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RELATIONSHIP BETWEEN METHYLATION STATUS AND EXPRESSION OF AN EPSTEIN-BARR VIRUS (EBV) CAPSID ANTIGEN GENE

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SUMMARY: The methylation status of the 160 kD viral capsid antigen (VCA) gene promoter was determined by hybridization analysis. The semi-permissive marmoset cell line FF41-1 lacked cytosine methylation in approximately three quarters of the VCA promoter CpG dinucleotide residues. In the stringently infected HH514CL16 cell line the same CpG residues were methylated in three quarters of the genomes. 5'deoxy-5'-S-isobutyladenosine (SIBA), a DNA methylase inhibitor, was utilized to disrupt the EBV latent state. As determined by flow cytometry, SIBA treatment significantly increased expression of VCA. The VCA promoter was hypomethylated in VCA-positive FF41-1 cells sorted by flow cytometry. While hypomethylation alone was not sufficient for VCA transcriptional activity, the absence of methylation of VCA promoter CpG dinucleotide residues was associated with expression of VCA.

The outcome of B lymphocyte infection by EBV is dependent on differential expression of the viral genome. Latent EBV infection is characterized by restricted gene expression and generation of continuously proliferating lymphoblastoid cell lines (LCLs) (1). Expression of viral genes associated with lytic infection results in the synthesis of structural proteins, the production of infectious virus particles, and subsequent death of the infected cell (1). The switch from the latent to lytic infection can occur spontaneously (1) or be induced with phorbol esters (2), nucleoside analogs (3), or S-adenosylhomocysteine (SAH) analogs (4).

Previously we showed that inhibitors of DNA methylase, such as the SAH analogs SIBA and sinefungin (SF), inhibit EBV-induced transformation of human B lymphocytes (4). SIBA or SF treatment of the FF41-1 marmoset cell line induces a significant increase in the number of cells expressing VCA and hypomethylation of the EBV EcoRI J fragment (4). In this study, the relationship between the methylation status of the promoter region of the 160 kD VCA gene and its expression in selected EBV-carrying cell lines was examined. SIBA, which metabolically inhibits DNA methylation (5), was used as to disrupt the EBV

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latency. Flow cytometry was used to sort SIBA-induced FF41-1 cells in order to examine CpG dinucleotide methylation within the VCA promoter region in VCA-expressing and nonexpressing cell populations. The results indicate a correlation between hypomethylation of CpG dinucleotide residues within the promoter region and VCA expression.

MATERIALS AND METHODS

Cell Lines: FF41-1 (6), a permissively infected marmoset LCL, and HH514CL16 (7), a transformation-defective Burkitt's lymphoma cell line, were provided by Dr. George Miller, Yale University, New Haven, CT. All cell lines were maintained as exponentially growing cultures as described (6,7).

Induction of Viral Antigen Expression: Exponentially growing cells were plated at 5 x 10^5 cells per ml of fresh RPMI 1640 medium with 10% fetal calf serum (Gibco, Grand Island, N.Y.) with or without 10 ug/ml SIBA (Sigma Chemical Co., St. Louis, Mo.) for 24 h (4). Flow cytometry was performed using the technique of Mann et al. (8) and a Coulter EPICS V flow cytometer (Coulter Electronics, Hialeah, FL).

Plasmids: Plasmids of subcloned EBV genomic fragments, EcoRI E fragment, pCCYC184-Eco E, and EcoRI H fragment pCCYC184-Eco H, were obtained from Dr. George Miller. The VCA promoter (EHL1 before BcLF1) (9) contained within a 1450 bp EcoRI-HindIII fragment (1450H) of the EcoRI H genomic fragment, as well as downstream coding sequences within the 310 bp HindIII-EcoRI fragment (310E) of the EcoRI E genomic fragment, were subcloned (Fig. 1). The 1450H fragment includes 965 bp upstream from the consensus promoter sequence 5'-TATTAAA-3', as well as 485 bp downstream. EcoRI-HindIII (New England Biolabs, Beverly, MA) - digested pCCYC184-Eco H DNA was electrophoretically separated on a 1% agarose gel and the 1450 bp fragment was electroluted onto DEAE paper (Schleicher and Schuell, Keene, NH). Following elution from the DEAE paper, the fragment was ligated into EcoRI-HindIII-digested pGemI plasmid (Promega, Biotec, Madison, WI) with T4 DNA ligase (New England Biolabs) and used to transform E. coli strain TB-1. The 310E fragment which encodes 310 bp of translated VCA mRNA (9) was similarly subcloned into the pGemI plasmid.

Southern Hybridization Analysis: DNA was isolated from unfixed cells by the method of Painter and Schaeffer (10). DNA from cells fixed with formalin/methanol for flow cytometry was isolated by the method of Goelz et al (11). DNAs were digested to completion with restriction endonucleases according to the manufacturers specified buffer conditions at 37°C (New England Biolabs). Digested DNA was electrophoretically separated in 2.5% agarose gels. Blotting and hybridization were carried out as described (12). Probes for hybridization were radiolabeled by oligonucleotide-primed DNA synthesis using 50 uCi of [alpha-^{32}P]dCTP (Amersham, Arlington Heights, IL) and 200 ng of fragment in combination with random oligonucleotides (Pharmacia, Piscataway, NJ) and Klenow polymerase (Pharmacia). Hybridized filters were exposed to Kodak XAR-5 x-ray film with two Quanta III intensifying screens (Dupont Cronex, Wilmington, DE) for one to four days at -80°C.

RESULTS AND DISCUSSION

Analysis of VCA Expression

As determined by flow cytometry, the proportion of FF41-1 cells expressing VCA increased from 3.5% to 28% following stimulation with 10 ug/ml SIBA for 24 h (Fig. 2A). An increase in VCA fluorescence from 0.4% to 2.4% was observed following SIBA stimulation of HH514CL16 cells (Fig. 2B).

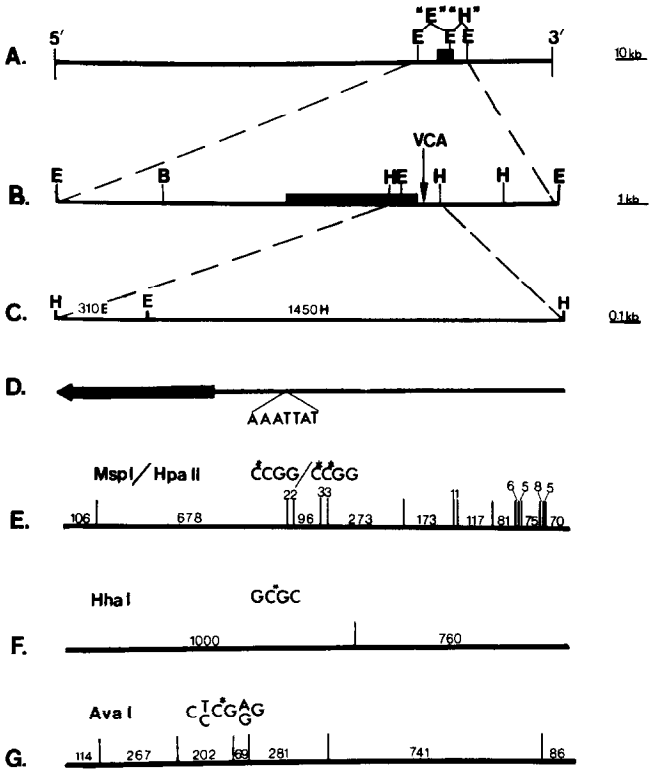

Figure 1. Physical map of the 5′ region of the VCA gene determined from the published DNA sequence of the B95-8 strain of EBV (11). (A) Linear map of the EBV genome, 5′ to 3′, left to right denoting the EcoRI "E" and "H" fragments. Coding region of VCA gene marked by raised bar. (B) Expanded map of EcoRI "E" and "H" fragments. Arrow points to VCA promoter (EHL1 before BcLF1). (C) Expanded map of the 1760 bp fragment contained within HindIII sites. 310E and 1450H represent the two fragments subcloned into pGemI. (D) EHL1 promoter (5′TATTAAA 3′) 244 bp upstream from the coding region of the VCA gene. MspI/HpaII (E), HhaI (F), and AvaI (G) restriction map of the 1760 bp Hind III fragment. Asterisk denotes sites of 5-methyldeoxycytosine which inhibits restriction. E = EcoRI; B = BamHI; H = HindIII.

Methylation Status of VCA Promoter

Approximately one-quarter of the VCA promoter region sequences in uninduced FF41-1 cells were methylated as evidenced by the presence of an undigested band migrating at 1760 bp in HhaI-HindIII and HpaII-HindIII digests (Fig. 3, A & B). The remaining bands represented completely digested restriction fragments. The HpaII isoschizomer MspI, which is not methylation sensitive, did not generate the 1760 bp fragment. In the AvaI-HindIII digest approximately one-third of the hybridized probe was located in the undigested 1760 bp band as well as a 1293 bp band representing a partial digest. The remaining fragments were complete digestion products. Thus, in the VCA region of the collective viral population of uninduced FF41-1 cells, approximately three-quarters of the CpG dinucleotide residues analyzed were not methylated within the HpaII (15 residues), HhaI (1 residue), and AvaI (6 residues) restriction endonucleases recognition sites (Fig.

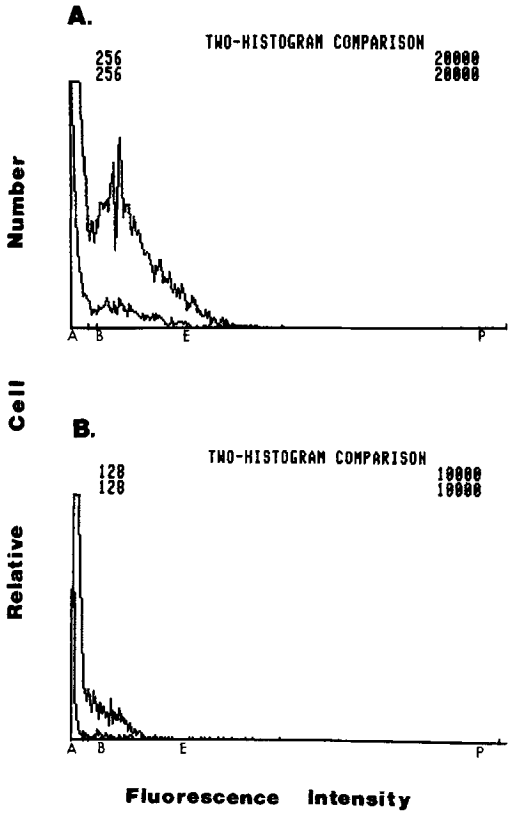

Figure 2. Flow cytometry two-histogram comparisons of VCA expression in FF41-1 and HH514CL16 cells. SIBA-induced (upper curves) and uninduced cells (lower curves). Profiles (A) 20,000 FF41-1 cells, maximum vertical axis = 256 cells and (B) 10,000 HH514CL16 cells, maximum vertical axis = 128 cells. Logarithmic representation of green fluorescence on horizontal axis divided into channels A through P. Cells in channel A display no fluorescence.

3, C). The methylation status of the FF41-1 VCA promoter did not appear to change following stimulation with SIBA for 24 h (Fig. 3, A & B).

The constitutive methylation status of the VCA promoter region in HH514CL16 cells differed from that of FF41-1 cells. MspI-HindIII digestion generated the same fragments observed in FF41-1 (data not shown). However, the 678 bp fragment clearly seen in HpaII-HindIII digests of FF41-1 was barely visible in the same digest of HH514CL16 (Fig. 4, A). The 273 and 173 bp fragments were not generated in the HpaII-HindIII digests of HH514CL16. Approximately three-quarters of the hybridized signal bound to the 1760 bp fragment representing undigested VCA promoter region in both the HpaII-HindIII and HhaI-HindIII (note HhaI restriction polymorphism) digests. Despite the apparent hypermethylation of the majority of the HpaII and HhaI sites, there were two CpG dinucleotide residues that lacked methylation in approximately three-quarters of the collective viral genomes. AvaI-HindIII digestion generated a major band of 1293 bp. The AvaI sites which generate this band are located 879 bp before and 414 bp after the TATA box (Fig. 4, B). The 281 or 202 bp fragments found in FF41-1 were not generated in the

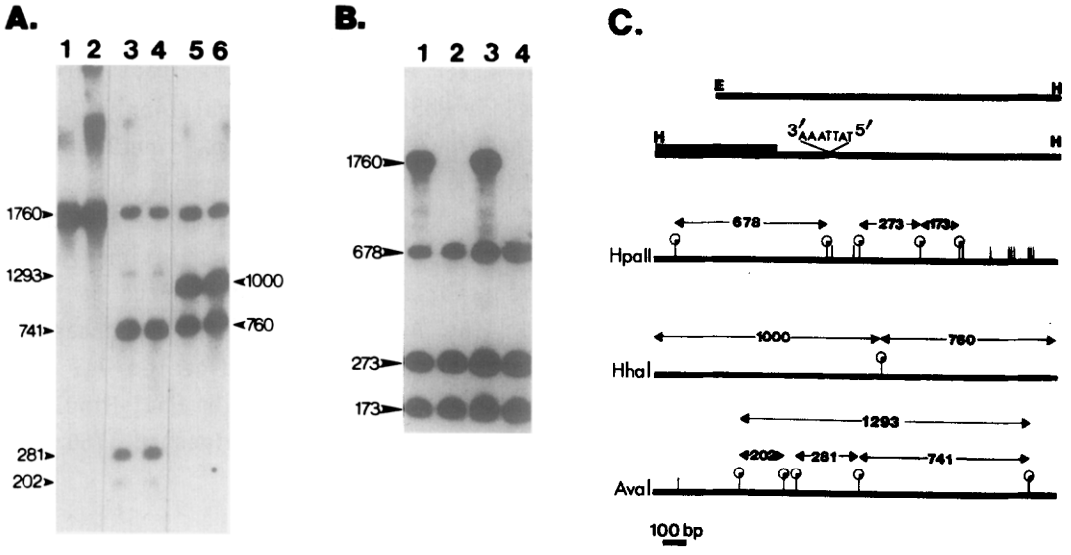

Figure 3. Southern blots of DNA from uninduced and SIBA-induced FF41-1 cells hybridized with the 1450H probe. Blot A lanes contain DNA digested with HindIII (1) uninduced and (2) SIBA-induced; AvaI-HindIII (3) uninduced and (4) SIBA-induced; or HhaI-HindIII (5) uninduced and (6) SIBA-induced. Blot B lanes contain DNA digested with HpaII-HindIII (1) SIBA-induced and (3) uninduced; or MspI-HindIII (2) SIBA-induced and (4) uninduced. DNA fragment sizes in base pairs calculated from BstEII-digested lambda phage DNA and HaeIII-digested ϕX174 DNA markers are indicated. Map (C) represents constitutive methylation status of specific CpG dinucleotide residues in the HpaII, HhaI, and AvaI restriction sites in the 5′ region of FF41-1 EBV VCA gene. Quarter-closed circles, ◔, represent CpG dinucleotide residues methylated in approximately 25% of the genomes in the collective population based on relative intensity of hybridization signal.

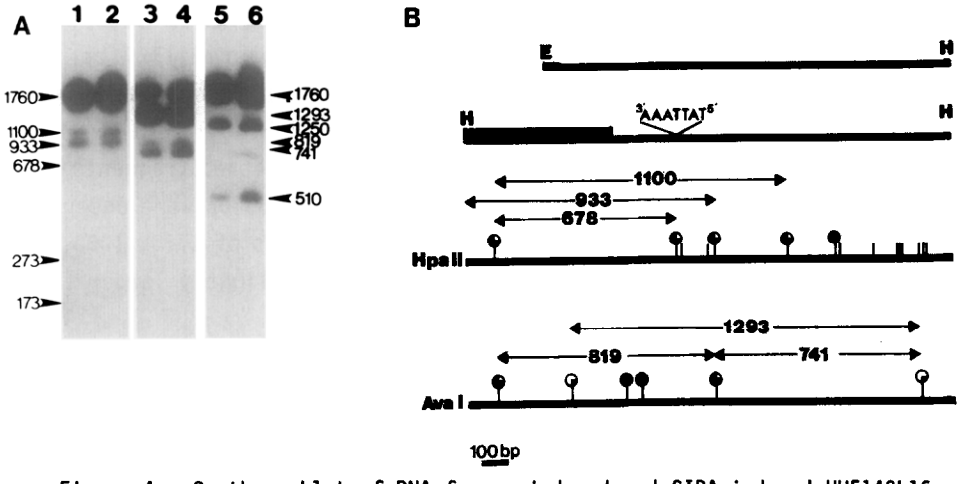

Figure 4. Southern blot of DNA from uninduced and SIBA-induced HH514CL16 cells hybridized with the 1450H probe. Lanes contain DNA digested with HpaII-HindIII (1) uninduced and (2) SIBA-induced; AvaI-HindIII (3) uninduced and (4) SIBA-induced; or HhaI-HindIII (5) uninduced and (6) SIBA-induced. DNA fragment sizes calculated as in legend for Fig. 3. Map (B) represents constitutive methylation status of specific CpG dinucleotide residues in the HpaII, HhaI, and AvaI restriction sites in the 5′ region of HH514CL16 EBV VCA gene. Quarter-closed circles, ◔, represent CpG dinucleotide residues methylated in approximately 25%; three-quarter-closed circles, ◕, represent CpG dinucleotide residues methylated in approximately 75%; and fully closed circles, ●, represent CpG dinucleotide residues methylated in approximately 100% of the genomes in the collective population, respectively.

HH514CL16 AvaI-HindIII digest, suggesting that the three AvaI restriction sites within this 1293 bp region of the VCA gene are methylated in at least three-quarters of the viral genomes in the collective HH514CL16 cell population. Thus, in uninduced HH514CL16 cells, at least three-quarters of the CpG dinucleotide residues analyzed were methylated within the HpaII (15 residues), HhaI (1 residue), and AvaI (6 residues) restriction endonucleases' recognition sites contained within the VCA promoter region of the collective viral population (Fig. 4, B). Following SIBA stimulation, the amount of 1450H probe hybridized to the partial digestion product of 1293 bp in the AvaI-HindIII digest decreased slightly, whereas the partially digested 819 and completely digested 741 bp fragments increased in proportion (Fig. 4, A). The 1250 and 510 bp HhaI-HindIII digestion products bound proportionally more probe than the undigested 1760 bp fragment.

Methylation Status of VCA-Sorted Cell Populations

The previous hybridization experiments were performed on cell populations consisting of both VCA-positive and negative cells. Hybridization analysis of flow cytometer sorted FF41-1 cells (Fig. 5, A) allowed us to examine the distribution of 5-methyl CpG dinucleotide residues within the VCA promoter region of VCA-expressing and non-expressing cell populations. MspI-HindIII digests of both VCA-positive and negative cells generated fragments of 678, 273, and 173 bp which hybridized with the 1450 H probe and an additional 933 bp fragment which hybridized with the 310 E probe. Substituting the 5-methyl CpG-sensitive isoschizomer HpaII for MspI produced the same bands plus a 1760 bp band, indicating DNA not digested by HpaII. A partially digested 1379 bp fragment appeared only in the digests from VCA- negative cells. Differences between VCA-positive and negative cells were also noted in the AvaI-HindIII and HhaI-HindIII digests. With AvaI-HindIII digestion the 1450 H probe detected fragments of 1760, 1091, and 741 bp. Two additional fragments of 1293 and 933 bp were observed only with VCA-negative cells. HhaI-HindIII generated fragments of 1760, 1000, and 760 bp which hybridized with the 1450 H probe. Novel bands slightly larger than 1000 and 1300 bp were seen only with the VCA-negative cells.

Restriction digests of DNA from sorted cell populations performed under the same conditions utilized for unsorted cells generated a different set of bands (compare Fig. 5 with Fig. 3). Sorted cells were fixed in formalin/methanol prior to separation. The DNA from these cells may have retained nuclear proteins which altered its susceptibility to digestion by restriction enzymes (11,13). Thus, the hybridized viral DNA fragments that were unique to non-fluorescent cells, may have resulted from either incomplete digestion due to the presence of specific 5-methyl CpG dinucleotide residues or pronase-resistant, formalin-fixed histone and non-histone proteins bound to specific regions of the VCA promoter region. Regardless, the absence of these unique bands in VCA-fluorescent cell DNA digests reflect a more highly ordered VCA promoter region

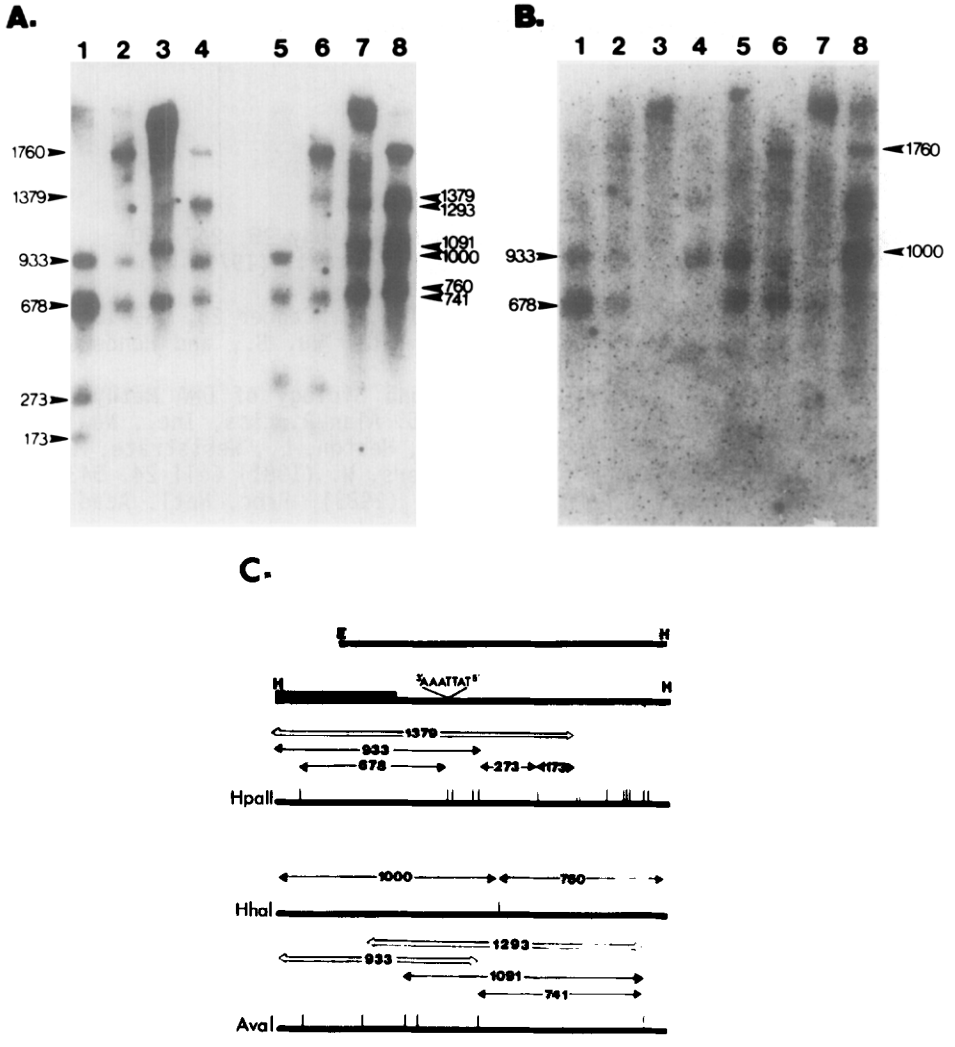

Figure 5. Southern blot of DNA from SIBA-induced, VCA-sorted FF41-1 cells hybridized with the 1450H (A) or 310E (B) probes. Lanes contain DNA digested with MspI-HindIII (1) VCA-positive and (5) VCA-negative; HpaII-HindIII (2) VCA-positive and (6) VCA-negative; AvaI-HindIII (3) VCA-positive and (7) VCA-negative; or HhaI-HindIII (4) VCA-positive and (8) VCA-negative. DNA fragment sizes calculated as in legend for Fig. 3. Map (C) represents HpaII, HhaI, and AvaI restriction sites in the 5' region of the FF41-1 EBV VCA gene Open arrows indicate hybridized restriction fragments unique to viral DNA from non-fluorescent cells.

chromatin structure in non-fluorescent cells. This finding is consistent with other unexpressed eucaryotic genes (14,15)

Undigested VCA sequences were detected in VCA fluorescent cells in the HpaII-HindIII, AvaI-HindIII, and HhaI-HindIII digests. Likewise, viral DNA sequences from the VCA region in VCA non-fluorescent cells which represented complete digests were also detected. Thus, not all of the viral episomes were hypomethylated in a lytically infected cell. Similarly, not all of the viral episomes were hypermethylated in latently infected cells. This finding supports the notion that hypomethylation is associated with but not sufficient for EBV gene transcription (16).

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