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Differences in *in Vivo* Methylation Patterns of Tyrosine and Histidine Transfer Ribonucleic Acids from Rat Liver and Novikoff Hepatoma†

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ABSTRACT: The methylation of tRNA from rat liver and Novikoff hepatoma has been studied using *in vivo* labeling of the tRNAs with [*Me*-^{3}H]methionine. Tyrosine tRNA and histidine tRNA have been purified from both tissues by means of chromatography on DEAE-Sephadex and benzoylated DEAE-cellulose. Essentially no difference in the methylation of unfractionated tRNAs from these two tissues was found. However, a clear difference in the methylated base patterns appeared when tyrosine tRNA and histidine tRNA from Novikoff hepatoma were compared to the same specific tRNAs from normal rat liver. The possible correlation of these findings with previous observations on alterations in chromatographic profiles of tRNAs (Baliga, B. S., Borek, E., Weinstein, I. B., and Srinivasan, P. R. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 899) and in tRNA methyltransferase activity (Sharma, O. K. (1973), *Biochim. Biophys. Acta 299*, 415) in those tissues is discussed.

A high level of tRNA methyltransferase activity has been observed in all tumor tissues which have been studied up to now (for a review, see Borek and Kerr, 1972). However, there is no conclusive evidence concerning the actual level of methylation in tumor tRNA. Some authors reported a very high content of methylated bases in tRNA from neoplastic tissues (Bergquist and Matthews, 1962; Craddock, 1969; Viale *et al.*, 1967). Other investigators found qualitative differences in the methylation patterns of tumor tRNAs (Inose *et al.*, 1972). Analysis of tRNA from tumor cell in culture (Iwanami and Brown, 1968) or from brain tumors (Randerath *et al.*, 1971) failed to demonstrate any significant variation, either qualitative or quantitative, of the methylation patterns. It thus appears that the activity of the tRNA methyltransferase has no direct relationship with the overall level of methylation of the tRNAs.

On the other hand, many investigators have reported alterations in chromatographic patterns of tumor specific tRNAs compared to their normal counterparts. (See, for instance, Taylor *et al.*, 1967; Gonano *et al.*, 1971; Srinivasan *et al.*, 1971; Hayashi *et al.*, 1973.) In each case, the differences, either quantitative or qualitative or both, were restricted to a few tRNA species; most specific tRNAs showed the same chromatographic profiles in tumor and in normal tissue.

Since it is known that methylation may alter the chromatographic behavior of a tRNA (Capra and Peterkofsky, 1968), there is a possibility that some of the alterations observed in tumor tRNAs correspond to modifications in their methylation pattern. According to this hypothesis, the elevated tRNA methyltransferase activity in tumors would not be correlated to an overall hypermethylation, but rather to aberrant methylation of some specific tRNAs; this would not give rise to significant differences when methylation of bulk tRNA is examined.

In order to test this hypothesis, we decided to analyze the methylation of tyrosine tRNA and histidine tRNA, whose chromatographic profiles on MAK columns[1] have been shown to differ in normal rat liver and in Novikoff hepatoma (Baliga *et al.*, 1969). The tRNA was labeled *in vivo* with [*Me*-^{3}H]-methionine, the specific tRNAs were isolated, and their methylated base content was determined. This system was chosen for several reasons. First, Novikoff hepatoma is a very fast growing tumor, where high label incorporation may be expected. Second, the only specific tRNAs which differ significantly in this tumor from the normal ones are tyrosine,

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[1] Abbreviations used are: MAK, methylated albumin kieselguhr; BD-cellulose, benzoylated DEAE-cellulose.

histidine, and asparagine tRNAs: the former two are very easy to separate by DEAE-Sephadex chromatography (Nishimura and Weinstein, 1969). Asparagine tRNA, whose purification is much more difficult, was not analyzed. Finally, tyrosine tRNA is of special interest, since it has been shown to vary in many tumors and differentiating tissues, while its chromatographic profile is remarkably constant in normal adult organs from different mammalian species (Holland *et al.*, 1967).

Materials and Methods

Novikoff hepatomas were grown on the peritoneum of young rats (*ca.* 100 g) of the Holtzman strain.

In Vivo Labeling. Four days after tumor transplantation the animals were fasted for 12 hr. Then they received, at 2-hr intervals, two intraperitoneal injections of 0.5 mCi of [*Me*-^{3}H]-methionine (Schwarz BioResearch, 2.5 mCi/mmol), in 1 ml of 0.5 M sodium formate. The rats were killed 2 hr after the last injection, the hepatomas and the livers were rapidly removed, and the tRNA was extracted immediately.

In Vivo Labeling of Liver Slices. Livers from normal animals of the same strain were rapidly removed and cut into thin slices (~1 mm thick) with a razor blade. This operation was carried out in the cold room.

The slices were incubated in 250-ml bottles containing 20 ml of Krebs–Ringer phosphate medium (Cohen, 1959) supplemented with sodium formate (10 mM final concentration), adenosine and guanosine (15 mg/100 ml each), and 0.1 mCi/ml of [*Me*-^{3}H]methionine (C.E.A., France; 2 Ci/mmol). Each bottle received about 3 g of tissue slices. The medium was previously saturated with pure oxygen, and additional oxygen flushings were made at the beginning of the incubation, after 90 min and after 3 hr.

The incubation was carried out in a water bath at 37°, with gentle shaking. After 4 hr, the medium was removed, the slices were rinsed once with cold medium, and tRNA was extracted immediately.

The extraction of tRNA was performed by the method described by Rogg *et al.* (1969) with only slight modifications. The phenol extraction was carried out without bentonite and in the presence of 0.5% sodium dodecyl sulfate. Since relatively small amounts of material were available, the tissues were homogenized using a glass-Teflon Potter-Elvehjem homogenizer, and the DEAE-cellulose chromatography was performed in a column instead of batchwise.

Upon gel filtration on Sephadex G-100, the purified tRNA eluted as a symmetrical peak, essentially free of high and low-molecular weight ultraviolet (uv) absorbing material.

DEAE-Sephadex chromatography was performed as described by Nishimura and Weinstein (1969). The columns (120 × 0.75 cm) were equilibrated with 0.02 M Tris-HCl (pH 7.5), 0.008 M $MgCl_2$, and 0.375 M NaCl (buffer A). tRNA (500–700 A_{260} units) was dissolved in buffer A and applied to the column. The elution was carried out at room temperature with a linear salt gradient, using 400 ml of buffer A in the mixing chamber, and 400 ml of 0.02 M Tris-HCl (pH 7.5), 0.012 M $MgCl_2$, and 0.450 M NaCl in the reservoir. The flow rate was 10 ml/hr, and the volume of the fractions was 8 ml.

BD-Cellulose Chromatography. The columns (10 × 0.5 cm) were operated at room temperature. A peristaltic pump was used in order to obtain a flow rate of 4 ml/hr. Fractions (1 ml) were collected.

Two sets of elution conditions were used: (a) equilibration in 0.05 M sodium acetate (pH 5.0)–0.4 M NaCl; elution with a linear gradient (100 ml) of 0.4–1.0 M NaCl in the same buffer; (b) equilibration in 0.05 M sodium acetate (pH 4.0), 0.4 M NaCl, and 0.010 M $MgCl_2$; elution with a linear gradient (100 ml) 0.4–1.25 M NaCl in the same buffer.

Aminoacyl-tRNA synthetases were obtained by homogenization of rat livers in 2 vol of 0.1 M Tris-HCl (pH 7.2), 0.005 M $MgCl_2$, 0.005 M KCl, 0.02 M 2-mercaptoethanol, and 15% glycerol. After centrifugation at 30,000*g* for 30 min and at 105,000*g* for 90 min, the supernatant was made up to 50% in glycerol and kept in small vials at −20°. This preparation was used as a source of aminoacyl-tRNA synthetases.

Aminoacylation was performed in 0.1–0.3 ml of reaction mixture containing 0.1 M sodium cacodylate (pH 7.0), 0.012 M $MgCl_2$, 0.008 M Na_2ATP, 0.010 M KCl, 0.005 M reduced glutathione, 0.05–0.1 mM ^{14}C-labeled amino acid (sp act. 40–60 mCi/mol), 0.010–0.030 ml of liver extract, and 0.05–0.2 ml of column eluate. This mixture was incubated for 30 min at 37°; then carrier RNA (0.5 mg) was added, together with cold 5% trichloroacetic acid. The precipitates were collected onto Whatman GF/C glass fiber filters, washed with cold 5% trichloroacetic acid and then with 95% ethanol, dried, and counted in a scintillation spectrometer (Nuclear-Chicago or Intertechnique).

Since the tRNA was already tritium labeled, channel settings were used which eliminated completely the tritium counts. Under those conditions, counting efficiency for ^{14}C was about 20%.

Analysis of Methylated Bases. The tRNA, supplemented with a mixture of unlabeled methylated bases, was hydrolyzed with 70% perchloric acid for 60 min at 100°. Free bases are released by this procedure, and O-methylated ribose is converted into insoluble products which are eliminated during the neutralization step. The overall recovery of radioactivity in bases was determined by submitting a sample of ^{3}H-labeled thymine to the whole procedure, in the same conditions as the tRNA. It was found to vary between 55 and 65%.

The bases were then separated by cellulose thin-layer two-dimensional chromatography, as described previously (Nau *et al.*, 1972). The total radioactivity recovered on the plate was about 22% of the input. This was due to quenching by the cellulose, since no radioactivity could be found outside the uv-absorbing spots.

Results

In Vivo Labeling. In a typical experiment, from a batch of 24 rats, we extracted 660 A_{260} units of tRNA from the hepatomas, with a specific radioactivity of 9000 cpm/A_{260} unit, and 510 A_{260} units of tRNA from the livers, with a specific radioactivity of 1800 cpm/A_{260} unit. This ratio of approximately 5 between the specific radioactivities of hepatoma and liver tRNA was very constant; it was found also in some experiments where we used ^{14}C-labeled methionine.

The specific radioactivity thus obtained in the liver tRNA of the same animals is too small for analysis of methylated bases by thin-layer chromatography. For this reason labeled tRNA was prepared by incubating liver slices. Under these conditions, we could reach a specific radioactivity of 3900 cpm/A_{260} unit.

Purification of Tyrosine and Histidine tRNA. The method of purification we followed was exactly the same for liver and for hepatoma tRNA, and the results were identical. Figure 1 shows the first step of the purification, which consists of DEAE-Sephadex chromatography, according to Nishimura and Weinstein (1969). Tyrosine and histidine tRNAs elute as

TABLE I: Relative Content of Methyl Groups in *in Vivo* Methylated tRNAs.

	Before Hydrolysis[a]			After Hydrolysis[b]		Methyl Group Distribution	
	A_{260} Units	cpm[c]	cpm/A_{260} Unit	cpm[d]	cpm/A_{260} Unit	In Bases (%)	In Ribose (%)
Liver							
Total tRNA	5.8	21,840	3765	14,060	2424	64	36
Tyr tRNA fraction	2.9	6,865	2370	6,092	2100	89	11
His tRNA fraction	1.4	6,280	4490	3,561	2543	57	43
Hepatoma							
Total tRNA	6.1	51,590	8460	34,560	5680	67	33
Tyr tRNA fraction	1.85	10,590	5720	9,030	4881	85	15
His tRNA fraction	0.9	6,915	7680	3,300	3666	48	52

[a] Radioactivity of methylated bases *and* methylated ribose. [b] Radioactivity of methylated bases only. [c] Corrected for tritium incorporation into the purine rings. [d] Total radioactivity recovered after thin-layer chromatography, corrected for quenching and for losses during hydrolysis.

sharp peaks, and are completely separated from each other. Fractions 20–32 and 50–60 were pooled. tRNA was recovered by ethanol precipitation; then each fraction was submitted to BD-cellulose chromatography at pH 5.0 (condition a). The most active fractions were pooled and chromatographed again on BD-cellulose, at pH 4.0 in the presence of 0.01 M $MgCl_2$ (condition b). The two or three fractions with the highest specific activity were pooled and dialyzed extensively against water. They were used to determine specific radioactivity and methylated base content. In Table I are shown the amounts of tRNA thus obtained, as well as their radioactivity.

Purity of the Fractions. Due to the very small amount of material we obtained after fractionation, it was impossible to measure the amino acid acceptor activity of the fractions. In order to determine a minimal value for their purity, we pooled the two tubes immediately preceding and the two tubes immediately following each peak. These fractions had a rather poor amino acid acceptor activity, ranging between 165 and 360 pmol of amino acid per A_{260} unit. This is probably due to two factors: inactivation of the tRNA during the purification procedure, which was relatively lengthy and involved some drastic concentration steps, and assay conditions which were far from optimal (the concentration of the tRNA in the reaction mixture was always very low and a crude liver extract was used as a source of aminoacyl-tRNA synthetases).

However, for each fraction, the radioactive amino acid incorporation was exactly the same when tested with either [^{14}C]tyrosine (or [^{14}C]histidine) alone, or with a mixture of 15 ^{14}C-labeled amino acids containing tyrosine and histidine at the same specific activity. Furthermore, in the last chromatographic steps, there was a good coincidence between the absorbance and the amino acid acceptor activity profiles. Finally, the methylated base compositions of the purified fractions were completely different from each other, and from total tRNA methylated base patterns; this could not be expected from a mixture of several tRNA species.

We feel therefore that these three independent sets of data provide good evidence that the fractions we studied were essentially free of major contamination by other tRNA species. There is still the possibility of some contamination by inactivated and/or degraded tRNAs. This might account for the fact that we do not obtain whole numbers for methylated base patterns, as will be discussed later.

Methylated Ribose Content. Table I presents an estimation of the methylated ribose content of the different tRNA fractions. It is obtained very simply by assuming that the radioactivity which is lost during the hydrolysis (after correction for the incomplete recovery of methylated bases) corresponds to the destruction of ribose moieties. It can be seen that the methyl groups in ribose may account for 10–50% of the total methyl group content, depending upon the tRNA.

Specific Radioactivity of the Purified tRNA Fractions. Table I includes also the specific radioactivity of the tRNAs, which reflects the total number of methyl groups per molecule. No quantitative comparison can be made between tRNAs from liver and from hepatoma, since the labeling conditions were different for each tissue. However, it can be seen that large differences exist between specific tRNA fractions and total tRNA in both cases. Their significance will be discussed later.

Methylated Base Content. The results shown in Table II are expressed as percentages of total radioactivity found in methylated bases. Some radioactivity was found in adenine and guanine (about 5% of the total counts). It was subtracted from total radioactivity, but no correction was made for tritium incorporation into the purine rings of the methylated bases.

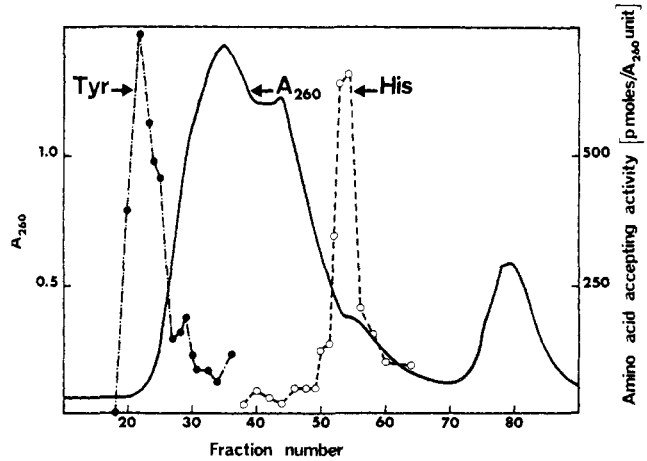

FIGURE 1: DEAE-Sephadex chromatography of Novikoff hepatoma tRNA (660 A_{260} units). See text for conditions. Fractions 20–32 and 50–60 were used for further purification of tyrosine tRNA and histidine tRNA, respectively.

TABLE II: Methylated Base Composition of tRNAs from Normal Liver and Novikoff Hepatoma.[a]

Methylated Base	Liver			Novikoff Hepatoma		
	Total tRNA	Tyr tRNA Fraction	His tRNA Fraction	Total tRNA	Tyr tRNA Fraction	His tRNA Fraction
1-Methyladenine	17.9 ± 0.4	15.0	20.4	18.5 ± 1.7	15.5	21.4
1-Methylguanine	0	0	0	0.2 ± 0.2	3.9	0
N_2-Methylguanine	15.4 ± 0.7	15.25	12.9	13.7 ± 0.8	14.4	14.0
N_2,N_2-Dimethylguanine	21.7 ± 0.3	27.9	11.4	21.4 ± 0.3	35.0	16.3
7-Methylguanine	4.8 ± 0.5	14.3	6.9	6.7 ± 0.8	15.3	8.4
1-Methylhypoxanthine	2.0 ± 0.1	2.9	0	2.7 ± 0.5	3.2	0
5-Methylcytosine	29.3 ± 0.1	17.3	41.1	27.2 ± 1.0	8.8	31.6
Thymine	9.0 ± 0.5	7.7	6.9	9.9 ± 0.4	4.1	6.5
Total radioact (cpm)	1828	792	463	4573	1174	429

[a] Data are expressed as percentages of the total radioactivity found in methylated bases.

Three separate determinations were made with total tRNA, and only one with each tRNA fraction (except for the tyrosine tRNA fraction from liver where we obtained enough material to perform two analyses: their results were identical).

Discussion

After *in vivo* labeling, the specific radioactivity of tRNA was considerably higher in hepatoma than in liver. However, no indication can be drawn from this observation concerning the actual level of methylation, in terms of the number of methyl groups per molecule. The specific radioactivity of a tRNA depends upon at least four factors: the number of methyl groups per molecule, the specific activity of the *S*-adenosylmethionine pool in the tissue, the rate of tRNA synthesis, and the activity of the tRNA methyltransferases. Each of these factors may vary independently in different tissues.

On the other hand, a comparison between different tRNAs from the same tissue is valid. In liver as well as in hepatoma, the specific radioactivity of the tyrosine tRNA fraction is much lower than the specific radioactivity of total tRNA (see Table I). This correlates very well with the low amount of methylated ribose (10–15% of the total methyl groups) in this fraction as compared to the average (about 35%). However, the specific radioactivities after hydrolysis are about the same, which means that the tyrosine tRNA fraction contains a number of methylated bases per molecule which is close to the average.

The amount of methylated ribose is significantly larger in the histidine tRNA fraction than in total tRNA. The specific radioactivity of this fraction is consequently higher than the average in liver. But in hepatoma the number of methylated bases per molecule in this fraction is smaller than in total tRNA: its specific radioactivity before hydrolysis is thus only 80% of the specific radioactivity of total tRNA.

Small differences in the molar extinction coefficients of the various tRNA species may exist, which should be taken into account if actual numbers of methyl groups (either in bases or in ribose) were to be derived from the data in Table I. It should be noted, however, that the uv spectra of the fractions (not shown here) are essentially identical.

The methylation patterns of total tRNA from liver and from hepatoma are almost identical. Assuming an average content of five–six methylated bases per molecule, each one should contribute about 17–20% to the total radioactivity if they were evenly distributed. Any base whose contribution is less than this value must, therefore, be absent in some tRNA species. This is the case with thymine, which has been shown to be lacking—or incompletely modified—in some mammalian tRNAs (Piper and Clark, 1973; Keith *et al.*, 1973). Our results are in general agreement with the minor base compositions of total tRNA previously published by Iwanami and Brown (1968) for HeLa cells, by Craddock (1969) for normal rat liver, and by Randerath *et al.* (1971) for normal human brain. However, we failed to detect any significant amount of 1-methylguanine in our preparations (except in the hepatoma tyrosine tRNA fraction).

The qualitative identity between methylation patterns in liver and in hepatoma total tRNA favors the hypothesis that the methylation changes in hepatoma are discrete and restricted to a few tRNA species. This is confirmed by the analysis of tyrosine and histidine tRNA fractions.

The methylation pattern of the tyrosine tRNA fraction from liver is quite suggestive of a single species, containing one 1-methyladenine, one N_2-methylguanine, one N_2,N_2-dimethylguanine, one 7-methylguanine, one 5-methylcytosine, and about half a residue of thymine (this might arise from an incomplete modification, as discussed above). In the same fraction isolated from hepatoma, the percentages of 5-methylcytosine and of thymine are one-half of those in liver. Furthermore, 1-methylguanine, which is completely absent from liver tRNA, represents a small but significant percentage in hepatoma. A simple hypothesis, which fits closely with these observations, is that a new species of tRNA appears in hepatoma *in addition* to the one in normal liver; it lacks 5-methylcytosine and thymine, and perhaps contains 1-methylguanine. This hypothesis is in good agreement with the observation by Baliga *et al.* (1969) that on MAK chromatography tyrosyl-tRNA from hepatoma elutes in two peaks, one of them corresponding to the single peak of tyrosyl-tRNA from liver, the other one eluting at a higher salt concentration. Our purification system most probably does not separate those two species.

In the case of histidine tRNA fractions, two major differences between liver and hepatoma appear in the level of 5-methylcytosine and N_2,N_2-dimethylguanine. However, the interpretation of these methylation patterns is not as simple as in the case of the tyrosine tRNA fraction.

Determination of the methylated base composition of a tRNA which is based only upon percentage of radioactivity cannot be considered as definitive, and only nucleotide sequence determination will achieve certain resolution. Contamination of our fractions by other tRNA species may in particular explain the presence of some bases in very low

amount, although it is very unlikely that it may account for the large differences observed between liver and hepatoma when purified tRNA fractions are compared.

The physiological significance of our results—as well as of all previous reports on heterogeneity of tRNAs—remains unclear. Novikoff hepatoma is a highly undifferentiated tumor, which no longer resembles normal liver by any histological or biochemical criteria.

In conclusion, it is of interest that the tRNA species which have distinct chromatographic behavior have different methylation patterns as well. This is by no means a proof of a causal relationship between the two phenomena: other alterations than base methylation (including, of course, differences in the primary structure) might also produce a change in elution profiles. However, the correlation we observed provides some evidence in favor of "aberrant" methylation of some tRNA species in tissues where the tRNA methyltransferase activity is aberrantly elevated.

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