Methylation of the SV40 *Hpa*II site does not affect late viral gene expression in microinjected tissue culture cells

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Intranuclear coinjection of the late SV40 *Kpn*I/*Bcl*I DNA fragment and the early promotor/enhancer *Hpa*II/*Bgl*I DNA segment into permissive monkey and non-permissive mouse cells allows late SV40 gene expression without T-antigen synthesis and DNA replication. These conditions were chosen to analyse the effect of DNA methylation on V-antigen synthesis detached from the process of DNA replication. We found that in vitro methylation of a single cytosine nucleotide proximal to the major late mRNA cap site by the *Hpa*II methylase does not block capsid protein synthesis. This result is in contrast to reported data obtained in *Xenopus laevis* oocyte injection experiments [(1982) Proc. Natl. Acad. Sci. USA 79, 5142–5146].

SV40 DNA *Methylation* *Microinjection* *V-antigen synthesis*

1. INTRODUCTION

Authors in [1] recently reported that DNA methylation inhibits the expression of SV40 genes. They found that methylation of a single cytosine nucleotide at the late SV40 map position 0.725 by *Hpa*II methylase blocked the synthesis of the capsid proteins (V-antigen) after microinjection of DNA into *Xenopus* oocytes [1]. We showed in [2] that complete methylation of SV40 and polyoma virus (PV) DNA by either the *Hpa*II or rat liver methylase did not affect the biological activity of these viral DNAs. When microinjected into cultured mouse and monkey cells, T-antigen synthesis, viral DNA replication and synthesis of V-antigen occurred with the same efficiency with both methylated and unmethylated DNAs [2]. We have thus proven that expression of the early SV40 and PV genes as well as viral DNA replication are methylation-insensitive. In view of the fact that DNA replication causes demethylation of injected DNA, nothing could be said about the methylation-insensitivity of late viral gene expression which depends on the replication step [2]. In frog oocytes V-antigen synthesis seems to occur in a manner independent of DNA replication [1].

We describe here conditions in which expression of late SV40 genes is obtained in the absence of viral DNA replication. SV40 capsid protein synthesis is induced efficiently by coinjection of the late promoter-free *Kpn*I/*Bgl*I DNA fragment [3,4], and the early SV40 'promoter/enhancer' *Hpa*II/*Bgl*I DNA fragment [5] regardless of whether the *Kpn*I/*Bcl*I fragment was methylated by the *Hpa*II enzyme. About 20 h after the microinjection, 20–30% of the recipient cells synthesized V-antigen even when DNA synthesis was blocked by ara C. Thus methylation of the *Hpa*II site does not prevent late SV40 gene expression in mammalian cells, unlike the situation reported for *Xenopus laevis* oocytes.

2. MATERIALS AND METHODS

2.1. *Cell culture and microinjection*

For all experiments TC7 cells, a subline of the CV1 line of African green monkey kidney cells and primary mouse kidney cells isolated from newborn

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NMRI mice were used. Cells were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (Gibco Europe). Details of the microinjection technique are described elsewhere [6].

2.2. *Methylation of SV40 DNA and preparation of DNA fragments*

Forty μg SV40 DNA were incubated with 400 units *Hpa*II methylase (Bio Labs New England) in 100 μl reaction buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM EDTA, 80 μM *S*-adenosylmethionine (Sigma) and 5 mM 2-mercaptoethanol at 37°C for 16 h. After incubation the reaction mixture was treated twice with 0.5% SDS and Tris–HCl (pH 7.0) saturated phenol. DNA was precipitated from the aqueous phase with ethanol. Completion of methylation was checked by incubation of the DNA with *Hpa*II and *Msp*I endonucleases as in [2]. To remove further any contaminating amounts of unmethylated DNA, the preparation was cleaved by *Hpa*II and *Bcl*I enzyme treatment. Under these conditions methylated DNA is converted into DNA III and unmethylated DNA into two fragments (see fig.1). After isolation from the agarose gel (1%), DNA III was further incubated with *Kpn*I and *Taq*I endonucleases. The reaction mixture was electrophoresed and the *Kpn*I/*Bcl*I DNA-A fragment isolated from the gel and dissolvd in 10 mM Tris–HCl (pH 7.4) buffer at a concentration of 0.2 μg/10 μl. The *Hpa*II/*Bgl*I fragment cloned in the *Sal* site of pBR322 was liberated from the plasmid DNA by *Sal*I digestion and purified by agarose gel electrophoresis. For microinjection *Kpn*I/*Bcl*I and *Hpa*II/*Bgl*I fragments were mixed at a concentration of 0.1 μg/10 μl each in Tris–HCl (pH 7.4).

3. RESULTS

As shown in fig.1, the *Kpn*I/*Bcl*I fragment (map position 0.715–0.189) of SV40 DNA contains the late coding region and the *Hpa*II site at the map position 0.725. This fragment was microinjected into the nuclei of both permissive monkey cells (TC7) and non-permissive mouse cells (primary mouse kidney cells) cultured in vitro. At different times after the injection (12, 24 and 36 h), cells were fixed and none stained positive for either T- or V-antigen as expected [3,4].

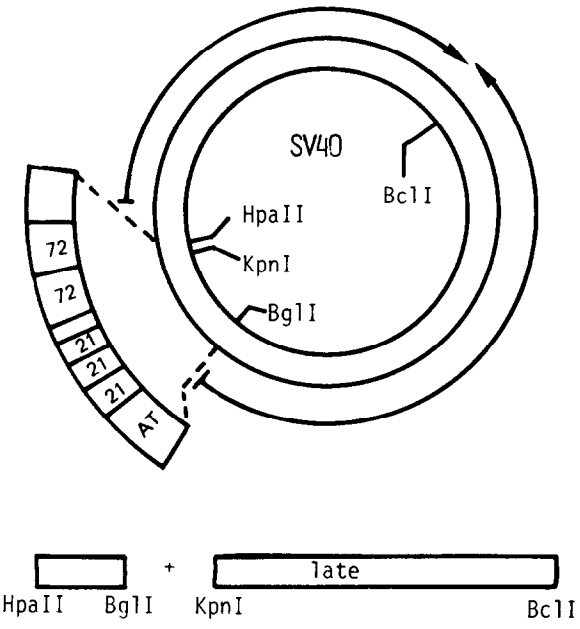

Fig.1. Transcription map and the early control region of SV40. The arrow-headed circles around the SV40 genome indicate the localisation and orientation of the early and late transcripts (spliced regions not indicated). The localisation of the two 72-bp enhancers and the three 21-bp repeats as well as the AT-rich sequence (TATA-box) within the *Hpa*II/*Bgl*I DNA fragment are shown schematically. The bottom part shows the two SV40 DNA fragments used for microinjection.

The SV40 DNA *Hpa*II/*Bgl*I fragment (map position 0.725–0.661) contains the early promotor/enhancer region; the cap site for early SV40 mRNA is distal to the *Bgl*I site [5]. When this fragment is coinjected with the promotor-minus *Kpn*I/*Bcl*I fragment of SV40, synthesis of V-antigen results (fig.2). In this experiment, fragments were only mixed in the injection buffer at 0.01 mg/ml each and in vitro ligation was found to be unnecessary. We found from DNA reextraction experiments and blot analysis that coligation of these fragments occurs immediately after the injection inside the recipient cell (in preparation). Following coinjection of these fragments, V-antigen positive cells were first demonstrable within 6–8 h and that about 25–30% of the cells exhibited a strong intranuclear V-antigen fluorescence 20 h after injection (table 1), while T-antigen synthesis could not be shown.

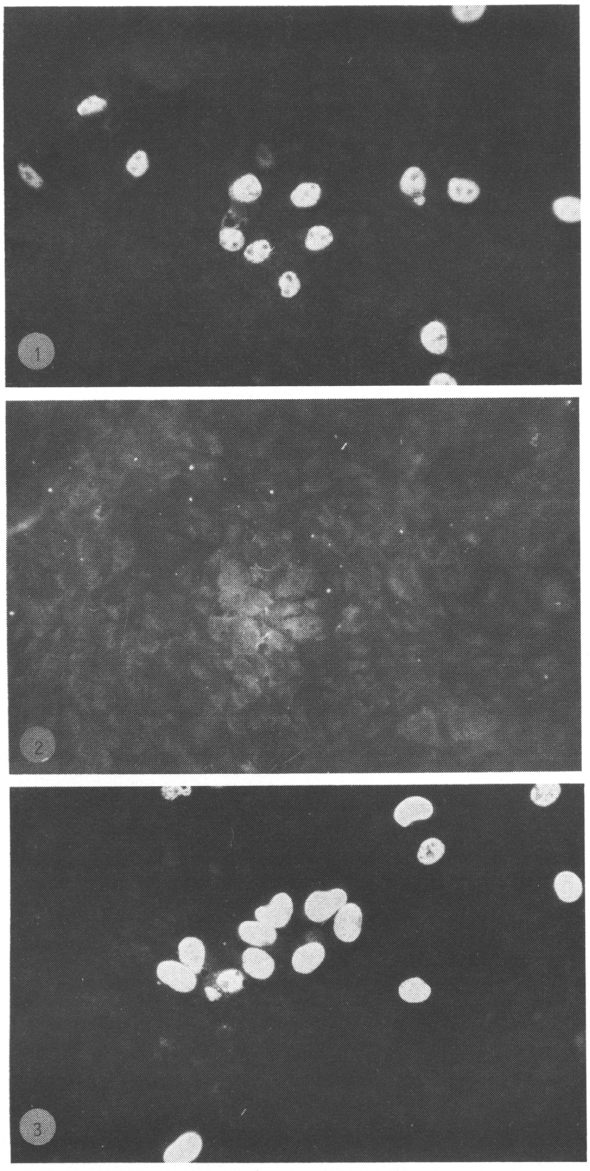

Fig.2. Immunofluorescence staining of microinjected TC7 cells. (1) V-antigen positive cells microinjected with the *KpnI/BclI* + *HpaII/BglI* fragment mixture. (2) The same cells stained for T-antigen. (3) V-antigen positive cells after microinjection of the methylated *KpnI/BclI* and *HpaII/BglI* promotor/enhancer fragment. Cells were fixed and stained 24 h after microinjection.

To ascertain whether inhibitors of DNA replication affect the synthesis of V-antigen, ara C was added to the medium (final conc. 40 μg/ml) 2 h prior to the injection and maintained throughout the culture period. We again found that in these conditions V-antigen synthesis occurred (table 1) as in the absence of ara C.

To determine whether methylation of the *KpnI/BclI* fragment blocks V-antigen synthesis, SV40 DNA I was methylated by exhaustive treatment with *HpaII* methylase. To remove possible trace amounts of unmethylated molecules, this DNA was further incubated with *HpaII* and *BclI* endonucleases, electrophoresed in agarose and SV40 form III DNA was eluted from the gel. This DNA was then digested with *KpnI* restriction endonuclease, reelectrophoresed in agarose and the *KpnI/BclI* methylated fragment was then coinjected with early SV40 promotor/enhancer DNA fragment. Again we found that V-antigen synthesis occurred with the same efficiency as in the experiments using the unmethylated *KpnI/BclI* DNA fragment, and that ara C did not prevent V-antigen synthesis under these conditions (table 1).

4. DISCUSSION

The biological significance of DNA methylation is still an open question although some of the experiments do indicate that methylation may be involved in the regulation of gene expression in eukaryotes. This is based on the observation that many active genes are less methylated than their inactive counterparts. More direct evidence supporting the hypothesis that DNA methylation influences gene expression has come from DNA transfection and microinjection experiments showing that the biological activity of methylated DNA is significantly lower than the unmethylated form when transferred to cultured mammalian cells or frog oocytes (review [7]).

Data exist which do not support such a generalization. For example, authors in [8] have shown that activation of the H2K gene(s) in teratocarcinoma cells is correlated with increased DNA methylation. Similarly, we have shown that complete methylation of SV40 and PV DNA does not affect the expression of early genes as well as viral DNA replication [2]. Here, we further show that methylation of the *HpaII* position in the *KpnI/BclI* fragment of SV40 DNA containing the late coding region in no way affects the expression of V-antigen. Considering our data as well as those of others, we postulate that if DNA methylation is involved in the regulation of eukaryotic gene ex-

Table 1

Biological activity of the methylated and unmethylated SV40 *KpnI/BclI* DNA fragment

Injection of	Antigen formation (%)			
	Monkey cells		Mouse cells	
	T	V	T	V
SV40 DNA I[a]	99	99	99	00
SV40 DNA I (ara C)	99	00	99	00
SV40 DNA fragments:				
KpnI/BclI	00	00	00	00
HpaII/BglI	00	00	NT	NT
KpnI/BclI + *HpaII/BglI*	00	25–30	00	25–30
KpnI/BclI + *HpaII/BglI* (ara C)	00	25–30	NT	NT
KpnI/BclI$^{methyl.}$ + *HpaII/BglI*	00	25–30	00	25–30
KpnI/BclI$^{methyl.}$ + *HpaII/BglI* (ara C)	00	25–30	00	25–30

[a] In these experiments the SV40 DNA I concentration was 0.05 mg/ml injection buffer

NT, not tested. Cells were fixed and stained for T- and V-antigen 20 h after microinjection by the indirect immunofluorescence technique

pression, then there must be at least two categories of genes, one of which is methylation-insensitive and the other methylation-sensitive. This clearly raises the question as to the nature of the difference between these two categories at least in terms of the site specificity for methylation-effectiveness. Analysis of the methylatable sites (CG) of SV40 and PV DNA and their distribution within the putative promotor/enhancer region of viral DNAs has shown that the CG dinucleotide sequence is not under-represented in this region as compared to the genes which have been reported to be methylation-sensitive. Of the 27 CG dinucleotides in the SV40 genome, 19 are located within the early promotor region. Similarly, 23 out of 96 such dinucleotides are in the promotor region of the PV genome [9]. This implies that methylation-sensitivity is not determined by the absolute number of CG but, rather, by a specific position within the gene. This implication is well supported by the report [1] that methylation of a single cytosine nucleotide distal to the late mRNA cap site blocked synthesis of the SV40 capsid proteins in frog oocytes. We have shown here that methylation of this specific site by the *HpaII* methylase does not impart methylation-sensitivity for the expression of V-antigen to either permissive monkey cells or non-permissive mouse cells.

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