N²-Guanine specific transfer RNA methyltransferase II from rat liver

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ABSTRACT

N²-guanine methyltransferase II was purified from rat liver. This enzyme methylated bulk *E. coli* tRNA to an extent of 7.6 nmoles of methyl groups/mg tRNA. Oligonucleotide analysis showed that N²-methylated guanines were present in the modified tRNA in two sequences, namely Y-m²G-Cp and Y-m²G-Cp in the ratio 4:3.

Two pure tRNA^{Leu} species, and tRNA^{Met} from *E. coli* were methylated with the enzyme to extents of 17, 11, and 8 nmoles of methyl groups incorporated per mg tRNA, respectively. When the methylated tRNAs were analysed no m²G was detected and the m²G occurred in the tRNAs specific for leucine in a Y-m²G-Cp sequence and in the tRNA^{Met} in a sequence Y-m²G-Up.

It is concluded that the mammalian enzyme specifically recognizes the interstem unpaired guanylate residue between the dihydrouridine arm and the anticodon arm. The absence of any detectable m²G methylation of individual tRNA species is discussed.

INTRODUCTION

N²-dimethylguanosine is not a natural component of *E. coli* tRNA¹. However, m²G occurs in about 50% of yeast and rat liver tRNAs²⁻⁴. It is found always at a specific position, namely between the dihydrouridine arm and the anticodon arm (N²-guanine methylation site II) most frequently in a sequence G-C-m²G-Y. Recently tRNA^{Met} from three mammalian sources has been sequenced^{5,6} which has two m²G residues, one was located in the 10th nucleotide from the 5'-end (site I) and the other at site II in a sequence G-U-m²G-C. These findings indicate that the guanylate residue at site II is, in some mammalian tRNAs, only monomethylated whilst in others (e.g. tRNA^{Ser}₁ from rat liver) it is dimethylated. It is not clear whether in mammalian cells there are two enzymes for site II modification, a mono-

methyltransferase and dimethyltransferase or, whether the level of N^2 -guanine methylation, using one enzyme is determined by the tRNA structure.

13 out of 20 sequenced yeast tRNAs contain a m_2^2G residue at site II. It has been shown that there are two separate genes in yeast, each coding for a different N^2 -guanine methyltransferase. tRNA from the mutant strains lacked m_2^2G but contained normal amounts of m^2G ⁷. No sequence analysis was made at that time so it can be only assumed that all m^2G was present at site I in the tRNA. The m_2^2G formation in the mutant tRNA was observed in vitro using an enzyme preparation from the wild type cells. However, m_2^2G formation was accompanied by a certain proportion of m^2G formation. All these results indicate that the eukaryotes contain one N^2 -guanine mono-methyltransferase I responsible for the modification of the site I and one or more enzymes (N^2 -guanine methyltransferase II) which mono- and dimethylates the guanylate at site II.

We have described in the preceding paper⁸ a purification of the N^2 -guanine methyltransferase I from rat liver and leukemic rat spleen which methylated G to m^2G in E. coli tRNAs containing a U-A-G-C sequence at site I. Another enzyme (N^2 -guanine methyltransferase II) is obtained when the methylating activities present in the high-speed supernatant are purified and fractionated on the column of hydroxyapatite. Using bulk E. coli tRNA and homogenous tRNA species we have studied the tRNA site and sequence specificity of N^2 -guanine methyltransferase II from rat liver partially purified on the hydroxyapatite column.

MATERIALS AND METHODS

Purification of N^2 -guanine methyltransferase II: An enzyme catalyzing the formation of N^2 -methylguanosine as well as of N^2 -dimethylguanosine was prepared by a modification of the procedure described by Kuchino and Nishimura⁹.

The procedure for preparation of the high-speed supernatant from rat liver (40 g) was essentially the same as described in the preceding paper⁸. The pH 5 precipitation step was omitted. The high-speed supernatant of rat liver homogenate was, after it had been passed through a plug of glass wool, directly applied to a column of hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories). Column fractions were assayed for tRNA methylating activity, the most active column fractions were pooled and solid ammonium sulphate was slowly added to 60% saturation and the precipitate was collected by centrifugation. The precipitate was dissolved in a small volume of buffer C (0.01 M Tris-HCl (pH 8.0) - 1 mM EDTA - 0.1 mM dithiothreitol) and dialyzed for 4 hours against buffer C with one intermediate change of buffer.

Mitochondrial tRNA from rat liver mitochondria was kindly provided by Mr. H. Hartmann (University of Basel).

All other materials and techniques employed for methylation of tRNA and for the enzymic analysis of [Me-¹⁴C]-tRNA were described in the preceding paper⁸. Unless otherwise stated, all tRNA samples were methylated in the presence of 20 µg rat liver tRNA.

RESULTS

Enzyme purification: The methylating activities present in the high-speed supernatant were purified and fractionated on the column of hydroxyapatite. Figure 1 shows that the bulk of the proteins had not been bound to the hydroxyapatite and had been washed out with the buffer before the phosphate gradient was started. tRNA methylating activity was then eluted in two peaks with a linear gradient of potassium phosphate approximately at 0.015 M and 0.035 M salt concentrations. The fractions corresponding to the first and second methylating activity peaks were pooled as indicated in the figure. The methylating activities were recovered by ammonium sulphate precipitation and dialysis. The overall yield of enzyme activity

Fig. 1

Fractionation of the high-speed supernatant from rat liver on a hydroxyapatite column. The column (2 x 28 cm) was packed with hydroxyapatite and equilibrated with buffer C. The high-speed supernatant (80 ml) was applied to the column which was then washed with buffer C (540 ml). The elution was carried out with a linear gradient of 0-50 mM potassium phosphate in buffer C (720 ml) at a flow rate of 0.9 ml/min. Fractions (4.5 ml) were collected, the absorbance at 280 nm was measured (—) and 0.2 ml aliquots were assayed for tRNA methylating activity using 50 μ g *E. coli* tRNA (.....).

was about 43% with a 5-fold degree of purification, if only pool I was considered.

Methylation of tRNA with enzyme pools from the hydroxyapatite column:

Both enzyme pool I and pool II from the hydroxyapatite column were assayed using the same *E. coli* tRNA concentration with increasing protein concentrations (Fig.2, curve 1 and 2). As it can be seen, the plateau appeared at a relatively low protein concentration. This effect was ascribed to the action of ribonucleases present in the enzyme pools, since it was not observed at higher tRNA-protein ratios in the assay and became more manifest with the decreasing tRNA-protein ratio. In order to increase the tRNA-protein ratio without increasing the amount of methyl-accepting tRNA substrate, rat liver tRNA was added to the incubation mixture. Curve 3 demonstrated that some methylation of rat liver tRNA by rat liver methyltransferase took

place, but the methylation extent represented only 3-4% of that of E. coli tRNA. Thus, if E. coli tRNA was methylated in the presence of "protector" rat liver tRNA an almost two times higher methylation extent was reached, namely 7.6 nmoles methyl group/mg tRNA (curve 4).

Fig. 2
Dependence of ¹⁴C-methyl incorporation on amount of enzyme protein in assay mixture. Assay tubes with hydroxyapatite enzyme pool I contained 20 µg of E. coli tRNA (curve 1), 20 µg of E. coli tRNA and 20 µg of rat liver tRNA (curve 4) and 20 µg of rat liver tRNA (curve 3). Assay tubes with hydroxyapatite enzyme pool II contained 20 µg of E. coli tRNA (curve 2). All tubes were incubated at 37° for 3 hours.

Nucleoside analysis of bulk E. coli tRNA methylated with N²-guanine

methyltransferase II: Hydroxyapatite pool I and pool II were used to methylate E. coli tRNA to an extent of 4.6 resp. 4 nmoles methyl group/mg tRNA. At the same time E. coli tRNA was methylated with hydroxyapatite pool I in the presence of rat liver tRNA to an extent of 7.6 nmoles methyl group/mg tRNA. The methylated tRNA was isolated from the incubation mixture, desalted, digested to nucleosides and separated by thin layer chromatography. The radioactive nucleosides were detected by autoradiography and identified as shown in Table I.

N²-guanine methylation represented approximately 70-80% of the total

methylation of E. coli tRNA by the hydroxyapatite enzymes. In contrast to the N²-guanine methyltransferase I purified on DEAE cellulose and Sephadex G200 the hydroxyapatite enzymes are capable of not only N²-guanine mono-methylation but of N²-guanine dimethylation as well. The ratio of m²G/m₂G was approximately 2:1 and remained constant during the fractionation of the high-speed supernatant on the column of hydroxyapatite. The second peak of methylating activity contained relatively more of 5-methylcytosine methyltransferase. In E. coli tRNA methylated in the presence of rat liver tRNA, the percentages of methylated nucleosides were almost identical to those in E. coli tRNA methylated alone, thus indicating that the presence of liver tRNA did not favor the methylation of one nucleoside more than of the others. The bulk E. coli tRNA was methylated in the presence of rat liver tRNA to an extent of nearly 8 nmoles methyl group/mg tRNA. This represents an incorporation of 0.2-0.3 residues per tRNA molecule or very approximately 0.1-0.2 residues m²G and 0.05-0.1 residues m₂G per tRNA molecule.

Table I
Products of tRNA methylation with hydroxyapatite enzymes*

Enzyme source	m ¹ A	m ⁵ C	m ¹ G	m ² G	m ₂ G
Hydroxyapatite pool I (N ² -guanine methyltransferase II)	15	11	0	48	26
Hydroxyapatite pool I**	14	8	0	52	26
Hydroxyapatite pool II	5	25	0	44	26

* Since m₂G accounts for two radioactive methyl groups the results are expressed at % of total nucleosides formed rather than % of total ¹⁴C incorporation.

** Methylation of E. coli tRNA in presence of rat liver tRNA.

Oligonucleotide analysis of bulk E. coli tRNA methylated with N²-guanine

methyltransferase II: In order to identify oligonucleotides in which the ¹⁴C-labelled m²G and m₂G occur, bulk E. coli tRNA was methylated with N²-guanine.

methyltransferase II to an extent of 7.6 nmoles methyl group/mg tRNA and the methylated tRNA was subjected to digestion with pancreatic RNase. The mixture of oligonucleotides was then fractionated on a column of DEAE cellulose. Fig. 3 shows that almost all the radioactivity was recovered in the dinucleotide region of the column.

The radioactivity present in peak 1 represented a mixture of radioactive decomposition products of ^{14}C -S-adenosylmethionine excess which were precipitated together with tRNA after the incubation.

In thin layer chromatography, these ^{14}C -SAM decomposition products were not detected when tRNA samples had been routinely desalted prior to digestion. Some 5-methylcytidine-3'-phosphate was found in the Cp peak and was identified by thin layer chromatography and detected by autoradiography.

Fig. 3
DEAE-cellulose chromatography of a pancreatic RNase digest of *E. coli* tRNA previously methylated with N^2 -guanine methyltransferase II. Optical density at 260 nm (—); radioactivity (.....).

Peaks 3 and 4 were found by sequence analysis to correspond to peaks 5 and 6 but containing cyclic 2', 3'-phosphate groups. They were found only because the tRNA was not exposed sufficiently long to the action of the

pancreatic RNase. The radioactive oligonucleotides from peaks 3 and 4 were isolated by thin layer chromatography in two dimensions. After another treatment with the pancreatic RNase they showed the same R_f positions as samples of peaks 5 and 6. After treatment with alkaline phosphatase and venom diesterase they yielded N^2 -dimethylguanosine and cytidine and N^2 -methylguanosine and cytidine, respectively, indicating that the original dinucleotides were $m_2^2G-C \gt p$ and $m^2G-C \gt p$. The two major peaks of radioactivity in the dinucleotide region were identified as follows.

The radioactive dinucleotide from peak 5 was isolated from the oligonucleotide mixture by thin layer electrophoresis and detected by autoradiography. Nucleoside analysis of this oligonucleotide yielded radioactive N^2 -dimethylguanosine and non-radioactive cytidine. If the radioactive dinucleotide was first dephosphorylated and then treated with venom diesterase, the two-dimensional chromatography yielded a radioactive N^2 -dimethylguanosine and cytidine 5'-phosphate. The structure of the dinucleotide was therefore m_2^2G-Cp .

The radioactive dinucleotide from peak 6 was first partly separated from the accompanying oligonucleotides, mainly from A-Cp by thin layer electrophoresis and detected by autoradiography. The final isolation was accomplished by thin layer chromatography. The further elucidation of the structure was the same as in the case of peak 5 (see above). The structure of the radioactive dinucleotide from peak 6 was m^2G-Cp .

The structures of radioactive oligonucleotides present in peak 7 of the trinucleotide region were not identified but contained N^2 -methylguanosine and 1-methyladenosine in approximately equal amounts. This indicates that the hydroxyapatite enzyme contained traces of N^2 -guanine methyltransferase I and of 1-adenine methyltransferase. As shown in Table II, the N^2 -guanine specific methylation and subsequent digestion of bulk tRNA yielded the following oligonucleotides: m^2G-Yp , m_2^2G-Cp , and $R-m^2G-Yp$, representing over

80% of the total methylation. In contrast to N^2 -guanine methyltransferase I⁸, where the m^2G was recovered upon tRNA digestion entirely in a trinucleotide A- m^2G -Cp, the N^2 -methylated guanosines were found this time mainly in dinucleotide sequences. This indicates that the hydroxyapatite enzyme (N^2 -guanine methyltransferase II) methylates other sites in tRNA than the N^2 -guanosine methyltransferase I, since the target N^2 -methylated guanylate residues have been adjacent to pyrimidine and were not only monomethylated but dimethylated as well.

Methylation of individual tRNAs with N^2 -guanine methyltransferase II.

Methylated nucleoside analysis: N^2 -guanine methyltransferase II was used to methylate several individual tRNAs, some of them of known primary structure. The methylation extents were measured and the nucleosides of tRNAs which accepted methyl groups were determined (Table II).

Table II shows that various amino acid specific tRNAs had different abilities to serve as methyl acceptors for N^2 -guanine methyltransferase II from rat liver. Four different leucine tRNA species were used: $tRNA_{1}^{Leu}$ and $tRNA_{2}^{Leu}$ were not substrates for this enzyme; $tRNA_{3}^{Leu}$ and $tRNA_{4}^{Leu}$ together with $tRNA_{f}^{Met}$ were the best substrates of all the *E. coli* tRNAs used.

The incorporation of 17 nmoles methyl group per mg $tRNA_{3}^{Leu}$ yielded approximately 0.5 residues N^2 -methylguanosine per $tRNA_{3}^{Leu}$ molecule, assuming that 1 mg tRNA is equal to about 33 nmoles²⁶. It was previously demonstrated that both $tRNA_{f}^{Met}$ and all four leucine specific tRNAs were not substrates for N^2 -guanine methyltransferase I³⁹. On the other hand, $tRNA^{Arg}$ and $tRNA^{Phe}$ which were extensively methylated by N^2 -guanine methyltransferase I were not methyl acceptors for N^2 -guanine methyltransferase II. The methylation of bulk *E. coli* with N^2 -guanine methyltransferase II yielded both m^2G and m_2^2G residues in tRNA chains and, as the oligonucleotide analysis showed, the ratio was 4:3 (expressed in methylated guanylate residues found). In contrast the guanylate residue in $tRNA_{3}^{Leu}$, $tRNA_{4}^{Leu}$, and $tRNA_{f}^{Met}$ was methylated

Table II

Methylation of various tRNAs with N²-guanine methyltransferase II

tRNA	Methylation extent nmoles CH ₃ / group mg tRNA	Methylation products % total nucleosides formed			
		⁵ mC	¹ mA	² mG	² m ₂ G
<u>E. coli</u> total	7.6	8	14	52	26
<u>E. coli</u> total*	7.3				
Yeast total	0.4				
Rat liver total	0.2				
Rat liver mitochondr. total	0.2				
Yeast Asp	2.2				
Yeast Asp**	2.0				
<u>E. coli</u> :					
Leu I	0.6				
Leu II	0.7				
Leu III	17.0	0	12	87	1
Leu IV	11.0	0	11.5	85.5	3
Ser I	4				
Ser III	3				
Phe	2.5				
Tyr	0.7				
Glu II	3				
Met F	8	0	0	100	0
Arg	0.6				

* Bulk E. coli tRNA was dialyzed 24 hours against 1 mM EDTA, 10 mM sodium cacodylate buffer pH 6.9 and after dialysis incubated at 60°C for 5 min.¹⁰

** tRNA^{Asp} from yeast (10 O.D.) was incubated for 11 min at 37° in buffer (0.5 ml) containing 9 mM Na₂HPO₄, 1 mM NaH₂PO₄, 10 M formaldehyde, 1 M NaCl and afterwards precipitated from the solution with 1 ml of ethanol, dissolved in 0.2 M NaCl, reprecipitated and washed twice with ethanol and once with ether.¹¹

almost exclusively to N²-methylguanosine. The nucleoside analysis of methylated tRNA^{Met}_f yielded radioactive N²-methylguanosine as the only methylated product. N²-methylguanosine was also the main product upon methylation of yeast tRNA^{Asp} but since the methylation extent was rather low, no further analysis was performed. Finally an attempt was made to modify the tRNA conformation in such a way that the ability to accept methyl

groups would be changed. A brief heat EDTA treatment speeds up the denaturation of some tRNAs, which occurs at all temperatures in an EDTA containing solvent of low ionic strength. As it was shown in Table II this treatment had no effect on either the methyl acceptance of bulk *E. coli* or on the incorporation of second methyl group into guanylate residue. Similarly a treatment with a solution containing 10 M formaldehyde had no significant effect on the methyl acceptance of tRNA^{Asp}.

Oligonucleotide analysis of *E. coli* tRNA₃^{Leu}, tRNA₄^{Leu}, and tRNA_f^{Met} methylated with N²-guanine methyltransferase II: In order to confirm the results of the oligonucleotide analysis of bulk *E. coli* tRNA and also to decide at what site the methylated oligonucleotides occur in the tRNA molecule, the tRNA₃^{Leu}, tRNA₄^{Leu}, and tRNA_f^{Met} were methylated with N²-guanine methyltransferase II and the oligonucleotide analysis was carried out as follows.

tRNA₃^{Leu}: *E. coli* tRNA₃^{Leu} was methylated to an extent of 17 nmoles methyl group/mg tRNA and digested with a pancreatic RNase. The digest yielded a mixture of oligonucleotides which was fractionated on a DEAE cellulose column (Fig. 4). The bulk of the radioactivity was found in peak 3. The dinucleotide was identified as m²G-Cp.

The radioactive oligonucleotide present in peak 4 contained 1-methyladenosine. Radioactive oligonucleotide containing 1-methyladenosine was separated from other oligonucleotides by thin layer electrophoresis, heated for 45 minutes at 100° in 0.1 M triethylammonium bicarbonate pH 8.6 to convert m¹A quantitatively to m⁶A,¹² and then further purified by two-dimensional thin layer chromatography, and located by autoradiography.

Digestion with alkaline phosphatase and venom diesterase yielded radioactive N⁶-methyladenosine and non-radioactive guanosine, adenosine and uridine in equal amounts. Digestion with T₁-ribonuclease yielded a non-radioactive uridylic 3' acid and a radioactive trinucleotide, indicating

Fig.4

DEAE-cellulose chromatography of a pancreatic RNase digest of *E. coli* tRNA^{Leu} previously methylated with N²-guanine methyltransferase II. Optical density at 260 nm (—); radioactivity (.....).

that guanosine had been in the second nucleotide from 3'-end in the original tetranucleotide sequence.

Following dephosphorylation, the trinucleotide was digested with venom phosphodiesterase to yield non-radioactive adenosine and guanosine 5'-phosphate and radioactive N⁶-methyladenosine 5'-phosphate. The sequence of the original radioactive tetranucleotide was therefore A-m¹A-G-Up.

tRNA^{Leu}₄, tRNA^{Met}_f: If the oligonucleotide mixture after pancreatic RNase digest of methylated bulk tRNA and tRNA^{Leu}₃ was separated both by thin layer chromatography and by chromatography on a DEAE cellulose column, the results of both analyses coincided (Table III) and therefore the oligonucleotide analysis of tRNA^{Leu}₄ methylated to the extent of 11 nmoles CH₃ groups/mg tRNA and tRNA^{Met}_f methylated to the extent of 8 nmoles CH₃ groups/mg tRNA was carried out only by means of thin layer chromatography.

The R_{Ap} values of the radioactive spots located by autoradiography were exactly the same for both tRNA^{Leu}₃ and tRNA^{Leu}₄ (Fig. 5a). The R_{Ap}

values are shown in Table I of the preceding paper.⁸ The percent distribution of the labelled oligonucleotides is given in Table III.

Fig. 5a, b

Oligonucleotide analysis of $\text{tRNA}_{3}^{\text{Leu}}$, $\text{tRNA}_{4}^{\text{Leu}}$ (a) and $\text{tRNA}_{f}^{\text{Met}}$ (b) previously methylated with N^{2} -guanine methyltransferase II and digested with pancreatic RNase. The oligonucleotides were separated by thin layer chromatography in two dimensions, in the first dimension in solvent system A, in the second dimension in solvent system C. Closed spots indicate positions of ^{14}C -methylated oligonucleotides located by autoradiography.

The $\text{tRNA}_{f}^{\text{Met}}$ methylated with N^{2} -guanine methyltransferase II was digested with pancreatic RNase and the mixture of oligonucleotides was separated by two dimensional thin layer chromatography. One radioactive spot was identified by autoradiography (Fig. 5b). The radioactive oligonucleotide was dephosphorylated with phosphatase, and the dephosphorylated sample was digested with snake venom diesterase. The radioactivity was associated with N^{2} -methylguanosine and in addition non-radioactive uridylic 5' acid was detected, indicating that the uridine was not in the 5' terminal nucleotide.

The structure of the only radioactive dinucleotide was therefore $\text{m}^{2}\text{G-Up}$. The results of oligonucleotide analysis of $\text{tRNA}_{3}^{\text{Leu}}$, $\text{tRNA}_{4}^{\text{Leu}}$ and $\text{tRNA}_{f}^{\text{Met}}$ showed similarly to those of bulk *E. coli* tRNA (Table III) that

Table III
Products of methylation of *E. coli* tRNAs with N²-guanine methyltransferase II*

Methods of analysis	tRNA from <i>E. coli</i>	Mono- m ⁵ Cp	N u c l e o t i d e s					Tetra- R-m ¹ A-R-Yp**
			m ² G-Cp	m ² G-Cp	m ² G-Up	R-m ² G-Yp	Tri	
Pancreatic	bulk	6	27.5	41	2.5	10.5		12.5
RNase	Leu III	0	1	86.5	0	0		12.5
TLC	Leu IV	0	3	84.5	0	0		12.5
	fMet	0	0	0	100	0		0
Pancreatic	bulk	5	29	41	0	12		13
RNase	Leu III	0	1.5	4.5	0	0		14
DEAE column								

* % total nucleotides formed

** identified as A-m¹A-G-Up in tRNA^{Leu}₃ and tRNA^{Leu}₄

about 80-90% of the incorporated radioactivity was found in dinucleotides, mostly in the sequence m^2G-Cp in the $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$ and 100% in the sequence m^2G-Up in the $tRNA_f^{Met}$. The radioactive sequence m_2^2G-Cp represented a very small amount of the incorporated radioactivity and was detected probably only due to a contamination of $tRNA^{Leu}$ species by other tRNAs.

DISCUSSION

When bulk E. coli tRNA was methylated with N^2 -guanine methyltransferase II from rat liver and digested with pancreatic RNase two dinucleotides m^2G-Cp and m_2^2G-Cp were obtained in the ratio 4:3. The methylation extent was increased in the presence of rat liver tRNA in the assay to nearly 8 nmoles of methyl group incorporated/mg tRNA. The same dinucleotide sequence m_2^2G-Cp , that we have found after the methylation of E. coli tRNA in vitro, occurs in yeast tRNAs (specific for tyrosine, phenylalanine, alanine, leucine, methionine) between the dihydrouridine arm and the anticodon arm at the site II. The fact that 90% of N^2 -methylated guanine was found in dinucleotide sequences in pancreatic RNase digest corresponds also under in vitro conditions to the methylation at site II since quanylate residue occurs in E. coli tRNAs in this position always in a sequence C-G-Y with the exception of $tRNA_1^{Gly}$.

Four different E. coli $tRNA^{Leu}$ species were used in this study. $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ were the most lipophilic species,¹³ probably due to ms^{2,6},¹⁴ responding to codons of UU series.¹⁵ $tRNA_3^{Leu}$ and mainly $tRNA_4^{Leu}$ were more polar, responding probably to codons of CU series as the two sequenced $tRNA^{Leu}$ species.¹⁶ None of them served as a substrate for N^2 -guanine methyltransferase I.⁸ $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ were also not substrates for N^2 -guanine methyltransferase II. $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$ on the other hand were methylated (0.5 and 0.3 methyl group per tRNA chain, respectively). The methylation of $tRNA_3^{Leu}$, $tRNA_4^{Leu}$, and $tRNA_f^{Met}$ with N^2 -guanine methyl-

transferase II yielded N^2 -methylguanosine as the only methylated guanylate residue. The N^2 -methylguanosine was liberated in pancreatic RNase digests of $tRNA_{3}^{Leu}$ and $tRNA_{4}^{Leu}$ in the sequence m^2G-Cp and of $tRNA_f^{Met}$ in the sequence m^2G-Up . The trinucleotide Y-G-C which can be liberated following the tRNA modification and digestion as m^2G-Cp occurs in one of the known $tRNA^{Leu}$ three times and twice in the other species. Similarly the sequence Y-G-U occurs in $tRNA_f^{Met}$ twice. The only identical position where Y-G-Y sequence occurs both in $tRNA_f^{Met}$ and in $tRNA^{Leu}$ is the position between the dihydro-uridine arm and the anticodon arm and this is most probably the only site where the guanylate residue can be recognized by N^2 -guanine methyltransferase II. The presence of a trinucleotide Y-G-Y in the primary sequence is clearly not the sole factor necessary for enzyme recognition since this short non-distinctive trinucleotide is present in different regions of $tRNA^{Phe}$, $tRNA^{Tyr}$, $tRNA_2^{Glu}$, and $tRNA^{Arg}$ and was not recognized there.

Methylation of guanylate at site II to m^2G in $tRNA_f^{Met}$ using a purified methylase from rat liver was already reported by Kuchino and Nishimura.⁹ The same enzyme catalysed the formation of m^2G in $tRNA_3^{Ser}$, although later the existence of two enzymes was considered.¹⁷ Using the same $tRNA_f^{Met}$ as a substrate, Shersheva¹⁸ working with crude methylase from rat hepatoma, Agris¹⁹ with a purified enzyme from HeLa cells, and Pegg²⁰ with extracts from normal mouse colon and colon tumours observed, similarly to Kuchino and Nishimura⁹, only m^2G formation at the site II. In addition, Pegg²¹ showed that the methylation of $tRNA_3^{Ser}$ yielded m^2G and only traces of m^2G . Pegg²² also found only m^2G at site II in $tRNA^{Asp}$ methylated with crude liver methylase thus demonstrating that the guanylate can also be recognized in a sequence $U-G^*-G$, however, the extent of methylation was rather low as it was confirmed in our experiment (Table II). Quite different results were obtained when methylase from wheat embryo was used to methylate E. coli tRNAs (T.C. Kwong and B.G. Lane, unpublished work). The N^2 -guanine

methylation at site II in the presence of 4.8 mM Mg^{++} yielded in $tRNA_3^{Ser}$ mostly m_2^2G , approximately the same amounts of m^2G and m_2^2G in $tRNA_1^{Leu}$ and $tRNA^{Ile}$, twice as much m^2G than m_2^2G in $tRNA_f^{Met}$ and only m^2G in yeast $tRNA^{Asp}$. The amount of m_2^2G found was significantly higher in tRNAs containing the target G^* in a sequence $G-C-G^*-C$.

The N^2 -guanine methylation at site II with a mammalian enzyme yielded in several *E. coli* tRNAs only m^2G . This explains why the m^2G was found in much greater amount than m_2^2G in bulk *E. coli* tRNA methylated with unfractionated methyltransferase preparation⁸, since in the nucleoside analysis it was derived both from site I and site II and not only from site I as it might have been expected from its occurrence in vivo.

It is not clear at present why the methylation of bulk tRNA yielded both m_2^2G and m^2G at the site II and stopped at the level of m^2G in $tRNAs^{Leu}$ and $tRNA_f^{Met}$ using the same enzyme preparation. The formation of m^2G and m_2^2G at the same site can be explained in several ways. (i) Either there are two methylating activities in eukaryotes, one responsible for formation of m^2G and the other responsible entirely for formation of m_2^2G ; or there is one activity catalysing the formation of m^2G and another activity adds the second methyl group in some cases; or only one of the two methylating activities can recognize the given tRNA substrate; (ii) or there is only one enzyme and the product is determined in some way by the tRNA substrate.

The experiments with a yeast mutant⁷ lacking m_2^2G do not necessarily suggest that single enzyme is responsible for the biosynthesis of m_2^2G .²³ It is possible that two enzymes need to act in succession and the mutant lacks the first enzyme of the pathway. There also may be a difference between the lower and the higher eukaryotes. Yeast and wheat embryo may possess only one enzyme which in vivo gives only m_2^2G and in vitro mostly m_2^2G ⁷ with some intermediate formation of m^2G in heterologous tRNAs,²³ whereas in mammals the enzyme(s) produce in vivo both m^2G and m_2^2G and

in vitro mostly m^2G in prokaryotic tRNAs. So far only a few tRNAs were tested and it must be remembered that in many studies ^{9,18-20} E. coli tRNA_f^{Met} was used as a substrate for the mammalian enzyme and it was no other than the tRNA_f^{Met} from mammals that was recently ^{5,6} shown to contain m^2G in vivo. This dilemma of one or two enzymes responsible for the N²-guanine methylation at site II and also the observation of elevated levels of N²-guanine dimethylase in cancer tissues²⁴ can perhaps be solved by using mutant tRNAs lacking the modification at site II or completely undermethylated tRNA species²⁵ and by variation of methylation conditions in favour of m^2G formation.

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