Transfer RNA Methylase Alterations in Polyoma Transformed Rat Embryo Culture Cells[1] (35372)

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The tRNA methylases are a group of cytoplasmic enzymes which transfer a methyl group from an *S*-adenosyl-L-methionine intermediate to a specific tRNA base position. Preparations of these enzymes from malignant tissues have been demonstrated to methylate both heterologous and homologous tRNA's more completely than an excess of enzyme(s) from normal tissues (1–4). The increased methylating capacity of tumor extracts indicates the methylation of additional tRNA sites and suggests the presence of different species of tRNA methylases in malignant cells. This correlates with the finding of hypermethylated tRNA in some natural tumors in which the degree of hypermethylation and of dedifferentiation appear to be directly related (5, 6). It has been suggested that methylase modifications of tRNA may lead to aberrations of protein synthesis and, therefore, to alterations in cellular control mechanisms (7, 8). Furthermore, tRNA (9, 10), and possibly some of its breakdown products (11, 12), may serve other regulatory functions not directly involving protein synthesis which could also be altered through modification of tRNA bases (13). Such changes in tRNA may be of importance in the transformation to and the maintenance of malignant growth (1, 14–17).

Virus-induced neoplastic transformation of normal cells in tissue culture seems a particularly favorable system for evaluating the significance of alteration in methylation of tRNA, as well as other tRNA changes, for several reasons: first, cells may be prepared under controlled conditions; second, a virus-coded change in methylase specificity or in tRNA structure may be more readily identified and evaluated than in other forms of neoplastic transformation; third, evidence suggests that phage-specific tRNA methylases are important in bacterial lysogeny (18), a process with some similarities to the neoplastic transformation of mammalian cells by oncogenic DNA viruses (19).

In this communication we report an increase in the tRNA methylase capacity following neoplastic transformation of rat embryo cells by polyoma virus in an *in vitro* system.

Materials and Methods. Cell culture system. Cells from 12-day-old embryos of inbred BN rats were established in tissue culture. The third to tenth passages were used in present experiments as control cells. This cell line was infected with polyoma virus SE3049, and the transformed cells were injected into adult BN rats. The third transplant of the resulting tumor was reestablished in tissue culture and was passaged several times prior to use. All cells were cultured in Eagle's medium with 10% fetal calf serum. They were harvested in the log phase of growth, 1 day after feeding, washed, and stored at −170° until use. The control cells grew in homogeneous monolayers; the polyoma-transformed cells grew in an overlapping pattern typical for cells with a loss of contact inhibition. Cytogenetic examination of 50 metaphase figures from each cell line disclosed the normal diploid number of chromosomes from the rat (*i.e.*, 42). However, while the control cells displayed a normal karyotype, the polyoma-transformed cells contained one large acrocentric chromosome (slightly larger than a pair 2 chromosome) and one minute submetacentric chromosome,

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i.e., this line is pseudodiploid. The incidence of polyploidy and of chromosome breaks or fragmentation was approximately 10% for each cell line.[2] The polyoma-transformed cells were free of infectious virus and were positive for both the transplantation and T antigens (20). No other type of carrier virus was detectable in either cell line by electron microscopy.

Transfer RNA methylase assay. Tissues were homogenized manually in 2 to 3 vol of 0.3 *M* sucrose containing 1 m*M* dithiothreitol, 0.01 *M* $MgCl_2$, 0.1 m*M* EDTA, and 0.01 *M* tris (pH 8.0), at 4°. The homogenate was centrifuged at 105,000*g* for 1 hr, and the supernatant fluid was used for enzyme assays. Protein concentrations were determined by the method of Lowry *et al.* (21). The procedure used for tRNA isolation is described elsewhere (17). The final reaction mixtures in a total volume of 0.15 *M* Tris–HCl, 0.24 *M* ammonium acetate, 2.5 m*M* dithiothreitol, 0.1 m*M* EDTA, 6 m*M* $MgCl_2$, 98 μmoles of [^{14}C-methyl]-*S*-adenosyl-L-methionine, variable amounts of protein from control and tumor cell supernatants and tRNA. In the control system, 30 μg of *E. coli* tRNA and in the polyoma-transformed system 1 μg of *E. coli* tRNA were employed for extent conditions. In Fig. 1A, the curves were normalized by calculating methyl groups incorporated per milligram of tRNA. Although similar data were recorded using 1 μg of tRNA with the control enzyme, they were not as reliable quantitatively due to the small amount of radioactivity incorporated. With *E. coli* tRNA as methyl acceptor, 30 μg of tRNA was used for all experiments under rate conditions. In the experiments presented in Fig. 1B, 60 μg of yeast tRNA was used with both the control and polyoma-transformed enzymes. Reactions were carried out at 37° for 90 min, and terminated by adding 5 ml of ice-cold 10% trichloroacetic acid (TCA). The precipitates were collected on filter disks (Millipore Corp., Bedford, Mass., type HA, 4.5-μ pore size) and washed with 25 ml of 5% TCA. After drying with an infrared lamp, the filters were placed in 10 ml of toluene Liquifluor scintillation medium and counted in a Packard Tri-Carb scintillation counter at an efficiency of 82%. The amount of methyl groups incorporated into tRNA was determined for each enzyme concentration as the difference between TCA-precipitable counts between samples with and without tRNA.

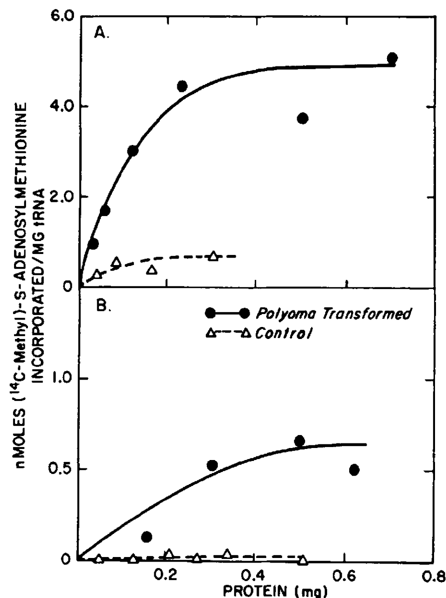

FIG. 1. Comparison of the tRNA methylase activity of control cell and polyoma-transformed tumor cell extracts with (A) hypomethylated *E. coli* $K_{12}W6$ tRNA; and (B) yeast tRNA. Experimental conditions are defined in Methods.

Results. With *E. coli* tRNA as methyl acceptor and with excess amounts of enzyme(s) a sevenfold increment in the extent, *i.e.*, *capacity*, of methylation was demonstrated with the tumor cell system compared to the control cell system (Fig. 1A). The differences in the plateaus of methylase activity (extent) shown here have been repeatedly demonstrated with enzyme preparations from different harvests of culture cells and indicate that more tRNA sites are being methylated by the tumor cell extracts. A comparison of the ability of the paired extracts to incorporate methyl groups with time and with tRNA in excess showed a three-to-sixfold difference in *rate* of reaction (Fig. 2). With yeast tRNA as the methyl acceptor, methylation was almost undetectable with the

[2] We thank Dr. Jacqueline Whang-Peng for performing the cytogenetic analyses.

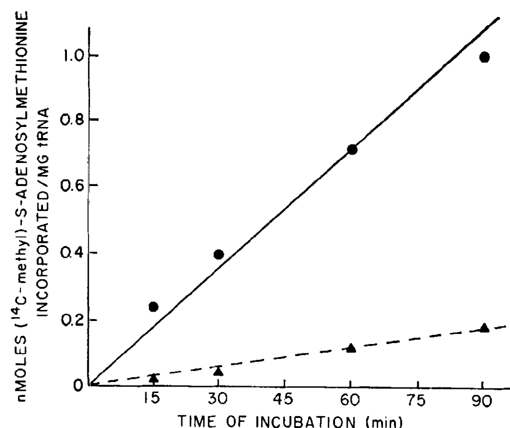

FIG. 2. Comparison of the rates of methylation of hypomethylated *E. coli* $K_{12}W6$ tRNA by control cell extracts (▲); and by polyoma-transformed cell extracts (●).

control preparation but reached an extent of approximately 0.7 nmoles/mg of tRNA with the tumor preparation (Fig. 1B).

The possibility was investigated that the differences in methylase activity might be due to certain adventitious factors. First, it was demonstrated that *S*-adenosyl-L-methionine was not limiting at all protein concentrations. Second, selective inactivation of the control enzymes was excluded by comparing the rate of tRNA methylation of samples preincubated at 37° for 90 min to the rate noted without preincubation. Although there was some decrease in subsequent rate of methylation in the samples preincubated without tRNA, this decrease was no greater in control than in tumor cell reactions. Third, a greater loss of acceptor tRNA in the control system due to ribonuclease degradation was excluded, as follows: tRNA, labeled with ^{14}C-uridine, was prepared from log phase *E. coli* by standard procedures. The ^{14}C-tRNA was incubated with either control of tumor extract. After 90 min incubation under extent conditions, the control system retained 92% of the acid precipitable counts, whereas the tumor system retained only 61%. This indicates that the sevenfold difference in extent of methylation shown in Fig. 1A may be an underestimate of the true difference.

The system was investigated for the presence of inhibitors or activators by mixing equal concentrations of protein from extracts of the control and polyoma-transformed cells with hypomethylated *E. coli* tRNA. The averages of duplicate determinations from different preparations of enzyme are shown in Table I. The results indicate that the differences in extent of methylation shown in Fig. 1A are not due to the presence of an inhibitor in the control cell extracts.

It has been reported in one system that the extent differences in methylation between normal and neoplastic tissues disappear in the presence of a high concentration of ammonium ions (22). In the present studies, no significant differences in the rate of reaction were found in the absence or in the presence of 0.24 *M* ammonium acetate. With the higher ammonium ion cencentration approximately a twofold depression of the extent of reaction was noted in both systems; however, there was no significant change in the relative capacities for methylation (Table II).

Discussion. The demonstration of marked differences in the extent of methylation between the control and tumor methylase preparations utilizing two different tRNA substrates, suggests that changes in the specificity of the tRNA methylases occurs after polyoma virus-induced malignant transformation. Recently, similar data have been reported for SV40-transformed cell culture lines (23). Whether such enzyme differences are related to the direct expression of a viral gene(s) or to the altered expression of host

TABLE I. Transfer RNA Methylase Activities of BN Rat Embryo Cells: A Comparison of Extracts from Control, Polyoma-Transformed and a Mixture of Control and Polyoma-Transformed Cells.

Control (cpm)	Polyoma (cpm)	Control + polyoma (cpm)	
		Actual	Predicted
302[a]	916	1448	1218

[a] Values represent the averages of duplicate determinations from two experiments in which equal protein concentrations of extracts from control and polyoma-transformed cells were assayed under standard rate conditions as described in Methods. Individual determinations showed less than 10% variation from the averages.

TABLE II. The Effect of Ammonium Acetate on the tRNA Methylase Activity of Control Cell Polyoma-Transformed Cell Extracts.

	Rate of methylation[a]		Extent of methylation[b]	
	No ammonium acetate	0.24 *M* Ammonium acetate	No ammonium acetate	0.24 *M* Ammonium acetate
Polyoma	0.75	0.80	4.50	2.6
Control	0.15	0.16	0.72	0.49

[a] (^{14}C-Methyl)-*S*-adenosyl-L-methionine (nmoles) incorporated/mg of *E. coli* tRNA/hr under rate conditions as defined in Methods.

[b] (^{14}C-Methyl)-*S*-adenosyl-L-methionine (nmoles) incorporated/mg of *E. coli* tRNA under extent conditions as defined in Methods.

genes, they may be of importance in the conversion to and/or maintenance of the malignant state.

A serious problem in the interpretation of experiments which purport to demonstrate a biochemical difference between the malignant and normal states is the adequacy of the controls. Although we cannot state that our control line was completely homogenous or normal, the continuous division of these cells gives more opportunity for expression of the pertinent genetic material than experiments in which extracts from tumors have been compared with extracts from fully-differentiated tissues. Despite our efforts to insure that the control and tumor cell lines were treated identically, the growth rates were not the same. This could affect tRNA methylase activity either by altering culture conditions or by changing the effective concentration of a cell-cycle specific enzyme. These possibilities seem unlikely to be the prime factors, however, because a control and polyoma-transformed mouse cell culture system studied in our laboratory in which the cell doubling times were equalized by varying the fetal calf serum content of the culture medium continued to show greater methylase capacity in the transformed cells (24).

Summary. A polyoma-transformed cell line of proven tumorigenicity is demonstrated to have greater tRNA methylase activity than its nontumorigenic parent cell line when cultured, harvested, and assayed under controlled conditions. The marked differences in capacity to methylate heterologous tRNA substrates suggests, but does not prove, that qualitative differences may exist between the methylases of the paired cell lines.

1. Tsutsui, E., Srinivasan, P. C., and Borek, E., Proc. Nat. Acad. Sci. U.S.A. **58**, 1003 (1966).

2. Hancock, R. L., Cancer Res. **27**, 646 (1967).

3. Gantt, R., and Evans, V. S., Cancer Res. **29**, 536 (1969).

4. Mittleman, A., Hall, R. H., and Yahn, D. S., Cancer Res. **29**, 536 (1969).

5. Berquist, P. L., and Matthews, R. E. F., Biochem. J. **85**, 305 (1962).

6. Viale, G. L., Restelli, A. F., and Viale, E., Tumori **53**, 533 (1967).

7. Capra, J., and Peterkofsky, A., J. Mol. Biol. **21**, 445 (1966).

8. Shugart, L. B. H., Chastain, G., Novelli, D., and Stulberg, M. P., Biochem. Biophys. Res. Commun. **31**, 404 (1968).

9. Silbert, D. F., Fink, G. R., and Ames, B. N., J. Mol. Biol. **22**, 335 (1966).

10. Eidlic, L., and Neidhardt, F. C. Proc. Nat. Acad. Sci. U.S.A. **53**, 539 (1965).

11. Gallo, R. C., Whang-Peng, J., and Perry, S., Science **165**, 400 (1969).

12. Skoog, F., Hamzi, H. Q., Szweykowska, A. M., Leonard, N. J., Carraway, K. L., Fujii, T., Helgeson, J. P., and Leoppky, R. N., Phytochemistry **6**, 1169 (1967).

13. Gefter, M. L., and Russell, R. I., J. Mol. Biol. **39**, 145 (1969).

14. Gallo, R. C., J. Cell. Physiol. **74** (Suppl. 1), 149 (1969).

15. Baliga, B. B., Borek, E., Weinstein, I. B., and Srinivasan, P. R., Proc. Nat. Acad. Sci. U.S.A. **62**, 899 (1969).

16. Yang, W., Hellman, A., Martin, D. H., Hellman, K. B., and Novelli, G. D., Proc. Nat. Acad. Sci. U.S.A. **64**, 1411 (1969).

17. Gallo, R. C., and Pestka, S., J. Mol. Biol. **52**, 195 (1970).

18. Wainfam, E., Virology **35**, 282 (1968).

19. Dulbecco, R., Cold Spring Harbor Symp. Quant. Biol. **33**, 777 (1968).

20. Takemoto, K. K., Malmgren, R. A., and Habel, K., Science **153**, 1122 (1966).

21. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

22. Kaye, A. M., and Leboy, P. S., Biochim. Biophys. Acta **15**, 289 (1968).

23. Kit, S., Nakajima, K., and Dubbs, D. R., Cancer Res. **30**, 528 (1970).

24. Wright, S., Ting, R. C. Y., and Gallo, R. C., Clin. Res. **18**, 477 (1970).

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