Methylation of *Escherichia coli* Transfer Ribonucleic Acids by Adenylate Residue-Specific Transfer Ribonucleic Acid Methylase from Rat Liver†

Yoshiyuki Kuchino and Susumu Nishimura*

ABSTRACT: To study recognition by tRNA methylase, adenylate residue-specific tRNA methylase was partially purified from the high-speed supernatant of rat liver by treatment at pH 5 and then DEAE-cellulose column chromatography. This preparation was free from other nucleotide-specific tRNA methylase. The rate of incorporation of the [^{14}C]methyl group into tRNA from *S*-adenosyl-L-methionine was assayed using numerous purified *Escherichia coli* tRNAs. *E. coli* $tRNA^{Ser}_3$, $tRNA^{Glu}_2$, $tRNA^{Met}$, $tRNA^{Thr}$, $tRNA^{His}_1$, $tRNA^{Tyr}_2$, and $tRNA^{Ser}_1$ were found to be good acceptor molecules, while $tRNA^{Ile}$, $tRNA^{Leu}_1$, $tRNA^{fMet}$, $tRNA^{Val}_1$, and $tRNA^{Gly}$ were not well methylated. The location of the 1-methyladenylate residue in $tRNA^{Ser}_3$ and $tRNA^{His}_1$ was determined as position 19 from the 3′-OH end. The common nucleotide sequence found in the six tRNAs that were well methylated is G-T-ψ-C-G-A-A-U-C in the GTψC loop region. This common sequence is also present in rat liver $tRNA^{Ser}$ that naturally contains 1-methyladenosine. Thus it is suggested that the recognition site of tRNA by adenylate residue-specific tRNA methylase is an oligonucleotide sequence containing the adenylate residue to be methylated.

The structure-function relationship of tRNA for recognition of aminoacyl-tRNA synthetase is not yet completely understood (Zachau, 1972). However, several workers recently suggested that aminoacyl-tRNA synthetase must recognize several regions located in different parts of the tRNA molecule (Roe and Dudock, 1972; Yamada and Ishikura, 1973; Dube, 1973). In contrast to the complexity of recognition by aminoacyl-tRNA synthetase, it seems that recognition by tRNA methylase must be rather simple, since unlike aminoacyl-tRNA synthetase, tRNA methylases catalyze the methylations of many tRNA species. In fact, as we previously reported, guanylate residue-specific tRNA methylase III from rat liver catalyzes the methylation of the guanylate residue at the 10th position in *Escherichia coli* $tRNA^{Val}_1$, $tRNA^{Phe}$, and $tRNA^{Met}$, forming N^2-monomethylguanosine (Kuchino and Nishimura, 1970). The common nucleotide sequence in these tRNAs is s^4U-A-G-C-U-C-A-G, in which the guanylate residue to be methylated is at position 10. This sequence is present in several eukaryotic tRNAs that normally contain N^2-methylguanosine (Zachau, 1972). Therefore, it was suggested that the methylase recognizes a short oligonucleotide sequence containing the susceptible nucleotide itself.

Using partially purified adenylate residue-specific tRNA methylase isolated from rat leukemic spleen tissue, Staehelin and his coworkers showed that adenylate in the 19th position from the CCA end of yeast $tRNA^{Ser}_1$ was methylated (Baguley *et al.*, 1970). The nucleotide sequence of yeast $tRNA^{Ser}_1$ near the methylated adenylate is A-m^1A-A-U, which is different from the corresponding sequence of G-m^1A-A-U present in natural rat liver $tRNA^{Ser}$ (Ginsberg *et al.*, 1971). Baguley and Staehelin (1969) also isolated a variety of methylated oligonucleotides, such as G-m^1A-Up, A-m^1A-Up, G-m^1A-Cp, G-m^1A-A-Up, and G-m^1A-A-Cp from a pancreatic RNase digest of unfractionated natural rat liver tRNA. These results suggest that the short segment of the nucleotide sequence containing the adenylate residue to be methylated is not an important factor for recognition by adenylate residue-specific methylase. It seems more likely that the methylase recognizes a specific nucleotide sequence located in a different part from the adenylate residue at position 19. In support of this yeast $tRNA^{Ser}_1$ and rat liver $tRNA^{Ser}$ possess an identical sequence in the GTψC stem (Zachau *et al.*, 1966; Ginsberg *et al.*, 1971), so that the GTψC stem may be the recognition site for adenylate residue-specific tRNA methylase.

To clarify the problem of recognition by adenylate residue-specific tRNA methylase, we studied the methylations of a large number of purified *E. coli* tRNAs *in vitro*. Results showed that the extents of formation of 1-methyladenylate in each tRNA were related to the nucleotide sequences in the GTψC loop. *E. coli* tRNAs which served as good methyl acceptors all contained the sequence G-T-ψ-C-G-A-A-U-C in the GTψC loop, although their nucleotide sequences in other regions such as the GTψC stem and extra-region differed widely. These results suggested that the main nucleotide sequence recognized by adenylate residue-specific tRNA methylase is an oligonucleotide sequence containing the adenylate residue to be methylated.

In this work, a simple method was developed to obtain adenylate residue-specific tRNA methylase completely free from other nucleotide-specific tRNA methylases.

Experimental Section

Methods

Preparation of Adenylate Residue-Specific tRNA Methylase. All purification procedures were carried out at 0–4°, and within 24 hr. Adult female rats of the Donryu strain (150–200 g body weight) were killed by decapitation, and their livers

† From the Biology Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan. *Received April 1, 1974.* This work was supported in part by grants from the Japanese Ministry of Education (S. N.) and the Naito Foundation (Y. K.).

[1] Abbreviations used are: m^1A, 1-methyladenosine; m^6A, 6-methyladenosine; m^2G, 2-methylguanosine; Ser 3, *E. coli* $tRNA^{Ser}_3$ Glu 2, *E. coli* $tRNA^{Glu}_2$; Met, *E. coli* $tRNA^{Met}$; His 1, *E. coli* $tRNA^{His}_1$; Tyr 2, *E. coli* $tRNA^{Tyr}_2$; Ser 1, *E. coli* $tRNA^{Ser}_1$; Leu 2, *E. coli* $tRNA^{Leu}_2$; A_{260} unit, an amount of material with an absorbance of 1.0 at 260 mμ when dissolved in 1 ml of water and measured with a 1-cm light path.

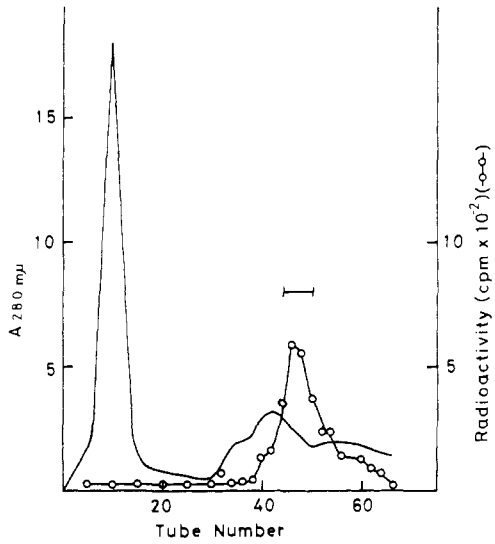

FIGURE 1: Fractionation of pH 5 supernatant enzymes from rat liver by DEAE-cellulose column chromatography: (—) uv absorbance; (O) radioactivity. Reaction mixtures (0.1 ml) in 0.1 M potassium phosphate buffer (pH 7.5) contained 0.01 M $MgCl_2$, 4 mM *S*-adenosylmethionine-*methyl*-^{14}C (specific activity, 25 Ci/mol), 0.05 A_{260} unit of *E. coli* $tRNA^{Ser}_3$, and 60 μl of test fraction. Incubation was carried out at 37° for 1 hr. Values were corrected for the incorporation of radioactivity in the absence of tRNA.

were quickly excised; 15 g of liver was homogenized in a Potter-Elvehjem type glass–Teflon homogenizer in 60 ml of buffer containing 10 mM Tris-HCl buffer (pH 8.0) containing 0.25 M sucrose, 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol. The homogenate was centrifuged at 18,000*g* for 10 min. The supernatant was recentrifuged at 105,000*g* for 60 min. The high-speed supernatant thus obtained was adjusted to pH 5.0 with 0.1 M acetic acid and immediately centrifuged at 18,000*g* for 10 min. The resulting supernatant was readjusted to pH 8.0 with 0.1 M NaOH and applied to a column (2 × 20 cm) of DEAE-cellulose. The column was washed with 100 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1 mM β-mercaptoethanol, and 10% ethylene glycol (buffer A). Then linear gradient elution was carried out by placing 150 ml of buffer A in the mixing chamber and 150 ml of buffer A containing 0.3 M NaCl in the reservoir. The activity of adenylate residue-specific methylase in the effluent was measured using *E. coli* $tRNA^{Ser}_3$ as methyl acceptor. Fraction 44–50 (Figure 1) was pooled and solid ammonium sulfate was added to give 50% saturation. The precipitate was collected by centrifugation, and dialyzed against buffer A for 3 hr with two changes of the buffer. The final enzyme preparation was stored at −80°.

Purified E. coli tRNAs. Unfractionated *E. coli* tRNA was prepared from *E. coli* B cells harvested in the late-log phase, by the procedure described by Zubay (1962), except that alkaline treatment was omitted. Amino acid specific *E. coli* tRNAs were isolated using combinations of various column chromatographic procedures, such as chromatography on DEAE-Sephadex A-50 at pH 7.5 or 4.0, and on benzoylated DEAE-cellulose and reversed phase partition column chromatography, as previously described (Nishimura, 1971). All the tRNAs used in this study were estimated to be more than 90% pure judging from their abilities to accept corresponding amino acids or from the chromatographic profiles of RNase T_1 digests.

Assay of tRNA Methylase Activity and Isolation of the ^{14}C-Methylated tRNA Product. The procedures used for assay of tRNA methylase and preparation of the ^{14}C-methylated product were as described previously, except that 0.1 M potassium phosphate buffer (pH 7.5) was generally used instead of Tris-HCl buffer (pH 8.0) for the methylation reaction (Kuchino and Nishimura, 1970).

Characterization of Structure of ^{14}C-Methylated Nucleotide and Its Location in tRNA. ^{14}C-methylated tRNAs were completely hydrolyzed by RNase T_2, and the digests were analyzed by Dowex 1 column chromatography as described previously (Kuchino *et al.*, 1971). To determine the chain length of the radioactive oligonucleotide obtained by digestion with RNase T_1 or pancreatic RNase, the digest was fractionated by DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea as described before (Kuchino and Nishimura, 1970). The radioactive oligonucleotide isolated was further degraded by RNase, *E. coli* alkaline phosphomonoesterase, or snake venom phosphodiesterase to determine the location of the radioactive adenylate, following the procedures described previously (Kuchino *et al.*, 1971; Harada *et al.*, 1971.

Materials

S-Adenosyl-L-methionine-*methyl*-^{14}C (specific activity, 56 Ci/mol) was purchased from the Radiochemical Centre, Amersham, England. RNase T_1 and RNase T_2 were obtained from Sankyo Co. Ltd., Tokyo. Bovine pancreatic RNase (recrystallized five times), *E. coli* alkaline phosphomonoesterase, and snake venom phosphodiesterase were obtained from Sigma Chemical Co., Miles Laboratories, Inc., and Worthington Biochemicals, respectively. Filter paper, No. 51A, equivalent to Whatman No. 1, was purchased from Toyo Roshi Co., Ltd., Tokyo. DEAE-Sephadex A-25 (capacity, 35 ± 0.5 mequiv/g; particle size, 40–120 μm), Dowex 1-X2, and DEAE-cellulose (capacity, 0.91 mequiv/g) were products from Pharmacia Laboratories, Inc., Dow Chemical Co., and Brown Co., Ltd., respectively. Authentic samples of 1-methyladenosine and N^6-methyladenosine were kindly supplied by Dr. M. Saneyoshi of our Institute.

Results

Purification of Adenylate Residue-Specific tRNA Methylase. As described in the Experimental Section, adenylate residue-specific tRNA methylase was purified by treatment at pH 5, followed by DEAE-cellulose column chromatography. It was found that most of the other tRNA methylases, such as predominant guanylate residue-specific tRNA methylases and cytidylate residue-specific tRNA methylase, were precipitated at pH 5. The tRNA methylase activity in the supernatant after treatment at pH 5 was largely that of adenylate residue-specific tRNA methylase as shown by the following experiments.

To characterize the ^{14}C-methylated nucleotide, $tRNA^{Ser}_3$ or $tRNA^{His}_1$ was methylated using methylase purified by DEAE-cellulose column chromatography. The resulting ^{14}C-methylated tRNAs were hydrolyzed to nucleotides by digestion with RNase T_2, and the products were analyzed by Dowex 1 column chromatography. As shown in Figure 2, almost all the radioactivity was eluted before cytidylic acid, indicating that the radioactive nucleotide was probably either 1-methyladenylic acid or 2′,3′-cyclic nucleotides. To characterize ^{14}C-methylated nucleotide further, the RNase T_2 digests were dephosphorylated by treatment with *E. coli* phosphomonoesterase. The radioactive products obtained were analyzed by paper electrophoresis with authentic 1-methyladenosine and N^6-methyladenosine as markers, as described previously (Kuchino *et al.*, 1971). Approximately 80% of the radioactivity coincided with the spot of 1-methyladenosine, and the rest was located in the position of N^6-methyladenosine. 1-Methyladenosine was probably partly

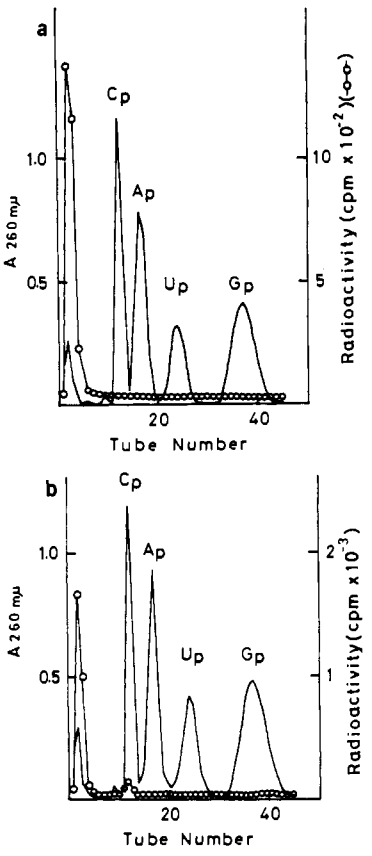

FIGURE 2: Dowex-1 column chromatography of RNase T_2 digests of ^{14}C-methylated *E. coli* $tRNA^{Ser}_3$ (a), and $tRNA^{His}_1$ (b): (—) uv absorbance; (O) radioactivity. ^{14}C-methylated tRNA containing approximately 10,000 cpm of radioactivity was incubated for 18 hr at 37° in 0.2 ml of 0.05 M potassium acetate buffer (pH 4.7) with 10 units of RNase T_2 and 10 A_{260} units of unfractionated *E. coli* tRNA. The hydrolysate was placed on a column (0.2 × 15 cm) of Dowex 1, and gradient elution was performed by placing 130 ml of 0.0035 N HCl in the mixing chamber and 200 ml of 0.25 M NaCl in the reservoir. Fractions of 1.5 ml were collected.

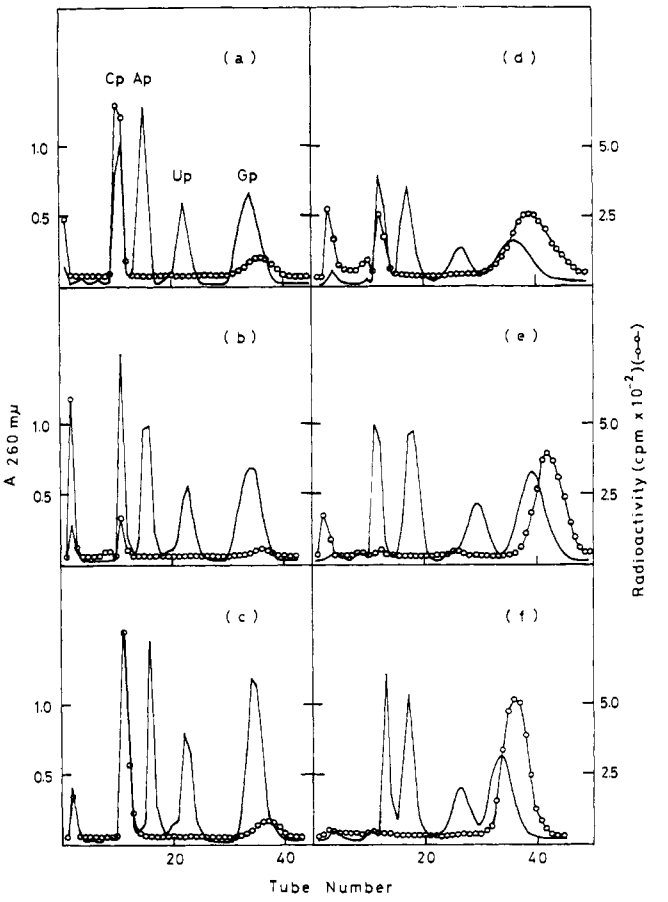

FIGURE 3: Dowex-1 column chromatography of RNase T_2 digests of ^{14}C-methylated *E. coli* $tRNA^{His}_1$ and $tRNA^{fMet}$: (—) uv absorbance; (O) radioactivity; (a) $tRNA^{His}_1$ methylated by the 105,000*g* supernatant; (b) $tRNA^{His}_1$ methylated by the pH 5 supernatant; (c) $tRNA^{His}_1$ methylated by the pH 5 precipitate; (d) $tRNA^{fMet}$ methylated at pH 7.5 by the 105,000*g* supernatant; (e) $tRNA^{fMet}$ methylated at pH 8.0 by the 105,000*g* supernatant; (f) $tRNA^{fMet}$ methylated at pH 9.0 by the 105,000*g* supernatant. The assay conditions were as described in Figure 2.

converted to N^6-methyladenosine during incubation with *E. coli* alkaline phosphomonoesterase at pH 7.8 (Brookes and Lawley, 1960; Baguley and Staehelin, 1969). When the radioactive product was treated with alkali (pH 9.0) before electrophoresis, all the radioactivity was found in the position of N^6-methyladenosine. These results indicated that the original ^{14}C-methylated nucleotide was in fact 1-methyladenylic acid.

The isolation of adenylate residue-specific tRNA methylase from other nucleotide-specific methylases by treatment at pH 5 and the effect of pH on the methylation are further illustrated in Figure 3.

Using a methylase preparation that had not been treated at pH 5 for the methylation reaction, the cytidylate and guanylate residues of *E. coli* $tRNA^{His}_1$ were also methylated considerably (Figure 3a). On the other hand, methylation of $tRNA^{His}_1$ by the pH 5 supernatant yielded predominantly 1-methyladenylic acid (Figure 3b), while methylated cytidylate and guanylate were major products on methylation with the pH 5 precipitate (Figure 3c). The pH 5 supernatant contained methylase activities other than that of adenylate residue-specific methylase, but they could be completely removed by subsequent DEAE-cellulose column chromatography, since they were eluted from the column later in tubes 52–62 (results not shown). Figure 3d–f illustrates the effect of pH on the methylation reaction. When methylation was carried out at pH 8.0 or 9.0 using the 105,000*g* supernatant, only the guanylate residue of $tRNA^{fMet}$ was methylated, as described previously (Figure 3e and f) (Kuchino *et al.*, 1972). However, when methylation was performed at pH 7.5, considerable amounts of 1-methyladenylate and 5-methylcytidylate were formed (Figure 3d). Therefore, in this study the methylation reaction was carried out at pH 7.5 instead of pH 8.0.

Determination of the Location of the Radioactive 1-Methyladenylate Residue in E. coli $tRNA^{Ser}_3$ and $tRNA^{His}_1$. The exact location of the ^{14}C-methylated nucleotide formed in *E. coli* $tRNA^{Ser}_3$ and $tRNA^{His}_1$ by adenylate residue-specific tRNA methylase was determined by examining the elution of radioactivity in a digest with RNase T_1 or pancreatic RNase from a DEAE-Sephadex A-25 column.

As shown in Figure 4a, the radioactive oligonucleotide derived from a pancreatic RNase digest of $tRNA^{Ser}_3$ was eluted in front of the trinucleotide fraction. The radioactive peak shifted to the tetranucleotide fraction when chromatography was carried out after heating the digest at 100° for 30 min at pH 9.0 (Figure 4b). The shift of the elution position of the radioactive oligonucleotide upon alkaline treatment is due to conversion of 1-methyladenylate to N^6-methyladenylate in the oligonucleotide, as discussed before. Since G-A-A-Up is the only tetranucleotide obtained on digestion of *E. coli* $tRNA^{Ser}_3$ with pancreatic RNase (Yamada and Ishikura, 1973), results show

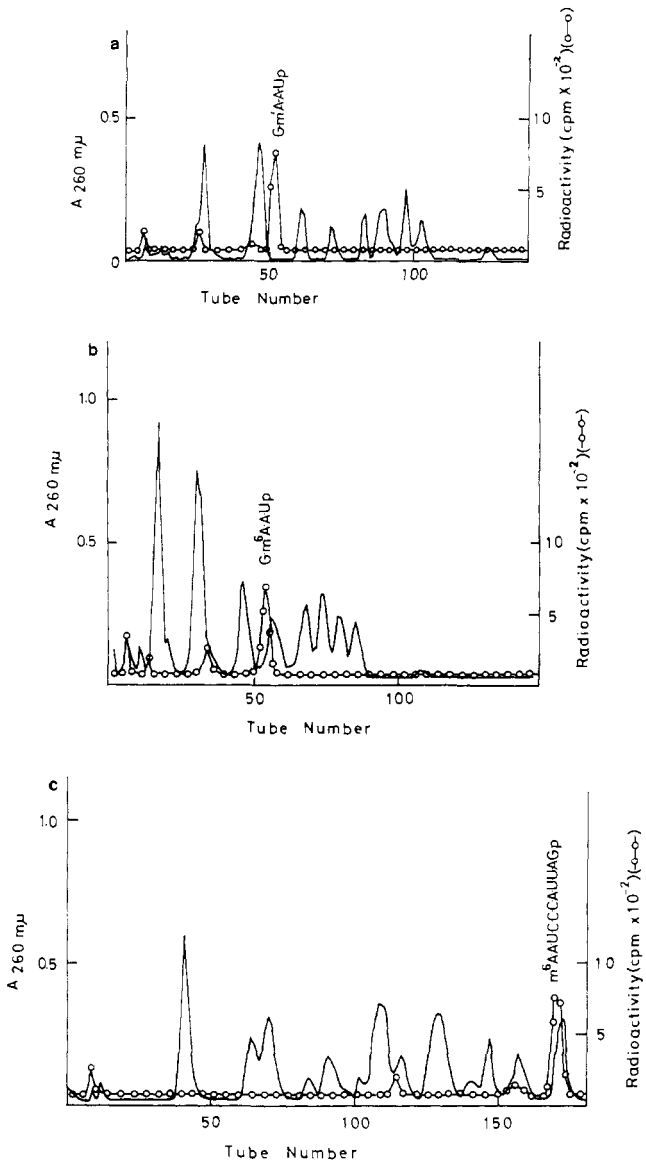

FIGURE 4: DEAE-Sephadex A-25 column chromatography of RNase digests of ^{14}C-methylated *E. coli* tRNAs: (a) pancreatic RNase digest of $tRNA^{Ser}_3$; (b) pancreatic RNase digest of $tRNA^{Ser}_3$ after treatment with alkali; (c) RNase T_1 digest of $tRNA^{His}_1$ after treatment with alkali; (—) uv absorbance; (O) radioactivity. ^{14}C-methylated tRNA containing approximately 10,000 cpm of radioactivity was dissolved with 20 A_{260} units of the respective carrier tRNA in 0.2 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 20 μg of RNase T_1 or 40 μg of bovine pancreatic RNase. Mixtures were incubated for 18 hr at 37°. Then the digests were adsorbed on a column (0.3 × 150 cm) of DEAE-Sephadex A-25 in the presence of 7 M urea. Elution was performed with a linear salt gradient obtained by placing 200 ml of 0.02 M Tris-HCl buffer (pH 7.5) containing 7 M urea and 0.14 M NaCl in the mixing chamber and 200 ml of the same buffer containing 7 M urea and 0.7 M NaCl in the reservoir. Fractions of 2 ml were collected.

that an adenylate residue in G-A-A-Up was methylated. To determine which adenylate residue of G-A-A-Up was methylated, the alkali-treated ^{14}C-methylated tetranucleotide, G(m^{6}A,A)Up, was treated with RNase T_1 and *E. coli* alkaline phosphomonoesterase. The resulting radioactive trinucleotide, (m^{6}A,A)U, was separated by descending paper chromatography with isobutyric acid–0.5 N NH_4OH (7:3, v/v) as solvent. The trinucleotide was then completely hydrolyzed by snake venom phosphodiesterase, and the hydrolysate was analyzed by paper electrophoresis. The radioactive component coincided

TABLE I: Methyl Group Acceptor Capacities of Individual *E. coli* tRNAs with Adenylate Residue-Specific tRNA Methylase from Rat Liver *in Vitro*.[a]

E. coli tRNA	Extent of Methylation (nmol/A_{260} unit of tRNA)	*E. coli* tRNA	Extent of Methylation (nmol/A_{260} unit of tRNA)
Ser 3	1.93	Leu 1	0.35
Glu 2	1.36	fMet	0.33
Met	0.96	Val 1	0.30
Thr	0.80	Gly	0.28
His 1	0.76	Leu 2	0.20
Tyr 2	0.72	Phe	0.19
Ser 1	0.61	Asp	0.13
Ile	0.45		

[a] Reaction mixtures were standard except that 0.6 mg of enzyme–protein was used. Assay conditions were as described in Figure 1.

with the N^6-methyladenosine used as a marker, indicating that radioactive N^6-methyladenosine was located at the 5′-OH end of the trinucleotide. Thus the site of methylation of $tRNA^{Ser}_3$ by adenylate residue-specific tRNA methylase was unambiguously identified as the 19th position from the CCA end.

The only radioactive oligonucleotide separated from an alkali-treated RNase T_1 digest of *E. coli* $tRNA^{His}_1$ was found in the fraction corresponding to A-A-U-C-C-C-A-U-U-A-Gp, as shown in Figure 4 (Harada *et al.*, 1972a; Singer and Smith, 1972). This radioactive oligonucleotide was dephosphorylated by *E. coli* alkaline phosphomonoesterase, and then hydrolyzed by snake venom phosphodiesterase, and the resulting mixture of nucleoside and 5′-nucleotide was analyzed by paper electrophoresis. The radioactive component coincided with N^6-methyladenosine. This indicated that the adenosine at the 5′-OH end of the nonanucleotide was methylated. Therefore, it was concluded that the site of methylation of *E. coli* $tRNA^{His}_1$ was also the adenylate residue at the 19th position from the CCA end.

Extents of Methylation of Individual E. coli tRNAs with Adenylate Residue-Specific tRNA Methylase. To examine the relationship between the structure of tRNA and its capacity as a methyl acceptor, numerous purified *E. coli* tRNAs were tested in the methylation reaction. The primary structures of most of the *E. coli* tRNAs used are already known, and their sequences vary considerably, so they seemed useful for determining the specific sequence recognized by the methylase.

As shown in Table I, the extents of methylation of individual *E. coli* tRNAs varied greatly, although all the tRNAs with known sequences contained an adenylate residue at the 19th position. *E. coli* $tRNA^{Ser}_3$ and $tRNA^{Glu}_2$ were the best acceptors and $tRNA^{Met}$, $tRNA^{Thr}$, $tRNA^{His}_1$, $tRNA^{Tyr}_2$, and $tRNA^{Ser}_1$ were also extensively methylated. It was calculated that 100% of the $tRNA^{Ser}_3$ was methylated assuming that 1 A_{260} unit of tRNA is equivalent to 1.66 nmol (Hoskinson and Khorana, 1965). On the contrary, $tRNA^{Asp}$, $tRNA^{Phe}$, and $tRNA^{Leu}_2$ were found to be very poor methyl acceptors, and incorporation of methyl groups into these tRNAs was approximately 10% of that into $tRNA^{Ser}_3$. Appreciable amounts of radioactivity were incorporated into $tRNA^{Ile}$, $tRNA^{Leu}_1$, $tRNA^{fMet}$, $tRNA^{Val}_1$, and $tRNA^{Gly}$, methylation being approximately 20% of that of $tRNA^{Ser}_3$.

The methylations of *E. coli* tRNAs were examined in more detail by measuring the incorporation of methyl groups after shorter times of incubation. As shown in Figure 5, the initial velocities of methylation of $tRNA^{His}_1$, $tRNA^{Tyr}_2$, and $tRNA^{Ser}_1$ were nearly equal to those of $tRNA^{Ser}_3$ and $tRNA^{Glu}_2$. However, methylations of the former tRNAs stopped after incubation for 10–20 min, while methylations of $tRNA^{Ser}_3$ and $tRNA^{Glu}_2$ continued for 60 min, resulting in complete methylation. It seems likely that some tRNAs, such as $tRNA^{His}_1$, $tRNA^{Ser}_1$, and $tRNA^{Tyr}_2$, may be appreciably degraded during prolonged incubation with the methylase preparation which is contaminated with RNase.

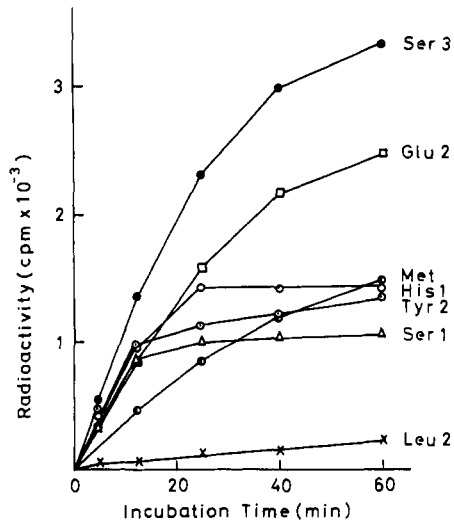

FIGURE 5: ^{14}C-methylated incorporation into *E. coli* $tRNA^{Ser}_3$, $tRNA^{His}_1$, $tRNA^{Ser}_1$, $tRNA^{Glu}_2$, $tRNA^{Tyr}_2$, $tRNA^{Met}$, and $tRNA^{Leu}_2$ on incubation for various times with adenylate residue-specific tRNA methylase from rat liver. The assay conditions were as described in Table I.

Discussion

A simple method was developed to obtain adenylate residue-specific tRNA methylase from rat liver free from other nucleotide residue-specific tRNA methylases. The method involves precipitation of other methylases at pH 5 and then DEAE-cellulose column chromatography.

The site of *in vitro* methylation of *E. coli* $tRNA^{Ser}_3$ and $tRNA^{His}_1$ was unambiguously determined as the adenylate residue at the 19th position from the CCA end, and the methylated product was identified as 1-methyladenylate. Thus the position of methylation of tRNA *in vitro* with a heterogeneous system was the same as that found in natural tRNAs from eukaryotic cells (Zachau, 1972). This supports previous findings by Baguley *et al.* (1970) with yeast $tRNA^{Ser}_1$ as methyl acceptor.

The primary sequences of *E. coli* tRNAs were compared, to find the structure common to all the tRNAs which were good methyl acceptors *in vitro* in the methylation reaction yielding 1-methyladenylate (Table I and Figure 5). *E. coli* $tRNA^{Ser}_3$ (Yamada and Ishikura, 1973; Ish-Horowicz and Clark, 1973), $tRNA^{Glu}_2$ (Ohashi *et al.*, 1972), $tRNA^{His}_1$ (Harada *et al.*, 1972a; Singer and Smith, 1972), $tRNA^{Ser}_1$ (Ishikura *et al.*, 1971), $tRNA^{Met}$ (Cory and Marcker, 1970), and $tRNA^{Tyr}_2$ (Goodman *et al.*, 1970; RajBhandary *et al.*, 1969) were extensively methylated. These all contain the sequence, G-T-ψ-C-G-A-A-U-C, in the GTψC loop region, although their sequences in other regions are quite different. For example, $tRNA^{Ser}_3$, $tRNA^{Tyr}_2$, and $tRNA^{Ser}_1$ possess a long extra arm, while $tRNA^{Glu}_2$, $tRNA^{His}_1$, and $tRNA^{Met}$ contain a short extra arm. The D stem and the fourth nucleotide, which are thought to be important for recognition of aminoacyl-tRNA synthetase (Roe and Duduck, 1972; Yamada and Ishikura, 1973; Chambers, 1971; Shimura *et al.*, 1972; Celis *et al.*, 1973), also differ in these tRNAs. It is unlikely that the GTψC stem, which is not near the methylated adenylated residue in the 19th position, is the recognition site for the methylase, because the nucleotide sequences in the GTψC stems of $tRNA^{Ser}_3$, $tRNA^{Ser}_1$, $tRNA^{Glu}_2$, $tRNA^{His}_1$, $tRNA^{Met}$, and $tRNA^{Tyr}_2$ differ greatly, and also differ from that of rat liver $tRNA^{Ser}$. It is also noteworthy that the *E. coli* tRNAs which are poor methyl acceptors almost all have a different sequence in the GTψC loop [the references giving the sequences of these *E. coli* tRNAs are: $tRNA^{Ile}$ (Yarus and Barrell, 1971), $tRNA^{fMet}$ (Dube and Marcker, 1969), $tRNA^{Asp}$ (Harada, *et al.*, 1972b), $tRNA^{Val}_1$ (Yaniv and Barrell, 1969; Kimura *et al.*, 1971), $tRNA^{Gly}$ (Squires and Carbon, 1971), $tRNA^{Leu}_1$ (Dube *et al.*, 1970), and $tRNA^{Leu}_2$ (Blank and Söll, 1971)]. Our hypothesis that only the sequence of the GTψC loop is important for recognition of the adenylate residue-specific methylase is in accordance with the finding of Baguley and Staehelin that 80% of the ^{14}C-methylated adenylate formed by rat liver tRNA methylase was in the G-m^{1}A-A-Up sequence (Baguley and Staehelin, 1968).

Baguley *et al.* (1971) showed that yeast $tRNA^{Ser}_1$, which contains the sequence G-T-ψ-C-A-A-A-U-C in the GTψC-loop, was 70% methylated by adenylate residue-specific tRNA methylase. Spremulli *et al.* (1972) reported that *E. coli* $tRNA^{fMet}$, which also has the sequence G-T-ψ-C-A-A-A-U-C in the GTψC-loop, was fully methylated to give 1-methyladenylate in the 19th position by a methylase isolated from HeLa cells. On the contrary, in our experiments, methylation of *E. coli* $tRNA^{fMet}$ was only 20% of that of *E. coli* $tRNA^{Ser}_3$. It is very likely that the adenylate residue-specific tRNA methylase can recognize several alternative sequences in addition to G-T-ψ-C-G-A-A-U-C in the GTψC loop with low efficiencies in terms of K_m or V_{max}.

Baguley and Staehelin (1969) reported that natural rat liver unfractionated tRNA contained the sequences, py-G-m^{1}A-U, py-A-m^{1}A-U, py-G-m^{1}A-C, and py-G-m^{1}A-A-C as well as py-G-m^{1}A-A-U and py-A-m^{1}A-A-U, that were all presumably derived from the GTψC loop. However, in our experiments $tRNA^{Phe}$ and $tRNA^{Val}_1$ were not well methylated *in vitro* by the adenylate residue-specific methylase, although these tRNAs possess the py-G-A-U sequence in the GTψC loop. There are several possible explanations of this discrepancy between *in vivo* and *in vitro* results. We previously showed that the conformation of tRNA is important for methylation (Kuchino *et al.*, 1971). The adenylate residue in the 19th position in *E. coli* $tRNA^{fMet}$ reconstituted from fragments is more susceptible to methylation than that in native $tRNA^{fMet}$. Moreover, it has been shown that mammalian precursor tRNA is already methylated (Burdon and Clason, 1969; Bernhardt and Darnell, 1969; Kay and Cooper 1969; Choe and Taylor, 1972). Therefore, it is reasonable to assume that tRNAs containing the py-G-m^{1}A-U sequence are better methylated at the precursor level. Another explanation is that there is an adenylate residue-specific tRNA methylase with different recognition specificity in the cells, but that it is excluded or inactivated during purification of the methylase. A third possibility is that there is sufficient methylase in the cells to methylate adenylate residues in other sequences than py-G-A-A-U in tRNA, since tRNAs with other sequences were methylated to some extent

in vitro. These problems must be studied using precursor tRNA or tRNA fragments.

References

Baguley, B. C., and Staehelin, M. (1968), *Biochemistry 7,* 45.

Baguley, B. C., and Staehelin, M. (1969), *Biochemistry 8,* 257.

Baguley, B. C., Wehrli, W., and Staehelin, M. (1970), *Biochemistry 9,* 1645.

Bernhardt, D., and Darnell, J. E., Jr. (1969), *J. Mol. Biol. 42,* 43.

Blank, H. U., and Söll, D. (1971), *Biochem. Biophys. Res. Commun. 43,* 1192.

Brookes, P., and Lawley, P. D. (1960), *J. Chem. Soc.,* 539.

Burdon, R. H. and Clason, A. E. (1969), *J. Mol. Biol. 39,* 113.

Celis, J. E., Hooper, M. L., and Smith, J. D. (1973), *Nature (London), New Biol. 244,* 261.

Chambers, R. W. (1971), *Progr. Nucl. Acid. Res. Mol. Biol. 11,* 489.

Choe, B. K., and Taylor, M. W. (1972), *Biochim. Biophys. Acta 272,* 275.

Cory, S., and Marcker, K. A. (1970), *Eur. J. Biochem. 12,* 177.

Dube, S. (1973), *Nature (London), New Biol. 243,* 103.

Dube, S. K., and Marcker, K. A. (1969), *Eur. J. Biochem. 8,* 256.

Dube, S. K., Marcker, K. A., and Yudelevich, A. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 9,* 168.

Ginsberg, T., Rogg, H., and Staehelin, M. (1971), *Eur. J. Biochem. 21,* 249.

Goodman, H. M., Abelson, J. N., Landy, A., Zadrazil, S., and Smith, J. D. (1970), *Eur. J. Biochem. 13,* 461.

Harada, F., Kimura, F., and Nishimura, S. (1971), *Biochemistry 10,* 3269.

Harada, F., Sato, S., and Nishimura, S. (1972a), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 19,* 352.

Harada, F., Yamaizumi, K., and Nishimura, S. (1972b), *Biochem. Biophys. Res. Commun. 49,* 1605.

Hoskinson, R. M., and Khorana, H. G. (1965), *J. Biol. Chem. 240,* 2129.

Ish-Horowicz, D., and Clark, B. F. C. (1973), *J. Biol. Chem. 248,* 6663.

Ishikura, H., Yamada, Y., and Nishimura, S. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 16,* 68.

Kay, J. E., and Cooper, H. L. (1969), *Biochim. Biophys. Acta 186,* 62.

Kimura, F., Harada, F., and Nishimura, S. (1971), *Biochemistry 10,* 3277.

Kuchino, Y., Endo, H., and Nishimura, S. (1972), *Cancer Res. 32,* 1243.

Kuchino, Y., and Nishimura, S. (1970), *Biochem. Biophys. Res. Commun. 40,* 306.

Kuchino, Y., Seno, T., and Nishimura, S. (1971), *Biochem. Biophys. Res. Commun. 43,* 476.

Nishimura, S. (1971), *Proced. Nucleic Acid Res. 2,* 542.

Ohashi, Z., Harada, F., and Nishimura, S. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 20,* 239.

RajBhandary, U. L., Chang, S. H., Gross, H. J., Harada, F., Kimura, F., and Nishimura, S. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28,* 409.

Roe, B., and Dudock, B. (1972), *Biochem. Biophys. Res. Commun. 49,* 399.

Shimura, Y., Aono, H., Ozeki, H., Sarabhai, A., Lamfrom, H., and Abelson, J. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 22,* 144.

Singer, C. E., and Smith, G. R. (1972), *J. Biol. Chem. 247,* 2989.

Spremulli, L. L., Agris, P. E., Brown, C. M., and RajBhandary, U. L. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 450.

Squires, C., and Carbon, J. (1971), *Nature (London), New Biol. 233,* 274.

Yamada, Y., and Ishikura, H. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 29,* 231.

Yaniv, M., and Barrell, B. G. (1969), *Nature (London) 222,* 278.

Yarus, M., and Barrell, B. G. (1971), *Biochem. Biophys. Res. Commun. 43,* 729.

Zachau, H. G. (1972), *in* The Mechanism of Protein Synthesis and Its Regulation, Bosch, L., Ed., Amsterdam, North-Holland Publishing Co., 173.

Zachau, H. G., Dütting, D., and Feldmann, H. (1966), *Hoppe-Syler's Z. Physiol. Chem. 347,* 212.

Zubay, G. (1962), *J. Mol. Biol. 4,* 347.