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Effect of methylation on expression of microinjected genes

(herpes simplex virus/*tk* gene/simian virus 40 large tumor antigen/microinjection)

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ABSTRACT **The cloned genes for the simian virus 40 large tumor antigen and for herpes simplex virus (HSV) thymidine kinase (TK) were methylated with *Eco*RI methylase. The genes were microinjected into the nuclei of TK-deficient (tk^-) cells, and expression of the genes was determined by immunofluorescence staining for the simian virus 40 large tumor antigen and by [^{3}H]thymidine incorporation followed by autoradiography for HSV TK. We found that methylation of the simian virus 40 gene, under *Eco*RI or *Eco*RI* conditions, resulting in methylation at sites within the gene and in the surrounding sequences, has no effect on expression of the large tumor antigen when the gene is manually microinjected into mammalian nuclei. However, methylation of the HSV *tk* gene at the two *Eco*RI sites markedly reduces or abolishes the expression of this gene. One of the *Eco*RI sites of HSV *tk* is ≈1.1 kilobases downstream from the 3′ end of the gene and is believed to have no regulatory function in the expression of the *tk* gene. The other *Eco*RI site is 79 base pairs upstream from the 5′ end of the gene and has considerable homology to the regulatory sequence proposed by [Benoist C., O'Hare, K., Breathnach, R., & Chambon, P. (1980) *Nucleic Acids Res.* 8, 127–142]. Our results are direct proof that methylation can alter gene expression and also that the effect depends strictly on the sites that are methylated.**

Recently, a number of observations have suggested that methylation may be involved in gene expression (for review, see refs. 1–3). Although the results have not always been devoid of contradictions, evidence has been accumulating that nonexpressed genes are more heavily methylated than genes that are being expressed (4–10). A direct approach to the problem is to methylate cloned genes and microinject them manually into the nuclei of mammalian cells in which their expression can be studied (11–13). We report here that methylation of the simian virus 40 (SV40) A gene at up to 24 different sites has no effect whatsoever on its expression, as measured by immunofluorescence, when it is microinjected into mammalian cells. On the other hand, a single methylation, at a restriction site ≈70 base pairs (bp) upstream from the cap site, completely abolishes or markedly reduces expression of microinjected herpes simplex virus (HSV) thymidine kinase gene (*tk*).

METHODS AND MATERIALS

Cells. tk^-ts13 cells are a mutant derived in our laboratory from ts13 cells, which, in turn, are a temperature-sensitive mutant of the cell cycle derived by Talavera and Basilico (14) from BHK21-13 cells. This mutant is deficient in tk activity, does not grow in hypoxanthine/aminopterin/thymidine medium, and has a very low reversion frequency ($\approx 1 \times 10^{-6}$). It is routinely grown in Dulbecco's minimal essential medium/10% calf serum (13). TC-7 monkey cells were grown in Dulbecco's minimal essential medium/10% fetal calf serum. Cells used for microinjection were plated on small cover glasses marked with small circles. Microinjection was carried out as described by Graessmann and Graessmann (11) under direct visual control on a fixed stage of an inverted phase-contrast microscope (Leitz Diavert 400) with a micromanipulator.

Recombinant Plasmids. pSV2G, a plasmid constructed in our laboratory and containing the early region of SV40, extending from map unit 1 counterclockwise to map unit 0.14, has been described and characterized (12). This plasmid has been shown to induce positive immunofluorescence for the large tumor (T) antigen and cellular DNA synthesis when microinjected into TC-7, ts13, and 3T3 cells (12).

pTK, a plasmid containing the *Bam*HI fragment of the *tk* gene of HSV cloned in pBR322, was generously given by Carlo Croce, The Wistar Institute, and it has been described in a paper from his laboratory (15). The third plasmid, pC6, which contains the full SV40 DNA and the HSV *tk* gene in pBR322, was also obtained from Carlo Croce and has been described in the same paper (15).

Plasmids were grown in HB101 cells. After amplification with chloramphenicol, plasmid DNA was isolated and purified on a CsCl gradient (16).

Enzymes and Reagents. Restriction enzymes and *Eco*RI methylase were purchased from New England BioLabs or from Bethesda Research Laboratories.

DNA Modification. Restriction or methylation of plasmid DNA at the canonical *Eco*RI sites (G-A-A-T-T-C), was done as suggested by the suppliers of the enzymes.

Methylation of plasmid DNA at the noncanonical *Eco*RI* sites (N-A-A-T-T-N′) was done as described by Woodbury *et al.* (17). Plasmid DNA (15–20 μg) was incubated with 150 units of *Eco*RI methylase (one unit corresponds to the amount required to incorporate 1 pmol of [^{3}H]methyl groups per min to a form that binds to DE81 at 37°C) in 200 μl of 25 mM Tris·HCl, pH 9.0/5 mM dithiothreitol/1 mM EDTA/50% (wt/vol) glycerol/1 μM *S*-adenosyl-L-methionine for up to 12 days at 37°C. Every 24 to 36 hr, *S*-adenosyl-L-methionine, which is unstable at alkaline pH, was replenished by addition of 1 μl of a 200 μM solution made from a 10 mM stock solution adjusted to pH 2.5 with H_2SO_4 (18) and an additional 15 units of fresh *Eco*RI methylase was added. The methylated DNA was purified by a short gel column/centrifugation technique (19) using a 1-ml packed bed of Bio-Rad P-4 beads equilibrated with TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA). The purified DNA was extracted once with buffer-saturated phenol and then three times with chloroform. The aqueous phase was made 0.2 M in NaCl, and the DNA was precipitated by addition of 2.2 vol of cold ethanol. The precipitate was collected by centrifugation. The

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Abbreviations: SV40, simian virus 40; bp, base pair(s); HSV, herpes simplex virus; *tk*, thymidine kinase gene; T antigen, SV40 large tumor antigen.

dried pellet was suspended in 20 μl of 10% TE buffer and stored at 4°C. For microinjection, the DNA was diluted to 0.1–0.2 μg/μl with 10 mM Tris·HCl, pH 7.4. The extent of methylation was determined by digestion of plasmid DNA with *Eco*RI under conditions for cleavage at noncanonical sites as described by Woodbury *et al.* (20). DNA (1 μg) was incubated in 50 μl of 20 mM Tris·HCl, pH 8.0/2 mM $MgCl_2$ containing 25 units of *Eco*RI for 4 hr at 37°C. The intactness and the restriction patterns of the plasmid DNA were monitored by agarose gel electrophoresis; 1.0% horizontal agarose gels were subjected to electrophoresis for 4 hr at 125 mA or for 15 hr at 40 mA in 40 mM Tris base/5 mM NaOAc/1 mM EDTA adjusted with HOAc to pH 7.9.

Detection of T Antigen by Immunofluorescence. Cells were fixed in cold methanol for 15 min at 4°C. SV40 T antigen was visualized by the indirect immunofluorescent technique of Pope and Rowe (21) using hamster anti-T antiserum and, as second antibody, fluorescein-conjugated goat anti-hamster gamma globulin.

Autoradiography. Cells were labeled for 24 hr with [^{3}H]thymidine (6.7 Ci/mol, 0.7 μCi/ml; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear), added immediately after microinjection. Autoradiographs were made and analyzed as described by Baserga and Malamud (22).

RESULTS

In experiment 1, the HSV-*tk* gene was microinjected into *tk*$^-$ ts13 hamster cells. As shown in Table 1, when the unmethylated gene is microinjected into these cells, ≈150% of the cells become capable of incorporating [^{3}H]thymidine. That means that, for each 100 cells microinjected, ≈150% were labeled with [^{3}H]thymidine 24 hr later. This is due to the fact that the microinjected cells were growing exponentially, so that a number of them divided during the 24-hr period (the background is 0 in such short-term experiments). When the same HSV *tk* gene, methylated by *Eco*RI methylase under canonical conditions, is microinjected into the same cells, ≈50% of the cells become capable of incorporating [^{3}H]thymidine. This experiment was carried out several times, even at intervals of several months, always giving essentially the same results.

The SV40 *A* gene does not have canonical *Eco*RI sites but it does have several noncanonical *Eco*RI (*Eco*RI*) sites. Instead of SV40 DNA, we used the plasmid pSV2G (12), which contains an intact early region—i.e., the gene coding for the T and small tumor antigens. When pSV2G methylated at the noncanonical *Eco*RI* sites was microinjected into TC-7 cells (Table 1), the number of cells positive for SV40 T antigen by immunofluorescence was the same as that in cells microinjected with unmethylated pSV2G. The percentage of T-positive cells was always consistently less than the percentage of cells positive for the *tk* gene. From unpublished data, we believe that this is due to indirect immunofluorescence being less sensitive than [^{3}H]thymidine incorporation (see below).

Table 1. Effect of methylation on expression of cloned genes

	Cells, %	
	Labeled	T positive
Experiment 1		
Unmethylated pTK	154 ± 20	
pTK methylated at *Eco*RI sites	53 ± 11	
pTK methylated at *Eco*RI sites and then digested with *Eco*RI	60	
Experiment 2		
Unmethylated pSV2G		61 ± 7
pSV2G methylated at *Eco*RI* sites		63 ± 12

In experiment 1, pTK DNA (0.2 mg/ml) was microinjected into tk$^-$ts13 hamster cells. Cells were labeled for 24 hr with [^{3}H]thymidine at 0.5 μCi/ml and the number of labeled cells was determined autoradiographically. In experiment 2, pSV2G DNA (0.2 mg/ml) was microinjected into TC-7 monkey cells; 24 hr later, the number of T-positive cells was determined after immunofluorescence staining. Results are mean ± SEM.

Table 2. tk$^-$ts13 cells expressing the gene for T antigen versus cells expressing the *tk* gene after comicroinjection of unmethylated or methylated SV40 and pTK DNAs

DNA	T-positive cells/TK-positive cells
Unmethylated SV40 and pTK	0.64 ± 0.07
*Eco*RI*-methylated SV40 and pTK	1.31 ± 0.14

DNAs were methylated separately, mixed together, and comicroinjected (each, 0.11 μg/μl) into nuclei of exponentially growing tk$^-$ts13 cells. Cells were then labeled for 24 hr with [^{3}H]thymidine at 0.2 μCi/ml, and the numbers of cells expressing the genes for T antigen or HSV-*tk* were determined after immunofluorescence staining followed by autoradiography. Results are mean ± SEM.

In another experiment, pTK (HSV *tk* gene) plasmid and SV40 DNA were microinjected together into tk$^-$ts13 cells. When the genes microinjected together were unmethylated, more cells were capable of incorporating [^{3}H]thymidine than became positive for SV40 T antigen by immunofluorescence (Table 2). The fact that the percentage of cells positive for T antigen is always less than the percentage of tk$^-$ cells capable of incorporating [^{3}H]thymidine is due to the fact that immunofluorescence detection of SV40 T antigen requires at least 50,000 T molecules (11) whereas incorporation of [^{3}H]thymidine depends on TK, which is probably required in a much smaller number of copies. When the two genes were separately methylated at the *Eco*RI* site and microinjected into tk$^-$ts13 cells, the number of cells positive for SV40 T antigen was significantly larger than the number of cells capable of incorporating [^{3}H]thymidine (Table 2).

A conclusive experiment was then carried out using plasmid pC6, which contains both the HSV *tk* gene and the SV40 genome cloned in pBR322 (15).

When this plasmid is microinjected into tk$^-$ts13 cells, ≈300% of the cells become capable of incorporating [^{3}H]thymidine and ≈150% of the cells become positive by immunofluorescence for SV40 T antigen (Table 3). As a general rule, no T-positive cells were incapable of incorporating [^{3}H]thymidine while a number of T-negative cells became labeled. When pC6 methylated at *Eco*RI* sites was microinjected into the same cells, the same percentage of cells became positive for T antigen by immunofluorescence whereas expression of the HSV *tk* gene was completely abolished. These experiments were repeated four times with the same results.

Fig. 1 is a photograph of an agarose gel, to show the extent of methylation of our microinjected genes. The figure shows that, when these genes were noncanonically methylated, they became completely refractory to cleavage by *Eco*RI.

Table 3. tk$^-$ts13 cells expressing the T-antigen or HSV *tk* genes after microinjection of plasmid pC6, either unmethylated or methylated at the *Eco*RI* sites

	Cells expressing gene, %	
	T antigen	*tk*
Unmethylated	169 ± 1%	327 ± 11%
Methylated	122 ± 15%	0%

pC6 was microinjected at 0.25 μg/μl. Results are mean ± SEM.

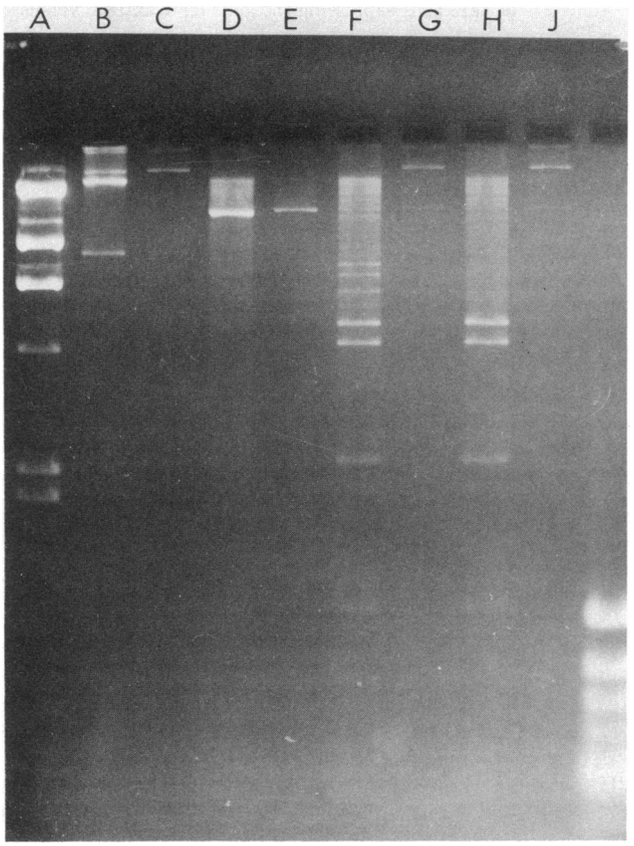

FIG. 1. Protection of pC6 DNA methylated at *Eco*RI* sites from *Eco*RI digestion. Samples were prepared and subjected to electrophoresis. Lanes: A, *Hin*dIII digest of λ DNA; B, D, F, and H, unmethylated pC6 DNA; C, E, G, and J, pC6 DNA methylated at *Eco*RI* sites. DNA in lanes D and E was digested with *Sal* I for 2 hr (linearizes plasmid), that in lanes F and G was digested with 2 units of *Eco*RI for 2 hr, and that in lanes H and J was digested with 4 units of *Eco*RI for 4 hr.

DISCUSSION

Our results show that methylation of a cloned gene affects its expression when it is microinjected into mammalian cells. Although extensive methylation of the SV40 *A* gene has no effect on its expression, methylation of the HSV *tk* gene decreases or completely abolishes its expression when microinjected into tk⁻ts13 cells. Our results, therefore, confirm and extend previous reports that DNA methylation plays a role in gene expression (1–10, 23, 24). Pollack *et al.* (25) and Wigler *et al.* (26) have also shown that methylation of the HSV *tk* gene reduces the number of clones that can be derived from tk⁻ cells in long-term genotypic experiments. Our experiments verify that, indeed, methylation of the HSV *tk* gene can abolish its expression when it is microinjected into mammalian cells.

The discrepancy between the results obtained with the SV40 *A* gene and the HSV *tk* gene, even when the two were methylated together and microinjected in the same plasmid into the same cells, raises the question whether the difference may be due not only to methylation but also to the sequences that were methylated. pSV2G has 39 *Eco*RI* (A-A-T-T) sites. Of these, 24 are within the coding and the 5′-flanking sequences. The sites can be deduced by the nucleotide sequence published by Weissman and coworkers (ref. 27; for review, see ref. 28). Thus, extensive methylation does not seem to affect expression of the SV40 *A* gene when it is microinjected into mammalian cells. Two of *Eco*RI* sites of pSV2G are in the promoter region (Fig. 2). One is ≈40 bp and the other is 135 bp upstream from the cap site for the T-antigen mRNA. Although these two sites are located in the prelude region (29) of the SV40 *A* gene, their methylation seems to be without effect on the expression of T antigen.

On the contrary, the HSV *tk* gene is methylated, whether under canonical or noncanonical conditions, only at two sites. These sites are deduced from the HSV *tk* gene sequence of McKnight (30) and Wagner *et al.* (31). For an interpretation of these results, the structure of the *tk* gene is shown in Fig. 3. There are two *Eco*RI sites in the flanking sequences of the gene. One *Eco*RI site is ≈1100 bp downstream from the 3′ end of the gene. McKnight and Davis (32) studied the expression of the *tk* gene after injection of specific deletion mutants of HSV *tk* into frog oocytes. They found that the 3′ limit of the gene is 1.32–1.6 kilobases downstream from the *Bam*HI site and that the *Eco*RI site on the 3′ side (3 kilobases downstream from this site) can be deleted without affecting expression of the HSV *tk* gene. However, the other *Eco*RI site is situated 80 bp 5′ to the beginning of the *tk* mRNA. It will be noted that the *Eco*RI site (G-A-A-T-T-C) is in a region ≈70 bp upstream from the cap site and shows the sequence G-G-C-G-A-A-T-T-C. This sequence is 66.6% homologous to the proposed consensus sequence of Benoist *et al.* (33), which has been proposed as G-G-$\frac{T}{C}$-C-A-A-T-C-T and found as a sequence homology in many promoter sequences of eukaryotic genes (adenovirus early 1A, chicken ovalbumin, chicken conalbumin, adenovirus major late, rabbit globin, mouse globin, *Strongylocentrotus purpuratus* histone H2b, silk fibroin). The sequence is considered important in gene expression and as a part of the prelude region described by Grosschedl and Birnstiel (34, 35), which includes other elements as, for instance, the Hogness–Goldberg T-A-T-

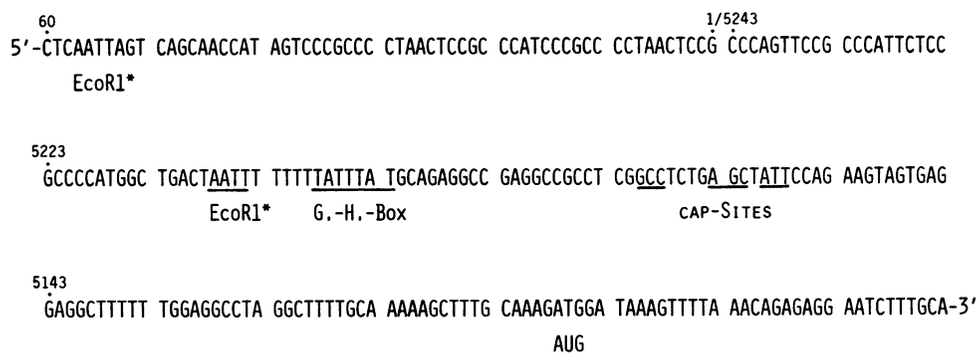

FIG. 2. DNA sequences around the three cap sites of the SV40 early mRNAs. Positions of two noncanonical *Eco*RI sites (N-A-A-T-T-N′), the Goldberg–Hogness box (G.-H.-Box), and the initiation codon for the T antigen are indicated. The numbering of the nucleotides was taken from Reddy *et al.* (27).

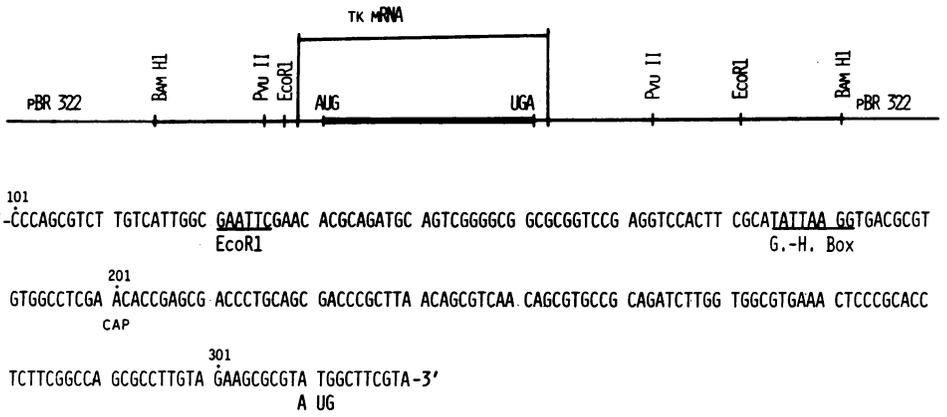

FIG. 3. Genetic map of plasmid pTK containing the HSV *tk* gene cloned into pBR322 and nucleotide sequence around the cap site of the *tk* mRNA. Positions of the *Eco*RI site in that region, the Goldberg–Hogness box (G.-H.-Box), and the initiation codon for the tk protein are indicated. The numbering of the nucleotides was taken from McKnight (30).

T-A box. Indeed, Dierks *et al.* (36) have shown that the sequences ≈70 bp upstream from the cap site are necessary for correct initiation of β-globin mRNAs. The importance of this prelude region for gene expression has also been pointed out by others (32).

*Eco*RI methylase catalyzes monomethylation of the N^6-exocyclic amino group of the central adenine residues of the G-A-A-T-T-C recognition sequence via methyl transfer from S-adenosylmethionine (37). Although methylated cytidine residues are much more abundant in eukaryotic cells, the presence of methylated adenosine residues has been demonstrated in a number of cells and viruses (38). For instance, ≈0.02% of the adenosine residues in HEK-human DNA and in baby hamster kidney DNA are methylated. It can therefore be calculated that each of these genomes contains 100,000 methylated adenosine residues. At any rate, methylation of a single site ≈70 bp upstream from the cap site of the HSV *tk* gene decreases or completely abolishes its transcription. On the other hand, extensive methylation of the SV40 *A* gene at various places in the coding region and the 5'-flanking sequences, but excluding the site described as a consensus sequence in several eukaryotic promoters (33), has no effect on its transcription when the gene is microinjected into mammalian cells. Recombinant plasmids with pBR322 do not replicate in mammalian cells (39, 40), so that gene expression in our experiments depends exclusively on microinjected copies.

Generalized conclusions cannot be drawn from these experiments in which only two genes have been examined. Furthermore, the genes were microinjected as naked DNA whereas, in the cell, genes are largely covered with proteins. However, the results make two important points: They show that (*i*) methylation has different effects on different genes and (*ii*) methylation has different effects, depending on the sites in the genes that are methylated. Whether methylation of the Benoist sequence (33) is critical for the expression of genes remains to be seen. In the meantime, one should realize that this technique of direct methylation and microinjection of cloned genes into mammalian cells can be helpful in elucidating the role of methylation in gene expression.

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