNA and individual mutants of the T-arm corresponding to those in the 16S rRNA 17mer are given in Table 1; methylation of the G53C or U54C mutant of the T-arm was undetectable. These assays were performed at 15 °C with identical concentrations of enzyme and RNA, and values reported are not intended to indicate kinetic constants.

DISCUSSION

In this study, we have shown that RUMT catalyzes the AdoMet-dependent methylation of both wild-type 16S rRNA and *in vitro* transcribed 16S rRNA, yielding about 1 mol of m^5U per mole of 16S rRNA.

The site of Syn 16S rRNA methylation was identified to be U788 as follows. First, fragments of 16S rRNA were tested as substrates for RUMT, and only those containing the 759–823 sequence yielded m^5U. It was subsequently shown that a 17mer oligoribonucleotide corresponding to nts 783–799 of 16S rRNA served as a substrate for RUMT. Second, RNAse T1 digestion of [^{3}H-Me]16S rRNA fragment F4 (nts 705–823) or Fa (nts 783–799) yielded a pentanucleotide containing tritium. Since complete RNase T1 digestion of these fragments contain only one pentanucleotide containing U (nts 787–791), we localized the site of methylation to either U788 or U789. Third, nearest-neighbor analysis of 16S rRNA fragment F4 labeled with [5′-α-^{32}P]UTP showed that the methylated 3′ m^5UMP product contained ^{32}P, which must have arisen from an adjacent 5′ ^{32}P in a UMP in the RNA; since the only UU sequence is at 788–789, U788 was identified as the methylated base. Finally, we verified that the same site was methylated in Syn 16S rRNA by demonstrating hybrid protection of [^{3}H]Me-16S rRNA with an oligonucleotide complementary to nts 783–799.

Evidence that the methylation was indeed catalyzed by RUMT and not some contaminant of the preparation was obtained by showing complete inhibition of the reaction by FUra-$tRNA^{Phe}$, a potent inhibitor of RUMT, and inhibition of binding of Syn 16S rRNA by a normal substrate tRNA.

Mechanism of 16S rRNA Methylation. We have previously shown that the methylation of tRNA by RUMT involves nucleophilic attack of Cys 324 at the C-6 of U54 to form transient Michael adducts, which are subsequently methylated by AdoMet (Kealey & Santi, 1991). In the absence of AdoMet, RUMT still forms reversible covalent Michael adducts with tRNA and T-arm substrates; these can be isolated on nitrocellulose filters and SDS–PAGE, and they result in exchange of the 5-H of the target U with protons of water (Gu & Santi, 1992). Specific binding of RUMT to Syn 16S rRNA (K_d = 1.8 μM) has been demonstrated by nitrocellulose filter binding assays. Moreover, in the absence of AdoMet, RUMT catalyzes the exchange of tritium of [5-^{3}H]Ura-16S rRNA for protons of water, and RUMT-Syn 16S rRNA complexes are stable on SDS–PAGE. These observations indicate a binding mechanism which involves Michael adduct formation between RUMT and 16S rRNA. It is reasonable to conclude that the complete mechanism of methylation of 16S rRNA is analogous to that previously established for the RUMT-catalyzed methylation of tRNA (Gu & Santi, 1992).

RUMT–16S rRNA Recognition. The primary determinants for the RUMT–tRNA interaction and subsequent methylation are contained within the structure of the T-arm of tRNA (Gu & Santi, 1991). By using synthetic analogs of the T-arm of tRNA to define minimal substrate size, and known tRNA substrates to define substrate sequences, a minimal consensus sequence for T-arm methylation was defined (Figure 1). This consists of a 2–5 base-pair stem and a 7-base loop, with certain constraints on base substitutions within the loop, and in the first two bases which close the loop.

Although 16S rRNA is a poor substrate of RUMT (40-fold poorer than tRNA), we have analyzed how it relates to the reported T-arm consensus sequence. In the predicted 16S rRNA secondary structure, U788 is the second nucleotide (5′ to 3′ direction) in a 9-base loop closed by a G786–C796 base pair to give a 17-base hairpin (nts 783–799) (Noller, 1984). If it is assumed that an A787–C795 base pair can form, and that substitution of Ado at positions 56 and 60 are allowable, the putative stem–loop structure from 16S rRNA does not fit within and expands the current consensus sequence for RUMT.

The fact that the 17mer corresponding to nts 783–799 of 16S rRNA serves as a substrate for RUMT confirms that these substitutions are permissible, albeit the rate of reaction is reduced over 30-fold compared to the model T-arm of tRNA. Further, corresponding single mutations made in the loop of the model T-arm substrate (i.e. C56A; C60A) and the first base of the stem (G53A converts the GC base-pair loop closure to an AC base pair) provide substrates for RUMT, with 6–20-fold lower activity than the T-arm under the same conditions. Interestingly, these substitutions are not found in *E. coli* tRNA, showing that the consensus sequence derived from tRNAs is a minimal one. This is not surprising since the T-arm sequences of tRNAs must not have evolved for the sole purpose of acting as RUMT substrates.

The T-arm double mutants G53C/C61G and G53U/C61A are good substrates for the enzyme (unpublished data), so the presence of a purine at position 53 is not required for substrate activity. However, we have shown that the G53C mutant of the substrate T-arm, which cannot form a loop–closure base pair, has no detectable substrate activity, and we conclude that the ability to form a 7-base loop in the active site of is essential. Although uncommon, A–C base pairs have been observed in stem loop structures (Puglisi et al., 1990). The low activity of 16S rRNA as a substrate for RUMT may thus be attributed to (a) in inaccessibility of the stem–loop structure, (b) the unfavorable A residues of the loop, and (c) the concentration of 16S rRNA that is in the appropriate 7-base loop conformation (A–C base pair closing the loop). The latter depends on the equilibrium constant for the 9 base to 7 base loop interconversion which may be affected or modified by the enzyme.

Relevance of U788 Methylation of 16S rRNA. Since m^5U has not been observed in wild-type 16S rRNA, and mature 16S rRNA isolated from *E. coli* is a substrate for RUMT, it is clear that U788 is not highly methylated *in vivo*. One must therefore question the physiological relevance of the RUMT-catalyzed methylation fo 16S rRNA. One possibility is that a low level of methylation of 16S rRNA could exist *in vivo* and be relevant, but has gone undetected. Indeed, the oligomer A-modU-U-A-G had been identified in RNase T1 digestion products of some bacteria (Gibson et al., 1979; Woese et al., 1982), although not in *E. coli*. Another possibility is that the methylation of 16S rRNA is so inefficient that it does not occur *in vivo* and is simply an *in vitro* artifact. Finally, it is possible that covalent adduct formation between RUMT and 16S rRNA has some biological role in the absence of methylation. Indeed, in extracts of *E. coli*, RUMT and 16S

rRNA are found in a covalent complex (Gustafsson & Bjork, 1993); however, the reported region of attachment of 16S rRNA to RUMT is far from the U788 site in the primary structure we have identified, so the relationship between these observations remains unclear. Regardless, the present study clearly demonstrates that, as previously proposed (Gu & Santi, 1991), RUMT can methylate RNAs other than tRNA and encourages further studies directed at identifying substrates which may be methylated by RUMT and which may be relevant *in vivo*.

REFERENCES

Buck, M., Connick, M., & Ames, B. N. (1983) *Anal. Biochem. 129*, 1–13.

Cunningham, P. R., Weitzmann, C. J., Nurse, K., Masurel, R., Knippenberg, P. H. V., & Ofengand, J. (1990) *Biochim. Biophys. Acta 1050*, 18–26.

Denman, R., Weitzmann, C., Cunningham, P. R., Negre, D., Nurse, K., Colgan, J., Pan, Y.-C., Miedel, M., & Ofengand, J. (1989) *Biochemistry 28*, 1002–1011.

Gibson, J., Stackebrandt, E., Zablen, L. B., Gupta, R., & Woese, C. R. (1979) *Curr. Microbiol. 3*, 59–64.

Grodberg, J., & Dunn, J. J. (1988) *J. Bacteriol. 170*, 1245–1253.

Gu, X., & Santi, D. V. (1991) *Biochemistry 30*, 2999–3002.

Gu, X., & Santi, D. V. (1992) *Biochemistry 31*, 10295–10302.

Gustafsson, C., & Bjork, G. R. (1993) *J. Biol. Chem. 268*, 1326–1331.

Hsuchen, C.-C., & Dubin, D. T. (1980) *J. Bacteriol. 144*, 991–998.

Ivanetich, K. M., & Santi, D. V. (1992) *Prog. Nucleic Acids Res. Mol. Biol. 42*, 127–156.

Jemiolo, D. K., Taurence, J. S., & Giese, S. (1991) *Nucleic Acids Res. 19*, 4259–4265.

Kealey, J. T., & Santi, D. V. (1991) *Biochemistry 30*, 9724–9728.

Krzyzosiak, W., Denman, R., Nurse, K., Hellmann, W., Boublik, M., Gehrke, C. W., Agris, P. F., & Ofengand, J. (1987) *Biochemistry 26*, 2353–2364.

Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783–8798.

Negre, D., Weitzmann, C., & Ofengand, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4902–4906.

Noller, H. F. (1984) *Annu. Rev. Biochem. 53*, 119–162.

Ny, T., Lindstrom, P. H. R., Hagervall, T. G., & Bjork, G. R. (1988) *Eur. J. Biochem. 177*, 467–475.

Persson, B. C., Gustafsson, C., Berg, D. E., & Bjork, G. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3995–3998.

Puglisi, J. D., Wyatt, J. R., & Ignacio Tinoco, J. (1990) *Biochemistry 29*, 4215–4226.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) Cold Spring Harbor Laboratory, New York.

Sampson, J. R., & Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033–1037.

Santi, D. V., & Hardy, L. W. (1987) *Biochemistry 26*, 8599–8606.

Santi, D. V., McHenry, C. S., & Perriard, E. R. (1974) *Biochemistry 13*, 467–471.

Silberklang, M., Gillum, A. M., & RajBhandary, U. L. (1979) *Methods Enzymol. 59*, 58–109.

Sprinzl, M., Hartmann, T., Meissner, F., Moll, J., & Vorderwulbecke, T. (1987) *Nucleic Acids Res. 15*, r53–r115.

Thomas, C. L., Gregory, R. J., Winslow, G., Muto, A., & Zimmermann, R. A. (1988) *Nucleic Acids Res. 16*, 8129–8146.

Weitzmann, C., Tumminia, S. J., Boublik, M., & Ofengand, J. (1991) *Nucleic Acids Res. 19*, 7089–7095.

Woese, C. R., Blanz, P., Hespell, R. B., & Hahn, C. M. (1982) *Curr. Microbiol. 7*, 119–124.

Zimmermann, R. A. (1979) *Methods Enzymol. LIX*, 553–582.