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Intraspecies Variation in Transfer RNA Methyltransferases of Inbred Mice[1]

Mark Dizik, Noel M. Relyea, and Elsie Wainfan

The Lindsley F. Kimball Research Institute of the New York Blood Center [M. D., E. W.], and Memorial Sloan-Kettering Cancer Center [N. M. R.], New York, New York 10021

ABSTRACT

Intraspecies differences in transfer RNA (tRNA) methyltransferases have been investigated by comparing the activities of the liver tRNA-methylating enzymes of two inbred strains of mice. Since the tRNA methyltransferases are known to exhibit altered activities during carcinogenesis, it seemed of interest to compare the characteristics of these enzymes from inbred mice with either high (AKR/J) or low (C57BL/6J) incidence of spontaneous leukemia.

The activities of liver enzymes from AKR/J mice were found to be about 35% higher than those of the enzymes from mice of the C57BL/6J strain when assayed *in vitro* with heterologous mixed *Escherichia coli* tRNAs as substrate. When either *E. coli* $tRNA^{Nfmet}$ or $tRNA^{Leu_2}$ was used as methyl group acceptor in the *in vitro* reaction, the activities of the enzymes from AKR/J mice were observed to be almost double those from the C57BL/6J animals. This contrasts with results obtained using *E. coli* $tRNA^{Tyr}$ as the methyl group acceptor, where no significant difference was observed between the methylase activity of enzymes from the livers of the two mouse strains. Under our assay conditions, *E. coli* $tRNA^{Nfmet}$ and $tRNA^{Leu_2}$ are virtually base-specific substrates for the enzymatic transfer of methyl groups to guanine moieties in tRNA, while the products of $tRNA^{Tyr}$ methylation are 3 methylcytidine and 5 methylcytidine. The results of these experiments, using tRNA substrates of differing specificities, indicate that in these mice there are preferential differences in the guanine tRNA methyltransferases of liver. Enzymes from kidneys of AKR/J and C57BL/6J mice showed a similar 2-fold difference in activity with $tRNA^{Nfmet}$ as substrate.

The liver tRNA methyltransferase activity of F1 progeny of a cross between AKR and C57BL/6 showed intermediate levels of activity to those of the parental strains. This observation is consistent with additive inheritance. Further studies are under way to elucidate the mechanisms for genetic control of this enzyme system.

INTRODUCTION

The tRNA methyltransferases from widely divergent species differ in their activity levels and base specificities. Some of these enzyme differences have been found to be reflected in the methylated base content of the tRNAs (3, 17, 22, 23, 30, 34). Species specificity of tRNA methylation is shown also by the ability of the enzymes from any given source to catalyze methyl group transfer *in vitro* to tRNAs from heterologous but not homologous sources (14, 22, 23). However, very little is known about intraspecies differences in activities of these enzymes, except for those found in bacterial mutants which lack certain base-specific tRNA methylases (3, 34) and in rat liver tRNA methylases which exhibit sex-specific activity differences (28).

In order to determine whether there are genetically determined intraspecies differences in mammalian tRNA methyltransferases, we are investigating the characteristics of these enzymes in inbred strains of mice. Inbred mice provide a system for the study of variation in the expression of normal gene function (1, 24). We report here the results of experiments in which the *in vitro* activities of liver and kidney tRNA methyltransferases from 2 strains of mice have been compared. Differences in specificity as well as overall activity were investigated by using several amino acid-specific tRNAs and mixed tRNAs from *Escherichia coli* as substrates for these enzymes.

MATERIALS AND METHODS

Mice. Animals from the strains of inbred mice used, C57BL/6J, hereafter called B6, and AKR/J, hereafter called AK, were obtained from The Jackson Laboratory (Bar Harbor, Maine) or from mice bred in our laboratory using parental mice from The Jackson Laboratory. Mice were fed *ad libitum* Ralston Purina Rodent Laboratory Chow No. 5001 (Ralston Purina Co., St. Louis, Mo.) containing 23.0% minimum crude protein, 4.5% minimum crude fat, and 6.0% maximum crude fiber. Water was given *ad libitum*.

Enzyme Preparations. Young adult mice (approximately 3 months old) were sacrificed by cervical dislocation, and organs were quickly excised and weighed. All subsequent operations were carried out at 0–4°. The organs were homogenized in 2 volumes of 0.05 M Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 5.0 mM $MgCl_2$, using a 13-mm Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland) at a setting of 5 for 30 sec. The homogenates were centrifuged at 12,000 × *g* for 20 min. The resulting supernatant was centrifuged at 105,000 × *g* for 90 min. The supernatant fraction from the high-speed centrifugation containing the tRNA methyltransferases was stored in small aliquots at −20° until used. These preparations were stable under these conditions for about 6 months (31).

tRNA Substrates. tRNA from *E. coli* B was prepared by a modification of the procedure of Fleissner and Borek (7) as described earlier (30, 31). Amino acid-specific tRNAs from *E. coli* were obtained commercially from either Sigma Chemical Co. or from Research Plus, Inc.

Enzyme Assays. Each incubation tube contained 25 μg of mixed tRNAs from *E. coli* or, when individual amino acid-specific tRNAs were used, 0.08 to 0.2 absorbance unit of tRNA. Each tube also contained 0.15 μCi Ado-Met[2] (specific activity, 55 mCi/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) as methyl donor and enzyme extract as indicated in a volume of 0.5 ml in a final buffer concentration of 10 mM Tris-HCl:5.0 mM $MgCl_2$:5.0 mM mercaptoethanol, pH 8.0, containing 0.3 M ammonium acetate. Incubation was carried out at 35° for 30 to 60 min. The reaction was terminated by the addition of 0.2 ml of 1.5 M NH_2OH, pH 8, to each tube, and the incubation was continued for 10 min longer. Samples were chilled, and 2 mg carrier tRNA followed by 4 ml of 0.5 M NaCl in 70% ethanol were added. Precipitates collected by centrifugation in the cold and washed as described earlier (30, 31) were assayed for radioactivity in a scintillation counter. Unless otherwise indicated, enzyme preparations were made from pooled organs from 2 or more animals.

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[2] The abbreviations used are: Ado-Met, [*methyl*-^{14}C]-*S*-adenosylmethionine; 2MeG, 2 methylguanosine; 7 MeG, 7 methylguanosine.

Ado-Met and tRNA^{Nfmet} Dependence Assays. Assays of tRNA methyltransferase activity were performed also at varying Ado-Met and/or tRNA^{Nfmet} concentrations. For these assays, Ado-Met concentrations ranged from 1.8 to 21 μM. tRNA^{Nfmet} concentrations ranged from 0.1 to 1.0 absorbance unit/tube. All other assay conditions remained the same as described under enzyme assays.

Base Specificity of tRNA Methylation. *E. coli* amino acid-specific tRNA (0.2 absorbance unit) was methylated under the same conditions described under enzyme assays. Samples were carried through the same NaCl extraction and washing procedures as described above. This methylated tRNA was hydrolyzed and analyzed by either a combination of high-voltage electrophoresis and paper chromatography or by high-performance liquid chromatography.

High-Voltage Electrophoresis and Paper Chromatography. The isolated tRNA was hydrolyzed with 0.4 N alkali or with venom phosphodiesterase, and the methylated base nucleotide content was determined by a combination of high-voltage electrophoresis and paper chromatography. Details of these procedures have been described in previous publications (30, 31).

High Performance Liquid Chromatography. tRNA was digested to nucleosides by a mixture of *E. coli* alkaline phosphatase and venom phosphodiesterase. Nucleosides were separated by reverse-phase chromatography on a Partisil 5 ODS-3 column (4.6 mm x 250 mm) (Whatman, Inc., Clifton, N. J.). Elution was carried out by using a 40-min linear gradient of 0–10% methanol in 0.005 M $NH_4H_2PO_4$ buffer, pH 4.8, followed by 20 min of 50% methanol:50% 0.005 M $NH_4H_2PO_4$, pH 4.8 (v/v). The flow rate was 0.9 ml/min, collecting 0.5-ml fractions. Absorbance was measured at 254 nm using a 24-μl UV flow cell. This procedure is a modification of the protocols described by Coward and Crooks (5).

RESULTS

The overall activities of liver tRNA methyltransferases of young adult male mice of the AK and B6 strains were assayed using mixed tRNAs from *E. coli* as substrate. Chart 1 shows the results of a typical experiment. The specific activity of the enzymes that transfer methyl groups were higher in AK mice than those of enzymes from mice of the B6 strain. The maximum methyl group transfer catalyzed by enzymes from livers of AK mice was observed to be about 35% higher than that of enzymes from B6 animals.

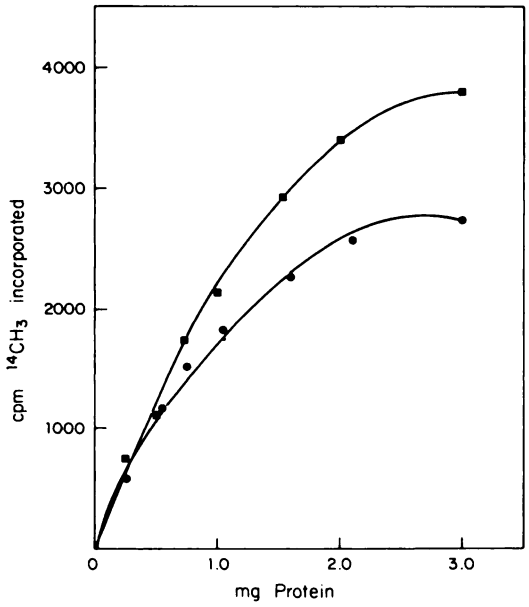

Chart 1. Methylation of mixed *E. coli* tRNA (25 μg/tube) catalyzed by mouse liver enzymes. ●, B6 enzyme; ■, AK enzyme. Assay conditions were as described in "Materials and Methods." The average coefficient of variation for *data points* shown here and in the other charts is 6%.

The activities of the liver enzymes were investigated further by using several amino acid-specific tRNAs from *E. coli* as substrates. When tRNA^{Nfmet} was the methyl group acceptor, the specific activity of the AK mouse liver enzymes was found to be approximately double those of the B6 mice (Chart 2). The 2-fold difference between the activities of enzymes from the 2 mouse strains was seen when Ado-Met concentration was varied over the range 1.8 to 21 μM (Table 1). This same 2-fold difference was observed when the amount of tRNA^{Nfmet} per sample was varied from 0.1 to 1.0 absorbance unit using enzyme extract containing 2 mg protein and an Ado-Met concentration of 5.2 μM (data not shown). The activities observed when AK and B6 enzyme extracts were mixed were found to be additive, with no evidence for inhibition. This was found in experiments using mixed tRNAs from *E. coli* and also in experiments using tRNA^{Nfmet} as acceptor. RNase activity was mea-

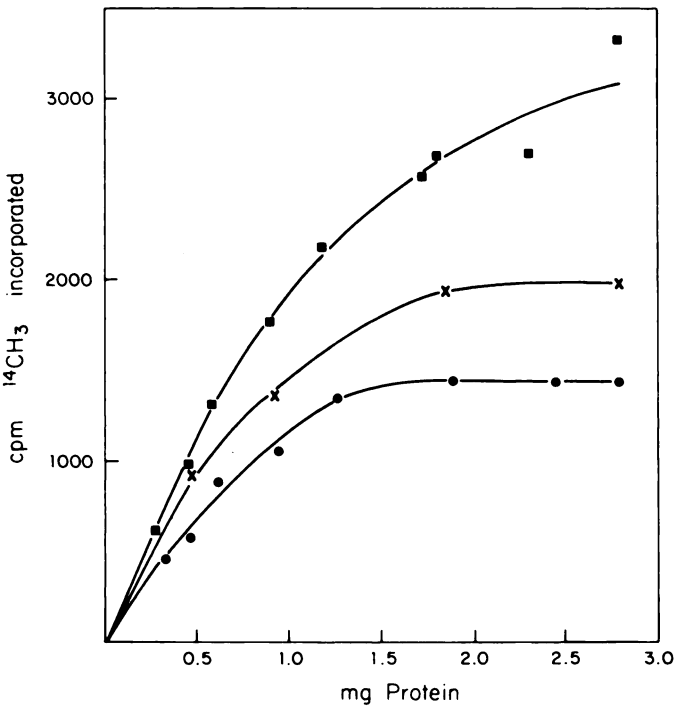

Chart 2. Methylation of *E. coli* tRNA^{Nfmet} (10 μg/tube) catalyzed by mouse liver enzymes. ●, B6 enzyme; ■, AK enzyme; ×, AKB6F$_1$ enzyme. Incubation was for 50 min at 35°.

Table 1

Methyl group transfer to E. coli tRNA^{Nfmet} catalyzed by mouse liver enzymes at various Ado-Met concentrations

Assay conditions were as described in "Materials and Methods" under "Ado-Met and tRNA^{Nfmet} Dependence Assays."

Ado-Met concentration (μM)	Sources of enzymes		Relative activity (AK/B6)
	AK (cpm)[a]	B6 (cpm)[a]	
1.8	843	326	2.6
2.7	1355	529	2.6
3.6	1775	791	2.2
5.2	2625	1147	2.3
10.5	4030	1671	2.4
15.7	4765	2253	2.1
21.1	5104	2539	2.0

[a] cpm transferred in 30 min at 35° to 10 μg tRNA^{Nfmet} by enzyme extract containing 2 mg protein.

sured in extracts of both strains and levels were found to be negligible.

Studies of enzyme activity using other tRNA substrates were carried out under the same conditions that resulted in maximum methyl group transfer to *E. coli* $tRNA^{Nfmet}$ as shown in Chart 2. The results of these experiments indicated that the relative activities of the liver tRNA methyltransferases from AK and B6 mice differed with the tRNA used as substrate (Table 2). The specific activities of the liver enzymes from AK mice that catalyze methyl group transfer to $tRNA^{Leu_2}$ as well as to $tRNA^{Nfmet}$ were seen to be double those of the enzymes from B6 mice. In contrast, there was little or no apparent difference in activity when $tRNA^{Tyr}$ acted as substrate.

The base specificity of methyl group transfer to these various amino acid specific tRNAs is shown in Table 3. $tRNA^{Nfmet}$ and $tRNA^{Leu_2}$ are virtually specific substrates for guanine methylation catalyzed by liver enzymes from mice of either the AK or B6 strains. Ninety % or more of the product of methylation of $tRNA^{Nfmet}$ catalyzed by the mouse liver enzymes is 2MeG. For $tRNA^{Leu_2}$, 86 to 91% of the product was recovered as 2MeG and 7MeG, while $tRNA^{Tyr}$ acted as a specific substrate for methylation of cytosine residues. The products of methyl group transfer to methyl-deficient mixed *E. coli* tRNAs were found to be 75 ± 5% (S.D.) methylated guanines, with the remaining 25% in other methylated bases.

These results indicate that the strain-specific differences seen in activities of liver tRNA methyltransferases of these mice represent selective differences in some of the tRNA-methylating enzymes with particular substrate specificities rather than overall higher activities of all the methylating enzymes in the AK strain. The quantitative data shown in Table 2 and the base analyses shown in Table 3 are consistent with a 2-fold difference between activities of N_2-guanine tRNA methyltransferase(s) of the 2 strains.

The activities of the tRNA methyltransferases from kidneys of B6 and AK mice were assayed using $tRNA^{Nfmet}$ as substrate. The data, shown in Chart 3, indicate that the activities of the kidney enzymes from AK mice are approximately twice as high as those from mice of the B6 strain. Ninety % of the product of these methylations was found to be 2MeG. These results which are similar to those obtained when the activities of liver tRNA methyltransferases were studied demonstrate that differences between the tRNA methyltransferase activities of these 2 mouse strains are not confined to the liver only.

The activity of enzymes from AKR × C57BL/6 animals, hereafter called $AKB6F_1$, was studied. The liver enzymes from a pooled sample of male F_1 progeny showed an activity level intermediate to that of the parental strains (Chart 2). The same intermediate levels were seen when activities from individual male or female $AKB6F_1$ animals were assayed. Comparisons of liver tRNA methyltransferase activities were made separately for each sex in order to eliminate the possible effects of hormones on the activities of these enzymes (18, 21, 28).

DISCUSSION

Species-specific differences in activities of tRNA methyltransferases were reported first by Srinivasan and Borek in 1963 and 1964 (22, 23), who observed the ability of enzymes from normal sources to catalyze methyl group transfer to some heterologous tRNAs but not to homologus tRNAs. Since that time, these observations have been confirmed in many laboratories where heterologous tRNAs have been used as substrates for *in vitro* studies of tRNA methyltransferase activities (4, 12, 13, 17). The isolation of bacterial mutants deficient in certain base-specific tRNA-methylating enzymes indicated the possibility for intraspecies variation in these enzymes (3, 34). Until now, however, it has not been known whether genetically determined intraspecies variations in the activities of these enzymes occur in mammals.

Because of the availability of their stable, reproducible genotypes (1, 24), inbred strains of mice seemed to offer a suitable system in which to look for variations in tRNA methyltransferases. We have compared the activities of tRNA methyltransferases from the AK and B6 strains of mice and found that mice of the AK strain exhibit high liver and kidney guanine tRNA

Table 2

Methyl group transfer to various E. coli tRNAs catalyzed by enzymes from mouse liver

Assay conditions were as described in "Materials and Methods" under "Enzyme Assays."

tRNA	Sources of enzymes: AK (cpm)[a]	Sources of enzymes: B6 (cpm)[a]	Relative activity (AK/B6)
Leu_2	2166	1077	2.0
Nfmet	2700	1450	1.9
Tyr	875	987	0.9

[a] cpm transferred in 50 min at 35° to 10 μg tRNA by enzyme extract containing 2 mg protein.

Table 3

Base specificities of methyl group transfer to several E. coli tRNAs catalyzed by mouse liver enzymes

tRNA	Enzyme source	% of total methylated nucleosides: 3MeC[a]	5MeC	2MeG	7MeG
Nfmet	B6			97	3
	AK			90	10
Leu_2	B6		14	64	22
	AK		9	81	10
Tyr	B6	9	91		
	AK	7	91	2	

[a] 3MeC, 3 methylcytidine; SMeC, 5 methylcytidine.

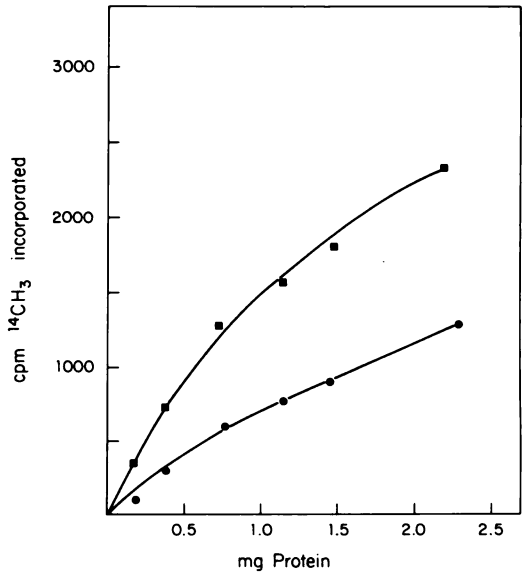

Chart 3. Methylation of *E. coli* $tRNA^{Nfmet}$ (10 μg/tube) catalyzed by mouse kidney enzymes. ●, B6 enzyme; ■, AK enzyme. Assay conditions were the same as described for liver enzymes. Incubation was for 50 min at 35°.

methyltransferase activity relative to those of B6 mice. By using mixed *E. coli* tRNAs to measure overall activity, *E. coli* $tRNA^{Nfmet}$ and $tRNA^{Leu_2}$ to measure guanine methylation, and $tRNA^{Tyr}$ for non-guanine-specific methylation, we have shown that a major portion of these strain-specific activity differences can be accounted for by altered activity of guanine tRNA methyltransferases.

The liver tRNA methyltransferase activity of F_1 progeny of a cross between AK and B6 mice showed activity intermediate to that of the parental types. The difference in tRNA methyltransferase activity between AK and B6 mice is consistent with additive inheritance.

Mice of the AK strain exhibiting high tRNA methyltransferase activity relative to B6 mice have a high incidence of spontaneous lymphatic leukemia (almost 100% at 1 year) while the B6 strain is noted for its low spontaneous tumor incidence (1, 24). It appears unlikely that the strain specific differences in tRNA methyltransferase activities are due to the presence of murine leukemia provirus since Gelb *et al.* (8) have shown that both of the mouse strains studied here carry similar amounts of murine leukemia virus-specific DNA per cell. However, at this time, it is not possible to exclude the influence of other viral factors.

We are investigating the possibility that the strain-specific differences in guanine tRNA methyltransferase activities between B6 and AK mice may be related to the strain-specific differences in susceptibility to cancer demonstrated by these animals. The activities of tRNA methylating enzymes obtained from hepatic tumors are higher than those of enzymes obtained from normal livers (20, 25). Increased activities of these enzymes have been found to occur also in response to carcinogens (2, 4, 6, 10, 11, 19, 26, 29, 31–33), several cancer promoters (16, 27, 29), and estrogens (18, 21, 28). As reported earlier, the increases in liver tRNA methyltransferase activities measured *in vitro* in each of these circumstances reflected primarily changes in guanine methylation and substrate specificity alterations that closely resembled those seen for the strain-specific differences reported here (26, 27, 28). Observations by Kuchino and Nishimura (15) and Glick *et al.* (9) indicate that there are several separate species of N_2-guanine tRNA methyltransferases with different substrate specificities in normal rodent liver. It seems possible to us that genetic determinants responsible for regulating activities of guanine-methylating enzymes and their relative resistance to change in response to certain stimuli may be factors in determining cancer susceptibility.

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