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DNA Methyltransferase Induced by Frog Virus 3

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Over 20% of the cytosine bases in frog virus 3 DNA are methylated at the 5-carbon position. To determine whether this high degree of methylation is the result of a virus-specific enzyme, we examined the kinetics of induction and the substrate specificity of a DNA methyltransferase from frog virus 3-infected fathead minnow cells. A novel DNA methyltransferase activity appeared in the cytoplasm of infected cells at 3 h postinfection. This activity was induced in the absence of viral DNA replication and was therefore probably an early viral enzyme. In contrast to the methyltransferase activity extracted from uninfected cell nuclei, the cytoplasmic enzyme showed a strong template preference for double-stranded over single-stranded and for unmethylated over hemimethylated DNA. The dinucleotide sequence dCpdG was a necessary and sufficient exogenous substrate for methylation in vitro. A mutant of frog virus 3, isolated as resistant to 5-azacytidine and having unmethylated virion DNA, did not induce cytoplasmic DNA methyltransferase, leading to the conclusion that this activity is coded for by the virus.

The presence of 5-methylcytosine (5mC) in eucaryotic DNA has been widely discussed as having a possible role in the control of gene expression (25). Methylation of specific genes appears to prohibit their transcription, e.g., ovalbumin sequences in nonoviduct tissue (19), β-globin genes in nonerythroid cells (20), metallothionein genes in mouse lymphoid cells (6), certain integrated adenovirus genes (28), and endogenous mammary tumor virus (5). Christy and Scangos (4) recently extended these findings by demonstrating that methylated herpesvirus thymidine kinase genes cannot be expressed in transfected cells. In addition, adenovirus EII genes methylated with *Hpa*II methylase in vitro are not transcribed upon microinjection into nuclei of *Xenopus laevis* oocytes, whereas unmethylated genes are expressed (29).

Frog virus 3 (FV3), genus *Ranavirus* of the family *Iridoviridae* (nomenclature approved by the International Committee on Taxonomy of Viruses, Strasbourg, 1981), is a large icosahedral virus with a linear, double-stranded DNA genome approximately 107 kilobase pairs in length (17). We have shown that FV3 virion DNA is heavily methylated at the cytosine residues of dCpdG sequences, with more than 20% of the total cytosine residues in the form of 5mC (34). FV3 exhibits a well-regulated series of transcriptional controls (33) in which both host RNA polymerase II and viral proteins are involved (9, 32). After infection with FV3, host mRNA transcription is reduced by 95% (32). It is not clear how the virus alters the host polymerase so that it stops transcribing cellular genes and begins to transcribe highly methylated viral DNA. Possibilities include active demethylation of the virion DNA, making new sites available for transcription; interaction of virion proteins with host RNA polymerase II, making the cellular enzyme competent to transcribe methylated DNA; or "melting" of the methylated viral DNA template by virion proteins to allow transcription by host RNA polymerase. Alternatively, FV3 DNA methylation may not be involved in transcription at all but may be part of a restriction-modification system (26) or be involved in DNA replication or packaging (24).

As a first step in determining the function of FV3 DNA methylation, we establish in this report that methylation of virion DNA results from a virus-coded enzyme activity and that methylation takes place in the cytoplasm after the DNA has been transported there from its initial site of synthesis, the nucleus (10).

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MATERIALS AND METHODS

Cells and virus. Fathead minnow (FHM) cells were propagated at 33°C as monolayers in roller bottles or 100-mm tissue culture dishes with Eagle minimal essential medium containing 5% fetal calf serum. A clonal isolate of FV3 was used to infect cells at a multiplicity of 1 PFU/cell. Virus was harvested and assayed as previously described (20).

Quantitation of 5mC. FHM monolayers in 100-mm dishes were exposed to heat-inactivated FV3 (ΔFV3) for 3 h at 30°C to inhibit host cell DNA synthesis (11), after which they were infected with 20 PFU of active virus per cell and incubated for 3.5 h at 30°C. At this time, when viral DNA synthesis was maximum (11), the cultures were pulse-labeled for 30 min with 100 μCi of [6-^{3}H]uridine (15 Ci/mmol) per ml, an isotope which is proportionately incorporated into both cytosine and 5mC, as well as into thymidine (13). The medium was removed after the pulse and replaced with medium containing 2 mM each nonradioactive deoxycytosine and thymidine to dilute the amount of label in the intracellular pools. At 0, 1, and 2 h after the chase with cold nucleotides, cells were scraped into the overlying medium, centrifuged (5 min at 600 × *g*), and washed twice in 0.01 M phosphate-buffered saline (pH 7). The cells were resuspended in 5 volumes of hypotonic buffer (RSB: 0.01 M NaCl, 0.01 M Tris [pH 8], 0.001 M $MgCl_2$) with 0.5% Triton X-100, after which the nuclei were collected by sedimentation at 1,000 × *g* for 5 min. The nuclei were washed once in 5 volumes of RSB, and the wash was added to the cytoplasmic fraction. The nuclei were resuspended in 5 volumes of RSB, and both cytoplasmic and nuclear fractions were digested for 4 h with 100 μg of proteinase K per ml in the presence of 0.1% sodium dodecyl sulfate. RNA was removed by alkaline hydrolysis, and DNA was extracted from each fraction or from unfractionated cells and prepared for thin-layer chromatography (TLC) as described by Gunthert et al. (13). The spots corresponding to the markers for cytosine and 5mC were cut out and counted by liquid scintillation photometry.

Preparation of labeled virions. FV3 was propagated for 3 days in the presence of 10 μCi of [*methyl*-^{3}H]thymidine (20

Ci/mmol) per ml. DNA was extracted from purified virions as previously described (34).

Radioactive labeling of viral DNA in infected cells. Monolayers of FHM cells were infected with 20 PFU of FV3 per cell after host cell nucleic acid synthesis had been inhibited with ΔFV3 as described above. Viral DNA was labeled with 20 μCi of [*methyl*-^{3}H]thymidine (20 Ci/mmol) per ml from 4 to 5 and from 6 to 7 h after infection with active virus. DNA was extracted from the infected cells as previously described (10).

Restriction enzyme analysis. Restriction endonuclease digestions were carried out for 4 h at a ratio of 3 U of enzyme per μg of DNA according to the conditions specified by the supplier. Approximately 0.1 to 2 μg of DNA was subjected to electrophoresis on a horizontal 0.7% agarose gel submerged in TAE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA) for 18 h at 50 V. Gels were stained with 1 μg of ethidium bromide per ml and viewed under UV light. Gels containing [^{3}H]DNA were impregnated with En^{3}Hance autoradiography enhancer (New England Nuclear Corp.), dried under vacuum, and exposed to Kodak X-AR5 X-ray film for 3 to 7 days.

Preparation of nuclear and cytoplasmic extracts. Phosphate-buffered saline-washed pellets of infected and uninfected FHM cells were resuspended in 3 volumes of ice-cold RSB–Triton X-100 and centrifuged (1,000 × *g* for 10 min) to separate the nuclei and cytoplasm. The supernatant (cytoplasmic extract) was made 50 mM in Tris (pH 8)–10 mM EDTA–1 mM dithiothreitol–20% glycerol (D buffer). The nuclear pellet was washed twice in RSB–Triton X-100 and extracted with KCl by the method of Simon et al. (27). After dialysis at 4°C overnight against D buffer, both nuclear and cytoplasmic extracts were centrifuged at 5,000 × *g* to remove insoluble debris and frozen in liquid N_2 until use. Protein concentration was determined by a modified Coomassie blue assay (2).

DNA methyltransferase assay. Methylation of DNA in vitro was carried out in a 50-μl volume in a reaction mixture consisting of 50 mM Tris (pH 8), 10 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 2 μCi of *S*-adenosyl[*methyl*-^{3}H]methionine (15 Ci/mmol), 5 to 10 μg of DNA, and 10 to 50 μg of protein. After 2 h at 30°C, the reaction was stopped by the addition of 0.5% sodium dodecyl sulfate and 50 μg of proteinase K. Methyl groups incorporated into protein and RNA were eliminated, and the samples were processed as described by Jones and Taylor (16).

Preparation of hemimethylated DNA. A hemimethylated DNA substrate was synthesized in vitro with M13 single-stranded phage DNA and the 15-mer primer (Collaborative Research, Inc.) as described by Gruenbaum et al. (12). Klenow DNA polymerase I (Boehringer Mannheim Corp.) was used to extend the primer for 3 h at 25°C with 500 mM each dATP, dGTP, TTP, and either dCTP or d5mCTP (PL Laboratories). After phenol extraction and ethanol precipitation, the DNA was used as a substrate for enzymatic methylation in vitro.

RESULTS

Methylation of FV3 DNA takes place in the cytoplasm. Viral DNA synthesis in FV3-infected cells takes place in a unique two-stage fashion; initiation and synthesis of unit-length genomes occurs in the nucleus, but elongation into concatemers and packaging into virions takes place in the cytoplasm (10). The first experiment was designed to find out in which of these two sites methylation of FV3 DNA occurred. If the viral DNA were methylated in the nucleus, a cellular enzyme could be responsible. On the other hand, methylation in the cytoplasm would suggest a virus-coded enzyme, since all known eucaryotic DNA methyltransferases are nuclear enzymes (1, 27). We determined the percentage of 5mC (in relation to total cytosine, methylated and unmethylated) in the DNA of both compartments immediately after a 30-min pulse at 3.5 h postinfection with [6-^{3}H]uridine, which serves as a labeled precursor of both forms of cytosine (13), and after 1 and 2 h of a chase in the presence of unlabeled deoxycytidine and thymidine. The distribution of radioactive cytosine is presented in tabular form (Table 1), and the percentage of 5mC is graphed in Fig. 1. It should be recalled that [6-^{3}H]uridine is converted to [6-^{3}H]deoxycytidine and to [6-^{3}H]thymidine, which are both incorporated into DNA and must be separated by TLC. As methylation of cytosine is a postreplicative event, label will move from cytosine to 5mC over a period of time.

Immediately after the pulse, 83% of the total counts were found in the nuclear fraction (Table 1), but only 2% of the nuclear cytosine was methylated (Fig. 1). In contrast, out of the 17% of the total labeled cytosine in the cytoplasm (Table 1), 18% was methylated (Fig. 1). After 1 h of chase, radioactive cytosine + 5mC was divided evenly between nucleus and cytoplasm, and after 2 h of chase the division was 18.5% in the nucleus and 81.5% in the cytoplasm, confirming the movement of newly synthesized viral DNA from nucleus to cytoplasm (10). The total number of counts incorporated into cytosine + 5mC in the unfractionated cells increased by 50% during the 2-h chase, which indicates that the chase was not completely effective (Table 1). However, during the 2-h chase, methylated cytosine in total cellular DNA rose from 4 to 22%, an increase of fivefold. Over the same period of time, the percentage of 5mC only increased from 2 to 4.8% in nuclear DNA (Fig. 1). DNA isolated from the cytoplasm immediately after the pulse already had over 18% 5mC; at 2 h, this percentage had risen to 22.8%, the value previously reported for virion DNA (34). The marked increase with time in the percentage of 5mC in the DNA extracted from unfractionated infected cells, but not from either of the two fractions, can be explained by the relative increase in the amount of label found in cytoplasmic DNA versus the amount of label remaining in the nucleus. The label associated with the nucleus 2 h after the chase probably represents continued synthesis resulting from the pool of

TABLE 1. Distribution of radioactive cytosine between FV3-infected cell nucleus and cytoplasm after pulse and chase[a]

Fraction	cpm [^{3}H]cytosine + 5mC (%)		
	0 h	1 h	2 h
Total cell	5,169 (100)	6,793 (100)	7,956 (100)
Nucleus	4,320 (83.3)	3,086 (55)	1,475 (18.5)
Cytoplasm	8,66 (16.7)	2,527 (45)	6,521 (81.5)

[a] FHM cell monolayers were exposed (3 h at 30°C) to ΔFV3 to inhibit host cell DNA synthesis, infected with 20 PFU of active virus per cell, and pulse-labeled with [6-^{3}H]uridine for 30 min at 3.5 h postinfection. The radioactive medium was removed and replaced with medium containing 2 mM each unlabeled deoxycytosine and thymidine. At 0, 1, and 2 h after the chase, samples were separated into nuclear and cytoplasmic fractions, and DNA was extracted from the fractions as well as from unfractionated cells. The purified DNA was hydrolyzed to the constituent bases in formic acid, and the hydrolysate was analyzed by two cycles of TLC (12). Spots corresponding to cytosine or 5mC were cut out and counted in a liquid scintillation system. The values represent the combined radioactivity of cytosine + 5mC.

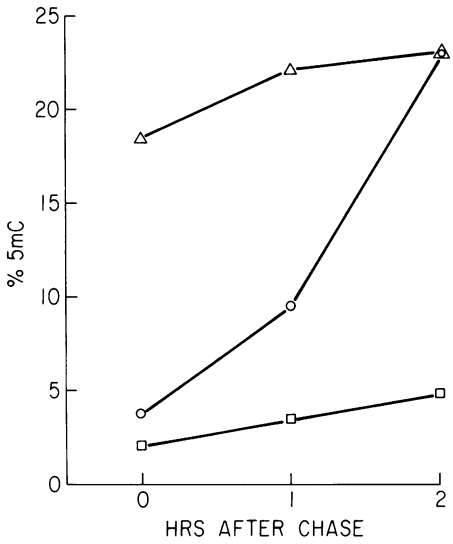

FIG. 1. FV3 DNA is methylated in the cytoplasm. The experimental protocol is described in footnote *a* of Table 1; data are plotted as counts per minute in 5mC over counts per minute in cytosine + 5mC. ○, Total cell; △, cytoplasm; □, nucleus.

radioactive precursors that was not completely diluted by the chase. Since most of the cytoplasmic DNA was methylated, whereas nuclear DNA was not, we infer that viral DNA was methylated shortly after transport to the cytoplasm.

Further confirmation that DNA methylation is a postreplicative event was obtained by examining the sensitivity of newly synthesized viral DNA to the isoschizomeric restriction endonucleases *Hpa*II and *Msp*I, which can distinguish between methylated and unmethylated DNA (30). Figure 2 shows the restriction endonuclease digests of FV3 DNA labeled from 4 to 5 h postinfection, when DNA synthesis is predominantly nuclear, and from 6 to 7 h postinfection, when viral DNA synthesis is cytoplasmic (10). Much of the DNA synthesized between 4 and 5 h was still sensitive to *Hpa*II, whereas the DNA synthesized between 6 and 7 h was not, supporting our conclusion that methylation of FV3 DNA is separated from its synthesis in both time and site.

Induction of DNA methyltransferase of FV3. As viral DNA methylation appeared to be taking place in the cytoplasm, we believed that we might be able to detect an increase in DNA methyltransferase activity in the cytoplasm of infected cells, in contrast to the situation reported for several lines of uninfected eucaryotic cells, where DNA methyltransferase activity is confined to the nucleus (1, 16, 27). The recent isolation of an FV3 mutant resistant to 5-azacytidine (AzaC^r mutant) provided us with an additional opportunity to check the viral specificity of the FV3-induced methyltransferase. This mutant was isolated by serial passage in the presence of 10 μM 5-azacytidine, a concentration of drug that inhibits DNA methylation (15) and reduces the infectivity of wild-type FV3 by >95% (R. Goorha, unpublished observations). The DNA of this mutant is not methylated; therefore, it seemed highly probable that the mutation is in the gene for the putative virus-induced DNA methyltransferase. Figure 3 shows that DNA methyltransferase activity in the cytoplasm of infected FHM cells increased as a consequence of time after infection, a maximum being attained at 3 h postinfection. A smaller, but still significant increase occurred in the absence of viral DNA synthesis, a condition achieved by infecting the cells in the presence of 10^{-3} M cytosine arabinoside (araC). The increase in methyltransferase activity in the absence of viral DNA synthesis suggests that the induction of DNA methyltransferase is an early viral function. Most important, the DNA methyltransferase level in cells infected with the AzaC^r mutant virus never exceeded that of uninfected cells, demonstrating that this activity is both a virus-induced and a virus-coded function.

Substrate specificities of FV3 DNA methyltransferase. DNA methyltransferases from different sources have been reported to have different substrate specificities. For example, single-stranded DNA is a relatively poor substrate for *Hpa*II methylase (23), whereas it is an excellent substrate for the DNA methyltransferase isolated from the nuclei of Krebs ascites cells (1). In addition, hemimethylated DNA has been shown to be a 100-fold better substrate for eucaryotic nuclear DNA methyltransferases than either single-stranded or unmethylated double-stranded DNA (12, 16).

If the FV3-induced methyltransferase is a virus-coded enzyme, it might have substrate specificities different from those of the host cell enzyme. Therefore, we compared the activity of extracts from the nucleus and cytoplasm of both FV3-infected and uninfected cells on both single-stranded and double-stranded DNA substrates (Table 2). The results with uninfected FHM cells confirmed the observations of others in that the cellular enzyme was primarily confined to the nucleus and preferred a single-stranded substrate in vitro. DNA methyltransferase activity was found in both the nucleus and cytoplasm of cells infected with wild-type FV3, but the magnitude of the nuclear values in Table 2 may be

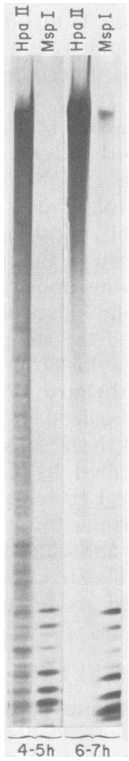

FIG. 2. Viral DNA methylation is a postreplicative event. Infected cells were treated with ΔFV3 to inhibit host DNA synthesis, and viral DNA was labeled with [*methyl*-^{3}H]thymidine from 4 to 5 and from 6 to 7 h after infection with active virus. DNA was extracted from whole cells and analyzed by restriction enzyme digestion electrophoresis and autoradiography.

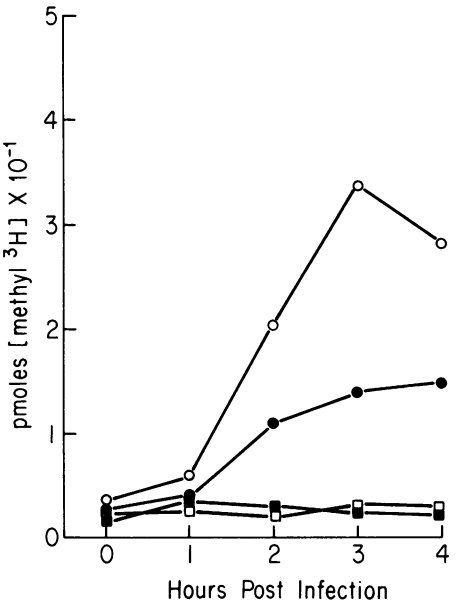

FIG. 3. Induction of DNA methyltransferase activity by FV3. One hour after adsorption of virus, FHM monolayers were overlaid with minimal essential medium with or without 10^{-3} M cytosine arabinoside and incubated at 30°C. At the indicated times, cytoplasmic extracts were prepared as described in the text, and 50 μg of protein from each extract was assayed for DNA methyltransferase activity, using 10 μg of double-stranded λ phage DNA as substrate. ○, Wild-type FV3; ●, FV 3 + araC; ■, AzaC^r mutant; □, uninfected FHM cells.

misleading since there was more protein in the cytoplasmic extracts. On a cell-equivalent basis, the amount of DNA methyltransferase activity extracted from the nuclei of infected cells was fivefold lower than that from the cytoplasm. The enzyme from the cytoplasm was virus induced, as cells infected with the AzaC^r mutant had the same pattern of enzyme distribution and substrate preference as uninfected cells. When a sample of the DNA that had been methylated in vitro by the virus-induced methyltransferase was purified, hydrolyzed in formic acid, and analyzed by TLC (13), the only labeled product was 5mC (results not shown).

Having established the preference of the FV3-induced methyltransferase for double-stranded DNA, we compared the methylating activity of this enzyme with various naturally occurring DNA substrates as well as with synthetic polynucleotides to determine whether there were any differences that could be attributed to specific recognition signals for the viral enzyme. Simon et al. (27) have shown that rat liver methyltransferase does not methylate simian virus 40 (SV40) DNA efficiently. They concluded that there is a specific sequence recognition signal for enzyme attachment, after which the enzyme "walks" along the DNA, methylating as it moves, and that this signal is missing in SV40. Our results with the FV3-induced enzyme (Table 3) demonstrate that it actively methylated a wide variety of naturally occurring DNAs, including SV40. The most notable exception was calf thymus DNA, which is already extensively methylated (7). As one would predict, DNA extracted from wild-type FV3 virions could not be further methylated, but the DNA extracted from the AzaC^r mutant was an excellent substrate.

A comparison of the specificity of the FV3-induced enzyme for several synthetic substrates (Table 3) shows that the sequence dCpdG was sufficient for methylation in vitro. In fact, this synthetic polymer was a better substrate than any of the naturally occurring DNAs, due to its large dCpdG content. If there is a specific sequence longer than a dCpdG dinucleotide required for recognition of substrate by enzyme (other than a polymer of *n* nucleotides), it is not operative in vitro.

Two groups of investigators have established the strong substrate preference of the eucaryotic nuclear enzyme for hemimethylated DNA (12, 16). This preference, they say, is because the enzyme functions as a "maintenance methylase," only methylating C in a dCpdG sequence opposite a strand that is already methylated, in order to conserve the methylation pattern that was established during differentiation. De novo DNA methyltransferase activities (those active on native unmethylated DNA) appear to exist only in embryonal tissues (14). To determine whether the FV3-induced methyltransferase was of the maintenance or of the de novo variety, we compared the activity of the extracts from virus-infected cells on a hemimethylated substrate prepared by repair synthesis of single-stranded M13 phage with d5mCTP against that of a control substrate synthesized with dCTP. The results in Table 3 demonstrate that the substrate prepared with the unmethylated nucleotide incorporated almost twice as many methyl groups as the hemimethylated substrate, as would be expected from an enzyme with de novo rather than maintenance activity. The experiment was carried out three times with three separate extracts, and the activity using the hemimethylated substrate was consistently 58 to 62% of that with the unmethylated substrate. Extracts from uninfected FHM nuclei preferred the hemimethylated DNA (results not shown), consistent with the maintenance type of activity reported for most eucaryotic cells.

Parental DNA is not demethylated after infection. Because DNA methylation has become synonymous with the inability of such DNA to be transcribed (6a, 25), we investigated the possibility that the parental FV3 genomes were demethylated before their transcription. Our approach was to grow virus in the presence of [*methyl*-^{3}H]thymidine, infect cells with labeled virus, extract DNA from infected cells at 1 and 4 h after infection, and compare the restriction endonuclease digestion patterns with *Hpa*II and *Msp*I. Figure 4 shows that

TABLE 2. FV3-induced methyltransferase prefers double-stranded DNA substrate[a]

Extract source	DNA methyltransferase activity[b]	
	Native DNA	Denatured DNA
Uninfected FHM cells		
Nucleus	0	10.62
Cytoplasm	0	2.25
Wild-type FV3-infected cells		
Nucleus	39.01	27.39
Cytoplasm	17.88	5.88
AzaC^r mutant-infected cells		
Nucleus	3.63	10.38
Cytoplasm	0.40	1.74

[a] Ten micrograms of *Micrococcus luteus* DNA, either in its native double-stranded form or denatured by boiling in distilled water for 5 min and freezing in a dry ice-acetone bath, were incubated (2 h at 30°C) with cytoplasmic or nuclear extracts and *S*-adenosyl[*methyl*-^{3}H]methionine as described in the text.

[b] Expressed as picomoles of [*methyl*-^{3}H]DNA transferred per milligram of DNA per milligram of protein per hour.

TABLE 3. Substrate specificities of FV3-induced DNA methyltransferase[a]

DNA substrate	cpm [*methyl*-^{3}H]DNA transferred/50 μg of protein
Naturally occurring DNA	
M. luteus	28,255
Lambda	21,145
Calf thymus	4,633
Wild-type FV3	403
AzaC^r FV3	21,385
SV40	10,065
pBR322	20,369
Synthetic DNA	
poly(dC · dG) · poly(dC · dG)	49,216
poly(dA · dC) · poly(dG · dT)	650
polydG · polydC	0
M13 (dCTP repair)	9,892
M13 (d5mCTP repair)	5,651

[a] DNA methyltransferase activity was extracted from the cytoplasm of cells 4 h after infection with FV3 as described in the text. Enzyme activity was assayed in 50-μl volumes using 50 μg of protein, 5 μg of the natural DNA substrates or 1 absorbance unit at 260 nm of the synthetic substrates, and 2 μCi of *S*-adenosyl[*methyl*-^{3}H]methionine. Counts from controls incubated without substrate were subtracted from all values.

at early times (1 h) and at late times (4 h) after infection, *Hpa*II failed to cut the labeled parental DNA, whereas *Msp*I digested it into small fragments. A *Hin*dIII digest was included as a control. By these criteria, no measurable demethylation of parental DNA had taken place by 4 h after infection, a time at which late RNA is being actively transcribed (33). Therefore, bulk demethylation of parental DNA cannot be evoked as a mechanism for its transcription by host RNA polymerase II, although demethylation at a few critical control regions remains a possibility.

DISCUSSION

The results reported in this paper show that FV3 induces a novel DNA methyltransferase activity in the cytoplasm of infected cells. The substrate specificities support the conclusion that this enzyme is a de novo methyltransferase completely distinct from the "maintenance methylase" found in the nuclei of uninfected cells. As an AzaC^r mutant of FV3 failed to induce DNA methyltransferase, it seems virtually certain that this enzyme is coded for by the virus, although the possibility exists that the viral mutation is in the gene for turning on a cellular methyltransferase. Many important questions concerning FV3 DNA methylation remain to be answered, among them, what is its function and does it have any relation to the phenomenon of methylated DNA in eucaryotes in general?

Razin and Friedman (24) have listed four possible functions for methylated bases in eucaryotic DNA: (i) restriction and modification, (ii) DNA replication, (iii) recombination and repair, and (iv) control of gene activity. Our data do not yet rule out any of these possibilities for FV3.

The only example of a restriction-modification system in eucaryotes is the process of zygote formation of chloroplast DNA in *Chlamydomonas* sp. (26). For the most part, all of the potentially methylatable sites in eucaryotic DNA are not methylated, questioning the generality of such systems in eucaryotes (24). However, since all of the dCpdG sequences in FV3 DNA appear to be methylated (34), they could restrict many nucleases. Infection of FHM cells with FV3 in the presence of 5-azacytidine, a drug which prevents methylation, substantially reduced the size of viral DNA packaged into virions, as well as the number of infectious progeny produced (unpublished observations). Therefore, it seems possible that FV3, which can grow in cells of piscine, avian, and mammalian origin (11), and itself carries endonuclease activity (22), has evolved a restriction-modification system to protect its own DNA.

Among the more interesting roles assigned to methylated DNA is the one of regulation of cellular differentiation and transcription. This idea has steadily gained credence because of the consistently observed inverse correlation between methylation and gene expression (6). Recently, a causal as well as a correlative function has been proposed after the observation that thymidine kinase genes methylated in vitro are not as efficiently expressed in transfected cells as their unmethylated counterparts (30). Compere and Palmiter (6) have also demonstrated that DNA methylation prevents the inducibility of metallothionein in mouse lymphoid cells. Once the gene becomes demethylated by several rounds of replication in the presence of 5-azacytidine, it can be induced by cadmium as in other cell lines in which the gene is normally unmethylated. Ley et al. (18) used 5-azacytidine to turn on quiescent embryonal globin genes. More recently, Busslinger et al. (3) have shown that cells transformed with

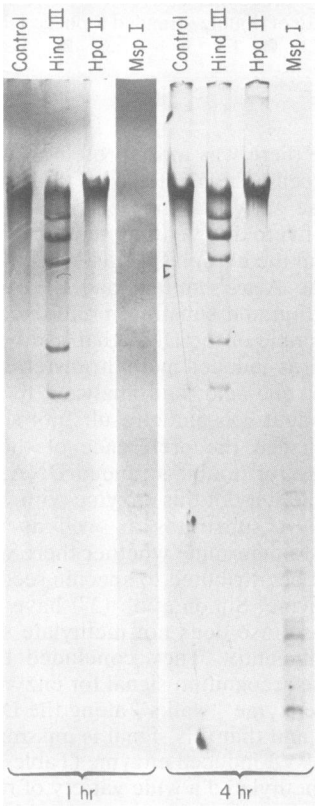

FIG. 4. Parental FV3 genomes are not demethylated after infection. FV3 DNA was labeled by growing virus stocks in the presence of [*methyl*-^{3}H]thymidine (10 μCi/ml). Labeled virions were used to infect cells at a multiplicity of 20 PFU/cell. At 1 and 4 h after infection, DNA was extracted from the infected cells, digested with restriction endonuclease, and subjected to electrophoresis on a 0.7% agarose gel. The gels were impregnated with En^{3}Hance, dried, and exposed to Kodak XAR-5 film at −70°C for 5 days.

DNA methylated throughout the 5′ region of the γ globin gene do not express the gene, whereas this gene is expressed in cells transformed with unmethylated DNA. Thus, the inability of eucaryotic RNA polymerase II to transcribe methylated DNA seems well established.

How, then, does FV3, which has been shown to use host RNA polymerase II for early transcription (7), manage to transform this enzyme so that it behaves in this unprecedented way? As purified virion DNA transfected into host cells is not infectious unless cotransfected with virion proteins (31), it is clear that a virion protein is somehow involved. We propose that the virion protein(s) interacts—directly or indirectly—with either the polymerase or the viral DNA to make such anomalous transcription possible. In addition, if there is a viral protein that can accomplish such a feat, the cell itself may have evolved a similar mechanism. Genes that have been turned off by methylation during differentiation may be turned on by such a protein, rather than by failure to methylate a daughter strand. Recent results reported by Gerber-Huber et al. (8), who have shown that the *Xenopus* vitellogenin genes are expressed when totally methylated, in contrast to other *Xenopus* genes, strongly support this concept. We now have an enzyme with de novo methyltransferase activity that can be purified and used to methylate a wide variety of genes in vitro. With the availability of systems to assay for gene expression both in vitro and in vivo, we can perhaps exploit the DNA methyltransferase of FV3 to discover the role of DNA methylation in the regulation of transcription.

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