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Evidence for DNA Methylation-Sensitive Transactivator Mediated Regulation of the Expression of an Epstein-Barr Virus Neutralizing Antigen

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**EVIDENCE FOR DNA METHYLATION-SENSITIVE TRANSACTIVATOR
MEDIATED REGULATION OF THE EXPRESSION OF AN
EPSTEIN-BARR VIRUS NEUTRALIZING ANTIGEN**

by

Sridhar Chalasani

**A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biological Sciences**

**Western Michigan University
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EVIDENCE FOR DNA METHYLATION-SENSITIVE TRANSACTIVATOR
MEDIATED REGULATION OF THE EXPRESSION OF AN
EPSTEIN-BARR VIRUS NEUTRALIZING ANTIGEN

Sridhar Chalasani, M.S.

Western Michigan University, 1994

DNA methylation has a profound effect on the expression of a number of eukaryotic genes. This study demonstrates that an Epstein-Barr virus (EBV) positive Burkitt's lymphoma cell line, Raji, contains the gene encoding gp350/220 but is not expressed. Frog virus 3 (FV3), an iridovirus, has the ability to overcome the inhibitory effect of DNA methylation. Fusion of FV3-infected cells with Raji cells resulted in expression of gp350/220, suggesting that gp350/220 is regulated by DNA methylation. Further evidence for this hypothesis was obtained by transfecting B95.8 cells with *HpaII/HhaI* methylated plasmid pLATE-CAT, containing the gp350/220 promoter. Southern blot analyses and a modified polymerase chain reaction revealed that the gp350/220 promoter is not methylated in Raji cells. These results, taken together, provide evidence that gp350/220 in Raji cells is regulated by a methylation-sensitive transactivator. Since gp350/220 is a neutralizing antigen, these results imply that perhaps EBV takes advantage of DNA methylation to evade the host immune system.

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Sridhar Chalasani

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INTRODUCTION

Epstein-Barr virus (EBV) is a human pathogen that has world wide distribution and is associated with a number of clinical conditions. There is currently no effective vaccine against EBV despite many years of vigorous efforts. The most suitable target antigen for the development of a vaccine against EBV is gp350/200, an envelope glycoprotein (Dolyniuk, Pritchett & Kieff, 1976; Dolyniuk, Wolff & Kieff, 1976). This glycoprotein acts as a ligand for CD21, a B lymphocyte surface receptor, which facilitates virus adsorption and entry into B lymphocytes (Nemerow, Mold, Keivens-Schwend, Tollefson & Cooper, 1987; Tanner, Weis, Fearon, Whang & Kieff, 1987). Gp350/220 is also a virus neutralizing antigen, since blocking the binding of gp350/220 to CD21 prevents the entry of the virus into the B lymphocyte (Tanner, Whang, Sample, Sears & Kieff, 1988; Thorley-Lawson & Geilinger, 1980). Several studies have targeted gp350/220 for the development of anti-EBV vaccines and have resulted in varying degrees of success (Epstein, Morgan, Finerty, Randle & Kirkwood, 1985; Epstein, Randle, Finerty & Kirkwood, 1986; Morgan *et al.*, 1988; Pither *et al.*, 1992). Since this glycoprotein is an integral part of virus infectivity, it would be useful to understand the molecular mechanisms that regulate its expression.

DNA methylation is known to play a regulatory role in the expression of many eukaryotic genes. There appears to be an inverse relationship between the

methylation of the regulatory region of a gene and its expression (reviewed in Razin & Cedar, 1991). It has been demonstrated that DNA methylation plays a significant role in the expression of a number of EBV genes (Ben-Sasson & Klein, 1981; Masucci *et al.*, 1989). This study attempts to evaluate the possible role of DNA methylation in the transcriptional regulation of gp350/220.

Raji cells, an EBV positive Burkitt's lymphoma cell line, contain but do not express, the gene for gp350/220. Results presented here, demonstrate that DNA methylation plays a role in the suppression of gp350/220 gene in Raji cells as shown by immunofluorescence and *in vitro* transient transfection assays. Southern blotting and polymerase chain reaction analyses revealed that the promoter for gp350/220 was not methylated. These results lead to the logical conclusion that gp350/220 is regulated by a DNA methylation-sensitive transactivator in Raji cells.

REVIEW OF LITERATURE

DNA Methylation

Eukaryotic gene regulation is a complex process that involves the precise interaction of a number of factors at precise times. DNA methylation is one such regulatory factor involved in transcriptional regulation. It has been established that DNA methylation plays a significant role in the regulation of the expression of a number of eukaryotic genes. It has also been shown that in addition to the four usual bases in eukaryotic genomic DNA, there are certain other modified bases. Of these modified bases, 5-methylcytosine (5mC) is believed to constitute about 3% of the cytosines in the mammalian genome (Lindhal, 1981). The presence of 5mC in the dinucleotide CpG has been known to alter the expression profile of a number of eukaryotic genes (reviewed in Razin & Cedar, 1991). The occurrence of methylated CpGs was reported to be localized in areas rich in CpGs referred to as CpG islands. In general, the methylation of CpGs at the 5' end (the promoter region) of a gene resulted in the suppression of its expression as described below. Methylation of the CpGs in the adenovirus type 12 promoters linked to the chloramphenicol acetyltransferase (CAT) gene resulted in suppression of CAT activity (Kruczek & Doerfler, 1983). Similarly decreased expression of *gamma*-globin gene (Busslinger, Hurst & Flavell, 1983), rat α -actin gene (Paroush, Keshet, Yisraeli & Cedar, 1991;

Yisraeli *et al.*, 1986), and β -globin gene (Yisraeli, Frank, Razin & Cedar, 1988) was observed after methylation of their 5' ends. Some isolated exceptions have been reported where methylation of the gene or its promoter had no demonstrable effect on its expression. Thus, the chicken lysozyme gene was transcribed *in vitro* even though it was heavily methylated (Wölfl, Schröder & Wittig, 1991). Examples of exceptions where methylation of the gene itself, but not its promoter, resulted in suppression of expression include the herpes simplex virus (HSV) thymidine kinase (TK) gene (Buschhausen, Wittig, Graessmann & Graessmann, 1987), and the mouse α -fetoprotein gene (Opdecamp, Rivière, Molné, Szpirer & Szpirer, 1992).

DNA methylation, being one of the regulatory factors for gene expression, has been implicated in the developmental process of an organism. Analysis of mouse sperm DNA showed that all known tissue specific genes were highly methylated (reviewed in Cedar & Razin, 1990). These genes were expressed only in specific cells during embryonic development after demethylation (Benvenisty, Mencher, Meyuhas, Razin & Reshef, 1985). Similarly, some genes have been demonstrated to undergo *de novo* methylation at certain stages of development when their expression was no longer biologically necessary. DNA methylation has also been shown to be involved in the inactivation of the X-chromosome (reviewed in Razin & Cedar, 1991), genetic imprinting (reviewed in Barlow, 1993), and cell memory (Morimoto & Koshland, 1991; Riggs, 1989). The presence of 5mC in a sequence also resulted in point mutations, a transition from C to T, by spontaneous (Lindhal & Nyberg, 1974; Bird, 1980) or enzymatic deamination (Cambarelli, Jensen,

Schabtach & Selker, 1989).

The experiments described above have clearly established a role for DNA methylation but have provided little insight into the molecular mechanism(s) of transcriptional suppression caused by DNA methylation. Unavailability of a cell-free methylation-sensitive transcription assay was a major technical obstacle in resolving these mechanisms. Such an assay is now available (Dobrzanski, Hoeveler & Doerfler, 1988) and has begun to uncover molecules responsible for the transcriptional suppression of methylated genes (Boyes & Bird, 1992; Meehan, Lewis & Bird, 1993).

The basic mechanism(s) by which DNA methylation exerts its regulatory effects appeared to be through altered DNA-protein interactions (reviewed in Razin & Cedar, 1991). Two major hypotheses, namely "direct" and "indirect", have been proposed to explain the mechanisms by which DNA methylation mediates transcriptional suppression (Boyes & Bird, 1991; reviewed in Razin & Cedar, 1991; Selker, 1990). It has been proposed that the presence of the methyl group itself interfered with the binding of transcriptional factors to the promoter region. This was shown to be the case in regulation of the tyrosine aminotransferase gene where genomic footprinting has demonstrated that transcription factors were unable to bind to the methylated promoter (Becker, Ruppert & Schütz, 1987). Similarly, two transcription stimulating factors present in HeLa cell extracts were unable to bind to methylated adenoviral promoters (Kovesdi, Reichel & Nevins, 1987; Watt & Molloy, 1988). On the contrary, the heavily methylated chicken lysozyme gene was

transcribed *in vitro* (Wölfl *et al.*, 1991). Similarly, Sp1 transcriptional factor has been shown to bind to DNA and activate transcription even when the binding site was CpG methylated (Holler, Westin, Jiricny & Schaffner, 1988). In experiments involving *in vitro* transfection of methylated foreign genes, a time lag has been reported between the introduction of a methylated gene into a cell and the onset of its down-regulation (Buschhausen *et al.*, 1987). It has been demonstrated that the methylated HSV-TK gene was transcribed initially when microinjected into TK-negative rat cells and was turned off only after its incorporation into chromatin (Buschhausen *et al.*, 1987). This data gave rise to another hypothesis, put forward by Cedar (1988), suggesting that the presence of the methyl group renders the methylated DNA unavailable for transcription by altering chromatin conformation where the methylated genes are buried inside the chromosome.

Recent studies using cell-free methylation-sensitive transcription assays have indicated the presence of two nuclear proteins, MeCP1 and MeCP2, that preferentially bind to methylated CpG islands (Boyes & Bird, 1992; Meehan *et al.*, 1993). Both these proteins bind to methyl CpG pairs and MeCP1 has been shown to interfere with the binding of transcriptional factors (Boyes & Bird, 1991). Because of this property, MeCP1 has been demonstrated to act "directly" to inhibit the transcription of methylated genes both *in vitro* and *in vivo* (Boyes & Bird, 1992). The same study also showed that strong promoters and the presence of enhancers tended to overcome the inhibitory effect of methylation and the binding of MeCP1 was dependent on the density of methyl CpGs (12 pairs) in the promoter. Results

from another study (Gutekunst, Kashanchi, Brady & Bednarik, 1993) that further emphasized this point showed the importance of methyl CpG density in the regulation of HIV-LTR. MeCP2 has been shown to differ from MeCP1 in that it requires only one pair of methyl CpGs for binding and has an additional non-specific DNA binding domain. MeCP2 was also shown to be much more abundant in the nucleus than MeCP1 and is thought to play a role in the protection of DNA containing methyl CpGs against nucleases (Meehan *et al.*, 1992).

The occurrence of methyl CpGs is not random and neither is the process of methylation. Attempts have been made to investigate factor(s) that determine DNA sequences to be methylated and the sequences to be left unmethylated (Benvenisty *et al.*, 1985; Selker, Cambareri, Jensen & Haack, 1987; Selker & Garrett, 1988; Selker, Jensen & Richardson, 1987; Yisraeli *et al.*, 1986). These studies also investigated the processes of *de novo* methylation and demethylation. Tissue specific genes have been reported to be highly methylated in mouse sperm (Monk, Boubelik & Lenhart, 1987) and expressed only in specific cells during embryonic development after demethylation (Benvenisty, *et al.*, 1985). *In vitro* methylated α -actin gene, a muscle cell specific gene, when introduced into L8 myoblast cells, was demethylated and expressed (Yisraeli *et al.*, 1986). In parallel experiments, *in vitro* methylated non-muscle genes were not demethylated or expressed when introduced into myoblasts. It has been suggested that the location of a gene in the genome and its surrounding sequences play a role in determining its methylation status (Selker *et al.*, 1987; Selker & Garrett, 1988; Selker, *et al.*, 1987). When pUC8 sequence was

inserted into the genome of *Neurospora crassa* at certain positions the insert was methylated but when inserted into other positions it was not methylated. Furthermore, when methylated *Neurospora* genes were introduced into bacterial or animal cells they were unmethylated but were methylated again when reintroduced into *Neurospora* cells (Selker, *et al.*, 1987). All of these studies suggest that the process of methylation and demethylation is regulated by the presence or absence of certain cellular factors and specific signals from specific sequences within the genome (Selker, 1990).

Methylation patterns have been reported to be stable and inheritable in cell lines, even after many passages in culture has been shown by transfection of methylated foreign genes and subsequent evaluation of their methylation states in daughter cells (Holliday 1987; Yisraeli & Szyf, 1984). DNA methyltransferase, the enzyme responsible for post-replicative maintenance methylation, has high affinity for hemimethylated DNA (Gruenbaum, Cedar & Razin, 1982). This enzyme recognizes the methylated parent strand as template to methylate the daughter strand. This result explains how methylation is inherited but it does not explain how *de novo* methylation can occur. A possible explanation has been suggested by Szyf (1991) who proposed the presence of "centers of methylation" which initiate *de novo* methylation and "centers of demethylation" which facilitate demethylation and possibly gene expression. Recently, it has been demonstrated that the presence of hemimethylated DNA initiated *de novo* methylation in a non-specific manner (Adams & Lindsay, 1993). This implies that the methyltransferase is not specific when dependent on

only methyl CpGs, and may require additional factors and conditions for the methylation process to be sequence specific.

A cytosine analog, 5-azacytidine (5-AzaC), binds irreversibly to DNA methyltransferase and prevents methylation of the daughter strand after DNA replication (Jones & Taylor, 1980). Several passages of cells containing methylated DNA in medium containing 5-AzaC results in cells with unmethylated DNA and making this drug particularly useful in methylation studies. A number of genes have been studied where previously methylated genes were expressed following passage of the cells in medium containing 5-AzaC. Some of these genes include the chick *ev-1* gene (Groudine, Eisenman & Weintraub, 1981), Human immunodeficiency virus (Bednarik, Cook & Pitha, 1990) and EBV genes (Ben-Sasson & Klein, 1981; Masucci *et al.*, 1989). Another interesting example includes hamster-human cell hybrids containing inactive human X chromosome, where 5-AzaC treatment induced the reactivation of the phosphoglycerate kinase-1 (*PGK1*) gene (Hansen & Gartler, 1990). All the clones expressing *PGK1* were unmethylated at the 5' ends of *PGK1* while the non-expressing clones showed discontinuous patterns of methylation. These results strongly suggested that methylation may be responsible for silencing the inactive X chromosome. Similarly, 5-AzaC treatment of 10T1/2 or NIH 3T3 cells resulted in three mesodermal cell types (Taylor & Jones, 1979).

Apart from so-called demethylation of genes, as a prerequisite for expression, some DNA viruses with methylated genomes have evolved other mechanisms for transcribing methylated genes. An interesting example is frog virus-3 (FV3), an

iridovirus, with a genome in which almost every CpG is methylated (Willis & Granoff, 1980). Although this virus has been able to transcribe exogenously methylated foreign genes, the mechanisms involved have not yet been elucidated (Thompson, Granoff & Willis, 1987; Thompson, Granoff & Willis, 1988; Willis, Essani, Goorha, Thompson & Granoff, 1990).

Epstein-Barr Virus

EBV is a human gamma herpesvirus and has been associated with a number of malignant and non-malignant clinical conditions (reviewed in Miller, 1990; Kieff & Liebowitz, 1990). This is an enveloped virus containing a 172,000 bp linear double stranded DNA genome. EBV is an infectious agent with world wide distribution and is spread mainly through the oral route (Miller, 1990) although blood transfusion (Gerber, Walsh, Rosenblum & Purcell, 1969) and sexual intercourse (Naher, Gissmann, Freese, Petzoldt & Helfrich 1992) have also been implicated.

EBV initially establishes infection in the human oropharyngeal epithelium which is permissive for EBV replication (Sixbey, Nedrud, Raab-Traub, Hanes & Pagano, 1984; Sixbey *et al.*, 1983). The virus then secondarily infects and establishes a latent infection in B lymphocytes which are relatively abundant in the oropharyngeal tissues. To gain entry into B lymphocytes, EBV utilizes an envelope glycoprotein, EBV gp350/220, to bind to the B lymphocyte surface complement receptor CD21 (previously known as CR2) (Fingerroth *et al.*, 1984). Upon gaining entry into a host cell EBV is capable of inducing two types of infections: (1)

productive or lytic infection in which expression of the viral particle is seen, and (2) non-productive or latent infection in which the production of the viral particle is not seen even though almost all of the viral genome is present in the infected cells.

EBV has been shown to induce a lytic infection following entry into oropharyngeal epithelial cells which expressed a receptor that appeared to be immunologically related to CD21 (Sixbey *et al.*, 1987; Young, Clark, Sixbey & Rickinson, 1986). However, other cells expressing CD21 following transfection were unable to initiate a lytic infection (Ahearn, Hayward, Hickey & Fearon, 1988; Cantaloube *et al.*, 1990). This result clearly suggested that the intracellular biochemical environment was also important for EBV lytic infection.

BLLF1, the open reading frame (ORF) for gp350/220 and its promoter, BL-L1, have been located on the *Bam*HI-L segment of the viral genome (Biggin, Farrell & Barrell, 1984; Hummel, Thorley-Lawson & Kieff, 1984). Although gp350/220 gene been detected in Raji cells, its expression has not been reported. The ORF, BLLF1, has been shown to give rise to two mRNAs, a 3.4 kb and a 2.8 kb encoding gp350 and gp220 respectively. It has been clearly shown that gp350 and gp220, products of the same ORF, resulted from differential processing of the mRNA. The 2.8 kb mRNA lacks almost all of the repeat sequences (Biggin *et al.*, 1984). Stimulation of B95.8 cells, an EBV positive marmoset lymphocyte cell line, with 12-O-tetradecanoylphorbol-13-acetate, dramatically increased the expression of the 3.4 kb mRNA while the effect was not so drastic for expression of the 2.8 kb mRNA species.

The existence of EBV genome in latently infected cells has been reported in both integrated (Matsuo, Heller, Petti, Oshiro & Kieff, 1984) and episomal (Adams & Lindahl, 1975) forms. A small set of EBV genes have been reported to be differentially expressed in different latently infected cells (Ernberg, 1990). These include Epstein-Barr nuclear antigens (EBNA)-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP, latent membrane protein (LMP)-1 and LMP-2. Two untranslated mRNAs termed EBER-1 and EBER-2 have also been detected in latently infected cells (Rymo, 1979). These latent gene products have been thought to be necessary for the maintenance of latent infection and cell transformation (Fähraeus, Rymo, Rhim & Klein, 1990; reviewed in Kieff & Liebowitz, 1990).

DNA Methylation and Its Role in the Latency of Epstein-Barr Virus

EBV has been etiologically associated with a variety of clinical conditions ranging from sub-clinical infections to malignant lymphomas and nasopharyngeal carcinomas. Differential expression of EBV genes may explain the wide variety of diseases associated with the virus. DNA methylation may be one of the factors that regulates EBV gene expression. The viral genome has been shown to become progressively methylated in EBV transformed cells (Kintner & Sugden, 1981). Ernberg *et al.*, (1989) have reported that the degree EBV genomic DNA methylation was dependant on the host cell line. For example, only EBNA 1 was expressed in Burkitt's lymphoma cells (Rowe *et al.*, 1986) whereas all the EBNAs and LMPs were

expressed in lymphoblastoid cell lines (Rowe & Gregory, 1989). Analyses of the DNA region encoding the EBERs demonstrated that it was hypomethylated in transformed cells regardless of the methylation status of the EBV genome as a whole (Minarovits *et al.*, 1992).

Evidence that methylation may indeed regulate the expression of latent genes was evident when treatment of latently infected cells with 5-AzaC resulted in expression of increased levels of the latent gene products (Masucci *et al.*, 1989). It was also observed that the use of 5-azaC resulted in the upregulation of LMP and all EBNA genes, except EBNA 1, in EBV positive Burkitt's lymphoma cell line designated Rael (Masucci *et al.* 1989). Ben-Sasson & Klein (1981) demonstrated that treatment of latently infected cell lines with 5-AzaC dramatically increased the expression of EBV early antigens and thus converted a latent infection into a lytic infection. When EBV infected P3HR1 cells were treated with phorbol 12-myristate 13-acetate and *n*-butyrate, widespread cellular and viral genomic hypomethylation was observed (Szyf, Eliasson, Mann, Klein & Razin, 1985). Cuomo *et al.*, (1993) have recently demonstrated that there was an increased response from T cells towards EBV positive Burkitt's lymphoma cell lines after these cells lines had been treated with 5-AzaC. These findings suggested that EBV perhaps uses DNA methylation to suppress certain genes thus avoiding cytotoxic T cell recognition and clearance (Straus, Cohen, Tosato & Meier, 1993).

From the above examples, it is evident that DNA methylation plays a significant role in suppressing EBV gene expression in latently infected B

lymphocytes. Any B lymphocytes that express the EBV antigens on the surface are promptly removed by cytotoxic T cells. If, for some reason, the T cells are rendered ineffective, as the case for immunodeficient states the incidence of EBV induced lymphoproliferative disorders increases. Methylation appears to be an important genetic factor in maintaining the infected cell in a latent state and thereby allowing a carrier state for EBV infected B lymphocytes. It appears that these B lymphocytes act as reservoirs of EBV in an infected individual rather than nasopharyngeal epithelial cells as previously thought (Straus *et al.*, 1993).

MATERIALS AND METHODS

Cell Lines and Media

The cell lines used included EBV transformed human B lymphocytes (Raji) (Epstein *et al.*, 1966), EBV transformed marmoset B lymphocytes (B95.8) (Miller & Lipman, 1973) and an epithelial cell line from the fat head minnow (FHM). All the above cell lines were cultured in RPMI 1640 (GIBCO/BRL, Gaithersburg, Maryland) containing 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate and 0.25 μ g/ml amphotericin B). A set of Raji cells were also grown in complete RPMI medium, as described above, containing 6.0 μ M 5-AzaC (Sigma, St. Louis, Missouri). Raji cells and B95.8 cells were incubated at 37°C and the FHM cells were incubated at 33°C. Raji cells were a gift from John Sixbey, St. Jude's Children's Hospital, Memphis, Tennessee and B95.8 cells were a gift from Donna Patton, University of Nebraska, Lincoln, Nebraska.

Plasmids

The plasmid pDK39 (Dambaugh *et al.*, 1980), which contains the EBV *Bam*HI-L fragment in pBR322, was a gift from Elliot Kieff, Harvard Medical School, Boston, Massachusetts. The EBV *Bam*HI-L fragment contains the ORF (BLLF1) and

the promoter (BL-L1) for EBV gp350/220. The plasmid pLATE-CAT (Kenney *et al.*, 1989), a gift from Shannon Kenney, University of North Carolina, Chapel Hill, North Carolina, contains the promoter for EBV gp350/220 linked to the CAT gene.

Fusion Protocol

Monolayers of FHM cells were infected using 20 pfu/cell of FV3. Following adsorption at 4°C for 1 hour, the infected cells were incubated at 30°C for 4 hours. The cells were then scraped gently and resuspended in RPMI 1640 medium by pipetting. These resuspended cells were then fused with Raji cells in a ratio of 10 FHM cells:1 Raji cell using the polyethylene-glycol (PEG) (GIBCO/BRL, Gaithersburg, Maryland) protocol (Geftter, Margiules & Scharff, 1977). A mixture of FHM and Raji cells were placed in a 37°C water bath and 0.8 ml of PEG was added over a period of 1 minute with continuous slow shaking. Shaking was continued for an additional 2 minutes and 1.0 ml of serum free medium was added and shaken for 1 minute. This step was repeated twice, 20.0 ml of medium at 37°C was added and the suspension was centrifuged at 100 xg (IECPR-6000, 500 rpm) for 5 minutes. The supernatant was removed by aspiration and cells were resuspended in RPMI medium containing 20% FCS and incubated at 30°C for 4 hours prior to immunofluorescence. The controls consisted of unfused Raji cells incubated at 37°C and 30°C, unfused FV3 infected FHM cells incubated at 37°C and 30°C and Raji cells fused with FV3 infected FHM cells but incubated at 37°C, a non-permissive temperature for FV3.

Immunofluorescent Staining

The cells were washed three times with ice cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄; pH 7.04) and fixed for 30 seconds using ethanol precooled to -20°C. The fixed cells were inundated with an anti-gp350/220 mouse monoclonal antibody, 72A1 (Hoffman, Lazarowitz & Hayward, 1980), and incubated at 37°C in a humidified incubator for 2 hours. The cells were then washed five times with ice cold PBS and labeled with FITC-conjugated anti-mouse IgG (Sigma, St. Louis, Missouri) for 1 hour at 37°C. The cell smears were then washed five times with ice cold PBS and observed under an ultraviolet microscope.

In Vitro DNA Methylation

The reaction mixture contained 5.0 - 7.0 µg DNA, 80 µM S-adenosylmethionine, 4 units of *Hpa*II methylase (New England Biolabs, Beverly, Massachusetts), 8 units of *Hha*I methylase (New England Biolabs, Beverly, Massachusetts) and 3 µl of 10X methylase buffer (500 mM Tris-HCl, 100 mM EDTA, 50 mM 2-mercaptoethanol; pH 7.5) adjusted to a final volume of 30.0 µl with deionized water. This mixture was incubated at 37°C for 12 hours after which the methylase activity was terminated by heating the mixture at 65°C for 20 minutes. The methylated DNA was then ethanol precipitated. *Hpa*II and *Hha*I methylases methylate the internal cytosine of the sequences 5'-CCGG-3' and 5'-GCGC-3'

respectively. The methylation of the DNA was confirmed by digesting the DNA with the isoshizomeric restriction endonucleases *HpaII* and *MspI* (GIBCO/BRL, Gaithersburg, Maryland) which recognize the sequence 5'-CCGG-3' and have the capability of differentiating between methylated and unmethylated sequences (Waalwijk & Flavell, 1978).

Transfection

Transfections were performed using TransfectACE or Transfectin (GIBCO/BRL, Gaithersburg, Maryland), a liposome mediated DNA transfer system, as suggested by the supplier. Two to five micrograms of pLATE-CAT was gently mixed with 12.0 μ l of TransfectACE/Transfectin reagent in 1.0 μ l of Opti-MEM (GIBCO/BRL, Gaithersburg, Maryland) at room temperature (RT) for 15 minutes. The DNA-liposome complex was added to 2.5×10^6 - 1.0×10^7 cells pre-washed with Opti-MEM and incubated at 37°C for 5 hours. An additional 1.0 ml of Opti-MEM was added and the incubation at 37°C was continued for another 10 hours.

Chloramphenicol Acetyltransferase Assay

CAT assays were performed with a commercially available kit (Promega, Madison, Wisconsin). The cells were washed three times with ice cold PBS and incubated at RT for 5 minutes with 0.6 ml of TEN buffer (40 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 7.5). After incubation, the cells were centrifuged at 100 xg (Eppendorf 5415C; 1000 rpm) for 10 minutes, resuspended in 75.0 μ l of 250 mM

Tris-HCl (pH 8.0), subjected to three freeze-thaw cycles, heated at 65°C for 10 minutes to inactivate endogenous acetylase activity and centrifuged at 100 xg (Eppendorf 5415C; 1000 rpm) for 10 minutes. The supernatant (75.0 μ l) was mixed with 2.0 μ l [14 C]-chloramphenicol (specific activity 54.2 mCi/nmol) (New England Nuclear, Wilmington, Delaware), 10.0 μ l n-butyryl Coenzyme A (5mg/ml) and 38.0 μ l 250 mM Tris-HCl, pH 8.0. The final volume of the mixture was brought up to 125.0 μ l with deionized water and incubated at 37°C for 18 - 20 hours. The CAT reaction was terminated by adding 125.0 μ l of ethyl acetate. The mixture was vortexed for 1 minute and centrifuged at 12,000 xg (Eppendorf 5415C; 14,000 rpm) for 3 minutes. The upper organic phase was transferred to a new eppendorf tube and evaporated in a vacuum oven. The residue was then resuspended in 15.0 μ l of ethyl acetate and spotted on a silica gel thin layer chromatography (TLC) plate (EM Industries, Gibbstown, New Jersey). The plate was placed in a closed tank containing one-hour pre-equilibrated chloroform:methanol (97:3) running solvent. Following a 1.5 hours run, the plates were removed from the tank, air dried and covered with plastic wrap. The CAT activity was detected by exposing the TLC plate to X-OMAT AR X-ray film (Eastman Kodak Company, Rochester, New York).

DNA Extraction

Nuclear DNA was extracted from Raji cells treated with 6.0 μ M 5-AzaC and untreated Raji cells using the following procedure. After the cells were washed three times in ice cold PBS and subjected to three freeze-thaw cycles, 0.5 ml of digestion

buffer (100 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 0.5% SDS, pH 8.0) and 100 μ l of proteinase K (10 mg/ml) (BRL, Gaithersburg, Maryland) were added and the mixture was incubated at 50°C overnight. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the eppendorf tube, vortexed briefly, centrifuged at 12,000 xg (Eppendorf 5415C; 14,000 rpm) for 1 minute and the upper phase transferred to a new eppendorf tube. This step was repeated three times and then 1/10 volume 3 M sodium acetate and 2 volumes of 100% ethyl alcohol (precooled to -20°C) were added and the tubes were incubated in an ethanol-dry ice bath for 10 minutes. This mixture was centrifuged at 12,000 xg (Eppendorf 5415C; 14,000 rpm) for 10 minutes. The supernatant was discarded and the pellet was washed in 70% ethyl alcohol. The pellet was then air dried and resuspended in 50.0 μ l of deionized water.

Southern Blotting

The probe was obtained by digesting pDK39 with the restriction endonucleases *Bam*HI, *Eco*RI and *Hind*III (GIBCO/BRL, Gaithersburg, Maryland). Three micrograms of the plasmid was digested with 15 units of each enzyme in a buffer (50 mM Tris-HCl, 10 mM NaCl; pH 8.0) and the mixture was incubated at 37°C overnight prior to electrophoresis in a 1.2% agarose gel. A 648 bp DNA fragment, containing the promoter sequence for gp350/220 was isolated, labeled with [α -³²P]-dCTP by the random primer method (Feinberg & Vogelstein, 1983) and used to probe the blot. The nuclear DNA extracted from Raji cells was digested with the

indicated restriction endonuclease(s), electrophoresed in a 1% agarose gel and transferred to nylon membrane (ICN, Irvine, California). The buffer used for *Bam*HI digestions contained 500 mM Tris-HCl (pH 8.0), 10 mM MgCl₂ and 100 mM NaCl, while the buffer used for the double digestions (*Bam*HI/*Hpa*II and *Bam*HI/*Msp*I) contained 50 mM Tris-HCl (pH 8.0) and 10 mM MgCl₂. The membrane was soaked in 6X SSC for 2 minutes, air dried, baked at 80°C in a vacuum oven for 2 hours and soaked in 6X SSC for 5 minutes. Prehybridization of the membrane was done for 6 hours in a solution containing 6X SSC, 0.2% Denhardt's solution, 50 µg of depurinated salmon sperm DNA (Sigma, St. Louis, Missouri) and 1% SDS. The prehybridizing fluid was completely drained from the plastic bag and hybridization was done for 12 hours in a solution containing 6X SSC, 50% deionized formamide, 50 µg of depurinated salmon sperm DNA, 1% SDS and the radioactive probe. The blot was then washed successively in 6X SSC with 0.2% SDS at RT for 5 minutes, 2X SSC with 0.1% SDS at RT for 15 minutes and finally 6 times in 0.1X SSC with 0.1% SDS at 42°C (30 minutes each cycle). The membrane was allowed to drip dry, wrapped in plastic film and autoradiography was done at -20°C.

Polymerase Chain Reaction (PCR)

PCR was done according to the procedure described by (Saiki *et al.*, 1988). A 1.0 µg sample of Raji DNA extracted from 5-AzaC treated and untreated cells was used in each PCR. The reaction mix consisted of 200 µM each of dGTP, dCTP, dATP and dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 1 µM

of each primer and 2.5 units of Taq polymerase (Perkin-Elmer, Norwalk, Connecticut) in a total volume of 100.0 μ l. The primers (5'-GGATCAGGCTCTGGATGGTGTACTGACA-3' and 5'-GAAGCTGGATTTCTCCGACGATCTCTAAT-3') (National Biosciences, Plymouth, Minnesota) were selected from sequences flanking either end of the gp350/220 promoter region. The DNA was initially denatured at 94°C for 3 minutes followed by 30 cycles each of 94°C for 1 minute, 55°C for 2 minutes and 72°C for 3 minutes. The samples were analyzed using a 1.4% agarose gel.

RESULTS

Expression of EBV gp350/220 in Raji Cells

EBV is known to regulate the expression of at least some of its genes by DNA methylation (Ben-Sasson & Klein, 1981; Szyf, Eliasson, Mann, Klein & Razin, 1985; Masucci *et al.*, 1989; Cuomo *et al.*, 1993; see review of literature for details). Raji cells contain the ORF and promoter for gp350/220 (Biggin, Bodescot, Perricaudet & Farrell, 1987; Hatfull, Bankier, Barrell & Farrell, 1988) but do not express the gene (Kirchner, Bornkamm & Polack, 1991). FV3 contains a highly methylated genome and has the ability to induce the expression of foreign genes previously silenced by DNA methylation (Thompson, Granoff & Willis, 1986; Thompson, Granoff & Willis, 1988; reviewed in Willis, Essani, Goorha, Thompson & Granoff, 1989). It was therefore reasoned that if gp350/220 expression was suppressed via DNA methylation, FV3 infection of Raji cells should be able to induce the expression of the gene. Difficulty in infecting Raji cells with FV3 necessitated an indirect approach where Raji cells were fused with FV3 infected FHM cells. Raji cells fused with FV3 infected FHM cells and incubated at 30°C consistently expressed gp350/220 (Fig. 1B) as demonstrated by indirect immunofluorescence using 72A1, a mouse monoclonal anti-gp350/220 antibody (Hoffman, Lazarowitz & Hayward, 1980). In parallel experiments, unfused FHM cells or Raji cells (Fig. 1A and 1B) and

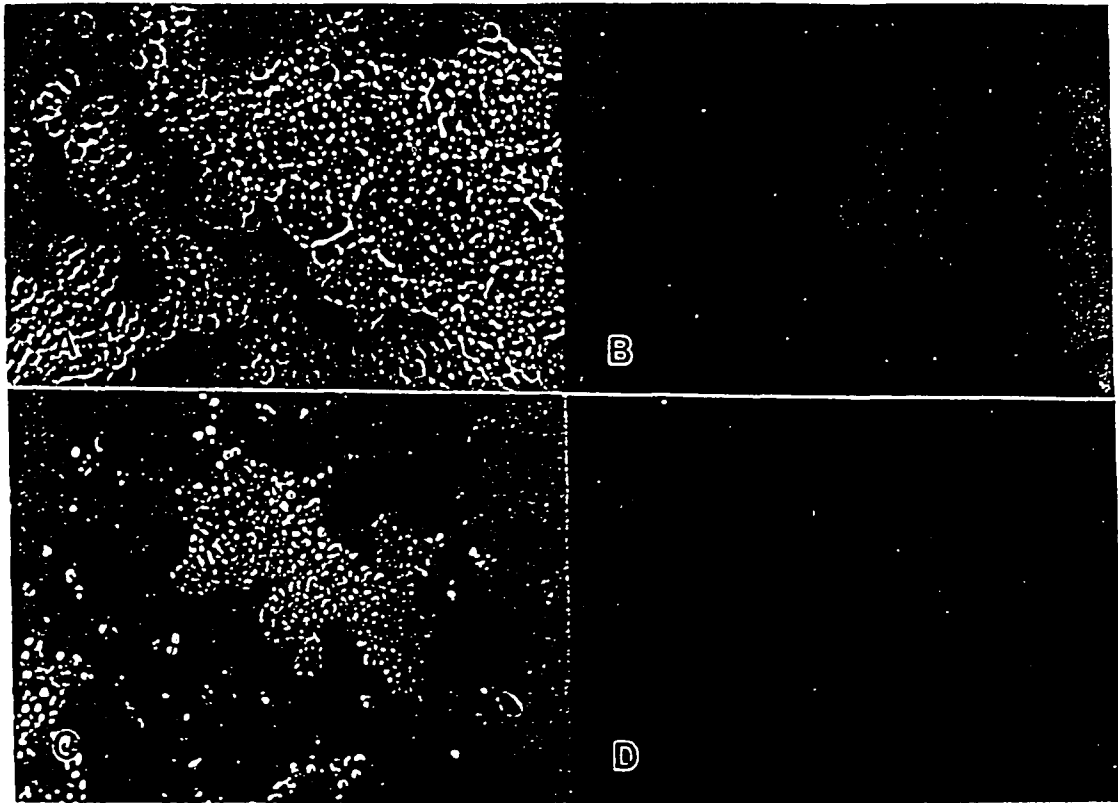


Figure 1. Expression of gp350/220 in Raji Cells Fused With FV3 Infected FHM Cells.

Only Raji cells fused with FV3 infected FHM cells show high levels of gp350/220 expression (B), as compared to Raji cells fused with uninfected FHM cells (D) or unfused Raji cells (B and D). A and C represent phase contrast micrographs of the corresponding B and D immunofluorescent images.

uninfected FHM cells fused with Raji cells (Fig. 1D) failed to express gp350/220. Furthermore, when FV3 infected FHM cells were fused with Raji cells and incubated at 37°C (non-permissive temperature for FV3), expression of gp350/220 was not observed (results not shown). These results confirmed that a potentially functional gp350/220 gene is present in Raji cells but is not expressed. Since FV3 is capable of inducing the expression of this gene it may be possible that gp350/220 expression is regulated by DNA methylation. Attempts were made to induce the expression of gp350/220 using 5-Azac, a potent inhibitor of DNA methyltransferase (Jones & Taylor, 1980), which has been shown to induce the expression of a number of EBV genes (Ben-Sasson & Klein, 1981; Masucci *et al.*, 1989). However, the use of 5-AzaC (6.0 μ M) resulted in inconsistent expression of gp350/220. This finding was unexpected and may have resulted from different mechanisms by which 5-AzaC and FV3 facilitated the expression of genes silenced by DNA methylation. Treatment with 5-AzaC results in irreversible inactivation of the DNA methyltransferase (Jones & Taylor, 1980). Replicating cells treated with 5-AzaC contain unmethylated newly synthesized DNA which should now be accessible to transcriptional factors and therefore transcribed. FV3, on the other hand, allows methylated DNA to be transcribed by yet unknown mechanism(s) (Willis, Essani, Goorha, Thompson & Granoff, 1989).

In Vitro Methylation of pLATE-CAT Suppresses CAT Activity in Transient Transfection

In vitro transfection studies involving a promoter linked to a reporter gene are very helpful in evaluating the effect of DNA methylation. If DNA methylation had a repressive effect on the methylated promoter, expression of the reporter gene should not be detected. *In vitro* methylation of pLATE-CAT and its transfection into B95.8 cells provided further evidence that the expression of gp350/220 may indeed be suppressed by DNA methylation. Transfection of unmethylated pLATE-CAT into B95.8 cells resulted in the expression of CAT activity as expected (Fig 2. lane 2), while transfection of *in vitro* methylated construct into B95.8 cells resulted in significant transcriptional suppression (Fig. 2. lane 3). The role of DNA methylation in these experiments was further corroborated by fusing FV3 infected FHM cells with B95.8 cells transfected with the methylated construct. The methylated construct now expressed CAT activity (Fig. 2. lane 4) at comparable levels with the unmethylated construct (Fig. 2. lane 2). This reversal of transcriptional suppression of the methylated construct by FV3 suggests that gp350/220 gene is sensitive to sequence specific DNA methylation. Untransfected FHM and B95.8 cells, either individually (Fig. 2. lanes 5 and 6) or fused (Fig. 2. lane 7) failed to show CAT activity as expected. The differences in CAT activity seen between the unmethylated and methylated plasmids and also the reversal of suppression of the methylated plasmid by FV3 infection demonstrate that methylation may be a regulatory factor in gp350/220 expression.



Figure 2. *In Vitro* Methylation of gp350/220 Promoter Suppresses Transcriptional Activity of the Reporter Gene.

In vitro methylation of pLATE-CAT and subsequent transfection into B95.8 cells results in significant suppression of CAT activity (lane 3) as compared with the unmethylated construct transfected into B95.8 cells (lane 2). The suppressive state of the methylated construct was reversed and significantly higher levels of transcriptional activity was observed when B95.8 cells transfected with the methylated construct were fused with FV3 infected FHM cells (lane 4). No transcriptional activity of the CAT gene was detected in untransfected B95.8 cells (lane 5), FHM cells (lane 6), or when untransfected B95.8 cells were fused with uninfected FHM cells (lane 7). Lane 1 represents a positive control with exogenous CAT enzyme.

Promoter for gp350/220 is Not Methylated

Immunofluorescent and transient transfection experiments described above strongly suggest that methylation does have an inhibitory effect on the expression of gp350/220. To provide direct evidence for the involvement of sequence specific DNA methylation in the expression of gp350/220 gene, Southern blot analyses were undertaken. DNA from Raji cells treated with 5-AzaC (6.0 μ M) and untreated cells was probed with BL-L1 promoter sequence. When Raji nuclear DNA was digested with *Bam*HI (Fig. 3. lanes 2 and 3), the probe specifically hybridized to a DNA fragment of similar size (5053 bp) to EBV *Bam*HI-L fragment (Fig. 3. lane 1). This suggested that a full length EBV *Bam*HI-L fragment existed in Raji cells (Fig. 3. lanes 2 and 3), confirming earlier studies (Hatfull, Bankier, Barrell & Farrell, 1988). To analyze the methylation status of the gp250/220 promoter region, Raji cell DNA was double-digested with either *Bam*HI/*Hpa*II or *Bam*HI/*Msp*I. *Hpa*II and *Msp*I are isoschizomeric enzymes which recognize the sequence CCGG. These enzymes are very useful in analyzing the methylation status of sequences as *Hpa*II does not restrict this sequence if the internal cytosine is methylated, while *Msp*I restricts the sequence irrespective of the methylation status of the internal cytosine (Waalwijk & Flavell, 1978). *Hpa*II digestion of DNA from 5-AzaC treated and untreated Raji cells indicated that the BL-L1 promoter is not methylated. This is evident in Figure 3 by the disappearance of the 5053 bp and the appearance of a \approx 200 bp band in the DNA from 5-AzaC treated (lane 3) and untreated (lane 4) Raji cells. *Msp*I digestions (lanes

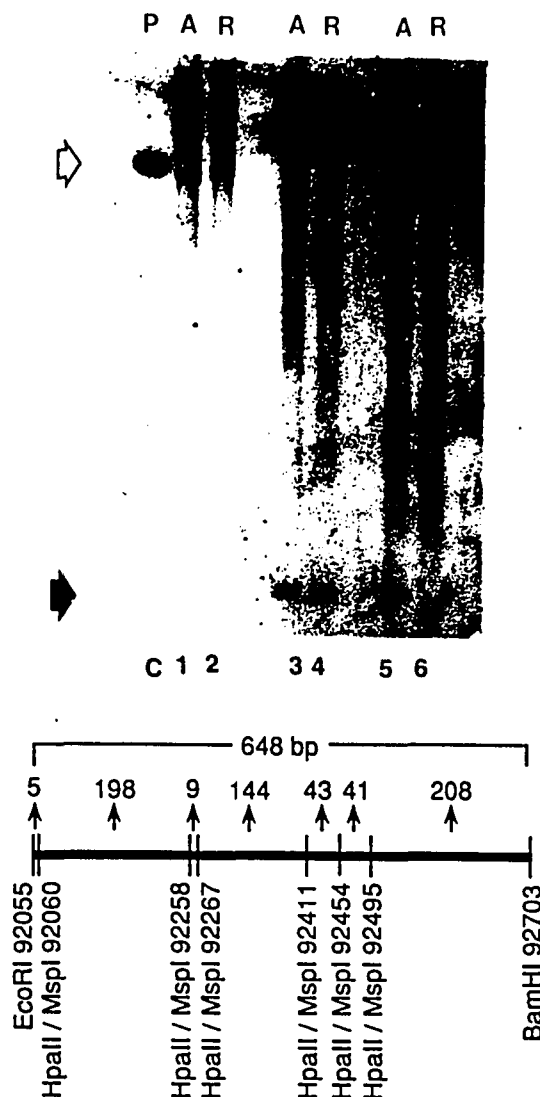


Figure 3. The BL-L1 Promoter Sequences in Normal Raji Cells Are Not Methylated.

Southern blot analyses show that BL-L1 promoter sequences in normal Raji cells were not methylated. A and R represent DNA from 5-AzaC treated and untreated Raji cells respectively. P represents the plasmid pDK39 containing EBV *Bam*HI-L fragment where BLLF1 and BL-L1 are located. Lane C shows the EBV *Bam*HI-L fragment (5053 bp) obtained by restricting pDK39 with *Bam*HI. A similar fragment (open arrow) resulted when total nuclear DNA from Raji cells (A and R) was digested with *Bam*HI (lanes 1 and 2). Double digestion of A and R DNA with *Bam*HI/*Hpa*II or *Bam*HI/*Msp*I revealed that the BL-L1 promoter was not methylated as evident by the complete digestion of the 5053 bp fragment (lanes 3, 4 and 5, 6 respectively), and the appearance of a ≈ 200 bp fragment (solid arrow). The bold horizontal line represents the probe showing restriction sites and the resulting fragments.

5 and 6) while showing the disappearance of the 5053 bp band as expected, did not show the 200 bp band seen in *HpaII* digestions. Since identical results were not obtained with *MspI* digestions, a modified PCR was used to confirm the methylation status of BL-L1 promoter. The idea was to select two primers flanking either end of the BL-L1 sequence and to see whether this DNA sequence could be amplified. In these experiments, the DNA from 5-Azac treated and untreated Raji cells was digested with either *HpaII* or *MspI* prior to PCR. If every internal cytosine in the sequence 5'-CCGG-3' was methylated, *HpaII* would not be able to digest the DNA and therefore amplification should occur. In contrast, *MspI* restricts the DNA regardless of its methylation status and amplification should not be seen. In control experiments to ascertain the feasibility of this experimental approach, Raji cell DNA was exogenously methylated *in vitro* prior to digestion with *HpaII* or *MspI*. PCR analyses of this *in vitro* methylated DNA (as confirmed with *HpaII* and *MspI* restrictions), indicated that the experimental approach was indeed feasible. This is evident in Figure 4, where methylated Raji DNA showed amplification after digestion with *HpaII* (lane 8) while there was no such amplification after digestion with *MspI* (lane 9). Figure 4 also shows that DNA from Raji cells, regardless of treatment with 5-AzaC, was restricted by *HpaII* and hence not methylated (lanes 4 and 5). Similar results were obtained when this DNA was restricted with *MspI* (lanes 6 and 7). Confirmation that the BL-L1 is present in Raji cells was obtained when unrestricted Raji cell DNA was amplified (arrowhead in Fig. 4, lanes 2 and 3). The size of the amplified fragment was ≈ 686 bp, as theoretically calculated from the EBV genomic

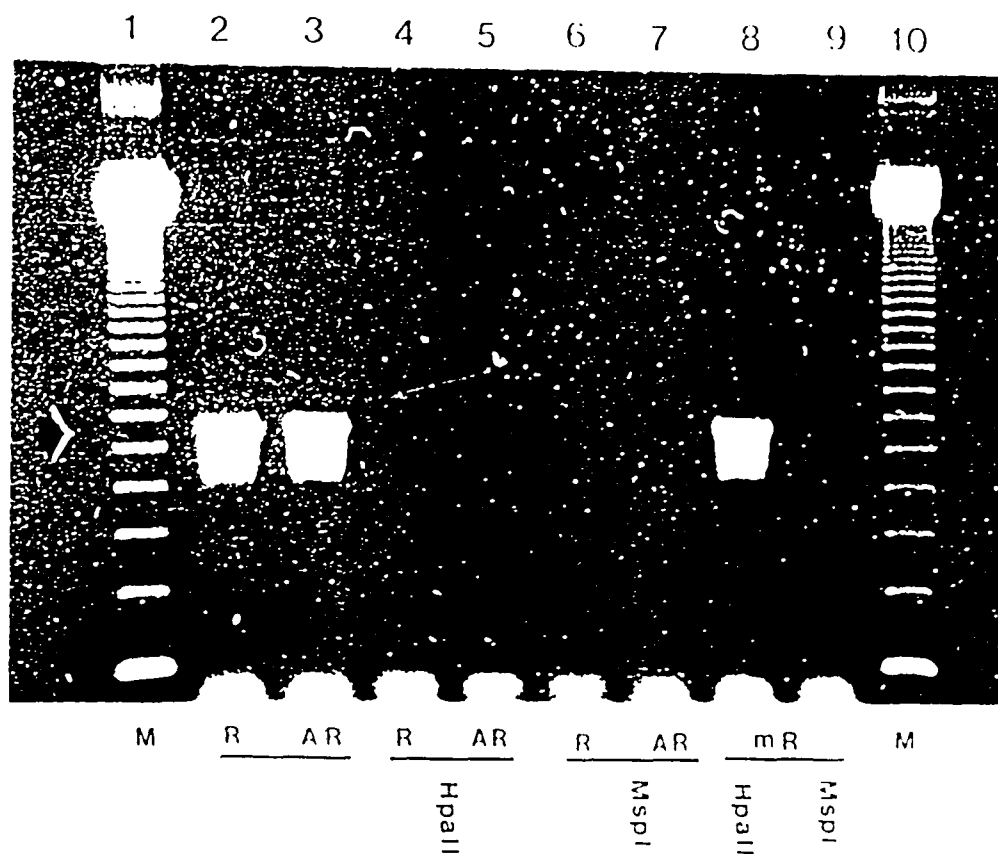


Figure 4. Polymerase Chain Reaction Failed to Amplify BL-L1 Promoter Sequences Following Restriction With *HpaII*.

The primers used were selected from sequences on either side of the BL-L1 sequence. Lanes 2 and 3 containing DNA from untreated (R) and 5-AzaC treated (AR) Raji cells was amplified (arrow head pointing to the ≈ 686 bp band) when subjected to PCR indicating that the BL-L1 sequence is present in Raji cells. *HpaII* or *MspI* restricted DNA, from untreated and 5-AzaC treated cells (lanes 4, 5 and 6, 7 respectively) did not show any amplification indicating that the DNA was not methylated. Lanes 8 and 9 represent a control experiment where Raji cell DNA was methylated *in vitro* using *HpaII* and *HhaI* methylases (mR). This methylated DNA when digested with *HpaII* (lane 8) showed amplification, while digestion with *MspI* prior to PCR did not result in amplification (lane 9). DNA markers are shown in lanes 1 and 10.

sequence. Furthermore, this fragment hybridized specifically to the 648 bp probe described earlier (results not shown). These results in conjunction with the southern blot analyses indicate that perhaps none of the internal cytosines in the sequence 5'-CCGG-3' are methylated in Raji cells. These results were rather surprising and when taken together with earlier results from the immunofluorescent and transfection experiments provided evidence that the promoter region of gp350/220 is regulated by the presence of a yet unidentified transactivator that is sensitive to DNA methylation.

DISCUSSION

We used two different experimental approaches, fusion experiments and *in vitro* transient transfection experiments, to demonstrate that the expression of gp350/220 is regulated by DNA methylation. This study also provides clear evidence that the promoter for gp350/220 is not methylated in Raji cells. The data presented here, therefore, leads to the logical conclusion that DNA methylation regulates the expression of a methylation-sensitive transactivator which in turn regulated the expression of gp350/220 gene in Raji cells. Thus, the transcriptional regulation of the EBV genome appears to be much more complicated than previously thought (reviewed in Kieff & Liebowitz, 1990). Simple analyses of the methylation status of individual promoters and their relationship with the expression of genes, though valid in some cases, may be an over simplistic view of the role of DNA methylation and the mechanisms by which it regulates EBV gene expression.

Although the existence of transactivators in the EBV genome has reported earlier (reviewed in Kieff & Liebowitz, 1990), the existence of a transactivator for gp350/220 appears to be contradictory to previously published studies where gp350/220 has been expressed in a variety of expression systems. These expression systems include rodent and primate cells (Whang, Silberklang, Morgan & Kieff, 1987), varicella-zoster virus (Lowe, Keller & Keech, 1987), and insect cells (Nuebling, Buck, Boos, von Deimling & Mueller-Lantzsch, 1992). A particularly

interesting study involved transfection of pZip-MA, a plasmid containing the promoter and ORF for gp350/220, into Raji cells (Khyatti, Patel, Stefanescu & Menezes, 1991). These transfected Raji cells were reported to have expressed high levels of gp350/220. This laboratory obtained these cells (courtesy of Josè Menezes, University of Montreal), and in our hands the level of expression of gp350/220 decreased over several generations of passage even in the presence of G418 selection pressure. This suggests that simple integration of a sequence that codes for gp350/220 does not perfectly correlate to its expression. Fusion of pZip-MA transfected Raji cells with FV3 infected FHM cells significantly increased the expression of gp350/220. These results, taken together with the fact that complete *HpaII* and *HhaI* methylation does not result in 100% suppression of CAT activity in pLATE-CAT transfected cells (Fig. 2. lane 3) suggests that a high copy number of the transfected gene may result in some degree of expression of the gene.

Transactivators (BZLF1 and BRLF1) play an important regulatory role in the induction of EBV early lytic cycle genes (reviewed in Kieff & Liebowitz, 1990). It seems probable that some of these transactivators may be regulated by DNA methylation in latently infected cells. A study done in this laboratory (Tao, 1992) showed that methylation played a role in the regulation of both the transactivator and the gene of interest. In this study a set of known EBV transactivators (BZLF1 and BRLF1) and a set of EBV early gene promoters (EBV immediate-early and early antigen-diffuse) linked to the CAT gene were chosen. The transactivators and the plasmids were either methylated *in vitro* or left unmethylated. Different combinations

of the transactivators and the plasmids were transfected into different cell lines. It was shown that in some cases the methylation of the transactivator down regulated gene expression in a cell specific manner.

As mentioned earlier, the suppression of lytic cycle genes may help evade the host immune system. It is of advantage to the virus to suppress the expression of gp350/220, a major neutralizing antigen, in order to avoid immune elimination. It is possible that the virus utilizes DNA methylation mediated suppression of the transactivator to achieve this goal.

The experiments described here also provide a unique example of interactions between two unrelated viruses and how one virus may modulate the gene expression profile of another virus in the same host cell. Extensive comparative analysis of the cloned EBV genomic DNA using IntelliGenetics software showed that EBV does not appear to encode its own DNA methyltransferase but utilizes the host DNA methyltransferase. These results therefore emphasize the importance of host factors and the presence of other organisms (in this case, an unrelated virus) which may alter the outcome of a viral infection.

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