A Fully 5'-CG-3' but Not a 5'-CCGG-3' Methylated Late Frog Virus 3 Promoter Retains Activity

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Several lines of evidence demonstrate that the DNA of the iridovirus frog virus 3 (FV3) is methylated in all 5'-CG-3' sequences both in virion DNA and in the intracellular viral DNA at late times after infection. The 5'-methyldeoxycytidine residues in this viral DNA occur exclusively in 5'-CG-3' dinucleotide positions. We have cloned and determined the nucleotide sequence of the L1140 gene and its promoter from FV3 DNA. The gene encodes a 40-kDa protein. The results of transcriptional pattern analyses for this gene in fathead minnow fish cells document that this gene is transcribed exclusively late after FV3 infection. The L1140 gene and its promoter are fully methylated at late times after infection. We have been interested in resolving the apparent paradox that the methylated L1140 promoter is methylated and active late in FV3-infected cells. Of course, the possibility cannot be excluded that one or a few 5'-CG-3' sequences outside restriction endonuclease sites escaped de novo methylation after FV3 DNA replication. We have devised a construct that places the chloramphenicol acetyltransferase gene under the control of the L1140 promoter. Upon transfection, this construct exhibits activity only in FV3-infected BHK-21 hamster cells, not in uninfected BHK-21 cells. The fully 5'-CG-3' or 5'-CCGG-3' (HpaI) methylated, HpaII-mock-methylated, or unmethylated L1140 promoter-chloramphenicol acetyltransferase gene construct is active in FV3-infected BHK-21 cells, whereas the same construct 5'-CCGG-3' (HpaII) methylated has lost activity. Apparently, complete methylation of the late L1140 promoter in FV3 DNA is compatible with activity. However, a very specific 5'-CCGG-3' methylation pattern that does not naturally occur in authentic FV3 DNA in infected cells abrogates promoter function. These results further support the notion that very specific patterns of methylation are required to inhibit or inactivate viral promoters.

The iridovirus frog virus 3 (FV3) (21) provides an exceptional opportunity for studies on the functional significance of DNA methylation. The viral genome has an estimated length of 166 kbp (15, 24). FV3 DNA is probably completely methylated in all 5'-CG-3' sequences (38, 39). Both in virion DNA and in intracellular viral DNA at late times after infection, all 5'-CG-3' sequences are found methylated by genomic sequencing, and 5'-methyldeoxycytidine occurs only in 5'-CG-3' sequences (28).

Since the sequence-specific methylation of eukaryotic promoters is involved in long-term gene inactivation (for reviews, see references 4 and 5), the presumably complete methylation of the FV3 genome raises questions about the role of 5-methyldeoxycytidine in this virus system. Two relevant observations might help to solve the apparent paradox. (i) In FV3-infected cells, trans-acting functions that can overcome the transcriptional block due to DNA methylation have been detected (32, 40). (ii) The newly synthesized FV3 DNA is initially not methylated but becomes de novo methylated soon after replication (28, 38). It is unknown whether all late transcription proceeds during the time span between FV3 DNA replication and its de novo methylation. It is conceivable that 5'-CG-3' dinucleotides in functionally essential positions of viral promoters can escape de novo methylation. Lastly, the essential signals in late FV3 promoters may be devoid of 5'-CG-3' sequences.

We have studied the structure and function of, and the effect of promoter methylation on, the late FV3 gene L1140. The nucleotide sequence of the L1140 gene, which codes for a 40-kDa protein, has been determined. This gene is transcribed exclusively late in the infection cycle, although all 5'-CG-3' sequences up to about 770 nucleotide pairs (ntp) upstream from the AUG site are methylated in the intracellular viral DNA (28). We have also determined the complex effects of in vitro methylation on the L1140 promoter in transient expression experiments.

MATERIALS AND METHODS

Cells, virus, and plaque assay. The fathead minnow cell line FHM (ATCC CCL-42) was originally derived from epithelial tissue of Pimephales promelas (12). As described earlier (28), cells were propagated at 34°C in Eagle medium supplemented with 10% fetal calf serum on 75-cm² surface plastic dishes. Cell line BHK-21 (ATCC CCL10) (31) was grown in monolayer culture in Dulbecco medium–10% fetal calf serum at 37°C.

FV3 was replicated on monolayers of either cell line at the pretested temperature optimum of 30°C after inoculation of the cells with 1 to 5 PFU per cell. Virus in cell culture fluid was harvested at 48 to 65 h postinfection (p.i.). Infectivity titers were determined by FV3 plaque assays on 75% confluent monolayers of FHM fish or BHK-21 hamster cells as described elsewhere (11).

Determination of the nucleotide sequence of the cloned FV3 DNA fragments p21A and pXbal-D. The p21 (p21A) and Xbal (pXbal-D) fragments of FV3 DNA had been cloned into pBluescript II KS as described earlier (28). Using appropriate sequential oligodeoxyribonucleotide primers, the nucleotide sequence of parts of these DNA fragments was determined by using the dyeoxy-chain termination procedure (27). Synthetic oligodeoxyribonucleotides were prepared in an Applied Biosystems 381A DNA synthesizer.

Extraction and analysis of cytoplasmic RNA from mock-infected or from FV3-infected FHM cells. Cells grown in monolayers to semiconfluence were infected (11) with 10 to 20 PFU of FV3 per cell or mock-infected with phosphate-buffered saline (PBS) (6). At 3 to 48 (in some cases to 65) h p.i., measured from the time of addition of the inoculum, the cytoplasmic RNA was extracted by the hot phenol technique (29) after the nuclei had been liberated by Nonidet P-40 treatment and sedimented. Total RNA was prepared by the guanidinium isothiocyanate method (2).

Three different RNA preparations were analyzed by electrophoresis on a 1%
agarose gel in 2.2 M formaldehyde–20 mM MOPS (3-[N-morpholino]propane-sulfonate acid) pH 5.5. After completion of the electrophoretic fractionation, the RNA was transferred to a nitrocellulose membrane for 6 h in 20% SSC (1× SSC is 0.15 M NaCl plus 0.015 mM sodium citrate) by the downward blotting procedure (17).

RNA analysis by primer extension. Cytoplasmic RNA (6 μg) was treated with DNase, annealed to a L1140 promoter-specific synthetic 23-base oligodeoxyribonucleotide primer at 40°C for 16 h in 1 M NaCl-0.17 M HEPE (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid [pH 7.5])–0.5 mM EDTA, and subjected to ethanol precipitation. The oligodeoxyribonucleotide primer was elongated for 90 min at 37°C with reverse transcriptase (20 U) from Moloney murine leukemia virus (Stratagene) in 50 mM Tris-HCl (pH 8.3)–50 mM KCl–1 mM MgCl₂–1 mM DTT (dithiothreitol)–1 mM EDTA–10 μg of bovine serum albumin per ml–0.5 mM spermidine–4 mM sodium pyrophosphate–5 U of RNase inhibitor (Sigma) in the presence of 1 μM each of the deoxyribonucleoside triphosphates (dNTPs). Prior to the extension reaction, the reverse transcriptase was preincubated on ice for 30 min in 10%(vol/vol) glycerol–10 mM Tris-HCl (pH 8.3)–1 mM MgCl₂–1 mM DTT (1,4-piperazinediethanesulfonic acid [pH 6.4]). The primer was extended for 3 h at 42°C in 50 mM Tris-HCl (pH 8.3)–20 mM KCl–10 mM MgCl₂–5 mM DTT–25 mM each of the dNTPs–4.8 U of RNase inhibitor–25 U of reverse transcriptase from avian myeloblastosis virus. Prior to electrophoretic analysis (see above), the reaction product was incubated for 15 min at 37°C with 2 μg of RNase A per ml in 50 mM EDTA.

Construction of the chloramphenicol acetyltransferase (CAT) expression vector with the L1140 promoter. The 1.2-kbp KpnI-SalI fragment (box in Fig. 1b) containing the 5′ region of the L1140 gene of FV3 DNA was excised from the pXbaI-D clone. This fragment was ligated into the HindIII-SalI site in the multiple cloning segment of the linearized and dephosphorylated pBLCAT6 vector (1). The ligase reaction mixture contained 0.5 μg each of the DNA fragments and 20 μl of 10×T4 DNA ligase buffer (Promega) and 0.2 mM each dNTP. The reaction mixture was annealed at 94°C for 5 min for initial denaturation and then subjected to 30 cycles of 1 min at 94°C, 1 min at 66°C, and 2 min at 72°C. Endonucleolytic cleavage at the 5′-CCGG-3′ site would fail to yield a product; absence of cleavage by HpaII, i.e., presence of a methylated C in the 5′-CCG-3′ position, would produce a reaction product of 134 nt. The analysis of products on an acrylamide gel revealed that the HindII site was completely methylated (data not shown): upon HpaII cleavage, PCR yielded a 134-nt fragment; upon MspI cleavage, no product was amplified. These data attested to the completely methylated state of the in vitro-methylated L1140 promoter-CAT construct.

Transfection of promoter-CAT constructs and CAT assays. For the transfection of FHM or BHK-21 cells, established protocols (10) were followed. Cells were grown to semiconfluence and subsequently FV3 infected or PBS mock infected, allowing a 1-h adsorption period at 30°C. Cells were transfected with the unmethylated, mock-methylated, or specifically methylated construct 6 h p.i.; 24 h later, the cells were washed with PBS and fresh Dulbecco medium with 10% fetal calf serum was added. Incubation at 30°C was continued for another 24 h. At 48 h p.i., the cells were treated with ultrasonic for 5 to 10 s. Thereafter, the CAT assay proceeded as described previously (9, 18). Total cell extracts were incubated at 37°C for 60 min with [3H]-labeled chloramphenicol as previously described (18). Reaction products were separated by thin-layer chromatography on silica gel. Portions of the chromatograms were visualized by autoradiography. The results were quantitated by excising the 3H-labeled spots and counting 3H in a Beckman liquid scintillation spectrometer.

RESULTS

Nucleotide sequence, signal, and amino acid sequence analyses of the L1140 gene in FV3 DNA. The FV3 DNA restriction map was published elsewhere (8, 33). We determined more refined restriction maps of the two previously cloned DNA fragments p21A and pXbaI-D (28) (Fig. 1b and c) and the nucleotide sequence of a 2,082-ntp segment of FV3 DNA comprising the 1,140-ntp open reading frame of the L1140 gene (Fig. 1a). In the 5′ upstream region of this gene, we had earlier assessed the pattern of complete 5′-CG-3′ methylation by using the genomic sequencing method (28). In addition to the nucleotide sequence of the L1140 gene, Fig. 1a also shows its computer-derived amino acid sequence. In the nucleotide sequence, all 5′-CG-3′ (enhanced horizontal lines in Fig. 1a) and in the 5′ upstream region, the 5′-CCGG-3′ (HpaII; under- mprints) and 5′-CGCC-3′ (HhaI; circle) sequences were indicated. The overall CG content in the sequenced region was 58%; the 5′-CG-3′ content among all C residues was 20%. The 5′-CG-3′ dinucleotides marked by roman numerals had been included in the earlier genomic sequence determination (28). Figure 1a also shows the locations of various oligodeoxyribonucleotide primers (horizontal arrows) used in different experiments detailed below and the transcriptional start site (arrowhead at nt 475). The far 5′ upstream nucleotide sequence was characterized by a partly degenerate sixfold repeat of the motif 5′-ATTATCTTAAGATACT-3′ with dyad symmetry whose functional significance is unknown. Between nt 126 and 387, at only seven positions, between nt 388 and 505 (frame), no 5′-CG-3′ sequences could be detected. It is conceivable that the L1140 gene is related to the late 43-kDa protein gene recently mapped (8).

Current searches for sequence homologies between the L1140 nucleotide and amino acid sequences in the EMBL DNA and Swissprot protein data banks, respectively, revealed no significant similarities to any previously published proteins.

Transcription of the L1140 nucleotide sequence. We next investigated the transcriptional activity of the L1140 gene in FHM cells infected with FV3 at 10 to 20 PFU per cell. Cytoplasmic RNA (Fig. 2a) or total cell RNA (Fig. 2b) was prepared and analyzed as described at different time points after infection. The data presented in Fig. 2a and b revealed that L1140-specific RNA could not be detected prior to 24 h p.i. At 48 h p.i., RNA size classes of about 1,700, 1,400, and 900 nt could be discerned in total cellular DNA (Fig. 2b). These results demonstrated that, at least by the criteria of that method, the L1140 gene of FV3 DNA was a late-transcribed gene.

By using the primer extension method as described in Materials and Methods, and analyses of the extension products by electrophoresis in a 6% denaturing polyacrylamide gel in the presence of 7 M urea, the major site of transcriptional initiation of L1140 RNA isolated 24 h after the infection of FHM cells with FV3 was located to the thymidine residue in position 475 on the rightward-transcribed strand (filled arrowhead in the nucleotide sequence in Fig. 1a). The 342-nt band apparent on the autoradiograms of Fig. 2c corroborated this map position (13).

Methylation of the 5′-CG-3′ sequences in the L1140 gene of intracellular FV3 DNA. One of the objectives of this study was to determine the transcriptional activity of a 5′-CG-3′ methylated late FV3 promoter in FV3-infected FHM cells. At 24 h
p.i., the L1140 gene was transcribed in FHM cells (Fig. 2). By using the methylation-sensitive restriction endonuclease HpaII or MspI as a control restrictase, the methylation status at the 5′-CCGG-3′ sites in the L1140 gene and its promoter was determined at 2, 4, 8, and 24 h p.i. The autoradiogram in Fig. 3 and a comparison of HpaII restriction fragment lengths with the restriction map in Fig. 1b documented that HpaII did not cut any of the sites in the L1140 gene, whereas the 5′-CCGG-3′ sites were cleaved by MspI. Hence, at least these restriction sites assessible by restriction enzyme analyses were methylated in FV3 DNA starting at least 4 h p.i. Moreover, the genomic sequencing data reported earlier (28) demonstrated that the 5′-CG-3′ sites I through XIII in the L1140 promoter (Fig. 1a) were all methylated in intracellular FV3 DNA late after infec-
tion. Thus, it was likely that the L1140 promoter and gene were extensively, probably fully, methylated at all 5'CG-3' sites. The possibility that one or a few 5'CG-3' dinucleotides essential for L1140 gene transcription had remained unmethylated could not be excluded. We therefore tested the L1140 promoter activity at various levels of methylation in transfection and transient expression experiments.

**L1140 promoter methylation and genetic activity.** We prepared constructs that carried the prokaryotic CAT gene with (pK/SCAT1) or without (pBLCAT6) the L1140 promoter sequence, i.e., ntp 1 to 859 from the sequence shown in Fig. 1a plus about 340 ntp (~340 in Fig. 4) whose sequence had not been determined. Construct pK/SCAT1, a derivative of the eukaryotic promoterless construct pBLCAT6 (1), carried a multiple cloning site and the CAT gene with the simian virus 40 polyadenylation site (not shown in Fig. 4) (9). The nucleotide sequences of both constructs were redetermined and found to be identical to the authentic nucleotide sequences (Fig. 1a and reference 9). In that way, the unmethylated or differently 5'CG-3' methylated L1140 promoter could be examined for its functionality in uninfected or FV3-infected eukaryotic cells. Since the L1140 gene was found to be transcribed late in the FV3 infection cycle, it was necessary to test this promoter activity primarily in FV3-infected cells in order to mimic the conditions of an authentic FV3 infection. In preliminary experiments, we transfected uninfected or FV3-infected FHM fish or BHK-21 hamster cells at 6 h after infection or mock infection. CAT assays were performed at 48 h after infection, i.e., 42 h after transfection. FHM cells did not stay attached to the plastic dish surface for the full period of the experiment. Both uninfected and infected FHM fish cells produced low CAT activities even when still attached to the plastic support and were therefore not used further for these transfection experiments. It had been shown previously that BHK-21 hamster cells were fully permissive for FV3 replication (22). Hence, the transfection studies with the unmethylated or the methylated L1140 promoter were restricted to BHK-21 cells.

CAT activities obtained with different promoter constructs could be compared only as long as the reaction was still in the linear phase. In Fig. 5a and b, the CAT reaction kinetics for the standard pSV2CAT construct (30) and for the constructs pK/SCAT1 and pBLCAT6, respectively, were determined. It was apparent that the activity of the L1140 promoter construct pK/SCAT1 was strikingly higher than that of the promoterless control but only about 1/10 the activity of the standard pSV2CAT plasmid. Linear CAT activity was attained at 60 min of incubation (Fig. 5a and b). For all further experiments, extracts were incubated for 60 min with 1 μCi of 14C-labeled chloramphenicol under standard conditions.

In Fig. 5c, the results of multiple (n = 1 to 16) CAT assays of constructs as indicated in uninfected and FV3-infected
BHK-21 cells are summarized. The following conclusions were drawn from the data obtained.

(i) In uninfected BHK-21 cells, the constructs tested exhibited no or very low activities, whereas in FV3-infected cells, some of the plasmids had significant activities. These findings were in keeping with the previously documented conclusion that the L1140 gene belonged to the late-transcribed group of FV3 genes. Stimulation of the L1140 promoter could be due to viral functions or to cellular functions that were available only in FV3-infected cells.

(ii) The promoter-free vector construct had low activity.

(iii) In construct pK/SCAT1, in vitro 5'-CCGG-3' (HpaII) premethylation decreased CAT activity very markedly, while 5'-GGCG-3' (HhaI) premethylation or HpaII mock methylation had no significant effects. Premethylation at 5'-CG-3' sequences might appear to reduce activity slightly (Fig. 5c). However, the results of additional experiments did not support the notion of a significant reduction in activity.

The results of a number of typical transfection and CAT activity experiments using the unmethylated or differently methylated construct are summarized in Table 1.

It was important to demonstrate that after transfection into FV3-infected BHK-21 cells, transcription of the unmethylated pK/SCAT1 construct was initiated at the authentic site in the L1140 promoter at nt 475 (arrowhead in Fig. 1a; Fig. 2c). Upon transfection of the unmethylated or the 5'-CCGG-3' methylated pK/SCAT1 construct into mock-infected or FV3-infected BHK-21 cells, total RNA was extracted at 42 h after transfection. The RNA was then analyzed by primer extension using the primer between nt 569 and 590 in the L1140 promoter (Fig.

**TABLE 1.** Summary of transfection experiments using the pK/SCAT1 construct in different states of methylation

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*For details, see Materials and Methods and the legend to Fig. 5.*
1a) as described in Materials and Methods. After transfection of the unmethylated construct into FV3-infected BHK-21 cells, a fragment of 116 nt in length was produced in primer extension experiments (data not shown), indicating that the authentic site in the L1140 promoter (arrowhead at position 475 in Fig. 1a) was used for transcriptional initiation. With RNA from uninfected BHK-21 cells transfected with the unmethylated construct or from BHK-21 cells transfected with the 5'-CCGG-3' methylated construct, specific signals were absent or hard to detect. The Hpa II-methylated promoter was silenced or severely inhibited.

Somewhat surprisingly, complete 5'-CG-3' methylation of the late L1140 promoter slightly decreased but did not abolish its activity. This finding was consistent with the activity of FV3 genes in infected cells despite their being completely methylated. Since the construct had been in vitro methylated by the M·SssI DNA methyltransferase (25), it was not reasonable to assume that any of the 5'-CG-3' sequences had remained unmethylated. In previously published work, we had demonstrated by the genomic sequencing method that the M·SssI DNA methyltransferase modified all 5'-CG-3' dinucleotides (14). In contrast, exclusive methylation of the 5'-CCGG-3' sequences in the L1140 gene seriously interfered with its activity. It was likely that structural alterations due to sequence-specific methylation of this promoter affected its activity. We recently published direct evidence to this effect for the late E2A promoter of adenovirus type 2 (Ad2). Upon its sequence-specific in vitro methylation with three different DNA methyltransferases, M·HpaII, M·HhaI, and M·SssI, the bending of this promoter sequence was altered, as documented by striking changes in its electrophoretic mobility in agarose gels (23). We interpreted this observation as evidence for direct effects of promoter methylation on DNA structure.

DISCUSSION

We set out to investigate whether the promoter of the late FV3 gene L1140 would function even if it was methylated in all 5'-CG-3' dinucleotide sequences. In addition to the completely methylated 5'-CG-3' positions I to XIII (Fig. 1a), the data presented in Fig. 3 provided evidence that all HpaII sites were methylated in the L1140 promoter of intracellular FV3 DNA at late times after infection. At 4 h p.i., some of the intracellular FV3 DNA was cut by HpaII; i.e., the FV3 DNA was not yet completely methylated. In spite of the high level of methylation, the L1140 gene was transcribed late in the viral infection cycle. Since the possibility still existed that individual 5'-CG-3' sequences in the L1140 promoter, that were not accessible to HpaII restriction analyses, had remained unmethylated, we in vitro methylated specifically all 5'-CG-3' sequences or the 5'-CCGG-3' or 5'-GCCG-3' sequences in the L1140 promoter-CAT gene construct and tested promoter activity with the CAT indicator gene after transfection into uninfected or into FV3-infected BHK-21 hamster cells. Significant L1140 promoter-CAT gene activity was observed only in BHK-21 cells FV3 infected 6 h prior to transfection. When all 5'-CG-3' or 5'-GCCG-3' sequences were methylated, L1140 promoter activity remained comparable to that of the unmethylated construct (Fig. 5c). However, when the 5'-CCGG-3' (HpaII) positions were modified, promoter activity was decreased to low levels (Fig. 5c). Five of the HpaII sites in the L1140 promoter region were located far upstream; three 5'-CCGG-3' sequences were located between the site of transcriptional initiation and the first AUG of the L1140 open reading frame.

The mechanism of this promoter inactivation due to highly sequence-specific methylation is not understood. As shown for a different viral promoter, methylation affected bending (23). It was conceivable that additional topological aspects of a promoter sequence were also altered and contributed to its inactivation.

In earlier work on the functionality of methylated viral and eukaryotic promoters (4, 5, 23), we did not encounter a comparable situation that a completely methylated promoter still exhibited full activity in transient expression experiments. FV3 promoters might be unique in that respect. We could not decide whether this exceptional behavior of the late FV3 promoter L1140 was related to a very particular nucleotide sequence that induced promoter structures resistant to the inhibitory effect of DNA methylation. Alternatively, viral and/or cellular functions present in FV3-infected BHK-21 cells might be capable of balancing or eliminating the inhibitory effect of a certain mode of promoter methylation. Obviously, this cancelling effect depended very critically on the sequence-specific positioning of 5-methyldeoxycytidine residues in the promoter, since HpaII methylation still caused its shutdown. The promoter segment between nts 388 and 505 which contained the transcriptional start site (Fig. 1a) was devoid of 5'-CG-3' sequences and thus could not be methylated. Perhaps, this promoter segment played an important role in the transcriptional regulation of the L1140 promoter.

The viral and/or cellular factors responsible for keeping the fully 5'-CG-3' methylated L1140 promoter of FV3 DNA active in BHK-21 cells are not known. It was demonstrated previously for an early E1A Ad12 promoter that its functionality was retained even after 5'-CCGG-3' methylation in FV3-infected CHO or HeLa cells (32), whereas in uninfected HeLa cells, the same methylated promoter had been shown to be inhibited (18). It was suggested that functions present in FV3-infected cells might have counteracted in an unknown way the inactivating effect of 5'-CCGG-3' methylation on the E1A promoter of Ad2 DNA. For an FV3 promoter, the present study is the first demonstration of apparently full activity in the completely methylated state in FV3-infected cells.

It will be interesting to elucidate the biochemical nature of the viral and/or cellular functions that are capable of this cancelling effect. For the 5'-CCGG-3' methylated late E2A promoter of Ad2 DNA, our model promoter for previous studies on the functional consequences of sequence-specific promoter methylation, we documented that in cells expressing the E1 region of Ad2 DNA, specifically the 289-residue E1A transactivator protein (37), the 5'-CCGG-3' methylated late E2A promoter was at least partly active (16, 20, 37). In the Ad2-transformed hamster cell line HEI (3), the late E2A promoter of Ad2 DNA in integrated, partly truncated Ad2 genomes (36) was methylated in all 5'-CG-3' sequences (34, 35) and inactive (7, 13), although the E1A region of Ad2 DNA was expressed in this cell line (16). We also showed by in vitro methylation studies that the late E2A promoter, which was methylated in all 5'-CG-3' sequences, remained inactivated in E1A-expressing human 293 cells (23a). These results suggested that the mode of methylation of a viral promoter determined the level of inactivation and also influenced the extent to which this inactivation due to methylation might be overcome by a transactivator, e.g., by the 289-residue E1A protein of Ad2 DNA. In this respect, the results on the late E2A promoter of Ad2 DNA might resemble the complexity of the present data on the L1140 promoter of FV3 DNA. Much further work will be required before we understand these complex interdependencies between methylated viral promoters and cellular transactivating functions.
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