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Methylation of the Promoter for an Immediate-Early Frog Virus 3 Gene Does Not Inhibit Transcription

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Methylation of critical sites within the promoter region of eucaryotic genes has been shown to inhibit transcription by RNA polymerase II. However, although the large DNA virus frog virus 3 (FV3) has a highly methylated genome, it uses host RNA polymerase II for at least the immediate-early stage of transcription. We have previously shown that an FV3-induced *trans*-acting protein allows transcription from adenovirus promoters inactivated by methylation. Since FV3 immediate-early genes are transcribed in the absence of de novo protein synthesis, it appears that the virus-induced *trans*-acting protein that allows transcription from methylated templates is not required for transcription of the immediate-early FV3 genes, possibly because they are not methylated in critical regulatory sequences. In this study, we used site-directed mutagenesis to alter the three CpG dinucleotide sequences in the promoter region of an immediate-early FV3 gene and thereby created sites recognized by bacterial methylases. Transient-expression assays demonstrated that neither the mutations nor methylation of the mutated sites inhibited transcription from the FV3 promoter in FV3-infected cells. These findings support the hypothesis that the immediate-early genes of FV3 do not contain methylatable sites in regions critical for transcription. The function of the virus-induced *trans*-acting protein that can override the inhibitory effect of methylation may therefore be to facilitate the transcription of methylated delayed-early or late FV3 genes.

Gene expression in cells infected with the iridovirus frog virus 3 (FV3) is sequentially ordered and coordinately regulated to produce distinct classes of transcripts at different stages of infection (25). Immediately after infection, and in the absence of de novo protein synthesis, at least seven immediate-early infected-cell RNAs (ICRs) are transcribed (25). Using an α-amanitin-resistant Chinese hamster ovary (CHO) cell line, Goorha (5) showed that host RNA polymerase II is required for transcription of this class of viral genes. However, purified FV3 DNA is not infectious and requires one or more virion-associated proteins to initiate transcription (9, 29). Willis and Granoff (32) have demonstrated an absolute requirement for a virion protein to *trans* activate the promoter of an immediate-early gene that encodes an ICR of 169,000 daltons (ICR 169). The *trans* activation of this immediate-early gene involves the recognition of an FV3-specific *cis* regulatory element located within the promoter (26). At least two other proteins, induced in temporal order after FV3 infection, are required for synthesis of the delayed-early and late classes of FV3 RNA (28).

An additional complexity in the expression of genes from the FV3 genome is extensive methylation of virion DNA (31). About 20% of the cytosine residues are methylated by a virus-encoded DNA methyltransferase (30). In cells infected with UV-inactivated FV3, or with active FV3 in the absence of protein synthesis, the input genomic DNA remains methylated (30). However, the synthesis of immediate-early mRNA takes place under both of these conditions (25, 32), which suggests that the methylated genome serves as a template for synthesis of immediate-early RNA. Methylation of critical sites within the promoter region of a gene has been shown to inhibit transcription by RNA polymerase II (4). We have previously shown that in FV3-infected cells, a virus-induced *trans*-acting protein allows transcription of adenovirus promoters inactivated by in vitro methylation and that the promoter remains methylated after infection with either active or UV-inactivated virus (24). This virus-induced protein is different from the virion-associated *trans*-acting protein that stimulates transcription of the ICR 169 gene. The finding that the *trans*-acting protein that overrides methylation is not a component of the virion suggests that the immediate-early genes, transcribed in the absence of de novo protein synthesis, do not require this protein and therefore either are not methylated or are methylated at sites that are not critical to transcription. However, the promoter of the ICR 169 gene contains three CpG doublets, sites that appear to always be methylated in FV3 DNA (27, 31).

In this communication, we provide data showing that the promoter of the gene for ICR 169 is indeed methylated in virion DNA but that this methylation does not inhibit transcription. These findings support the hypothesis that the transcription of immediate-early FV3 genes is not inhibited by methylation. This lack of inhibition permits the production of an immediate-early gene product that allows the subsequent transcription of any delayed-early and late FV3 genes that are inhibited by methylation.

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 10% fetal calf serum. A clonal isolate of FV3 was used to prepare virus stocks at 25°C; virus was harvested and plaque assayed by the method of Naegele and Granoff (18). FV3 was UV inactivated as described elsewhere (9, 29).

Enzymes and reagents. Restriction enzymes and T4 DNA ligase (New England BioLabs, Inc., Beverly, Mass.; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used according to directions of the suppliers. T4 polynucleotide kinase and DNA polymerase I, Klenow

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fragment, were supplied by United States Biochemical Corp. [^{14}C]chloramphenicol, [γ-^{32}P]ATP, [α-^{35}S]dATP, and [α-^{32}P]dCTP, as well as GeneScreen and GeneScreen Plus nylon membranes, were obtained from Dupont, NEN Research Products, Boston, Mass.

Genomic sequencing. The methylation state of the ICR 169 promoter (32) from virion DNA was determined by genomic sequencing (3). Purified FV3 DNA (6) was cut to completion with *Xba*I, which cleaves about 50 base pairs (bp) upstream of the ICR 169 promoter (32). The cleaved DNA was extracted with phenol-chloroform, precipitated with ethanol, and suspended at 10 μg/μl in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. Portions (5 μl) were then treated with standard G, A+G, C, and C+T chemistries (15), and the fragments generated were electrophoresed on a denaturing 8% polyacrylamide-urea gel. The DNA was then electrophoretically transferred to a GeneScreen Plus nylon membrane (3).

To identify the fragments containing the ICR 169 promoter, a riboprobe was synthesized from a 2-kilobase *Xba*I-*Bgl*II FV3 restriction fragment excised from mp10XB2 with *Hin*dIII and *Eco*RI (27). This fragment was subcloned into the procaryotic transcription vector SP18 (Bethesda Research Laboratories) in an orientation that would give a sense transcript consisting of mp18 vector sequences, the ICR 169 promoter, and the entire ICR 169 coding sequence. The riboprobe was generated by linearizing the plasmid with *Eco*RI and synthesizing a radioactive transcript. Hybridization conditions were as described by Church and Gilbert (3).

Bacterial strains and plasmids. The recombinant phage mp18-169P*cat* contains the promoter of the immediate-early FV3 gene that encodes ICR 169 linked to the reporter gene for chloramphenicol acetyltransferase (CAT) (32). This promoter consists of a 78-bp *Hin*dIII-*Bam*HI fragment that flanks the 5′ end of the coding sequences of this gene and has been shown to promote transcription when *trans* activated by a virion-associated FV3 protein (32).

The plasmid pAd12-E1a *cat* contains the left-terminal 525 bp of adenovirus type 12 DNA linked to the *cat* gene in a pBR322 vector (13). This adenovirus type 12 fragment contains the E1a promoter, with two TATA motifs starting at bp 276 and 414 of this fragment. The promoter carries two *Hpa*II and three *Hha*I sites upstream of the leftmost TATA signal. Methylation of these sites results in transcriptional inactivation (13, 24). Plasmid XK-15 contains the 3.5-kbp *Xba*I fragment K from FV3 DNA cloned into MBV17 (27).

Escherichia coli JM101 was used for replication of all recombinant M13 bacteriophages. Replicative-form DNA was purified from cells by the technique of Ish-Horowicz and Burke (10). Single-stranded DNA was prepared from phage as described by Messing and Vieira (16).

Oligonucleotide-directed mutagenesis. A 14-mer, complementary to the region of the ICR 169 promoter containing the upstream CpG dinucleotide (27), and a 25-mer, complementary to the portion of the ICR 169 promoter that spans the two downstream CpG dinucleotides (27), were used to create site-directed mutations (33) in the ICR 169 promoter. These oligonucleotides, of sequences 5′-CTGGCGCTATCTCA-3′ and 5′-CTATTTTCCGGGACAAGCGCCTTCA-3′, contained single base substitutions adjacent to the CpG doublets; they were synthesized with a DNA synthesizer (Applied Biosystems, Foster City, Calif.) and purified by polyacrylamide gel electrophoresis. Mutant plaques were identified by hybridization at the T_m to the end-labeled 14-mer, and the sequence of the putative mutants was confirmed by the dideoxy-chain termination method (22). Single-stranded mutant DNA was purified and used as template for second-strand synthesis, with the 25-mer used as a primer. Mutant phage containing three point mutations were identified as described above.

In vitro methylation of mutant mp18-169P*cat*. The bacterial methyltransferases *Hpa*II and *Hha*I were used to methylate the mutated ICR 169 promoter. *Hpa*II and *Hha*I methylated the internal cytidine residues of the sequences 5′-CCGG-3′ and 5′-GCGC-3′, respectively. Replicative-form DNA was incubated with 0.5 U of *Hpa*II methyltransferase and *Hha*I methyltransferase per μg of DNA in 50 mM Tris hydrochloride (pH 7.5)–10 mM EDTA–80 μM *S*-adenosylmethionine–5 mM 2-mercaptoethanol. Methylation reactions were incubated at 37°C for 48 h to ensure complete methylation. To test methylated samples for complete methylation, the DNA was cut with *Hpa*II and *Hha*I restriction endonucleases and analyzed by agarose gel electrophoresis. Only samples that were completely methylated were used in transfection experiments.

Transfection of eucaryotic cells. DNA was transfected as $CaPO_4$ precipitates as described previously (8). Four hours after transfection, cells were subjected to a 2-min 15% glycerol shock (20) and incubated for an additional 18 h at 33°C. Cells were then mock infected or infected with FV3 (10 to 20 PFU per cell) and incubated for 4 h at 30°C (optimal temperature for virus replication). Transfected cells were harvested for CAT assay or nucleic acid purification as described below.

Assay for CAT. Cell extracts were assayed for CAT by the method of Gorman et al. (7) with 0.2 μCi (1 Ci = 37 GBq) of [^{14}C]chloramphenicol per reaction. After 30 min, the assay was stopped within the linear part of the reaction. Each assay was performed at least three times.

Isolation of RNA and dot blot analysis. Total RNA was extracted by the procedure of Chirgwin et al. (2). Ten micrograms of RNA was spotted onto GeneScreen as recommended by the manufacturer and hybridized to a ^{32}P-labeled *cat*-specific probe. RNA containing *cat*-specific sequences was visualized by autoradiography.

RESULTS

CpG doublets found within the FV3 ICR 169 promoter are methylated in virion DNA. Previous sequencing of the ICR 169 gene and flanking regions revealed that the promoter region contained three CpG doublets, two just downstream of the TATA motif and another approximately 25 bp upstream (27). Since an earlier study of 5-methylcytosine content in FV3 DNA indicated that apparently all CpG dinucleotides within the genome are methylated (31), it seemed likely that the promoter for this immediate-early gene contained methylated bases at sites that might inhibit transcription by RNA polymerase II. Numerous investigations have shown that methylation of critical sites within a promoter region, directly upstream of a promoter region, or in the 5′ end of the coding sequences of a gene may lead to transcriptional inactivation (1, 11, 23). To show conclusively that the CpG dinucleotides found within the ICR 169 promoter were methylated, we used the technique of genomic sequencing (3). FV3 DNA cleaved with the restriction endonuclease *Xba*I was treated with the standard A, A+G, C, and C+T chemistries (15). In the C+T reaction, hydrazine reacts poorly with 5-methylcytosine relative to its reaction with cytosine and thymine residues (17, 19). Thus, sites of cytosine methylation result in a missing band in the sequencing ladder. To visualize the genomic sequencing pattern of

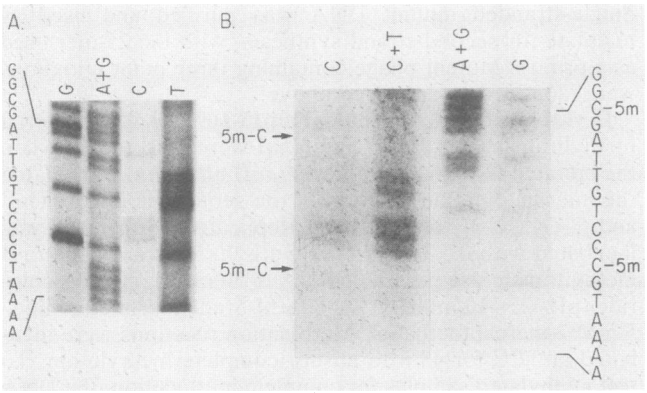

FIG. 1. Genomic sequencing showing the presence of 5-methylcytosine in the ICR 169 promoter. (A) Sequence analysis of a portion of the ICR 169 promoter from the clone XK-15 (27). The *Xba*I K fragment was purified from XK-15 by cleavage with *Xba*I and ultracentrifugation over sucrose gradients. The 3′ ends of the K fragment were labeled with [α-^{32}P]dCTP and Klenow fragment of DNA polymerase. The fragment was then cleaved with *Bgl*II, and a 2.0-kilobase fragment containing the ICR 169 promoter was purified by electrophoresis on a 1.2% low-melting-temperature agarose gel. The fragment was treated with the standard G, A+G, and C chemistries (15). A potassium permanganate reaction that cleaves after thymine and 5-methylcytosine residues was also used (21). The fragments generated were electrophoresed on an 8% polyacrylamide-urea gel, and the sequence pattern was visualized by autoradiography. (B) Genomic sequencing pattern of the same region of the ICR 169 promoter. Purified FV3 DNA was cut to completion with *Xba*I and treated with the standard G, A+G, C, and C+T chemistries (15). The potassium permanganate reaction failed and is not shown. The resulting fragments were electrophoresed on an 8% polyacrylamide-urea gel and electrophoretically transferred to a nylon membrane. The genomic sequencing pattern of the ICR 169 promoter was visualized by hybridization with a radiolabeled mRNA-sense riboprobe transcript as described in Materials and Methods. 5-Methylcytosine is indicated as 5m-C on the left side of panel B and as C-5m on the right.

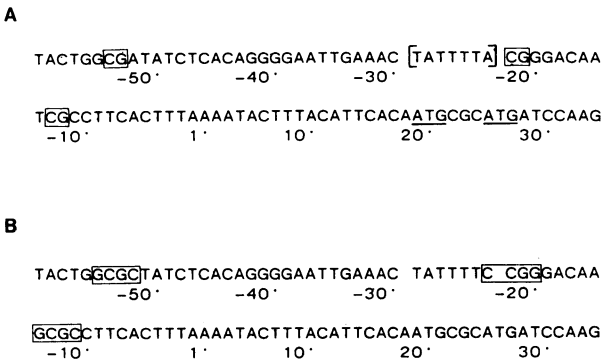

FIG. 2. Sequence of wild-type ICR 169 promoter (A) and ICR 169 promoter altered by oligonucleotide-directed mutagenesis (B). (A) The three CpG dinucleotides found within the ICR 169 promoter are boxed. The TATA motif is in brackets, and two potential translational start sites are underlined. (B) The tetranucleotide sequences created by oligonucleotide-directed mutagenesis are boxed. For each tetranucleotide, one base adjacent to a CpG doublet was changed to create a site that would be recognized by *Hpa*II or *Hha*I methyltransferase.

the ICR 169 promoter, we probed the sequence ladder of the *Xba*I-cut FV3 DNA with a transcription vector SP6 riboprobe specific for the *Xba*I fragment that contained the ICR 169 promoter (Fig. 1B). For comparison, we sequenced the same region of the ICR 169 promoter from the unmethylated DNA of plasmid XK-15 (Fig. 1A) (27). The genomic sequencing pattern of the ICR 169 promoter revealed that at least two of the CpG dinucleotides found within the promoter were methylated in virion DNA (Fig. 1). The proximity of the upstream CpG doublet to the *Xba*I cleavage site used as an endpoint for this subset of fragments in the sequence ladder prevented visualization of the sequence in this portion of the promoter. Although we could not demonstrate that this 5′-proximal doublet was methylated, the finding that the two distal CpG sites were methylated, coupled with earlier results (31) showing no cleavage with a panel of restriction enzymes sensitive to C methylation, strongly suggest that this site too was methylated in virion DNA.

Mutated ICR 169 promoter that can be methylated in vitro is still transcriptionally active in an FV3-dependent manner. To determine the effect of methylation on the transcriptional activity of the ICR 169 promoter, we used an M13 construct, mp18-169P*cat*, that contains the ICR 169 promoter linked to the reporter gene for CAT (7, 32). The 78 bp that precede the start site of ICR 169 transcription and supply all of the information needed to initiate transcription in the presence of FV3 (32) are contained within this construct. Since all cloned FV3 DNA is derived from a 5-azacytidine-resistant mutant that contains unmethylated DNA (R. Goorha and G. Kitchingman, J. Virol., in press) and is propagated in bacteria, the methylation found in wild-type FV3 DNA is no longer present. To methylate the ICR 169 promoter in the construct in a manner that would mimic the methylation pattern in virion DNA, we altered the sequence of the promoter by oligonucleotide-directed mutagenesis so that the three CpG sites methylated in virion DNA would be recognized by commercially available bacterial methylases. For each CpG doublet, we changed one adjacent nucleotide to create either an *Hpa*II (CCGG) or an *Hha*I (GCGC) recognition site. The original sequence of the ICR 169 promoter and the sequence of the mutated promoter are shown in Fig. 2.

Before we could investigate the effect of methylation on the ICR 169 promoter, we had to show that the three changes we made in the promoter did not alter the ability of the promoter to initiate transcription. To test the transcriptional activity of the mutated promoter, we introduced the unmethylated wild-type and mutant promoter-containing constructs into FHM cells by DNA-mediated gene transfer. Half of the cultures were then infected with FV3 as described in Materials and Methods. Cytoplasmic extracts were prepared and assayed for CAT activity (Fig. 3). Three separate experiments showed that the ability of the promoter to initiate transcription of the *cat* gene was not altered by the three changes made by oligonucleotide-directed mutagenesis. Furthermore, the mutated promoter, like the wild-type promoter, was transcriptionally active only when cells were infected with FV3.

Methylation of the ICR 169 promoter does not inhibit synthesis of CAT. We have shown that no CAT synthesis occurs in cells transfected with wild-type or mutated mp18-169P*cat* unless the cells are infected with FV3 (Fig. 3) (32). The ICR 169 promoter contains *cis* regulatory elements that are *trans* activated by one or more virion-associated proteins (26). Thus, in cells transfected with wild-type or mutated mp18-169P*cat*, the promoter is inactive regardless of its methylation state unless the virion-associated proteins needed for *trans* activation of the promoter are present.

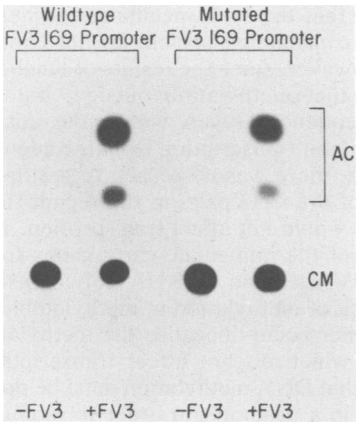

FIG. 3. Results of CAT assays showing that the three changes made in the ICR 169 promoter by oligonucleotide-directed mutagenesis did not destroy promoter activity. FHM cells were transfected with either wild-type mp18-169P*cat* or mp18-169P*cat* containing the mutated ICR 169 promoter. After 24 h, one dish from each set was infected with 10 to 20 PFU of FV3 per cell. Four hours later, cytoplasmic extracts were prepared and assayed for CAT activity. This experiment was typical of several assays, all of which were taken for thin-layer chromatography during the linear portion of the reaction; the amount of CAT activity induced from the mutated promoter varied from 85 to 100% of that induced from the wild-type promoter. The degree of CAT activity was proportional to the amount of [^{14}C]chloramphenicol converted to acetylated forms. Abbreviations: CM, unacetylated chloramphenicol; AC, acetylated chloramphenicol.

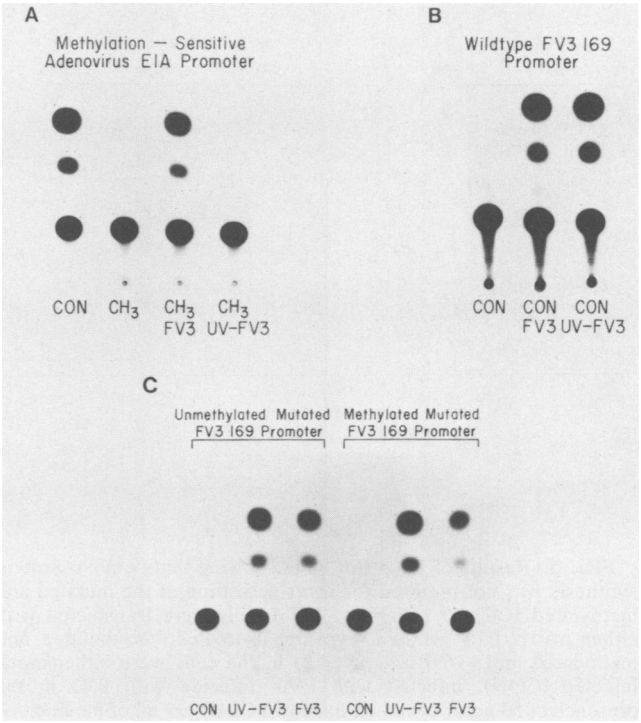

FIG. 4. Results of CAT assays showing that the methylated ICR 169 promoter could be *trans* activated by UV-inactivated FV3. Virus suspensions were inactivated as described in the text. FHM cells were transfected with either unmethylated pAd12-E1a *cat*, methylated pAd12-E1a *cat*, wild-type mp18-169P*cat*, unmethylated mp18-169P*cat* containing the mutated ICR 169 promoter, or methylated mp18-169P*cat* containing the mutated ICR 169 promoter. After 24 h, replicate cultures were mock infected (CON), infected with FV3, or treated with UV-inactivated FV3. Cell extracts were prepared and assayed for CAT activity.

To test the effect of methylation on transcription from the ICR 169 promoter, we had to supply the virion-associated protein(s) needed to turn on the promoter without inducing the protein that overrides inhibitory methylation. In this way, we could determine whether the methylation in the ICR 169 promoter was inhibitory to transcription. To provide these conditions, we treated transfected cells with UV-inactivated FV3.

An earlier study had shown that some viral protein synthesis continues to occur in cells treated with UV-inactivated FV3 (14). To determine whether UV-inactivated FV3 would provide virion proteins without inducing the protein required to transcribe methylated DNA, we tested UV-inactivated FV3 for its ability to *trans* activate the wild-type ICR 169 promoter and the methylation-sensitive adenovirus E1a promoter (13, 24). For the CAT assay, FHM cells were transfected as described in Materials and Methods with either mp18-169P*cat* or pAd12-E1a *cat*. In the case of pAd12-E1a *cat*, cells were transfected with either unmethylated or methylated plasmid DNA. The unmethylated pAd12-E1a *cat* construct was readily expressed in transfected cells, whereas the methylated plasmid DNA in mock-infected cells was essentially inactive (Fig. 4A). Cells transfected with the methylated pAd12-E1a *cat* construct and subsequently infected with FV3 produced an abundant amount of CAT. In contrast, treatment of similarly transfected cells with UV-inactivated FV3 did not induce transcription of the *cat* gene.

Cells transfected with mp18-169P*cat* alone did not synthesize CAT. When similarly transfected cells were infected with FV3 or treated with UV-inactivated FV3, an abundant amount of CAT was synthesized (Fig. 4B). These results demonstrate that UV-inactivated FV3 can be used to provide virion-associated proteins without inducing the protein needed to override inhibitory methylation.

Next, we tested the ability of UV-inactivated FV3 to *trans* activate the methylated ICR 169 promoter. The plasmid construct containing the mutated ICR 169 promoter was methylated with *Hpa*II and *Hha*I methylases and transfected into FHM cells. The cells were then mock infected, infected with active FV3, or treated with UV-inactivated FV3. As expected, the mock-infected cells did not produce CAT, whereas the cells infected with FV3 synthesized CAT (Fig. 4C). The cells transfected with methylated mp18-169P*cat* and treated with UV-inactivated FV3 also synthesized CAT. Southern blots of methylated plasmid DNA extracted from the methylated, mutated m18-p169P*cat*-transfected, FV3-infected cells and probed with radioactive ICR 169 promoter demonstrated that no detectable demethylation took place (results not shown), consistent with our earlier findings that the methylated adenovirus promoters were not demethylated after infection with active or UV-inactivated FV3 (24). These results show that methylation of the ICR 169 promoter at the three CpG dinucleotides that were methylated in virion DNA did not inhibit the ability of the promoter to initiate transcription of the *cat* gene.

Protein synthesis inhibitors do not block *trans* activation of the methylated ICR 169 promoter by FV3. We also tested the transcriptional activity of the methylated ICR 169 promoter by infecting transfected cells in the absence of de novo protein synthesis and measuring the production of CAT-specific RNA. In contrast to many other virus-cell systems,

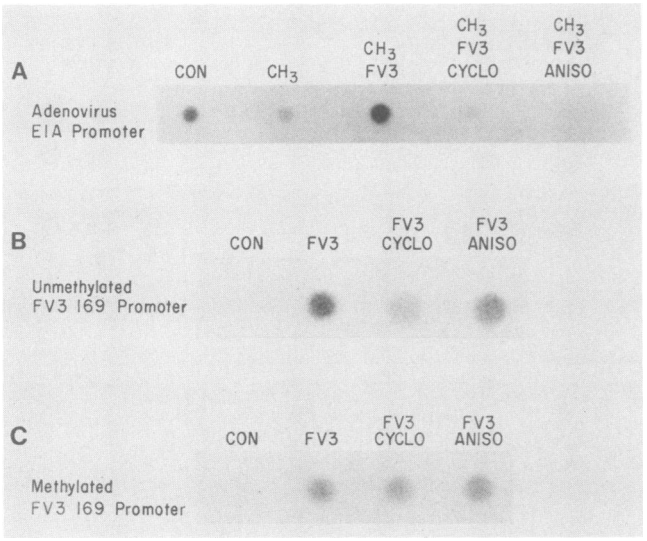

FIG. 5. Results of RNA dot blots showing that de novo protein synthesis was not required for *trans* activation of the mutated and methylated ICR 169 promoter. FHM cells were transfected with either pAd12-E1a *cat*, wild-type mp18-169P*cat*, or mutated and methylated mp18-169P*cat*. After 24 h, the cells were either mock infected (CON), infected with FV3, infected with FV3 in the presence of 50 μg of cycloheximide (CYCLO) per ml of medium, or infected with FV3 in the presence of 100 μM anisomycin (ANISO). Total RNA was extracted after 4 h of infection. Ten micrograms of RNA was spotted onto a nylon membrane and hybridized overnight to a ^{32}P-labeled *cat*-specific probe. RNA with *cat*-specific sequences was visualized by autoradiography.

protein synthesis is irreversibly inhibited by >99% by cycloheximide and anisomycin in FV3-infected FHM cells (25). Treatment with cycloheximide or anisomycin completely abolished transcription of the methylated adenovirus E1a promoter in FV3-infected cells (Fig. 5A). The presence of these drugs did not inhibit transcription from the unmethylated ICR 169 promoter in infected cells (Fig. 5B). Transcription from the *cat* gene also continued in the presence of cycloheximide and anisomycin in infected cells that had been transfected with the methylated mp18-169P*cat* construct (Fig. 5C). These results show that de novo protein synthesis was not required for *trans* activation of the methylated ICR 169 promoter, thereby confirming that the virus-induced protein that allowed transcription from promoters inactivated by methylation was not required for the transcription of the methylated ICR 169 promoter. These results support the conclusion that the methylation found within the ICR 169 promoter in virion DNA does not inhibit transcription by RNA polymerase II.

DISCUSSION

We previously showed that a protein induced during FV3 infection is capable of overriding the inhibitory effect of methylation on transcription from adenovirus promoters (24). However, the immediate-early FV3 genes are transcribed in the absence of de novo protein synthesis (25), indicating that this subset of genes does not require this virus-induced protein. We show here that although at least one of the immediate-early FV3 genes is methylated at sites within the promoter region, the methylation found within this promoter is not inhibitory to transcription. This finding substantiates those of other investigations from this laboratory showing that the FV3-specific sequences that respond to the virion *trans*-acting protein do not contain CpG doublets (26). However, since the results of Knebel and Doerfler (12) showed that methylation outside, but close to, *cis*-responsive sequences (even within the adjacent plasmid vector) can inhibit transcription from the adenovirus type 12 E1a promoter, there was no reason to assume a priori that methylation of the CpG pairs in the region surrounding the 169 promoter would not affect transcription. It appears that transcription of the immediate-early genes from the highly methylated FV3 genome by RNA polymerase II is not the result of a lack of methylation at methylatable sites in these genes but rather occurs because the methylation is at noncritical sites which do not affect transcription. It is well documented that DNA methylation must be present at highly specific sites in a promoter in order to modulate transcriptional activity (12). Site-specific promoter methylations are thought to interfere with promoter activities by affecting specific DNA-protein interactions (4). At this time, no pattern correlating methylation of specific regions of a promoter to the effect on promoter activity has been established. Knebel and Doerfler (12) have postulated that each promoter may have its individual set of methylation-specific sites that can affect promoter function and that the location of these sites may depend on the three-dimensional structure of a promoter. If this is the case, it appears that the conformational structure of the immediate-early FV3 promoters is such that the methylation found within them does not modulate promoter activity.

In all probability, the *trans*-acting virus-induced protein that allows transcription from promoters that are inhibited by methylation is a product of one of the immediate-early genes. Only after expression of this protein would promoters that are inhibited by methylation become transcriptionally active. Thus, the only promoters that could be inhibited by methylation are those of delayed-early and late genes.

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