Vol. 104, No. 4, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1982 Pages 1489-1494

DNA METHYLATION LEVELS IN NORMAL AND CHEMICALLY-TRANSFORMED MOUSE 3T3 CELLS.

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Received January 12, 1982

Normal mouse embryo 3T3 cell cultures and those oncogenically transformed by the chemical carcinogens benzo(a)pyrene and methylcholanthrene were analyzed by high performance liquid chromatography to determine the 5-methylcytosine to cytosine base ratios in their total genomic DNA. The DNA methylation levels appear to be approximately equal in the three cell lines examined.

INTRODUCTION

While there has been an increased interest in investigating a possible correlation(s) between DNA methylation and oncogenic transformation, conflicting published reports have indicated an increase (1-5), a decrease (6-8) or no change (9-10) in the extent of methylation associated with oncogenic transformation.

In particular, we found little or no change in total genomic DNA methylation levels associated with oncogenic transformation by simian virus 40 in human fibroblast cells when measured by high performance liquid chromatography (HPLC) (11). We then wished to ask what the effect of chemical transformation was on total genomic DNA methylation as measured by HPLC. To answer this intriguing question, we have measured the levels of DNA methylation in mouse 3T3 cells transformed by benzo(a)pyrene (12) and methylcholanthrene (13) and in normal mouse 3T3 cells. The results of these investigations are presented in this report.

MATERIALS & METHODS

Cell Lines and Growth Conditions - The normal mouse embryo 3T3 cell culture was obtained from Professor Jerry A. Schneider (University of California at San Diego School of Medicine). The benzo(a)pyrene-transformed Balb 3T3 (BP-3T3) cells were kindly provided by Professor Robert W. Holley (The Salk Institute, La Jolla, CA.) and the methylcholanthrene-transformed Balb 3T3

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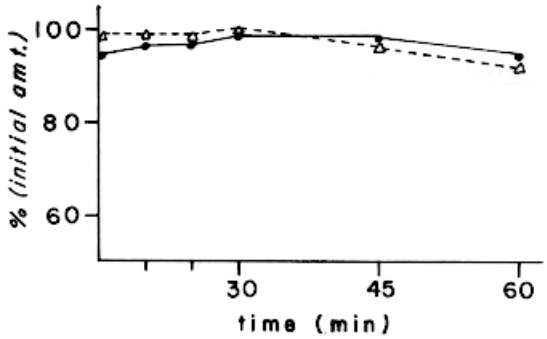

Figure 1. Hydrolysis of 5-methylcytosine and cytosine base standards.

1mM standards of 5-methylcytosine (solid circle/line) and of cytosine (open triangle/broken line) were acid hydrolyzed at 180C for the times shown. Description is in Methods section.

(MC5-5) cell line was generously provided by Professor Robert A. Weinberg (Massachusetts Institute of Technology).

All of the cell cultures were grown and maintained in Corning 850 cm^2 roller bottles containing approximately 100 mls of modified Eagle's MEM (14) supplemented with 10% dialyzed fetal calf serum and Garamycin (10 µg/ml, Schering Corp.). Cell monolayers were detached with ATV solution (0.5% trypsin, 0.2% EDTA), washed with phosphate buffered saline, counted in a Coulter-counter and finally pelletted by centrifugation at 4C.

DNA Isolation - DNA was extracted from isolated whole nuclei by standard techniques (15), treated with ribonuclease A (CalBiochem), extracted with a solution of phenol/chloroform/isopentyl alcohol (ratios were 25:24:1), and precipitated with cold 95% ethanol. The final DNA product was lyophilized and then hydrolyzed to bases by resuspending in 200 µl of 88% formic acid, sealing the mixture in a glass ampoule and heating at 180C for 20 min.(10). The hydrolysate was evaporated to dryness under nitrogen and resuspended in 200 µl of 100 mM HCl.

In control experiments with cytosine (C) and 5-methylcytosine (m^5Cyt), which is the only major methylated base found in vertebrate DNA, we have found that this method of acid hydrolysis does not affect these bases. This is in agreement with the method of Riggs (10) but not with the results of Ford (16). 1mM concentrations of individual 5-methylCytosine and cytosine standards were acid hydrolyzed at 180C for 15,20,25,30,45 and 60 min. The hydrolyzed samples were evaporated and resuspended as described above and analyzed by HPLC to check for loss of product. The results clearly show that any possible breakdown of m^5Cyt or cytosine was negligible (Figure 1.).

After treatment of the DNA samples with ribonuclease A during the isolation procedure, uracil was not detected in our preparations by HPLC analysis indicating that any RNA contamination was below interfering levels.

Analysis by High Performance Liquid Chromatography - The bases released by formic acid hydrolysis were analyzed by HPLC. Samples (20 to 50 µl) were injected into an Altex Ultrasil-10CX column

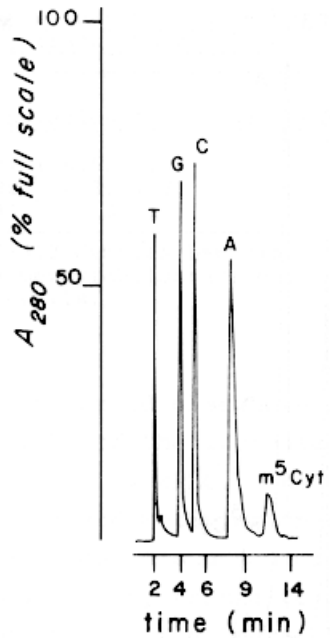

Figure 2. DNA base chromatogram from a normal mouse 3T3 cell line.
As indicated in the text, 20 to 50 µg (micrograms) of hydrolyzed DNA were analyzed in each HPLC run. The flow rate of the elution buffer was 2.5 ml per minute at a pressure of 1500 pounds per square inch. All samples were run at an initial sensitivity scale of 0.08 and subsequently increased to 0.01 sensitivity scale after 8 minutes to ensure detection of m^5Cyt. T represents Thymine, G, Guanine, C, Cytosine, A, Adenine and m^5Cyt, 5-methylcytosine. UV detection was at 280 nanometres.

(0.46 x 25 cm) and eluted with 0.02M ammonium phosphate (monobasic) buffer, adjusted to a pH of 2.3 with HCl. The column was run at ambient temperature and connected to a Beckman/Altex programmable system. The bases were identified relative to the elution of authentic standards (Sigma and CalBiochem) with detection at 280 nanometres, using a method modified from Riggs (10). The data were processed by a Shimadzu C-R1A chromatopac integrator and calculated using standard curves.

Calculations - The percent methylation was calculated by the ratio of nanomoles (nM) m^5Cyt to nM m^5Cyt plus nM cytosine multiplied by 100. Calculations of the nanomole concentrations of m^5Cyt and of cytosine were determined by linear regression analysis using values supplied by standard curves.

RESULTS and DISCUSSION

The DNA bases released after formic acid hydrolysis are represented in the elution patterns obtained from high pressure liquid chromatography, as demonstrated in Figures 2 and 3. In

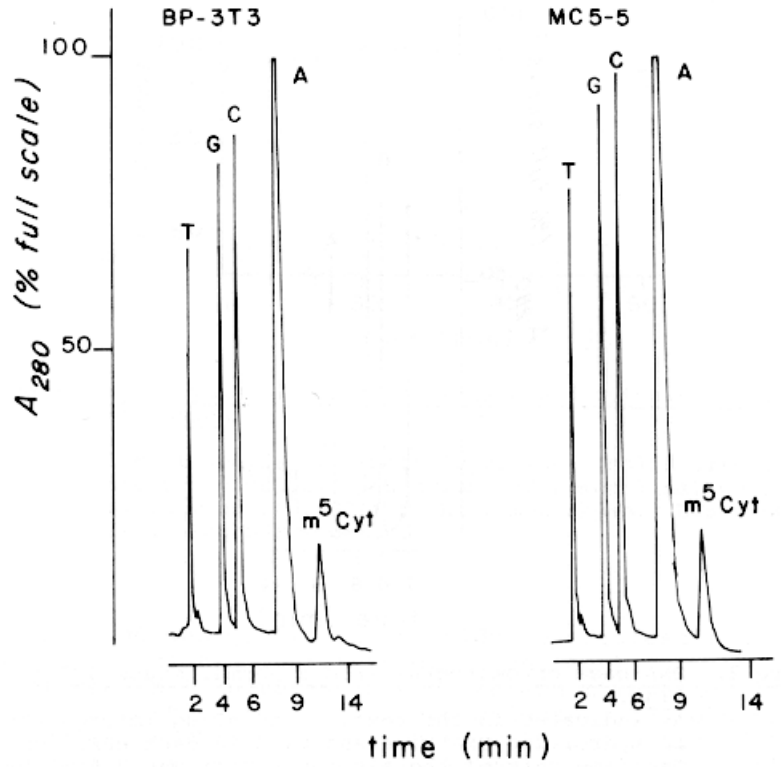

Figure 3. DNA base chromatograms from 2 chemically-transformed 3T3 cell lines.
DNA samples ranging from 20 to 50 µg were analyzed under exactly the same conditions as described in the legend to Fig. 2 and in the text.

Fig. 2, a representative chromatogram of DNA prepared from a normal strain of mouse 3T3 cells is shown, while Fig. 3 demonstrates the DNA patterns of benzo(a)pyrene- and methylcholanthrene-transformed 3T3 cells, respectively. Detection of the base m^5Cyt is easily facilitated by increasing the sensitivity scale from 0.08 to 0.01 during each HPLC run.

The data obtained by HPLC analysis are summarized in Table 1. The mean for 10 HPLC determinations of the normal 3T3 cells was 2.87% with a standard deviation of $\pm$0.58, while that for the BP-3T3-transformed cells and the MC5-5-transformed cells was 2.88% $\pm$0.31 (13 determinations) and 2.81 $\pm$0.18 (15 determinations), respectively. These results have led us to conclude that there is no real difference in the extent of total genomic DNA methylation between normal and chemically-transformed 3T3 cells when measured by HPLC.

These results, together with our earlier reported findings in virally-transformed human fibroblast cell cultures (11), suggest

Table 1. Summary of extent of DNA methylation in normal and chemically-transformed 3T3 cells.

Cell Line	% methylation ±stand.dev. and (# determinations)
normal 3T3	2.87 ± 0.58 (10)
BP-3T3	2.88 0.31 (13)
MC5-5	2.81 0.18 (15)

The mouse cell line BP-3T3 denotes benzo(a)pyrene-transformed 3T3 cells while MC5-5 is a methylcholanthrene-transformed 3T3 cell line. See text for further details.

that methylation studies reported elsewhere may not be entirely accurate especially in light of the increased sensitivity of our analysis of total genomic DNA by HPLC. The advantages of HPLC over radioactive pulse-labelling and methylation-sensitive restriction-endonuclease analysis have been discussed elsewhere (11). However, we do not dismiss the possibility that alterations in site-specific DNA methylation may have important effects on oncogenic transformation and we are pursuing the problem.

ACKNOWLEDGEMENTS. This work was supported by grant CA27564 from the National Institutes of Health; The Council for Tobacco Research-USA, Inc.; The United Cancer Council, Inc.; The Cancer Research Coordinating Committee of the University of California; Grants from the Academic Senate, University of California, San Diego; and a Special Fellowship to R.M.H. from the Leukemia Society of America. E.S.D. is a NIH Postdoctoral Fellow and is supported by USPHS grant AM07318-03 and the Council for Tobacco Research-USA, Inc. We stand indebted to Professor Jerry A. Schneider for his generosity.

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