

# Comparative Transfer RNA Methylase Capacity in Mouse Ascites Tumors and in Their Derived Tumorigenic and Nontumorigenic Cell Cultures<sup>1</sup>

R. T. Dell'Orco,<sup>2</sup> D. G. R. Blair,<sup>3</sup> and J. F. Morgan<sup>4</sup>

Department of Cancer Research, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

## SUMMARY

The transfer RNA (tRNA) methylase activity from two types of mouse ascites tumor cells (TA3 adenocarcinoma and 6C3HED lymphosarcoma) was compared to that of derived tumorigenic and nontumorigenic cultured cells of the same cell types. The nontumorigenic cells had lost their ability to produce tumors on back transplantation by extended passage in cell culture. The two ascites cell types differed in their capacity to methylate *Escherichia coli* B tRNA. The TA3 cells had an approximately 30% greater capacity than did the 6C3HED cells. There was no difference in the methylating capacity of either cell line during *in vivo* tumor development. Cell extracts from 9-day TA3 ascites tumors and from TA3 tumorigenic cultured cells exhibited between 50 and 60% more methylating ability than did extracts from nontumorigenic cultured cells of the same cell line. Contrary to this, 9-day 6C3HED ascites cell extracts showed no difference in tRNA methylase activity when compared to extracts from 6C3HED nontumorigenic cultured cells. These results indicate that a quantitative alteration in tRNA methylase activity may not necessarily be associated with loss of tumorigenicity in this ascites cell system.

In a control experiment, it was found that the tRNA methylase activity of a solid, s.c., 3-methylcholanthrene-induced transplantable mouse tumor was 35% greater than that of normal mouse liver or kidney and 80% greater than that of normal mouse lung. This observation is in accord with previous reports by other authors that tRNA methylase activity of some neoplastic tissues and cells is increased in comparison with normal tissue.

## INTRODUCTION

The function of the methylated bases that are found in the nucleic acids, particularly tRNA, has been the source of much investigation and speculation in recent years (2, 21, 23, 24, 26).

<sup>1</sup> Supported by the National Cancer Institute of Canada.

<sup>2</sup> Present address: The Samuel Roberts Noble Foundation, Inc., P. O. Box 878, Ardmore, Okla. 73401.

<sup>3</sup> Research Associate of the National Cancer Institute of Canada and the person to whom requests for reprints should be addressed.

<sup>4</sup> Present address: Department of Biochemistry, University of Saskatchewan, Saskatoon, Canada.

Received September 21, 1970; accepted January 15, 1971.

Associated with the basic role of these minor components in the function of tRNA is the question of the part they play in the conversion of "normal," nonneoplastic cells to "abnormal," neoplastic cells. Beginning with the original report of Berquist and Matthews (1) on the increased number of methylated purines present in tumor tissue as compared to normal mouse tissue, numerous investigators have studied the activities of the specific enzymes which catalyze the methylation of tRNA bases in various neoplastic systems. These have included virus-induced tumors in hamsters (18) and chickens (17), virus-transformed cultured cells (15), rat hepatoma (11, 25), a variety of mouse and human tumors (27), and a neoplastic cultured cell line (9). In all of these systems, it is reported that there is an increase in the tRNA methylase activity in neoplastic material in contrast to normal controls.

Previous studies from this laboratory (20) have shown that ascites tumor cells of the mouse become nontransplantable after long-term cultivation *in vitro*, although they maintain an undiminished rate of proliferation. A 3-component tumor model system based on this observation has been developed consisting of the tumors passaged *in vivo* together with their derived tumorigenic and nontumorigenic cell cultures. The availability of this model system made it possible to investigate whether decreases in tRNA methylase activity might be associated with loss of tumorigenicity. Such a finding would provide confirmatory evidence for the increased tRNA methylase activity observed by other workers during the transformation of cells from the normal to the neoplastic state.

Our studies revealed that, while there was a difference in the tRNA methylase activity between the TA3 tumorigenic and nontumorigenic cells, the 6C3HED cells exhibited no such difference. These results indicated that a quantitative alteration in the total tRNA methylase activity did not parallel tumorigenicity in this system.

## MATERIALS AND METHODS

TA3 adenocarcinoma cells were carried by weekly passage as ascites tumors in female Swiss mice of the Connaught Laboratories strain, and the 6C3HED lymphosarcoma cells were carried in the same manner in female C3H mice (7). Both cell types were passaged *in vitro* with Medium 199

supplemented with 5% calf serum. The 2 cell types differed in the relative time required for loss of tumorigenicity during passage in cell culture; while the 6C3HED cells become nontumorigenic within 6 months, the TA3 line required from 3.0 to 3.5 years of propagation *in vitro* (10). Tumorigenicity was determined by back transplantation into the host strain of origin. Cells were judged to be nontumorigenic when an inoculum of  $10^8$  cells failed to give rise to ascites tumors after 60 days. In contrast, the tumorigenic cells produced ascites tumors within 20 days in 100% of the mice inoculated with  $10^2$  cells (20). Solid tumors were induced s.c. in male Swiss mice (20 to 24 g) with a single 0.25-mg dose of 3-methylcholanthrene in tricapylin (3, 22). Extracts from this type of tumor tissue were compared with those of normal mouse tissues (kidney, liver, and lung).

Mouse ascites cells were obtained from mice which had been injected with 0.25 ml of ascites fluid (approximately  $2 \times 10^6$  cells) 13 days previously. The cells were harvested 6, 9, and 13 days after inoculation by washing the peritoneal cavities of mice which had been killed by cervical fracture. The cells were then pelleted and washed once with hypotonic salt solution (0.05 M KCl, 0.00166 M EDTA, and 0.00166 M mercaptoethanol) to remove any red blood cells. This was followed by 2 washes with cold Hanks' BSS.<sup>5</sup>

Tumorigenic and nontumorigenic TA3 cultured cells and nontumorigenic 6C3HED cultured cells were harvested from 7- to 10-day logarithmic phase cultures which had not become confluent monolayers, by scraping them into cold Hanks' BSS with a rubber policeman. The cells were then pelleted and washed twice with cold Hanks' BSS. Washed cells from all sources were suspended in a cold solution of 0.25 M sucrose containing 0.01 M magnesium chloride. Cell-free extracts were prepared by methods essentially the same as those used by Kit *et al.* (15). In preliminary experiments, homogenization of cells in a glass homogenizer with Teflon pestle (9) at 4°, even for extended periods of time, failed to break all cells. Consequently, the cell suspensions were subjected to the minimum length of sonic disruption at 4° (MSE sonicator; 2.5 min) required for disruption of all cells. These suspensions were then centrifuged at  $105,000 \times g$  at 4° for 1 hr. The supernatant was withdrawn with a syringe, with precautions taken to avoid removing any of the lipid layer, and used without further purification. Extracts from the 3-methylcholanthrene-induced solid tumors and the normal mouse tissues were prepared in the same manner, with the exception that these tissues were homogenized for 2 min with a motor-driven Dounce homogenizer before sonic disruption. The protein content of the extracts was determined by the Folin phenol method of Lowry *et al.* (16). All extracts were assayed for tRNA methylase activity immediately or were frozen at -20° and assayed within 4 days. There was a 10% decrease in activity in extracts which had been stored at -20° for more than 4 days.

The assay for tRNA methylase activity was performed in a manner similar to that described by Gantt and Evans (9). The reaction mixtures were made up as follows: 0.15 ml of a solution of 0.10 M Tris buffer, 0.10 M magnesium chloride,

and 0.04 M reduced glutathione (Mann Research Laboratories, New York, N. Y.); 0.04 ml of a 15 mg/ml solution of *E. coli* B tRNA (Mann); 0.03 ml of <sup>3</sup>H-methyl-labeled *S*-adenosylmethionine (Amersham/Searle, Toronto, Canada), which contained 50  $\mu$ Ci <sup>3</sup>H and 600  $\mu$ g *S*-adenosylmethionine/ml; and distilled water and cell extract to obtain a final volume of 0.30 ml. The <sup>3</sup>H-labeled *S*-adenosylmethionine (specific activity, 4.7 Ci/mmmole) was diluted with unlabeled *S*-adenosylmethionine (Calbiochem, Los Angeles, Calif.) to arrive at the working solution that was added to the reaction mixtures. The final reaction mixtures contained 2.0 mg of tRNA and 60  $\mu$ g of *S*-adenosylmethionine per ml at a pH of 8.2. All reaction mixtures were incubated for 1 hr at 37°. During this period the reaction rate was linear with time. At the end of the incubation period, 0.1 ml was withdrawn from each tube and adsorbed on a filter paper disc (Whatman No. 3). The discs were placed on a filter manifold and washed 4 times with 5.0 ml of cold 10% trichloroacetic acid followed by four 5.0-ml washes with ethanol:ether (1:1). The discs were then counted in 5.0 ml of toluene:PPO:POPOP scintillation solution (0.4% PPO and 0.005% POPOP in toluene) on a Nuclear-Chicago Unilux liquid scintillation spectrometer. Each experimental sample was assayed in duplicate, and a control sample, which did not contain tRNA, was assayed at the same time. The radioactive counts from these controls, which ranged from 8 to 20% of the experimental sample counts, were subtracted from the experimental sample counts before the data were plotted.

## RESULTS

Chart 1 compares the ability of cell extracts from TA3 and 6C3HED ascites cells to incorporate radioactive methyl groups from *S*-adenosylmethionine-<sup>3</sup>H into the nonspecific receptor, *E. coli* B tRNA. In order to determine whether the methylase

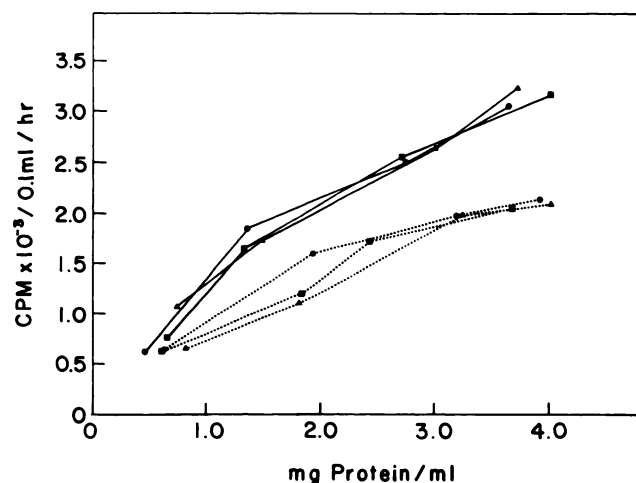


Chart 1. Comparison of tRNA methylase capacity of extracts from TA3 (—) and 6C3HED (---) ascites cells harvested at different times after inoculation: 6-day cells (●), 9-day cells (■), and 13-day cells (▲). Ordinate, rate of incorporation of radioactivity from <sup>3</sup>H-labeled *S*-adenosylmethionine into tRNA; abscissa, protein content/ml of reaction mixture. Components of the reaction mixture are given in "Materials and Methods." Numbers must be multiplied by the exponent to obtain correct value.

<sup>5</sup>The abbreviation used is: BSS, balanced salt solution.

activity from the 2 ascites cell types was altered during the development of the tumors *in vivo*, extracts were prepared from ascites cells taken 6, 9, and 13 days after inoculation. The tRNA methylase activity for both cell types was constant during the growth of the tumor *in vivo*. In all subsequent experiments, 9-day ascites cells were used in comparing the tRNA methylase activity from ascites cells to that derived from the cultured cell systems. The data of Chart 1 also show that the TA3 ascites cell extracts can incorporate approximately 30% more radioactivity into the tRNA than do the 6C3HED extracts. This difference in methylating ability may be due to either the origin of the tumor cells, 6C3HED lymphosarcoma and TA3 adenocarcinoma, or the difference in the host in which they were grown, 6C3HED from C3H mice and TA3 from Swiss mice. This difference in activity is similar to those reported by Tsutsui *et al.* (27), who showed comparable differences in the methylase activities of normal tissues of different strains of mice. They also reported differences in activity between different tumor types, both from the same mouse strain and from different strains.

The data presented in Chart 2 compare the tRNA methylase activity contained in extracts prepared from 9-day TA3 ascites cells, tumorigenic cultured cells, and nontumorigenic cultured cells. The results indicated that there was no significant difference between the methylase activity from the ascites cell extracts and from those prepared from the tumorigenic cultured cells; however, there was between 50 and 60% less activity in the nontumorigenic cell extracts. These results are comparable with those published by other investigators who used similar assay methods in comparing a wide range of malignant and normal tissues from a variety of sources (9, 17, 18, 25, 27). Therefore, the TA3 cell line, in agreeing with the previous reports, appears to have an elevated tRNA methylase activity associated with tumorigenicity as determined by transplantability.

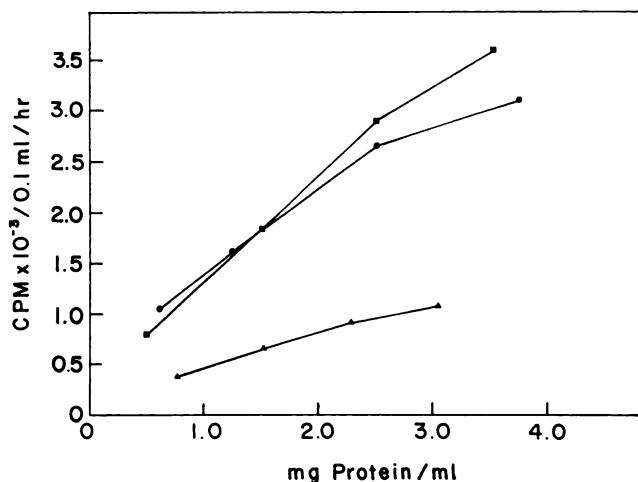


Chart 2. Comparison of the tRNA methylase capacity of extracts from 9-day TA3 ascites cells (●), tumorigenic cultured TA3 ascites cells (■), and nontumorigenic cultured TA3 ascites cells (▲). Other details are as given in Chart 1. Numbers must be multiplied by the exponent to obtain correct value.

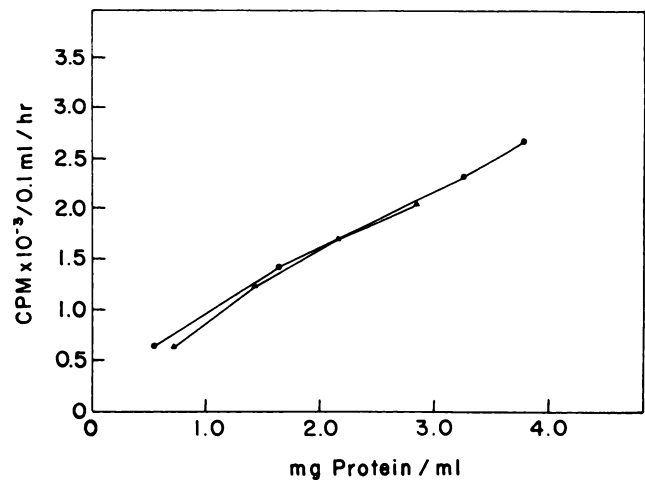


Chart 3. Comparison of the tRNA methylase capacity of extracts from 9-day 6C3HED ascites cells (●) and nontumorigenic cultured 6C3HED ascites cells (▲). Other details are as given in Chart 1. Numbers must be multiplied by the exponent to obtain correct value.

Chart 3 shows the data from the assay of the tRNA methylase activity from 9-day 6C3HED ascites cells and nontumorigenic cultured cells. Data for this cell line did not include the tumorigenic cultured cells, because the 6C3HED cells convert to the nontumorigenic state before the stage of rapid propagation is reached; it was impossible to collect enough tumorigenic cultured cells to perform a representative number of assays. However, by comparison of the data from the ascites cells to those from the nontumorigenic cultured cells, it was obvious that this cell line behaved differently than the previously described TA3 cell line. There was no quantitative difference in the methylating capacity of the ascites cell and the nontumorigenic cell extracts of this cell line. Although there was no difference in the overall methylating ability of the 2 extracts, the possibility remains that individual methylating enzymes could have modified activities.

An experimental control system was used to check the validity of the assay procedures which were used in the preceding experiments. The tRNA methylase activity from a chemically induced solid mouse tumor was compared to the methylase activity derived from normal mouse tissues. The tumors were induced in male Swiss mice by the s.c. injection of 0.25 mg of 3-methylcholanthrene (see "Materials and Methods"). The resulting tumors were transplanted into other male Swiss mice by the s.c. injection of 0.3 ml of a 25% homogenate of the original tumors. The transplanted tumors developed in 3 to 6 weeks in all of the mice that were given injections. The transplanted tumors were excised, necrotic areas were removed, and the remaining tumor tissue was treated as described in "Materials and Methods." Normal mouse kidney, liver, and lung were treated in the same manner. The results obtained from these tissues are shown in Chart 4. The tRNA methylase activity in the tumor tissue is approximately 35% greater than that of liver and kidney and 80% greater than that of lung. The activities from all of the tissues in this experiment were considerably lower than those

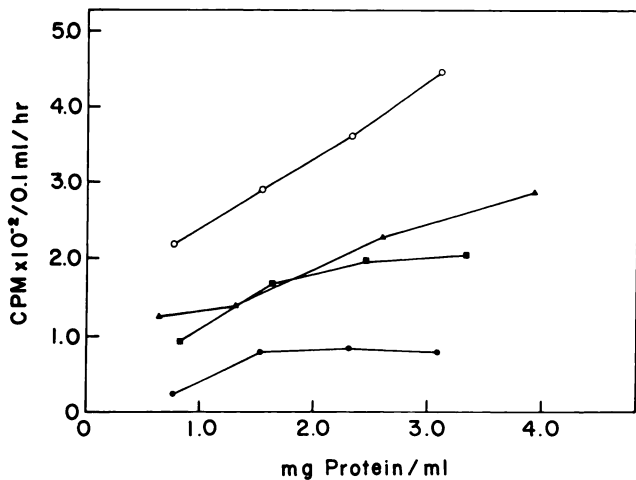


Chart 4. Comparison of the tRNA methylase capacity of extracts from a solid tumor (○), which was induced with 3-methylcholanthrene, and normal mouse lung (●), liver (■), and kidney (▲). Other details are as given in Chart 1. Numbers must be multiplied by the exponent to obtain correct value.

observed in either the ascites cells or the cultured cells. This difference in activity could be due to natural inhibitors which may be present in the whole-tissue extracts (14) but which may be absent from the ascites and the cultured cell systems.

## DISCUSSION

The loss of transplantability by tumor cells after long-term propagation *in vitro* was first observed by De Bruyn and Gey (6) in 1952 and has since been confirmed by many other investigators. The mechanism of this phenomenon has not been established, although some change in chromosome complement has been observed (12, 19), and hybrid cells between Ehrlich ascites and polyoma virus-transformed hamster cells have been found to be nontransplantable (29). In most instances, loss of transplantability has been gradual and not complete, but in the present model system the cells appear to be truly nontumorigenic (20) while still retaining a high immunoprotective capacity (8). These cells, accordingly, provided an opportunity to conduct comparative enzyme studies and to look for changes associated with loss of malignancy which might serve as biochemical markers to identify the nontumorigenic cells. These studies were envisaged as the reverse of the experiments conducted by Gantt and Evans (9) with paired neoplastic and nonneoplastic cell lines during transformation to the malignant state *in vitro*. The data of Chart 3 indicate that a quantitative difference to tRNA-methylating ability is not necessarily concomitant with the loss of tumorigenicity. This result is in partial disagreement with the hypothesis of Srinivasan and Borek (23) regarding the correlation of hypermethylation and neoplasia. While the TA3 cell line agrees with the published reports concerning the quantitative increase in tRNA methylase activity in neoplastic tissue, the 6C3HED cell line does not exhibit this overall increase. However, since it has been accepted that there is more than 1 tRNA methylating enzyme (5, 13), it is possible

that the activity of 1 or more of these enzyme proteins has been increased while that of others has been decreased during the reversion of the 6C3HED cells to the nontumorigenic state. If these 2 alterations balanced each other, there would be no quantitative change in total methylase activity; there would be a very definite qualitative change. This is very likely if a correlation can be made between these results and those of Craddock (4) and Turkington and Riddle (28). Craddock found that the various methylated bases in the tRNA of rat liver were labeled to different extents during carcinogenesis caused by dimethylnitrosamine and aflatoxin. Also, Turkington and Riddle have reported that the individual methylating enzymes of a mouse mammary carcinoma are altered to different extents when compared to normal tissue. Although both of these investigators did find a net increase in methylating capacity, neither investigator found all of the methylating enzymes increased to the same extent.

The results presented in this report point up the basic problems concerning the relationship between hypermethylation of the nucleic acids, particularly tRNA, and neoplasia. Primarily, there is a lack of knowledge concerning the precise function of these minor base components, not only in neoplastic cells, but also in normal cells. Before any true understanding of the effects of hypermethylation on cellular metabolism can take place, the normal function of the methylated bases on the nucleic acids must be elucidated. Secondly, the majority of the work that has been carried out on this problem has been concerned with the total methylating capacity of normal and tumor tissue. Again, before any proposal can be made concerning the effects of hypermethylation, all of the individual enzymes involved in the methylation of tRNA will have to be separated and characterized to at least a limited degree. The activities can then be compared in neoplastic and nonneoplastic cells in order to determine if selective alterations in the characteristics of these enzymes or in the enzyme patterns themselves can lead to neoplastic conversion.

## ACKNOWLEDGMENTS

The assistance of Mr. Leonard Kleine is gratefully acknowledged.

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