The Specificity of Transfer Ribonucleic Acid Methylases from Rat Liver*

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ABSTRACT: Transfer ribonucleic acid (tRNA) methylating enzymes were isolated in high-speed supernatants from rat liver extracts and partially purified by DEAE-cellulose chromatography. The purified enzyme methylated guanine in the 2*N* position and adenine in the 1 position using *Escherichia coli* methyl-deficient tRNA as substrate and *S*-adenosylmethionine as the methyl donor. The tRNA could be methylated to an extent of more than 0.5 methylated base of each type per molecule. The question of whether the methylase recognizes a base by virtue of its surrounding nucleotide sequence was examined by fractionation of a pancreatic ribonuclease digest of ^{14}C-methylated tRNA. Almost all of the radioactivity was found in two sequences, one of which was identified as adenylyl-(3′,5′)-2*N*-methylguanylyl-(3′,5′)-cytidylic 3′-acid and the other as an oligonucleotide containing methyladenine in a nonterminal position.

Transfer ribonucleic acid contains, besides the four major nucleotides, a number of minor nucleotides, many of which are methylated derivatives of the major ones (Hall, 1965). A group of enzymes, called methylases, has been found to transfer the methyl group from *S*-adenosylmethionine to accepting positions in tRNA (Fleissner and Borek, 1962). Methylation may therefore be studied *in vitro*, using as substrate for the methylases either submethylated tRNA, usually prepared from a methionine-starved culture of a particular *Escherichia coli* mutant (Mandel and Borek, 1963), or tRNA from a heterologous organism (Srinivasan and Borek, 1963).

The function of these modifications of tRNA is unknown. However, since only 3–6% of the bases in the tRNA molecule can be methylated, the methylases recognize only a few specific accepting sites. The mechanism by which the bases are recognized has not yet been elucidated.

In the present communication, we report the purification from rat liver of an enzyme preparation containing 2*N*-guanine methylase and 1-adenine methylase activity. *E. coli* tRNA methylated with this enzyme preparation was hydrolyzed with pancreatic RNase, and only two major sequences were found to be methylated. The results suggest a strict sequence specificity for these enzymes.[1]

Materials and Methods

Preparation of tRNA. E. coli K-12 met$^-$ RC^{rel}, kindly provided by Dr. F. Gros (Institut de Biologie Physico-Chimique, Paris) was grown in minimal medium containing 0.0003% L-methionine to stationary phase in order to produce methyl-deficient tRNA (Hurwitz *et al.*, 1964). The authors are grateful to Dr. J. Gruner for the growth of the bacteria. tRNA was isolated as described by Zubay (1962) except that the pH 10.3 incubation was omitted.

Preparation of Rat Liver Enzyme. Adult Fisher rats were killed, the livers were excised, either used immediately or frozen in liquid nitrogen, and stored at −15°. Liver (6 g) was cut into small pieces and homogenized in four volumes of cold 0.25 M sucrose containing 10 mM Tris-Cl (pH 8.0) and 1 mM dithiothreitol, using a motor-driven glass–Teflon homogenizer. The extract was centrifuged successively at 5000*g* for 5 min, 30,000*g* for 10 min, and 105,000*g* for 60 min to remove, in turn, nuclei and cell fragments, mitochondria, and microsomes. The high-speed supernatant was used for study.

DEAE-cellulose (Serva Entwicklungslabor, Heidelberg, capacity 0.49 mequiv/g) was packed in a column (2 × 10 cm) and equilibrated with Tris buffer (50 mM Tris-Cl (pH 8.0) containing 0.1 mM EDTA and 0.1 mM dithiothreitol). The high-speed supernatant (24 ml) was applied to the column, which was then washed at 0° with Tris buffer (70 ml) followed by a 0–0.3 M sodium chloride gradient in Tris buffer (400 ml). Fractions (12 ml) were collected, the absorbance at 280 mμ was measured, and aliquots were assayed for RNA methylating activity.

The eight column fractions of highest activity were pooled and dialyzed against three volumes of 30% polyethylene glycol containing 1 mM EDTA and 0.1 mM dithiothreitol. When the volume was reduced to

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[1] Abbreviations used: mononucleotides: Ap, Gp, Cp, Up, 1MeAp, 2MeGp: adenylic, guanylic, cytidylic, uridylic, 1-methyladenylic, 2-methylguanylic 3′-acids, respectively; oligonucleotides: *e.g.*, ApGpCp: adenylyl-(3′,5′)-guanylyl-(3′,5′)-cytidylic 3′-acid.

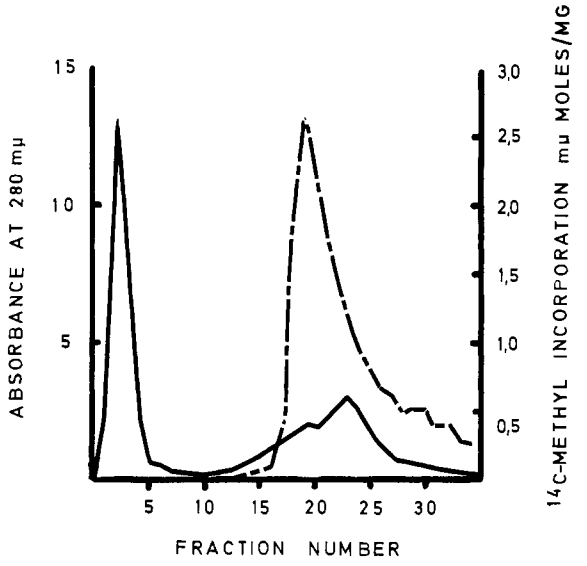

FIGURE 1: Fractionation of a high-speed supernatant from rat liver on a DEAE-cellulose column. The column was first washed with 0.05 M Tris-Cl (pH 8.0) and then with a sodium chloride gradient commencing at fraction 11. Fractions (12 ml) were collected and assayed for tRNA methylating activity using 50 μg of tRNA and 0.2 ml of enzyme solution per 0.3-ml assay. Calculated yield of activity in the column was about 80%. Fractions 18–25 were concentrated and used as the purified enzyme. Optical density at 280 mμ (———); [^{14}C]methyl incorporation per assay (–·–·–).

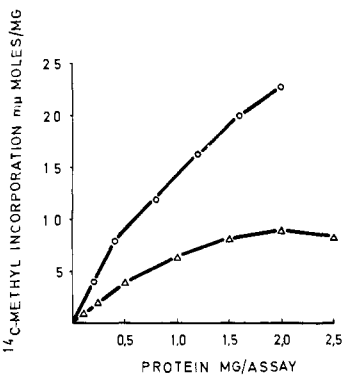

FIGURE 2: Dependence of [^{14}C]methyl incorporation on amount of enzyme protein in assay mixture. Assay tubes contained 10 μg of tRNA in 0.3 ml and were incubated for 60 min at 37°. Curve for supernatant enzyme (△—△); curve for purified enzyme (○—○). Results are corrected for enzyme blank values (20% of total counts for crude enzyme; 10% for purified enzyme).

about 1 ml, the dialysis sac was dialyzed for 3 hr against Tris buffer. The final enzyme was diluted to a protein concentration of 10 mg/ml and made 1 mM in dithiothreitol. It was stored at 0° for no more than 3 days before being used, and lost activity at the rate of 10%/day. Protein estimations were made according to Warburg and Christian (1941).

Assay of RNA Methylating Capacity. The conditions used were similar to those of Rodeh *et al.* (1967). [^{14}C]Methyl-*S*-adenosylmethionine (International Chemical and Nuclear Corp., 40 mc/mmole) was used at a concentration of 10 μM. *E. coli* methyl-deficient tRNA was used at concentrations of 10–100 μg/0.3 ml of assay. Following incubation at pH 9 and at 37°, assay tubes were cooled and 1 ml of cold 2 N HCl was added. The precipitates were collected on glass fiber filter disks, washed with a total of 20 ml of 0.01 N HCl, and dried at 80°. The filters were placed in toluene containing 0.6% 2-(4′-*t*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole and counted at an efficiency of 70% in a Packard Tri-Carb liquid scintillation spectrometer.

Methylated Nucleotide Analysis. tRNA was isolated from the reaction mixture, after addition of further tRNA to a concentration of at least 300 μg/ml, by deproteinizing with an equal volume of water-saturated phenol. Following centrifugation, the aqueous phase was collected and the RNA was precipitated by addition of one-tenth volume of 20% potassium acetate (pH 5) and two volumes of ethanol at 0°. The precipitate was collected by centrifugation, dissolved in 2% potassium acetate, reprecipitated with ethanol, and washed twice with ethanol and twice with ether.

Such isolated RNA (200–400 μg) was digested with 0.3 N NaOH at 37° for 18 hr. The alkali was removed by chromatography on carboxymethylcellulose paper. The nucleotides were chromatographed on thin layer cellulose plates in 1-propanol–NH_3–H_2O (60:30:10) (Hanes and Isherwood, 1949) in the first dimension, and in isopropyl alcohol–HCl–H_2O (68:17.6:14.4) (Wyatt, 1951) in the second dimension.

Autoradiographs were made of the plates with Gevaert X-ray film. The positions of the radioactive spots were compared to those of the ultraviolet light absorbing major nucleotides. The radioactive spots were eluted and further identified following conversion to the corresponding nucleosides by bacterial alkaline phosphatase (Worthington Biochemical Corp., Freehold N. J.) (25 μg of enzyme/0.1 ml, 0.05 M ammonium carbonate (pH 9.2), 1 mM $MgCl_2$, 2 hr at 37°) and to the corresponding bases by acid treatment (1 N HCl, 100°, 60 min).

Oligonucleotide Analysis. ^{14}C-Methylated RNA was hydrolyzed with pancreatic RNase (Boehringer, Mannheim) and the digest was fractionated on a DEAE-cellulose column (1 × 50 cm) according to the method of Staehelin (1961). Electrophoresis of oligonucleotides was carried out in 0.1 M ammonium formate (pH 2.5) containing 1 mM EDTA, using a water-cooled thin layer electrophoresis apparatus and a potential of 50 v/cm (Bergquist, 1965).

Oligonucleotides were analyzed by digestion with T_1 ribonuclease (Sankyo Biochemical Co., Tokyo, Japan) (20 μg of enzyme/0.1 ml, 0.02 M ammonium

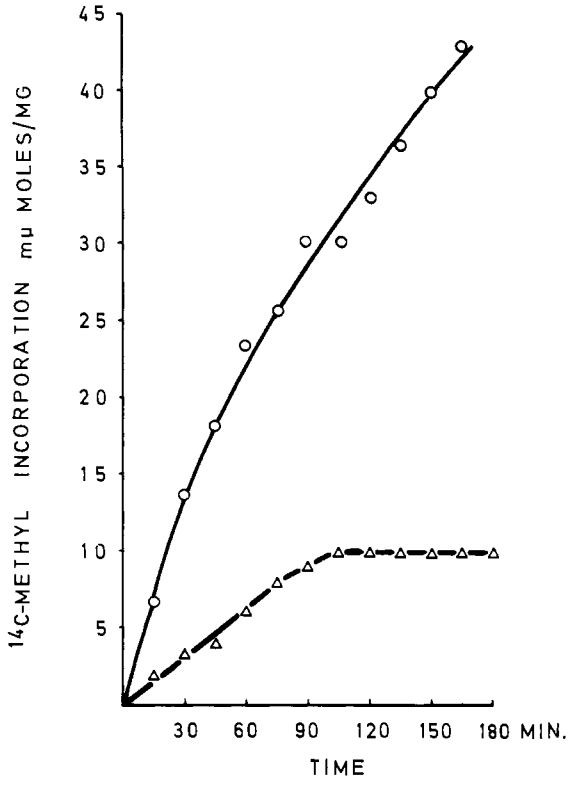

FIGURE 3: Dependence of [^{14}C]methyl incorporation on time. Assay tubes contained 33 μg of tRNA/ml. Curve for supernatant enzyme at concentration of 10 mg/ml (△—△). Curve for purified enzyme at concentration of 6.6 mg/ml (○—○). Results are corrected for enzyme blank values.

formate (pH 7.5), 2 hr at 37°). Dephosphorylated oligonucleotides were analyzed by digestion with snake venom phosphodiesterase (Worthington Biochemical Corp., Freehold, N. J.) (Staehelin, 1961) (25 μg of enzyme/0.1 ml, 0.1 M ammonium carbonate (pH 9.2), 5 mM $MgCl_2$, 1.5 hr at 37°). Products were analyzed by electrophoresis.

Results

Properties of the tRNA Methylating Enzymes. Homogenates of rat livers were divided by centrifugation into fractions containing nuclei, mitochondria, microsomes, and supernatant proteins. Suspension of the pellets in 0.01 M Tris buffer (pH 8.0) were compared with the high-speed supernatant fraction for RNA methylase activity. Activity (85%) was found in the supernatant fraction, and 10–12% was found in the nuclear suspension. The finding confirmed that of others (Rodeh *et al.*, 1967; Zeleznick, 1967; Simon *et al.*, 1967; Tsutsui *et al.*, 1966) that the bulk of the tRNA methylating activity is in the cytoplasm of mammalian cells. It was also found that prior rapid freezing and storage of rat livers did not quantitatively affect the level of methylase activity of the extracts.

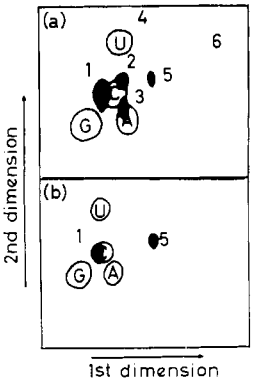

FIGURE 4: Analysis of nucleotide products of tRNA. (a) tRNA was methylated to a level of 6 mμmoles/mg using crude liver enzyme. The tRNA was isolated, hydrolyzed with alkali, and chromatographed in two dimensions. The thin-layer plate, containing about 20,000 cpm of radioactivity, was exposed to X-ray film for 5 days. Positions of marker nucleotides are also indicated. (b) tRNA was methylated to a level of 18 mμmoles/mg using DEAE-purified liver enzyme, then treated as above.

Since rat liver enzymes are capable of methylating different bases of tRNA, fractionation of these enzymes is advantageous to their study. A three- to fivefold purification of enzyme specific activity was achieved by DEAE-cellulose chromatography of the high-speed supernatant (Figure 1). Column fractions were assayed directly; the presence of sodium chloride in the assay mixture inhibited the reaction rate by about 25%. The eight fractions containing the highest activity (fractions 18–25) were concentrated by dialysis against polyethylene glycol. This DEAE-purified enzyme was compared to the unfractionated enzyme in its methylating capacity at different protein concentrations (Figure 2), in its time course of methylation at high protein concentration (Figure 3), and in its specificity for base methylation (Figure 4).

Both the protein concentration curve and the time curve for the unpurified enzyme show a plateau of about 9 mμmoles of methyl group/mg of tRNA. This corresponds to an incorporation of about 0.25 methyl group/tRNA chain. That this plateau is not due to saturation of the tRNA is demonstrated by the corresponding curves for the DEAE-purified enzyme, which methylated tRNA to levels of about one methyl group per molecule (Figure 3). Using DEAE-purified enzyme, it was found that, on a basis of reaction rate, normally methylated *E. coli* tRNA, yeast tRNA, and rat liver tRNA were, respectively, 80, 6, and 0% as effective as methyl-deficient tRNA in accepting methyl groups.

Products of Methylation. When tRNA, methylated to a level of 6 mμmoles/mg using unfractionated liver enzyme, was isolated, alkali hydrolyzed, and chromatographed in two dimensions, six radioactive compounds

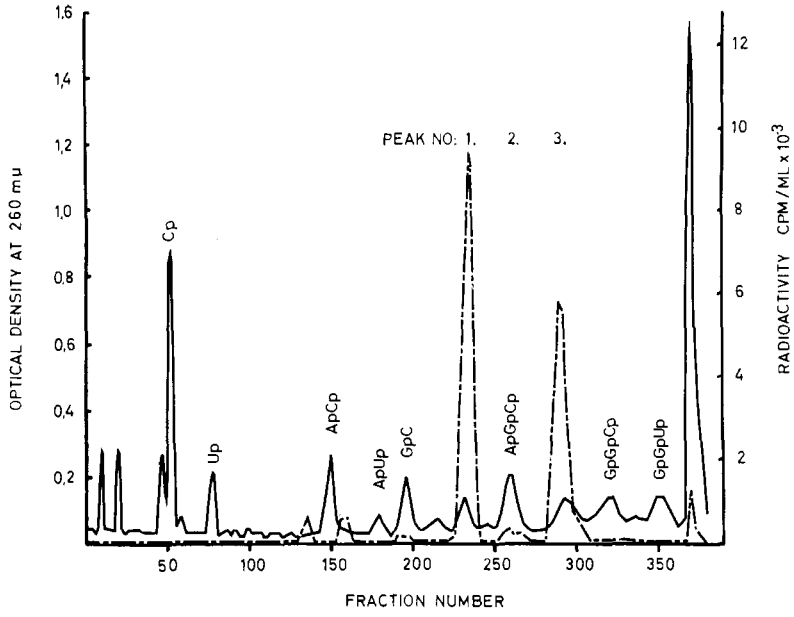

FIGURE 5: DEAE-cellulose chromatography of a pancreatic RNase digest of tRNA (8 mg) previously methylated with DEAE-purified liver enzyme. The incubation mixture for methylation contained enzyme protein (2 mg/ml), tRNA (333 μg/ml), and *S*-adenosylmethionine of specific activity 10,000 cpm/mμmole. The column was eluted with a linear gradient of triethylammonium bicarbonate (pH 8.6) from 0.02 to 0.3 M. At fraction 365 the column was eluted with 0.8 M buffer. Aliquots from each 6-ml fraction were dried in scintillation vials which were then filled with toluene scintillation fluid and their radioactivity was measured. The positions of some of the oligonucleotides and the radioactive peaks are indicated in the figure. Optical density at 260 mμ (———); radioactivity (-·-·-).

were detected by autoradiography (Figure 4a). Spot 1 was identified as 2*N*-methylguanylic acid since after conversion to the nucleoside by alkaline phosphatase it cochromatographed in two dimensions with authentic 2*N*-methylguanosine isolated from yeast tRNA (Staehelin, 1964) and was also distinguishable from the alkaline degradation product of 7-methylguanosine and from 2*N*-dimethylguanosine. Spot 3 was identified in a similar fashion as 1-methylguanylic acid, possibly containing some dimethylguanylic acid. Spot 5 was identified as 6-methyladenylic acid. The radioactive nucleotide obtained after enzymatic digestion with snake venom phosphodiesterase chromatographed in isopropyl alcohol–HCl behind adenylic acid and with synthetic 1-methyladenylic acid (kindly provided by Dr. W. Rittel), whereas the nucleotide obtained after alkaline digestion chromatographed with synthetic 6-methyladenylic acid and ahead of adenylic acid. It was concluded that spot 5 was derived from 1-methyladenylic acid by an alkali-catalyzed rearrangement (Brookes and Lawley, 1960). Spots 2 and 4 corresponded in position to 5-methylcytidylic acid and ribothymidylic acid. Spot 6 was unidentified, but could correspond to an alkali degradation product of *S*-adenosylmethionine which had not been completely removed from the tRNA during isolation.

The analysis of the radioactive products confirms the identifications made by Rodeh *et al.* (1967) and Zeleznick (1967). The analysis of individual DEAE column fractions from 15 to 26 showed that 1-guanine methylase activity was located in fractions 15–17, 1-adenine methylase activity in fractions 18–21, and 2*N*-guanine methylase activity in fractions 17–26. When tRNA, methylated to levels of 18–44 mμmoles/mg, using DEAE-purified enzyme, was analyzed for methylated nucleotides, the autoradiographs showed 2*N*-methylguanylic acid and 6-methyladenylic acid in approximately equal amounts, with only traces of others (Figure 4b).

Oligonucleotide Analysis of Methylated RNA. tRNA (8 mg), methylated to an extent of 18 mμmoles/mg with [^{14}C]methyl-*S*-adenosylmethionine using DEAE-purified enzyme, was digested with pancreatic RNase and chromatographed on a DEAE-cellulose column (Figure 5) to fractionate the oligonucleotides (Staehelin, 1961). A portion of the digest was also fractionated by electrophoresis (Figure 6a). Practically all of the radioactivity chromatographed on the DEAE column in the trinucleotide region in two main peaks and one small one (Figure 5, peaks 1–3). The pattern was further simplified by the finding that the first of these peaks contained 2*N*-methylguanylic acid and the second two peaks contained 1-methyladenylic acid as the only radioactive nucleotides. On electrophoresis at pH 2.5, the material from the first peak moved close to the trinucleotide ApGpCp (Figure 6b). Digestion with T_1 ribonuclease gave a product which moved with ApGp on electrophoresis. After dephosphorylation of a

portion of the starting material with alkaline phosphatase, venom diesterase released a radioactive component which moved as guanylic acid on electrophoresis, indicating that the radioactive nucleotide was not at the 5′-terminal end of the sequence. The probable sequence of the trinucleotide is therefore Ap2MeGpCp.

Material from the second peak gave as the major component on electrophoresis a band moving close to ApApUp (Figure 6c). Venom diesterase treatment, as above, indicated that the methylated adenylic acid was not at the 5′-terminal end. Material from the third peak moved on electrophoresis at pH 2.5 just behind GpApUp (Figure 6d). T_1 RNAse digestion gave a radioactive product migrating close behind ApUp. Venom diesterase treatment of the starting material indicated that the methylated adenylic acid was not 5′ terminal.

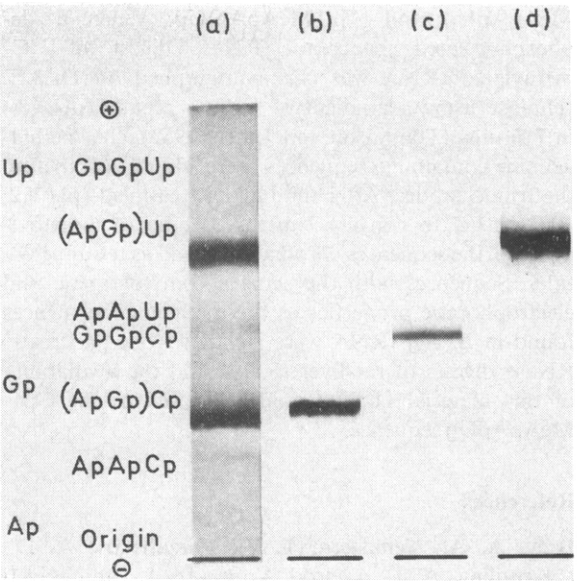

FIGURE 6: Autoradiograms of electrophoresed radioactive oligonucleotides. (a) Portion of pancreatic digest applied to the column in Figure 5 (b–d). Portions of components 1–3, respectively, obtained from the column in Figure 5. Electrophoresis at pH 2.5 and at 50 v/cm for 45 min (current about 10 ma). Positions of marker mono- and trinucleotides are indicated at the left.

Discussion

Enzymes capable of methylating in *E. coli* tRNA guanine at the 2*N* position and adenine at the 1 position have been isolated from rat liver homogenates. The highest extent of methylation of *E. coli* methyl-deficient tRNA obtained corresponds to about 0.5 group of each methylated base per molecule (Figure 3). This is not necessarily the highest extent because a plateau value was not obtained. The apparent plateau obtained both in enzyme saturation curves and time curves (Figures 2 and 3) using unpurified enzyme illustrates the caution which must be exercised in the determination of the maximum extent of methylation of tRNA.

Since similar results were obtained using normally methylated *E. coli* tRNA, rat liver enzymes must methylate bases in *E. coli* tRNA not recognized by *E. coli* methylases. It is interesting to compare this result with determinations of methylated base content in *E. coli* and rat liver tRNA. 1-Methylguanine, 2*N*-methylguanine, 2*N*-dimethylguanine, and 1-methyladenine occur in rat liver and yeast tRNA at levels of 0.5–1.7%, but occur in *E. coli* tRNA in much smaller amounts (Dunn, 1959, 1961; Staehelin, 1966; Dubin and Günalp, 1967). The reverse is true for 2-methyladenine, 6-methyladenine, and 7-methylguanine, which occur in *E. coli* tRNA at levels of 0.2–0.6% (Dunn, 1961; Dubin and Günalp, 1967).

RNA methylases from *E. coli* were fractionated and studied by Hurwitz and coworkers (1964), who showed that one or more specific enzymes were required for the formation of each methylated base. However, the question as to whether bases of the tRNA molecule were susceptible to methylation by virtue of their surrounding sequence, or by the shape of the tRNA molecule, was not answered.

The analysis of a T_1 RNase digest of ^{14}C-methylated *E. coli* tRNA by Littauer *et al.* (1963) revealed no simple pattern of sequence specificity. In contrast, the results reported here demonstrate a very strict sequence specificity when methylated tRNA is analyzed following pancreatic RNase digestion. This sequence specificity of methylases parallels the observations of commonly occurring methylated base containing sequences in tRNA (Staehelin, 1964; Zamir *et al.*, 1965). The determination of complete primary structures for several individual yeast tRNA species has not only demonstrated methylated base containing sequences similar to those reported here, but has shown that they are located in specific positions in the tRNA molecules. Yeast tyrosyl- and phenylalanyl-tRNA contain the sequence Ap2MeGpCp in the region between the 5′-terminal region and the dihydrouracil-containing loop (Madison *et al.*, 1966; RajBhandary *et al.*, 1967). Yeast phenylalanyl- and valyl-tRNA contain the sequence Gp1MeApUp in the thymine-containing loop (RajBhandary *et al.*, 1967; Baev *et al.*, 1966).

In conclusion, the present results indicate that there exists in *E. coli* tRNA two sequences which can be specifically methylated by 2*N*-guanine methylase and 1-adenine methylase, respectively, from rat liver. The degree of methylation of the tRNA shows that these accepting sequences occur at the level of at least 0.4–0.5/tRNA molecule. It is therefore possible that both of these accepting sequences are commonly occurring structural elements in many different tRNA molecules. The results also suggest that each tRNA methylase recognizes a specific sequence in many different tRNA species.

Added in Proof

The 1-methyladenine-containing sequences were subsequently identified as the tetranucleotides Gp1-

MeApApUp and Ap(1MeApAp)Up. When a dephosphorylated, pancreatic RNase digest of ^{14}C-methylated tRNA was chromatographed on DEAE-cellulose using a gradient of sodium acetate (pH 7.0) in 7 M urea (Tomlinson and Tener, 1963), the methyladenine-containing sequences were eluted just before the trinucleotides. After mild alkali treatment (pH 9.2, 100°, 1 hr) to convert 1-methyladenine to 6-methyladenine, the sequences chromatographed as tetranucleotides. Sequences with the same chromatographic and electrophoretic properties as the methylated sequences found in *E. coli* tRNA were isolated from pancreatic RNase digests of rat liver tRNA, and the availability of this material facilitated identification of the Gp1-MeApApUp sequence.

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