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Inhibition of herpes simplex thymidine kinase gene expression by DNA methylation is an indirect effect

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ABSTRACT

The biological activity of in vitro methylated HSV-TK DNA was analysed after microinjection into thymidine kinase negative rat 2 cells. It was found that the fully methylated DNA (HpaII methylase) was as active as the non methylated control DNA for about 48 hours after injection. DNA reextraction experiments and blot analysis showed that DNA demethylation was not the reason for the observed TK activity. With prolonged cultivation time the methylated DNA becomes rapidly inactive and 100 hrs after injection thymidine incorporation was no longer detectable in the recipient cells. In transformed cells, obtained after coinjection with SV40 DNA, the HSV-DNA was partially demethylated and inactive. Addition of 5-azacytidine to the culture medium induced further demethylation and reactivation of the thymidine kinase gene.

INTRODUCTION

Change of the DNA methylation pattern is considered as one possible gene control mechanism in eukaryotic cells. The current model proposes that demethylation is a necessary although not a sufficient step to allow transcription of the DNA by the RNA polymerase II (reviewed in 1). This assumption is based on the observation that different genes are less methylated in their active, than in their inactive state (2-4). Furthermore it was shown that some in vitro methylated genes (e.g. HSV-TK DNA) were inactive following transfection or microinjection into culture cells (5-10). So far it is not known how methylation affects gene expression (11). To gain more understanding of the molecular basis of this mechanism, we asked whether DNA methylation per se is sufficient, or whether additional events are required to inhibit TK-gene expression after the transfer into TK-negative rat 2 cells.

For this purpose Herpes simplex virus I thymidine kinase (HSV-TK) DNA was methylated in vitro by the HpaII enzyme and expression of the DNA was tested by autoradiography at various points in time after microinjection and transformation efficiency.

MATERIAL AND METHODS

HSV-TK DNA containing plasmid vectors

The following HSV-TK plasmids were used in our experiments: i) the pHSV-106 (12), ii) the heavy metal inducible ΔEK (the HSV-promotor is replaced by the mouse metallothionine I gene promotor) (13), and iii) pML-BPV-TK4 (14). The pML-BVP-TK4 plasmid contains the Bovine papilloma virus DNA (BPV), which enhances the transcription of the HSV-TK gene (Fig. 1).

In vitro methylation of the HSV TK-DNA

5 µg of the HSV-TK-plasmid DNA were incubated with 50 units HpaII methylase (Biolabs, New England) in 100 µl reaction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 mM 2-mercaptoethanol at 37°C and 80 µM S-adenosylmethionine (Sigma) for 16 hrs. 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 the HpaII and MspI endonucleases and blot analysis as described (15).

Cells, microinjection and autoradiography

For all experiments, thymidine kinase negative rat 2 cells were used (16). Details of the microinjection technique are described (17). To assay the HSV-TK enzyme activity at the cellular level, (^{3}H)-thymidine (Amersham, specific activity: 40-60 Ci/mMol) was added to the medium (DMEM plus 5% fetal calf serum) to a final concentration of 1 µCi/ml for 24 hrs. After incubation, cells were washed with phosphate-buffered saline (PBS) and fixed in a mixture of aceton/methanol (2:1) for 10 min, covered with Kodak nuclear track emulsion NTB, exposed for one week at 4°C, developed (Kodak D 19) and fixed with Kodak Unifix. For activation of the metallothionine-I promotor in ΔEK injected cells, $CdSO_4$ (10 µM) or $ZnCl_2$ (100 µM) was added to the medium for about 6-20 hrs (18).

DNA reextraction (modified Hirt method (19/20), cellular DNA isolation and blot analysis

For DNA reextraction experiments, rat cells were grown on small glass slides (2x2 mm). After microinjection, cells were further cultivated under standard conditions. Before lysis, slides with the cells were washed in PBS and transferred into Eppendorf tubes filled with 200 µl lysis buffer (0.6% SDS, 10 mM EDTA, pH 7.5). Tubes were left at room temperature for 10 to 15 minutes and 50 µl of 5 M NaCl were added. Cellular DNA was precipitated at 4°C for at least 8 hrs. After centrifugation for 15 min in an Eppendorf centrifuge, supernatant was collected and plasmid DNA was precipitated by addition of 3 volumes ethanol and 1/10 volume of 1 M NaCl. The dryed DNA pellet was dissolved in TE buffer (10 mM Tris HCl, pH 7.4, 1 mM ETDA). DNA was further purified by RNase treatment and phenol extraction. After digestion with restriction endonucleases, DNA was separated by standard agarose gel electrophoresis, transferred to nitro cellulose filters and hybridized with nick translated ^{32}P-HSV-DNA (specific activity: 1-2x 10^8 cpm/µg DNA) (21).

High molecular weight DNA was isolated from the culture cells by standard procedure (22). Genomic DNA (5-10 µg) was digested with restriction endonucleases and the product was analysed by electrophoresis on agarose gels and Southern blotting technique (23).

RESULTS AND DISCUSSION

The plasmids shown in Fig. 1 were methylated in vitro by the HpaII methylase as described in Material and Methods, and completeness of the methylation was tested by blot analysis of the HpaII or MspI endonuclease restricted DNA, as shown in Fig. 2. To test if inhibition of TK gene expression occurs immediately after the transfer into recipient cells, the methylated pHSV-106 DNA (pHSV-106-CH_3) was microinjected into the nuclei of the thymidine kinase negative rat 2 cells (16) at a multiplicity of 200-400 DNA molecules per cell (20). At different times after injection, cells were labeled with (^{3}H)-thymidine for 24 hr periods, fixed thereafter and pro-

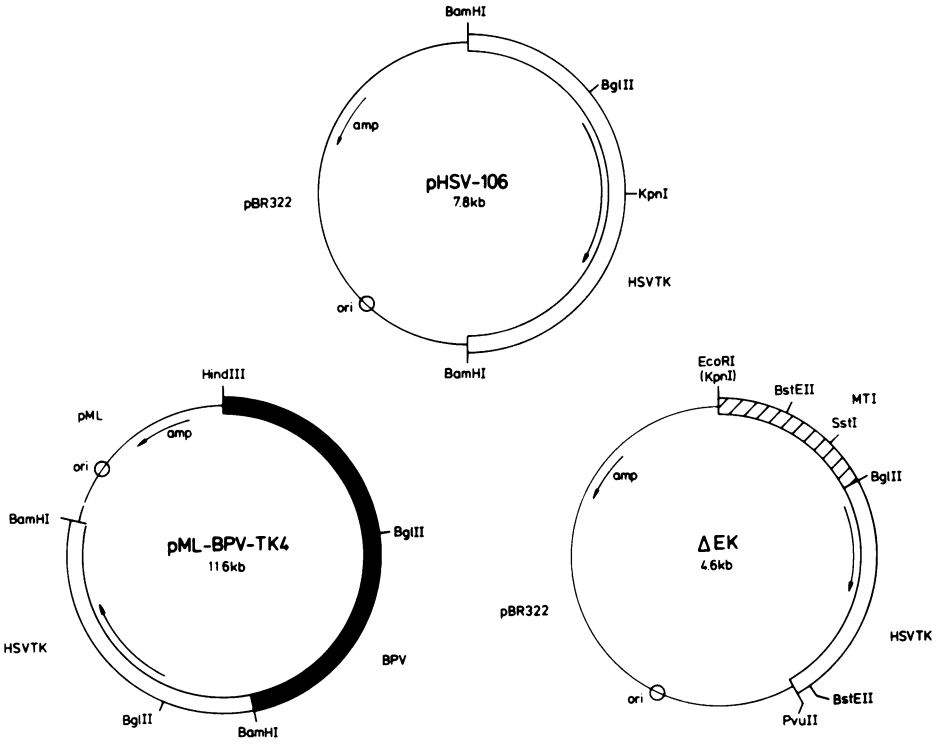

Figure 1. Structure of the plasmids used for the microinjection experiments. The open boxes represent the HSV-TK-DNA segment, the solid black box of the pML-BPV-TK4 plasmid the BPV-DNA and the hatched segment of the ΔEK plasmid the MTI promotor upstream from the HSV-TK coding region.

cessed for autoradiography (Fig. 3). These experiments showed that pHSV-106-CH_3 DNA was as active as the non methylated pHSV-106 DNA during the first 48 hrs after microinjection. Both the number of TK positive cells obtained and the amount of silver grains detectable per nuclei were comparable during this time period (Fig. 3, Fig. 4). Only with prolonged cultivation of injected cells did the methylated DNA became progressively inactive and cells incubated with (^{3}H)-thymidine later than 100 hrs after injection were always negative for TK activity.

We further asked if an enhanced transcription of the injected DNA could prolong the TK gene expression over 48 hrs. For this

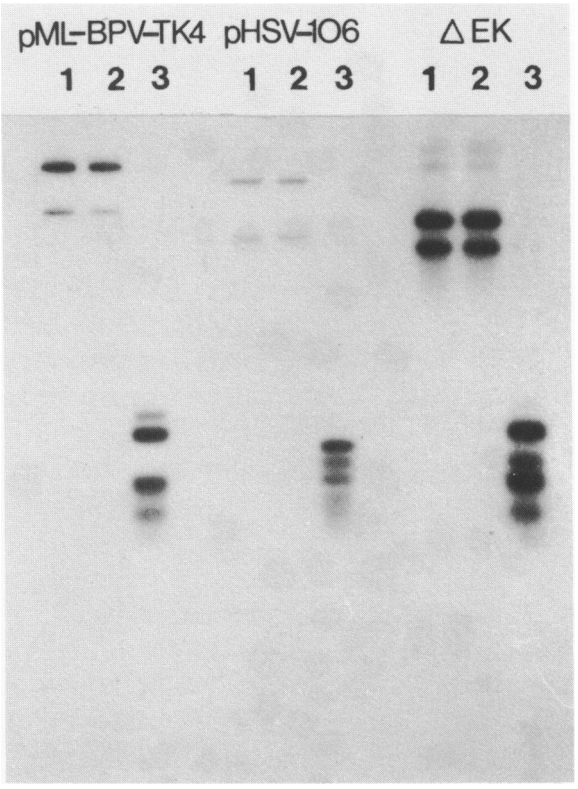

Figure 2. Southern blot analysis of the used methylated plasmids. (1) Methylated DNA, (2) the same DNA treated with the HpaII endonuclease, (3) methylated DNA cleaved with the MspI enzyme.

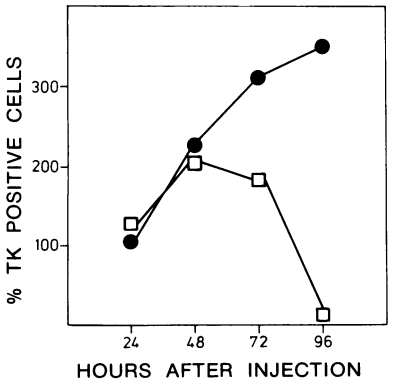

Figure 3. Time course of (^{3}H)-thymidine incorporation into rat 2 cells after microinjection of the pHSV-106-CH_3 and pHSV-106 DNAs. DNA was microinjected into the nuclei of rat 2 cells and (^{3}H)-thymidine was added to the medium for 24 hrs interval. The number of injected cells are counted as 100%. The data are the meanvalues from three independent injection experiments with 100 injected cells each.

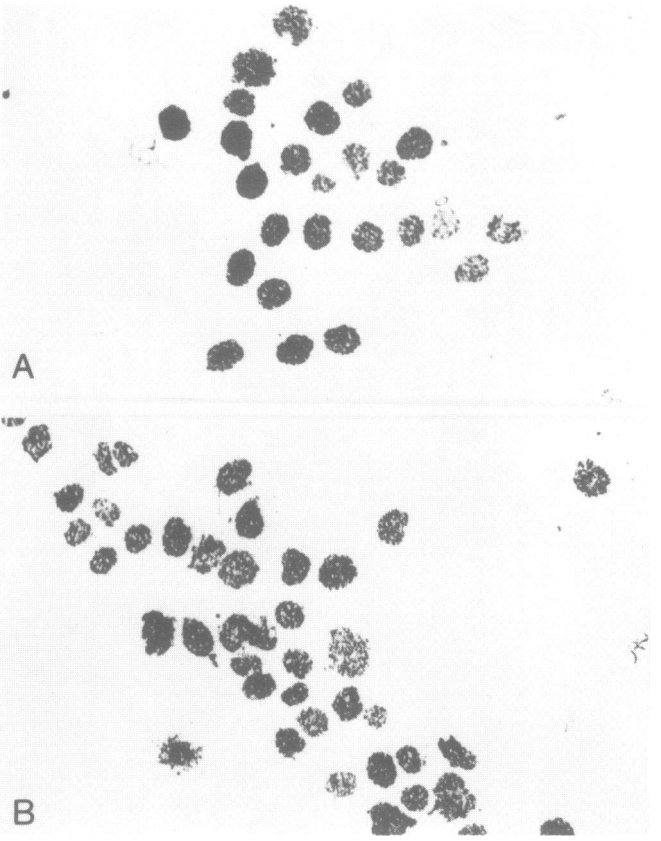

Figure 4. Autoradiogram of rat 2 cells after injection of: (A) pHSV-106 DNA and (B) pHSV-106-CH_3 DNA. (^{3}H)-thymidine was added to the medium directly after injection. Cells were fixed 24 hrs later and processed for autoradiography.

reason we repeated the experiments with the methylated pML-BPV-TK4 DNA. We found that the enhanced transcription of the HSV-TK gene, mediated by the BPV-DNA (24), did not affect the time course of TK expression. Similar results were obtained after injection of the methylated ΔEK DNA, regardless whether divalent cations ($CdSO_4$ 10 μM or $ZnCl_2$ 100 μM) were added to the medium or not (13).

To ascertain that the observed TK-activity was not due to the contamination of the DNA with a low number of under-methylated molecules, not detectable by blot analysis (Fig.

2), experiments with lower DNA concentrations were performed. As summarized in table 1, TK activity was also demonstrable with a high level of efficiency after transfer of only 2-4 methylated DNA molecules per cell.

Expression of the TK DNAs was also not caused by partial or total demethylation of the DNA inside of the recipient cells. We reextracted the DNA at various points after injection and analysed the methylation pattern of the HpaII and MspI restricted DNA by Southern blotting technique (15). As shown in Fig. 5, the ΔEK DNA reextracted 8 hrs after injection was still entirely resistant to HpaII endonuclease digestion, but sensitive to the MspI enzyme.

In a second set of experiments we analysed the transformation efficiency of the methylated, as well as the non methylated HSV-TK DNAs. Single rat 2 cells, grown on small glass slides subdivided into numbered squares, were microinjected into the nuclei with 20-40 DNA molecules per cell (20). Two days after injection, slides with the cells were transferred into HAT medium and TK positive clones were counted two to three weeks later. As summarized in table 2, stable TK positive cell lines were obtained with a frequency of 20-30%. This high transformation rate was obtained with all three

TABLE I: The biological activity of methylated and non methylated HSV-TK-plasmid

Material injected	% of TK-positive cells after injection: number of DNA molecules injected/cell		
	200-400	20-40	2-4
pHSV-106	120-140	120-140	80-120
pHSV-106-CH_3	120-140	120-140	80-120
ΔEK	120-140	120-140	80-120
ΔEK-CH_3	120-140	120-140	80-120
pML-BPV-TK4	120-140	120-140	80-120
pML-BPV-TK4-CH_3	120-140	120-140	80-120

In all experiments the number of injected cells were counted as 100%. Rat 2 cells were labeled with ^{3}H-thymidine (1 μCi/ml) for 24 hours directly after injection.

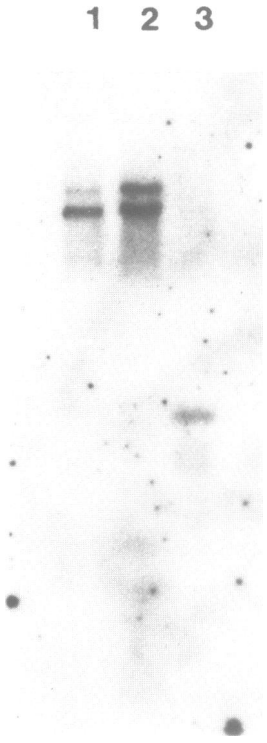

Figure 5. Methylated ΔEK DNA reextracted from 500 rat 2 cells 8 hours after injection. Isolated DNA was subdivided into three samples: (1) served as control, (2) treated with HpaII- and (3) with MspI restriction endonucleases. Southern blot was hybridized with the nick translated ^{32}P-TK gene.

TABLE II: Transformation capacity of methylated and non methylated HSV-TK-plasmids

Material injected	% of transformed colonies selected in HAT medium	by SV40* induced cell transformation
pHSV-106	20-30	20-30
pHSV-106-CH_3	0	20-30
ΔEK	20-30	N.T.
ΔEK-CH_3	0	20-30
pML-BPV-TK4	20-30	N.T.
pML-BPV-TK4-CH_3	0	20-30

* SV40 DNA (20-40 DNA molecules/cell) was coinjected with the HSV-TK-plasmids (20-40 DNA molecules/cell). The numbers given represent the T-antigen positive cell lines isolated. N.T. = not tested.

types of the HSV-TK-plasmids (Fig. 1). Addition of $CdSO_4$ or $ZnCl_2$ to the culture medium of ΔEK plasmid injected cells did not increase the transformation rate. Using HAT medium for selection we could not obtain TK positive clones from cells microinjected with the methylated DNAs (Table 2). Therefore, we coinjected the methylated HSV-TK-DNA with SV40 DNA and selected for SV40 transformants. The SV40 DNA induced transformation rate was again 20-30%. In this way, more than 20 independent T-antigen positive cell lines were isolated. They did not grow in HAT medium. To analyse the state and the methylation pattern of the HSV-TK DNA in the transformants, cellular DNA was extracted and analysed by DNA blotting. For these experiments one cell line was randomly chosen from each category of transformants as listed in table 2. In all cases the HSV-TK DNA was found to be covalently integrated into the host genome but partially demethylated. However reactivation of the HSV-TK gene in the transformed cells was obtained after addition of 5-azacytidine (2-10 μM for 48 hrs) to the culture medium which was connected with a further degree of DNA demethylation. As shown in Fig. 6, many of the HSV-TK HpaII sites are demethylated in the pHSV-106 transformants after induction with 5-azacytidine and selection in HAT medium.

So far, we do not know which site of the HSV-DNA has to be demethylated to allow reactivation of the TK-gene. Our experiments demonstrate that inhibition of HSV-TK gene expression by DNA methylation occurs after a latency period of several hours in microinjected rat 2 cells. This observation indicates that DNA methylation per se does not block gene expression, but requires a further event(s) which occur(s) late after the microinjection of nacked methylated DNA. This might be the association of the DNA with cellular proteins, change of the DNA conformation, or its integration into the host genome.

The exact time point of the transition from methylation insensitivity to sensitivity cannot be determined from our experiments, since our results are based on thymidine kinase activity and not on the transcription rate of the injected DNA.

Remarkable is also the high transformation efficiency. After intranuclear microinjection of the DNA, 20-30% of the

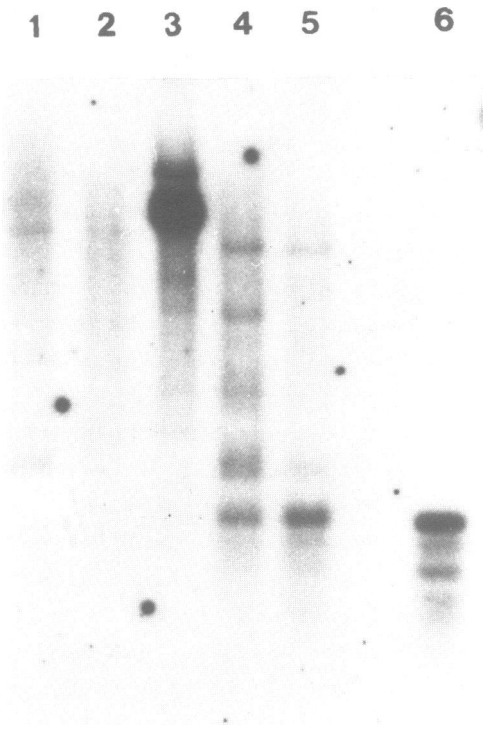

Figure 6. (1) Cellular rat 2 DNA pretreated with SstI and XhoI restriction endonucleases was further cleaved with HpaII, (2) with MspI endonucleases. (3) Rat 2 cells transformed by micro-injection of SV40 DNA and pHSV-106-CH_3 DNA. Cellular DNA was extracted from the cells after treatment with 5-azacytidine and selection in HAT medium. DNA was cleaved with the SstI and XhoI restriction endonucleases. These enzymes do not cleave the pHSV-106 DNA. (4) This cellular DNA further digested with HpaII, (5) with MspI, (6) marker MspI-pHSV-106-CH_3 DNA fragments. DNA blot was hybridized with the nick translated ^{32}P-TK gene.

recipient cells were transformed into permanent TK positive cell lines, regardless of whether the injected DNA contained enhancer sequences or not (pML-BPV-TK 4, pHSV-106). Also the addition of divalent cations to the medium of the ΔEK injected cells did not increase the number of transformants. These observations support the assumption that the transcription rate is not essential for the high transformation efficiency of the injected HSV-TK-DNA in rat 2 cells.

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