Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 7688–7692, October 1986
Biochemistry

Trans-activation of a methylated adenovirus promoter by a frog virus 3 protein

(DNA-mediated gene transfer/chloramphenicol acetyltransferase/transcriptional initiation sites/gene regulation/DNA methylation)

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Communicated by David M. Prescott, July 7, 1986

ABSTRACT **The high degree of methylation of the frog virus 3 (FV3) genome suggests that FV3-infected cells are capable of transcribing highly methylated DNA. We tested this hypothesis by assaying the transcriptional activity of adenovirus promoters known to be inhibited by methylation. Plasmid constructs containing the E1a and E2aE promoters of adenovirus type 12 linked to the gene for chloramphenicol acetyltransferase [(CAT) EC 2.3.1.28], when methylated and introduced into eukaryotic cells, promoted CAT synthesis only when the cells were subsequently infected with FV3. Mapping of transcriptional initiation sites revealed that the same sites in the E1a promoter were used for the initiation of transcription in uninfected and infected cells. Moreover, Southern blots showed that transfected plasmid DNA from FV3-infected cells was not demethylated. The absence of CAT-specific RNA in transfected cells infected with FV3 in the presence of protein synthesis inhibitors demonstrated that a virus-induced protein was responsible for the trans-activation. Inhibition of transcription from the methylated template by α-amanitin indicated that a functional host RNA polymerase II is required for transcription of methylated DNA in FV3-infected cells. The virus-induced trans-acting protein presumably alters either host RNA polymerase II or the methylated DNA template to allow transcription from the methylated adenovirus promoters.**

The role of DNA methylation in the regulation of eukaryotic gene expression has received considerable attention over the past several years (1–4). Numerous examples indicate that there is an inverse correlation between the extent of DNA methylation and the level of gene expression; hypermethylation of the promoter region of a gene may lead to gene inactivation, whereas hypomethylation of this region is associated with transcriptional activity (5–8). DNA methylation is believed to be involved in the long-term inactivation of genes (9). No active demethylating mechanism has been observed (9), and no mechanism has been described for the transcription of eukaryotic DNA inactivated by methylation.

The DNA of the iridovirus frog virus 3 (FV3) is the most highly methylated of all known animal viruses, with ≈20% of the cytosine residues methylated by a virus-encoded DNA methyltransferase (10, 11). This high degree of methylation and the fact that the virus uses host RNA polymerase II for at least the immediate-early stage of transcription (12) suggest that RNA polymerase II of FV3-infected cells is capable of transcribing highly methylated DNA.

We now present evidence that (*i*) a FV3-specific protein induces transcription from a promoter inactivated by methylation and that (*ii*) the transcriptional initiation sites for RNA synthesis from the methylated promoter are the same as those utilized by the unmethylated promoter in uninfected cells. Therefore, FV3-infected cells are capable of overriding the inhibitory effect of methylation on transcription. The data presented not only are significant for gaining an understanding of the control of FV3 gene expression but also may extend to eukaryotic gene expression in general.

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's minimal essential medium containing 5% fetal calf serum. HeLa cells were maintained at 37°C in Eagle's minimal essential medium containing 5% fetal calf serum. Wild-type α-amanitin-sensitive Chinese hamster ovary (CHO) cells and a mutant CHO line with a well-characterized α-amanitin-resistant RNA polymerase II (13) were maintained as monolayers at 37°C in α-medium with 10% fetal calf serum. A clonal isolate of FV3 was used to prepare virus stocks at 25°C; virus was harvested and plaque-assayed as described (14). Adenovirus type 5 (Ad5) was grown and purified as described (15).

Transfection of Eukaryotic Cells. Published procedures for transfection of eukaryotic cells were followed (16). Four hours after transfection, cells were subjected to a 2-min 10% glycerol shock (17) and incubated for an additional 18 hr at their optimum temperature (33°C for FHM, 37°C for mammalian cells) before infection with FV3 or Ad5. Cells were mock-infected or infected with virus [10–20 plaque-forming units (pfu) per cell for FV3, 10 pfu per cell for Ad5] and incubated (4 hr for FV3, 10 hr for Ad5) at the optimum temperature for virus replication (30°C for FV3, 37°C for Ad5). Transfected cells were harvested for chloramphenicol acetyltransferase [(CAT) EC 2.3.1.28] assay or nucleic acid purification as described below.

Assay for CAT. Cell extracts were assayed for CAT by the method of Gorman *et al.* (18) with 0.2 μCi (1 Ci = 37 GBq) of [^{14}C]chloramphenicol per reaction.

Plasmids. The plasmid pAd12-1a CAT contains the left-terminal 525 base pairs (bp) of adenovirus type 12 (Ad12) DNA linked to the bacterial gene for CAT in a pBR322 vector (19). This Ad12 fragment contains the E1a promoter with two TATA motifs starting at bp 276 and 414 of this fragment. This promoter carries two *Hpa* II sites and three *Hha* I sites upstream from the leftmost TATA signal. pSVO CAT is identical to pAd12-1a CAT but without the E1a promoter.

The plasmid pEC-113 contains a *Sau*3A fragment of Ad5 linked to the CAT gene (20). This fragment contains the E2aE promoter element of Ad5. Numerous *Hpa* II and *Hha* I sites are found in the 100-bp region immediately upstream of the major transcriptional start site of this promoter.

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Abbreviations: Ad2, -5, and -12, adenovirus types 2, 5, and 12; CAT, chloramphenicol acetyltransferase; FV3, frog virus 3; FHM, fathead minnow; CHO, Chinese hamster ovary; bp, base pair(s); pfu, plaque-forming units.

***In Vitro* Methylation of the pAd12-1a CAT or pEC-113 Construct.** The bacterial methyltransferases *Hpa* II and *Hha* I were used to methylate the constructs. *Hpa* II and *Hha* I methylated the internal cytidine residues of the sequences 5′ CCGG 3′ and 5′ GCGC 3′, respectively. Plasmid DNA was incubated with 0.5 unit of *Hpa* II methyltransferase and *Hha* I methyltransferase per μg of DNA in 50 mM Tris·HCl, pH 7.5/10 mM EDTA/80 μM *S*-adenosylmethionine/5 mM 2-mercaptoethanol. All methylation reaction mixtures were incubated at 37°C for 48 hr to ensure complete methylation. To test methylated samples for complete methylation at 5′ CCGG 3′ and 5′ GCGC 3′ sites, 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 utilized in transfection experiments.

Analysis of the Methylation State of Transfected DNA. HeLa cells were transfected with either unmethylated or methylated pAd12-1a CAT as described above. After transfection, half of the cultures transfected with methylated plasmid were infected with FV3. Four hours after FV3 infection, DNA was extracted from all cultures by using the Hirt procedure (21). The DNA was restricted with either *Hpa* II or *Msp* I, electrophoresed on a 1.2% agarose gel, and Southern blotted to a nylon membrane (22). A ^{32}P-labeled probe specific for the E1a promoter of Ad12 was used to visualize the restriction pattern by autoradiography.

Isolation of RNA and Dot-Blot Analysis. Extraction of total RNA was accomplished following the procedure of Chirgwin *et al.* (23). 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.

Analysis of Transcriptional Initiation Sites of CAT Transcripts in Transfected Cells by Primer Extension. Total RNA was extracted from transfected cells as described above. The poly(A)$^+$ fraction of the RNA was selected using Hybond mAP paper (Amersham). A single-stranded oligonucleotide of sequence 5′ ATGAG CTGGC AGAGA 3′ was synthesized with an Applied Biosystems (Foster City, CA) apparatus and purified by polyacrylamide gel electrophoresis. This oligonucleotide, complementary to nucleotides 472–486 of the E1a promoter (24), was end-labeled by using [^{32}P]ATP (7000 Ci/mmol) and T4 polynucleotide kinase. Cloned Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), the labeled primer, and poly(A)$^+$ RNA were used to generate primer extension products following the protocol provided with the enzyme. After extension, the samples were brought to 200 μl with 10 mM Tris·HCl, pH 8.0/0.1 M NaCl/1 mM EDTA, extracted with phenol/chloroform, and analyzed by electrophoresis on a 6% polyacrylamide/urea gel, followed by autoradiography.

RESULTS AND DISCUSSION

FV3-Infected Cells Can Transcribe Methylated DNA That Is Inactive in Uninfected Cells. To determine if transcription of methylated DNA occurred in FV3-infected cells, we used two adenovirus promoters (E1a and E2aE) known to be suppressed by methylation (6, 19). The E1a promoter from Ad12 contains two *Hpa* II and three *Hha* I sites upstream from a pair of TATA signals (19). Kruczek and Doerfler have shown that methylation of these sites *in vitro* results in inactivation of the promoter (19). Moreover, a cell line transformed by the cloned left terminus of Ad5 DNA that carries the E1a region in a highly methylated state expresses viral genes at a reduced rate (19). These results indicate that information obtained from *in vitro* methylation studies with this promoter may apply to *in vivo* methylation as well.

In the present study, a plasmid construct (pAd12-1a CAT) that contains the E1a promoter linked to the CAT gene was used to measure transcriptional activity (18). This plasmid was methylated with the bacterial methylases *Hpa* II and *Hha* I and introduced into HeLa cells by DNA-mediated gene transfer. After 18 hr, cells were mock-infected or infected with FV3 or Ad5 and the CAT activity of cell extracts was assayed 4 hr (FV3) or 10 hr (Ad5) after infection. The results are shown in Fig. 1. The unmethylated pAd12-1a CAT construct was readily expressed in transfected cells (lane 1), whereas the methylated plasmid DNA in the mock-infected cells was essentially inactive (reduction by a factor of >10) (lane 2). Cells transfected with methylated plasmid DNA and subsequently infected with FV3 produced an abundant amount of CAT (lane 3). In contrast, Ad5 infection of similarly transfected cells did not induce transcription from the methylated E1a promoter (lane 4).

To demonstrate that the effect was not cell specific, other cell types were tested in the CAT assay. Trans-activation was also found to occur in FHM cells and CHO cells (data not shown). Similarly, the effect was not promoter specific, as a plasmid construct containing the E2aE promoter of Ad5 linked to the CAT gene gave results identical to those obtained with pAd12-1a CAT (Fig. 1, lanes 5–8).

There are several explanations that may account for the transcription of the methylated constructs in FV3-infected cells. One could involve the use of a cryptic promoter on the plasmid, devoid of *Hpa* II and *Hha* I sites, to initiate transcription. An examination of this possibility is paramount in light of the recent finding that the E1 region of adenovirus trans-activates prokaryotic promoter-like sequences in pBR322 (25). If an FV3 protein functioned in a similar manner, the transcription observed from the methylated constructs could be due to the activation of these pBR322 sequences rather than an actual override of the inhibitory effect of methylation on the adenovirus promoters. As numerous studies have shown that only methylation at critical sites within the promoter region of a gene inhibits transcription (7, 8, 19, 26–30), initiation of transcription at an

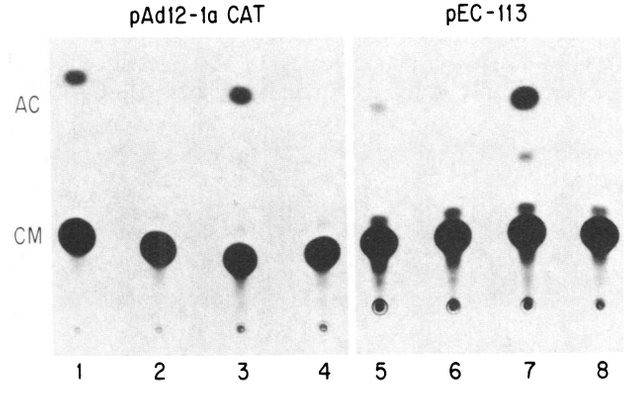

FIG. 1. FV3 induces transcription from adenovirus E1a and E2aE promoters inactivated by methylation. HeLa cells were transfected with either unmethylated or methylated plasmid constructs containing the E1a (pAd12-1a CAT) or E2aE (pEC-113) promoters linked to the CAT gene. After 18 hr, transfected cells were either mock-infected, infected with FV3, or infected with Ad5. The cells were incubated at 30°C (FV3-infected cells) or 37°C (Ad5-infected cells) and collected for CAT assay 4 hr (FV3-infected cells) or 10 hr (Ad5-infected cells) after infection. The presence of acetylated derivatives of chloramphenicol in the enzyme assay indicates transcriptional activity from the adenovirus promoter. Lane 1, unmethylated pAd12-1a CAT (uninfected); lane 2, methylated pAd12-1a CAT (uninfected); lane 3, methylated pAd12-1a CAT (FV3-infected); lane 4, methylated pAd12-1a CAT (Ad5-infected); lane 5, unmethylated pEC-113 (uninfected); lane 6, methylated pEC-113 (uninfected); lane 7, methylated pEC-113 (FV3-infected); and lane 8, methylated pEC-113 (Ad5-infected). CM = chloramphenicol; AC = acetylated derivatives of chloramphenicol.

unmethylated site on the constructs would allow expression of the CAT gene. We tested this possibility by analyzing the effect of FV3 infection on a plasmid devoid of eukaryotic promoter sequences and by directly analyzing the initiation sites used in FV3-infected cells.

pSVO CAT Is Not Trans-Activated in FV3-Infected Cells. To determine if initiation of transcription was occurring within the methylated E1a promoter and not at a cryptic promoter on the plasmid devoid of *Hpa* II and *Hha* I recognition sequences, we tested an unmethylated plasmid (pSVO CAT) identical to pAd12-1a CAT but without the E1a promoter (18, 19). This plasmid, which lacks eukaryotic promoter sequences, is able to express the CAT gene upon transfection into human or hamster cells that harbor and constitutively express the E1 region of Ad2 or Ad5 DNA (25). We found, however, that the FV3-infected cells were unable to express the CAT gene from pSVO CAT (Fig. 2). These results indicate that the trans-activation of methylated DNA by FV3 occurs via a mechanism different from that of the E1 products of adenovirus, in which prokaryotic promoter-like sequences within pBR322 are utilized to initiate transcription.

Transcriptional Initiation Sites for the E1a-CAT Construct Are Identical in Infected and Uninfected Cells. To determine if there were any differences in transcriptional initiation sites between uninfected and infected cells, we analyzed the CAT transcripts in cells transfected with pAd12-1a CAT by primer extension. Primer extension analysis of RNA from cells transfected with unmethylated pAd12-1a CAT and from cells transfected with methylated pAd12-1a CAT and subsequently infected with FV3 revealed that the same sites were utilized for initiation of transcription in infected cells as in uninfected cells (Fig. 3). The two smaller primer extension products correspond to transcriptional initiation downstream from the two TATA motifs present in the E1a promoter. These sites are readily utilized in the FV3-infected cells transcribing methylated DNA. In addition to these sites, there are upstream start sites that are utilized in uninfected and infected cells. Evidence for minor upstream start sites has been obtained (31–34). The finding that the same start sites are utilized in uninfected and FV3-infected cells indicates that transcription from the methylated E1a-CAT construct in FV3-infected cells involves overriding the inhibitory effect of methylation on the promoter.

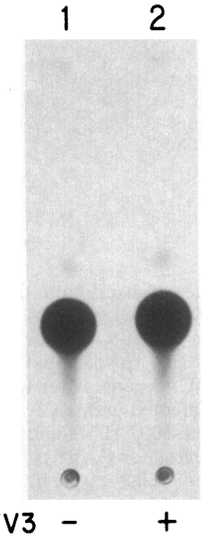

FIG. 2. FV3-infected cells do not utilize a cryptic promoter on the plasmid to initiate transcription. HeLa cells were transfected with the unmethylated plasmid pSVO CAT and half were subsequently infected with FV3. Cells were collected for CAT assay 4 hr after infection with FV3. Lane 1, pSVO CAT (uninfected); and lane 2, pSVO CAT (FV3-infected).

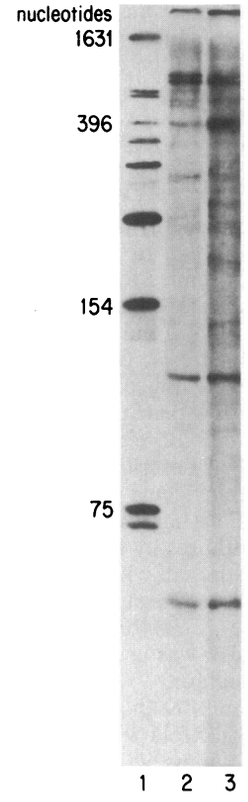

FIG. 3. Transcripts from methylated template utilize the same sites for initiation of transcription as transcripts from unmethylated template. FHM cells were transfected with unmethylated or methylated pAd12-1a CAT. Cells transfected with methylated plasmid DNA were subsequently infected with FV3. Four hours after infection, total RNA was extracted. The poly(A)$^+$ portion of the RNA was selected and analyzed by primer extension. Lane 1, marker pBR322 cut with *Hin*fI; lane 2, primer extension products from RNA extracted from cells transfected with unmethylated plasmid DNA; and lane 3, primer extension products from RNA extracted from cells transfected with methylated plasmid DNA and subsequently infected with FV3.

Demethylation of Template Cannot Account for Transcription of Methylated DNA. One way by which the inhibitory effect of methylation could be overcome is demethylation of the template prior to transcription. The methylation state of the transfected DNA from infected cells was examined by restriction analysis to determine if demethylation of template was occurring. Transfected DNA was extracted from cells, subjected to the isoschizomeric restriction enzymes *Hpa* II and *Msp* I (35), and analyzed by Southern hybridization (22). Whereas *Msp* I will cleave at the recognition sequence 5′ CCGG 3′ regardless of the methylation state of the internal cytidine residue, DNA methylated at this cytidine is refractory to cleavage by *Hpa* II. Fig. 4 is the Southern blot of DNAs from cells transfected with unmethylated and methylated plasmid DNA and analyzed with a probe specific for the E1a promoter. It shows that unmethylated plasmid DNA is cut by both restriction enzymes (lanes 6 and 7), whereas methylated plasmid DNA from uninfected cells is cut only by *Msp* I (lanes 8 and 9). Fig. 4 also shows that transfected methylated plasmid DNA from FV3-infected cells is completely refractory to cleavage by *Hpa* II (lane 10), indicating that the methylation state of transfected DNA is not altered by FV3 infection. Thus, demethylation cannot account for the induction of transcription from the methylated E1a promoter.

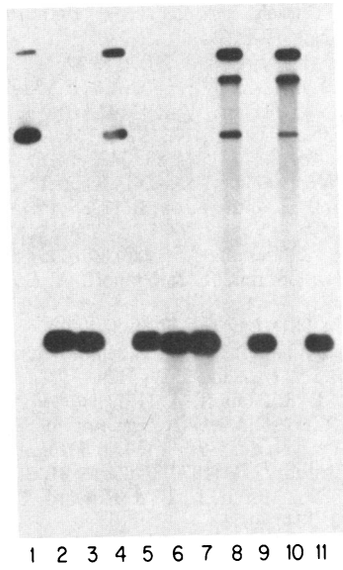

FIG. 4. Infection with FV3 does not alter the methylation state of transfected DNA. HeLa cells were transfected with either unmethylated or methylated pAd12-1a CAT. After transfection, half of the methylated plasmid-containing cultures were infected with FV3. Four hours after FV3 infection the transfected DNA was extracted from all cultures using the Hirt procedure (21). The DNA was digested with either *Hpa* II or *Msp* I, electrophoresed on a 1.2% agarose gel, and Southern blotted to a nylon membrane (22). A ^{32}P-labeled probe specific for the E1a promoter was used to visualize the restriction pattern by autoradiography. Lane 1, uncut pAd12-1a CAT DNA; lane 2, *Hpa* II-cut pAd12-1a CAT DNA; lane 3, *Msp* I-cut pAd12-1a CAT DNA; lane 4, *Hpa* II-cut methylated pAd12-1a CAT DNA; lane 5, *Msp* I-cut methylated pAd12-1a CAT DNA; lane 6, unmethylated transfected DNA cut with *Hpa* II; lane 7, unmethylated transfected DNA cut with *Msp* I; lane 8, methylated transfected DNA cut with *Hpa* II; lane 9, methylated transfected DNA cut with *Msp* I; lane 10, methylated transfected DNA from FV3-infected cells cut with *Hpa* II; and lane 11, methylated transfected DNA from FV3-infected cells cut with *Msp* I.

Competition for a Repressor That Binds to Methylated DNA Cannot Account for Transcription from the Methylated Adenovirus Promoters. One mechanism by which methylation of DNA could inhibit transcription is by binding of a repressor that prevents transcription. If this were the case, then the excess of highly methylated DNA introduced by FV3 infection would compete with the methylated plasmid constructs for the repressor, thereby allowing transcription of the methylated template. We tested this possibility by analyzing the ability of a FV3 mutant that has unmethylated DNA (11) to induce transcription from the methylated adenovirus promoters. We found that the mutant virus also was able to induce transcription from the methylated plasmid constructs (data not shown). This result shows that competition for a repressor protein that binds to methylated DNA is not the mechanism whereby methylated DNA is transcribed in FV3-infected cells.

A Virus-Induced Protein Is Required for Transcription of Methylated DNA. To determine if the trans-acting protein responsible for induction of methylated DNA is virion-associated or virus-induced, we tested the ability of FV3-infected cells to induce transcription of methylated DNA in the presence of protein synthesis inhibitors. In contrast to many other virus–cell systems, protein synthesis is irreversibly inhibited >99% by cycloheximide and anisomycin in FV3-infected FHM cells (36). Treatment with cycloheximide or anisomycin completely abolished transcription of methylated DNA in FV3-infected cells (Fig. 5). These results showed that *de novo* protein synthesis is required for trans-activation of methylated template, thereby indicating that the protein responsible for trans-activation of methylated DNA is virus-induced.

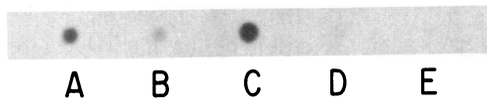

FIG. 5. *De novo* protein synthesis is required in infected cells for trans-activation of the methylated E1a promoter. FHM cells were transfected with unmethylated pAd12-1a CAT (A) or methylated pAd12-1a CAT (B–E); 16 hr later the cells were mock-infected (B), infected with FV3 (C), infected with FV3 in the presence of 50 μg of cycloheximide per ml (D), or infected with FV3 in the presence of 100 μM anisomycin (E). Total RNA was extracted after 4 hr of incubation at 30°C. Ten micrograms of RNA was spotted onto a nylon membrane (New England Nuclear) and hybridized overnight to a ^{32}P-labeled CAT-specific probe following the directions provided by the manufacturer. RNA with CAT-specific sequences was visualized by autoradiography.

As shown in Fig. 5, the cells transfected with methylated pAd12-1a CAT did contain some CAT-specific RNA. The amount of RNA in these cells, however, was greatly reduced compared to that of the cells transfected with unmethylated plasmid DNA. This finding is consistent with the data from our CAT assays, which reveal a low level of CAT activity in cells transfected with methylated DNA. Either the constructs were not completely methylated or the inhibitory effect of methylation on the promoters studied is not absolute.

Functional Host RNA Polymerase II Is Required for Trans-Activation of Methylated DNA. The fact that adenovirus and FV3 use host RNA polymerase II for transcription (12, 37, 38) suggests that this enzyme is responsible for transcription of the methylated template. The requirement for a functional host RNA polymerase II was tested by assaying CAT production in the presence of α-amanitin, an inhibitor of RNA polymerase II. As shown in Fig. 6, the presence of α-amanitin abolished CAT activity in α-amanitin-sensitive cells, whereas resistant cells were unaffected. One explana-

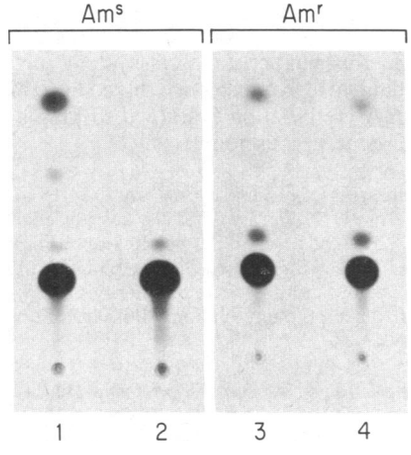

FIG. 6. Functional RNA polymerase II is required for induction of transcription from the methylated template by FV3. CAT assays were performed by using α-amanitin-sensitive (Am^s) and α-amanitin-resistant (Am^r) CHO cells transfected with methylated pAd12-1a CAT and infected with FV3. The α-amanitin-resistant cells have a well-characterized mutant RNA polymerase II (13). The two cell lines were transfected with methylated pAd12-1a CAT and incubated at 37°C for 18 hr. One-half of each set was treated with 5 μg of α-amanitin per ml and after 6 hr all cultures were infected with 20 pfu of FV3 per cell, incubated at 30°C, and assayed for CAT activity after 4 hr. Lane 1, α-amanitin-sensitive cells infected with FV3; lane 2, α-amanitin-sensitive cells pretreated with 5 μg of α-amanitin per ml and infected with FV3; lane 3, α-amanitin-resistant cells infected with FV3; and lane 4, α-amanitin-resistant cells pretreated with 5 μg of α-amanitin per ml and infected with FV3.

tion for this result is that host RNA polymerase II is responsible for transcription of the methylated template. Alternatively, α-amanitin could act indirectly by inhibiting transcription of the gene that encodes the trans-acting protein required for transcription of methylated DNA.

If RNA polymerase II of the host cell is responsible for transcription of the methylated template, the trans-acting protein could act in one of two ways. Either the DNA template itself could be altered by the trans-acting protein to allow transcription or the host polymerase could be modified to enable it to transcribe methylated DNA. A previous study has shown that RNA polymerase II in FV3-infected cells is modified (39). Although this investigation did not address the functional significance of this modification, the fact that such a modification occurs in FV3-infected cells suggests that this is the mechanism whereby methylated DNA is transcribed.

CONCLUSIONS

The iridovirus FV3 has evolved a mechanism for transcription of methylated DNA, necessary because of its highly methylated genome. This mechanism involves a direct override of the inhibitory effect of methylation on transcription. The available evidence suggests that RNA polymerase II is modified in FV3-infected cells to allow transcription of methylated DNA. The protein that allows transcription of methylated template by RNA polymerase II is virus-induced.

The fact that the trans-acting protein responsible for transcription of methylated DNA is not a structural component of the virion suggests that the earliest genes transcribed in the FV3-infected cell contain unmethylated promoters. Since it appears that every dCdG dinucleotide in the FV3 genome is methylated (10), the promoters of the earliest genes transcribed must be devoid of this sequence or contain this sequence only at sites not critical for transcription. Only after expression of the protein that allows transcription of methylated DNA would any genes with methylated promoters be transcribed. Thus, the transcription of the gene whose product allows transcription of methylated DNA could be one form of control in the regulation of gene expression in FV3.

The evolution of a mechanism for transcription of methylated DNA in this eukaryotic viral system suggests that a similar mechanism also may have evolved in cellular systems in which DNA methylation is known to play a role in the regulation of gene expression.

The generous gifts of plasmids pAd12-1a CAT from W. Doerfler and pEC-113 from J. Nevins are gratefully acknowledged. This work was supported by Public Health Service Research Project Grant CA 07055, Cancer Center Support (CORE) Grant CA 21765, and the American Lebanese Syrian Associated Charities (ALSAC). J.P.T. is a recipient of Cancer Biology National Research Service Award 2 T32 CA09346-06.

1. Razin, A. & Riggs, A. D. (1980) *Science* **210,** 604–610.
2. Doerfler, W. (1983) *Annu. Rev. Biochem.* **52,** 93–124.
3. Riggs, A. D. & Jones, P. A. (1983) *Adv. Cancer Res.* **40,** 1–30.
4. Doerfler, W. (1984) *Curr. Top. Microbiol. Immunol.* **108,** 79–98.
5. Keshet, I., Yisraeli, J. & Cedar, H. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2560–2564.
6. Langner, K.-D., Vardimon, L., Renz, D. & Doerfler, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2950–2954.
7. Kruczek, I. & Doerfler, W. (1982) *EMBO J.* **1,** 409–414.
8. Stein, R., Sciaky-Gallili, N., Razin, A. & Cedar, H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2422–2426.
9. Doerfler, W., Langner, K.-D., Knebel, D., Weyer, U., Dobrzanski, P. & Knust-Kron, B. (1985) *Prog. Clin. Biol. Res.* **198,** 133–155.
10. Willis, D. B. & Granoff, A. (1980) *Virology* **107,** 250–257.
11. Willis, D. B., Goorha, R. & Granoff, A. (1984) *J. Virol.* **49,** 86–91.
12. Goorha, R. (1981) *J. Virol.* **37,** 496–499.
13. Chan, V. L., Whitmore, G. F. & Siminovitch, L. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 3119–3124.
14. Naegele, R. F. & Granoff, A. (1971) *Virology* **44,** 286–295.
15. Nevins, J. R. (1980) *Methods Enzymol.* **65,** 768–785.
16. Graham, F. & van der Eb, A. (1973) *Virology* **52,** 456–457.
17. Parker, B. & Stark, G. (1979) *J. Virol.* **31,** 360–369.
18. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051.
19. Kruczek, I. & Doerfler, W. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7586–7590.
20. Imperiale, M. J., Hart, R. P. & Nevins, J. R. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 381–385.
21. Hirt, B. (1967) *J. Mol. Biol.* **26,** 365–369.
22. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
23. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
24. Sugisaki, H., Sugimoto, K., Takanami, M., Shiroki, K., Saito, I., Shimojo, H., Sawada, Y., Uemizu, Y., Uesugi, S. & Fujinaga, K. (1980) *Cell* **20,** 777–786.
25. Langner, K.-D., Weyer, U. & Doerfler, W. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1598–1602.
26. Flavell, R. A., Grosveld, F., Busslinger, M., de Boer, E., Kioussis, D., Mellor, A. L., Golden, L., Weiss, E., Hurst, J., Bud, H., Bullmann, H., Simpson, E., James, R., Townsend, A. R. M., Taylor, P. M., Schmidt, W., Ferluga, J., Leben, L., Santamaria, M., Atfield, G. & Festenstein, H. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **47,** 1067–1078.
27. Ott, M.-O., Sperling, L., Cassio, D., Levilliers, J., Sala-Trepat, J. & Weiss, M. C. (1982) *Cell* **30,** 825–833.
28. La Volpe, A., Taggart, M., Macleod, D. & Bird, A. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **47,** 585–592.
29. Haigh, L. S., Owens, B. B., Hellewell, S. & Ingram, V. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5332–5336.
30. Wilks, A. F., Cozens, P. J., Mattaj, I. W. & Jost, J.-P. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4252–4255.
31. Berk, A. J., Lee, F., Harrison, T., Williams, J. & Sharp, P. A. (1979) *Cell* **17,** 935–944.
32. Sambrook, J., Greene, R., Stringer, J., Mitchison, T., Hu, S.-L. & Botchan, M. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **44,** 569–584.
33. Grodzicker, T. & Klessig, D. F. (1980) *Cell* **21,** 453–463.
34. Osborne, T. F., Gaynor, R. B. & Berk, A. J. (1982) *Cell* **29,** 139–148.
35. Waalwijk, C. & Flavell, R. A. (1978) *Nucleic Acids Res.* **5,** 3231–3236.
36. Willis, D. & Granoff, A. (1978) *Virology* **86,** 443–453.
37. Price, R. & Penman, S. (1972) *J. Virol.* **9,** 621–626.
38. Wallace, R. D. & Kates, J. (1972) *J. Virol.* **9,** 627–635.
39. Campadelli-Fiume, G., Costanzo, F., Foá-Tomasi, L. & La Placa, M. (1975) *J. Gen. Virol.* **27,** 391–394.