Johnson, R., & Chenoweth, D. E. (1985) *J. Biol. Chem. 260*, 7161-7164.
Johnson, R., & Chenoweth, D. E. (1987) *Biochem. Biophys. Res. Commun. 148*, 1330-1337.
Jose, P. J., Forrest, M. J., & Williams, T. J. (1981) *J. Immunol. 127*, 2376-2380.
Karnik, S. S., & Khorana, H. G. (1990) *J. Biol. Chem. 265*, 17520-17524.
Kozak, M. (1987) *Nucleic Acids Res. 15*, 8125-8148.
Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105-132.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simons, M., Dumont, J. E., & Vassart, G. (1989) *Science 244*, 569-572.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Meuer, S., Ecker, U., Hadding, U., & Bitter-Suermann, D. (1981) *J. Immunol. 126*, 1506-1509.
Miki, I., Watanabe, T., Nakamura, M., Seyama, Y., Ui, M., Sato, F., & Shimizu, T. (1990) *Biochem. Biophys. Res. Commun. 166*, 342-348.
O'Dowd, B. F., Hnatowich, M., Regan, J. W., Leader, W. M., Caron, M. G., & Lefkowitz, R. J. (1988) *J. Biol. Chem. 263*, 15985-15992.
Okajima, F., & Ui, M. (1984) *J. Biol. Chem. 259*, 13863-13871.
Rollins, T. E., & Springer, M. S. (1985) *J. Biol. Chem. 260*, 7157-7160.
Rosenthal, H. E. (1967) *Anal. Biochem. 20*, 525-532.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Scheid, C. R., Webster, P. M., Henson, P. M., & Findlay, S. R. (1983) *J. Immunol. 130*, 1997-1999.
Seed, B., & Aruffo, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3365-3369.
Shirato, M., Takahashi, Nagasawa, S., & Koyama, J. (1988) *FEBS Lett. 234*, 231-234.
Siciliano, S. J., Rollins, T. E., & Springer, M. S. (1990) *J. Biol. Chem. 265*, 19568-19574.
Stimler, N. P., Brocklehurst, W. E., Bloor, C., & Hugli, T. E. (1981) *J. Immunol. 126*, 2258-2261.
Strader, C. D., Sigal, I. S., & Dixon, R. A. F. (1989) *FASEB J. 3*, 1825-1832.
Young, D., Waitches, G., Birchmeier, C., Fasano, O., & Wigler, M. (1986) *Cell 45*, 711-719.

The T-arm of tRNA Is a Substrate for tRNA (m^5U54)-Methyltransferase[†,‡]

Xiangrong Gu and Daniel V. Santi*

Departments of Biochemistry and Biophysics and Pharmaceutical Chemistry, University of California, San Francisco, California 94143

Received December 26, 1990; Revised Manuscript Received January 29, 1991

ABSTRACT: Fragments of *Escherichia coli* FUra-$tRNA_1^{Val}$ as small as 15 nucleotides form covalent complexes with tRNA (m^5U54)-methyltransferase (RUMT). The sequence essential for binding includes position 52 of the T-stem and the T-loop and extends toward the 3′ acceptor end of FUra-tRNA. The in vitro synthesized 17mer T-arm of *E. coli* $tRNA_1^{Val}$, composed of the seven-base T-loop and 5-base-pair stem, is a good substrate for RUMT. The K_m is decreased 5-fold and k_{cat} is decreased 2-fold compared to the entire tRNA. The T-arm structure could be further reduced to an 11mer containing the loop and two base pairs and still retain activity; the K_m was similar to that of the 17mer T-arm, whereas k_{cat} was decreased an additional 20-fold. The data indicate that the primary specificity determinants for the RUMT-tRNA interaction are contained within the primary and secondary structure of the T-arm of tRNA.

tRNA (m^5U54)-methyltransferase [EC 2.1.1.35, tRNA (uracil-5-)-methyltransferase] catalyzes the methylation by AdoMet of U54 all prokaryotic and most eukaryotic tRNAs. The function of m^5U (ribothymidine) in tRNA remains uncertain, but it appears to improve the fidelity of protein synthesis (Kersten et al., 1981), stabilize the structure of tRNA (Davenloo, et al., 1979), and show a selective advantage in *Escherichia coli* cell growth (Bjork & Neidhardt, 1975). The catalytic mechanism of RUMT[1] is analogous to those of dTMP synthase and the DNA (m^5C)-methyltransferases (Santi & Danenberg, 1984; Santi & Hardy, 1987; Wu & Santi, 1987); it involves initial formation of a covalent Michael adduct between an enzyme nuclophile, probably a Cys thiol, and the 6-position of the target U54 of tRNA to activate the 5-position for subsequent one-carbon transfer. We have reported that, in the presence of AdoMet, RUMT and FUra-tRNA form a covalent complex that can readily be monitored by a gel-shift assay of RUMT on SDS-PAGE (Santi & Hardy, 1987); free RUMT migrates as a 42-kDa protein, whereas the covalent enzyme-tRNA complex migrates as a 65-kDa band (Santi & Hardy, 1987). It has been surmised that in this complex the 6-position of FUra-54 is covalently bound to the enzyme and the 5-position of FUra-54 is methylated.

[†] This work was supported by USPHS Grant CA-14394.

[‡] This paper is dedicated to Robert H. Abeles on the occasion of his 65th birthday.

* To whom correspondence should be addressed.

[1] Abbreviations: FUra-$tRNA_1^{Val}$, $tRNA_1^{Val}$ containing substitution of Ura by FUra; AdoMet, *S*-adenosylmethionine; Py, pyrimidine; Pu, purine; RUMT, *E. coli* tRNA (m^5U54)-methyltransferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, tlc, thin-layer chromatography.

0006-2960/91/0430-2999$02.50/0

Little is known about RUMT–tRNA interactions. However, the fact that RUMT methylates a single Ura residue in most tRNAs suggests there are common structural features of tRNAs that guide recognition and catalysis. Here, we report studies directed at determining the minimal structure of tRNA necessary for recognition by RUMT, with the aim of ultimately understanding aspects of the protein–RNA interaction. We show that fragments of the T-arm of FUra-tRNA are sufficient to form covalent complexes with RUMT and that the T-arm of tRNA serves as a substrate for the enzyme.

Materials and Methods

FUra-$tRNA_1^{Val}$ was a gift from J. Horowitz (Department of Biochemistry and Biophysics, Iowa State University), and the plasmid p67YFO used for preparation of $tRNA^{Phe}$ was a gift from O. C. Uhlenbeck (Department of Chemistry and Biochemistry, University of Colorado). 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 & Dann, 1988) except that S-Sepharose (Pharmacia) was used as the first column instead of Trisacryl SP. Oligonucleotides were prepared at the UCSF Biomolecular Resource Center. [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham. RNase A (Boehringer Mannheim), RNase T_2 (Sigma), and nuclease P_1 (Boehringer Mannheim) digestion and cellulose tlc were performed as described (Silberklang, et al., 1979). Urea–PAGE was used for RNA purification (Ogden & Adams, 1987). RUMT was the purified preparation previously described (Gu & Santi, 1990).

RNA Synthesis. T7 RNA polymerase catalyzed in vitro RNA synthesis was performed with appropriate templates and primers as described (Chu & Horowitz, 1989; Milligan et al., 1987). Products were fractionated on 7 M urea–20% polyacrylamide gels and eluted overnight with a buffer of 50 mM KOAc, 20 mM EDTA, and 100 mM KCl at 4 °C. To remove urea, about 1 A_{260} unit of oligoribonucleotide was applied to a TSK-gel Toyopearl DEAE-650 C (Supelco) column (0.5 × 0.5 cm) previously equilibrated with 20 mM Tris-HCl buffer pH 7.0, and 0.1 M NaCl; RNA was eluted with 0.5 M NaCl in the same buffer and concentrated by precipitation with 3 volumes of ethanol. The RNAs were dissolved in a buffer of 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 2 mM $MgCl_2$, heated to 80 °C for 3 min, and allowed to cool to room temperature.

5′-Labeling of tRNA. In vitro synthesized tRNA was treated with calf intestinal phosphatase (Boehringer Mannheim) to remove the 5′ triphosphate and labeled at the 5′ end with polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (3000 Ci/mmol)(Silberklang, et al., 1979). The 5′-end-labeled tRNA was resolved by electrophoresis on 7 M urea–12% polyacrylamide gels.

Isolation of tRNA Fragments. Limited digestions of FUra-tRNA were performed in deionized formamide at 100 °C for 2 min (Stanley & Vassilenko, 1978). After spin evaporation and 5′-^{32}P-labeling using polynucleotide kinase and [γ-^{32}P]ATP, the ^{32}P-labeled fragments were purified by 7 M urea–15% PAGE, excised, eluted, and then purified again on 4 M urea–20% PAGE. For analysis, the labeled fragments were hydrolyzed with nuclease P_1 and the 5′-^{32}P-labeled nucleotides were identified by tlc on cellulose plates (Kodak).

Determination of Ribothymidine. Oligoribonucleotides (100 pmol) were methylated with RUMT (25 pmol) and excess AdoMet (10 nmol) in 5 μL of the standard buffer for the RUMT assay for 3 h at 15 °C (Santi & Hardy, 1987). The methylated product was hydrolyzed with RNase T_2, labeled with [γ-^{32}P]ATP and polynucleotide kinase, and digested with nuclease P_1; the 5′-mononucleotides were analyzed by two-dimensional tlc.

Gel Shift Assays. Gel shift assays of RUMT–FUra-tRNA complexes on SDS–PAGE were performed as described (Santi & Hardy, 1987). Typically, reaction solutions (40 μL) containing the 5′-^{32}P-labeled fragment (ca. 2 × 10^4 cpm), 1 μM RUMT, and 250 μM AdoMet in the binding buffer were incubated at 15 °C for 3 h. A 20-μL aliquot was mixed with an equal volume 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), incubated at 95 °C for 3 min, and analyzed by SDS–12% PAGE. The remaining 20 μL was incubated with 1 μL of 1 μg/μL RNAse A (Boehringer Mannheim) at 37 °C for 1 h, mixed and heated with an equal volume of 2× loading buffer, and analyzed by SDS–PAGE.

Enzyme Assays. Assay reactions (60 μL) contained varying concentrations of oligoribonucleotide or tRNA, 50 μM [*methyl*-^{3}H]AdoMet (2 Ci/mmol), and 0.1 μM RUMT in the standard 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) were incubated at 15 °C (Santi & Hardy, 1987). Aliquots (18 μL) were removed at 1, 2, and 30 min and allowed to permeate DEAE-cellulose filter disks, and the disks were washed five times with 2 mL of cold 50 mM glycine hydrochloride (pH 2.3) and then two times with 2 mL of cold ethanol The filters were dried and counted under Aquasol II (New England Nuclear). Activity measurements were made during initial velocity, usually at 2 min. The lower limit of detection of methylation (control levels) was estimated to be about 0.01 pmol. Kinetic parameters were assessed by nonlinear least-squares fit of the data to the Michaelis–Menten equation and the data displayed as double-reciprocal plots.

Results and Discussion

In our initial approach, we wished to determine whether fragments of degraded FUra-tRNA retained the ability to form covalent RUMT–RNA complexes. The work began when we attempted to analyze the RUMT–FUra-$tRNA_1^{Val}$ complex by using 5′-end-labeled FUra-tRNA that was prepared by in vitro transcription. The calf intestinal phosphatase preparation used to remove the 5′ triphosphate of the FUra-tRNA was contaminated with a RNase. After 5′-labeling with polynucleotide kinase and [γ-^{32}P]ATP, [5′-^{32}P]tRNA and about 20 radioactive RNA fragments of varying size were resolved on 7 M urea–20% PAGE. Six of these fragments that varied from 15 to 40 nucleotides were arbitrarily chosen, eluted, incubated with excess RUMT and AdoMet, and tested for the formation of covalent complexes by the SDS–PAGE gel shift assay followed by autoradiography. Surprisingly, most of the fragments chosen, including one of 15 nucleotides in length, caused a shift in RUMT mobility to about 48–55 kDa on SDS–PAGE. Also, the complex formed with the 15-nucleotide fragment could be converted to a 42-kDa band by RNase A treatment prior to SDS–PAGE. We concluded that fragments of FUra-tRNA as small as 15 nucleotides could form covalent complexes with RUMT.

We next designed an experiment to define the minimum linear sequence of FUra-tRNA extending from the 3′ end that would bind to RUMT. In vitro transcribed FUra-$tRNA_1^{Val}$ was subjected to limited partial digestion in deionized formamide (100 °C for 2 min) to cause approximately one cleavage per molecule, and the free 5′ends were labeled with [γ-^{32}P]-ATP and polynucleotide kinase (Stanley & Vassilenko, 1978); this produced a series of 5′-labeled fragments extending from

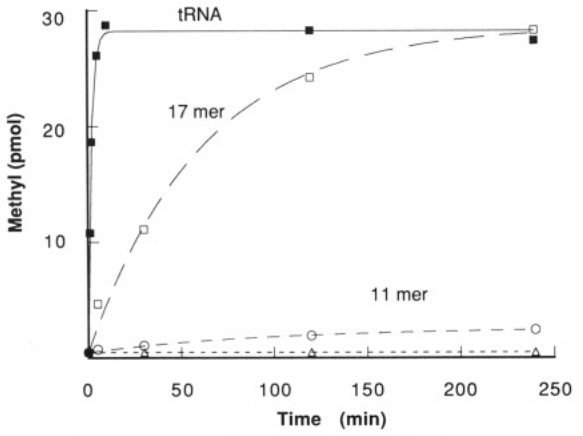

FIGURE 1: Methylation of in vitro transcribed tRNA^{Phe} (■), 17mer T-arm (□), 11mer T-arm (○), and U54C 17mer T-arm (△, nonsubstrate) derivatives. Assays were as described under Materials and Methods with 1.5 μM RNA, 25 μM [*methyl*-^{3}H]AdoMet, and 0.2 μM RUMT in 100 μL; the control omitted RNA and was subtracted from data points. Twenty-microliter aliquots were removed and assayed.

various 5′ nucleotide ends to the 3′ acceptor terminus. Radioactive fragments of length 21–28 nucleotides were purified by 7 M urea–15% PAGE and then 4 M urea–20% PAGE, and their identities were verified by 5′-end-group analysis (Silberklang, et al., 1979). The analysis demonstrated high purity of each of the radioactive fragments, with only minor contamination. The isolated fragments were then tested for covalent binding to RUMT in the presence of AdoMet by the SDS–PAGE gel shift assay. We found that fragments containing 25 or more nucleotides from the 3′ end formed covalent complexes with RUMT, but fragments containing 24 nucleotides or less from the 3′ end did not. Thus, the recognition sequence is contained within nucleotides from position 52 to the 3′ end A76 and contains the methylation site at residue 54; the first two residues of the putative binding sequence, G52 and G53, are involved in forming two complementary base pairs of the T-stem of tRNA adjacent to the loop. This result, together with the aforementioned finding that a sequence of only 15 nucleotides can bind to RUMT, indicates that the essential binding sequence exists within the T-arm (G52–A66) of FUra-tRNA$_1^{Val}$.

We next prepared a 17-base oligoribonucleotide corresponding to the T-arm of tRNA$_1^{Val}$ (nucleotides 49–65 of tRNA$_1^{Val}$; 5′-pppGGCGGUUCGAUCCCGUC) and tested it as a substrate for RUMT. The enzyme catalyzed the transfer of the methyl group of [*methyl*-^{3}H]AdoMet to this oligoribonucleotide (Figure 1); the 17mer T-arm U54C mutant, which is not a substrate, is also shown. The product of a reaction taken to completion was hydrolyzed with RNase T$_2$, labeled with [γ-^{32}P]ATP and polynucleotide kinase, digested with nuclease P$_1$, and analyzed by two-dimensional tlc. The spots were excised and counted, and ribothymidine was shown to be present in an amount that corresponds within experimental error to stoichiometric reaction at U54 (calculated, A/U/T = 1/3/1; found, A/U/T = 1.1/3.4/1.0). The T_m of the 17mer was determined to be 45 °C, so it is reasonable to conclude that the stem–loop structure binds to the enzyme under the assay conditions.

Kinetic properties of the T-arm 17mer were measured at pH 8.4 and 15 °C with 50 μM [*methyl*-^{3}H]AdoMet (Table I); a low assay temperature was used to ensure predominance of hairpin structures. Under these conditions, K_m for the T-arm was about 6-fold higher and k_{cat} was about 3-fold lower than for unmodified yeast tRNA^{Phe}. The k_{cat}/K_m is 17-fold

Table I: Kinetic Parameters for Methylation of RNAs with RUMT[a]

substrate	K_m	k_{cat}	k_{cat}/K_m
tRNA^{Phe}	0.8	5.5	6.9
17mer T-arm	5.0	2.0	0.40
11mer T-arm	4.0	0.090	0.023

[a] Reactions were performed as described under Materials and Methods.

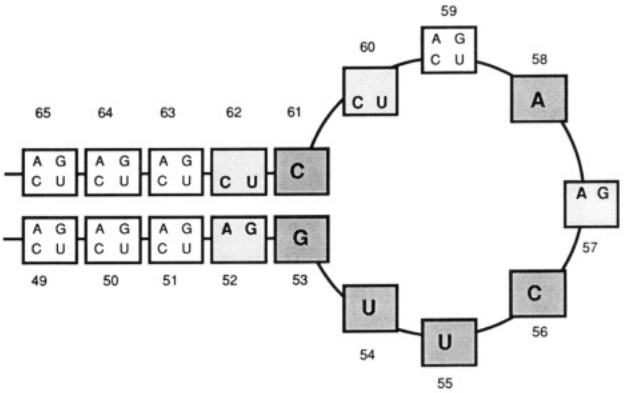

FIGURE 2: Consensus sequence of T-arm of tRNA derived from the structures of *E. coli* tRNAs. Dark shading indicates complete conservation of base, light shading indicates conservation of Py or Pu, and no shading indicates nonconserved positions.

lower for the T-arm (0.40 μM^{-1} min^{-1}) than for tRNA^{Phe} (6.9 μM^{-1} min^{-1}), indicating a higher specificity for tRNA than for the T-arm alone. These results show that other aspects of the structure of tRNA may play a role in interactions with RUMT, but the primary specificity determinants are contained within the primary and secondary structure of the T-arm.

The T-arm could be further truncated to 11 bases consisting of only the loop and two adjacent base pairs of the stem (nucleotides 52–62 of tRNA$_1^{Val}$; 5′-pppGGUUCGAUCCC) and still retain activity (Figure 1). For the 11mer, the K_m was about the same as the 17mer, but k_{cat} and k_{cat}/K_m values were decreased about 20-fold. We determined that the T_m of the 11mer was 38 °C and concluded that the stem–loop is the predominant secondary structure at the temperature used for enzyme assays. Substrate activity of the 11mer is in accord with aforementioned results from covalent binding of FUra-tRNA fragments to RUMT, which indicated a requirement for G52 on the 5′ end of fragments containing the 3′ acceptor end. Thus far, the 11mer is the smallest sequence we have found that is methylated and the smallest known oligoribonucleotide that is a substrate for a RNA modifying enzyme; smaller fragments have not yet been tested because of difficulties in preparation (Milligan et al., 1987).

Having shown that the determinant for recognition is present within the T-arm of tRNA, we compared the sequences of over 40 tRNAs from *E. coli* that are substrates for the methyltransferase (Sprinzl et al., 1987). This allowed us to deduce a consensus sequence for recognition and catalysis by RUMT (Figure 2). We reasoned that the nonconserved residues of the T-arm of tRNA pobably do not provide information regarding specificity, unless some unrecognized feature of their context was involved. By this rationale, and the observed substrate activity of the 11mer, we excluded the first three nonconserved base pairs of the stem and the nonconserved base at position 59 as being important in recognition; of course, these could play roles in higher order structure important for binding that are base-independent, such as the helical hairpin structure and conformation of the loop. Thus, the consensus sequence for methylation derived from tRNA homology and the result reported here includes only 11 bases: the Pu52-Py62

and G53-C61 base pairs adjacent to the loop; U54, U55, C56, and A58, which are completely conserved components of the loop; and Pu57 and Py60 as semiconserved bases in the loop. This sequence may contain elements necessary for tRNA function other than methylation, so the consensus sequence for methylation may be less stringent.

Recently, it was reported that tRNA analogues comprising short helical hairpins that resemble the acceptor stem and a T-arm (minihelix) or the acceptor stem and a 6–7-base loop (microhelix) of $tRNA^{Ala}$ or $tRNA^{His}$ are charged in vitro by *E. coli* Ala- or His-tRNA synthetase (Francklyn & Schimmel, 1989, 1990; Shi et al., 1990). The finding that the 17 base T-arm of tRNA, and analogues containing as little as 11 bases, can serve as a substrate for RUMT represents another example of a truncated tRNA that serves as a substrate for an enzyme that uses tRNA as substrate. The small size of the oligoribonucleotide substrates of RUMT will greatly facilitate detailed studies of the enzymatic reaction.

It is intriguing to speculate that the consensus sequence described here might also provide the specificity required for other modifications that occur on the T loop, such as formation of pseudouridine at position 55 and 1-Me-A at position 58. Also, the T-arm consensus sequence for methylation of *E. coli* tRNA is found in all eukaryotic tRNAs that contain ribothymidine, and it is reasonable to suggest that the eukaryotic counterpart of RUMT has a similar specificity. A search of the Genbank data base for RNA sequences other than tRNA revealed several RNAs that contain the aforementioned 11-base T-arm consensus sequence for methylation (Figure 2). In the bacterial sublibrary, three such matches were seen for *E. coli*; intriguingly, one was in the coding sequence for EF-G (*fus*) and the other two were in the coding sequences for EF-Tu (*tufA* and *tufB*). It is interesting and possibly relevant that RUMT may be tightly bound to one or more RNAs in crude extracts of *E. coli* (Ny et al., 1988). In the structural sublibrary, no *E. coli* non-tRNA matches were found. In the eukaryotic case, many matches were found in non-tRNA sequences known to be expressed as RNA, including rRNA (e.g., a conserved sequence in 18S RNA), other noncoding cellular RNAs [e.g., 7SL RNA (*Schizosaccharomyces pombe*) and 4.5S RNA (mouse)], viral small RNAs [e.g., VAI (adenovirus)], and mRNAs for cellular (e.g., glutathione peroxidase and myoglobin) and viral (e.g., HSV-1 DNA polymerase) proteins. RNA pol III transcripts in particular were frequent sources of matches to the consensus, which may be attributed to the fact that the consensus sequence in the tRNA is in a region that in the DNA is an internal RNA pol III promoter. Ribothymidine would have gone undetected in most of these, since the sequences were deduced from DNA sequencing. Provided the appropriate secondary structure is adopted, modification of such sequences could occur, serving a thus far unrecognized function for tRNA modification enzymes.

ACKNOWLEDGMENTS

We thank Ralph Reid for his data base search and thoughtful suggestions and Kathryn Ivanetich for comments on the manuscript.

REFERENCES

Bjork, G. R., & Neidhardt, F. C. (1975) *J. Bacteriol. 124*, 99–111.

Chu, W., & Horowitz, J. (1989) *Nucleic Acids Res. 17*, 7241–7252.

Davenloo, P., Sprinzl, M., Watanabe, K., Albani, M., & Kerstin, H. (1979) *Nucleic Acids Res. 6*, 1571–1581.

Francklyn, C., & Schimmel, P. (1989) *Nature 337*, 478-481.

Grodberg, J., & Dann, J. J. (1988) *J. Bacteriol. 170*, 1245-1253.

Gu, X., & Santi, D. (1990) *DNA Cell Biol. 9*, 273-278.

Kersten, H., Albani, M., Mannlein, E., Praisler, R., Wurmbach, P., & Nirhaus, K. (1981) *Eur. J. Biochem. 114*, 451-456.

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

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

Ogden, R., & Adams, D. (1987) *Methods Enzymol. 152*, 61.

Santi, D. V., & Danenberg, P. V. (1984) in *Folates and Pterins* (Blakely, R. L., & Benkovic, S. J., Eds.) pp 343-396, Wiley, New York.

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

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.

Stanley, J., & Vassilenko, S. (1978) *Nature 274*, 87-89.

Wu, J. C., & Santi, D. V. (1987) *J. Biol. Chem. 262*, 4778–4786.