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Interaction of Normal and Tumor Transfer RNA Methyltransferases with Ethionine-induced Methyl-deficient Rat Liver Transfer RNA[1]

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SUMMARY

The tRNA methyltransferases from normal rat liver and Novikoff hepatoma have been compared with respect to their base specificity, capacity to methylate, and reaction kinetics, using mixed *Escherichia coli* B transfer RNA (tRNA) and ethionine-induced partially methyl-deficient rat liver tRNA.

The pattern of base methylation of the two substrates is different with the use of enzymes from either source. In particular, N^1-methylguanine methylation is much greater in the methyl-deficient rat liver tRNA. The enzymes from the two sources also show differences in specificity of base methylation in either substrate, particularly in the percentage of N^2-methylguanine synthesized.

The Novikoff hepatoma enzymes have a greater capacity for methylation with either type of tRNA than do rat liver enzymes.

The methyl-deficient rat liver tRNA is a poorer substrate for the enzymes from both sources than is *E. coli* B tRNA in terms of rate of methylation as well as total acceptance of methyl groups. The affinity constants are somewhat higher for the methyl-deficient rat liver tRNA than for *E. coli* B tRNA. The Novikoff hepatoma enzymes, in general, have larger affinity constants than the rat liver enzymes.

Maximal velocities for the various base-specific enzymes are lower with the methyl-deficient rat liver tRNA, with the exception of the 1-methylguanine specific enzymes. These enzymes from either rat liver or Novikoff hepatoma exhibit approximately a 2.5-fold greater maximal velocity with methyl-deficient rat liver tRNA.

INTRODUCTION

The tRNA methyltransferases, a family of enzymes that modify the structure of preformed tRNA by the insertion of methyl groups, were first shown to be aberrant in a number of tumor tissues by Tsutsui *et al.* (9). Their results have been confirmed and extended to over 35 different neoplasms (2, 9).

In most instances, the studies of tumor cell enzymes have been performed with crude extracts measuring the total capacity of these extracts to incorporate methyl groups into a heterologous substrate. The crude extracts of various tumors could introduce anywhere from a 2- to a 10-fold greater number of methyl groups into tRNA, compared with the closest normal counterpart of the neoplastic tissue. Another method of characterizing the tRNA methyltransferases is by the pattern of base specificities exhibited by the enzymes from a particular tissue. By this parameter, tumor enzymes again differ from those of the normal counterparts (2, 4, 8).

A 3rd manner in which to delineate the enzyme specificities is to measure their activity, using specific tRNA species. For instance, normal homologous tRNA will not serve as a substrate for the enzymes from the same tissue. Mittelman *et al.* (4) have shown that the tRNA methyltransferases from an SV40-induced tumor of hamsters could hypermethylate the host hamster homologous tRNA. This is further evidence of qualitative changes in the specificities of the enzymes from tumor cells.

Sharma (8) has compared the tRNA methyltransferases from normal rat liver and Novikoff hepatoma both in total extracts and in fractions separated by hydroxylapatite column chromatography. Transfer RNA's from a variety of sources, as well as individual purified tRNA species, were tested as substrates. The enzymes from Novikoff hepatoma showed both quantitative and qualitative differences when tested against the various types of tRNA.

One of the uncertainties involved in all these assays was that homologous tRNA, presumably the natural substrate, could not be used. It has been questioned whether the differences observed would be seen if the natural tRNA substrate were used.

Rajalakshmi (7) has developed a method for inducing the synthesis of methyl-deficient mammalian tRNA by the administration of ethionine to rats. Even with natural submethylated tRNA as a substrate, she found differences in the enzymes from normal rat liver and from ethionine-induced tumors of rat liver. We have found that the differences observed between the enzymes from Novikoff hepatoma and rat liver are still apparent when using the methyl-deficient rat liver tRNA. We report here on these findings, including some kinetic parameters of the enzymes from the 2 sources.

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MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade. [*methyl*-^{14}C]-*S*-Adenosyl-L-methionine (54 mCi/mmole) was purchased from International Chemical and Nuclear Corporation, Irvine, Calif. Standard methylated purines were obtained from Cyclo Chemical Company, Los Angeles, Calif.

Animals. Female Holtzman rats (120 g) were obtained from Holtzman Farms, Madison, Wis. Novikoff hepatoma was grown on the omentum of the rats and carried by serial transplantation every 8 to 10 days.

Preparation of tRNA. Methyl-deficient rat liver tRNA was induced in female Holtzman rats by the method of Rajalakshmi (7). The tRNA was isolated from organs of treated rats by the procedure of Brunngraber (3) and further purified by gel filtration on Sephadex G-100. Commercially available *E. coli* B tRNA (General Biochemicals, Inc., Chagrin Falls, Ohio) was also subjected to gel filtration on Sephadex G-100 to eliminate high-molecular-weight contaminants. One A_{260} nm unit of tRNA was assumed to be 2×10^{-9} mole.

Preparation of Enzyme Extracts. Livers from untreated rats or Novikoff hepatoma were homogenized in 6 volumes of 0.01 M Tris-HCl-0.01 M NaCl-0.0015 M $MgCl_2$-0.001 M β-mercaptoethanol, pH 7.4. The homogenates were centrifuged successively at 30,000 $\times$ *g* for 15 min and at 105,000 $\times$ *g* for 60 min. The high-speed supernatant extracts were brought to pH 5.0 by the addition of 1 N acetic acid. The extracts were centrifuged at 10,000 $\times$ *g* for 10 min and the supernatant was discarded. The precipitate was extracted with 2 volumes of 0.05 M potassium phosphate-0.001 M β-mercaptoethanol, pH 6.0. The extract was recentrifuged at 30,000 $\times$ *g* for 10 min and the residue was discarded. The supernatant served as the source for tRNA methyltransferase activity. At least 85 to 90% of the enzyme activity is recovered in this procedure which also results in a 4- to 6-fold purification. All operations were carried out at 4°, and all enzyme extracts were prepared immediately before use and not stored in any manner in order to avoid loss of enzymatic activity.

Enzyme Assays. The standard assay mixture contained 50 mM Tris-HCl (pH 8.2)-5 mM spermidine-1 mM dithiothreitol-20 μM [*methyl*-^{14}C]-*S*-adenosyl-L-methionine, and varying amounts of tRNA and enzyme extract in a total volume of 0.2 ml. After incubation at 37° for varying times, the reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid. The insoluble material was collected by filtration through glass fiber filters (Whatman GF/C, 2.4 cm in diameter) after 10 min at 4°. The filters were washed with 5% trichloroacetic acid, air dried at 80°, and counted in a liquid scintillation counter.

For base analyses, the reaction mixtures were scaled up to 1.0 ml and the reaction was terminated by the addition of an equal volume of water-saturated phenol, 0.1% 8-quinolinol. Commercial *E. coli* B tRNA (300 μg) was added as carrier and the tRNA was precipitated from the aqueous phase with 2 volumes 95% ethanol. The precipitate was washed with 95% ethanol and dried in a vacuum. The tRNA was redissolved and hydrolyzed in 1 N HCl at 100° for 30 min. The hydrolysates were subjected to 2-dimensional thin-layer chromatography according to the method of Bjork and Svensson (1). The radioactive derivatives were located by autoradiography, scraped into scintillation vials, and counted.

A unit of tRNA methyltransferase activity is defined as the amount of enzyme that incorporated 1 pmole of ^{14}C-labeled methyl group into tRNA per 15 min.

RESULTS

Induction of Methyl-deficient tRNA. The tRNA was isolated from the livers of 3 individual rats that had been treated with ethionine and adenine according to the procedure of Rajalakshmi (7). The tRNA preparations, when tested against normal rat liver enzyme, showed equivalent levels of methyl acceptor activity, so the 3 preparations were combined for use in further experiments.

Other organs were also affected by the ethionine treatment. tRNA isolated from pancreas and kidney also showed methyl acceptor activity with homologous enzymes, although at a lower level than liver tRNA (30% and 20%, respectively, of the level reached with treated liver tRNA).

Specific Activity and Extent of Methylation. The activities of the pH 5 enzyme preparations from rat liver and from Novikoff hepatoma were compared, using both *E. coli* B tRNA and the methyl-deficient rat liver tRNA as substrates (Table 1). It can be seen that the enzymes from Novikoff hepatoma exhibit a greater specific activity than the rat liver enzymes toward both *E. coli* B tRNA and methyl-deficient rat liver tRNA, although the magnitude of difference is not as great with methyl-deficient rat liver tRNA as substrate.

In capacity to methylate, that is, when the tRNA substrate is limited in order to determine the absolute level of methylation, the enzymes from both sources approach the same level in *E. coli* B tRNA. However, the tumor enzymes can insert almost twice as many methyl groups into the methyl-deficient rat liver tRNA than can the homologous rat liver enzymes.

A comparison of the 2 types of tRNA substrate shows that, both in terms of reaction rate and total acceptance of methyl groups, the bacterial tRNA is a more receptive substrate for the enzymes from both sources.

Base Specificity. Base analyses of the enzymatically methylated tRNA's were carried out to determine which derivatives were synthesized by the methyltransferase preparations from liver and Novikoff hepatoma (Table 2). The greatest difference is seen in the N^1-methylguanine pattern where the percentage of incorporation is 5-fold greater into rat liver methyl-deficient tRNA than into *E. coli* B tRNA, using enzymes from either source. There is also the appearance of N^7-methylguanine which is not detected in the *E. coli* B tRNA.

The base patterns synthesized by the enzymes from the 2 sources, liver and hepatoma, also vary. The differences in the percentage of N^2-methylguanine and methylcytosine synthesized in tRNA of either type are the most obvious.

Kinetic Analysis. Kinetic measurements were carried out

Table 1

Methylation of E. coli B tRNA and methyl-deficient rat liver tRNA by enzymes from rat liver and Novikoff hepatoma

Values are the averages of the results from assays using 4 separate tumors and 4 individual livers. For specific activity, assays were incubated with 50 μg tRNA for 15 min. For extent of methylation, assays were incubated with 0.5 μg of *E. coli* B tRNA or 2 μg of methyl-deficient rat liver tRNA for 120 min.

	E. coli B tRNA		Methyl-deficient rat liver tRNA	
	Specific activity (pmoles/15 min/mg)	Extent (moles/mole)	Specific activity (pmoles/15 min/mg)	Extent (mole/mole)
Rat liver	195	0.94	65	0.09
Novikoff hepatoma	1400	1.10	252	0.16

Table 2

Base specificities of enzymes from rat liver and Novikoff hepatoma

Analyses of ^{14}C-methylated tRNA were carried out by previously published procedures (1). The results are presented as percentage radioactivity recovered and are the averages of 4 separate experiments with each tissue.

	% base						
	mUra	mCyt	m^1Ade	m^1Gua	m^2Gua	m_2^2Gua	m^7Gua
Rat liver							
E. coli B tRNA	0.96	13.7	15.1	5.3	49.7	14.5	
met⁻ RL tRNA[a]	1.1	19.2	16.5	25.2	25.5	8.4	4.3
Novikoff hepatoma							
E. coli B tRNA	0.3	7.2	16.8	5.1	60.8	10.1	
met⁻ RL tRNA	0.25	8.7	9.5	25.2	45.0	8.1	2.9

[a] met⁻ RL tRNA, methyl-deficient rat liver tRNA.

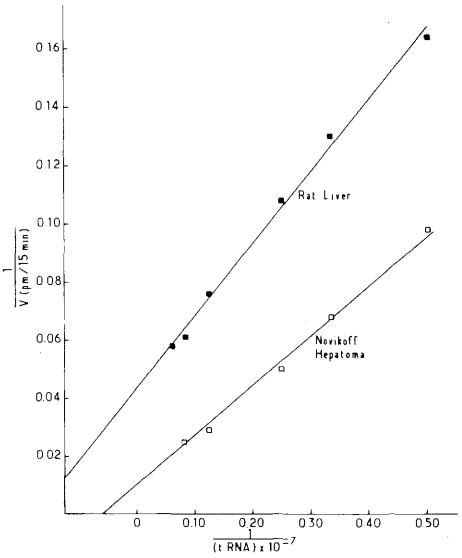

Chart 1. Lineweaver-Burk plots for the methylation at 37° of *E. coli* B tRNA by pH 5 enzymes from rat liver and from Novikoff hepatoma. Experimental conditions are described in "Materials and Methods." *Points*, average initial rate of triplicate reactions. The *lines* were fitted to the points by the method of least squares using a Smith-Corona Marchant 1016 PR computer with an IOTA-1 programmer.

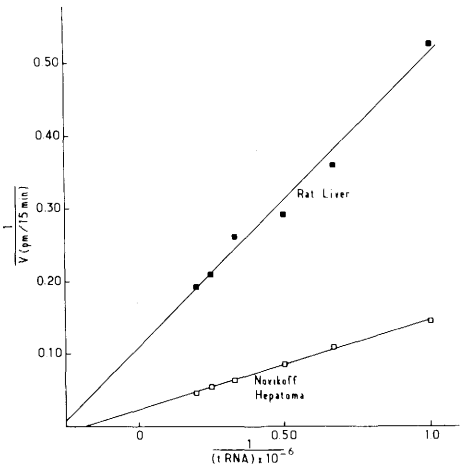

Chart 2. Lineweaver-Burk plots for the methylation at 37° of methyl-deficient rat liver tRNA by pH 5 enzymes from rat liver and from Novikoff hepatoma. Details as in Chart 1.

and Michaelis-Menten constants were calculated in order to measure the relative affinities of the enzymes for the 2 types of tRNA. This was done both as an average of the total mixture of enzymes from either source (Charts 1 and 2) and

also for the various base-specific enzymes within the preparations.

The results are presented in Table 3. All the K_m's are in the micromolar range. In the case of the enzymes from Novikoff hepatoma, the values obtained with rat liver methyl-deficient tRNA are consistently higher than when *E. coli* B tRNA was used as substrate. With the rat liver enzymes, this appears to be the case also with the total enzyme extract, but the various base-specific enzymes do not show a uniform trend.

It is apparent that the K_m values for the methylation of the individual bases do not bear a consistent relationship to the K_m value for methylation of total tRNA. This may stem from 2 sources. Losses of methylated derivatives during hydrolysis and chromatography may not be equivalent. Also, only total tRNA concentration was used to calculate the values, because the actual number of sites for individual base methylations in the bulk tRNA is unknown. The values obtained thus represent an approximation of the affinity of particular base-specific enzymes toward total tRNA.

From the calculation of V_{max} (Table 4), it is seen again that the homologous methyl-deficient rat liver tRNA reacts with the enzymes from either source more slowly than does the heterologous *E. coli* B tRNA. There is one exception. The N^1-methylguanine-specific enzymes from both liver and tumor react at a 2.5-fold greater rate with the rat liver tRNA.

For total enzymes and the other base-specific enzymes, the differences in V_{max} are variable. For rat liver enzymes, the rates show a 2- to 15-fold greater velocity with *E. coli* B tRNA. With the Novikoff hepatoma enzymes, the rates do not vary quite so much, showing differences in the range of 2.5- to 6-fold greater velocity with *E. coli* B tRNA.

A comparison of the maximal velocities of the enzymes from the 2 sources is not particularly useful, as these are relative measurements on crude preparations, and the exact concentrations of the various enzymes in the extracts are not known. The higher V_{max} values obtained with Novikoff hepatoma extracts probably reflect their greater specific activity.

DISCUSSION

We have investigated some of the interactions of the tRNA methyltransferases from normal and tumor tissue with methyl-deficient homologous mammalian tRNA as compared to their interactions with tRNA from a heterologous bacterial source.

One of the significant findings is that the abnormal activity exhibited by tumor enzymes with *E. coli* B tRNA as a substrate is also seen when methyl-deficient mammalian tRNA is used as substrate. Thus, the previous findings with many other tumors (2) is not merely an artefact caused by the use of an alien substrate.

A 2nd result is that the methyl-deficient rat liver tRNA is actually a poorer substrate in many respects than is the heterologous bacterial tRNA for mammalian enzymes. There are several possible reasons for this. First, the preparation of tRNA from the ethionine-treated rat liver of necessity contains at least 50% of previously synthesized, normally methylated tRNA, and inhibition of methylation by ethionine may not be complete. Thus, the tRNA obtained is probably less than 50% methyl deficient and is a

Table 3

K_m values for enzymes from rat liver and Novikoff hepatoma

Values were derived from Lineweaver-Burk plots of kinetic assays carried out in triplicate, as shown in Charts 1 and 2. Values for total tRNA are the mean ± S.E. of 4 experiments, while those for specific methylated derivatives are the averages of 2 experiments. Abbreviation is as in Table 2.

		K_m (μM)					
Enzyme source	tRNA	Total tRNA	mCyt	m^1Ade	m^1Gua	m^2Gua	m_2^2Gua
Rat liver	*E. coli* B	0.75 ± 0.09	1.22	0.45	1.11	0.92	0.59
	met⁻ RL	3.61 ± 0.42	1.70	1.45	1.57	0.78	2.55
Novikoff hepatoma	*E. coli* B	1.28 ± 0.14	0.83	0.40	0.50	0.95	0.68
	met⁻ RL	4.94 ± 0.68	1.90	2.18	2.55	3.00	2.00

Table 4

Maximal velocities of enzymes from rat liver and Novikoff hepatoma

Details are as in Table 3.

		V_{max} (pmoles/15 min/mg protein)					
Enzyme source	tRNA	Total tRNA	mCyt	m^1Ade	m^1Gua	m^2Gua	m_2^2Gua
Rat liver	*E. coli* B	298 ± 32	95.5	24.1	9.1	136	27.2
	met⁻ RL	85 ± 4	21.8	13.1	23.0	8.7	7.3
Novikoff hepatoma	*E. coli* B	2375 ± 293	326	242	58	1222	249
	met⁻ RL	442 ± 25	90	84	135	222	25

mixture of normal and partially methyl-deficient tRNA, and it is not the naked macromolecule the enzymes encounter in the cell. This makes interpretation of the higher K_m's obtained with the rat liver methyl-deficient tRNA ambiguous. Every tRNA species within the bulk tRNA is not a substrate for methylation. Thus, the apparent concentration of substrate used is not the actual substrate concentration. An apparent increase in K_m need not signify a lower affinity but could also be caused by a decrease in the number of tRNA species recognized.

This argument does not apply to the differences observed in V_{max} between the 2 substrates, because these values are essentially independent of apparent substrate concentration as they are extrapolated to infinite concentration. The slower reaction observed with the methyl-deficient rat liver tRNA may be due to factors such as the state of tRNA maturation, that is, the natural substrate may be some sort of larger precursor, or it may stem from the need for base-specific sequential modifications.

That the only base-specific enzyme which increases in reaction rate with the methyl-deficient rat liver tRNA is the N^1-methylguanine-specific enzyme activity may be an indication that this enzyme is more sensitive to inhibition by *S*-adenosylethionine *in vivo* than are the other base-specific enzymes and, as a result, the tRNA has a relatively greater number of sites for N^1-guanine methylation. Presumably, ethionine treatment induces methyl deficiency through the formation of *S*-adenosylethionine which has been shown previously to inhibit these enzymes *in vitro* (5, 6).

These studies will have to be extended, using purified enzyme fractions and, if possible, purified methyl-deficient mammalian tRNA species before any final conclusions on the interaction of mammalian tRNA methyltransferases with their homologous tRNA can be drawn. However, even with these first indications, alterations in the enzymes in tumor tissue are apparent, inviting further studies.

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