

# Transfer RNA Methylases and Transfer RNA-methylated Bases of Polyoma Virus-transformed and Spontaneously Transformed Mouse Culture Cells<sup>1</sup>

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## SUMMARY

The transfer RNA methylase enzyme activities and the methylated nucleic acid bases of pulse-labeled, cellular transfer RNA's were compared in a C3Hf mouse tissue-culture system, which included nontumorigenic control cells and both spontaneously and polyoma virus-transformed cells. Even when the growth rates of the three cell types were equalized, the transfer RNA methylase activities of the transformed cells were at least twofold greater than the activity of the control cells. A two- to fourfold relative increase in 3-methylcytosine was found in the transformed cells.

## INTRODUCTION

Abnormal methylation of tRNA has been proposed as a biochemical mechanism of oncogenesis (35). In support of this concept, tumor tissues have consistently been shown to have greater tRNA methylase enzyme activity than normal, mature tissues (12, 25, 41). Furthermore, tRNA methylases from many different types of neoplastic cells were shown to methylate heterologous (12, 25, 29, 41) or, in some cases, homologous tRNA (16, 18, 28, 38, 42) to a greater degree or extent than methylases from more differentiated, nonneoplastic cells. Some studies have correlated the increased extent of *in vitro* tRNA methylation by tumor cell extracts with the methylation of different nucleic acid bases in the tRNA substrate (16, 28, 29, 41); other investigations have failed to demonstrate differences in the methylated bases of substrate tRNA (1, 5, 25, 30). Thus the proposal by Tsutsui *et al.* (41) that the greater methylating capacity of extracts from tumor cells may be due to the presence of methylases with additional or altered specificities remains controversial.

Since several factors, including the ionic milieu (26) and various effectors and inhibitors (17, 22, 23), can alter the methylation of tRNA *in vitro* and probably *in vivo*, an alternative approach is to study the tRNA recovered from intact cells. In this regard, several studies have identified differences between tumorous and control cells in the cellular complement of isoaccepting tRNA species (4), and

such chromatographic variations may be due to differences in tRNA methylation (6, 15). However, direct comparisons of the methylated base composition of tRNA from normal and tumor tissues have been reported in only a few instances (2, 7, 31, 32, 43), and the results conflict.

Variations in tRNA methylases and tRNA species are at least partly a function of environmental growth conditions, cell growth rate, and cell differentiation (10, 12, 33, 34, 41, 44). In this study, we attempted to control these parameters in comparing the tRNA methylases and tRNA methylated bases of mouse tissue culture cells, including control (nontumorigenic) cells and both spontaneously and polyoma virus-transformed cells.

## MATERIALS AND METHODS

Cells from 16- to 18-day-old C3Hf mouse embryos were established in tissue culture, and cells from the 3rd to 10th *in vitro* passages were used as control cells. The transformed cell lines were derived from a cloned tissue culture cell line established from the parotid gland of a C3Hf adult male (40). A portion of the original clone was transferred repeatedly in tissue culture until the cells spontaneously transformed into a malignant cell line (line 4094). Another portion of the original clone was infected with polyoma virus, which resulted in rapid malignant transformation (line 4198). Both transformed cell lines were passed through the host (C3Hf mouse) and placed back into tissue culture. The 3 cell types, spontaneously transformed, virus-transformed, and nontransformed control, were cultured and harvested under identical conditions, except when serum concentration was altered to control growth rate.

**Assay of tRNA Methylases.** Cells were homogenized by 10 strokes with a motor-driven Teflon glass homogenizer in 2 to 10 volumes of homogenizing fluid, which consisted of 0.3 M sucrose, 1 mM dithiothreitol, 0.01 M MgCl<sub>2</sub>, 0.01 Tris (pH 8.0), and 0.01 mM EDTA. The homogenate was centrifuged at 105,000 × *g*, and the supernatant was used directly for tRNA methylase assays or stored at -170°. Protein concentrations were determined by the method of Lowry *et al.* (27). Undermethylated *Escherichia coli* K<sub>12</sub>W<sub>6</sub> tRNA was prepared and extracted, as previously described (33). (*E. coli* K<sub>12</sub>W<sub>6</sub> was kindly donated by Dr. T. Breitman, National Cancer Institute, Bethesda, Md.). With concentrations of *S*-adenosyl-L-methionine-

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methyl- $^{14}\text{C}$  (44.5 mCi/mmole; New England Nuclear, Boston, Mass.) shown to be saturating in preliminary experiments, the tRNA methylase activity was measured as incorporation of methyl groups into under-methylated *E. coli* tRNA. Each assay had a minus tRNA control. The reaction mixture contained 0.15 M Tris-HCl, 0.24 M  $\text{NH}_4\text{Ac}$ , 98  $\mu\text{M}$  S-adenosyl-L-methionine-methyl- $^{14}\text{C}$ , 0.1 mM EDTA, 6 mM  $\text{MgCl}_2$ , 2.5 mM dithiothreitol, variable amounts of tRNA, and enzyme. Reactions were performed at 37° for 90 min, terminated with ice-cold trichloroacetic acid, and processed, as described elsewhere (33).

**Analysis of tRNA-methylated Bases.** Cells were treated with 143 M methionine-methyl- $^{14}\text{C}$  (New England Nuclear), 9.1 mCi/mmole, for 16 hr in the presence of 20 mM sodium formate, to prevent utilization of the methyl- $^{14}\text{C}$  group in the synthesis of the ring structure of the bases of tRNA. Thus, methyl- $^{14}\text{C}$  incorporation represents only methylation of bases in preformed tRNA.

The methylated base analysis of  $^{14}\text{C}$ -labeled tRNA was carried out according to previously described methods (8, 21) after isolation of the tRNA (33). In brief, the procedure consisted of hydrolysis of the tRNA with HCl (1 ml of 1 N HCl at 100° for 30 min) and separation of the bases by AG 50W-X12 ( $\text{H}^+$  form; 200 to 400 mesh) (J. T. Baker Chemical Co., Phillipsburg, N. J.) column chromatography. Fractions of individual base groups were combined and lyophilized. Guanine and adenine bases, the 2nd and 3rd chromatographic fractions, respectively, were dissolved in 1 ml of 0.1 N HCl, and 0.015-ml aliquot samples were applied to paper (Whatman No. 1) and thin-layer chromatography (flexible cellulose with fluorescence; J. T. Baker Chemical Co.) The spots were neutralized with an equal volume of 0.1 N  $\text{NH}_4\text{OH}$ . The guanine and adenine bases were separated by running with Solvent 1 (68% isopropanol, 17.6% 12N HCl, 14.3%  $\text{H}_2\text{O}$ ) for 6 days and with Solvent 2 (85% 1-butanol, 15%  $\text{H}_2\text{O}$ ) for 14 hr, respectively. Pyrimidine nucleotides, which were contained in the 1st fraction eluting from the ion-exchange column, were hydrolyzed with 70% perchloric acid for 1 hr at 100°. Aliquots (0.015) were spotted on thin-layer chromatography paper, neutralized with an equal volume of NaOH, and run with solvent 3 [85 ml isopropanol, 15 ml  $\text{H}_2\text{O}$ , 13 ml 28% (aqueous) ammonia] for 14 hr at room temperature. Samples of known standards (80 nmoles each) were run simultaneously. Samples were located with UV, identified from the known standards, and cut out; radioactivity was determined by counting in a liquid scintillation counter with an efficiency for carbon-14 of 82% to a minimum counting accuracy of  $\pm 10\%$ .

## RESULTS

**tRNA Methylases of Control and Transformed Cells.** Methylation of *E. coli* tRNA with the control cell enzyme extract reached a plateau at 0.5 mg of protein, while methylation with the transformed cell extracts did not saturate the substrate tRNA under these assay conditions (Chart 1). This indicates that the transformed cell methyl-

ases can methylate more tRNA sites, i.e., they have greater methylase capacity, than the control cell methylases.

It was possible that the differences in tRNA methylase capacities were related to variation in growth rate, since the transformed cells grew 2- to 3-fold faster than control cells. Consequently, the cell growth rates of the 3 cell lines were equalized by lowering the serum concentration of the polyoma-transformed cell line to 2.5% and that of the spontaneously transformed cell line to 5%, while the control cell line serum concentration remained at 10%. Adjusted doubling times were as follows: control, 40 hr; spontaneously transformed, 41 hr; virus-transformed, 40 hr. With the same doubling times, tRNA methylase capacities of extracts from the malignant cell lines were still at least twice those of extracts from the control cells.

Consideration was given to adventitious factors that might account for the differences in tRNA methylase activity. A difference in RNase activity might result in differences due to more tRNA degradation in assays with the control cell extracts. However, as shown in Table 1, this was not the case and, in fact, the difference may be underestimated, since a slightly greater degradation of uridine- $^{14}\text{C}$ -labeled *E. coli* tRNA was found after incubation with the extract from the transformed cells. Further, preincubation of the tRNA with extracts from the normal cells did not diminish the ability of *E. coli* tRNA

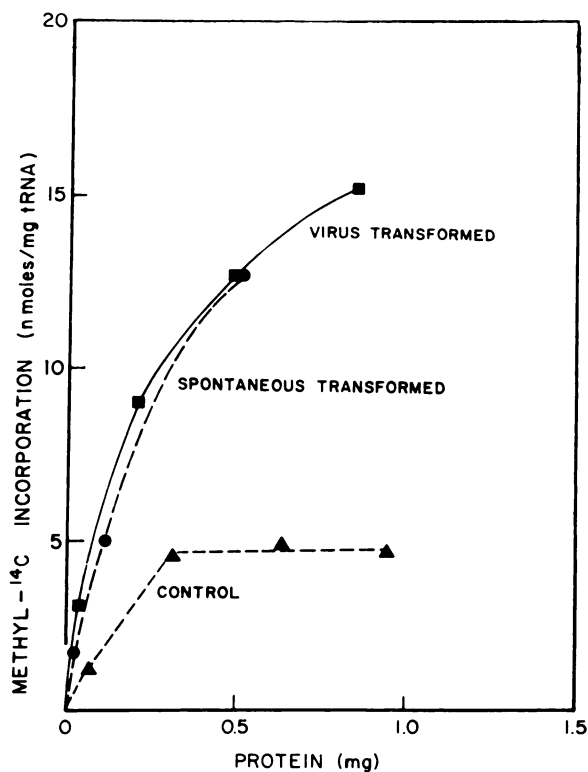


Chart 1. tRNA methylase activities as a function of protein concentration in control and transformed mouse culture cells. Cells were grown at different rates (doubling time in hr: control, 40; spontaneously transformed, 32; virus-transformed, 15) in the same (10%) fetal calf serum concentration. Assays were performed as described in "Materials and Methods," with 1  $\mu\text{g}$  of methyl-deficient *E. coli*  $\text{K}_{12}\text{W}_6$  tRNA per assay.

to be methylated subsequently with extracts from the transformed cells.

Mixing experiments were performed to determine whether differences could be explained by variation in the amount of inhibitors and/or activators. The absence of such factors is indicated by the data in Table 2, which show that mixing extracts from control and transformed cells resulted in additive tRNA methylase activity.

**Analyses of tRNA-methylated Bases.** tRNA-methyl-<sup>14</sup>C with the following specific activities was recovered from the 3 cell types: control, 730 dpm/ $\mu$ g; spontaneously transformed, 788 dpm/ $\mu$ g; and polyoma-transformed, 489 dpm/ $\mu$ g. If calculated per equivalent number of cells, methylation was the same for all cell types.

Very similar fractional amounts of the methylated bases were found in the 3 types of tRNA, except for 3-methylcytosine, which was increased 2-fold in the spontaneously transformed cells and 3- to 4-fold in the polyoma-transformed cells relative to the control cells (Table 3). 3-Methylcytosine was very clearly resolved from its closest neighbor, 5-methylcytosine, in this chromatographic system. No radiolabel was recovered in areas where non-methylated, major bases eluted, indicating effective blocking of methionine-methyl-<sup>14</sup>C incorporation into the ring

structures of the nucleic acid bases by sodium formate (see "Materials and Methods").

## DISCUSSION

These studies indicate that increased tRNA methylases in tumor cells are not strictly related to environmental conditions or to cellular growth rate. Also, we confirm other studies (9, 11, 24, 32, 33) that demonstrated that transformed culture cells have increased tRNA methylase activity, compared with the activity of replicating nontumorigenic cells, as well as with that of fully differentiated, non-replicating tissues. Differences in cytodifferentiation may, as previously suggested (12), be related to the differences in methylase activities, since the culture history and phenotypic properties of the control cell line differed from that of the 2 transformed cell lines.

Methylase preparations from the tumor cells also had a demonstrably greater capacity for methylating *E. coli*

Table 1

### *RNase assays of tRNA methylase preparations from mouse culture cells*

The assays were performed by incubation of 1  $\mu$ g tRNA-<sup>14</sup>C (Miles Laboratories Inc., Kankakee, Ill.; 0.01  $\mu$ Ci/ $\mu$ g) with enzyme extracts under maximum methylating conditions for the control cells.

Cell types	% tRNA- <sup>14</sup> C degraded at 90 min of incubation
Control	28
Spontaneously transformed	39
Virus-transformed	34

Table 2

### *tRNA methylase activities of mixtures of extracts from mouse culture cells*

Assays were performed under conditions of tRNA excess in which activity was a linear function of enzyme concentration. The predicted values represent the sum of the activities of the control and transformed cell extracts when assayed separately. The actual values indicate the activities when the control and transformed cell extracts were mixed and assayed together. The reported values represent the average of duplicate determinations which showed less than 15% variation from the mean. Reactions were performed as described in "Materials and Methods."

Cell types	cpm	
	Predicted	Actual
Spontaneously transformed and control	6122	6061
Virus-transformed and control	6778	6941

Table 3

### *Comparison of the methylated bases of 4 S RNA from control (nontumorigenic) and transformed mouse culture cells*

tRNA-methyl-<sup>14</sup>C was hydrolyzed, chromatographed, and analyzed as described in "Materials and Methods." The reported values indicate the percentage of the total applied dpm of acid-digested, tRNA-methyl-<sup>14</sup>C-labeled recovered in areas of the chromatogram, corresponding to the migration of purified methylated nucleic acid base standards. Chromatographic recovery exceeded 95% of applied dpm.

	% of total dpm with methionine-methyl- <sup>14</sup> C incorporation in					
	Control cells		Spontaneously transformed cells		Polyoma-transformed cells	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
1-Methylguanine	10.52	10.70	9.21	10.02	10.22	9.46
7-Methylguanine	7.81	8.24	8.18	8.10	7.69	8.01
N <sup>2</sup> -Dimethylguanine	12.86	13.96	13.28	12.02	12.09	12.78
N <sup>2</sup> -Methylguanine	12.81	13.43	12.99	12.12	12.01	12.85
1-Methyladenine	11.76	11.95	11.16	11.54	11.50	11.08
2-Methyladenine	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
N <sup>6</sup> -Methyladenine	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Unidentified material remaining at origin	17.52	15.86	16.77	17.78	17.12	16.78
5-Methylcytosine	16.90	17.58	17.57	17.75	17.55	18.95
3-Methylcytosine	0.74	0.96	1.90	1.89	2.97	2.52
5-Methyluracil	7.45	6.48	8.94	8.58	8.73	8.38

tRNA than did methylases from the control cells (Fig. 1). This does not necessarily signify the presence of tRNA methylases with altered specificity in the tumor cells since, as indicated in the "Introduction," several factors may affect the amount and possibly the sites of tRNA methylation. However, we detected no inhibitors in the control cells and no activators in the transformed cells in these experiments (Tables 2 and 3) or in studies with analogous cell culture systems (9, 11, 33). The lack of inhibitors may be related to the embryonic derivation of the control cell lines (23). Also, ammonium ions, which may suppress inhibitors (26), were used in the enzyme reactions. However, this seems an unlikely explanation for the failure to detect inhibitors, since ammonium ion concentration did not affect the relative activity or capacity of the methylases of control and transformed rat culture cells under similar reaction conditions (11). Thus, although ancillary factors rather than differences in tRNA methylase specificity may account for the disparity in methylase capacities of the control and transformed cell lines, we found no evidence of such factors in these tissue culture cells.

Breier and Holley (5) reported finding no differences in the methylated base composition of *E. coli* or rat liver tRNA, which was methylated *in vitro* with extracts from 3 paired control and polyoma virus-transformed tissue culture cell lines. However, the interpretation of some of their data has been challenged (4) and, furthermore, it must be considered that analysis of whole tRNA, which consists of at least 64 isoaccepting species (3, 44), may overlook significant methylation differences in individual isoaccepting tRNA species (29). Conversely, Mittleman *et al.* (28) reported differences in the methylated base composition of both heterologous and homologous tRNA after methylation *in vitro* with methylase preparations from nontumorigenic control and simian virus 40-transformed cells. [Simian virus 40 and polyoma virus are very closely related; both belong to the papovavirus group (3).]

In these experiments, tRNA-methyl-<sup>14</sup>C with approximately 30% less specific activity (dpm/μg tRNA) was recovered from the virus-transformed cells, as compared with the other 2 cell lines, which were approximately equal (see "Results"). This relative hypomethylation of the tRNA in the virus-transformed cells might be due to several factors, including differences in cellular uptake or intracellular distribution of methionine-methyl-<sup>14</sup>C, differences in rates of tRNA synthesis, or variations in the activity of the enzyme ATP:L-methionine S-adenosyltransferase (EC 2.5.1.6). However, since the pulse interval (16 hr) was long relative to the time taken for these processes to reach equilibrium (7, 39) and since the half-life of tRNA is considerably longer (5 days; Ref. 19), such factors should be minimized. This suggests the accumulation of a low-molecular-weight, nonmethylated RNA in the virus-transformed cells (37). In any event, there is no apparent hypermethylation of tRNA in either type of transformed cells. Perhaps, as proposed by G. D. Novelli (personal communication), the elevation of tRNA methylases in tumor cells is compensatory to a defect in tRNA methylation and processing.

Regardless of overall methylation, a 2- to 4-fold relative increase in methylation of cytosine in position 3' occurred in tRNA of the pulsed tumor cells (Table 3). This increase could be due either to increased 3'-methylation of cytosine in many tRNA's or, more likely, to increased methylation of a few isoaccepting species of tRNA with relatively high amounts of cytosine capable of being methylated in position 3'. Of the several species of tRNA which have been sequenced, only tRNA<sup>ser</sup><sub>rat</sub> has been shown to contain 3-methylcytosine, which occurs at 2 base sites in this tRNA species (36). Since neither yeast (45) nor *E. coli* (20) tRNA<sup>ser</sup> contains 3-methylcytosine, the presence of a site-specific methylase (29) in tumor cells in much larger quantities than in control cells could partially account for the increased capacity of tumor cell extracts to methylate heterologous tRNA (Fig. 1). Reversed-phase chromatography of seryl-tRNA from control and polyoma-transformed rat culture cells suggested some heterogeneity in the 6 isoaccepting species resolved (10). These observations emphasize the need, in future studies, to define methylation differences by detailed structural analysis of individual tRNA species.

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