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EXPRESSION OF INTEGRATED AND METHYLATED HIV-LTR IN HUMAN T-CELLS AND MONKEY KIDNEY CELLS BY FROG VIRUS 3 INFECTION

by

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DNA methylation has been implicated in the suppression of transcription of a large spectrum of eukaryotic genes. Frog virus 3 (FV3) contains genomic DNA that is the most extensively methylated of all known animal viruses. However, FV3 gene expression is tightly regulated in a sequential fashion in infected cells. Therefore, FV3 must have evolved mechanism(s) to overcome the inhibitory effects of DNA methylation. FV3 has been shown to induce expression of methylated foreign genes in transient transfections. This study was designed to establish if this FV3 induced expression of methylated genes could be demonstrated in stable cell lines which contain integrated foreign genes that are silenced by DNA methylation. Stably transfected simian Vero and human T-cells containing a single copy of the methylated and transcriptionally suppressed HIV-LTR CAT construct were either infected with FV3 or fused with FV3-infected fat head minnow cells. The results from these experiments lead us to conclude that FV3 infection does promote expression of a foreign, stably integrated gene (HIV-LTR) which was previously silenced by DNA methylation.

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INTRODUCTION

Following careful transcriptional analyses of adenovirus promoters (Doerfler, 1981; Kruczek & Doerfler, 1982), it was evident that sequence-specific methylation functions as a negative regulatory signal in eukaryotic gene expression. More recent data continue to support the notion that DNA methylation is involved in the down-regulation of eukaryotic gene expression (Meehan, Lewis, Cross, Nan, Jeppesen & Bird, 1992; Razin & Cedar, 1991; Selker, 1990; Volpe, Iacovacci, Butler & Eremenko, 1993). The methylated DNA sequences assembled into condensed inert chromatin are usually not expressed, either *in vivo* or *in vitro* (Riggs & Pfeifer, 1992). Conversely, unmethylated genes remain at the periphery of chromatin and are thus accessible to all essential transcription related molecules required for gene expression (Keshet, Lieman-Hurwitz & Cedar, 1986). Exceptions to this general rule include the chicken lysozyme gene where no apparent relationship between DNA methylation and transcription has been reported (Wölfl, Schräder & Wittig, 1991). In some cases, methylation of the cytosine in the dinucleotide sequence CG in the promoter region, immediately upstream of the gene, has been shown to be the most important site for suppression of gene expression (Bird, 1986; Lindsay & Bird, 1987). Significant transcriptional inactivation by methylation of CG sites in the human T-cell leukemia virus type 1 LTR (Saggioro, Forino & Chieco-Bianchi, 1991), the promoter region of the human retinoblastoma gene (Ohtani-Fujita, Fujita, Aoike, Osifchin,

Robbins & Sakai, 1993) and the human papillomavirus upstream regulatory region (Rösl, Arab, Klevenz & zurHausen, 1993) has been demonstrated. Additional studies have shown that methylation of cytosines within the open reading frame (ORF) or 3' to the ORF has little effect on gene expression. For example, methylation of every CCGG sequence in the 3' region of the E2a (adenovirus type 2) gene had no effect on transcription whereas methylation of three CCGG sites at the 5' end of the E2a gene rendered it inactive (Langner, Vardimon, Renz & Doerfler, 1984). However, the precise molecular mechanisms governing DNA methylation-mediated suppression still remain poorly understood (Boyes & Bird, 1991; Selker, 1990).

Frog virus 3 (FV3) is an iridovirus and contains double-stranded, linear, circularly permuted and terminally redundant DNA of approximately 170 kilobase pairs as its genome (Kelly & Avery, 1974; Goorha & Murti, 1982). FV3 replicates in a wide variety of cell types providing the incubation temperature is held at 30°; infected cells incubated at 37° fail to yield any detectable infectious virions (reviewed in Thompson, Granoff & Willis, 1986). FV3 genomic DNA has been shown to be highly methylated. Over 20% of the total cytosines and every internal C in the tetranucleotide sequence CCGG in FV3 genomic DNA has been reported to be methylated as established by isoschizomeric *Hpa*II and *Msp*I restriction analyses (Schetter, Grünemann, Hölker & Doerfler, 1993; Willis & Granoff, 1980). Both of these restriction endonucleases recognize and cut at the tetranucleotide sequence CCGG. *Hpa*II does not cut DNA if the internal C is methylated whereas *Msp*I cuts

regardless of methylation status (Waalwijk & Flavell, 1978). Inasmuch as FV3 DNA is highly methylated and DNA methylation generally inhibits gene expression, existence of FV3 in nature is puzzling. These observations clearly suggest that FV3 must have evolved one or more mechanisms to overcome the inhibitory effects of DNA methylation and may serve as a unique and useful model to elucidate molecular mechanisms involved in DNA-mediated transcriptional suppression. Initial suggestion for this notion originated from transient transfection assays using plasmid DNA, where it has been shown that FV3 induced the expression of previously inactive, methylated genes (Thompson, Granoff & Willis, 1986). Transiently transfected Chinese hamster ovary (CHO) cells containing the E1a (adenovirus type 12 promoter) linked to the bacterial reporter gene, chloramphenicol acetyltransferase (CAT), were used to demonstrate FV3's ability to induce expression of a methylated gene (Thompson, Granoff & Willis, 1986). CAT expression in these studies was only detected in cells with unmethylated construct or when cells transfected with the methylated construct were infected with FV3 and incubated at 30° (Thompson, Granoff & Willis, 1986). In this study, we demonstrate that FV3 infection can overcome the inhibitory effect of DNA methylation on chromosomally integrated genetic elements which more accurately reflect the true physiological state of the cell's genetic architecture.

REVIEW OF LITERATURE

DNA Methylation

Recent data continue to support the notion that DNA methylation is involved in the down regulation of eukaryotic gene expression (Meehan, Lewis, Cross, Nan, Jeppesen & Bird, 1992; Razin & Cedar, 1991; Selker, 1990; Volpe, Iacovacci, Butler & Eremenko, 1993). Methylation of cytosine, predominately in the dincleotide CpG in promoter regions, has been shown to be one of the most important transcriptional elements in the regulation of eukaryotic gene expression (Deobagkar, Liebler, Graessmann & Graessmann, 1990; Langner, Vardimon, Renz & Doerfler, 1984; Levine, Cantoni & Razin, 1991; Lewis & Bird, 1991; Ohtani-Fujita, Fujita, Aoike, Osifchin, Robbins & Sakai, 1993). Methylation in other regions of DNA has not been demonstrated to inhibit transcription (Keshet, Yisraeli & Cedar, 1985; Lewis & Bird, 1991). The distribution of methylcytosines in eukaryotic genomes is not random (Selker, 1990). Certain regions, such as CpG-rich islands and housekeeping genes, have been shown to be unmethylated whereas other regions, such as tissue-specific genes, have been found to be methylated (Bird, 1986).

The discovery of the restriction enzymes *Hpa*II and *Msp*I has been very useful in the study of DNA methylation. These isoschizomeric enzymes recognize and cut the DNA at the tetranucleotide sequence CCGG; however, *Hpa*II does not cut when the internal cytosine is methylated and *Msp*I does (Waalwijk & Flavell, 1978). The

use of these specific restriction enzymes has allowed productive study of the methylation status and the inheritance of methylation patterns. Transfection and microinjection experiments have demonstrated that immediately after DNA replication, the daughter strand was not methylated, regardless of the methylation status of the parental strand (Jones & Taylor, 1980; Razin & Cedar, 1991). If the parental strand was not methylated, the daughter strand usually remained unmethylated as well. If the parental strand was methylated, however, the daughter strand became methylated by specific methyltransferases. This pattern of inheritance has been shown to be very stable (Jones & Taylor, 1980).

The hypothesis that methylation regulates gene activity has also been supported with use of the drug 5-azacytidine (5-azaC) (Bednarik, Cook & Pitha, 1990b; Bednarik, Mosca & Raj, 1987; Razin & Cedar, 1991). This cytosine analog binds irreversibly to DNA methyltransferase and prevents methylation of the daughter strand after DNA replication (Jones & Taylor, 1980). Therefore, treatment of cells containing methylated DNA with 5-azaC for one or more generations results in a population of cells with unmethylated DNA. This unmethylated state is then stably maintained even after removal of the drug (Jones & Taylor, 1980). These 5-azaC treated cells are then able to express genes that were previously silent. Simian or murine cells containing a stably integrated and enzymatically methylated copy of HIV-LTR linked to CAT failed to express CAT (Bednarik, Mosca & Raj, 1987). Treatment of these cells with 5-azaC restored CAT activity.

DNA-Protein Interaction and Chromatin Structure

How does methylation inhibit transcription? Although the precise mechanisms of methylation-mediated transcriptional inactivity remain elusive, DNA-protein interactions have been shown to be involved (Boyes & Bird, 1992; Levine, Cantoni & Razin, 1991; Meehan, Lewis, Cross, Nan, Jeppesen & Bird, 1992). Two models have been proposed. First, the direct model proposes that specific transcription factors do not recognize the methylated bases and therefore do not bind. Second, the indirect model proposes that DNA binding proteins at the methylated sites render the DNA inaccessible to the transcriptional factors necessary for gene expression (Szyf, 1991). The methyl-CpG binding protein (MeCP1) has been shown to play a major role in supporting the indirect model (Boyes & Bird, 1991). Cells deficient in MeCP1 were shown to express genes previously silenced by DNA methylation *in vitro* as well as *in vivo*. The level of this expression was related to the amount of MeCP1 in cells. To further support the indirect model, competitive DNA was introduced that could bind MeCP1 and transcription of methylated genes occurred (Boyes & Bird, 1991). MeCP2 has been shown to be a second protein that binds to DNA containing methylated cytosine but differs from MeCP1 in that a single methylated CpG site is sufficient to promote binding; MeCP1 requires multiple methylated sites to bind (Nan, Meehan & Bird, 1993).

The density of methylation and CpG sites plays a role in level of gene expression (Boyes & Bird, 1992). Promoters with varying degrees of methylated sites

were used to measure the severity of repression. It has been previously demonstrated that low density of methylation inhibited expression in genes with weak promoters but not in genes with strong promoters (Boyes & Bird, 1992). Several genes with varying degrees of methylated CpG sites were studied. Results of the assays showed that sparsely methylated genes bound weakly to MeCP1 and were not expressed. Addition of an enhancer, which disrupted the unstable complex, overcame the effects of methylation and the genes were then expressed. With high density of methylated CpG sites, transcription was inhibited even in the presence of an enhancer (Boyes & Bird, 1992). This density dependent expression of methylated genes has been confirmed using HIV-LTR in combination with *tat*, a viral transacting factor (Bednarik, Cook & Pitha, 1990b; Gutekunst, Kashanchi, Brady & Bednarik, 1993).

DNA-protein interaction has also been shown to be responsible in part for chromatin structure. The conversion of chromatin into the tightly condensed form reflects transcriptional inactivity and can be distinguished under light and electron microscopes (Lewis & Bird, 1991). It has been suggested that DNA-protein interactions lead to the inability of the chromatin to obtain the active structure (Keshet, Lieman-Hurwitz & Cedar, 1986). The active form of chromatin is sensitive to the nuclease DNAaseI and conversely the inactive form is insensitive to DNAaseI. DNA modification has been thought to be responsible for the insensitivity to the nuclease, in other words, the methylated DNA becomes inaccessible in the chromatin (Keshet, Lieman-Hurwitz & Cedar, 1986).

Methylated and non-methylated thymidine kinase constructs were shown to be

expressed at the same level upon injection. After 8 hours, there was a sharp drop in the expression level of the methylated construct due to the assembly of the transfected DNA into chromatin. On the other hand, when the constructs were assembled in chromatin *in vitro* and then injected, gene expression was inhibited immediately (Buschhausen, Wittig, Graessmann & Graessmann, 1987). This and other studies (Deobagkar, Liebler, Graessmann & Graessmann, 1990; Keshet, Lieman-Hurwitz & Cedar, 1986; Lewis & Bird, 1991) further support the hypothesis that chromatin structure is important in the inhibition of gene expression.

DNA Methylation in Prokaryotes

The DNA of higher eukaryotes contains the modified base methylcytosine as well as the four unmodified bases. In lower eukaryotes and prokaryotes, methyladenine is found as well (Selker, 1990). Prokaryotes have been used as models to help understand the function of methylation in eukaryotes. However, there are several differences in methylation characteristics of eukaryotes and prokaryotes (Reviewed in Selker, 1990). For example, the methylation status at a specific site seems to be uniform throughout a population of eukaryotic cells whereas in prokaryotic cells, it is not uncommon to see differences at specific sites in a single population.

There has been evidence to suggest that the function of DNA methylation differs significantly in prokaryotes and eukaryotes (Doerfler, 1992; Reviewed in Selker, 1990). Protection of genomic DNA from endonuclease cleavage and DNA

repair appears to be the primary function of DNA methylation in prokaryotes. On the contary, DNA methylation plays an important role in the regulation of transcription in eukaryotes.

Frog Virus 3

General Characteristics

Frog virus 3 (FV3) has been classified in the genus *Ranavirus* of the family *Iridoviridae* and was first isolated from the leopard frog, *Rana pipiens* (Granoff, Came & Breeze, 1966). FV3 infects amphibians but replicates *in vitro* in a wide variety of cells including mammalian, piscine, amphibian and avian (Kelly & Robertson, 1973).

FV3 is a large, icosehadral virus falling in size between the pox and herpes virus groups and is similar to insect iridescent viruses in size and morphology (Houts, Gravell & Darlington, 1970). Beneath the icosahedral capsid is an inner lipid and protein membrane and a central core of DNA and proteins, as revealed by freeze-etching studies (Darcy-Tripier, Nermut, Braunwald & Williams, 1984). FV3 has been found to be ether sensitive (Kelly & Robertson, 1973). FV3 in crude suspension has been shown to be either enveloped or nonenveloped. Enveloped particles bud through the plasma membrane or cytoplasmic vacuoles whereas nonenveloped particles are released by cell lysis (Tripier-Darcy, Braunwald & Kirn, 1982). The envelope contains about 15% lipid (Kelly & Robertson, 1973). Both enveloped and nonenveloped

particles are infectious. The enveloped FV3 particle is 160-200 nm in diameter whereas nonenveloped particles measure 120-130 nm in diameter (Granoff, 1969).

The FV3 virion contains several enzymatic activities. Among them, nucleotide phosphohydrolase and endodeoxyribonuclease (pH 5.0) are located in FV3 cores and endoribonuclease and endodeoxyribonuclease (pH 7.5) are located in the outer capsid (Kang & McAuslan, 1972; Vilagines & McAuslan, 1971). These enzymes may be involved in DNA replication.

At least 12 virus specific proteins with affinity for DNA have been isolated from FV3 infected cells (Goorha, 1981a; Willis, Goorha & Chinchar, 1985). FHM cells were first treated with inactivated FV3 to inhibit host protein synthesis, infected with active FV3 and then the proteins were analyzed by DNA affinity chromatography. The function of these proteins has not been elucidated but DNA-dependent DNA polymerase and endonuclease activities were present in the proteins as well as in FV3 infected cells (Goorha, 1981a). One of the proteins isolated had much higher affinity for single-stranded DNA as opposed to double-stranded DNA. This property caused unwinding of the DNA and possible involvement in viral DNA replication has been suggested.

FV3 replicates at temperatures ranging from 12° to 31° with 30° being the optimum temperature (Goorha & Granoff, 1974b; Gravell & Granoff, 1970). At temperatures from 12° to 29°, the viral latent period is longer although high titers of infectious virus are obtained (Gravell & Granoff, 1970). Between 32° and 33°, viral

DNA and proteins are synthesized but DNA is not packaged and, therefore, no infectious virus particles are released (Goorha & Granoff, 1974b; Kucera, 1970). At 34-35°, early proteins are synthesized and initiation of DNA replication is blocked (Cordier, Tondre, Aubertin & Kirn, 1986). However, when initiation takes place at a lower temperature and then the temperature is shifted to 34°, replication of DNA takes place (Goorha & Granoff, 1974b). At 37°, immediate early proteins are the only viral proteins produced in FV3-infected cells (Cordier, Tondre, Aubertin & Kirn, 1986). The transition from immediate early to delayed early transcription is impaired at 37° (Lopez, Aubertin, Tondre & Kirn, 1986). These results suggest that one or more immediate early proteins involved in the synthesis of delayed early and late proteins is inactivated at 37° (Martin, Aubertin & Kirn, 1982).

The Genome

The genome of FV3 contains linear, double stranded DNA of 170 kilobase pairs (Murti, Goorha & Granoff, 1982) with a GC content of 56% (Kelly & Robertson, 1973). Restriction endonuclease and electron microscopic analyses have demonstrated that FV3 DNA has direct terminal repeats consisting of roughly 4% of the genome (Goorha & Murti, 1982). Complete denaturing of linear FV3 genomic DNA followed by slow renaturization resulted in circular molecules with two single stranded tails. These results show that the FV3 DNA is circularly permuted (Goorha & Murti, 1982). These two features of FV3 DNA are quite unique among animal

viruses. It has been shown that denatured FV3 DNA yields linear genome length single strands suggesting that FV3 genome does not have crosslinked termini as in poxvirus (Murti, Goorha & Granoff, 1982). Foldback structures are not formed upon reannealing suggesting that FV3 genome does not contain inverted repeats as in herpesvirus (Murti, Goorha & Granoff, 1982).

FV3 DNA is the most extensively methylated of any animal virus reported to date. Over 20% of the total cytosines, but not adenine, and every internal C in the sequence CCGG, has been demonstrated to be methylated as seen by isoschizomeric *Hpa*II and *Msp*I restriction analysis (Schetter, Grünemann, Hölker & Doerfler, 1993; Willis & Granoff, 1980). Indeed, the promoter region for an immediate early FV3 gene has been shown to be methylated at the three CG sequences contained in the promoter but transcription of this gene is not inhibited (Thompson, Granoff & Willis, 1988). Somehow, FV3 has evolved mechanism(s) to overcome the inhibitory effect of DNA methylation.

Methylation of FV3 DNA takes place within 1 hour of DNA synthesis by a virus-encoded DNA methylase and a 26,000 Da virus-specific protein has been implicated in this enzyme activity (Essani, Goorha & Granoff, 1987; Willis, Goorha & Granoff, 1984). Parental FV3 DNA remains methylated even at the time that late RNA is being transcribed (Murti, Goorha & Granoff, 1985). FV3 DNA replication occurs in two stages as described later (page 15). The first stage occurs in the nucleus and this newly synthesized viral DNA is not methylated (Goorha, 1982). The second stage occurs in the cytoplasm where FV3-encoded DNA methyltransferase is

synthesized and methylates the viral DNA (Goorha, 1982; Willis, Goorha & Granoff, 1984). However, recently, FV3-encoded DNA methylase has also been detected in the nucleus of infected cells at four, eleven and twenty hours post infection (Schetter, Grünemann, Hölker & Doerfler, 1993). This viral methyltransferase has been shown to be an early enzyme as methylation occurs in the absence of viral DNA replication. FV3 methyltransferase has been shown to be completely distinct from cellular maintenance methylase found in the nuclei of uninfected cells as demonstrated by substrate preferences of each enzyme (Willis, Goorha & Granoff, 1984). Maintenance methylase has affinity for double-stranded, hemimethylated DNA and works to conserve the methylation pattern established previously. Cellular methyltransferases prefer single-stranded DNA. FV3-induced methyltransferase, on the other hand, prefers double-stranded, unmethylated DNA (Willis, Goorha & Granoff, 1984).

FV3 DNA methylation may play a role in endonuclease protection (Goorha, Granoff, Willis & Murti, 1984). This has been demonstrated by infecting 5-azaC-treated cells with FV3 that resulted in a significant decrease in infectious virion production. Two possible explanations for this inhibition have been proposed. First, 5-azaC has a general inhibitory effect on viral DNA synthesis. However, this inhibition accounts for only a slight decrease in infectious virus production. The major affect of 5-azaC is to inhibit FV3 DNA methylation. It has been suggested that hypomethylated FV3 DNA is susceptible to endonucleases which cause nicks in the DNA and improper packaging into capsids thereby reducing the number of infectious virus particles (Goorha, Granoff, Willis & Murti, 1984).

Frog Virus 3 Infection

Upon infection with FV3, host DNA, RNA and protein synthesis is significantly decreased (Kelly & Robertson, 1973). Structural proteins of FV3 appear to cause the rapid inhibition of host DNA, RNA and protein synthesis (Tan & McAuslan, 1971). Heat and uv inactivated FV3 inhibits host protein synthesis by blocking initiation of translation of cellular mRNAs (Raghow & Granoff, 1979). Subsequent replication of infectious FV3 is not affected (Goorha & Granoff, 1974a). Host DNA synthesis is inhibited significantly within two to three hours and is completely inhibited five to six hours after infection (McAuslan & Smith, 1968). By six hours post-infection viral DNA foci are established in the cytoplasm (McAuslan & Smith, 1968).

The uncoating of FV3 has been studied by electron microscopy in rat Kupffer cells, the liver macrophages (Gendrault, Steffan, Bingen & Kirn, 1981). Phagocytic vesicles can be seen with virus particles in them two hours post-infection. Uncoating occurs in a single step and is not dependent on protein synthesis or incubation temperature (Armentrout & McAuslan, 1974; Kucera, 1970).

Enveloped FV3 particles are usually internalized by endocytosis via clathrin coated pits (Gendrault, Steffan, Bingen & Kirn, 1981). Enveloped particles can be seen shortly after infection in cytoplasmic vacuoles close to the plasma membrane and then later, partially degraded particles are seen in lysosomes. Nonenveloped FV3 particles usually fuse with the cell membrane rather than uptake at coated pits

(Braunwald, Nonnenmacher & Tripier-Darcy, 1985). The DNA is then released into the cytoplasm and the viral shell integrates into the cell membrane. The DNA reaches the nucleus where DNA replication and transcription begins. FV3 DNA can enter and replicate inside a host cell only if an active protein associated with a complete virion is present; purified FV3 DNA is not infectious (Willis, Goorha & Granoff, 1979).

DNA Replication

Animal viruses replicate and assemble in either the host nucleus or the cytoplasm and have been classified as such. FV3 was first classified as a cytoplasmic virus but later it was shown that FV3 DNA replicates in both the nucleus and the cytoplasm of host cells (Goorha, 1982). A functional host nucleus has been reported to be essential, as FV3 does not replicate in enucleated or uv-irradiated cells (Goorha, Murti, Granoff & Tirey, 1978). FV3 DNA replication occurs in two stages (Goorha, 1982). The first stage of DNA replication is restricted to the nucleus where genome or twice genome size molecules are synthesized (Goorha & Murti, 1982). Stage 1 DNA synthesis continues late into infection and apparently is not affected by stage 2 DNA replication. Stage 2, beginning 3 hours post-infection, occurs exclusively in the host cytoplasm where concatamers of replicating DNA are formed (Goorha, 1982). It has been suggested that viral protein(s) located in the cytoplasm are necessary for the switch between stage 1 and stage 2 of DNA replication (Goorha & Murti, 1982).

The nuclear-associating DNA has been shown to be the precursor of cytoplasmic DNA in infected cells. Both nuclear-associating DNA and cytoplasmic

DNA have a guanine plus cytosine content of 52% and they have similar sedimentation profiles in sucrose gradients (McAuslan & Smith, 1968).

Two stage replication for FV3 DNA has been confirmed by work with a temperature sensitive mutant defective in the second stage of DNA replication (Goorha & Dixit, 1984). At 30°, the nonpermissive temperature, this mutant synthesized genome or twice genome length DNA only in the nucleus. After a temperature shift to 25°, large cytoplasmic DNA was rapidly seen.

The two stage model of FV3 DNA replication was further supported by use of arginine starved cells, as it has been shown that arginine is required for the production of infectious FV3 (Aubertin, 1975; Martin, Aubertin, Tondre & Kirn, 1984). In arginine depleated cells, early proteins were synthesized and the first stage of DNA replication occured but the passage from first to second stage DNA replication did not occur, late proteins were not synthesized and mature virions were not produced (Aubertin, 1975; Martin, Aubertin, Tondre & Kirn, 1984). Subsequent addition of arginine restored normal growth conditions and infectious particles were produced.

Viral DNA synthesis begins approximately two hours post-infection and reaches a peak at four hours post-infection but continues to be made even after viral progeny appear (Armentrout & McAuslan, 1974; McAuslan & Smith, 1968). Protein synthesis is required at the initiation of viral DNA replication. Once initiated, viral DNA replication can continue in the absence of protein synthesis (McAuslan & Smith, 1968). At two hours post-infection, the size of the viral DNA is approximately

genome length. At four hours post-infection, however, the size of most of the viral DNA is very large concatameric structures. This concatameric DNA has been shown to be the precursor to mature virion DNA and protein synthesis is necessary for this conversion (Goorha, 1982). Concatamer processing is probably involved with DNA packaging and virus assembly which takes place in distinct areas of the cytoplasm called assembly sites (Chinchar, Goorha & Granoff, 1984). These sites form 6-7 hours post-infection and require at least one early protein and viral DNA synthesis but not late proteins (Chinchar, Goorha & Granoff, 1984). FV3 utilizes various components of the host cytoskeleton for formation and maintenance of the assembly sites (Murti, Goorha & Chen, 1985). It has been shown that packaging and maturation of FV3 is highly sensitive to protein synthesis inhibitors. Although DNA is synthesized, it is not packaged properly (McAuslan & Smith, 1968).

Protein Synthesis

FV3 replication takes place in an ordered and sequential fashion. Three classes of proteins can be detected; immediate early, early and late (Willis & Granoff, 1976b; Willis & Granoff, 1978). These proteins are synthesized and accumulate at different rates during the course of infection (Goorha & Granoff, 1974a). Immediate early and early transcription occur in the nucleus and host RNA polymerase II is necessary for this process to take place (Goorha, 1981b; Willis, Thompson, Essani & Goorha, 1989). Late transcription, on the other hand, probably occurs in the cytoplasm by a virus induced enzyme (Willis, Goorha, Miles & Granoff, 1977). It has been shown

that a structural protein of FV3 modifies the host RNA polymerase II for transcription of the viral genome (Goorha, 1981b). Viral structural protein synthesis is an early event in FV3 replication cycle and viral DNA synthesis is necessary to regulate this process (Goorha & Granoff, 1974a).

Viral DNA synthesis is not the only event that regulates protein synthesis. Experiments with temperature sensitive mutants or wild type virus infection with incubation at nonpermissive temperature show that even with DNA synthesis, synthesis of some proteins is not regulated (Goorha & Granoff, 1974b). With shifts to permissive temperatures, regulation soon commenced. This result suggests that some protein synthesis is regulated by a post-transcriptional viral protein. Viral transcription and viral protein synthesis are also controlled by viral regulatory proteins both quantitatively and qualitatively (Goorha, Naegele, Purifoy & Granoff, 1975; Goorha, Willis & Granoff, 1979; Willis & Granoff, 1976a).

By using an FV3 temperature sensitive mutant defective in DNA replication, viral protein synthesis has been studied (Goorha, Willis, Granoff & Naegele, 1981). This mutant had a nonpermissive temperature of 30° and a permissive temperature of 23°. At 30° all detectable proteins were synthesized, even though DNA replication did not take place, but the late proteins were delayed and did not reach the levels found in wild type FV3.

Frog Virus 3 Transactivation

It has been shown in transient transfection experiments that FV3 has the ability

to transactivate genes that were previously silenced by DNA methylation (Thompson, Granoff & Willis, 1987). This FV3 transactivation of methylated DNA is highly specific for cytosine methylation. FV3 is unable to induce transcription of DNA methylated at adenine residues (Thompson, Granoff & Willis, 1987). Various eukaryotic cell lines have been transfected with constructs containing a methylated or unmethylated promoter region linked to the bacterial reporter gene chloramphenicol acetyltransferase (CAT). CAT synthesis is usually seen only with unmethylated construct or with methylated construct and subsequent infection with frog virus 3 (Willis & Granoff, 1985).

Most cells with methylated genes do not express these genes. As stated earlier, upon infection of these cells with FV3, previously silent genes can be expressed (Willis & Granoff, 1985). It has been suggested that an FV3 protein alters either host RNA polymerase II or the methylated template to allow transcription to take place (Thompson, Granoff & Willis, 1987; Willis, Essani, Goorha, Thompson & Granoff, 1989).

Deletions and point mutations throughout the promoter region of an immediate early FV3 gene were used to determine what DNA sequences are required for transactivation of methylated genes (Willis, 1987). A 27 bp deletion at the 5' end of the promoter had no effect on FV3 induced CAT activity. Deletion of , and point mutations within, the AT rich region of this gene reduced CAT activity to 16-50% of the wild type FV3 promoter. Only deletion of this TATA sequence caused shifting of the transcription start site. Deletion of the region immediately 5' to the AT rich region

caused the most significant decrease in CAT synthesis showing that this area is critical for FV3 transactivation and that this TATA box acts to position the start site of transcription (Willis, 1987).

In summary, FV3 is a unique model for studying DNA methylation and its effects on gene expression. With its unusually high degree of methylation, use of host RNA polymerase II, two stage DNA replication and properties that resemble both eukaryotic and prokaryotic viruses, FV3 is an outstanding candidate for studying the mechanisms of transcriptional and possibly translational control in many biological systems.

MATERIALS AND METHODS

Cell Lines

The cell lines used include fat head minnow (FHM) cells, simian kidney cells (Vero), HIV-LTR CAT cells, A3.01 cells (human CD4+ T-cells) and A3N92.2 cells. HIV-LTR CAT cells are Vero cells containing a single copy of stably integrated methylated HIV-LTR linked to CAT (Bednarik, Cook & Pitha, 1990a). A3N92.2 cells are a derivative of A3.01 cells containing a single stably integrated methylated copy of HIV-LTR linked to CAT (Gutekunst, Kashanchi, Brady & Bednarik, 1993). HIV-LTR CAT, A3.01 and A3N92.2 cells were a gift from Dr. Daniel Bednarik (Centers for Disease Control, Atlanta, GA). Bednarik *et al* (1987, 1990a) and Gutekunst *et al* (1993) have clearly demonstrated that HIV-LTR in these cell lines is methylated. This observation was further confirmed in our laboratory prior to initiating this study. FHM, Vero and HIV-LTR CAT cells were grown in monolayers and A3.01 and A3N92.2 cells were grown in suspension. All cells were grown in RPMI 1640 medium (Gibco BRL, Gaithersberg, MD) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM glutamine and antibiotics (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) at 37° (or 33° for FHM cells) with 5% CO_2.

Virus

Frog virus 3 (FV3) was cultivated in confluent monolayers of FHM cells. Adsorption was carried out for 1 hour at room temperature on a rocker table. FV3-infected cells were incubated in maintenance medium (RPMI 1640, 2% FCS, glutamine and antibiotics) at 30° with 5% CO_2. Infected cells were harvested 7-10 days post-infection using a rubber policeman and pelleted by centrifugation at low speed (approximately 50 × *g*, Damon IEC HN-S centrifuge at 1,500 rpm) for 10 minutes at room temperature. The pellet was resuspended in autoclaved deionized water and subjected to three freeze/thaw cycles (dry ice-ethanol bath for 10 minutes followed by incubation in a 37° waterbath for 10 minutes). Cell debris was removed by centrifugation at low speed (approximately 50 × *g*, Damon IEC HN-S centrifuge at 1,500 rpm) for 10 minutes at room temperature. The supernatant was centrifuged at 85,000 × *g* (Beckman L8-70M ultracentrifuge using a Ti70.1 fixed angle rotor at 30,000 rpm) for 1 hour at 4°. The resultant virus pellet was resuspended in maintenance medium to a final concentration of 100X and quantitated as described below before being stored at -20°.

Plaque Assay

Plaque assays were carried out using the standard procedure in our laboratory (Essani, 1982). Briefly, virus dilutions from 10^{-1} to 10^{-9} were made in RPMI. FHM cells were plated out in 6-well dishes and grown to confluent monolayers as described

above. Duplicate wells of FHM cells were infected with 200 µl of the 10^{-3} to 10^{-9} dilutions of 100X FV3. Maintenance medium with 0.5% methylcellulose (4000 centipoise) was added and the 6-well dishes were placed at 30° for 7-10 days until plaques were visible using an inverted light microscope. The medium was then aspirated carefully and 0.1% crystal violet with 10% formaldyhyde in deionized water was added and incubated for 15 minutes at room temperature. The dishes were then rinsed in deionized water, air dried and the plaques were counted to determine the number of plaque forming units (pfu) per ml.

Cell Preparation and Fusions for CAT Assay

Vero and HIV-LTR CAT cells were planted in 60 mm dishes as described above 24-48 hours prior to CAT assay to obtain an 80-85% confluent monolayer. T-cells (A3.01 and A3N92.2) were split 24-48 hours prior to CAT assays. Where indicated, cells were treated with 10 µM 5-azacytidine (5-azaC) for several generations. For FV3-infected Vero and HIV-LTR CAT cells, the growth medium was removed and 20 pfu/cell FV3 was added. Adsorption was carried out for 1 hour at room temperature on a rocker table. The FV3-infected cells were incubated at 30° or 37° for 4 hours. For T-cells, fusions were carried out using polyethylene glycol (Essani, Satoh, Prabhakar, McClintock & Notkins, 1985). Briefly, FHM cells were infected with FV3 (20 pfu/cell). Following adsorption for 1 hour at room temperature, cells were incubated at 30° for 2 hours. FHM cells (infected or

uninfected) were then mixed with T-cells at a ratio of 100 to 1 in serum free RPMI medium. Prewarmed polyethylene glycol (0.5 ml; 50% PEG 4000; Gibco, Gaithersburg, MD) was added to the cells. RPMI medium (20 ml) was slowly added with gentle shaking to dilute the PEG, fused cells were centrifuged at low speed for 10 minutes at room temperature, resuspended in 2.5 ml RPMI medium with 20% FCS and incubated for 4 hours at 30° or 37°.

Preparation of Cell Extract for CAT Assay

CAT assays were performed using a CAT Assay Enzyme System as described by the manufacturer (Promega, Madison, WI). Briefly, cells were washed three times in phosphate buffered saline (PBS)(137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, final pH 7.3), 250 µl TEN buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl) was added and incubated 5 minutes, room temperature. Monolayered cells were scraped using a rubber policeman. Cells were transferred to a microcentrifuge tube, centrifuged at low speed (approximately 50 × *g*, Brinkman 5415C eppendorf centrifuge at 1,500 rpm) for 10 minutes, room temperature, and the supernatant was discarded. The pellet was resuspended in 50 µl Tris-HCl (0.25 M, pH 8.0) and subjected to three freeze/thaw cycles. Cell lysate was then heated to 60° for 10 minutes to inactivate endogenous deacetylase activity. An additional 90 minute freeze in dry ice/ethanol and thaw at 37° for 10 minutes followed. The lysate was then centrifuged at 6,000 × *g* (Brinkman 5415C eppendorf centrifuge at 10,000 rpm) for 10

minutes. The supernatant was used in the CAT assay.

CAT Reaction

Cell extract (50 µl) was mixed with 5 µl n-butyryl coenzyme A (5 mg/ml in H_2O), 0.1 µCi of [^{14}C]-chloramphenicol (58.1 mCi/mmol; NEN, Wilmington, DE) and 68 µl Tris-HCl (0.25 M, pH 8.0) to a final volume of 125 µl, and incubated at 37° for 18-22 hours. A positive control was run using one unit of CAT enzyme (10 units/µl in 100 mM Tris-HCl, pH 7.8) instead of cell extract. A negative control was also run using no cell extract and no CAT enzyme.

Thin Layer Chromatography

CAT reaction was terminated by adding 125 µl ethyl acetate, vortexed for 1 minute and centrifuged at 6,000 × *g* (Brinkman 5415C eppendorf centrifuge at 10,000 rpm) for 3 minutes. The upper organic phase was transferred to a new tube and evaporated to dryness. The residue was resuspended in 15 µl ethyl acetate, spot onto a silica gel plate and dried. Chromatography was run for approximately 1 hour in a pre-equilibrated (95:5, chloroform:methanol, 1 hour) closed tank, removed, air dried, covered with plastic wrap and exposed to X-OMAT AR film (Eastman Kodak Company, Rochester, NY), room temperature, for 3-7 days. The images were scanned in a Macintosh computer and printed without any image manipulation.

RESULTS

To assess the effect of FV3 infection on expression of an integrated gene previously suppressed by DNA methylation, we used a stably transformed simian kidney cell line (Vero) that contains a single integrated copy of enzymatically methylated HIV-LTR linked to CAT (Bednarik, Cook & Pitha, 1990a), herein referred to as HIV-LTR CAT cells. It has been shown earlier (Bednarik, Mosca & Raj, 1987) that treatment of these cells with 10 μM 5-azacytidine (5-azaC), a potent inhibitor of DNA methylation, resulted in CAT expression. In addition, direct evidence that HIV-LTR is methylated in these cell lines has also been published (Bednarik, Cook & Pitha, 1990a; Bednarik, Mosca & Raj, 1987; Gutekunst, Kashanchi, Brady & Bednarik, 1993) and duplicated in this laboratory. We have also duplicated the 5-azaC results by demonstrating that treatment of HIV-LTR CAT cells with 5-azaC at either 30° (Fig. 1, lane C) or 37° (Fig. 1, lane D) induced CAT activity. No CAT activity was detected in untransfected Vero cells treated with 5-azaC (10 μM for several generations) and incubated at 30° (Fig. 1, lane A) or 37° (Fig. 1, lane B) and untreated HIV-LTR CAT cells incubated at 30° (Fig. 1, lane E) or 37° (Fig. 1, lane F). This series of experiments established that HIV-LTR CAT cells contained HIV-LTR which has been silenced by DNA methylation.

The following experiments were designed to establish whether or not methylated HIV-LTR stably integrated into cellular DNA can be transcribed by FV3

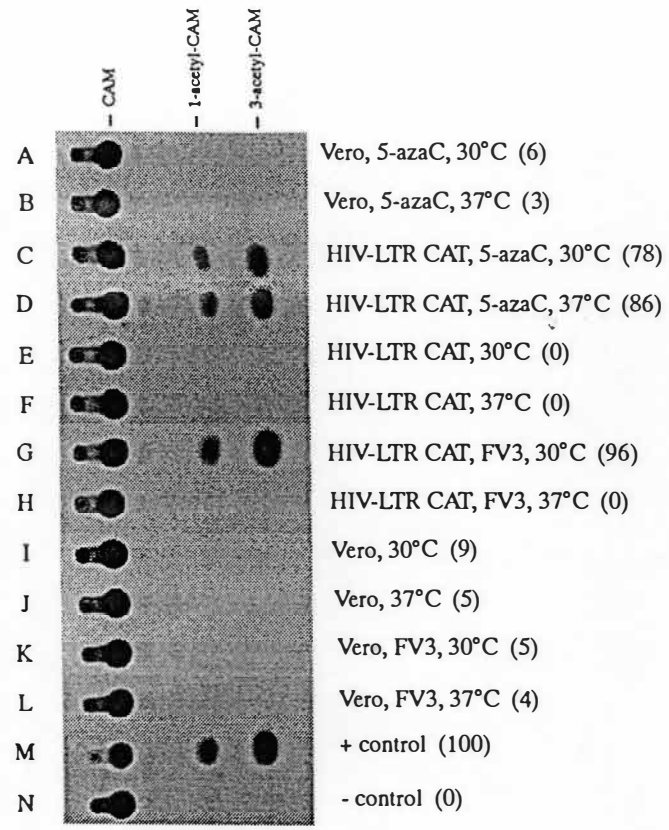

Figure 1. Transcription of Methylated HIV-LTR CAT in Monkey Kidney Cells.

Vero cells (untransfected) and HIV-LTR CAT cells (stably transfected with a single integrated methylated copy of HIV-LTR linked to CAT) were grown in monolayers. Wherever indicated cells were treated with 10μM 5-azaC for 3 or more generations. Cells were infected with 20 pfu/cell of FV3. Following adsorption for 1 hour at room temperature, cells were incubated for 4 hours at 30° or 37°. CAT assays were performed as described in Materials and Methods. Lanes A and B represent 5-azaC treated Vero cells incubated at 30° or 37°, respectively. Lanes C and D represent 5-azaC treated HIV-LTR CAT cells incubated at 30° or 37°, respectively. Lanes E and F represent untreated HIV-LTR CAT cells incubated at 30° or 37°, respectively. Lanes G and H represent FV3-infected HIV-LTR CAT cells incubated at 30° or 37°, respectively. Lanes I and J represent untreated Vero cells incubated at 30° or 37°, respectively. Lanes K and L represent FV3-infected Vero cells incubated at 30° or 37°, respectively. Positive (lane M) and negative (lane N) controls were run as described in Materials and Methods. The numbers in parentheses represent percent conversion of chloramphenicol (CAM). The percentage was computed by densitometry considering positive control as 100% conversion. 5-azaC: 5-azacytidine.

infection. HIV-LTR CAT cells, not expressing CAT, were infected with FV3 (20 pfu/cell) and incubated at 30° for 4 hours. Results shown in Figure 1, lane G demonstrate that when infected cells were incubated at 30° (permissive temperature for FV3 replication), CAT was expressed. In contrast, when these infected cells were incubated at 37° (restrictive temperature for FV3 replication), no CAT activity was detected (Fig. 1, lane H). This confirms our notion that it is one or more FV3 proteins which mediated this activity since we do not see CAT activity at 37°. A series of elegant experiments by Thompson, Granoff and Willis (1986) have shown that *de novo* protein synthesis is required for transactivation of methylated template. Therefore, the FV3 protein responsible for transcribing methylated DNA is virus-induced and is not part of FV3 virion. The results in Figure 1 also show that untransfected Vero cells incubated at 30° (lane I) or 37° (lane J) and FV3-infected Vero cells incubated at 30° (lane K) or 37° (lane L) failed to express CAT activity. CAT expression was only observed in the FV3-infected HIV-LTR CAT cells incubated at 30° (Fig. 1, lane G). These experiments strongly suggest that the putative FV3 protein(s) may enable transcription of integrated genes that are silenced by DNA methylation.

Since monkey kidney cells are not the natural host for HIV, attempts were made to see whether the same effect could be demonstrated in human T-cells. We have used A3.01 (CD4+, human T-cells) and A3N92.2 (human T-cells with a single integrated copy of methylated HIV-LTR CAT) (Gutekunst, Kashanchi, Brady & Bednarik, 1993). It had been shown in transient assays that 5-azaC failed to induce

expression of CAT in T-cells unless specific HIV trans-acting factors were present (Bednarik, Mosca & Raj, 1987). We have found that treatment of A3N92.2 cells with 5-azaC (10 μM for several generations) did not increase CAT expression (data not shown). In our hands, FV3 failed to infect human T-cells. To overcome this unexpected difficulty, a fusion protocol was adopted. FHM cells were infected with 20 pfu/cell FV3 or mock infected and incubated for two hours at 30°. Fusions were carried out using the polyethylene glycol method (Essani, Satoh, Prabhakar, McClintock & Notkins, 1985). Approximately $3x10^5$ T-cells were fused with $3x10^7$ FHM (infected or uninfected) cells. Microscopic examination of cells following fusion determined that all T-cells were fused but only 50% of FHM cells were fused. This calculation was based on microscopic counting of unfused fish FHM and human T-cells using cell size as a criterion. FHM cells are significantly smaller in size. Following fusion, incubation was carried out for 4 hours at 30° or 37° prior to CAT assay. CAT activity was seen at a significantly higher level in A3N92.2 cells fused with FV3-infected FHM cells and incubated at 30° (Fig. 2, lane M). In contrast, when such cells were incubated at 37°, no CAT activity was detected (Fig. 2, lane N). Results shown in Figure 2 also demonstrate that no CAT activity was detected in unfused A3.01 cells incubated at 30° (lane A) or 37° (lane B), unfused A3N92.2 cells incubated at 37° (lane D) and unfused FHM cells incubated at 30° (lane E) or 37° (lane F). Results in Figure 2 also demonstrate that no CAT activity was detected in A3.01 cells fused with uninfected FHM cells and incubated at 30° (lane G), or 37°

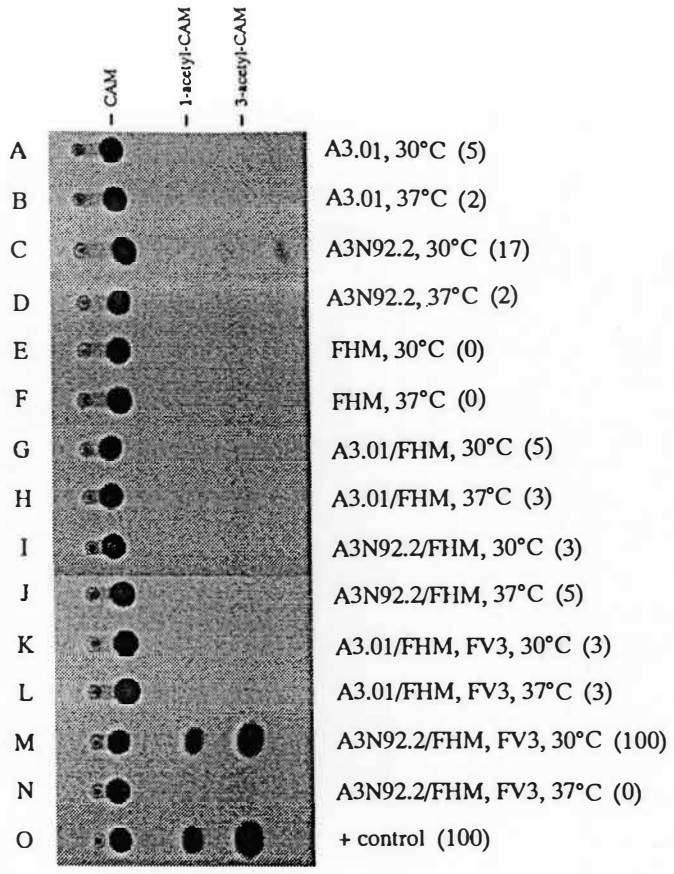

Figure 2. Transcription of Methylated HIV-LTR CAT in Human T-Cells.

A3.01 cells (untransfected), A3N92.2 cells (stably transfected with a single integrated methylated copy of HIV-LTR linked to CAT) and FHM cells were grown as described in Materials and Methods. FHM cells were infected with 20 pfu/cell FV3. Following adsorption for 1 hour at room temperature, cells were incubated at 30° for 2 hours. Fusions and CAT assays were carried out as described in Materials and Methods. Lanes A and B represent unfused A3.01 cells incubated at 30° or 37°, respectively. Lanes C and D represent unfused A3N92.2 cells incubated at 30° or 37°, respectively. Lanes E and F represent unfused FHM cells incubated at 30° or 37°, respectively. A3.01 cells were fused with uninfected FHM cells and incubated at 30° (lane G) or 37° (lane H). A3N92.2 cells were fused with uninfected FHM cells and incubated at 30° (lane I) or 37° (lane J). Lanes K and L represent A3.01 cells fused with FV3-infected FHM cells and incubated at 30° or 37°, respectively. Lanes M and N represent A3N92.2 cells fused with FV3-infected FHM cells incubated at 30° or 37°, respectively. A positive control (lane O) was run using exogenous CAT enzyme. The numbers in parenthses represent percent conversion of chloramphenicol (CAM).

(lane H), A3N92.2 cells fused with uninfected FHM cells incubated at 30° (lane I) or 37° (lane J) and A3.01 cells fused with FV3-infected FHM cells incubated at 30° (lane K) or 37° (lane L). A notable observation, however, was that human T-cells containing methylated HIV-LTR expressed significantly small but detectable CAT activity following incubation at 30° (Fig. 2, lane C) without any contribution from either FV3 or 5-azaC. Although the significance of this finding in HIV/T-cell pathogenesis system remains elusive, cold-shock and other stress responses are known to alter gene expression both in human (Holland, Roberts, Wood & Cunliffe, 1993) and bacterial (Qoronfleh, Debouck & Keller, 1992) cells.

DISCUSSION

The results from this study, taken together, provide compelling evidence that FV3 has evolved mechanism(s) to facilitate the transcription of integrated genes suppressed by DNA methylation. Site-specific DNA methylation has a strong silencing effect on most of the genes transcribed by eukaryotic RNA polymerase II (Riggs and Jones, 1983) and this enzyme is used by FV3 to transcribe its mRNA from a highly methylated genomic template (Goorha, 1981b). Using a plasmid containing the E1a promoter (already known to be transcriptionally inhibited by methylation) attached to an easily assayable reporter gene, it has been demonstrated that an early FV3 gene product can induce transcription from a methylated E1a-CAT template (Thompson, Granoff & Willis, 1986). In transient transfection assays, it has already been demonstrated that the transcription start site in the E1a promoter is the same in both uninfected and infected cells (Thompson, Granoff & Willis, 1986). It has been shown that only FV3 infection can induce the transcription of silent methylated genes. When other viruses such as vaccinia virus and adenovirus were used in identical conditions, no transcription was demonstrated (Thompson, Granoff & Willis, 1986). The putative FV3 trans-acting protein(s) may have an affinity for methyl CpG binding protein (Boyes and Bird, 1991), or perhaps it modifies the RNA polymerase II such that it now reads the methylated template within 2-4 hours following FV3 infection. It has been documented that FV3 infection does not alter the methylation status of the

DNA template (Thompson, Granoff & Willis, 1986). In contrast, 5-azaC inhibits DNA methylase and 5-azaC treated cells show maximally undermethylated DNA 48 hours after treatment (Jones and Taylor, 1980). Therefore, the mechanisms involved in the transcription of methylated template by 5-azaC and FV3 must be different.

The most popular hypothesis concerning the role of DNA methylation in the regulation of transcription predicts that demethylation of promoters is a necessary but not a sufficient condition for maximum transcription to occur, and that demethylation may be the result of active transcription rather than the cause (reviewed in Bird, 1986). If transcription results in demethylation rather than the opposite, then eukaryotic cells must have a means of transcribing methylated DNA. An activation of this mechanism may be involved in the turn-on of previously silent genes--especially important for cancer if the genes in question are altered cellular oncogenes. The oncogenes of neoplastic cells are often hypomethylated (reviewed in Hoffman, 1984); an illustration of this is that methylation of the human Ha-*ras* oncogene significantly reduces its transforming activity (Borrello, Pierotti, Bongarzon, Donghi, Mondellini & Porta, 1987). FV3, with its unrivaled capacity to override the inhibitory effect of DNA methylation on transcription in a non-cell specific (Figures 1 and 2) and non-promoter specific (Thompson, Granoff & Willis, 1986) manner, provides an exceptional opportunity to explore the question as to how cellular RNA polymerase II can be influenced to transcribe methylated DNA.

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