Complete DNA methylation does not prevent polyoma and simian virus 40 virus early gene expression

(in vitro methylation/liver DNA methylase/microinjection/methylation-insensitive genes)

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ABSTRACT The effect of DNA methylation on polyoma virus and simian virus 40 gene expression was investigated. For this purpose, the cytosines of all C-G dinucleotides of the viral DNAs were methylated by the use of rat liver methylase and the completeness of methylation was verified by dinucleotide analysis and restriction endonuclease treatment. The biological activity of unmethylated and fully methylated DNAs was tested by microinjecting them into tissue culture cells. The functions analyzed included early and late viral gene expression, viral DNA replication, oncogenic transformation efficiency, and virus maturation. No difference in the activity of these biological functions was observed between methylated and unmethylated DNA. Early gene expression of methylated DNA is not the result of demethylation because viral DNA reextracted from the injected cells, under nonpermissive conditions, retained the methylation pattern of the input DNA. In contrast, viral DNA extracted from transformed cells or from intact virus particles was partially or completely demethylated.

In animal cells, the only methylated DNA base is 5-methylcytosine, which is found almost exclusively in C-G dinucleotide sequences (1–3). The biological function of this methylation is still unclear, but there is increasing evidence that it plays a role in the regulation of gene expression. As a general rule, genes that are actively transcribed are undermethylated, whereas genes that are not transcribed are highly methylated (4–8). There are exceptions to this rule, although in only one sense. Some genes, such as the β-globin gene in the human placenta (9), the albumin gene in rat hepatoma cells (10), and the adenovirus 12 genes in hamster cells (11), are evidently not transcribed and yet are unmethylated. Moreover, the DNA of some organisms is totally devoid of methyl groups (12, 13), but no convincing examples have been reported to date of genes that can be transcribed in vivo in the fully methylated state. These correlations have suggested that undermethylation is a necessary but not sufficient condition for gene transcription.

More direct evidence for a causal connection between DNA methylation and transcription has come from experiments in which the biological activity of the same DNA was compared in the unmethylated and methylated condition by transfection or microinjection into recipient cells (14–16). In contrast to these findings, it has been reported recently that early simian virus 40 (SV40) and polyoma virus (PV) gene expression is not affected by DNA methylation (17–19). But the interpretations that can be drawn from these latter experiments are limited by the fact that the bacterial methylases (EcoRI or Hpa II) were used for in vitro methylation of viral DNAs. The EcoRI enzyme methylates only adenine but not cytosine, and the Hpa II enzyme (with its recognition sequence C-C-G-G) only methylates about 6% of the potential C-G methylation sites. Because there is no indication of a specific role of C-C-G-G, or of any other sequence more complex than C-G in eukaryotic gene regulation by methylation, it cannot be excluded that the regulation-relevant sites in the DNA were in fact undermethylated in these experiments.

In the study reported here we demonstrate that early SV40 and PV gene expression, viral DNA replication, and oncogenic cell transformation are not affected by methylation of all of the potential C-G methylation sites of the viral DNAs by treatment with the rat liver methylase. Upon microinjection into tissue culture cells the completely methylated viral DNA exhibited the same biological activity as the unmethylated DNA. From these results we conclude that the early genes of SV40 and PV represent a class of genes whose expression is methylation insensitive.

MATERIAL AND METHODS

Cells, Microinjection, and Viral DNA. The following cell types were used: monkey TC7 and CV1 cells (sublines of African green monkey kidney cells), rat 52.2 cells (Fisher rat embryo fibroblasts provided by W. C. Topp, Cold Spring Harbor Laboratory), and mouse 3T6 and primary mouse kidney cells. Cells were grown on glass slides subdivided into numbered squares of 2 × 2 mm² in Dulbecco's medium supplemented with 5% (vol/vol) fetal calf serum. For DNA reextraction experiments cells were grown on small glass pieces (3 × 3 mm). For this purpose a 50-μl drop of medium containing about 500 cells was placed on a sterile glass plate lying in a 60-mm Falcon dish. After incubation for 30 min at 37°C and attachment of the cells to the glass surface, 5 ml of medium with serum was added. On the next day cells were used for microinjection (20). SV40 (strain 776) and PV DNAs (A-2) were isolated from virus-infected cells by the Hirt method and further purified as described (21, 22).

Preparation of DNA Methylases. Rat liver DNA methylase was isolated from 120 g of regenerating rat liver, as described (23), and purified with the following modifications. After DEAE-Sephadex A-50 gradient chromatography the fractions containing the enzyme were diluted 1:2 with buffer A (20 mM Tris-HCl, pH 7.8/1 mM EDTA/0.5 mM dithiothreitol) and stirred with 5 ml of heparin-Sepharose (Pharmacia) for 1 h. The loaded gel was then packed into a column and the enzyme was eluted with a 60-ml KCl gradient from 0.085–0.32 M in buffer A with 10% glycerol. The pooled peak fractions were precipitated by 40–60% ammonium sulfate. About 4,000 units of enzyme (1 mg of protein) were then chromatographed on a Sephadex G-150 column (0.9 × 60 cm) in buffer A, supplemented with 0.6 M KCl and 15% glycerol. The most active enzyme fractions were fi-

Abbreviations: SV40, simian virus 40; PV, polyoma virus.

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nally concentrated 10-fold by filtration on an Amicon UM 20 membrane and stored at −70°C. The bacterial Hpa II methylase was prepared and purified as published elsewhere (24).

Methylation of Viral DNAs in Vitro. SV40 DNA or PV DNA (0.5 μg) was incubated for 1 hr at 30°C in a total reaction volume of 500 μl containing 20 mM Tris-HCl (pH 7.8), 60 mM KCl, 5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100, 200 μg of bovine serum albumin, 5 μM S-adenosyl-[methyl-3H]methionine (specific activity 15 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ Bq), and 500 units of rat liver DNA methylase (1 unit incorporates 1 pmol of CH₃ in denatured Micrococcus luteus DNA in 1 hr at 37°C). Under these conditions 0.5 μg of the synthetic polynucleotide (dC-dG), can be completely methylated. For preparations of mock-methylated DNAs S-adenosylmethionine was omitted from the reaction mixture. Under similar conditions SV40 DNA and PV DNA (5 μg) were methylated by 0.5 unit of Hpa II methylase (1 unit incorporates 10 pmol of CH₃ in denatured M. luteus DNA in 15 min at 37°C) in a 200-μl reaction mixture, containing 50 mM Tris-HCl (pH 7.8), 5 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 200 μg of bovine serum albumin, and 30 μM S-adenosyl-[methyl-3H]methionine for 16 hr at 33°C. After incubation the reaction mixtures were treated with 1% sodium lauryl sarcosinate and 250 μg of Pronase per ml for 40 min at 37°C; 1% NaDodSO₄ and 0.3 M NaCl were added and followed by two phenol and four ether extractions. The DNA was precipitated from the aqueous phase by ethanol. The in vitro methylated DNA was suspended in 10 mM Tris-HCl/1 mM EDTA, pH 7.4, to 1–2 μg/ml for injection experiments. DNA preparations were treated for completeness of methylation by cleavage with Hpa II, Msp I, and Hha I. DNA fragments were analyzed by electrophoresis on horizontal 1.4% agarose slab gels.

Dinucleotide analysis of methylated viral DNA was performed essentially as described by Sano and Sager (25). Methylated DNAs were digested with pancreatic DNAse I and the resulting dinucleotides were separated by paper electrophoresis. The isolated dinucleotides were further digested with alkaline phosphatase and spleen or snake venom phosphodiesterase and assayed by high-voltage paper electrophoresis. The migrating positions of authentic 5-methyl-2'-deoxycytidine-5'-monophosphate and 5-methyl-2'-deoxycytidine were identified by ultraviolet light quenching, and the spots were cut out and counted for radioactivity after elution with 0.6 M HCl.

Isolation of Cellular DNA and Blot Hybridization. High molecular weight DNA was isolated from the culture cells by standard procedures (26). Genomic DNA (3–10 μg) was digested with restriction endonucleases and the product was electrophoresed on horizontal 1.0% agarose gels. The fragments were transferred to nitrocellulose filter sheets and hybridized to nick-translated ³²P-labeled DNA probes (specific activity = 1–2 × 10⁶ cpm/μg) as described elsewhere (27, 28).

RESULTS

In Vitro Methylation of SV40 and PV DNAs. Under the experimental conditions described here, about 2.5% of the SV40 and 3.8% of the PV DNA nucleotides were methylated by the rat liver DNA methylase, as estimated from the incorporation of the ³H-labeled methyl group transferred from S-adenosyl-[methyl-³H]methionine into the DNA. Because cytosine as C-G dinucleotide represents only 0.5% of the entire SV40 genome (27 C-G pairs) and 1.9% of the PV DNA (96 C-G pairs), respectively, we studied the methylation pattern of the SV40 and PV cytosine dinucleotides by the method described by Sano and Sager (25). As summarized in Table 1, the rat liver methylase did not methylate cytosine exclusively in C-G dinucleotide; in fact, most of the methylated cytosine residues were in C-A and C-T dinucleotides. Methylation of cytosine in C-C dinucleotides was not observed. It should be mentioned that degradation of DNA with DNAse I does not result in a strictly quantitative distribution of the dinucleotides (25). The completeness of SV40 and PV DNA methylation was also tested by Hpa II and Hha I restriction endonuclease digestion. The tetranucleotide recognition sequences for both enzymes contain C-G, but the enzymes do not cleave the DNA if these sequences are methylated. SV40 DNA contains one cleavage site for Hpa II and two for Hha I endonuclease; the PV DNA contains eight for Hpa II and four for the Hha I endonuclease.

As analyzed by gel electrophoresis and the Southern blotting technique, the methylated SV40 and PV DNAs are totally resistant to both restriction endonucleases, as shown in Figs. 1 and 2. SV40 and PV DNA were also methylated with the DNA methylase of Haemophilus parainfluenzae II (Hpa II methylase). This treatment converted all of the SV40 and PV Hpa II cleavage sites into Hpa II endonuclease-resistant sites, while remaining cleavable by the Msp I endonuclease, a Hpa II isochizomer that cleaves methylated and unmethylated DNA.

The Biological Activity of the Methylated DNA. The biological activity of the methylated SV40 and PV DNA was tested...
after microinjection into either TC7 (SV40 DNA) or 3T6 cells (PV DNA). As summarized in Table 2, SV40 and PV DNA injected at a multiplicity of two to four DNA molecules per cell induced T-antigen and V-antigen synthesis in all recipient cells, as tested by direct immunofluorescence staining. Because viral DNA replication is a prerequisite for late viral gene expression, V-antigen synthesis indirectly shows that viral DNA replication occurred in the injected cells as well (29). Similar results were obtained after injection of mock-methylated SV40 and PV DNA (Table 2). Furthermore, the T- and V-antigen-specific immunofluorescence per cell was as intense in the cells injected with methylated DNA as in control cells injected with the unmethylated DNA. The time course of T- and V-antigen synthesis was also similar in all injected cells.

To test more directly if methylation affects viral DNA replication, single cells of confluent cell cultures were microinjected with one or two DNA molecules (DNA concentration, 0.005 mg/ml of injection buffer; injection volume, 1 × 10⁻¹¹ ml per cell). In these experiments CV1 cells were used for SV40 and primary mouse kidney cells for PV DNA injections. One day after injection of DNA, cultures were overlayed with agar (0.9%) and processed for plaque assay. In three replicate experiments methylated DNA as well as unmethylated SV40 and PV DNA induced plaque formation in 40–60% of the injected cells (Table 2). Plaque sizes and times of plaque appearance were also similar for all the experiments.

As a third biological test, the relative efficiency of oncogenic transformation of rat 52:2 cells by methylated and unmethylated SV40 and PV DNA was compared. For these experiments the recipient cells were grown to semiconfluence on glass slides subdivided into numbered 1-mm² squares (20). In each square only 1 cell was injected with two to four DNA molecules (50 cells for each experiment). One week after microinjection, the cells within the squares were isolated by manipulation under the microscope by use of glass capillaries and further cultivated under standard conditions in microculture dishes. After a second passage in 60-mm dishes isolates were analyzed for T-antigen-positive cells. In three replicate experiments T-antigen-positive cells were found in 20–30% of the isolates. This high transformation rate was observed after injection of both methylated and unmethylated SV40 and PV DNA. For further characterization two randomly chosen isolates of each category of transformed cells were recloned twice in soft agar. Cells of these clones were found to synthesize T antigen permanently and to fulfill all criteria for fully transformed cells (data not shown) (30).

**The Fate of Methylated DNA in Injected Cells.** To test whether T-antigen synthesis after injection of fully methylated viral DNA is the result of an initial demethylation we reisolated the injected DNA and analyzed the state of its methylation: (i) during the first 20 hr after injection; (ii) in PV- and SV40-transformed cells; and (iii) in virus particles obtained from 3T6 cells injected with methylated PV DNA.

(i) It is possible to determine the biochemical fate of injected DNA by analysis of a small number of injected cells (20). For the Southern blotting technique a DNA concentration of about 1 pg is sufficient to give a clear signal on the autoradiogram (20). At an injection volume of 2 × 10⁻¹¹ ml per cell and a DNA concentration of 1 mg/ml, 50–100 injected cells fulfill these conditions. To follow the intracellular fate of the DNA the methylated PV DNA was injected into 3T6 cells. To prevent DNA demethylation as a consequence of replication, 1-ß-D-arabinofuranosylcytosine (final concentration, 30 μg/ml) was added to the cells directly after injection. Under these conditions DNA replication and hence late viral gene expression were blocked, whereas T-antigen synthesis was not affected. At various times after injection (1, 4, 8, 16, and 20 hr) the cells were lysed and DNA was extracted by the Hirt method (21). Each DNA extract was subdivided into three samples. One of them served as a control and the other two were treated with either Hpa II or Msp I restriction endonuclease. After agarose gel electrophoresis the DNA was transferred to a nitrocellulose filter and hybridized with nick-translated PV DNA (specific activity, 1–2 × 10⁹ cpm/μg). These experiments showed that the reextracted DNA remained resistant to Hpa II and Hha I endonuclease digestion throughout the entire period monitored but was completely cleaved by the Msp I enzyme. Fig. 3 shows the Hpa II-

**Table 2. Biological activity of methylated and unmethylated SV40 and PV DNAs**

<table>
<thead>
<tr>
<th>Injection of two to four DNA molecules per cell</th>
<th>Antigen formation, %*</th>
<th>Virus production, % of injected cells†</th>
<th>Cell transformation, % of injected cells‡</th>
</tr>
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<tbody>
<tr>
<td>SV40 DNA I</td>
<td>T 100  V 100</td>
<td>40–60</td>
<td>20–30</td>
</tr>
<tr>
<td>SV40 DNA I methylated</td>
<td>With rat liver methylase 100  100</td>
<td>40–60</td>
<td>20–30</td>
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<tr>
<td>With Hpa II methylase</td>
<td>100  100</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PV DNA I</td>
<td>T 100  V 100</td>
<td>20–55</td>
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<td>PV DNA I methylated</td>
<td>With rat liver methylase 100  100</td>
<td>40–60</td>
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<tr>
<td>With Hpa II methylase</td>
<td>100  100</td>
<td>ND</td>
<td>ND</td>
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* T- and V-antigen formation was tested by the direct immunofluorescence technique 24 hr after DNA injection.
† Virus production was assayed by plaque formation.
‡ Transformation was assayed by colony formation in soft agar.

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**Fig. 2. Sensitivity of methylated PV DNA to Hpa II and Msp I endonucleases analyzed by Southern blotting technique. Methylated PV DNA undigested (lane 1) and after digestion by Hpa II (lane 2) or Msp I (lane 3). The positions of forms I and II of the PV DNA are indicated.**
methylated PV DNA reextracted 20 hr after injection. The higher molecular weight fractions in lanes 1 and 2 that migrated more slowly than the DNA II form are concatemers of the injected PV DNA. Formation of concatemers is regularly observed in microinjected cells and is not specific for methylated DNA (unpublished data).

(ii) To analyze the state of methylation in the transformed cells, cellular DNA was extracted from two PV DNA (clones 20 and 21) and one SV40 DNA transformed line (SV-CH2) at passages 20–25. These lines were obtained from rat 52.2 cells injected with methylated PV or SV40 DNA. The extracted DNA was digested with different restriction enzymes, separated by agarose gel electrophoresis, and analyzed by the Southern blotting technique. Fig. 4 shows the DNA isolated from clone 20 cells. Two conclusions can be drawn from this autoradiogram. First, clone 20 cells contain the PV DNA in a free episomal form only. PV sequences covalently integrated in the host DNA are not demonstrable. Cellular DNA treated with different restriction enzymes that do not cut the PV DNA (lane 5, Bgl II; lane 9, Sal I) shows the same PV-specific pattern as the "no DNase" treated extract (lanes 6 and 12). Similar results were obtained when the DNA was isolated by the Hirt method. Only the PV DNA yield was significantly higher (see Fig. 5 for clone 21). Second, the PV DNA from clone 20 cells is partially demethylated. The majority of the DNA molecules carry one to four Hpa II-sensitive sites (lanes 7 and 10). A smaller fraction of the PV DNA molecules could even be totally demethylated. After Msp I endonuclease treatment, only PV-specific fragments are demonstrable (lanes 8 and 11). In control experiments unmethylated PV DNA was added to the DNA extracted from normal rat 52.2 cells. Under these conditions PV DNA was completely cleavable by the endonuclease-treated Hpa II and Msp I (lanes 1–4). We reexamined the state of PV DNA of clone 20 cells again at passage 45 and obtained the same results as described above.

Most of the PV DNA in the clone 21 cell is also in the free episomal form. In contrast to clone 20 cells, this PV DNA is entirely sensitive to both the Hpa II and the Msp I endonuclease treatment, as shown in Fig. 5 for cellular DNA (lanes 4–8) and for Hirt extract DNA (lanes 1–3). The SV40 DNA transformed SV-CH3 cells contain the viral DNA only in the integrated form. Two out of three integrated SV40 DNA copies were found to be Hpa II endonuclease-sensitive and only one was resistant, but all three could be further cleaved by Msp I treatment (data not shown).

(iii) PV DNA extracted during the late period of the infection cycle (40 hr after injection) or from full virus particles obtained from microinjected cells was Hpa I and Hha I endonuclease-sensitive. These data show that after replication the viral DNA is demethylated.

**DISCUSSION**

If methylation of eukaryotic DNA is involved in regulation of gene expression, two categories of genes can be postulated, one whose expression is methylation-sensitive and another whose expression is methylation-insensitive.

Numerous examples of methylation-sensitive genes have been reported in the last few years (for review, see refs. 31 and 32). In contrast, we have shown here that early SV40 and PV gene expression as well as viral DNA replication are not sensitive to DNA methylation. Thus far, no definitive conclusions can be drawn about late viral gene expression, because capsid protein
synthesis requires DNA replication, after which the methyl groups have been lost. Methylation insensitivity of the expression of SV40 and PV genes had been previously reported, but in these earlier investigations bacterial enzymes had been used for in vitro methylation of the viral DNA. These enzymes methylate either only a subfraction of the C-G dinucleotides, as does the Hpa II methylase, or only adenosine instead of cytosine, as does the Escherichia coli methylase (17–19). Therefore, viral gene expression due to undermethylation could not be excluded. Our experiments are free from this objection because we used DNA methylase isolated from regenerating rat liver cells. This enzyme efficiently transfers the methyl group from S-adenosylmethionine to cytosine in the C-G sequence of double-stranded supercoiled, circular, and linear DNA in vitro (33).

Dinucleotide analysis and restriction enzyme digestion of our in vitro methylated SV40 or PV DNA showed complete methylation. Furthermore, the high rate of early viral gene expression in cells microinjected with the low multiplicity of two to four methylated DNA molecules per cell makes it unlikely that a subfraction of unmethylated DNA molecules is responsible for the observed biological activity. Evidently methylation of the injected viral DNAs is conserved in cells whose DNA synthesis is blocked by 1-β-D- arabinofuranosylcytosine. PV DNA reextracted from the cells was completely resistant to cleavage by the Hpa II endonuclease. In spite of the fact that this enzyme recognizes only 8 of the 96 PV DNA C-G dinucleotides, we consider this number as representative for all methylated sites. Verification of complete methylation would require sequence analysis of the DNA, which is difficult to carry out on molecules reextracted from a small number of microinjected cells. On the other hand, partially or totally demethylated PV and SV40 DNAs were found in the oncogenically transformed cells. Thus far, active demethylation by an enzyme cannot be excluded. A DNA demethylase has recently been found in Friend leukemia cells (34). Because we did not observe demethylation when DNA replication was blocked, a loss of methyl groups during replication by insufficient maintenance-methylation seems more likely.

Because our experiments have shown that exhaustive DNA methylation does not abolish expression of some viral genes, the question arises what might be the difference between methylation-sensitive and -insensitive genes. It has been assumed that methylation of C-G sites of the promoter region is sufficient to suppress transcription in vivo (1, 5, 6, 35). This is not the case for SV40 and PV. The observed gene expression is not due to underrepresentation of C-G sites that are capable of methylation because the promoter/enhancer region of SV40 contains about 68% (19 of 28) and the PV 24% (23 of 96) of all viral C-G dinucleotides (20). This does not exclude the possibility that the decisive role is played by a specific position of the C-G dinucleotides and not by the absolute number of the C-G dinucleotides within the promoter region. It is also conceivable that the promoter region of methylation-insensitive genes lacks essential sequence elements crucial for regulation or that specific properties of insensitive genes overcome the methylation block (or both). If C-G dinucleotides outside of the promoter region are the targets for regulation by methylation, the region downstream from the cap site could be a candidate for this. It is striking that the early SV40 and PV genes are lacking sites that are capable of methylation in the first 240 nucleotides downstream from the cap site. This fact is in contrast to the relative abundance of C-G dinucleotides in this region of genes known to be methylation-sensitive, as is the β-globin and the herpes simplex virus thymidine kinase genes (36–38). This working hypothesis is also supported by experiments reported by Fradin et al. (17). Methylation of the only SV40 Hpa II-sensitive site downstream from the major late mRNA site inhibited late transcription of the methylated DNA after microinjection into oocytes.

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