NUCLEOTIDE SEQUENCE SPECIFICITIES OF GUANYLATE RESIDUE-SPECIFIC tRNA METHYLASES FROM RAT LIVER

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Recent studies (1-3) have suggested that tRNA methylases recognize a specific region of tRNA. This seems probable since a particular methylated nucleotide is specifically located in the same region as in the clover-leaf structure (See review by Zachau (4) for examples). However, the positions of the methylated bases introduced *in vitro* are not yet known, since unfractionated tRNA was used as a methyl acceptor in previous experiments.

Numerous purified *E. coli* tRNA's are available in our laboratory so we could study the problem of recognition by tRNA methylase more precisely. This paper reports that there are at least two species of guanylate specific methylases in rat liver differing in their recognition sites on tRNA. One of these methylases exclusively catalyzes incorporation of methyl groups into *E. coli* $tRNA_1^{Val}$, $tRNA^{Phe}$, $tRNA^{Met}$ and $tRNA_1^{Ser}$. N^2-Methylguanosine was the sole product in $tRNA_1^{Val}$ and $tRNA^{Phe}$. The other methylates $tRNA^{fMet}$, $tRNA_3^{Ser}$ and $tRNA_2^{Leu}$, and also produces N^2-methylguanosine except with $tRNA_3^{Ser}$ where N^2-dimethylguanosine is the major product. Analyses of the oligonucleotides derived from individual ^{14}C-methylated tRNA's by RNase T_1 or pancreatic RNase clearly demonstrated that the former methylase catalyzes methylation of the guanylate residue located between the dihydrouridine-stem and the CCA-stem. The other methylase catalyzes methylation of the guanylate residue located between the dihydrouridine-stem and the anticodon-stem.

MATERIALS AND METHODS

Amino acid specific tRNA's from *E. coli* B were obtained by column chromatographies on DEAE-Sephadex A-50 (5), followed by benzoylated DEAE-cellulose (6) or/and reverse phase partition column chromatography (7). Details of the procedures were described previously (5,8-10) or will be published elsewhere. The tRNA's used were at least 70 to 95 % pure, judging from the capacities to accept individual amino acids and the chromatographic profiles of their digests with RNase T_1.

Unfractionated methyl deficient tRNA was prepared by the method of Zubay (11) from *E. coli* K12-58-161 RC^{rel} which was grown in a minimal medium containing 0.0003 %

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L-methionine. Rat liver tRNA was prepared as described by Zubay (11) with a slight modification. Authentic samples of N^2-methylguanosine, N^2-dimethylguanosine and N^1-methylguanosine were kindly supplied by Dr. M. Saneyoshi of our Institute.

Preparation of guanylate-specific methylase from rat liver: Eight g of liver from adult female rats were homogenized with four volumes of cold 0.25 M sucrose-0.01 M Tris-HCl (pH 8.0)-0.01 M $MgCl_2$-0.001 M β-mercaptoethanol. The homogenate was centrifuged at 105,000 x g for 1 hr. The crude extract thus obtained (25 ml) was applied to a column of hydroxylapatite (BIO-GEL HTP; column size: 1.5 cm x 20 cm), and washed with 150 ml of 0.01 M Tris-HCl (pH 8.0)-0.001 M EDTA (pH 8.0)-0.001 M β-mercaptoethanol (buffer A). Elution was achieved with a linear gradient obtained by placing 200 ml of buffer A in the mixing chamber and 200 ml of 0.01 M Tris-HCl (pH 8.0)-0.001 M EDTA (pH 8.0)-0.001 M β-mercaptoethanol-0.05 M potassium phosphate buffer (pH 8.0) (buffer B) in the reservoir. Solid ammonium sulfate was added to 60 % saturation to the eluate containing methylase activity. The precipitate was dissolved in a minimal volume of buffer A, and dialyzed against buffer A.

Assay of tRNA methylase activity: The procedure was essentially that described by Tsutsui et al. (12). The reaction mixture contained 10 μmoles of Tris-HCl (pH 8.0), 1 μmole of $MgCl_2$, 1 μmole of reduced glutathione, 4 mμmoles of ^{14}C-methyl labeled S-adenosylmethionine (specific activity: 20 C/M), 0.05 O.D. units of tRNA and an appropriate amount of methylase in a final volume of 0.1 ml. After incubation at 37°C for 1 hr, 0.05 ml of the reaction mixture was applied to a filter paper disk. Disks were washed three times with cold 5 % trichloroacetic acid and then successively with ethanol-ether mixture (1:1, v/v) and ether. Radioactivity was counted in a liquid scintillation counter.

Large scale preparation of methylated tRNA in vitro: Five ml of the reaction mixture described above were treated with an equal volume of 88 % phenol. tRNA was precipitated from the aqueous layer by adding 0.1 volume of 5 M NaCl, 10 O.D. units of the respective tRNA and 2.5 volumes of cold ethanol. tRNA was dissolved in a minimal volume of 0.01 M Tris-HCl (pH 7.5)-0.06 M KCl-0.001 M β-mercaptoethanol and dialyzed first against the same buffer, and then against distilled water.

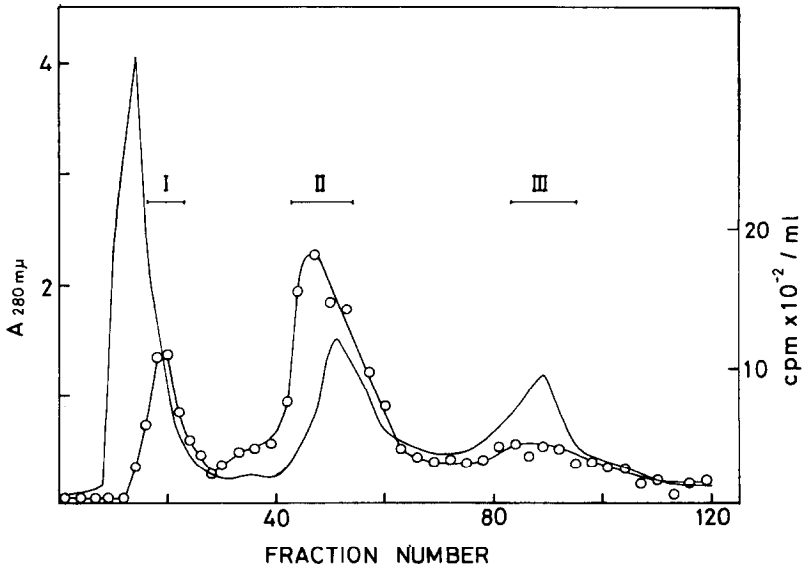

Fig. 1. Fractionation of rat liver tRNA methylase by hydroxylapatite column chromatography. ——, UV absorbance; -o-o-, radioactivity.

RESULTS

As shown in Fig. 1, rat liver tRNA methylase was separated into three species by hydroxylapatite column chromatography. Methylase activity was measured using E. coli methyl deficient tRNA as methyl acceptor. The abilities of various amino acid specific tRNA's isolated from E. coli B to serve as acceptors for these three methylases were tested. Table I shows that these tRNA's had different abilities to act as methyl acceptors, and that the extent of methylation depended on the type of methylase. For example, $tRNA^{fMet}$, $tRNA_3^{Ser}$ and $tRNA_2^{Leu}$ were extensively methylated by methylases I and II, while $tRNA_1^{Val}$, $tRNA^{Phe}$, $tRNA^{Met}$ and $tRNA_1^{Ser}$ were very poor methyl acceptors for these two methylases. On the contrary, methylase III catalyzed extensive methylation of $tRNA_1^{Val}$, $tRNA^{Phe}$, $tRNA^{Met}$ and $tRNA_1^{Ser}$ but it could not well methylate $tRNA^{fMet}$, $tRNA_3^{Ser}$ or $tRNA_2^{Leu}$. Table I also shows that $tRNA^{Asp}$, $tRNA_2^{Glu}$ and $tRNA_2^{Tyr}$ were inert with both methylases. These results clearly indicate that methylases I and II have rather similar properties, but that methylase III differs from methylases I and II with respect to its recognition site on tRNA.

To characterize the structure of the methylated nucleotides formed on methyl-

Table I. Methyl acceptor capacities of individual E. coli tRNA's with rat liver methylases in vitro

	Extent of methylation (cpm/0.05 ml of reaction mixture)		
tRNA	Methylase I	Methylase II	Methylase III
$tRNA^{Asp}$	210	290	150
$tRNA_2^{Glu}$	440	530	140
$tRNA_2^{Leu}$	1790	2340	170
$tRNA^{fMet}$	4580	4110	210
$tRNA_1^{Met}$	74	110	510
$tRNA^{Phe}$	33	240	770
$tRNA_1^{Ser}$	32	220	520
$tRNA_3^{Ser}$	2380	3270	110
$tRNA_2^{Tyr}$	260	480	210
$tRNA_1^{Val}$	64	350	710
E. coli tRNA	1440	1280	460
Methyl deficient E. coli tRNA	1730	1600	540
Rat liver tRNA	45	84	34
Yeast tRNA	230	240	70

Each reaction mixture contained 0.7 mg of the enzyme preparation. The numbers listed are averages of values obtained from two separate experiments using different enzyme preparations.

ation of each tRNA, ^{14}C-methylated tRNA^{fMet} and tRNA$_3^{Ser}$ with methylase II, and ^{14}C-methylated tRNA$_1^{Val}$ and tRNA^{Phe} with methylase III were completely hydrolyzed by RNase T_2 as described previously (9), and each digest was first analyzed by Dowex 1 column chromatography as described by Tada et al. (13). All the radioactivities were eluted close together with guanosine 3'-phosphate used as marker, indicating that in all cases the guanylate residue in tRNA's was methylated. To determine the structure of the methylated base, the RNase T_2 digest of tRNA was treated with E. coli alkaline phosphatase, and the resulting ^{14}C-methylated nucleosides were chromatographed with the markers N^2-methylguanosine, N^2-dimethylguanosine and N^1-methylguanosine as described by Hacker and Mandel (14). As shown in Fig. 2, N^2-methylguanosine was the only ^{14}C-methylated product from tRNA^{fMet}, tRNA$_1^{Val}$ and tRNA^{Phe}, while N^2-dimethylguanosine was the major product from tRNA$_3^{Ser}$. Although not shown in the figure, it was shown that N^2-methylguanosine was the major product from tRNA$_2^{Leu}$. Thus methylase II catalyzed the formation of two different methylated bases, N^2-methylguanosine and N^2-dimethylguanosine, depending upon the tRNA used as methyl acceptor.

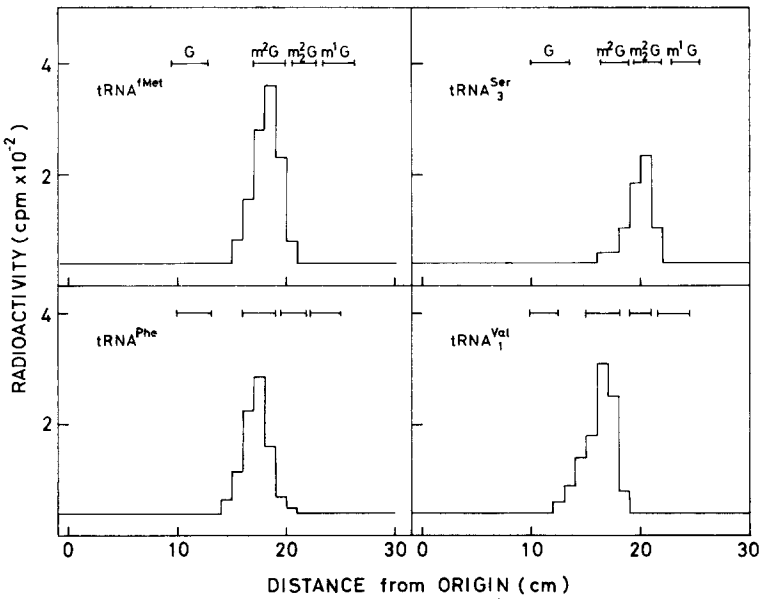

Fig. 2. Characterization of ^{14}C-methylated nucleoside by paper chromatography.

To determine the exact location of the nucleotides methylated in individual tRNA's, tRNA^{fMet}, tRNA$_1^{Val}$ and tRNA^{Phe} were used as methyl acceptors, since their primary sequences are known (15-18). The ^{14}C-methylated tRNA's were mixed with an excess of the corresponding control tRNA, and hydrolyzed either with RNase T_1 or pancreatic RNase. Each RNase digest was fractionated by DEAE-Sephadex A-25 column

chromatography in the presence of 7 M urea, as described previously (9). In the RNase T_1 digest of $tRNA^{fMet}$ methylated with methylase II, all the radioactivities were eluted in the tetranucleotide region where CpCpUpGp, CpUpCpGp and pCpGp were eluted (Fig. 3a). In the digest of $tRNA^{fMet}$ with pancreatic RNase, radioactivity was only detected in the dinucleotide region where GpCp, GpUp and ApUp were eluted (Fig. 3b). From these results and the known primary structure of $tRNA^{fMet}$, it was concluded that the oligonucleotide containing ^{14}C-methylated guanylate in the RNase T_1 digest of $tRNA^{fMet}$ was either $pCpm^2Gp$ or $CpUpCpm^2Gp$. To determine which oligonucleotide was actually methylated, the radioactive oligonucleotide obtained from the tetranucleotide fraction of the RNase T_1 digest was dephosphorylated by treatment with E. coli alkaline phosphatase, and chromatographed with markers using n-propanol-conc. NH_4OH-H_2O (35:10:35, v/v/v) as solvent. The radioactivity coincided with the marker CpUpCpG, indicating that the methylated oligonucleotide was $CpUpCpm^2G$. These results show unambiguously that the site of methylation of $tRNA^{fMet}$ by methylase II

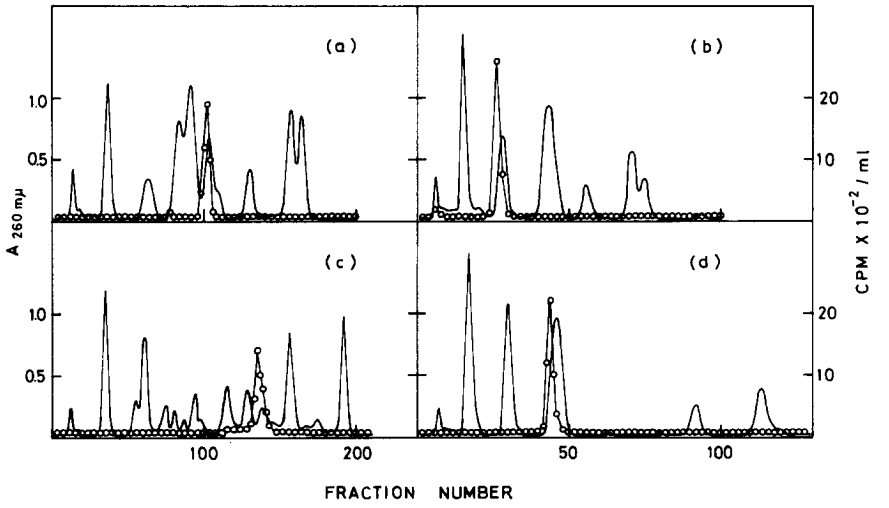

Fig. 3. DEAE-Sephadex A-25 column chromatography of RNase digests of ^{14}C-methylated E. coli tRNA's. a) RNase T_1 digest of $tRNA^{fMet}$, b) pancreatic RNase digest of $tRNA^{fMet}$, c) RNase T_1 digest of $tRNA_1^{Val}$, d) pancreatic RNase digest of $tRNA_1^{Val}$; ——, UV absorbance; -o—o-, radioactivity; column size, 0.3 x 150 cm; Gradient elution was achieved by placing 200 ml of 0.02 M Tris-HCl (pH 7.5)-0.14 M NaCl-7 M urea in the mixing chamber, and 200 ml of 0.02 M Tris-HCl (pH 7.5)-0.7 M NaCl-7 M urea in the reservoir. Each fraction contained 2 ml of effluent.

was the guanylate residue at the 27th position from the 5'-OH end, located between the dihydrouridine-stem and the anticodon-stem (Fig. 4).

Similarly, the site of methylation of $tRNA_1^{Val}$ by methylase III was determined from analysis of digests of ^{14}C-methylated $tRNA_1^{Val}$ with RNase T_1 or pancreatic RNase. In the RNase T_1 digest, the radioactivity was only present in the fractions contain-

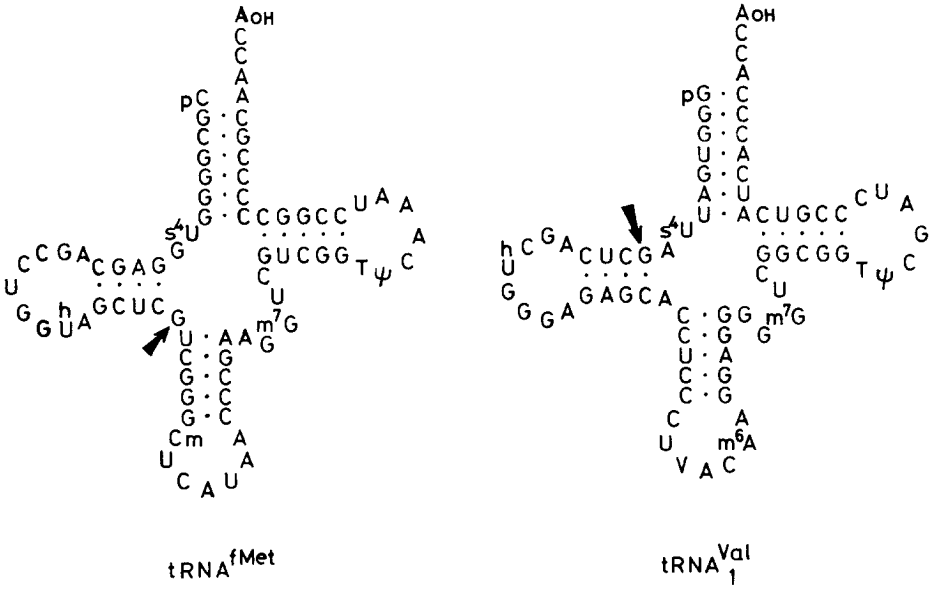

Fig. 4. Clover-leaf structure of E. coli $tRNA^{fMet}$ and $tRNA_1^{Val}$, indicating the site of methylation. V, uridin-5-oxyacetic acid (27).

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ing ApUpCpCpCpGp and ApUps^{4}UpApGp (Fig. 3c). In the pancreatic RNase digest, the radioactivity was eluted in the trinucleotide fraction containing ApGpCp, GpGpCp, GpApUp and GpGpTp (Fig. 3d). Therefore the site of methylation was clearly the guanylate residue at the 10th position from the 5'-OH end, namely in the region between the CCA-stem and the dihydrouridine-stem. Although not shown in the figure, a similar type of experiment using ^{14}C-methylated $tRNA^{Phe}$ showed that $tRNA^{Phe}$ was exclusively methylated at the guanylate residue at the 10th position from the 5'-OH end, which was exactly the same site as that methylated in $tRNA_1^{Val}$.

On methylation of $tRNA_3^{Ser}$ with methylase II the exact location of N^2-dimethylguanosine could not been determined, since the primary structure of this tRNA is not known yet. Analysis of digests with RNases showed that N^2-dimethylguanosine was present in the Cpm_2^2GpC sequence.

DISCUSSION

Methylase III catalyzed methylation of the guanylate residue at the 10th position in both E. coli $tRNA_1^{Val}$ and $tRNA^{Phe}$ resulting in the formation of N^2-methylguanosine. The common nucleotide sequence around the methylated guanylate in these tRNA's is s^{4}UAGCUCAG, suggesting that this nucleotide sequence is the site specifically recognized by the methylase. Evidence that E. coli $tRNA^{Met}$ which contains s^{4}UAGCUCAG in the same region (See ref. 19) was exclusively methylated by methylase III also supports the above conclusion. It is interesting to

note that the UAm^2GC sequence is present in the same region of yeast $tRNA^{Phe}$ (20), wheat germ $tRNA^{Phe}$ (21), and yeast $tRNA^{Tyr}$ (22,23). The trinucleotide sequence, AGC, by itself is not the sole factor determining the recognition by methylase III, since the guanylate residues in the AGC sequence located in a different region of E. coli $tRNA^{fMet}$, $tRNA_1^{Val}$, $tRNA^{Phe}$ and $tRNA_2^{Tyr}$ were not methylated by methylase III. It is also clear that the presence of only a guanylate residue at the 10th position from the 5'-OH end is not sufficient for methylation of this guanylate residue by methylase III, since $tRNA^{fMet}$ which contains a guanylate residue at this position was not methylated at all by methylase III. These results show that the methylase recognizes a guanylate residue in terms of a nucleotide sequence around the guanylate residue methylated, as well as in terms of the secondary structure of the molecule.

The guanylate residue at the 27th position in $tRNA^{fMet}$ was methylated by methylase II yielding N^2-methylguanosine. The nucleotide sequence around the methylated guanylate of $tRNA^{fMet}$ is CGU, namely pyrimidine-G-pyrimidine. N^2-Dimethylguanosine formed in $tRNA_3^{Ser}$ was also found in the sequence, pyrimidine-G-pyrimidine. This result agree with the fact that the same region is occupied by a pyrimidine-m_2^2G-pyrimidine sequence in several tRNA's from yeast and wheat germ (See ref. 20-26). As in the case of methylase III, the pyrimidine-G-pyrimidine sequence is not the sole determining factor for the recognition of methylase II, since a guanylate residue in a CGU sequence located in a different region in E. coli $tRNA_1^{Val}$ and $tRNA^{Phe}$ was not methylated by methylases I and II. The methylation product formed by methylase II differed depending upon the tRNA used as methyl acceptor. Namely N^2-methylguanosine was formed from ^{14}C-methylated $tRNA^{fMet}$, while N^2-dimethylguanosine was predominantly formed from ^{14}C-methylated $tRNA_3^{Ser}$. It is probable that a single enzyme participates in formation of both N^2-methylguanosine and N^2-dimethylguanosine. However, it is possible that methylase II is a mixture of two enzymes, as in the case of yeast in which different tRNA methylases seem to be involved in the synthesis of N^2-methylguanosine and N^2-dimethylguanosine respectively (27).

Further studies on the recognition of tRNA methylase using purified tRNA's and their fragments as methyl acceptors are in progress.

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