Volume 8 Number 11 1980 Nucleic Acids Research

DNA methylation and viral gene expression in adenovirus-transformed and -infected cells

L.Vardimon, R.Neumann, I.Kuhlmann, D.Sutter* and W.Doerfler

Institute of Genetics, University of Cologne, Cologne, GFR

Received 31 March 1980

ABSTRACT

The level of DNA methylation in adenovirus type 2 (Ad2) and type 12 (Ad12) DNA was determined by comparing the cleavage patterns generated by the isoschizomeric restriction enzymes HpaII and MspI.

As previously reported virion DNA of Ad2 and Ad12 is not methylated. Parental or newly synthesized Ad2 DNA in productively infected human KB or HEK cells is not methylated either, nor is the integrated form of Ad2 DNA in productively infected cells. Hamster cells and Muntiacus muntjak cells are abortively infected by Ad12. We have not detected methylation of Ad12 DNA in hamster or Muntiacus muntjak cells.

An inverse correlation between the level of methylation and the extent of expression of viral DNA in Ad12-transformed hamster cells has been described earlier. A similar relation has been found for the EcoRI fragment B of Ad2 DNA which is not methylated but is expressed as the Ad2 DNA-binding (72K) protein in the Ad2-transformed hamster line HE1. Conversely, the same segment is completely methylated in lines HE2 and HE3, and there is apparently no evidence for the expression of the 72K protein in these cell lines.

INTRODUCTION

With the integrated DNA sequences of adenovirus type 12 (Ad12) in Ad12-transformed hamster cells and in Ad12-induced rat brain tumor cells, an inverse correlation was observed between the extent of DNA methylation and the expression of specific Ad12 DNA sequences into messenger RNA (1,2). The integrated DNA segments in transformed and tumor cells that were expressed as mRNA were strikingly undermethylated in comparison to those regions of the Ad12 genome that were silent. It had been reported previously that the DNA isolated from purified adenovirions was not methylated to any significant extent (3,4). Ad12 DNA in transformed cells, however, was extensively methylated (5). The DNA from a baculovirus of insects, the Autographa californica

nuclear polyhedrosis virus (AcNPV), was not found to be methylated either (6).

In other virus-transformed systems, a functional correlation between methylation and genetic expression was suggested as well. The DNA of herpesvirus saimiri was found to be extensively methylated in non-producer lymphoid cells, whereas in cells producing herpesvirus saimiri the viral DNA was undermethylated (7). Chicken cells transformed by Rous sarcoma virus (RSV) allow active replication of the virus. The proviral DNA in these cells was found not to be methylated. On the other hand, in RSV-transformed rat cells, virus was not produced, and the proviral genes could be shown to be extensively methylated (cited in 9). In all these investigations the distinction between methylated and unmethylated DNA sequences was based on differential cleavage of DNA by the isoschizomeric pair of restriction endonucleases HpaII (haemophilus parainfluenzae II) and MspI (Moraxella species I). It was discovered by Waalwijk and Flavell (8) that MspI cleaved the 5'-C^{m}CGG-3' sequence, and that HpaII did not.

For a number of eukaryotic genes, differences in the levels of methylation were reported, and it was suggested in some cases that correlations to the functional state of these genes did exist. Low levels or absence of methylation of CCGG (HpaII site) or GCGC (HhaI site) sequences were associated with active gene expression for the ribosomal genes in HeLa cells (9) and in Xenopus laevis (10,11). This correlation was less striking for the globin gene in rabbits (12), but was well documented for the globin gene in chickens (13), for a variety of genes in Echinus esculentus (14), and for the conalbumin and ovomucoid genes in chickens (15). However, little or no differences were observed in the extents of methylation in DNAs from mouse embryos, from mouse teratocarcinomas, and adult mice (16). Although the mechanisms by which DNA methylation may influence gene expression in eukaryotes is unknown, many examples from viral and nonviral eukaryotic systems suggest a functional correlation of DNA methylation and gene expression although this correlation is not universal. It cannot be ruled out, however, that DNA methylation may be the consequence rather than the cause of the inactivation

of eukaryotic genes.

In the present study, we used several systems to investigate the extent of methylation of viral genes as a function of the activity of these genes. In human KB cells productively infected with adenovirus tpye 2 (Ad2), neither the parental nor the newly synthesized viral DNA was found to be methylated at any time after infection. It appeared that the integrated form of Ad2 DNA in productively infected cells (17) was not methylated either. We have previously reported on two cell systems that are abortively infected with Ad12, BHK21 cells (18) and Muntiacus muntjak cells (19). These cells did not support the replication of Ad12 DNA. In both systems Ad12 DNA remained unmethylated. On the other hand, Ad2 genomes covalently integrated into the genomes of Ad2-transformed hamster cells (20,21,22), were partly methylated depending on whether certain sections of the viral genome were expressed or remained silent.

We conclude that adenovirus DNA can escape methylation in mammalian cells, unless it is stably integrated into the cellular genome in transformed cells. The abortive state of Ad12 DNA in hamster and Muntiacus muntjak cells cannot be explained by DNA methylation.

MATERIALS AND METHODS

Cells: All virus-cell-systems investigated in this study were previously described. Human KB cells and human embryonic kidney cells were productively infected with Ad2 (17,18). Baby hamster kidney cells (subline B3) were infected with Ad12 at multiplicities between 1500 and 2000 plaque forming units (PFU) per cell (18,23). The abortive Ad12-Muntiacus muntjak cell system was also described elsewhere (19). Multiplicities of 500 PFU/cell were used.

We have also analyzed the DNA from five hamster cell lines HE1 to HE5 transformed with UV-inactivated Ad2 (20,21). Primary cultures of LSH hamster embryos had been used for transformation experiments.

Adenovirus and viral DNA: Ad2 and Ad12 were propagated in suspension cultures of human KB cells (18). The virus was purified and the viral DNA was extracted as previously reported

(18,23).

Preparation of Ad2 DNA fragments. Nick translation of Ad2 and Ad12 DNAs. Ad2 DNA was cleaved with the EcoRI restriction endonuclease, and the fragments were separated preparatively on cylindrical 0.5% agarose gels as described elsewhere (24,25). Adenovirus DNA and specific viral DNA fragments were [^{32}P]-labeled by nick translation (26) as outlined earlier (6).

Isolation of cellular DNA. Nuclear DNA from adenovirus-infected or transformed cells was extracted and purified as described elsewhere (5,34). The high molecular weight forms of DNA from human cells productively infected with Ad2 were purified as detailed before (27).

Analysis of the total intranuclear DNA from infected or transformed cells. Viral DNA sequences in adenovirus-infected or transformed cells were analyzed by cleavage with the HpaII or MspI restriction endonuclease. The DNA fragments were subsequently separated by gel electrophoresis on horizontal 1.5 % agarose slab gels. Viral DNA fragments were identified by Southern blotting (28), DNA-DNA hybridization (29) using [^{32}P]-labeled adenovirus DNA or viral DNA fragments followed by autoradiography. All methods employed were previously described in detail (1,2,5,24,25).

RESULTS

Absence of methylation of Ad2 virion DNA and of intranuclear viral DNA in productively infected cells.

We have reported previously that Ad2 and Ad12 DNA are not methylated to any significant extent (3). This conclusion was based on a biochemical analysis of viral DNA by two-dimensional thin layer chromatography. These results were confirmed by an enzymatic analysis of adenovirus DNA using the HpaII or MspI restriction endonuclease, followed by Southern blotting and DNA-DNA hybridization. Both enzymes yielded the same cleavage patterns with Ad2 DNA (Fig. 1) and also cut Ad12 DNA equally efficiently (Fig. 2 and references 1,2).

The intranuclear Ad2 DNA isolated from productively infected human cells was also analyzed by the same methods. The total intranuclear DNA was extracted between 1 h and 20 h postin-

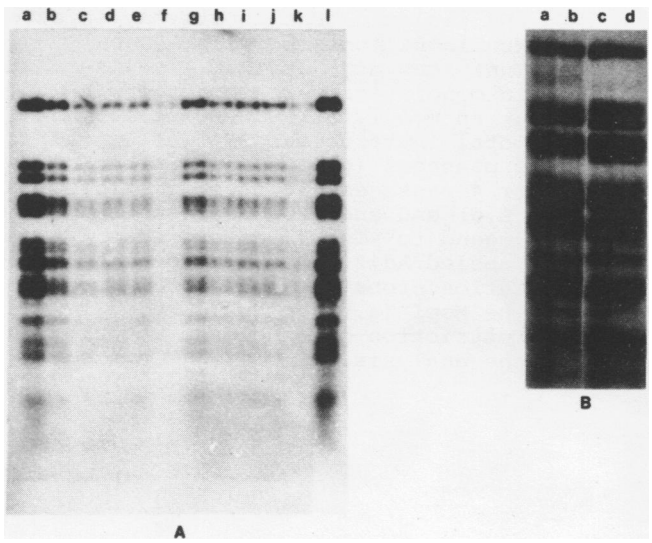

Fig. 1: Absence of methylation in Ad2 DNA extracted from purified virions or from productively infected human cells.
A: DNA was isolated from Ad2 virions (a,l) or from the nuclei of Ad2-infected KB cells at 1 h (f,k), 2 h (e,j), 3 h (d,i), 4 h (c,h) or 5 h (b,g) postinfection. KB cells were infected with 100 PFU/cell of Ad2.
B: The DNA was extracted 14 h (b,d) or 20 h (a,c) postinfection from the nuclei of Ad2-infected KB cells.
Subsequently, the DNA was cleaved with the HpaII (A: a-f; B: c,d) or the MspI (A: g-l; B: a,b) restriction endonuclease, the fragments were separated by electrophoresis on 1.5% agarose slab gels, and virus-specific DNA fragments were detected by Southern blotting (28) and DNA-DNA hybridization (29) using [^{32}P]-labeled, nick-translated Ad2 DNA probes followed by autoradiography.

fection of KB cells (Fig. 1 a,b) or between 1 h and 10 h postinfection of HEK cells with Ad2 (data not shown). Multiplicities of infection ranged from 5 to 100 PFU/cell in these experiments. It was important to analyze only intranuclear DNA in these experiments or else the DNA from virions unspecifically attached to the cytoplasma membrane might have obscured the cleavage patterns. Furthermore, low multiplicities of infection were used in some of the experiments. Upon cleavage with the HpaII or MspI restriction endonucleases the cleavage patterns of intranuclear Ad2 DNA extracted from KB or HEK cells under a variety of con-

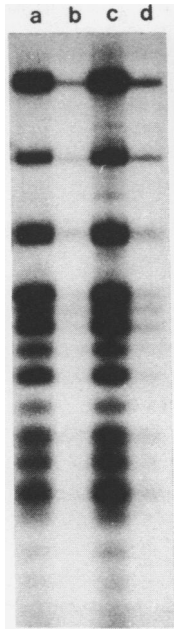

Fig. 2: Absence of methylation of Ad12 DNA isolated from abortively infected Muntiacus muntjak cells.
Muntiacus muntjak (M.m.) cells growing in monolayers were infected with Ad12 at an m.o.i. of 500 PFU/cell. The total intranuclear DNA was extracted 2 passages (6 days) postinfection (a,c) or 4 passages (12 days) postinfection (b,d) and analyzed as described in the legend to Fig. 1, except that [^{32}P]-labeled Ad12 DNA was used as hybridization probe.
The MspI (a,b) or HpaII (c,d) restriction endonuclease was used in the analysis.

ditions were identical (Fig. 1). The possibility existed that minor differences in cleavage patterns of the HpaII and MspI restriction endonucleases might have escaped detection when total Ad2 DNA was used as probe. We, therefore, employed the cloned HindIII fragments C, I, D, and E of Ad2 DNA as [^{32}P]-labeled probes also. No differences in cleavage patterns were obtained either (data not shown). These fragments are located in the BamHI B, D, C, and A fragments of Ad2 DNA, respectively (see map locations in Fig. 3). The HindIII DNA fragments of Ad2 DNA had been cloned in the E.coli plasmid pBR322 and were kindly provided by Dr. M. Mathews, Cold Spring Harbor, N.Y. We conclude that Ad2 DNA is not methylated in human cells at any time after productive infection.

It was of particular interest to investigate whether the high molecular weight forms of Ad2 DNA in KB cells were methylated as well. Previously, we had demonstrated that this form of Ad2 DNA was covalently linked to cellular DNA (17,27,30). The high molecular weight form of Ad2 DNA was isolated and the double-stranded DNA was selected as described (27), cleaved with

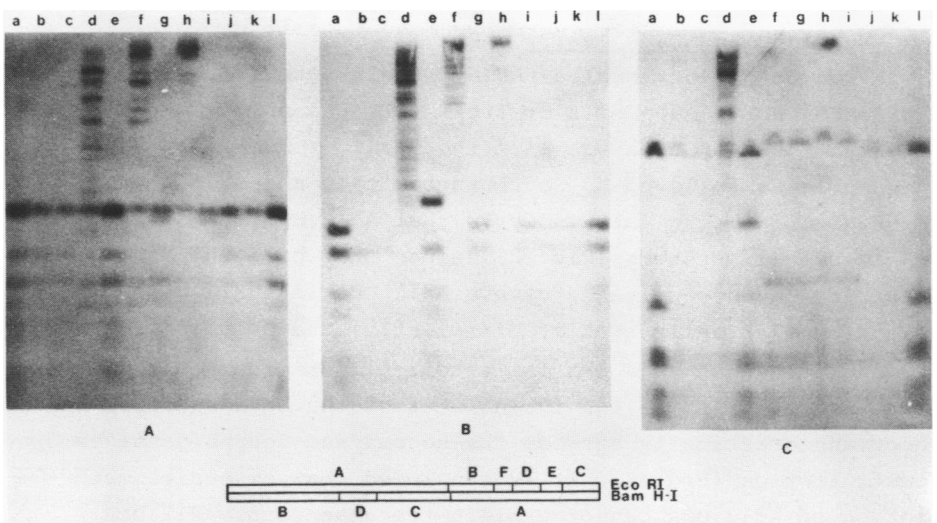

Fig. 3: Extent of methylation of Ad2 DNA integrated into the DNA of Ad2-transformed hamster cell lines HE1 to HE5.
Cell lines HE1 to HE5 were grown in monolayers, and the total intracellular DNA was extracted and analyzed as described in the legend to Fig. 1. The following [^{32}P]-labeled hybridization probes were used, the restriction endonuclease BamHI fragment B (A), the BamHI fragment D (B), and the EcoRI fragment C (C). The map positions of these fragments on the Ad2 genome are indicated in the scheme. In parts (A), (B), and (C) of this figure, DNA preparations were derived from Ad2 virions (a,l) or from cell lines HE1 (j,k), HE2 (h,i), HE3 (f,g), HE4 (d,e), or HE5 (b,c) and cleaved with the HpaII (a,b,d,f,h,j) or the MspI (c,e,g,i, k,l) restriction endonuclease. Further analyses were performed as described in the legend to Fig. 1.

the MspI or HpaII restriction endonuclease and analyzed by gel electrophoresis, Southern blotting and DNA-DNA hybridization with [^{32}P]-labeled Ad2 DNA followed by autoradiography. The data (not shown) demonstrate that the integrated Ad2 DNA sequences isolated between 1 and 20 h postinfection from KB cells are not methylated extensively.This finding would suggest that the Ad2 sequences linked to cellular DNA are expressed into messenger RNA. There is at present no further direct evidence to support this supposition.

Absence of methylation of Ad12 DNA in abortively infected hamster and Muntiacus muntjak cells.

The total intranuclear Ad12 DNA extracted from abortively infected BHK21 (B3) cells or from Muntiacus muntjak cells was also analyzed by cleavage with the HpaII or MspI restriction endonuclease followed by Southern blotting and DNA-DNA hybridization. The data indicate that Ad12 DNA is not methylated in these cell systems (Fig. 2), since the HpaII und MspI restriction endonucleases cleave Ad12 DNA in BHK21 cells or Muntiacus muntjak cells equally efficiently. The data in Fig.2 also demonstrate that Ad12 DNA fails to replicate in Muntiacus muntjak cells, since the intensities of the Ad12 specific bands decrease drastically between the second and fourth passages postinfection (Fig. 2). It is concluded that extensive methylation of Ad12 DNA cannot explain the absence of Ad12 DNA replication in hamster (18) or Muntiacus muntjak cells (29), since Ad12 DNA is not methylated in these systems.

Differential methylation of Ad2 DNA in Ad2-transformed hamster cells.

We have also investigated the extents of DNA methylation in Ad2 DNA sequences integrated into the genomes of the Ad2-transformed hamster cell lines HE1 to HE5 (20,21). The total intracellular DNA from these cell lines was cleaved with the HpaII or MspI restriction endonuclease, the fragments were separated by electrophoresis on horizontal 1.5% agarose slab gels and blotted according to the Southern procedure. Specific [^{32}P]-labeled fragments of the Ad2 genome were used as probes in DNA-DNA hybridization experiments. The data presented in Fig. 3 demonstrate that there are striking differences in the extent of DNA methylation in different parts of the Ad2 genome depending on the cell line and the region of the genome investigated. The BamHI fragment B (Fig. 3 A) is not methylated in lines HE1 and HE5, while it is methylated in the other three lines. Similar findings are observed for the BamHI fragment D (Fig. 3 B). The EcoRI fragment C (Fig. 3 C) is heavily methylated in line HE4, less extensively in lines HE2 and HE3, and apparently unmethylated in the other two lines. Some of the regions of the viral genome which were not investigated were

found to be absent from the genomes of some of the Ad2-transformed hamster lines (22).

Little is known as yet about the expression of the Ad2 genome in cell lines HE1 to HE5 (21). Therefore, functional correlations between methylated Ad2 DNA sequences and the expression of these sequences in the transformed lines HE1 to HE5 can be drawn only in a few instances. The scheme in Fig. 4 presents the functional map of Ad2 DNA in a highly simplified form. It is apparent that the DNA-binding protein of Ad2, the 72K protein, is encoded on the l-strand of the EcoRI fragment B of Ad2 DNA. The results on the expression of viral functions in the HE1 to HE3 lines published by Johansson et al. (21) suggest that the DNA-binding protein of Ad2 is synthesized in line HE1 and not in the other two Ad2-transformed hamster cell lines. Furthermore, preliminary results indicate that in vitro translation of polyadenylated cytoplasmic RNA from cell line HE1 gives rise to a polypeptide of about the size of the 72K protein,

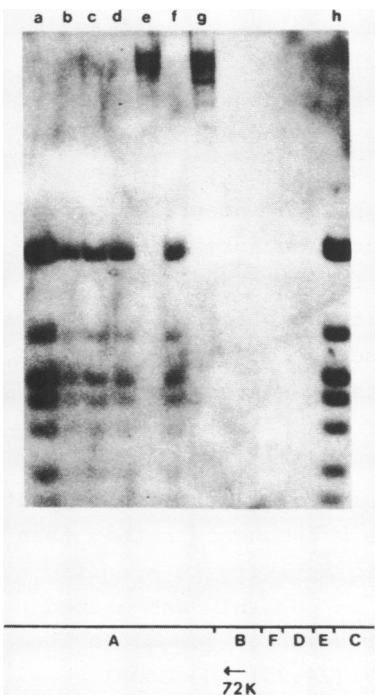

Fig. 4: Autoradiogram of Southern blots of HE1, HE2, and HE3 hamster cell DNA digested with the restriction endonuclease HpaII (c,e,g,h) or MspI (a,b,d,f). DNA preparations were extracted from Ad2 virion DNA (a,h) or from the Ad2-transformed hamster lines HE1 (b,c), HE2 (d,e) or HE3 (f,g). The DNA on the Southern blots was hybridized with the [32P]-labeled EcoRI fragment B of Ad2 DNA. The scheme indicates the EcoRI map of Ad2 DNA and the location of the 72K protein, the DNA-binding protein of Ad2.

whereas RNAs from cell lines HE2 or HE3 do not (H. Esche, unpublished experiments). It was therefore of particular interest to investigate the extent of DNA methylation in EcoRI fragment B in line HE1 in comparison to that in lines HE2 and HE3 (Fig. 4). We have determined the patterns of integration of Ad2 DNA in the DNA of lines HE1 to HE5 (22). The EcoRI fragment B of Ad2 DNA is present in its entire length only in cell lines HE1, HE2, and HE3. The restriction endonucleases HpaII and MspI cleave the DNA in EcoRI fragment B of line HE1 equally well, whereas in lines HE2 and HE3 the restriction endonuclease HpaII does not cleave any of the sequences in the EcoRI fragment B (Fig. 4). This finding demonstrates that the DNA in EcoRI fragment B is not methylated in line HE1, where it is expressed. In lines HE2 and HE3 the same segment of viral DNA is completely methylated, and there is no evidence at present that the DNA-binding protein is synthesized in these lines.

DISCUSSION

In Ad2- and Ad12-transformed hamster cells, as well as in Ad12-induced rat brain tumor lines (1,2), a very striking inverse correlation has been found between the degree of DNA methylation in specific segments of the viral genome and the level of expression of the same viral DNA segments into messenger RNA. This correlation appears to apply to integrated viral genomes (1,2,9) or to viral genomes stably anchored within the cell in an episomal state (7). Much further work will be required to elucidate the possibly quite complicated mechanisms relating modification of specific DNA sequences and their availability for expression. The results presented raise tantalizing questions as to the role of DNA methylation in the regulation of gene expression in transformed cells and perhaps in mammalian cells in general. Of course, it is conceivable that DNA methylation or demethylation is the consequence rather than the cause of different levels of genetic activity in specific regions of the genome. DNA methylation is possibly associated with long-term inactivation of viral or cellular genes. In the Ad12-transformed hamster cell line T637 (24,25) the EcoRI fragments B of the integrated Ad12 genomes are not expressed.

All 5'-CCGG-3' sites in this fragment in the integrated Ad12 genomes are methylated in line T637 (2). In preliminary experiments we have investigated whether the levels of methylation in specific parts of the integrated viral genome can be altered upon hormone treatment of the cells. Exposure of T637 cells, an Ad12-transformed hamster cell line, to the steroid hormone dexamethasone at a concentration of 10^{-5}M does not lead to a decrease of methylation in the genetically inactive EcoRI fragment B of the integrated Ad12 genomes. Obviously, this problem requires more detailed examination.

Viral DNA in productively or abortively infected cells or virion DNA (3,4,18) remains unmethylated, even at very early times postinfection when late viral functions are not yet expressed. If DNA methylation was in fact the cause or the consequence of genetic activity of specific DNA sequences, it would not be advantageous for viral replication to become subject to cellular regulatory mechanisms. It is, however, completely enigmatic, how viral DNA can escape modification by cellular methyl-transferases.

It is worth noting that the high molecular weight form of Ad2 DNA in productively infected cells, which represents viral DNA covalently linked to cellular DNA (17), does not become methylated either. It remains to be determined whether the integrated viral DNA sequences in productively infected cells are expressed as messenger RNA. Absence of methylation in integrated viral DNA might suggest that the integrated Ad2 DNA sequences in productively infected cells are genetically active.

In BHK21 cells abortively infected with Ad12, only the early Ad12 genes are expressed as messenger RNA (23). The entire viral genome, however, remains unmethylated as late as 24 h postinfection. These data indicate again that free viral DNA is not modified. We have not separately analyzed the integrated form of Ad12 DNA described in this system earlier (31).

ACKNOWLEDGMENTS

We thank Hanna Mansi-Wothke for the preparation of cells and media and Birgit Kierspel for typing this manuscript. This research was supported by the Deutsche Forschungsgemeinschaft

through SFB 74 and by the Ministry of Science and Research of the State of Northrhine-Westfalia.

*Present address: Department of Microbiology, State University of New York at Stony Brook, N.Y.

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