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Patterns of Frog Virus 3 DNA Methylation and DNA Methyltransferase Activity in Nuclei of Infected Cells

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The iridovirus frog virus 3 (FV3) can replicate in culture in fat head minnow (FHM) fish cells or in BHK-21 hamster cells. Viral DNA replication commences about 3 h after infection of FHM cells with FV3. Between 3 and 6 h postinfection (p.i.), a portion of the intranuclear FV3 DNA is partly unmethylated. At later times p.i., all of the viral DNA in the nuclear and cytoplasmic compartments is methylated at the 5′-CCGG-3′ sequences. Cytoplasmic FV3 DNA has not been found unmethylated. We have cloned viral DNA fragments from methylated virion DNA. By using the genomic sequencing technique, it has been demonstrated for segments of the FV3 DNA replicated both in FHM fish and BHK21 hamster cells that in a stretch encompassing a total of 350 bp, all of the analyzed 5′-CG-3′ dinucleotides are methylated. The modified nucleotide 5-methyldeoxycytidine is present exclusively in the 5′-CG-3′ dinucleotide combination. In the cloned FV3 DNA fragment p21A, an open reading frame has been located. The 5′ region of this presumptive viral gene is also methylated in all 5′-CG-3′ positions. DNA methyltransferase activity has been detected in the nuclei of FV3-infected FHM cells at 4, 11, and 20 h p.i. In the cytoplasmic fraction, comparable activity has not been observed. These data are consistent with the interpretation that FV3 DNA is newly synthesized and de novo methylated in the nuclei of infected FHM cells and subsequently exported into the cytoplasm for viral assembly.

Sequence-specific promoter methylation is one factor determining long-term promoter inactivation (5–7). The genomes of many eukaryotic DNA viruses are not methylated at a detectable level when the virion DNA is analyzed (17, 19, 37) but can become de novo methylated upon integration into the host cell genome (3, 9, 18, 25, 32, 33). In contrast, the genome of frog virus 3 (FV3) virions contains 5-methyldeoxycytidine (5-mC) in about 22% of the cytidine residues (41, 43). Similar results have been obtained with the virion DNA from fish lymphocystis disease virus (38).

FV3 DNA is a linear, circularly permuted, terminally redundant DNA molecule about 130 to 150 kbp in length (13). Immediate-early viral genes can be transactivated by a virion-associated protein (42). FV3 proteins can transactivate the methylated E1A adenovirus promoter (34). Methylated adenovirus promoters are inactive (21, 22) but can be transactivated by the 289-amino-acid E1A transactivator of adenovirus type 2 DNA (23, 39). The promoter of one of the immediate-early FV3 genes is not inhibited by the methylation of 5′-CG-3′ dinucleotides (35). In FV3-infected cells, a DNA methyltransferase is induced (10, 40). It is not clear yet what contributions viral or host genes can make to the synthesis of this enzyme system.

We have now initiated investigations on the modes of DNA methylation of FV3 DNA propagated in fish or in hamster cells and on the DNA methyltransferase induced after viral infection. The FV3 system provides a unique experimental tool with which to study the structural and enzymatic preconditions for de novo DNA methylation. By using the genomic sequencing technique (2, 28, 36), we have determined the patterns of FV3 DNA methylation in several segments of that genome when replicated in fish or hamster cells.

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MATERIALS AND METHODS

Cells and virus; virus propagation and purification. The cell line FHM from fat head minnow (*Pimephales promelas*) (15) (ATCC CCL42) was grown in monolayer cultures at 34°C in Eagle medium supplemented with 10% fetal calf serum. FV3 (ATCC VR567) (14) was propagated at 30°C on monolayers of FHM cells after inoculation with 0.04 to 4 PFU per cell (14, 40, 41). Plaque assays with FV3 were performed on monolayers of FHM cells, using standard protocols. In some experiments, BHK-21 hamster cells (31) were grown at 34°C and infected with FV3 at 30°C.

Intracellular virus particles were liberated by ultrasonic treatment. The extracts were layered on top of a CsCl step gradient in 10 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$ consisting of solutions of 1.55-, 1.35-, 1.25-, and 1.2-g cm^{-3} CsCl density (each 2.5 ml). The FV3 particles were sedimented in an SW41 Beckman ultracentrifuge rotor at 35,000 rpm at 4°C for 1 h to their buoyant density positions.

Extraction and analysis of virion, intranuclear, or cytoplasmic DNA. Purified virions were dialyzed versus 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, and the DNA was extracted by the standard proteinase K-sodium dodecyl sulfate (SDS)-phenol-chloroform method (32). Nuclei and cytoplasm from FV3-infected cells were separated by Nonidet P-40 treatment of cells, and DNA from either cell compartment was extracted by the proteinase K-SDS-phenol-chloroform method. Restriction analyses and Southern blotting (alkaline transfer) to nitrocellulose or Nylon (Hybond N^+) membranes were performed as previously described (20, 25, 30).

Cloning of FV3 DNA fragments. FV3 virion DNA was cleaved with *Pst*I or *Xba*I, and the generated fragments were ligated into the *Pst*I- or *Xba*I-cut plasmid pBluescript KS (Stratagene) vector. The ligation mixture (0.5 μg each of cut FV3 DNA and cut and dephosphorylated vector in 30 μl of 50 mM Tris-HCl [pH 7.5]–7 mM $MgCl_2$–1 mM dithiothreitol (DTT)–1 mM ATP–4 U of T4 ligase [Stratagene]) was incubated for 14 to 16 h at 16°C, and the DNA was directly transfected into competent *Escherichia coli* HB101/LM1035

cells. Difficulties in cloning fully methylated FV3 virion DNA fragments were not encountered. Positive colonies were identified by the Grunstein-Hogness method (16), using FV3 DNA probes.

Determination of nucleotide sequences in the FV3 clones p4A and p21A. By using appropriate oligodeoxyribonucleotide primers and the dideoxy-chain termination technique (29), parts of the nucleotide sequences in the two *Pst*I clones of FV3 DNA, the ~2.5-kbp p4A (see Fig. 2b) and the ~4-kbp p21A (see Fig. 3c), were determined.

Labeling of FV3 virion DNA with digoxigenin and identification of positive colonies. Heat-denatured FV3 DNA, random hexanucleotide primer, a mixture of the deoxyribonucleoside triphosphates (dNTPs) including one digoxigenated dNTP, and Klenow polymerase (2 U) were incubated for 20 h at 37°C (DIG kit; Boehringer). Hybridization (12 h) and washing of the membranes proceeded at 68°C. The digoxigenin-labeled DNA was detected by digoxigenin-specific antibodies coupled to alkaline phosphatase. Substrates for the phosphatase reaction were naphthol-AS (2-hydroxy-3-naphthoic acid anilide)-phosphate (0.02%) and the diazonium salt of fast blue BB (0.1%).

Nick translation or oligolabeling of DNA probes. The procedures were performed as previously described (11, 27).

Genomic sequencing of FV3 DNA segments. For genomic sequencing of FV3 DNA segments (2, 26, 28, 36), virion FV3 DNA or DNA isolated from FV3-infected cells was cleaved with *Pst*I, and the DNA was repurified and reacted with hydrazine in the presence of 1.5 M NaCl for 10, 15, or 20 min at 20°C (C reaction) or with dimethylsulfate for 4 min at 20°C (G reaction). After the C reaction was stopped with 0.3 M sodium acetate (pH 7.5)–0.1 mM EDTA and the G reaction was stopped with 1.5 M sodium acetate (pH 7.0)–1 M β-mercaptoethanol, the DNA was twice ethanol precipitated and subsequently reacted with 1 M piperidine at 90°C for 30 min. The DNA fragments were then resolved by electrophoresis on 6% polyacrylamide gels in TEB (89 mM Tris-HCl, 2 mM EDTA, 89 mM boric acid)–7 M urea. DNA fragments were next electroblotted (30 min at 0.5 A; 30 min at 1 A) onto GeneScreen (DuPont) membranes and cross-linked by UV light treatment for 3 min.

Specific segments of each of the cloned FV3 DNA fragments p4A and p21A were ^{32}P labeled by using specific primers as indicated, Klenow polymerase (15 U), and the four dNTPs with dCTP ^{32}P labeled (500 μCi, 6,000 Ci/mmol). Subsequently, the ^{32}P-labeled FV3 DNA inserts were excised and isolated by electrophoresis on 6 to 10% polyacrylamide gels in TEB–7 M urea. These hybridization probes were used at 68°C for about 16 h in 0.25 M sodium phosphate (pH 7.2)–7% SDS–0.25 M NaCl–10% polyethylene glycol–1 mM EDTA. Upon hybridization, the membranes were washed once for 10 min at 68°C in 500 ml of 0.05 M sodium phosphate (pH 7.2)–2.5% SDS–0.5 mM EDTA and then washed six times for 10 min each time at 68°C in 500 ml of 0.05 M sodium phosphate (pH 7.2)–1% SDS–1 mM EDTA. The membranes were then dried and exposed to X-ray films for autoradiography.

Assay for DNA methyltransferase activity. Uninfected or FV3-infected (multiplicity of infection, 1 to 5 PFU per cell) cells were washed with cold phosphate-buffered saline (8) and suspended in hypotonic buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) (4). The cytoplasmic membranes were broken by 10 strokes in a Dounce homogenizer with a B pestle, and the cytoplasmic and nuclear fractions were separated by low-speed centrifugation (10 min at 2,000 ×

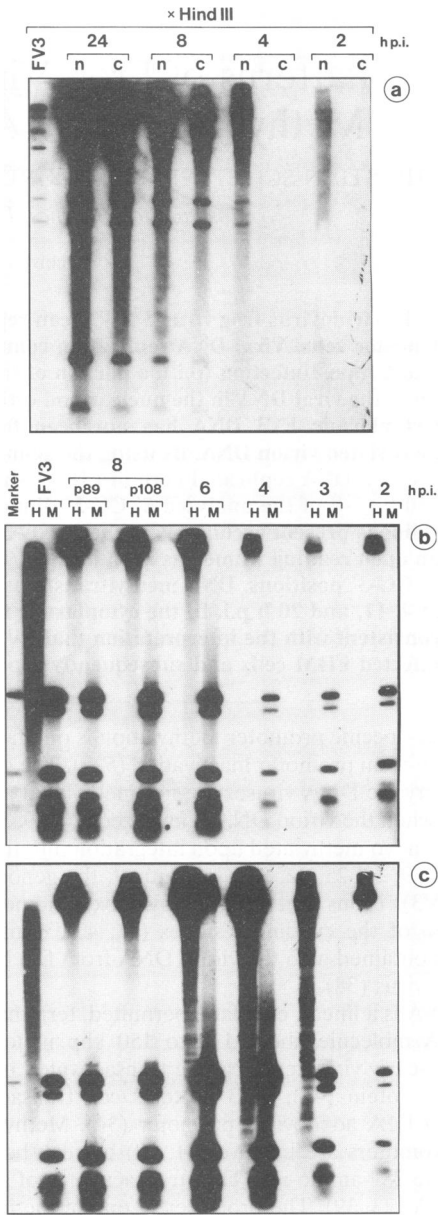

FIG. 1. Replication and de novo methylation of FV3 DNA in FHM cells. (a) Nuclear (lanes n) or cytoplasmic (lanes c) DNA was extracted at various times after FV3 infection of FHM cells as indicated and cut with *Hin*dIII, and the fragments were analyzed by electrophoresis on a 1% agarose gel. After blotting, the FV3 DNA fragments were visualized by hybridization to ^{32}P-labeled FV3 DNA followed by autoradiography. *Hin*dIII fragments of FV3 DNA were coelectrophoresed as markers. (b and c) FHM cells were infected with FV3, and at the time intervals indicated, the DNA was extracted from the cytoplasm (b) or the nuclei (c) of the cells. The DNA was then cleaved with *Hpa*II (lanes H) or *Msp*I (lanes M), and the fragments were separated by electrophoresis on 0.8% agarose gels, transferred to nylon membranes, and hybridized to the ^{32}P-labeled clone p21A of FV3 DNA. The washed and dried membranes were autoradiographed on Kodak XAR film. FV3 virion DNA or p21A DNA (Marker) cleaved with *Hpa*II or *Msp*I was coelectrophoresed. Amounts of total DNA cleaved and electrophoresed in each lane were as follows: 2 h p.i., 15 μg; 3 h p.i., 10 μg; 4 h p.i., 5 μg; 6 h p.i., 3 μg; and 8 h p.i., 0.5 μg. As the amounts of DNA used in each slot were very different, intensities of FV3 DNA hybridization signals could not be directly compared.

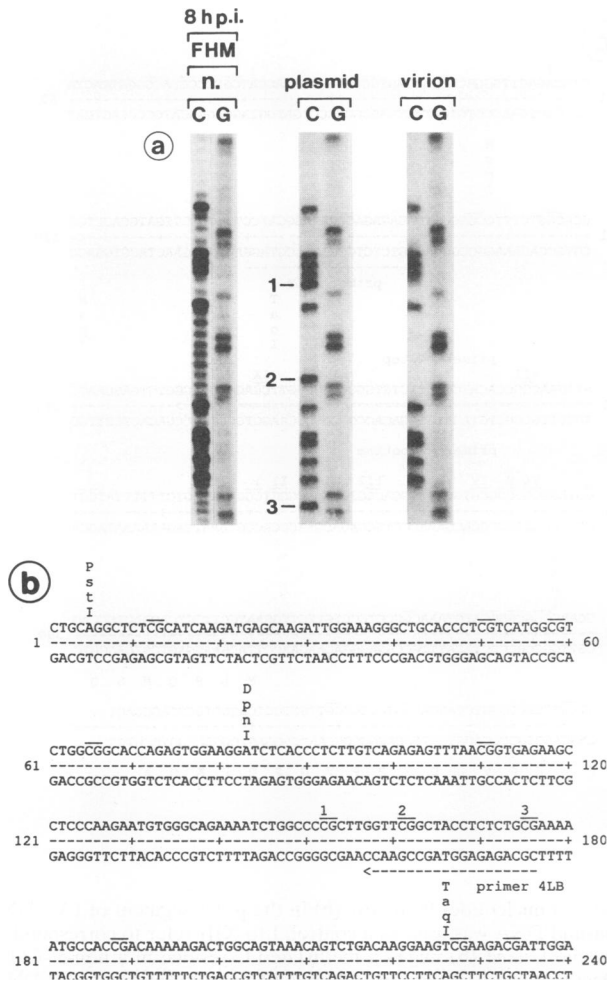

FIG. 2. Genomic sequencing of FV3 DNA from nucleotides 139 to 176 in clone p4A. The procedure used is described in Materials and Methods. (a) DNA was extracted at the indicated times after infection from FHM cells or from purified FV3 virions as described. Intranuclear (n.) or virion FV3 DNA was then reacted with hydrazine (C) or with dimethylsulfate (G); p4A plasmid DNA was treated similarly. The genomic FV3 DNA and plasmid p4A DNA sequence ladders were aligned such that equivalent positions could be directly compared. The 5′-CG-3′ positions in the segment analyzed are designated 1 to 3; these numbers correspond to the equivalent positions in the nucleotide sequence from the p4A segment presented (b). C band signals missing in the genomic sequencing ladders but present in plasmid DNA represented 5-mC residues in 5′-CG-3′ positions in intracellular or virion FV3 DNA, since 5-mC did not react with hydrazine (24). (b) Part of the nucleotide sequence of clone p4A. Primer 4LB and the *Pst*I site were used to prepare the probe used in Fig. 4. Analyzed 5′-CG-3′ sequences are marked by numbers above the sequence.

g). The cytoplasmic fraction was adjusted to buffer W (20 mM HEPES [pH 7.9], 20 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 U of aprotinin per ml [Boehringer]). The nuclear pellet was resuspended in buffer C (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 25% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 U of aprotinin per ml) and incubated with stirring for 45 min at 4°C. The nuclear suspension was subsequently centrifuged for 20 min at 4°C and 22,000 × *g*. The supernatant was dialyzed for 4 h at 4°C against several changes of buffer W. Both fractions were centrifuged at 5,000 × *g*; the supernatants were shock-frozen with liquid N_2 in 70-μl portions and stored at −80°C. Protein concentrations in extracts were determined by Coomassie brilliant blue staining (1).

In a total volume of 50 μl of 50 mM Tris-HCl (pH 8.0)–10 mM EDTA–1 mM DTT–20% glycerol–2 μCi of ^{3}H-labeled *S*-adenosylmethionine (15 Ci/mmol), 1 to 5 μg of DNA was incubated with 10 to 30 μg of protein from cytoplasmic or nuclear extracts at 30°C for 1 or 2 h. The DNA was then extracted by the SDS-proteinase K method, 20 μg of salmon sperm DNA was added as carrier, and the DNA was precipitated in 10% trichloroacetic acid. The precipitates were collected on glass fiber filters, washed with 10% TCA trichloroacetic acid and subsequently with ethanol, and dried. The amount of ^{3}H label transferred to DNA was measured in a Beckman scintillation spectrometer.

RESULTS AND DISCUSSION

Cloning of methylated FV3 DNA fragments. Virion FV3 DNA was cleaved with *Pst*I or *Xba*I, and 13 *Pst*I and 8 *Xba*I fragments were cloned into the pBluescript KS vector. Some of the Southern blot hybridization data documented that the FV3 virion DNA sequences in the cloned viral DNA fragments p4A and p21A could not be cut with *Hpa*II (Fig. 1b and c, lanes FV3) and thus were methylated in the viral DNA. Obviously, methylated FV3 DNA fragments can be cloned under these conditions.

Rapid methylation of intracellular FV3 DNA in the cytoplasmic and nuclear compartments of FHM cells. The results in Fig. 1a were obtained by Southern blot hybridization with total FV3 DNA as the probe on *Hin*dIII-cleaved nuclear or cytoplasmic DNA extracted from FV3-infected cells at time intervals after infection of FHM cells as indicated. FV3 DNA sequences were detected in the nucleus, not in the cytoplasm, of FHM cells at 2 h postinfection (p.i.) and were probably derived from parental FV3 genomes. At 3 h p.i., FV3 DNA started to replicate and was transported to the cytoplasm (12) later than 4 h p.i.

By using the cloned p21A segment as a more sensitive hybridization probe, FV3 DNA was shown to be present in both the cytoplasm (Fig. 1b) and the nucleus (Fig. 1c) of FV3-infected FHM cells at 2 h p.i. Replication appeared to commence at about 3 h p.i. (Fig. 1b and c). These DNA preparations were cleaved with *Hpa*II or *Msp*I to assess the extent of DNA methylation in the 5′-CCGG-3′ sequences of the FV3 genome. At 2 h p.i., the presumably parental sequences were completely methylated at these sites. Cytoplasmic FV3 DNA was never found to be unmethylated at the 5′-CCGG-3′ sequences (Fig. 1b). Between 3 and 6 h p.i., some of these sequences could be cut by *Hpa*II (Fig. 1c); hence, they were unmethylated in the nuclei and were probably in newly synthesized FV3 genomes. From 8 h p.i. onward, nuclear and cytoplasmic FV3 DNA was completely methylated at the 5′-CCGG-3′ sequences, as it could be cut with *Msp*I but not with *Hpa*II.

FV3 DNA methylation was also investigated by using the methylation-sensitive enzyme *Sal*I (5′-GTCGAC-3′) and the adenosine methylation-dependent enzyme *Dpn*I (5′-GATC-3′). At these sequences, FV3 virion DNA was not cleaved; i.e., the former sequences were completely methylated, and the latter sequences were unmethylated (data not shown).

It is concluded that under the experimental conditions used, FV3 DNA replication in FHM cells starts at about 3 h p.i. The newly synthesized viral DNA in the nuclei is partly unmethylated between 3 and 6 h p.i. Later, unmethylated FV3 DNA

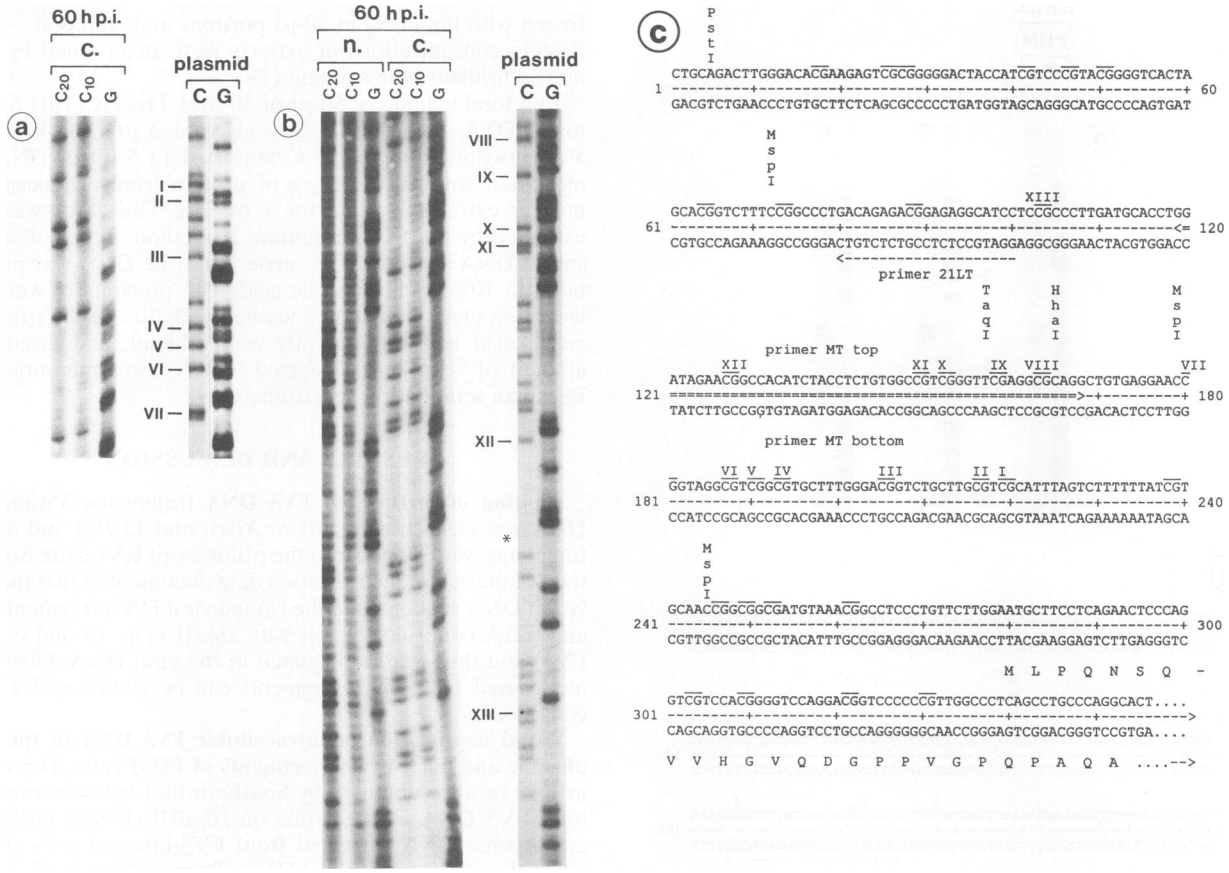

FIG. 3. Genomic sequencing of FV3 DNA from nucleotides 175 to 229 (a) and nucleotides 90 to 167 (b) in the p21A segment of FV3 DNA (c). For details, see the legend to Fig. 2 and Materials and Methods. p21A plasmid DNA was used as a control, I to XIII refer to corresponding 5′-CG-3′ dinucleotide positions of segment p21A (c). The asterisk in the plasmid C lane (b) marks a methylated C residue in a non-5′-CG-3′ position in plasmid DNA which was methylated in the bacterial host by the Dcm methylase system. In authentic FHM cell-replicated FV3 DNA, this atypical 5-mC was, of course, not present. (c) Part of the nucleotide sequence of clone p21A. Primer 21LT and the *Pst*I site were used to prepare the probe used in the genomic sequencing analyses (shown in panels a and b). The 5′-CG-3′ sequences marked with roman numerals were analyzed in panels a and b. Primers Mt top and Mt bottom were used unmethylated or methylated at all 5′-CG-3′ sequences for the assays shown in Fig. 4c.

cannot be detected any longer by restriction analysis in the nucleus or in the cytoplasm. There was no evidence for the presence of N^6-methyldeoxyadenosine in FV3 DNA.

Since de novo methylation appears to be a rapid process in this system, it is difficult to assess whether all of the newly synthesized FV3 DNA molecules are transiently unmethylated in both DNA strands. It is possible that some molecules are hemimethylated and become subject to maintenance methylation. The data in Fig. 1 suggest that FV3 DNA replicates in the nucleus of infected cells and is subsequently transferred to the cytoplasm for viral assembly.

Genomic sequencing data. For a more refined analysis, the genomic sequencing method was used. Figures 2 and 3 present the results of genomic sequencing analyses of the 5′-CG-3′ positions in the FV3 DNA sequences shown in Fig. 2b and 3c that were investigated for the presence of 5-mC residues in virion or in intracellular viral DNA. The autoradiograms present authentic sequencing data for nucleotide positions 139 to 176 in the p4A segment (Fig. 2) and of positions 175 to 229 (Fig. 3a) and 90 to 167 (Fig. 3b) in the p21A segment of FV3 DNA (Fig. 3c). Virion DNA was found to be completely methylated at all analyzed 5′-CG-3′ dinucleotides (Fig. 2a). Similar data were obtained for FV3 DNA replicated both in fish (FHM) cells (Fig. 2a) and hamster (BHK-21) cells (not shown). In the p21A segments of cytoplasmic FV3 DNA, all 5′-CG-3′ dinucleotides analyzed were methylated (Fig. 3a and b). The C signals in CpG dinucleotides were missing in all lanes, confirming the presence of 5-mC in all molecules in the reaction (Fig. 2 and 3). Nuclear FV3 DNA exhibited very similar methylation patterns; however, some of the 5′-CG-3′ sites in nuclear DNA might not be completely methylated. In dinucleotide sequences other than 5′-CG-3′, 5-mC residues were never observed in cytoplasmic or nuclear FV3 DNA from fish and hamster cells or from virion DNA. As positive controls, genomic sequencing results with the unmethylated plasmid DNAs (C and G reactions) are also shown (Fig. 2 and 3).

These data are in agreement with the results of the restriction-Southern blot hybridization experiments (Fig. 1). With the aid of the genomic sequencing technique, we have documented that the segments of FV3 DNA analyzed are methylated in all 5′-CG-3′ dinucleotide sequences and that 5-mC can be found only in this dinucleotide combination. This finding implies that the DNA methyltransferase system responsible for the de novo

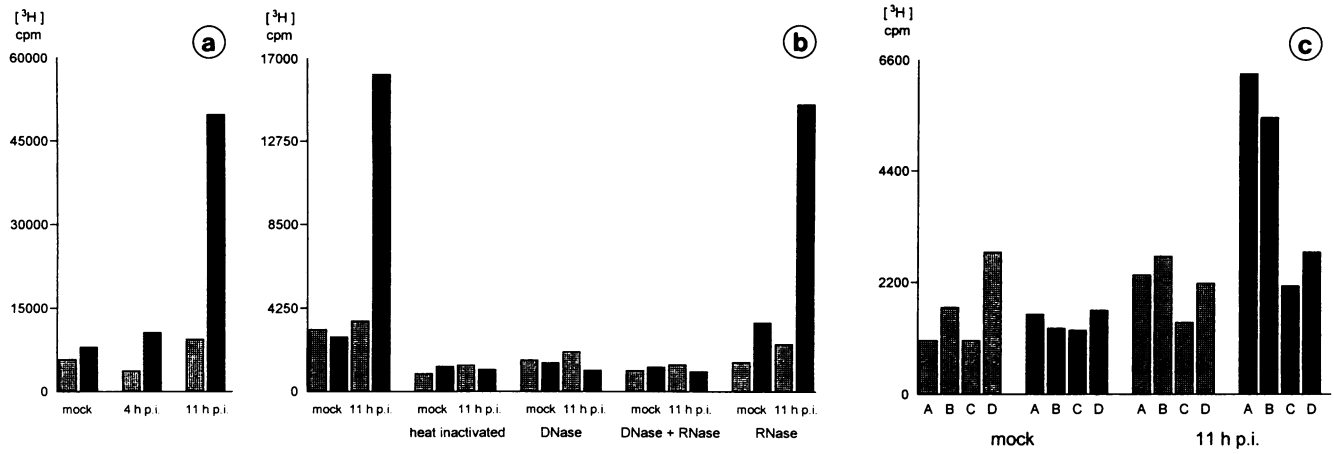

FIG. 4. DNA methyltransferase activity in nuclear (black bars) or cytoplasmic (cross-hatched bars) extracts of FV3-infected or mock-infected FHM cells. (a) Extracts were prepared from FV3-infected cells at 4 or 11 h p.i. or from mock-infected cells; the p21A fragment of FV3 DNA was used as the substrate. The DNA methyltransferase assay using ^{3}H-labeled *S*-adenosylmethionine as the methyl donor is described in Materials and Methods. In each assay, the amount of protein was 30 μg and the amount of p21A DNA was 5 μg in a total volume of 50 μl. Incubation time was 2 h at 30°C. (b) Nuclear (black bars) or cytoplasmic (cross-hatched bars) extracts were heat inactivated at 56°C for 20 min. After incubation of the p21A DNA with extracts as indicated, the reaction mixture was incubated for 30 min at 30°C with DNase (0.1 μg/μl), RNase (0.4 μg/μl), or DNase plus RNase before the ^{3}H activity incorporated in acid-insoluble form was determined. In each assay, the amount of protein was 10 μg and the amount of p21A DNA was 1 μg in a total volume of 50 μl. Incubation time was 1 h at 30°C. (c) Substrate specificity of DNA methyltransferase activity in nuclear (black bars) or cytoplasmic (cross-hatched bars) extracts. The different substrates (A to D) used as methyl group acceptors in these assays were unmethylated (A), hemimethylated top strand (B), hemimethylated bottom strand (C), and fully methylated (D). In each assay, the amount of protein was 10 μg and the amount of synthetic double-stranded DNA template was 2 μg in a total volume of 50 μl. Incubation time was 1 h at 30°C.

methylation of newly synthesized FV3 DNA exhibits high specificity.

DNA methyltransferase activity in nuclear extracts of FV3-infected FHM cells. FHM cells growing in monolayer cultures were infected with FV3. At various times p.i., nuclear and cytoplasmic extracts were prepared and assayed for DNA methyltransferase activity as described. The cloned p21A fragment of FV3 DNA was used as the methyl acceptor, and the [^{3}H]*S*-adenosylmethionine assay was employed. The DNA methyltransferase activity was found exclusively in nuclear extracts starting at 4 h p.i. and very markedly at 11 (Fig. 4a) and 20 (data not shown) h p.i. In the cytoplasmic extracts, only background activity was observed. At 11 h p.i., there was possibly a slight increase of DNA methyltransferase activity also in the cytoplasm (Fig. 4a and b). The incorporation of ^{3}H-labeled methyl groups was resistant to protease (see Materials and Methods) and RNase but was eliminated by DNase I (Fig. 4b), attesting to the fidelity of the DNA methyltransferase activity. Heat-inactivated (56°C for 20 min) extracts had lost all activity (Fig. 4b).

It is concluded that in FV3-infected cells, the DNA methyltransferase activity can be recovered almost exclusively from the nuclear extracts. This enzymatic activity methylates the p21A segment (Fig. 4) and a synthetic $(CGG)_{17}$ oligodeoxyribonucleotide substrate (data not shown) upon in vitro incubation.

The substrate specificities of the DNA methyltransferase activities in crude cytoplasmic or nuclear extracts prepared from mock-infected FHM cells or in FHM cells 11 h after infection with FV3 were assessed by using different synthetic oligodeoxyribonucleotides which represented the nucleotide sequence between positions 119 and 168 in segment p21A of FV3 DNA comprising the 5′-CG-3′ dinucleotides VIII to XII (Fig. 3). This polymer and its complement were prepared in an Applied Biosystems model 381A DNA synthesizer. The polymer was left unmethylated in both strands (Fig. 4c, substrate A), hemimethylated in all 5′-CG-3′ sequences in the top strand (Fig. 4c, substrate B), hemimethylated in the bottom strand (Fig. 4c, substrate C), or methylated in both complements (Fig. 4c, substrate D). The results demonstrated that the unmethylated oligodeoxyribonucleotide was the best substrate, followed by one of the hemimethylated constructs (5-mC in the top strand), whereas the methylated or the other hemimethylated substrates were inefficient methyl acceptors. These data were consistent with the notion that nuclear extracts from FV3-infected cells contained DNA methyltransferase activity proficient in de novo and maintenance methylations of FV3 DNA.

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