# *TRNA METHYLASES IN TUMORS OF ANIMAL AND HUMAN ORIGIN**

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At least ten different enzymes which methylate nucleic acids at the macromolecular level are now known to exist. All of these enzymes are species-specific, and consequently they confer an individuality on the number and distribution of methylated bases in the tRNA, rRNA, and DNA in each species.[1] In any organism the distribution of methylated bases in tRNA appears to be constant. However, Bergquist and Matthews have reported elevated levels of methylated bases in tRNA's isolated from some tumor tissues.[2]

The mechanism of neoplastic transformation is obscure. In the case of chemical carcinogenesis by alkylating agents, a causality between the aberrant alkylation of DNA and the subsequent tumor formation has been inferred. However, even in this instance, doubt has been cast on the molecular site of carcinogenesis by a finding of Magee and Farber, who showed that alkylating carcinogens alkylate tRNA much more than they do DNA.[3]

On the basis of the above information, we have proposed a hypothesis of possible oncogenesis by enzymes which methylate nucleic acids.[4, 5] We have postulated that, since all nucleic acid-methylating enzymes are species-specific, an oncogenic virus might introduce a capacity for the synthesis of nucleic acid-methylating enzymes which are foreign to the host. Should this occur, then the host would harbor enzymes which might methylate its nucleic acids aberrantly, just as alkylating carcinogens do. In this communication, studies on the tRNA methylases of tumors of animal and human origin are presented.

The tRNA methylases can be characterized by three different parameters: rates of methylation of the substrate, the total number of methyl groups introduced, and the sites of methylation. For *in vitro* studies of the levels of the base-specific tRNA methylases, almost any heterologous tRNA can be used as a substrate. We have used methyl-deficient tRNA from *E. coli* $K_{12}$ 58–161 $RC^{rel}$ simply because the total number of methyl groups introduced is amplified with this substrate. The use of methyl-deficient tRNA is justified because enzymes from a particular source ranging from microorganisms to animal organs introduced into the same preparation of methyl-deficient tRNA the same number of methyl groups in a constant ratio of the bases methylated.[6, 7]

Four different animal tumors and three different human malignancies served as the objects of this study. In the case of animal tumors, normal organs which are the closest counterparts to the tumor tissues served as the controls. With the solid tumors of human origin, extracts of the tumor tissue were compared to extracts obtained from the excised normal tissue of the same surgical specimen. Enzyme levels of normal white cells were determined in lymphocytes and leukocytes of normal male and female donors.

*Experimental.*—The tRNA methylase activity of normal and tumor tissue was assessed by previously described methods.[5] The activities at saturation levels of enzyme were obtained in the following manner. A preliminary experiment was performed to determine the saturation levels of enzyme extract, i.e., further addition of extract produced no significant change in the total $C^{14}$

incorporated per mg of tRNA. A large-scale incubation was then carried out using 5–10 mg of methyl-deficient tRNA. The tRNA was isolated, purified, and subjected to hydrolysis, chromatography, and autoradiography by methods published earlier.[15]

For the determination of tRNA methylase activity in blood samples from normal subjects and patients with blood dyscrasias, the following processing was carried out. Lymphocytes were isolated from approximately 350 ml of normal blood by Thierfelder's method[8] as modified by Hirschberg *et al.*,[9] and polymorphs were isolated from another 100-ml aliquot. Twenty ml of blood from leukemic patients was used for the isolation of the total population of white cells. Enzyme extracts were prepared from the isolated cells by mixing with 2–3 ml of Tris buffer (0.01 *M* pH 7.4) containing $10^{-3}$ *M* $MgCl_2$ and $10^{-2}$ *M* mercaptoethanol, and by freezing and thawing three times. The slurry was homogenized in a glass homogenizer for 1 min and centrifuged at 105,000 *g* for 1 hr. The clear supernatant was used for the assay of tRNA methylase activity.

The reaction mixture containing 100 $\mu$moles of Tris buffer, pH 8.2, 10 $\mu$moles each of $MgCl_2$ and reduced glutathione, 0.2 mg of methyl-deficient tRNA, 0.2 $\mu$c of (methyl-$C^{14}$)-S-adenosylmethionine (30 $\mu$c/$\mu$mole), and varying aliquots of enzyme extract in a total volume of 1 ml was incubated at 37° for 45 min. At the end of this period, 0.2 mg of bovine serum albumin was added to each tube followed by 5 ml of 6% TCA, and the precipitate was transferred to Millipore filters and repeatedly washed with cold 5% TCA. These were dried at 60° for 20 min and counted in a low-background gas-flow counter. A control incubation mixture without methyl-deficient tRNA was included with each enzyme concentration to evaluate the blank value.

The animal tumors were obtained from the laboratory of Dr. Eric Hirschberg of this institution. The Novikoff hepatoma, solid form, grew on the omentum of rats from Holtzman Farms. Melanoma S91 was transplanted into DBA × Swiss hybrid mice. Glioma 26 and mammary carcinoma 755 were carried in C-57 black mice.

*Results.*—In Table 1 results of the interaction of tRNA methylases from normal and tumor tissues are presented. The activities were obtained at saturation levels of enzyme extracts from both normal and tumor tissues. An elevation of methylase activity of two- to tenfold was observed in every tumor tissue compared to that of the appropriate normal controls. To determine whether such elevation is an intrinsic characteristic of tumor tissue or is a general concomitance of rapid growth, the enzyme activities of regenerating liver were determined. Regenerating rat liver was found to have a slight elevation compared to the normals but it still had only one fifth of the activity of Novikoff hepatoma. Comparisons were also made of normal chicken liver and embryos at various stages of development. No appreciable differences were noted among enzyme activities of extracts from 5- and 10-day-old embryos and extracts from the liver of normal young chicks.

TABLE 1

tRNA METHYLASE ACTIVITY IN NORMAL AND TUMOR TISSUES

| Normal control (cpm/mg tRNA) | Tumor (cpm/mg tRNA) |
|---|---|
| Rat liver: | Novikoff hepatoma: |
| I, 3400 | 28,000 |
| II, 2500 | 25,500 |
| III, 5200 | 25,800 |
| Regenerating rat liver: | |
| I, 4900 | |
| II, 5700 | |
| C-57 mouse brain: | Glioma: |
| 4400 | 18,300 |
| | 31,800 |
| C-57 mouse liver: | Mammary carcinoma: |
| I, 2200 | 15,600 |
| II, 1200 | 18,000 |
| DBA × Swiss mouse liver: | Melanoma: |
| I, 8800 | 13,000 |
| II, 9800 | 15,300 |

The elevated activities of tRNA methylases in tumor tissue may stem from a uniform increase of all of the base-specific enzymes or of only some of them. Two experimental approaches may be used to resolve this question. The type and quantity of methylated bases produced by extracts from normal and tumor tissue can be determined by isolation of the tRNA followed by hydrolysis and chromatography as described earlier. Such a

study is presented in Table 2. It is evident that the ratios of methyl A to methyl C, monomethyl G to methyl C, and dimethyl G to methyl C in the tRNA isolated after exposure to extracts of Novikoff hepatoma are significantly altered compared to those obtained with normal liver.

TABLE 2

LEVELS OF BASE-SPECIFIC tRNA METHYLASES IN NORMAL AND TUMOR TISSUE

| | Normal liver | | Novikoff hepatoma | | |
|---|---|---|---|---|---|
| MeA:MeC | 1.0 | 1.3 | 1.5 | 2.5 | 2.1 |
| MeG:MeC | 1.2 | 0.9 | 2.2 | 2.3 | 2.4 |
| $(Me)_2G$:MeC | 7.9 | 7.7 | 19.5 | 17.4 | 18.9 |

MeA = 6-methyladenylic acid, MeC = 5-methylcytidylic acid, MeG = monomethylguanylic acid, $(Me)_2G$ = dimethylguanylic acid.

The alteration of the methylated base ratios produced by enzymes from tumor tissues implies a qualitative change in the sequence of methylated bases in the tRNA. Conversely, the tRNA methylases from tumor tissue might have altered specificities with respect to the same substrate. Should this be the case, then tRNA methylases from normal and tumor tissue would seek out different sites for methylation in the same substrate. This can be explored by exposing tRNA sequentially to normal enzymes, and subsequently to enzymes from tumor tissue. Table 3 presents the data obtained from experiments of such a design. In these experiments, steps were taken to assure that both the enzymes and the adenosylmethionine were in excess. Moreover, the tRNA in each case was reisolated and purified by the usual methods prior to exposure to the battery of new enzymes. The results (expt. C) indicate that enzymes from the tumor tissue introduce methyl groups into the reisolated tRNA from experiment A to about 80 per cent of the level achieved in usual interactions (expt. B). Since the pattern of the results was the same with four widely different tumors, a change in the specificities of the tRNA methylases in tumor tissues is strongly implied.

Results of studies of the tRNA methylases of three different human malignant tissues are summarized in Tables 4–6. In Table 4, the rates of tRNA methylase activity of lymphocytes and polymorphs of normal subjects and of the leukocytes of patients suffering from leukemia are presented. The average rate of enzyme activity from normal lymphocytes is about one tenth of that of enzyme extracts from leukemic white cells. In subject 26 who had polycythemia vera prior to the onset of leukemia, the enzyme activity was found to be the highest. It seemed of interest therefore to ascertain the level of tRNA methylase activity in the white cells of subjects afflicted only with the former dyscrasia. Blood of donors 31 and 32,

TABLE 3

SEQUENTIAL METHYLATIONS OF tRNA BY EXTRACTS FROM NORMAL AND TUMOR TISSUES

| | Expt. A* | Expt. B† | Expt. C‡ |
|---|---|---|---|
| | (tRNA methylase activity, $\mu\mu$moles of methyl groups incorporated per mg of tRNA) | | |
| Normal liver: | | | |
| DBA × mice | 745 | | |
| C57 mice | 462 | | |
| Rat | 293 | | |
| Tumor: | | | |
| Melanoma transplanted in DBA × mice | | 1394 | 1820 |
| Glioma transplanted in C57 mice | | 3676 | 3188 |
| Mammary carcinoma transplanted in C57 mice | | 2380 | 2250 |
| Hepatoma transplanted in rats | | 2597 | 2309 |

* Interaction of liver extracts with methyl-deficient tRNA.
† Interaction of tumor extracts with methyl-deficient tRNA.
‡ Interaction of tumor extracts with tRNA reisolated from expt. A.

both of whom had polycythemia vera, was therefore examined but the level of tRNA methylase activity in their leukocytes was found to be normal. A case of leukocytosis (#30) was also examined. The normal enzyme levels in this instance probably indicate that the elevated enzyme activity is not necessarily a concomitance of rapid proliferation of white cells.

In Table 5, studies of rates of enzyme activity from nine solid tumors and corresponding normal tissues are presented. The tumor tissue was used as excised by the pathologists. Since no refined dissection was employed, the possibility of

TABLE 4

tRNA METHYLASE ACTIVITY OF WHITE CELLS*

| Normal Subjects | | | Leukemia Subjects | | |
|---|---|---|---|---|---|
| Subject no. | Polymorphs | Lymphocytes | Subject | Diagnosis | Leukocytes |
| 1 | 40 | 80 | 21 | Chronic leukemia | 2870 |
| 2 | 20 | 290 | 22 | Leukemia | 2270 |
| 3 | 55 | 540 | 23 | Leukemia | 670 |
| 4 | 20 | 80 | 24 | Acute myelogenous leukemia | 970 |
| 5 | 230 | 260 | 25 | Acute myelogenous leukemia | 940 |
| 6 | — | 50 | 26 | Polycythemia vera, subsequent acute leukemia | 3560 |
| 7 | 20 | 150 | 27 | Acute myelogenous leukemia | 2860 |
| 8 | — | 300 | | | |
| | | | 28 | " " | 1260 |
| 9 | 100 | 140 | | | |
| | | | 30 | Leukocytosis | 100 |
| 10 | 30 | 90 | | | |
| | | | 31 | Polycythemia vera | 50 |
| 11 | 50 | 50 | | | |
| | | | 32 | " " | 80 |
| 12 | 80 | 80 | | | |

* Cpm/mg of protein. For details see text.

TABLE 5

ACTIVITY OF tRNA METHYLASES OF NORMAL AND MALIGNANT TISSUE IN CARCINOMAS OF COLON, RECTUM, AND BREAST*

| Subject | Type of tumor | Adjacent normal tissue | Malignant tissue |
|---|---|---|---|
| 1 | Carcinoma of colon | 170 | 1080 |
| 2 | " " | 40 | 530 |
| 3 | " " | 70 | 310 |
| 4 | " " | 110 | 770 |
| 5 | Carcinoma of rectum | 110 | 420 |
| 6 | " " | 120 | 620 |
| 7 | Carcinoma of breast | 50 | 220 |
| 8 | " " | 70 | 360 |
| 9 | " " | 70 | 600 |

* Activity is expressed as cpm/mg of protein. For details see text.

TABLE 6

tRNA METHYLATING CAPACITIES OF NORMAL AND MALIGNANT TISSUE IN HUMAN MAMMARY CARCINOMA*

| Subject | Adjacent normal tissue | Malignant tissue |
|---|---|---|
| 21 | 510 | 6200 |
| 22 | 280 | 4150 |
| 23 | 380 | 1840 |
| 24 | 270 | 2200 |
| 25 | 220 | 940 |

* All activities were determined at saturation levels of enzyme extracts. The activity is expressed as cpm/mg of isolated tRNA.

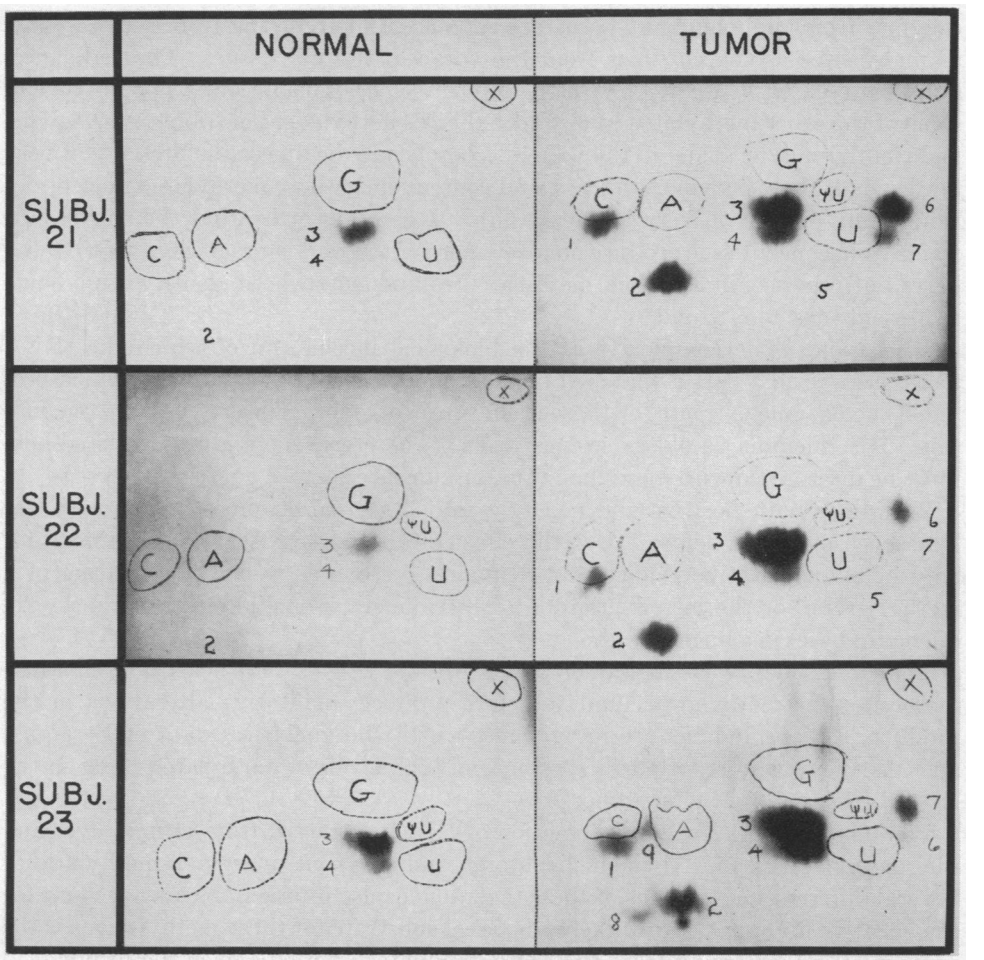


FIG. 1.—Radioautographs of the methylated nucleotides produced in tRNA by enzyme extracts from normal and tumor tissue of the human breast. The extracts were derived from cases 21, 22, 23 of Table 6. Period of exposure was 1 year for the normals and 3 months for the malignancies. *C* = cytidylic acid; *A* = adenylic acid; *G* = guanylic acid; and *U* = uridylic acid; *ψU* = pseudouridylic acid. Radioactive spot *1* = 5-methylcytidylic acid; radioactive spot *2* = 6-methyladenylic acid; radioactive spot *3* = dimethylguanylic acid; radioactive spot *4* = methylguanylic acid; radioactive spot *5* = ribothymidylic acid; radioactive spots *6*, *7*, *8*, and *9* are unidentified methylated nucleotides. Some of the numbered spots in the photographs are not visible because they are light radioactive areas in the radioautographs.

the presence of normal tissue or of capsular material cannot be excluded. In each case, an enzyme extract from normal tissue of the same surgical specimen was investigated parallel with the malignant tissue. The elevations of enzyme activity in the cancerous tissues range from 4- to 12-fold above normal. The large increments in enzyme activity in the first six cases listed are particularly significant, for in carcinomas of the bowel there is no change in the type of proliferating tissue; both the normal and the malignant tissues are epithelial.

In Table 6, results of studies of the extent of methylation by tRNA methylases from normal and malignant tissues of the human breast are presented. Since these data were obtained at saturation levels, the conclusion may be drawn that

enzymes from the malignant tissue are capable of methylating from 4 to 14 times as many sites as the enzymes from the adjacent normal tissue. The radioautographs presented in Figure 1 visually confirm such a conclusion. The absence of some of the other methylated nucleotides (i.e., 6-methyladenylic, 5-methylcytidylic, and ribothymidylic acids) in the normal controls may be due to the presence of very low levels of those enzyme activities, and consequently those methylated nucleotides would only emerge on very long exposure. It is noteworthy that the enzyme extracts from cancer tissue produce not only more of the well-characterized methylated bases but produce, in addition, methylated components whose identity is unknown at present (nos. 6, 7, 8, and 9).

*Discussion.*—The complexity of the biological mechanism of which the tRNA methylases form a part is matched by the complexity of the syndromes which provided the enzyme extracts for these studies. Moreover, causality versus concomitance is a dilemma as old as science itself. Therefore, the findings reported here must be interpreted with more than usual circumspection.

Variations both positive and negative among several enzymes in tumor tissues have been observed before. But the effect of altered tRNA methylases may not only be a quantitative change, but a qualitative one as well. The shifting of a methyl group, or the introduction of a supernumerary one, alters the primary sequence of bases in a nucleic acid.

The tRNA methylases of tumor tissue seek out *in vitro* in at least one substrate more sites for methylation, and therefore produce qualitative alterations in the product. These findings are in agreement with the analytical data of Bergquist and Matthews who reported the presence of high levels of methylated bases in the tRNA's isolated from some tumors.[2]

Preliminary studies of the interaction of tRNA extracted from normal liver and the methylases from Novikoff hepatoma indicate but marginal methylations. Several different mechanisms of these macromolecular interactions may account for the negative findings. Most likely, the ideal substrate for these methylations is the nascent tRNA emerging from the DNA template. Perhaps once the enzymes in normal liver methylate the product and thereby achieve the usual conformation of the tRNA, it may become resistant to overmethylations by the enzymes of tumor tissue. Or, the sequence of base specific methylations may have a directive role in the extent of methylation. Nor can the presence of an inhibitor associated with the tRNA from normal liver be excluded at the present.

All shifts in enzyme levels are the cumulative effects of changes in protein synthesis and in regulatory mechanisms. But since the products of the methylating enzymes are tRNA's which are cardinal components of the protein-synthesizing apparatus, altered tRNA methylases are not only the end products of protein synthesis, but they may also be primary effectors for changes in protein synthesis.

With the currently proposed mechanisms for protein synthesis, no function has been assigned for the methylated bases or, for that matter, for the other minor components of tRNA. Marcker[10] has shown that it is the tRNA with which methionine is esterified that determines whether the amino acid is formylated or not. Capecchi and Gussin[11] have shown that a serine-specific tRNA is the permissive determinant for a phage coat protein synthesis. Without information about the total base sequences of the various tRNA's, it is impossible to assess what roles the

minor components play in the subtle differences in function listed above. Since the tRNA methylases are species-specific, the distribution of methylated bases must have a species-specific individuality in the tRNA's from any source. Tumor tissue is but one of the altered biological systems in which changes in the tRNA methylases have been observed within a species. Wainfan *et al.*[7] have observed profound alterations in the tRNA methylases in *E. coli* infected by $T_2$ bacteriophage or in lysogenic *E. coli* after induction by heat or UV irradiation. Furthermore, Baliga *et al.*[12] have observed major fluctuations in the tRNA methylases in the insect *Tenebrio molitor* during its cycle of metamorphosis. These are biological systems that are undergoing profound shifts in regulatory mechanisms.

In turn, tumor tissues are characterized by pronounced lack of differentiation, stemming probably from a failure of regulatory mechanisms. Therefore, in four different biological systems—phage infection, induction, insect metamorphosis, and tumor tissue—in all of which radical changes occur, the tRNA methylases undergo measurable alteration which must affect the configuration of some of their substrates. Should the arrangement of methyl groups form some kind of code for recognition, then their redistribution by altered tRNA methylases might turn out to be a prelude to some regulatory process. There are two known instances of alteration of tRNA function by a deficiency of methylation. Revel and Littauer,[13] and Capra and Peterkofsky[14] have demonstrated coding ambiguities in methyl-deficient phenylalanyl and leucyl tRNA, respectively. Whether overabundant methylation of tRNA's of tumor tissue has similar effects on coding or other properties of tRNA remains to be explored.

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