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After microinjection hemimethylated DNA is converted into symmetrically methylated DNA before DNA replication

G. Sandberg, E. Guhl, M. Graessmann and A. Graessmann

Institut für Molekularbiologie und Biochemie der Freien Universität Berlin, Arnimallee 22, 1 Berlin 33, Germany

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In this investigation we analysed the maintenance methylation activity of the mammalian cell DNA methyltransferase by microinjection of hemimethylated HSV-tk DNA into thymidine kinase-negative rat 2 cells. We found that the hemimethylated DNA was efficiently converted into symmetrical methylated molecules before DNA replication. Furthermore, integration of the *trans*-DNA into the host genome is an early event after gene transfer.

Hemimethylated DNA; Microinjection; Maintenance methylation; DNA replication; 5-Azacytidine

1. INTRODUCTION

Transcriptional regulation in mammalian cells often requires a complex interaction of different *cis*- and *trans*-acting elements. One of the *cis*-acting elements crucial for gene activation is the change in the DNA methylation pattern. The current hypothesis is that under-methylation is a prerequisite for gene activation (for review see [1]). The most direct evidence that DNA methylation can indeed inhibit gene expression has been obtained by transfection and microinjection experiments [2,3]. Furthermore, it has been shown that the in vitro methylation pattern of the *trans*-DNA can be maintained in vivo for many generations [2]. In contrast to maintenance methylation, de novo methylation is a very rare process in somatic cells [4]. In mammalian cells a single protein possesses both de novo and maintenance methylation activity [5].

Enzymatic and immuno-fluorescence staining experiments revealed a non-random distribution of the methyltransferase during the cell cycle, with the highest activity at the S and G2 phase [6,7]. Resting cells exhibit a very low methyltransferase activity, but the activity increases rapidly upon mitotic stimulation [8]. This observation is consistent with the hypothesis that maintenance methylation is linked to DNA replication and that the methyltransferase uses the methylation pattern of the parental DNA strand to methylate the newly synthesised DNA strand [1].

In this investigation we microinjected hemimethylated HSV-tk DNA into the nuclei of thymidine kinase-negative rat 2 cells and obtained strong evidence that the hemimethylated DNA was efficiently converted into symmetrical methylated molecules before DNA replication.

Correspondence address: G. Sandberg, Institut für Molekularbiologie und Biochemie der Freien Universität Berlin, Arnimallee 22, 1 Berlin 33, Germany

2. MATERIALS AND METHODS

2.1. *Cell culture and microinjection*

Thymidine kinase negative rat 2 cells, grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum were used for all experiments; the HAT medium contains hypoxanthine, aminopterin and thymidine. For microinjection recipient cells were grown on glass slides [9]. Shake-off cells were isolated from confluent culture dishes by gentle shaking. The microinjection procedure has been described in detail elsewhere [9].

2.2. *Preparation of hemimethylated DNA*

For second strand DNA synthesis in vitro 25 ng of the 17-mer M13 primer (BRL) were added per 1 μg of the M19-tkI or M18-tkI DNA. For annealing of the primer with the single-stranded (ss) DNA, the mixture was kept for 5 min at 95°C, for 30 min at 55°C and 30 min at 37°C. For DNA synthesis 6 units of the Klenow fragment of the *E. coli* DNA polymerase (BRL) were added per 2 μg DNA. The reaction was carried out overnight at 15°C in 70 mM Tris-HCl, pH 7.5; 70 mM $MgCl_2$ with 1.5 mM for each dNTP. For synthesis of the hemimethylated DNA (M19-tkII-CH_3; M18-tkII-CH_3) 1.5 mM 2'-deoxy-5-methylcytosine 5'-triphosphate was used instead of dCTP [10]. Each preparation was tested by agarose gel electrophoresis and *Hpa*II and *Msp*I endonuclease treatment (Fig. 2).

3. RESULTS AND DISCUSSION

To obtain hemimethylated molecules (methylated in all cytosine residues of one DNA strand), the HSV-tk DNA was inserted into the multiple cloning site of the RF-M13 DNA and propagated in *E. coli*. The ss DNA was isolated from the phage (M19-tkI contains the non-coding strand and the M18-tkI the coding strand of the HSV-tk gene) (Fig. 1) and used as the template for in

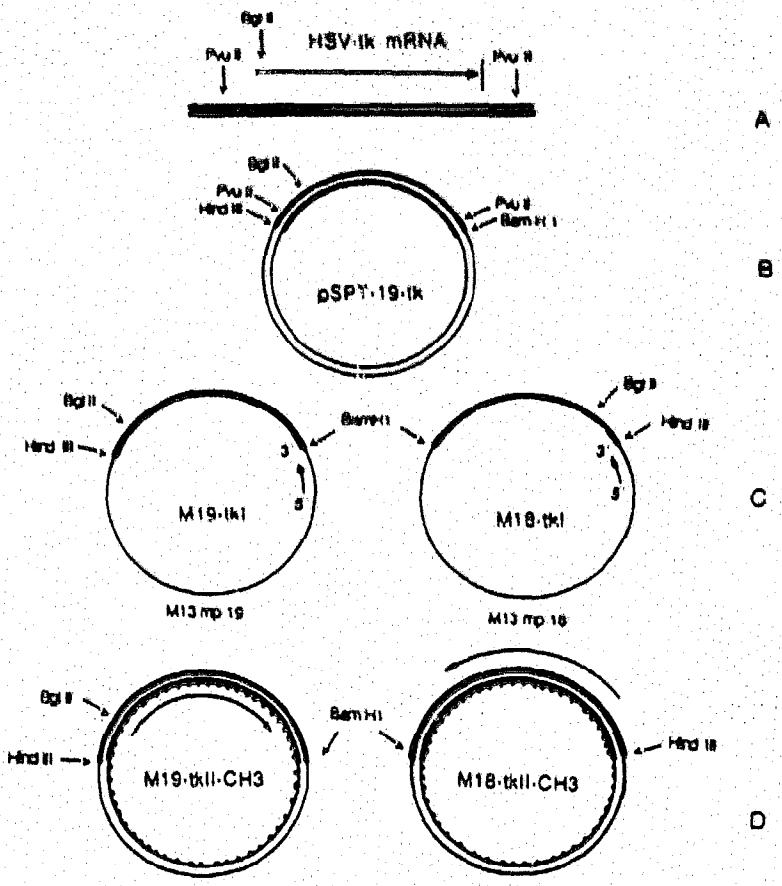

Fig. 1. Schematic representation of the in vitro second strand DNA synthesis. (A) The HSV-tk gene was isolated as a *PvuII* restriction fragment from the pHSV-106 (BRL). The transcription orientation is indicated by the arrow. (B) The *PvuII* fragment was inserted into the *PvuII* site of the pSPT19 (Pharmacia) and propagated in *E. coli* as pSPT-19-tk. (C) The tk gene was then isolated from the pSPT-19-tk DNA as a *HindIII-BamHI* DNA fragment and inserted into the M13mp19 RF and M13mp18 RF DNA and propagated in *E. coli*. The single stranded (ss) M19-tkI and M18tkI DNA were isolated from the bacteriophages. The location and the orientation of the universal M13 primer are indicated. (D) The complementary DNA strands were synthesized in vitro. The dots on the inner circle of the double strand (ds) M19-tkII-CH_3 and M18-tkII-CH_3 represent 5-methylcytosine residues.

vitro DNA synthesis. Unmethylated ds DNA was obtained using dCTP instead of 5-methyl CTP for second strand synthesis (Fig. 2.).

After purification the DNA was microinjected into thymidine kinase-negative rat 2 cells. One day after gene transfer the DMEM medium was replaced by the HAT medium and cell clones were counted 1–2 weeks later. As summarised in Table I, 25–30% of the cells, injected with the non-methylated DNA (M19-tkII, M18-tkII) become HAT-medium-resistant cell clones, which permanently express the HSV-tk gene as confirmed by thymidine incorporation and RNA blot analysis (data not shown).

In contrast, HAT-medium-resistant cell clones were not obtained after microinjection of either the hemimethylated M19-tkII-CH_3 or the M18-tkII-CH_3 DNA (Table I). This observation clearly demonstrates that the hemimethylated DNA was converted into symmetrical methylated molecules before DNA replication. If this were not so, the same number of HAT-medium-positive cell clones would be expected as after injection of the unmethylated DNA, because the first round of semiconservative DNA replication would generate unmethylated and hemimethylated DNA molecules and hence HAT-medium-resistant cells. We also transfected the hemimethylated DNA into rat 2 cells and obtained some HAT-medium-positive cell clones. Southern blot analysis of the cellular DNA revealed that the HSV-tk DNA was demethylated or highly undermethylated (data not shown). So far, we have not tested further whether the HSV-tk DNA was actively demethylated [12] or whether there was an insufficient maintenance methylation activity.

Since the HSV-tk constructs used in our experiments do not contain any eukaryotic replication origin, replication of the *trans*-DNA requires integration into the host genome. So far it is not certain when after injection the *trans*-DNA is integrated and replicated. To

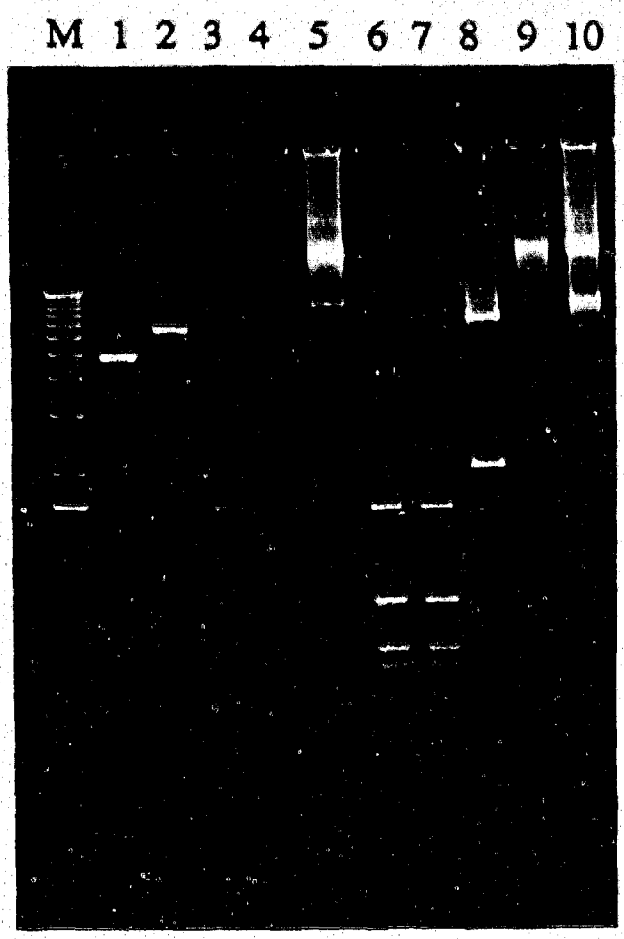

Fig. 2. The agarose gel contains: M, size marker; lane 1, M19-tkI DNA; lane 2, RF M19-tk DNA; lane 3, RFM19-tk DNA *Hpa*II enzyme digested; lane 4, RFM19-tk DNA *Msp*I digested; lane 5, M19-tkII DNA; lane 6, M19-tkII DNA *Hpa*II digested; lane 7, M19-tkII DNA *Msp*I digested; lane 8, M19-tkII DNA *Bam*HI-*Hin*dIII digested, the lower band is the HSV-tk DNA; lane 9, M19-tkII-CH_3; lane 10, M19-tkII-CH_3 *Msp*I enzyme-treated.

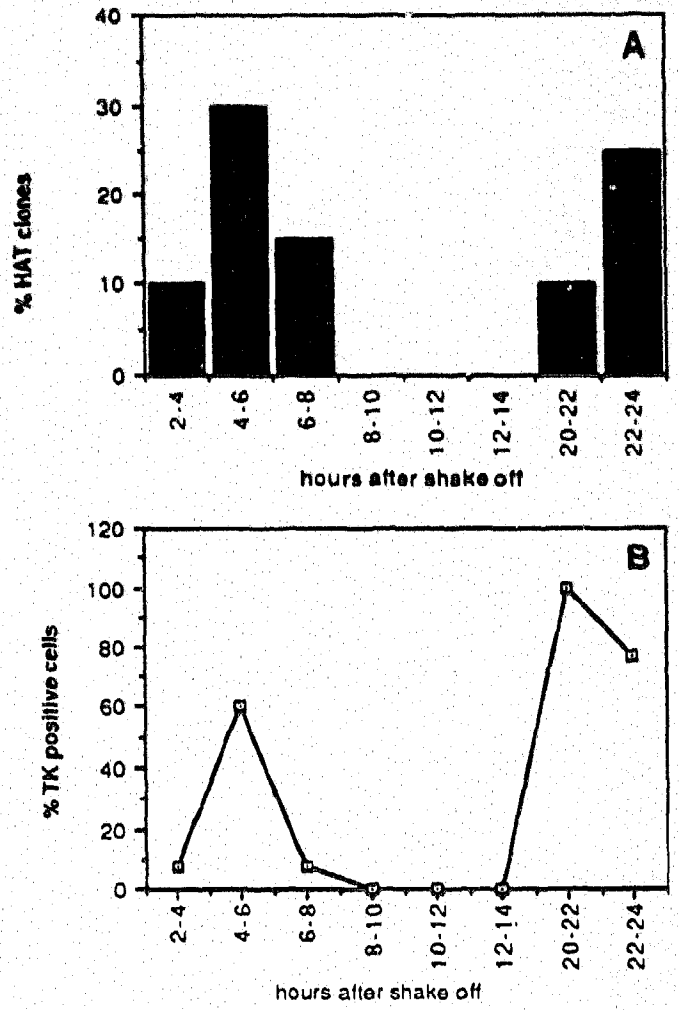

Fig. 3. Shake-off cells were microinjected at different times after isolation as indicated and subsequently treated for a further two hours with 5-azacytidine. Thereafter the cells were washed with DMEM medium and transferred into HAT medium. (A) shows the number of injected cells which grew out into HAT-medium-resistant cell clones. The number of injected cells were counted as 100%. (B) Shake-off cells were microinjected with the pSTP-19-tk at the time indicated. After injection, [^{3}H]thymidine (0.1 μCi/ml) was added to the culture medium for two hours. Thereafter the cells were fixed and processed for autoradiography.

Table I

Material injected	HAT-medium-positive cell clones (% of injected cells)
M18-tkII	25–30
M18-tkII-CH_3	0
M18-tkII-CH_3 + 5-azaC*	20–25
M19-tkII	20–30
M19-tkII-CH_3	0
M19-tkII-CH_3 + azaC*	20–25
pSPT-19-tk	25–30
pSPT-19-tk-CH_3	0
pSPT-19-tk-CH_3 + 5-azaC*	20–25

Rat 2 cells grown on glass slides were microinjected with 20–40 DNA molecules/cell and transferred into HAT medium as described in section 2.* After microinjection, 5-azacytidine was added for 20 h to the culture medium. Thereafter cells were transferred to HAT medium.

test whether this occurs during the first cell cycle after gene transfer, the demethylating agent 5-azacytidine (2 μM) was added to the cells directly after injection and left for 20 h. Thereafter the cells were washed twice with DMEM medium, in order to remove tracer amounts of 5-azacytidine and then further cultivated in HAT medium. As shown in Table I, the 5-azacytidine treatment caused demethylation of the HSV-tk gene, as confirmed by Southern blot analysis (data not shown), and hence growth of these cells in HAT medium.

To get more precise information on when after-injection integration of the *trans*-DNA occurred, synchronised cells were used as recipients. To avoid

chemical treatment for synchronisation shake-off cells were used for our experiments (M-phase cells). At different times after shake-off the cells were microinjected and treated for two-hour intervals with 5-azacytidine C (Fig. 3). Then the cells were washed and transferred to HAT medium and cell clones were counted as described above.

The earliest time that shake-off cells are accessible for microinjection is two hours after preparation. Before this time, the cells are still round and do not adhere well to the glass slides. Fig. 3 shows that 10% of the cells injected two hours after preparation were converted into HAT-medium-resistant cells by the subsequent two hours of 5-azacytidine treatment. The maximum number of HAT-medium-positive cell clones were obtained 4-6 h after shake-off, when about 30% of the cells grew in HAT medium. This is in contrast to the cells which were injected and treated with 5-azacytidine 8–18 h after shake-off. None of them were converted into HAT-medium-resistant cells. HAT-medium-positive clones were again obtained 20–22 h after shake-off. If 5-azacytidine was omitted, none of the recipient cells grew in HAT medium regardless of when after-shake-off microinjection occurred; however, positive clones were always obtained when 5-azacytidine remained for 20 h on the cells (data not shown). As shown by thymidine incorporation and autoradiography, shake-off cells enter into the S-phase as early as 2–4 h after preparation with reactivation of the hemimethylated DNA by 5-azacytidine treatment corresponding with DNA replication (Fig. 3).

Next we investigated whether reactivation of the in vitro methylated DNA by 5-azacytidine requires incorporation of the cytosine analogue into the DNA. For this we methylated the pSTP19-tk DNA (Fig. 1) with *Hpa*II methyltransferase (pSPT-19-tk-CH_3). Methylation of the HSV-tk gene by the *Hpa*II methyltransferase caused gene inactivation (Table I). However, the symmetrically methylated DNA was also reactivated after microinjection into the shake-off cells by the two-hour 5-azacytidine treatment as was observed after injection of the hemimethylated DNA (data not shown). These results indicate that the 5-azacytidine was incorporated into the DNA during the first round of DNA replication, generating hybrid DNA molecules with one methylated DNA strand and leaving the second strand with the cytosine analogue. The subsequent replication cycle then caused demethylation of the second DNA strand and HSV-tk gene activation.

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