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Methylation of simian virus 40 *Hpa* II site affects late, but not early, viral gene expression

(DNA methylation/simian virus 40 viral capsid protein VP-1 and tumor antigen synthesis/microinjection of *Xenopus laevis* oocytes)

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ABSTRACT **DNA methylation has been correlated with reduced gene expression in a number of studies, although evidence for a causal link between the two events has been lacking. Because microinjection of simian virus 40 (SV40) DNA into the nucleus of *Xenopus laevis* oocytes results in the synthesis of both early and late viral gene products, it was possible to test whether a specific methylation event can affect gene expression. The single SV40 *Hpa* II site at 0.72 SV40 map units was specifically methylated with *Hpa* II methylase. When this DNA was injected into oocytes, there was a marked reduction in the synthesis of the major late viral capsid protein VP-1, relative to the synthesis by an unmethylated control. However, production of the early proteins (the large and small tumor antigens) was not affected by *Hpa* II methylation. Therefore, methylation at a single site on the viral DNA located near the 5′ end of the late region can specifically repress late gene expression. The possible mechanisms by which this repression is mediated are discussed.**

The only modified base in eukaryotic DNA is 5-methylcytosine (for review, see refs. 1 and 2). This modification occurs in the DNA of a wide variety of species, from bacteria through vertebrates and plants, which suggests that it may function in gene regulation. The major 5-methylcytosine residue in eukaryotic DNA is located in the dinucleotide sequence C-G. Restriction enzymes *Hpa* II and *Msp* I both cleave the sequence C-C-G-G. However, the former cleaves the sequence only if the C-G is unmethylated whereas the latter will cut the sequence in either state. By using the Southern blotting procedure to compare the restriction patterns of DNA cleaved with one or the other enzyme, it is possible to locate methylated C-C-G-G sites in genomic DNA. In this way, a strong correlation between gene activity and the state of DNA methylation has been demonstrated in various systems. For example, tissue-specific differences in the methylation patterns of chicken (3) and human (4) globin, ovalbumin (5), and viral (6, 7) DNA have been observed which suggest that actively expressed genes are under-methylated. This is supported by the observation that actively transcribing chromatin is under-methylated relative to methylated transcriptionally inactive chromatin (8).

The experiments in this study were further prompted by the strikingly nonrandom fashion in which C-G residues are arranged on the simian virus 40 (SV40) genome (Fig. 1). Nineteen of the 27 C-G residues are located between 0.66 and 0.75 map units, a region comprising <10% of the viral genome. This region delineates the sequences that form the nucleosome-free "gap" region that has been identified in SV40 minichromosomes (9–11) and contains the majority of the known regulatory sequences of the viral genome including the origin of replication (reviewed in ref. 12), the early (13, 14) and late (15, 16) region promoters, and the 72-base-pair repeat sequence (17) that enhances transcription. In contrast, in the remaining 90% of the genome, which exists as a regular nucleosome-containing structure in the viral minichromosome and which contains the protein encoding sequences, there is a relative dearth (8 of 27) of C-G residues.

Both late (capsid protein) and early (tumor antigen) viral gene products are expressed after SV40 DNA is microinjected into *Xenopus laevis* oocyte nuclei (18, 19). The use of *Hpa* II methylase, which methylates the C-G sequence in the single SV40 *Hpa* II cleavage sequence C-C-G-G at 0.72 SV40 map units (Fig. 1), has provided the means to test whether methylation directly affects gene expression. *Hpa* II methylated SV40 DNA yielded greatly reduced quantities of the major late protein VP-1 after microinjection into oocyte nuclei when compared to unmethylated viral DNA. Therefore, a causal link between a specific methylation event and the expression of a unique transcription unit has been established.

MATERIALS AND METHODS

Materials. *X. laevis* mature females were purchased from Nasco (Fort Atkinson, WI). Restriction enzymes were obtained from New England BioLabs and SV40 form I DNA was bought from Bethesda Research Laboratories. [^{35}S]Methionine and ^{14}C-labeled protein markers were purchased from the Radiochemical Centre (Amersham, England). Collagenase type I was obtained from Sigma and inactivated *Staphylococcus aureus* (Pansorbin) was bought from Calbiochem. Anti-SV40 large tumor antigen (T antigen) antiserum was obtained from the Research Resources Branch, National Institutes of Health; and anti-SV40 capsid antiserum was a kind gift from H. Ozer.

Methylation of SV40 DNA. *Hpa* II methylase, initially a gift from M. Wigler, was prepared as described (20) except that the Bio-Gel column was replaced by a Sephadex G-100 column and the elution buffer contained 0.5 M NaCl rather than 1.0 M NaCl. The methylase was free of *Hpa* I methylase activity. Form I SV40 or pBR322 DNA remained >75% supercoiled after incubation with the enzyme. Methylated DNA could be digested with *Eco*RI, *Hpa* I, and *Msp* I but not with *Hpa* II whereas control unmethylated DNA was also sensitive to *Hpa* II digestion (Fig. 2). Experiments by ourselves and by others (20) have failed to provide evidence for methylation of additional sites on the DNA. DNA methylation reaction mixtures contained 5 μg of SV40 DNA (form I), 5 μl of Hpa II methylase, 50 mM Tris (pH 7.5), 10 mM EDTA, and 5 mM 2-mercaptoethanol with or without 0.2 mM *S*-adenosylmethionine in 0.05 ml. One microliter of enzyme was sufficient to completely methylate 1 μg of pBR322 DNA in 4 hr at 37°C, conditions also used in SV40 DNA methylation reactions. After treatment with the methylase, DNA was extracted twice with phenol and once with chloroform and then precipitated twice with ethanol.

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; t antigen, small tumor antigen.

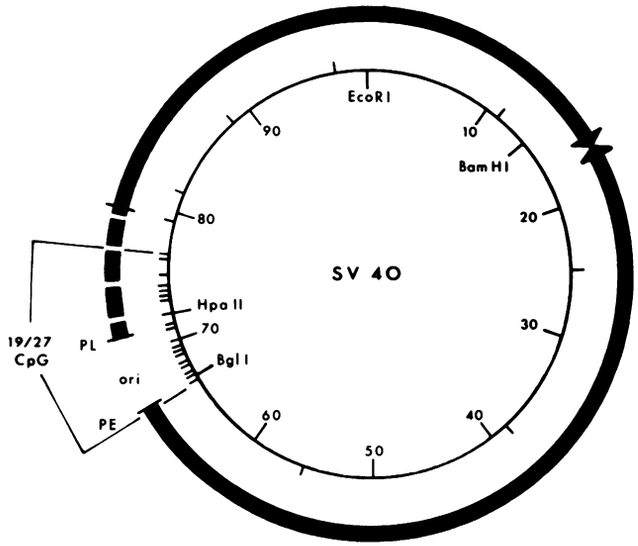

FIG. 1. Location of C-G residues in the SV40 genome. Inner circle refers to the map of the SV40 genome with positions of four single-cut restriction sites, including the *Hpa* II site at 0.726 SV40 map units. Lines emanating from the SV40 map refer to positions of C-G residues. Heavy outer circle with arrowheads shows the late (clockwise) and early (counterclockwise) transcription units. The nucleosome-free gap region is located between 0.66 and 0.75 map units. The heavy broken line refers to the area where 5′ termini of late mRNAs synthesized in infected monkey cells have been mapped.

X. *laevis* Microinjection System. Excised ovaries of *X. laevis* mature females were incubated in OR-2 medium (21) containing 0.2% collagenase (type 1) for 4 hr at 20°C or until the ovarian tissue was dissociated and follicle cells were digested. The defolliculated oocytes were then carefully washed and maintained in modified Barth's solution (22). In order to microinject accurately into the oocyte nucleus, a modification of procedures described by Kressmann *et al.* (23) was used. Oocytes in 6 ml of modified Barth's (MB) solution were placed in a 60-mm Petri dish and centrifuged in a Sorvall HS4 swinging bucket rotor at 2,000 rpm for 10 min. This causes the germinal vesicle (nucleus) to rise to the surface of the animal pole, creating a visible ring in the pigment into which microinjection can be directed. After centrifugation, stage V oocytes were chosen both for uniformity of size and accessibility of the germinal vesicle.

Micropipettes were pulled from glass capillary tubes to tips 20–30 μm in diameter by using a Leitz microforge. The micropipettes were calibrated so that 20 microinjections of 50 nl each would be sequentially delivered to the same number of oocytes. Microinjection equipment, a kind loan from G. Khoury, consisted of a stereo-zoom microscope (Bausch and Lomb), a micromanipulator (Singer Instruments, Somerset, England), and a micrometer syringe (Agla Wellcome Research Laboratories, Sheffield, England) attached to the micromanipulator. DNA samples in 70% ethanol/0.2 M ammonium acetate were precipitated by centrifugation at 15,000 rpm for 10 min. The salt-free pellet was dried under reduced pressure and then resuspended in the appropriate volume of H_2O such that each oocyte would receive 50 nl of DNA solution. Gel electrophoresis of aliquots of methylated and nonmethylated DNA showed that the concentrations of the two samples were identical.

After microinjection, oocytes in groups of 20 were placed in an Eppendorf tube containing 1.0 ml of MB solution, containing streptomycin, penicillin, and kanamycin (50 units/ml). After incubation for 24 hr at 20°C, the medium was replaced by 100 μl of MB solution containing 250 μCi (1 Ci = 3.7×10^{10} becquerels) ^{35}[S]methionine. After 24 hr, or more if indicated,

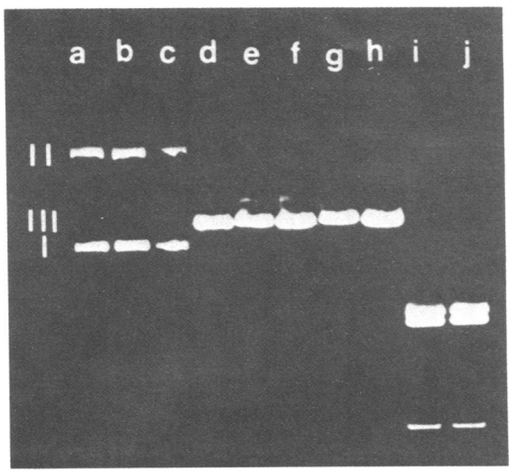

FIG. 2. Restriction enzyme analysis of methylated and nonmethylated DNA. SV40 form I DNA was incubated with *Hpa* II methylase in the presence (lanes a, c, e, g, and i) or absence (lanes b, d, f, h, and j) of *S*-adenosylmethionine. Aliquots (0.2 μg) of either DNA were then treated with no additional enzyme (lanes a and b), *Hpa* II (lanes c and d), *Msp* I (lanes e and f), *Eco*RI (lanes g and h), or *Hpa* I (lanes i and j) and then subjected to agarose gel electrophoresis.

the oocytes were washed several times in MB solution and once in water. The water was immediately removed and the oocytes were stored at −70°C until used.

Extraction and Immunoprecipitation of Oocyte Proteins. All procedures were performed at 4°C. Twenty oocytes were suspended in 1.0 ml of 0.1 M Tris·HCl/0.1 M NaCl/5 mM KCl/1 mM $CaCl_2$/0.5 mM $MgCl_2$/0.7 mM Na_2HPO_4, pH 9.0/0.5% Nonidet P-40 (19). After disruption by 10 strokes of a Dounce homogenizer with a loose-fitting pestle, the homogenate was sonicated for 1 min with a Branson L575 Sonicator, kept on ice for 20 min, and then centrifuged at 15,000 × *g* in an Eppendorf centrifuge for 15 min. Aliquots (5 μl) of the supernatant were stored for direct analysis on polyacrylamide gels, and the remainder was immunoprecipitated with the appropriate antiserum. For immunoprecipitation of capsid proteins, 250-μl aliquots from a 1.0-ml extract of 20 oocytes were incubated for 1 hr with 10 μl of rabbit anticapsid antiserum or nonimmune rabbit serum. This was followed by binding the antigen–antibody complex to formaldehyde-inactivated *S. aureus* according to published procedures (24). For immunoprecipitation of the tumor antigens, 500-μl aliquots of 1.0-ml extracts of oocytes were incubated with 20 μl of normal hamster serum for 16 hr. After addition of 100 μl of formaldehyde-inactivated *S. aureus* for 30 min followed by centrifugation at 10,000 × *g* for 2 min to remove the immune complexes, the supernatant was divided into two 250-μl aliquots to which was added either 10 μl of hamster anti-SV40 T antigen antiserum or nonimmune hamster serum for 1 hr, followed by precipitation with 50 μl of *S. aureus* bacteria. In all cases, polypeptides were released from immune complexes by heating in electrophoresis sample buffer and analyzed by polyacrylamide gel electrophoresis as described (24).

RESULTS

SV40 Gene Expression in *X. laevis* Oocytes. Microinjection of SV40 form I DNA into the nucleus or germinal vesicle of *X. laevis* oocytes has been shown to result in expression of the major capsid protein (VP-1) (18) and of the T antigen and small tumor antigen (t antigen) (19). We also have observed expression of the early and late SV40 gene products after introduction of SV40 DNA into the oocyte germinal vesicle. At all times and concentrations tested, VP-1 was synthesized in considerable

excess (5- to 10-fold) over the tumor antigens. In this way the viral expression resembles that observed in late lytic infection of monkey cells. In these experiments, VP-1 and the tumor antigens were identified by immunoprecipitation of extracts of microinjected oocytes with specific anti-SV40 capsid or anti-SV40 tumor antigen antisera, respectively. VP-1 synthesis increased in direct proportion to the concentration of microinjected DNA. Amounts ranging from 0.02 to 20 ng were injected per oocyte. At the higher DNA concentrations, or with increased times of labeling with ^{35}[S]methionine, it was possible to identify VP-1 directly among the proteins synthesized by the oocytes without immunoprecipitation (Fig. 3). It was also possible, at the higher DNA concentrations, to identify the minor capsid proteins VP-2 and VP-3 among the proteins specifically immunoprecipitated with anticapsid antiserum (data not shown). One of the major problems encountered in these oocyte microinjection experiments, and described previously (25), was variability in expression from animal to animal and even among oocytes of a single animal. Therefore, experiments were repeated several times to confirm observations, several additional parameters (such as total protein synthesis) were measured simultaneously to verify conclusions, and groups of 20 oocytes were used for each determination.

Despite this drawback, the *X. laevis* oocyte system has several specific advantages. Oocytes are large and hardy and can carry out most biosynthetic processes for several days after excision from the animal. Furthermore, oocytes do not replicate the microinjected DNA (26), thus removing the possible complication of lack of maintenance of methylation in progeny DNA molecules. It therefore is possible to test the direct effect of a specific methylation event on viral gene expression.

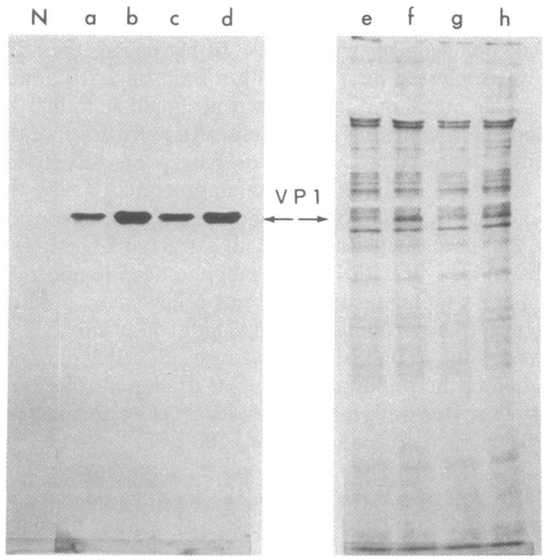

FIG. 3. SV40 VP-1 synthesis in *X. laevis* oocytes after microinjection of *Hpa* II-methylated and nonmethylated DNA. Groups of 20 oocytes that had been microinjected with different DNA samples were labeled from 24 to 72 hr with [^{35}S]methionine (2.5 mCi/ml) after injection. After disruption in 1 ml of extraction buffer, 250 μl of extract was immunoprecipitated with 10 μl of rabbit anti-SV40 capsid antiserum or 10 μl of nonimmune rabbit serum. Immune complexes were released by heating in electrophoresis sample buffer, and half of the final volume was analyzed by polyacrylamide gel electrophoresis. Lanes a and b, oocytes injected each with 0.25 ng of methylated (a) or unmethylated SV40 DNA (b); c and d, microinjected with 1.25 ng of methylated (c) or unmethylated DNA (d); N, normal rabbit serum immunoprecipitate of 250 μl of extract from lane d [similar results were obtained with control immunoprecipitates from lanes a, b, and c (not shown)]. Lanes e, f, g, and h: 2.5 μl of extracts of a, b, c, and d, respectively, analyzed directly by polyacrylamide gel electrophoresis without immunoprecipitation.

When *Hpa* II methylated SV40 DNA was microinjected into the oocyte germinal vesicle, a marked reduction in VP-1 synthesis was observed, compared to the unmethylated control (Fig. 3). The inhibitory effect was less at higher concentrations of microinjected DNA. This could be explained if there were a small (<5%) amount of DNA remaining unmethylated by the *Hpa* II methylase, which, as the concentration of microinjected DNA increased, contributed proportionally to the normal gene expression, and thus masked the methylation-induced inhibition. The total pattern of endogenous oocyte protein synthesis remained unaffected by the concentration or methylation state of microinjected SV40 DNA.

The effect of DNA methylation on VP-1 synthesis led to the obvious question of whether the early viral gene expression was similarly affected. Groups of 20 oocytes were microinjected with *Hpa* II-methylated or nonmethylated DNA. After incubation of oocytes in ^{35}[S]methionine, extracts of oocyte proteins were divided into two parts and then immunoprecipitated with anticapsid of antitumor antiserum. *Hpa* II methylation caused reduced expression of VP-1, in a particularly striking fashion at low concentrations of DNA (0.25 ng injected per oocyte) (Fig. 4). However, synthesis of both T and t antigens was unaffected by DNA methylation in these same groups of oocytes. As first described by Smith *et al.* (27), some anti-SV40 T antigen antisera immunoprecipitate minor quantities of VP-1. The antiserum used in these experiments was previously noted to have this property in early studies in this laboratory (28). As shown in Fig. 4, lane b, there was a small amount of VP-1 in the anti-T antigen immunoprecipitate of extracts of oocytes that had received unmethylated DNA, but it was absent from extracts of oocytes that had received methylated DNA (lane a). This confirms the observation that VP-1 synthesis is affected by *Hpa* II methylation whereas tumor antigen synthesis is not. Densitometer tracings of the autoradiogram of VP-1 and T antigen showed that, in the same group of oocytes in which T antigen was not detectably affected by *Hpa* II methylation, VP-1 synthesis was reduced by 80% (Fig. 5).

DISCUSSION

SV40 DNA methylated at a single C-G residue at 0.72 map units yields much less VP-1 than do similar quantities of mock-methylated DNA after microinjection into *X. laevis* oocyte nuclei. Despite its marked effect on late gene expression, this modification does not alter the amount of T and t antigens synthesized in the same oocytes. In addition to demonstrating the specificity of the methylation effect, the unchanged pattern of early viral gene expression provided a crucial internal control for the result obtained with the late viral protein synthesis. It confirms that identical amounts of DNA were injected in each case, that methylated DNA was not preferentially degraded after injection, and that the results were not due to the variability among animals or oocytes in their response to microinjected viral DNA. Although the mechanism by which methylation specifically reduces late gene expression is not known, various possibilities can be suggested, and all of them imply altered protein–DNA interaction. The SV40 *Hpa* II site occurs downstream of the late promoter but falls within the region in which 5′ cap sites of late mRNA synthesized in infected monkey cells have been mapped (Fig. 1). Thus, although *Hpa* II methylation is not likely to affect directly the binding of RNA polymerase to the late promoter, there may be a subsequent effect on the initial stages of RNA polymerization. Because some methylated residues have been identified in actively transcribed genes (1), an effect on RNA polymerase–DNA interaction may be sequence-specific. This can be tested directly by analyzing the effects of methylating other SV40 sequences.

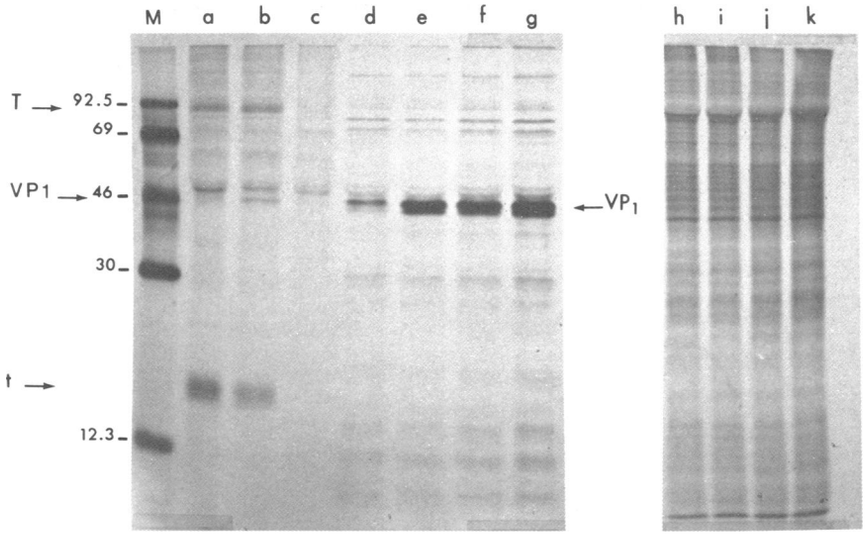

FIG. 4. Early and late viral polypeptide synthesis in *X. laevis* oocytes after microinjection of *Hpa* II-methylated and nonmethylated SV40 DNA. Conditions were as in Fig. 3 except that labeling period was from 24 to 48 hr after infection. Groups of 20 oocytes were microinjected with *Hpa* II-methylated or nonmethylated SV40 DNA and disrupted in 1.0 ml of extraction buffer. Lanes: M, ^{14}C-labeled protein markers, molecular weights are indicated at the left $\times 10^{-3}$; a and b, 250 μl of extracts of oocytes microinjected with 0.22 ng of methylated (a) or nonmethylated DNA (b) were immunoprecipitated with 10 μl of hamster anti-SV40 T antigen antiserum; 250 μl of b immunoprecipitated with 10 μl of nonimmune hamster serum; d and e, 250 μl of the same oocyte extract as in a and b, respectively, immunoprecipitated with 10 μl of anticapsid antiserum; f and g, 250 μl of extracts of oocytes microinjected with 2.2 ng of *Hpa* II-methylated (f) or unmethylated DNA (g) immunoprecipitated with 10 μl of anticapsid antiserum; h, i, j, and k, direct electrophoresis of 2.5 μl of extracts of labeled oocyte proteins before immunoprecipitation corresponding to d, e, f, and g, respectively.

Another mode by which methylation may influence gene expression in oocytes may be alteration in viral chromatin structure. Viral DNA has been shown to be assembled into chromatin after microinjection into *X. laevis* oocyte nuclei (29). Although the existence of the nucleosome-free gap observed in viral minichromosomes isolated from infected monkey cells has not yet been reported in *X. laevis* oocytes, the similarity of the viral gene expression observed in oocytes to that in monkey cells suggests that the chromatin structure may be similar in both cases. Because there is a relatively great abundance of C+G residues in the region of the genome where the nucleosome-free gap has been mapped (Fig. 1), there may be a link between minichromosome structure and methylatable sequences. One likely contributing factor to the gap structure is the binding of the viral T antigen to the *ori* sequences (30, 31) in minichromosomes. Viral DNA is not replicated in oocytes, but preliminary evidence suggests that T antigen autoregulates early mRNA synthesis in a similar fashion to its role in lytic infection (unpublished data). However, because the C+G-rich sequences of the gap extend beyond the T antigen-binding sites (32, 33) in the lateward direction, it is possible that cellular regulatory proteins also may have specific affinity for this region in a fashion that may or may not be influenced by the presence of T antigen at the origin. Thus, the possibility exists that methylation may interfere with the ability of nonhistone proteins to bind to viral DNA. Alternatively, it can be suggested that methylation directly affects nucleosome phasing, which may then influence rates of transcription. This effect would be localized because expression of the early region is unaffected by methylation at this site. Preliminary results have failed to show an effect of *Hpa* II methylation on transcription by RNA polymerase II when assayed in an *in vitro* transcription system (34). This strengthens the idea that methylation affects chromatin structure because the majority of templates in the *in vitro* transcription system exist as naked DNA.

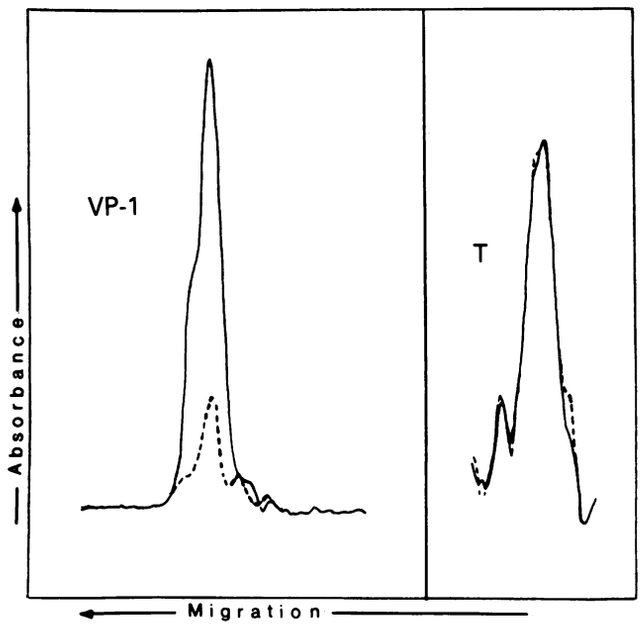

FIG. 5. Densitometer tracings of VP-1 and T antigen synthesized in oocytes injected with methylated or nonmethylated SV40 DNA. Densities of the bands corresponding to T antigen and VP-1 shown in a and b and in d and e of Fig. 4 were measured with a Gilford recording densitometer apparatus. ——, Polypeptides from oocytes injected with nonmethylated DNA; – – – –, polypeptides from oocytes injected with *Hpa* II-methylated DNA.

DNA methylation studies have shed light upon the complex question of gene regulation in eukaryotes. Actively expressed genes appear to be under-methylated and unexpressed regions of the genome are relatively hypermethylated. Other lines of evidence support the idea that methylation is related to decreased gene activity. Methylated DNA is severalfold less efficient in DNA-mediated gene transfer experiments than is nonmethylated DNA (20, 35). The correlation of methylation with gene regulation has led to theories that methylation functions in cell differentiation. Because mammalian sperm DNA has been shown to be hypermethylated (36–38), the current

hypothesis is that, during development, demethylation of specific sequences is responsible for the active state of unique genes in specialized tissues (1). Although the question of the initial developmental decision regarding unique demethylation events remains unanswered, the methylation studies provide a theoretical basis for explaining ongoing expression of differentiation-specific genes. Thus, it has been established that methylation patterns exist and are inherited (20, 35) and that there are methylases (39) that presumably serve the function of maintaining the methylated sequences through cell division and through the development of the animal.

Recently attention has focused on the existence and properties of the Z form of DNA (40). This conformation may be a transcriptionally inactive form of DNA because it has been shown that anti-Z-DNA antibodies react specifically with the interband region of polytene chromosomes (41). That methylation at the C-5 position of poly(dC-dG) results in its conversion from B form to Z form at physiological salt concentrations (42) suggests that methylation may influence the B-to-Z transition and, in this manner, repress gene activity.

Analysis of the methylation state of integrated and nonintegrated viral genes has been particularly supportive of these ideas. Sutter and Doerfler (7) have shown that adenovirus type 12 DNA integrated into the genome of transformed cells is extensively methylated at *Hpa* II sites whereas purified virion DNA is not. Furthermore, adenovirus genes integrated in cell lines expressing late viral proteins are under-methylated compared to the state of these genes in cell lines that do not synthesize these products. Results of a similar nature have been observed by Jaenisch *et al.* (43) with Moloney leukemia virus genomes in which endogenous retroviral DNA is highly methylated and is unexpressed whereas exogenous DNA from superinfecting virus is unmethylated and infectious. In another study (44) they have found that genomic mouse liver DNA containing hypermethylated Moloney murine leukemia virus is markedly less infectious than comparable quantities of proviral DNA cloned in a bacterial vector, which was thus unmethylated.

At present, the situation regarding the state of methylation of integrated compared to nonintegrated SV40 DNA is not well defined. *Hpa* II-methylated SV40 DNA functions as well as unmethylated DNA in transfection of monkey cells to produce progeny virus. Progeny DNA, however, is unmethylated (S. Weissman, personal communication). Thus, it is not yet clear whether or not methylation plays a role during lytic infection. For example, SV40 DNA may become methylated early after infection, thereby repressing late gene expression. As the infection proceeds, methylation might be reversed, either actively by a specific demethylase or passively by DNA replication. Therefore, although oocytes are useful for studying the causal link between methylation and reduced gene expression, it is of considerable importance to identify similar patterns of methylation or lack of methylation in both SV40-infected and transformed cells.

Note Added in Proof. While this manuscript was in preparation, we learned of similar results obtained by Vardimon *et al.* (45) which showed that *Hpa* II methylation of a cloned adenovirus gene inhibited its expression after microinjection into *X. laevis* oocytes.

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1. Razin, A. & Riggs, D. (1980) *Science* **210**, 604–610.
2. Wigler, M. H. (1981) *Cell* **24**, 285–286.
3. McGhee, J. D. & Ginder, G. D. (1979) *Nature (London)* **280**, 419–420.
4. van der Ploeg, L. H. T. & Flavell, R. A. (1980) *Cell* **19**, 947–958.
5. Mandel, J. L. & Chambon, P. (1979) *Nucleic Acids Res.* **7**, 2081–2103.
6. Desrosiers, R. C., Mulder, C. & Felckenstein, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3839–3843.
7. Sutter, D. & Doerfler, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 253–256.
8. Weintraub, H., Larsen, A. & Groudine, M. (1981) *Cell* **24**, 333–334.
9. Varshavsky, A. J., Sundin, D. & Bohn, M. (1979) *Cell* **16**, 453–466.
10. Saragosti, S., Moyro, G. & Yaniv, M. (1980) *Cell* **20**, 65–73.
11. Jakobovitz, E., Bratosin, S. & Aloni, Y. (1980) *Nature (London)* **285**, 263–265.
12. Bergsma, D. J., Olive, D. M., Hartzell, S. W. & Subramanian, K. N. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 381–385.
13. Berk, A. & Sharp, P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1276–1278.
14. Benoist, C. & Chambon, P. (1981) *Nature (London)* **290**, 304–310.
15. Lai, C. J., Dhar, R. & Khoury, G. (1978) *Cell* **14**, 971–982.
16. Ghosh, P. K., Reddy, V. B., Swinscoe, T., Liebowitz, P. & Weissman, S. M. (1978) *J. Mol. Biol.* **126**, 813–846.
17. Gruss, P., Dhar, R. & Khoury, G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 943–947.
18. DeRobertis, E. M. & Mertz, J. E. (1977) *Cell* **12**, 175–182.
19. Rungger, D. & Turler, H. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 6073–6077.
20. Wigler, M., Levy, D. & Perucho, M. (1981) *Cell* **24**, 33–40.
21. Eppig, J. J. & Steckman, M. L. (1976) *In Vitro* **12**, 173–179.
22. Gurdon, J. B. (1968) *J. Embryol. Exp. Morphol.* **20**, 401–414.
23. Kressmann, A., Clarkson, S. G., Telford, J. L. & Birnstiel, M. L. (1977) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 1077–1082.
24. Prives, C. & Shure, H. (1979) *J. Virol.* **29**, 1204–1212.
25. Mertz, J. E. & Gurdon, J. B. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1502–1506.
26. Harland, R. M. & Laskey, R. A. (1980) *Cell* **21**, 761–771.
27. Smith, A. E., Smith, R. & Paucha, E. (1979) *J. Virol.* **28**, 140–153.
28. Gidoni, D., Scheller, A., Barnet, B., Hantzopoulos, P., Oren, M. & Prives, C. (1982) *J. Virol.* **42**, 456–466.
29. Laskey, R. A., Honda, B. M., Mills, A. D., Morris, N. R., Willie, A. H., Mertz, J. E., DeRobertis, E. M. & Gurdon, J. B. (1977) *Cold Spring Harbor Symp. Quant. Biol.* **42**, 171–178.
30. Mann, K. & Hunter, T. (1979) *J. Virol.* **29**, 232–241.
31. Reiser, J., Renart, S., Crawford, L. V. & Stark, G. (1980) *J. Virol.* **33**, 78–87.
32. Tjian, R. (1978) *Cell* **13**, 165–179.
33. Shalloway, D., Kleinberger, T. & Livingston, D. M. (1980) *Cell* **20**, 411–422.
34. Manley, J. L., Fire, A., Cano, A., Sharp, P. A. & Gefter, M. L. (1980) *Proc. Natl. Acad. Sci. USA* **79**, 3855–3859.
35. Stein, R., Gruenbaum, Y., Pollack, Y., Razin, A. & Cedar, H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 61–65.
36. Waalwijk, C. & Flavell, R. A. (1978) *Nucleic Acids Res.* **5**, 4631–4636.
37. Mandel, J. L. & Chambon, P. (1979) *Nucleic Acids Res.* **7**, 2081–2103.
38. Singer, J., Roberts-Ems, J., Luthardt, F. W. & Riggs, A. D. (1979) *Nucleic Acids Res.* **7**, 2369–2375.
39. Roy, P. H. & Weissbach, A. (1975) *Nucleic Acids Res.* **2**, 1669–1684.
40. Wang, A. H. J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G. & Rich, A. (1979) *Nature (London)* **282**, 680–686.
41. Nordheim, A., Pardue, M. L., Lafer, E. M., Muller, A., Stoller, B. B. & Rich, A. (1981) *Nature (London)* **294**, 417–422.
42. Behe, M. & Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1619–1623.
43. Jaenisch, R., Jahner, D., Nobis, P., Simon, I., Lohler, J., Harbers, K. & Grotkopp, D. (1981) *Cell* **24**, 519–529.
44. Harbers, K., Schnieke, A., Stuhlmann, H., Jahner, D. & Jaenisch, R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7609–7613.
45. Vardimon, L., Kressmann, A., Cedar, H., Maechler, M. & Doerfler, W. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1073–1077.