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HYPOMETHYLATION OF HELA CELL DNA AND THE ABSENCE OF 5-METHYLCYTOSINE IN SV40 AND ADENOVIRUS (TYPE 2) DNA: ANALYSIS BY HPLC.

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Methylation of the purified virion DNA of both SV40 and adenovirus (type 2) was measured by high performance liquid chromatography (HPLC) and compared to their hosts, African green monkey kidney cells and HeLa cells, respectively. Essentially, no 5-methylcytosine was detected in either viral DNA. Implications for viral gene regulation by methylation are discussed. In comparison with normal human cell DNA methylation levels, HeLa cell DNA methylation is reduced significantly.

INTRODUCTION

Recent reviews (1-4) have documented the role of DNA methylation in the control of gene expression. An important aspect is how DNA methylation might regulate genes in tumor viruses, particularly in SV40 and adenovirus. This central problem has been examined initially. Ford et.al. (5) measured the methylation in SV40 DNA and concluded that methylation was absent. For adenovirus, studies from Doerfler's laboratory concluded a lack of viral DNA methylation (6,7).

However, the conclusions drawn from these studies may not have been justified by the data. The thin layer chromatograms (TLC) presented by Ford et.al. (5) of hydrolyzed, radioactively labelled DNA of SV40 reveal the presence of 5-methylcytosine (m^5Cyt) which may have been from the virus or contaminating DNA, thus leaving the question open if SV40 DNA is indeed unmethylated. For adenovirus DNA, Doerfler's first report (6) indicated significant amounts of

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m^5Cyt compared to cytosine in radioactively labelled bases separated by TLC. Their second study (7) was based on the susceptibility of viral DNA to methylation-sensitive DNA restriction endonucleases which can measure only a small proportion of the methylated cytosine sites.

To clear up the possible ambiguities, we used what we feel is the most accurate technique now available for measuring total genomic DNA methylation, namely high performance liquid chromatography (8,9,11), to measure the extent of DNA methylation in highly purified SV40 and adenovirus (type 2).

Utilizing this technique, we demonstrate conclusively the absence of DNA methylation in SV40 and adenovirus (type 2) DNA. Furthermore, in comparing the viral DNA methylation levels to that of their host-cell types, we find the unexpected result that the DNA from HeLa cells is hypomethylated when compared to previous measurements of both normal and SV40-transformed human cells (8).

MATERIALS & METHODS

Cell Lines and Culture Conditions - HeLa cells were the kind gift of Professor Theodore Friedmann's laboratory (University of California, San Diego). Professor Alexander Varshavsky (Massachusetts Institute of Technology) generously provided the African green monkey kidney (CV-1) cell line.

The cell lines were maintained in MEM media supplemented with Earle's salts, 10% fetal bovine serum and garamycin and subsequently harvested as described previously (9).

Viral DNA - SV40 strain 777 and adenovirus (type 2) virion DNAs were purified by and purchased from Bethesda Research Laboratories.

DNA Extraction Procedures - Using standard techniques (10), DNA from isolated whole nuclei was treated with Pronase and SDS overnight at 37°C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1, respectively) and precipitated with cold 95% ethanol. This DNA was then treated with pre-boiled RNase to remove any contaminating RNA, the pronase, phenolic extraction and ethanol precipitation steps repeated and the final product lyophilized. Hydrolysis to bases was by heating at 180°C for 20 min in sealed glass ampoules containing 88% formic acid. This method of acid hydrolysis does not affect these bases (9). The resulting hydrolysate was dried with nitrogen and resuspended in 0.1N HCl before HPLC analysis.

HPLC Methodology - DNA samples were injected into an Altex-Ultrasil-10CX column (ambient temperature) and eluted with either 0.02 or 0.03M ammonium phosphate (monobasic) buffer, pH2.3 (HCl adjusted). 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 (11). 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

Our study of the HPLC pattern of the individual bases from virion SV40 DNA demonstrates the absence of m^5Cyt (Fig. 1A). Our method can detect as little as 2 picomoles of m^5Cyt. In SV40 DNA, we have measured as much as 12 nanomoles of cytosine without concommittant detection of m^5Cyt. Our observation of less than 2 picomoles of m^5Cyt detected in the experiment where we measured 12 nanomoles of cytosine indicates less than one m^5Cyt per SV40 genome containing approximately 2120 cytosine bases (12,13). SV40 contains 27 CpG pairs, which is the usual methylation site (12,13). If all CpG pairs were methylated, this would yield a significant 1.3% methylation of total cytosines; however, in the virion DNA, none seems to be methylated.

The lack of methyl groups in SV40 is in direct contrast to monkey kidney cells in which SV40 is propagated. Figure 1C demonstrates the HPLC chromatogram of CV-1 DNA bases. Calculations of the data indicate a 3.72 ± 0.19 (12 runs) percent methylation of total cytosines.

Figure 1B shows the elution pattern of virion adenovirus (type 2) DNA bases. As with SV40, m^5Cyt is not present. We have measured up to 8.35 nanomoles cytosine without having detected any 5-methylcytosine. Even if 2 picomoles were detected, this would yield a figure of 2.4×10^{-2} percent methylation of

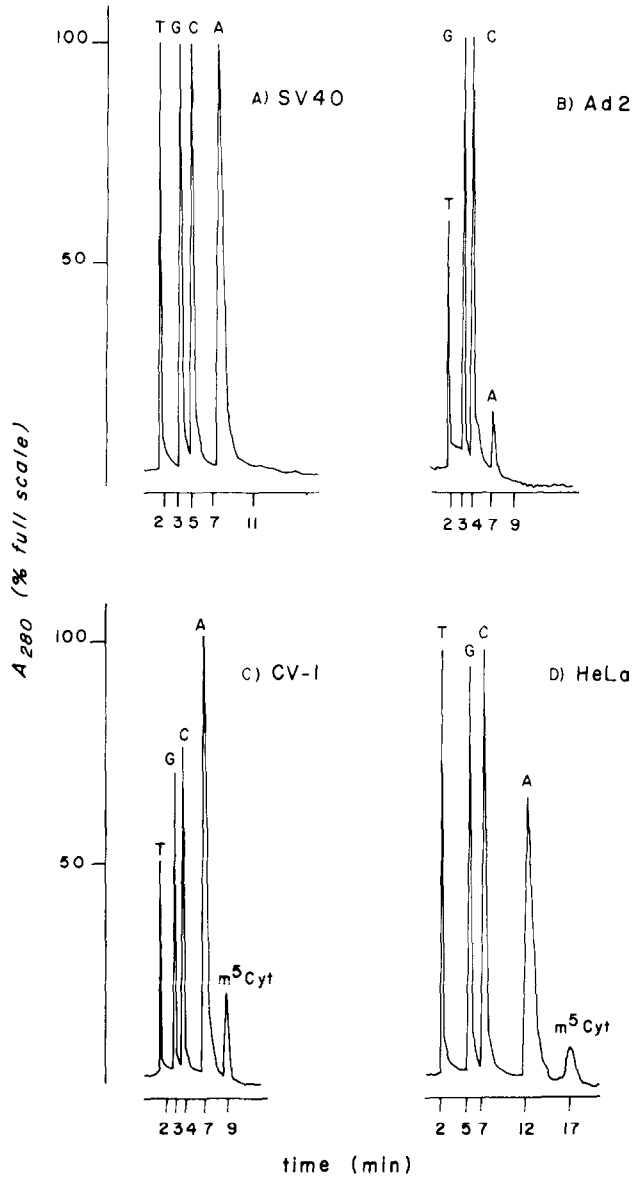

Figure 1. Chromatograms of DNA bases from SV40, Adenovirus, Monkey Kidney Cells and HeLa.

10 to 50 µg (micrograms) of hydrolyzed DNA were analyzed in each HPLC run. The flow rate of the ammonium phosphate eluting buffer (either 0.02 or 0.03M where indicated) was 2.5 ml per minute at a pressure of approx.1400 pounds per square inch. Samples were run at an initial sensitivity scale of 0.04 on an Altex Model 153 UV Detector (except for HeLa DNA which was initially started at 0.08 and Ad2 DNA which was run at 0.01 sensitivity for the entire run) and increased to a scale of 0.01 sensitivity after the complete elution of cytosine to maximize detection of m^5Cyt. The wavelength monitored was 280 nanometres. Abbreviations are T for Thymine, G, Guanine, C, Cytosine, A, Adenine and m^5Cyt, 5-methylcytosine, SV40, purified virion

the total of approximately 18,500 cytosines in adenovirus (type 2) DNA (6). This yields an upper limit of only 4-5 methylated cytosines in the virus. These results are in marked contrast to the HeLa cells in which adenovirus grows. In HeLa cells, there is a large amount of m^5Cyt in the DNA, as seen in Figure 1D. In HeLa, calculations of these data indicate that approximately 2.33% $\pm$ 0.22 (15 runs) of the total cytosines are methylated. However, this level of DNA methylation in HeLa cells is considerably lower than in normal and SV40-transformed human cells, which is about 3% (9).

A central question now raised is whether or not SV40 and adenovirus (type 2) gene activity is regulated by DNA methylation. For adenovirus (type 2), DNA methylation seems important to regulation because: 1) integrated adenovirus sequences are methylated in transformed cells (7,14); 2) there is an inverse correlation between integrated adenovirus gene expression and degree of methylation (7,14); and 3) *in vitro* methylation of a cloned gene of adenovirus (type 2) inhibits its transcription when microinjected into the nuclei of *Xenopus laevis* oocytes (15). Possibly the lack of methylation in virion DNA maximizes gene activity upon the entry of viral DNA in the cell. Adenovirus types 5 and 12 DNA may also be unmethylated in the virion (16,6,7). Considering SV40, the question is still open, but the clustering

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SV40 DNA, Ad2, purified virion adenovirus(type 2) DNA, and CV-1, African Green Monkey Kidney Cells.

For the elution patterns shown, the following data represent the concentration of the DNA bases (in nanomoles):

DNA SOURCE	A	T	G	C	m^5Cyt
A. SV40	22.68	19.35	12.15	11.8	--
B. Ad2	1.32	1.83	1.52	1.73	--
C. CV-1	15.97	12.27	8.18	7.81	0.30
D. HeLa	20.20	17.95	11.37	10.88	0.25

Retention times are later in the case of HeLa (D) due to column replacement and subsequent change to 0.03M $NH_4H_2PO_4$ buffer.

of CpG sequences near the beginning of the late genes (12,13) indicates the possibility of methylation involvement in late gene regulation when the viral DNA is integrated in transformed cells. Currently, this possibility is being pursued in our laboratory. The related polyoma virus DNA also seems unmethylated in the virion (17), and the question also remains as to whether its genes are controlled by methylation when the viral DNA is integrated in transformed cells.

Methylation also may be important in the regulation of the genes of some herpes viruses (16,18-21), a papilloma virus (22) and in retroviruses (23-27). Another important question is how it is determined if virus DNA is methylated or not upon entry into the cell. Perhaps non-permissiveness is associated with the ability of the cell to methylate incoming viral DNA. This indeed may be the case for avian sarcoma virus (23), and it must be investigated further whether this is a general phenomenon and what its possible basis is. Its implication for viral oncogenic transformation is most important.

In regard to cellular DNA, the hypomethylation of HeLa cell DNA is an unexpected result. This hypomethylation may explain the ectopic production of human chorionic gonadotropin (28-30) and the expression of an oncogene (31) in these cells. Whether or not the hypomethylation of the DNA is related or possibly causal to the oncogenic transformation of these cells remains unclear at the present time, but a most important question.

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