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Host-Cell-Determined Methylation of Specific Epstein-Barr Virus Promoters Regulates the Choice between Distinct Viral Latency Programs

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Epstein-Barr virus (EBV) is capable of adopting three distinct forms of latency: the type III latency program, in which six EBV-encoded nuclear antigens (EBNAs) are expressed, and the type I and type II latency programs, in which only a single viral nuclear protein, EBNA1, is produced. Several groups have reported heavy CpG methylation of the EBV genome in Burkitt's lymphoma cell lines which maintain type I latency, and loss of viral genome methylation in tumor cell lines has been correlated with a switch to type III latency. Here, evidence that the type III latency program must be inactivated by methylation to allow EBV to enter the type I or type II restricted latency program is provided. The data demonstrates that the EBNA1 gene promoter, Qp, active in types I and II latency, is encompassed by a CpG island which is protected from methylation. CpG methylation inactivates the type III latency program and consequently allows the type I or II latency program to operate by alleviating EBNA1-mediated repression of Qp. Methylation of the type III latency EBNA gene promoter, Cp, appears to be essential to prevent type III latency, since EBNA1 is expressed in all latently infected cells and, as shown here, is the only viral antigen required for activation of Cp. EBV is thus a pathogen which subverts host-cell-determined methylation to regulate distinct genetic programs.

Methylation of genomic DNA is observed in many species across the phylogenic spectrum, from unicellular prokaryotes to humans. While the principal function of methylation of bacterial genomes has been determined to be protection of self DNA from cleavage by endogenous restriction endonucleases (which act to prevent the acquisition of foreign DNA sequences), most evidence suggests that the primary function of genome methylation in higher eukaryotes (i.e., vertebrates) is the regulation of gene expression. Numerous studies over the past two decades have demonstrated a repressive effect of cytosine-methylated CpG dinucleotides in promoter sequences on gene expression. Thus, cytosine methylation has been widely regarded as a general mechanism by which eukaryotic promoters can be inactivated, perhaps by effecting changes in chromatin structure which make methylated promoters inaccessible to the transcriptional apparatus (reviewed in references 3 and 57). That the inactivity of hypermethylated loci is an effect, rather than a cause, of DNA methylation has been demonstrated by characterization of transgenic mice in which methylation of genomic DNA was impaired or eliminated via targeted disruption of the endogenous methyltransferase gene. In embryos derived from the methyltransferase-deficient mice, a strong correlation was observed between the absence of methylation and activated transcription of the characteristically hypermethylated and silent allele of certain (imprinted) genes (42).

Since methylation appears to play a role in the regulation of endogenous gene expression, it is reasonable to expect that promoter usage of at least some eukaryotic viruses may also be regulated by host cell methyltransferase activity. CpG methylation is believed to silence transcription from integrated retroviral genomes until later cellular events (e.g., cellular differentiation) trigger demethylation and reactivation of the retroviral genome (3, 19, 25, 26). Additionally, analyses of gene expression from integrated adenovirus genomes have demonstrated a strong correlation between CpG methylation of specific adenovirus promoters and silenced transcription from the corresponding viral genes (37, 39). Several other examples of repression of viral gene expression by host-cell-determined methylation of viral DNA sequences have been reported; these include Marek's disease virus (20, 31), simian foamy virus type 3 (72), and Epstein-Barr virus (EBV) (see below).

Several different groups have contributed to a detailed analysis of EBV genome methylation (1, 10, 27, 45, 49, 58, 67). These studies have revealed that whereas the EBV genome is nearly free of CpG methylation in lymphoblastoid cell lines (LCLs) established by in vitro infection of B cells (type III latency), the genome is heavily CpG methylated in the subset of Burkitt's lymphoma (BL) tumor cell lines which retain the phenotype of the freshly explanted tumor (type I latency). There is thus a correlation between heavy methylation of the EBV genome and restricted expression of latency-associated genes in BL cell lines which retain the tumor phenotype. In this restricted form of latency (referred to as type I latency [62]), Epstein-Barr virus nuclear antigen 1 (EBNA1) is the only latency-associated antigen expressed (60, 61, 64, 67). In EBV-associated Hodgkin's disease (8, 21, 53) and nasopharyngeal carcinoma (NPC) tumors (5, 12, 16, 23), a related form of latency (type II) in which one or more of the latent membrane proteins LMP1, LMP2a, and LMP2b are expressed in addition to EBNA1 is observed. In contrast, hypomethylation of the EBV genome is correlated with the characteristic LCL pattern of latency (type III), in which six nuclear antigens (EBNAs 1,

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2, 3a, 3b, 3c, and 4) and all three latent membrane proteins are expressed. A subset of these EBV-encoded type III latency antigens immortalize the infected cells, resulting in continuous proliferation. However, the vigorous cellular immune response directed against EBV-immortalized cells limits the proliferation and expansion of such latently infected cells in vivo to early stages of infection of a naive host or to immunocompromised individuals (for a review, see reference 36).

Detailed analyses of CpG methylation in type I latent cell lines have demonstrated heavy methylation of Cp and Wp, the promoters responsible for expression of all six EBNAs during type III latency (see Fig. 1A). Treatment of such cell lines with 5-azacytidine results in both demethylation and activation of Cp and Wp, providing evidence that methylation may be responsible for maintaining these promoters in an inactive state during type I latency (1, 10, 27, 45, 49, 58, 67). Other studies have provided evidence of hypomethylation of type I latent genomes at two functionally active elements: (i) *oriP* (10), which contains binding sites for EBNA1 (EBNA1 binding to *oriP* is required for replication of the EBV genome), and (ii) the EBER genes (48), which encode two small polymerase III (pol III) transcripts that are abundantly expressed in all three latency programs. Thus, in the cases of both EBV and eukaryotic genomic DNA, hypomethylation is correlated with transcriptional activity and hypermethylation is correlated with silenced transcription.

We have reported that transcription of the EBNA1 gene during type I latency is driven by a previously unrecognized promoter, Qp, which has an architecture suggesting that it may be a housekeeping promoter (69). Subsequent studies have confirmed the identification of Qp as the EBNA1 gene promoter active during restricted viral latency (51a). As a TATA-less promoter Qp might be expected to exhibit activity in virtually any cell type. Here, transfection of numerous cell lines supports this hypothesis.

Confirming previously published observations, we also found that Qp is very strongly repressed by the binding of EBNA1 to sites immediately downstream of the transcription initiation site in type III latent cells (65, 67, 69). Additionally, our data shows that the major type III latency EBNA gene promoter, Cp, is active in cells expressing EBNA1 from either an endogenous viral genome or an exogenously introduced plasmid, regardless of the cell type. In the absence of EBNA1, little or no Cp activity could be detected. EBNA1 expressed from Qp during type I or II latency would thus be expected to activate Cp. This evidence indicates that regardless of cell type, without *cis* inactivation of Cp by methylation (or a functionally equivalent mechanism), the type III latency program would be activated, repressing Qp and the type I or II latency program. The restricted (types I and II) and immortalizing (type III) programs of EBV latency therefore appear to be mutually incompatible, and activation of the type I or II latency program appears to be critically dependent upon inactivation of type III latency promoters via host-cell-determined methylation of the EBV genome. These results thus provide the first mechanistic evidence that methylation of the EBV genome is not simply associated with restricted latency but is most likely required for the establishment and maintenance of type I and type II latency.

MATERIALS AND METHODS

Cell lines and tissue culture. Akata (77), Mutu I (18), and Rael (35) are group 1 BL cell lines. DG-75 is an EBV-negative BL cell line. P3HR1 is a group 3 BL cell line (22). The LCLs X50-7, JC5, and JY have been described and characterized previously (81). The polyclonal LCL AW was established from thymic B lymphocytes (a kind gift from Ana Lena Spetz). U937 is a monocytic line established from a histiocytic lymphoma (76). The above cell lines were propagated in RPMI 1640 supplemented with 10% fetal bovine serum. NPC-KT and D98HR1 are EBV-positive adherent cell lines. NPC-KT was derived by fusion of freshly explanted NPC tumor cells with the EBV-negative epithelial line AdAH (79), and D98HR1 was the product of a fusion between the BL cell line P3HR1 and the epithelial cell line D98 (52). CNE1, CNE2 (41), and AdAH (78) are EBV-negative adherent cell lines derived from NPC biopsy specimens. The HeLa cell line was derived from a cervical carcinoma (information regarding the derivation of HeLa can be obtained from the American Type Culture Collection), and SAOS-2 is an osteosarcoma line (15). All adherent cells were cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum.

Generation of plasmids. The plasmids CW1CAT (82) and BGCAT (14) and the FQUCAT and FQUGlobin plasmids (69) have been described previously. The −122QUCAT construct was generated by *Sph*I/*Hin*dIII digestion of the FQUCAT construct, releasing a fragment containing all Q and U sequences downstream of +136, relative to Qp. PCR amplification with sequence-specific primers having either an appended *Sac*I site (5′ end) or the natural *Sph*I site at +136 (3′ end) was used to generate the −122/+136 fragment. This PCR fragment was digested with *Sac*I and *Sph*I and cloned with the *Sph*I/*Hin*dIII Q/U fragment (described above) into the *Sac*I/*Hin*dIII sites of the pGL2-CAT polylinker, in a three-part ligation. Constructs having site-directed mutations in the EBNA1 binding sites were generated by unique site elimination mutagenesis (9). *Bam*HI/*Pvu*II fragments (ca. 250 bp) containing mutagenized EBNA1 sites were subcloned via several intermediate steps into the desired QpCAT constructs. The mutagenic oligonucleotides, employed singly or in combination, were 5′-AAAGGCGCGGGATctaGaGCGCTACCGGAT-3′ (upstream EBNA1 site; UpE1mut) and 5′-GGCGGGTAATACcTcgagTCCTTACATTTTG-3′ (downstream EBNA1 site; DnE1mut).

QpGlobin constructs corresponding to QpCAT constructs described above were generated by replacing the chloramphenicol acetyltransferase (CAT) gene with the globin gene. The construct containing a deletion downstream of Qp was generated by the digestion of the −122QUCAT construct (described above) with *Nar*I. The largest fragment of the *Nar*I digestion was circularized by using T4 DNA ligase, resulting in the generation of −122QUdNarICAT having a deletion in the QU intron from +306 to +4994, relative to Qp. The CAT gene was then replaced with the rabbit β-globin gene to generate −122QUdNarIGlobin.

pcDNA3/EBNA1 was generated by ligating bases 255 to 2612 (*Bst*YI/*Pvu*II) of the B95.8 *Bam*HI K fragment, containing the entire EBNA1 open reading frame, to the *Bam*HI/*Eco*RV sites of the plasmid pcDNA3 (Invitrogen). The pSV40/Globin constructs were generated by replacing the luciferase gene in pGL2 Basic (Promega) with a cassette containing the simian virus 40 (SV40) promoter/enhancer cloned upstream of the rabbit β-globin gene. For the three constructs in which two EBNA1 sites were placed between the SV40 promoter and the globin gene, the following EBNA1 site synthetic double-stranded oligonucleotides were used (only the sense strand is shown, and sequences corresponding to EBNA1 binding sites are underlined): 5′-CGCGCGGGATAGCGTGCGCTACCGGATGGCGGGTAATACATGCTATCCT TAC-3′ (wt), 5′-CGCGCGGGATctaGaGCGCTACCGGATGGCGGGTAATACcTcgagTCCTTAC-3′ (mut), and 5′-C GCGAGGATAGCATATGCTACCCGATGGAGGGTAGCATATGCTACCCG TAC-3′ (ideal). These sequences were cloned approximately 50 bp downstream of the major transcription initiation sites of the SV40 promoter.

Transient transfections. Suspension cells were electroporated as follows. Cells were harvested, washed once in unsupplemented RPMI 1640 media, and resuspended at a density of 4×10^6 cells/ml in unsupplemented RPMI 1640. Fifteen (CAT reporters) or 20 (globin reporters) μg of reporter plasmid was added to 0.25 ml of cells in 0.4-cm-gap electroporation cuvettes. Cuvettes were then chilled on ice for 5 to 10 min. Cells were electroporated in a Bio-Rad gene pulser at 960 μF and 230 V (BL cells and LCLs) or 270 V (U937). Cuvettes were returned to ice after electroporation, and 0.7 ml of ice-cold phosphate-buffered saline was added to each cuvette immediately after all electroporations were completed. Samples were then transferred to 25-cm^2 flasks containing 10 ml of the appropriate prewarmed media. Transfectants were shaken vigorously several times to break up aggregates of cell debris and surviving cells and were placed in a 37°C, 5% CO_2 incubator for 40 to 48 h. In transfections with β-globin constructs, for each reporter three separate electroporations were pooled into 50 ml of media. Adherent cells were transfected in 60-mm-diameter dishes with 2 μg of CsCl-purified plasmid DNA by using the Lipofectamine reagent (Gibco-BRL) according to the instructions of the manufacturer.

CAT assays were performed as previously described (17). All CAT assay data shown represents the average of three or more transfections per cell line.

RNA preparation and S1 nuclease protection analysis. Total RNA was isolated from cells by the single-step method (6) with either Tri-Reagent (Molecular Research Center) or guanidium thiocyanate-phenol prepared according to the method of Chomczynski and Sacchi (6). RNA was then treated with RQ1 DNase (Promega) according to the manufacturer's instructions. Synthetic oligonucleotides were labeled with [γ-^{32}P]ATP according to established protocols (63). ^{32}P-labeled oligonucleotides were hybridized overnight to RNA samples, digested with S1 nuclease, and analyzed by electrophoresis on denaturing polyacrylamide gels as previously described (81). All hybridizations and digestions were performed at 37°C, with the exception of experiments employing the Qp and Qp-mut S1 oligonucleotides, in which hybridization and digestion were both performed at 45°C. Sequences of S1 oligonucleotides are listed in Table 1.

TABLE 1. Sequences of oligonucleotides used in this study

Probe or primer	Sequence (5′→3′)
S1 nuclease probes	
Qp-wt	CCGCCATCCGGTAGCGCACGCTATCCCGCGCCTTTTCAAGCACTTTCGTTTTCGCAAAGC
Qp-mut	CCGCCATCCGGTAGCGCTCTAGATCCCGCGCCTTTTCAAGCACTTTCGTTTTCGCAAAGC
β-Globin	CTGGACAGATGCACCATTCTGTCTGTTTTGGGGGATTGCATCAAGCGT
β-Actin	ACATAGGAATCCTTCTGACCCATGCCCACCATCACGCCCTGGGAAGGAAAGGACAAGA
PCR primers	
Bisulfite analysis[a]	
Cp-5′, 1°	CACCTACATCCTACAAAAA
Cp-3′, 1°	TTTAGGAGGTTTTTAGATTTA
Cp-5′, 2°	GCAGGTACCCAAACCTTACAAAACA
Cp-3′, 2°	GGCTCTAGAAGTGGGTTTATATGGT
Amplification of EBNA transcripts	
W0/W1	CAGGAGATCTGGAGTCCACACAAATCCT
W2	ACTGAAGCTTGACCGGTGCCTTCTTAGGAG
Q	ATATGAGCTCGGGTGACCACTGAGGGA
K	GGATCGAATTCCATTTCCAGGTCCTGTACCT

[a] 1°, primary PCR amplification primers; 2°, nested PCR amplification primers.

Immunoblotting. For detection of EBNA1 in EBV-positive cell lines, the indicated amount of protein was electrophoresed through an 8% Tris-glycine sodium dodecyl sulfate (SDS)-polyacrylamide resolving gel. The polyclonal antiserum, used at a 1:200 dilution, was obtained from a donor who has a high anti-EBNA1 titer but a lower titer of antibody against other EBV-encoded nuclear antigens (data not shown). The secondary antiserum was a peroxidase-conjugated goat anti-human immunoglobulin G (Fc fragment) (Sigma) and was used at a dilution of 1:2,000. Specific proteins were visualized by chemiluminescence with a Renaissance kit (New England Nuclear).

Restriction endonuclease analysis of EBV genome methylation. EBV-positive and EBV-negative cell lines were propagated as described above (see "Cell lines and tissue culture"), and approximately 10^8 cells were harvested by centrifugation and washed once in phosphate-buffered saline. Cell pellets were resuspended in 1 ml of TES (50 mM Tris [pH 7.5], 100 mM NaCl, 50 mM EDTA), and 0.1 ml of 10% SDS and 200 μg of proteinase K were added. This mixture was gently shaken at 37°C for 3 h and was extracted twice with phenol-chloroform (1:1) and once with chloroform (extractions were incubated for 20 min at 37°C with shaking). Genomic DNA was recovered by spooling with a bent Pasteur pipette following addition of 3 volumes of absolute ethanol. DNA was washed with 70% ethanol, dried briefly, and resuspended overnight in 1 ml of glass-distilled water. The following day, all the above steps (beginning with the addition of SDS) were repeated, and DNA was resuspended in 200 to 400 μl of Tris-EDTA buffer and stored at 4°C until used.

Restriction endonuclease digestion of genomic DNA (1 to 20 μg, depending on the cell line) was performed over a 16-h period with the indicated enzymes (New England Biolabs) at a concentration of 1 to 2 U/μl in a volume of 50 to 100 μl and with the buffer recommended by the manufacturer. DNA was then purified by phenol-chloroform (1:1) extraction followed by ethanol precipitation. Digested DNA was resuspended at a concentration of 0.5 μg/μl (with the exception of DNA from the producer cell line B95.8, at 0.05 μg/μl). The amount of DNA loaded for each cell line was varied according to the previously calculated EBV copy number per cell (data not shown). The per-lane loading was as follows: DG75, 10 μg; Akata, 5 μg; Rael, 2 μg; Mutu I, 2 μg; X50-7, 10 μg; JC5, 2.5 μg; Jijoye, 2.5 μg; B95.8, 0.125 μg. DNA was fractionated on a 1.5% agarose gel, transferred to Hybond-N membranes (Amersham), and Southern blotted according to established protocols (63). Three different EBV genome fragments spanning regions surrounding Qp (60,889 to 62,253 bp, 62,106 to 62,729 bp, and 63,408 to 62,557 bp) were used as probes. Probes were labeled by random priming, as described above. In Fig. 7, the compiled methylation analysis of the three group 1 BL cell lines is presented by enzyme site. The majority of sites examined were unmethylated in the type III cell lines tested. Sites upstream and downstream of the first and last coordinates (respectively) listed for a given enzyme either were not examined or could not be unambiguously determined from the data collected. All enzyme recognition sites which lie between the first and last listed coordinates have been included in Fig. 7 (i.e., no sites within the listed range have been omitted). Figure 8 is a graphic compilation of all of the data from Fig. 7, with the exception of the data listed as ND.

Bisulfite-PCR analysis of Cp methylation. Bisulfite PCR was performed according to the protocol of Clark et al. (7) and the modifications described by Raizis et al. (56). For each cell line, 20 μg of DNA was cut with *Eco*RV and *Bgl*II (which do not cut within the target region). Next, 5 μg of DNA was bisulfite treated, and 1 μg was used for primary PCR amplification of the Cp antisense strand. Following the 25-cycle primary PCR, a 15-cycle secondary PCR was performed with internal primers. Sequences of PCR oligonucleotides are listed in Table 1. All PCR amplifications employed *Taq* polymerase (Perkin-Elmer). The amplified region (EBV coordinates 10588 to 11422) was cloned into Bluescript-KS+ (Stratagene), and the region from 10953 to 11377 was sequenced to determine sites of cytosine methylation.

5-Azacytidine treatment of D98HR1 and RT-PCR. Equal numbers of logarithmically growing D98HR1 cells were split into four 100-mm-diameter dishes and grown overnight in complete media to approximately 20% confluence. The media were then replaced with fresh cell culture media supplemented with 4 μM 5-azacytidine (complete medium alone was added to the mock plate). Cells were harvested at the indicated time points (mock-treated cells were harvested with the 73-h sample), and total RNA was prepared as described above. Reverse transcriptase (RT) PCR was performed according to the method of Kawasaki (34). Two micrograms of total RNA from the indicated cell line or D98HR1 time point was reverse transcribed at 42°C with Superscript-II RT (Gibco-BRL) and random hexamers, according to the manufacturer's instructions. Following phenol-chloroform extraction, chloroform extraction, and ethanol precipitation, the RNA template was removed by hydrolysis with 0.15 N sodium hydroxide. The cDNA was then ethanol precipitated and resuspended in 50 μl of water. Two microliters of this reverse transcription product was then PCR amplified in a 25-μl volume with 20 pmol of each of the indicated primers, *Taq* polymerase (Perkin-Elmer), and the buffer supplied by the manufacturer. Sequences of PCR oligonucleotides are listed in Table 1. Following amplification, one-fifth of the PCR product was separated by electrophoresis on a 1.5% agarose gel, transferred to Hybond-N membranes (Amersham), and Southern blotted according to established protocols (63). Blots were probed with a random-primed ^{32}P-labeled *Bam*HI U exon probe (Q/U/K PCR) derived from bases 1250 to 1731 (*Xho*I to *Cla*I) of the EBV *Bam*HI U fragment, which includes the entire U exon, or with IB4-WY1 cDNA (W0-W1/W2 PCR) (75), which contains seven copies of the EBV W1/W2 repeat exons, as well as the Y1, Y2, and Y3 exons. The probes were labeled with a random-primed labeling kit (Boehringer-Mannheim) and with [α-^{32}P]dGTP and [α-^{32}P]dCTP (New England Nuclear). Standard conditions for hybridization and washing were employed (63).

RESULTS

Preferential use of the type III EBNA gene promoter, Cp, in the presence of EBNA1. Our previous studies of promoter usage in EBV-positive B cell lines (69) indicated that the type I latency EBNA1 gene promoter, Qp, and the type III latency EBNA promoter Cp are not simultaneously active. The locations of the type III latency promoters, Cp and Wp, and the type I latency promoter, as well as the previously determined structures of the Cp- and Qp-initiated EBNA1 transcripts, are indicated in Fig. 1A. It has been reported (65) that in type III latent cells, Qp is repressed by the binding of EBNA1 to sites downstream of the Qp transcription initiation site (see Fig. 1C

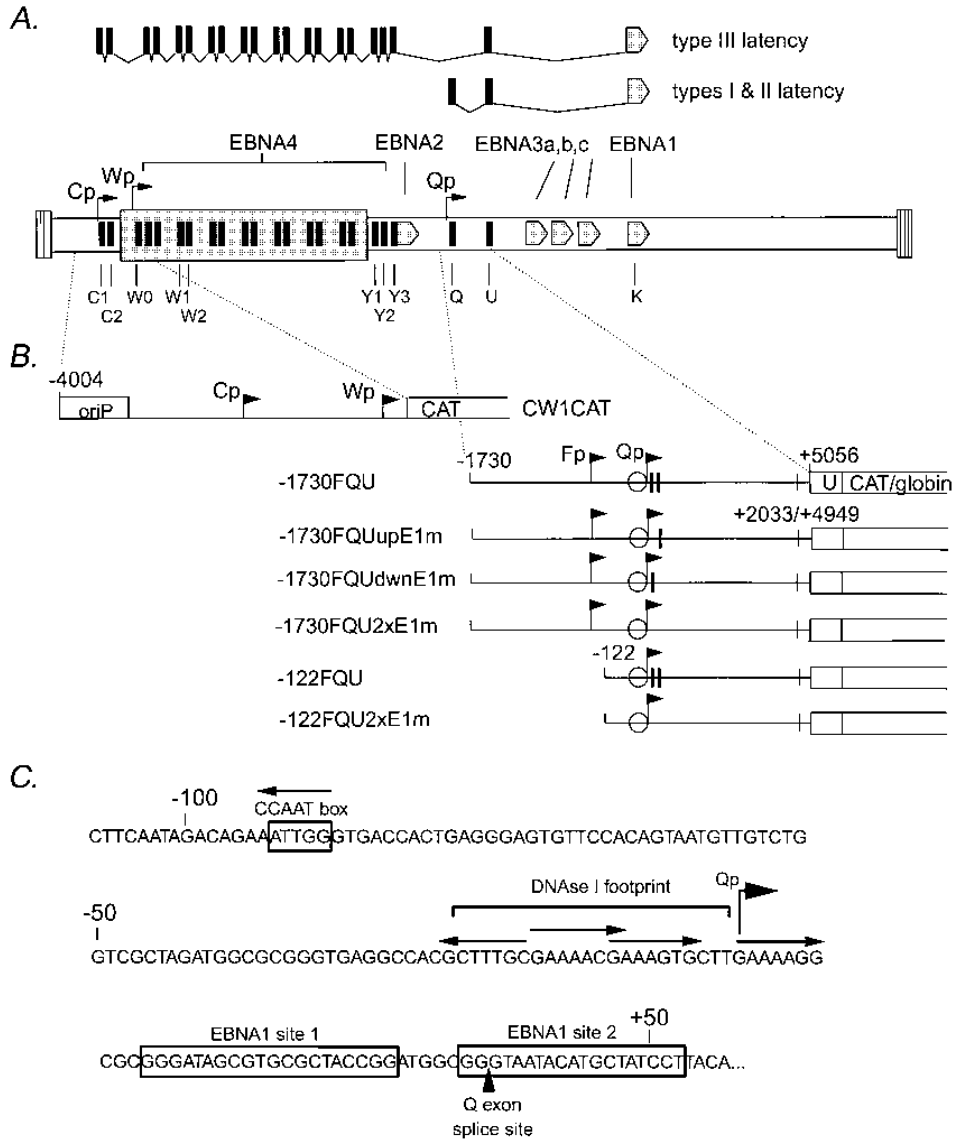


FIG. 1. (A) Simplified schematic representation of exon structures of EBNA1 gene transcripts expressed during either type III latency or type I or II latency. Regions encoding the various EBNA gene products are indicated on the viral genome. The large shaded rectangle depicts the major internal repeat (IR1) in EBV, which is composed of a variable number of 3-kb direct repeats. All EBNA transcripts expressed during type III latency have common 5′ exons (the W1 and W2 repeat exons encoded within IR1 and the Y1, Y2, and Y3 exons), which are alternatively spliced to downstream coding exons (for a review, see reference 75a). EBNA gene transcription in type III latently infected B cells is initiated from either Wp or Cp, while EBNA1 gene transcripts in type I and type II latently infected cells are initiated from Qp (69). (B) Schematic illustration of reporter constructs employed in the analysis of Cp and Qp activities. Also shown is the location of a viral promoter, Fp, located approximately 200 bp upstream of Qp, which is active during the early phase of the lytic cycle (40, 68, 69). The filled rectangles immediately downstream of the Qp transcription initiation site represent two low-affinity EBNA1 binding sites. Reporter constructs containing mutations in either the upstream EBNA1 site (upE1m), the downstream EBNA1 site (dwnE1m), or both EBNA1 sites (2×E1m) are depicted. The open circle immediately upstream of the Qp transcription initiation site depicts a region protected from DNase I digestion by nuclear extracts prepared from B cell lines. (C) Sequence of the Qp region of the EBV genome. The bent arrow indicates the major initiation site identified by S1 nuclease protection and by rapid amplification of cDNA ends (69). A region protected from DNase I digestion by crude nuclear extracts prepared from an EBV-negative BL cell line is indicated (70a). Horizontal arrows indicate a repeated sequence motif near the Qp transcription initiation site. The low-affinity EBNA1 binding sites, the Q exon (also referred to as the FQ exon) splice site, and an inverted CCAAT box are also indicated.

for the structure of Qp and locations of the EBNA1 binding sites). However, in type I BL cell lines, even though Cp of the endogenous virus is quiescent, Cp-driven reporter constructs transfected into these cell lines exhibit high levels of activity (69). Additionally, EBV superinfection of type I BL lines results in Cp-driven transcription from the newly introduced genomes, while Cp of the endogenous type I genomes remains transcriptionally silent (11). Since the endogenous viral Cp is activated in these cell lines by treatment with the CpG methylase inhibitor 5-azacytidine (1, 45), the cumulative evidence strongly suggests that methylation of the endogenous Cp is required in type I BL cell lines to maintain repression of Cp.

In addition to B lymphocytes, EBV is known to infect at least a subset of nasopharyngeal epithelial cells and has also recently been reported to be capable of infecting endothelial cells (30), gastric cells (73), and thymocytes (54). Since little information is available regarding the regulation of viral promoters active during different latency programs in non-B cells, we undertook

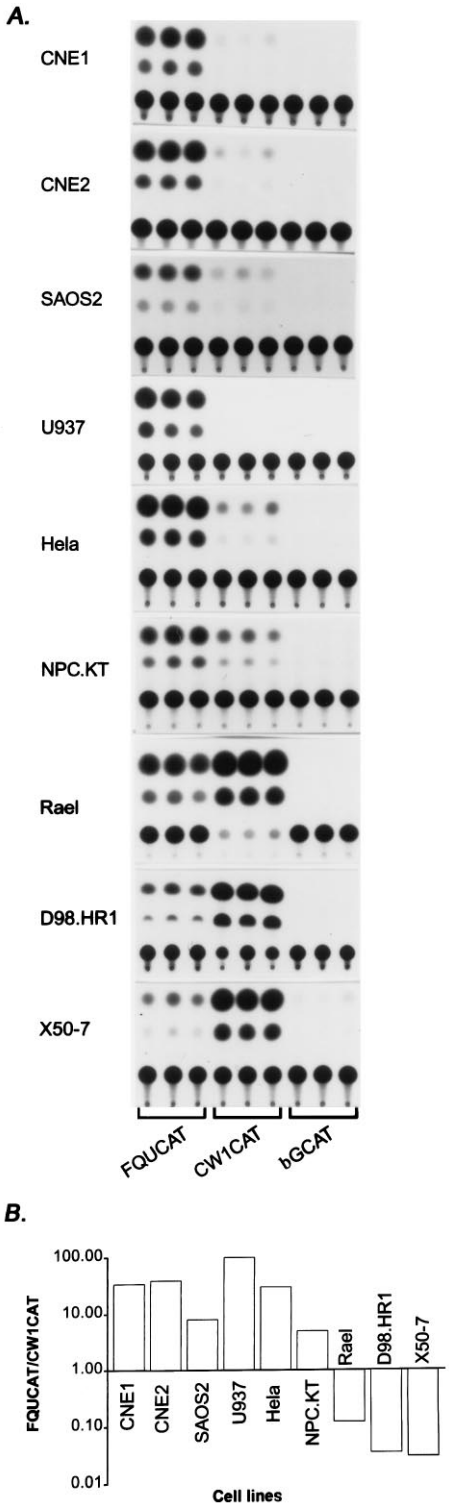


FIG. 2. (A) Activities of Qp- and Cp-driven CAT reporter constructs in a panel of EBV-negative and EBV-positive cell lines. The structures of the reporter constructs and the transfection of the indicated cell lines are described in Materials and Methods. Three independent transfections were analyzed for each reporter construct. The FQUCAT reporter construct is the −1730FQU reporter construct depicted in Fig. 1B. The structure of the CW1CAT reporter construct is also shown in Fig. 1B. The bGCAT control reporter construct contained the TATA box from the β-globin promoter driving the CAT reporter gene in a modified pGL2 vector (Promega). The origins of the indicated cell lines are described in Materials and Methods. CNE1, CNE2, SAOS2, U937, and HeLa are EBV-negative cell lines. NPC.KT, Rael, and D98.HR1 are EBV-positive cell lines which exhibit either the type I or type II latency pattern of viral gene expression. X50-7 is an EBV-positive LCL which exhibits the type III latency pattern of viral gene expression. The region of the thin-layer chromatograms where the diacetylated choloramphenicol species migrates has been cut off to conserve space in the figure. Those reactions in which more than 70% of the chloramphenicol was acetylated were repeated with smaller amounts of cell extract in order to obtain results in the linear range of the reaction. (B) Ratio of Qp to Cp activity observed in the indicated cell lines with the reporter constructs shown in panel A. Those CAT reactions shown in panel A which were out of the linear range were repeated with a smaller amount of cell extract to allow an accurate assessment of the level of reporter gene activity (not shown).

a study to characterize the activities of Qp and Cp in a wide variety of cell types (Fig. 2). Qp exhibited considerable activity in nearly all cell lines tested, with the exception of the EBV-infected, type III latent LCL X50-7 and the type I/II D98.HR1 fusion cell line. In the latter cases Qp is presumably downregulated by EBNA1-mediated repression (see below). The observation that Qp is active in a wide variety of EBV-negative cell lines is consistent with our previous hypothesis that Qp has the properties of a housekeeping promoter (69) (Fig. 1C) and should thus be active in most, if not all, cell types. In contrast, the EBV Cp promoter exhibited strong activity in the majority of EBV-positive cell lines (X50-7, D98.HR1, Rael, and NPC-KT) but weak activity relative to Qp in the EBV-negative cell lines tested (CNE1, CNE2, SAOS2, U937 and HeLa). The ratio of Qp to Cp activity with these reporter constructs is shown in Fig. 2B and, in general, demonstrates that EBV infection leads to activation of Cp-driven reporter constructs and repression of Qp-driven reporter constructs. Among the EBV-positive cell lines, NPC-KT exhibited a higher than expected Qp/Cp activity ratio, but this appears to reflect a very low level of EBNA1 expression in this cell line (70).

Since Cp transcription in LCLs has recently been shown to be entirely dependent upon transactivation by *oriP*-bound EBNA1 (55), we hypothesized that the requirement for EBNA1 transactivation to upregulate Cp activity may be a generalized phenomenon. To directly test this possibility, two EBV-negative cell lines were transfected with either a Qp-driven reporter construct (−122QUCAT) or a Cp-driven reporter construct (CW1CAT) in the presence of either an EBNA1 expression vector (pcDNA3-EBNA1) or the vector control (pcDNA3) (Fig. 3). In the absence of EBNA1, Qp was active and Cp was essentially inactive in both the HeLa and CNE2 cell lines. However, in the presence of EBNA1, Qp activity was repressed to nearly undetectable levels, while Cp activity was upregulated to a level which met or exceeded the activity of Qp observed in the absence of EBNA1 (Fig. 3). Thus, in the presence of EBNA1, Cp can also be active in non-B cells.

Characterization of EBNA1-mediated repression of Qp. To assess the role played by the binding of EBNA1 downstream of the Qp transcription initiation site, site-directed mutations were introduced into each of the EBNA1 binding sites individually and in combination. The altered binding sites were cloned into the −1730QUCAT reporter construct (see Materials and Methods, and reference 68, for details) and the double EBNA1 site mutant was also cloned into the −122QUCAT reporter (see Fig. 1B for structures of reporter constructs). Figure 4A shows that both the −1730Qp and −122Qp CAT constructs were inhibited by EBNA1 in the EBV-negative epithelial cell line AdAH, whereas the −122Qp reporter construct in which both EBNA1 sites were mutated (−122QU2×E1m) was not significantly inhibited by EBNA1. Additionally, activity of the −122QU reporter construct was almost undetectable in the type III latent LCL X50-7, whereas

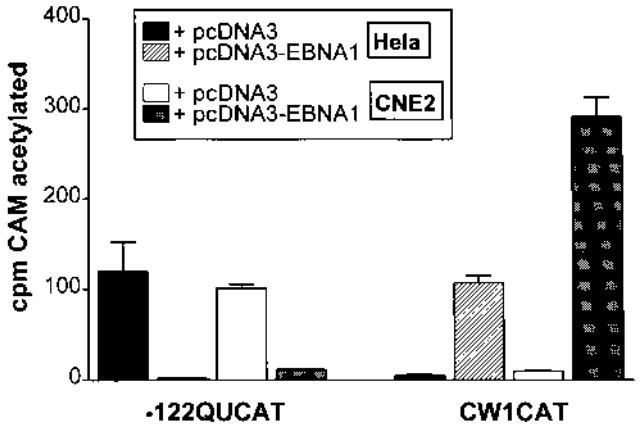


FIG. 3. EBNA1 upregulates Cp activity and downregulates Qp activity in EBV-negative epithelial cell lines. The structures of the reporter constructs and expression vectors and the transfection of the indicated cell lines are described in Materials and Methods. CAT activity is expressed as the amount of chloramphenicol (CAM) acetylated. The data shown represents the compiled results of three independent transfections. Error bars, standard error of the mean.

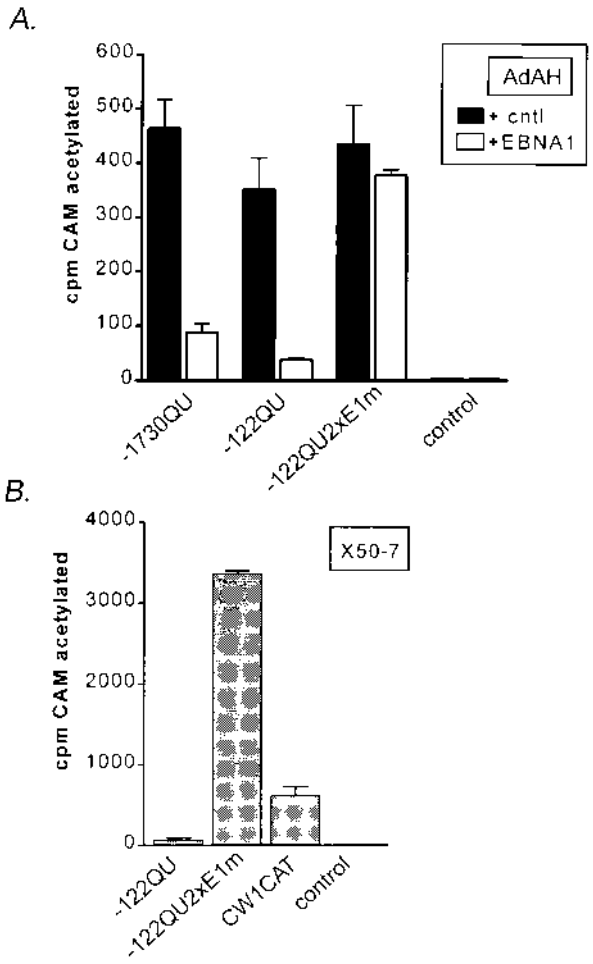


FIG. 4. Mutation of the low-affinity EBNA1 binding sites downstream of the Qp transcription initiation site abrogates EBNA1-mediated repression of Qp activity. (A) The indicated reporter constructs, described in Materials and Methods, were cotransfected into the EBV-negative epithelial cell line AdAH along with either a control expression vector (pcDNA3) or an EBNA1 expression vector (pcDNA3-EBNA1). (B) The indicated reporter constructs were transfected into the EBV-positive LCL X50-7 as described in Materials and Methods. CAT activity is expressed as the amount of chloramphenicol (CAM) acetylated. The data shown represents the compiled results of three independent transfections. Error bars, standard error of the mean.

the −122QU2×E1m reporter construct exhibited activity which exceeded that of the Cp-driven reporter construct, CW1CAT (Fig. 4B). The data in Fig. 3 and 4 thus confirms the observations of Sample et al. (65), as well as demonstrating that EBNA1-mediated inhibition of Qp reporter activity can also occur in non-B cells. Notably, the activities of the −1730QU and −122QU reporter constructs in the AdAH cell line were nearly the same, consistent with previous observations that the distal Fp promoter (present in the −1730QU reporter construct but not in the −122QU reporter construct [Fig. 1B]) does not contribute significantly to the observed activity.

To assess the relative contribution of each EBNA1 site to the observed inhibition of Qp, the wild-type (wt) −1730Qp reporter construct and derivatives containing site-directed mutations in one or both EBNA1 sites were transfected into the EBV-negative AdAH cell line with varying amounts of EBNA1 expression vector. The data in Fig. 5 indicated that when cells were transfected with low concentrations (<0.2 μg) of EBNA1 expression vector, the wild-type construct was inhibited more efficiently than constructs having a site-directed mutation in either the upstream or downstream Qp EBNA1 binding site. Over this range of EBNA1 concentrations, the degree of repression mediated by each EBNA1 site was roughly equivalent, and the combined repressive effect of both EBNA1 sites appeared to be additive rather than synergistic. This functional data correlates well with previous observations (29, 47) which indicated that the binding of EBNA1 was not cooperative, even when multiple high-affinity sites were used as the binding template. At higher concentrations (0.2 or 1.0 μg) of EBNA1 expression vector, little or no difference in the level of inhibition was observed between the constructs having one intact EBNA1 binding site and the wild-type construct. This observation suggests that full occupancy of either EBNA1 site results in maximal inhibition of Qp. The presence of two binding sites improves the probability that one of the two sites will be occupied, resulting in increased levels of inhibition at below-saturating concentrations of EBNA1.

To further define the mechanism of EBNA1-mediated inhibition of Qp-initiated transcription, we generated β-globin reporter constructs in which the SV40 early promoter and enhancer were placed upstream of either two consensus EBNA1 binding sites (SVp/eE1ideal; the spacing of these sites was identical to the spacing between the low-affinity EBNA1 sites downstream of Qp), the two wild-type Qp low-affinity EBNA1 binding sites (SVp/eE1wt), two mutant Qp EBNA1 binding sites (SVp/e2×E1m; same mutations as the 2×E1m QpCAT constructs), or no EBNA1 binding sites (SVp/e). The EBNA1 binding sites were placed approximately 50 nucleotides downstream of the major SV40 early promoter transcription initiation site. Thus, with these reporters, the binding of EBNA1 would be expected to decrease the steady-state level of globin transcripts if the mechanism of inhibition involves a physical blockade of RNA polymerase progression. Alternatively, if EBNA1 interferes with the initiation of transcription from Qp, then the placement of these sites 50 bp downstream of the SV40 promoter transcription initiation site would be unlikely to affect the level of globin transcripts generated. For comparison, Qp-driven reporter constructs containing either the functional low-affinity EBNA1 sites (−1730QU) or mutations in

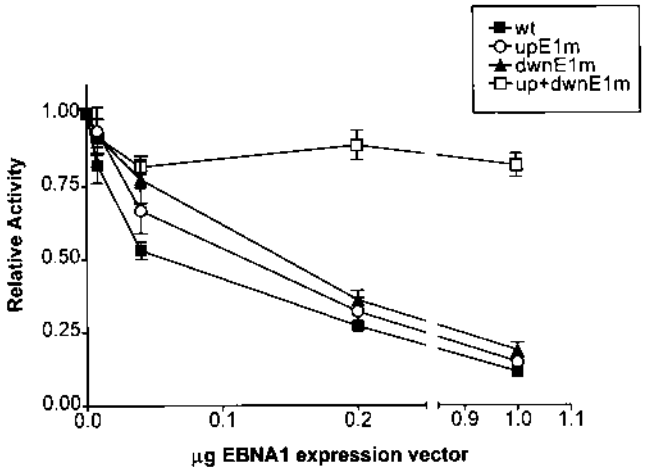


FIG. 5. Only a single low-affinity EBNA1 binding site is required downstream of the Qp transcription initiation site for EBNA1-mediated inhibition of Qp activity. The locations of the EBNA1 binding sites relative to the Qp transcription initiation site are shown in Fig. 1C. Site-directed mutations were introduced into the upstream (upE1m) or downstream (dwnE1m) or both (up+dwnE1m) EBNA1 binding sites in the context of the −1730QUCAT reporter construct, as described in Materials and Methods. The mutant and wild-type (wt) reporter constructs were transfected into the EBV-negative epithelial cell line AdAH along with the indicated amount of an EBNA1 expression vector (pcDNA3-EBNA1). A total of 1 μg of expression vector DNA was transfected in all cases by the addition of the appropriate amount of control expression vector lacking the EBNA1 gene (pcDNA3). Activities are expressed relative to the activity of each reporter construct in the absence of EBNA1, which was defined as 1.0.

both sites (−1730QU2×E1m) were assessed for the level of Qp-initiated transcripts.

S1 nuclease protection analyses of RNA prepared from Rael cells (a type I BL cell line) transfected with the Qp-driven reporter constructs showed that the level of Qp-initiated globin transcripts was increased severalfold by mutation of both Qp EBNA1 binding sites (Fig. 6A and B). This was assessed by using probes spanning the Qp transcription initiation site (Fig. 6A) or a probe specific for the level of β-globin transcripts (Fig. 6B). It should be noted that in order to detect the level of Qp-initiated transcripts in cells transfected with the reporter construct containing site-directed mutations in the EBNA1 binding sites (−1730QU2×E1m), a different S1 probe was required (Qp-mut oligo). To allow a direct comparison of the results, both the wild-type and mutant sequence probes were labeled to the same specific activity (determined by scintillation counting). In addition, it should be noted that the wild-type Qp probe also detects the steady-state level of Qp-initiated transcripts from the endogenous viral genome. The level of endogenous Qp-initiated transcripts is apparent with RNA prepared from Rael cells transfected with the CW1CAT control reporter construct (Fig. 6A) and assayed with the wild-type sequence probe (note that the mutant sequence probe does not detect any background with the same control RNA). Thus, this background level of Qp-initiated transcripts must be subtracted from the signal observed with RNA prepared from cells transfected with the −1730QU reporter construct. The results obtained with the Qp probes were confirmed by the β-globin probe (Fig. 6B).

Surprisingly, we observed that a deletion which removed most of the Q/U intron (the −122QUdNarI mutant) but did not affect the EBNA1 binding sites increased transcription of the globin gene as much as mutation of the Qp EBNA1 binding sites (Fig. 6B). However, the levels of specifically initiated Qp transcripts were only slightly elevated, whereas transcription from upstream of the Qp start site apparently accounted for most of the increased globin gene transcription (Fig. 6A). Thus, the deletion of Q/U intronic sequences may result in an enhancement of transcription initiation from upstream of the major Qp start site, or the deletion may remove intronic sequences which facilitate the degradation of transcripts which were not initiated at the +1 start site. Endogenous β-actin transcription was measured by S1 nuclease analysis to control for RNA quality, and nearly equivalent signals were obtained for all samples (Fig. 6C). The data shown in Fig. 6 has also been confirmed by using the same promoter regions upstream of the CAT reporter gene (data not shown).

In contrast to the results obtained with the Qp-driven reporter constructs, the level of SV40 early promoter-initiated globin transcripts was not affected by the Qp wild-type, Qp mutant, or consensus EBNA1 binding sites (Fig. 6B). These results support the hypothesis that the binding of EBNA1 to the EBNA1 sites immediately downstream of the Qp transcription initiation site inhibits the initiation of transcription from Qp rather than blocking polymerase progression. Thus, these results suggest that EBNA1 binding to the Qp sites specifically inhibits Qp-initiated transcription and does not interfere with transcription from the distal type III latency EBNA gene promoters, Cp and Wp (see Fig. 1 for relative locations of these promoters).

As discussed above, transcription initiation from Qp is very strongly repressed by EBNA1 in type III latently infected B cells (69) (Fig. 2A and 4B) but is repressed to a much lower degree by EBNA1 in type I BL cell lines (69) (Fig. 2A and 6A and B). To examine whether there is a difference in EBNA1 protein levels between type I and type III latently infected B cells, an immunoblot titration was performed with cell extracts prepared from representative cells of these types (data not shown). The type I BL cell lines Rael and Mutu I appeared to contain approximately two- to threefold less EBNA1 than the type III LCLs JY and AW. The type I BL line Akata was found to have ca. 10-fold less EBNA1 protein than the type III latent cell lines; this difference may be exaggerated, due to the progressive loss of EBV genomes which has been reported to occur in this cell line (74). The observation of essentially complete repression of Qp during type III latency, but only moderate repression during type I latency, is difficult to explain on the basis of an apparent two- to threefold difference in the level of EBNA1. Thus, the striking differences in the impact of mutating the Qp EBNA1 sites in type I and type III latently infected cells likely reflects the contribution of other factors in addition to differences in the levels of EBNA1 protein.

The EBV genome in type I BL cell lines is specifically hypomethylated in a region centered on Qp. Because EBV genomes in type I BL cell lines and in BL tumor biopsy specimens have been shown to be hypermethylated at CpG dinucleotides, whereas the EBV genomes in type III latent cell lines generally contain few methylated cytosines (10), it was of interest to determine the methylation pattern around Qp in type I BL cell lines. Cytosine methylation at CpG sequences in promoter regions is believed to be a general mechanism by which genes are specifically inactivated in eukaryotic cells (reviewed in references 3 and 38). Other investigators have previously observed that the type III latency EBNA gene promoters, Cp and Wp, are hypermethylated in type I BL cell lines but not in type III latent cell lines (1, 27), suggesting that the mechanism of Cp/Wp inactivation in BL tumors and type I BL cell lines may be promoter methylation.

Initial experiments were performed with the isoschizomer pair *Hpa*II (CCGG cleavage blocked by methylation at CpG)

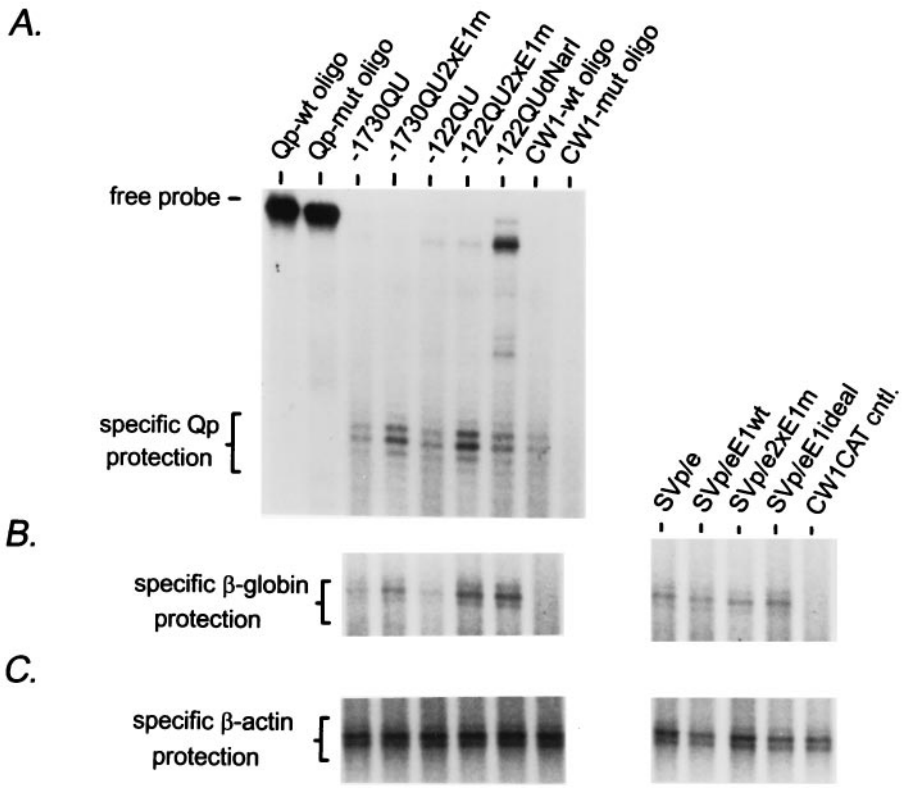


FIG. 6. EBNA1 inhibits transcription initiation but not transcription elongation. (A) Analysis of the steady-state level of Qp-initiated transcripts with reporter constructs containing either the functional or mutated Qp EBNA1 sites. The reporter constructs containing functional Qp EBNA1 sites (−1730QU, −122QU, and −122QUdNarI) or site-directed mutations which abrogate EBNA1 binding to the Qp EBNA1 sites containing mutations (−1730QU2×E1m and −122QU2×E1m) are described in Materials and Methods. The Rael cell line (type I latency) was transfected with the indicated reporter constructs and RNA was recovered from the transfected cells as described in Materials and Methods. Qp activity was assessed with a ^{32}P-labeled oligonucleotide probe which was complementary to the region spanning the Qp transcription initiation site. RNA was hybridized with the Qp-wt probe if the reporter construct contained functional EBNA1 binding sites; it was hybridized with the Qp-mut probe if the reporter construct contained a mutated EBNA1 binding site. This was followed by digestion with S1 nuclease and fractionation of the resulting products on a denaturing polyacrylamide gel (see Materials and Methods). To control for basal Qp activity from the endogenous viral genomes in Rael, RNA was prepared from Rael cells transfected with a control CAT reporter construct (CW1CAT) and analyzed with both the Qp-wt and Qp-mut probes. The level of Qp activity observed with the Qp-wt probe reflects the steady level of endogenous Qp-initiated transcripts (see CW1-wt oligo). Note that the Qp-mut probe did not cross-hybridize with endogenous Qp-initiated transcripts (see CW1-mut oligo). (B) S1 nuclease protection analysis of the level of β-globin reporter transcripts. A probe specific for the 5′ end of the β-globin gene was hybridized to RNA prepared from Rael cells transfected with the indicated Qp- and SV40 early promoter/enhancer-driven β-globin reporter constructs (the latter constructs are described in Results). For the Qp-driven reporter constructs, the same RNA analyzed in panel A was employed. (C) S1 nuclease protection analysis of cellular β-actin transcripts. The same RNAs analyzed in panels A and B for reporter gene expression were assessed for the level of β-actin expressed, as described in Materials and Methods.

and *Msp*I (CCGG cleavage insensitive to CpG methylation), for which there are numerous recognition sequences surrounding Qp. Genomic DNAs from three type I BL cell lines (Akata, Rael, and Mutu I), four type III cell lines (X50-7, JC5, Jijoye, and B95.8), and an EBV-negative control (DG75) were digested with *Hpa*II or *Msp*I. Digested DNAs were separated by electrophoresis in agarose, and fragments generated from sequences near Qp were identified by Southern hybridization with labeled EBV genome fragments (see Materials and Methods for details). The expected fragment pattern (based on the B95.8 EBV sequence [13]) was generated for all DNAs digested with *Msp*I and all type III cell lines digested with *Hpa*II. However, *Hpa*II digestion of DNAs prepared from type I cell lines generally did not yield the expected cleavage pattern but rather produced fragments much larger than the expected size, indicating frequent CpG methylation at *Hpa*II/*Msp*I sites in the viral genomic DNA surrounding Qp. Notably, a single fragment of the expected size was generated by *Hpa*II cleavage of all three type I cell line DNA samples, and these two *Hpa*II sites are those closest to the site of Qp transcription initiation (Fig. 7 and data not shown). This preliminary analysis thus provided evidence of hypomethylation in the immediate vicinity of Qp, whereas sequences upstream and downstream of Qp appeared to be hypermethylated.

Due to the large number of closely spaced *Hpa*II/*Msp*I sites in the vicinity of Qp, many fragments which were too small to be detected by Southern blotting were generated, and it was not possible to determine whether specific CpG sequences were cytosine methylated or unmethylated. Other restriction endonucleases which have fewer cleavage sites in the region surrounding Qp, recognize sequences that include a CpG dinucleotide, and cannot cut when CpG is modified to 5MeCpG were thus employed to generate a detailed methylation map of Qp and surrounding sequences (Fig. 7 and 8). These studies revealed that the region immediately surrounding Qp is hypomethylated in the type I cell lines, whereas the region upstream and downstream of Qp is hypermethylated (Fig. 7 and 8). Although there were some differences in digestion patterns seen in the three type I cell lines, there were also striking similarities, providing evidence that there is a specific and conserved pattern of EBV genome CpG methylation in type I latency. That the restriction sites were indeed present, but

Bst UI	*Eag I*	*Hae II*	*Hha I*	*Hpa II*	*Sal I/Ava I*
60,888 - Me	62,736 - Un	60,452 - Me[c]	60,451 - Me[c]	62,276 - Un	60,331 - Me
61,224 - Me	63,018 - Me	61,177 - Me[c]	61,176 - Me[c]	62,450 - Me	60,470 - Me
61,417 - Me	63,930 - Me	61,716 - Un[c]	61,715 - Me[c]		60,503 - Me
61,721 - ND	64,320 - Me	61,858 - Me[c]	61,723 - ND		60,550 - Me
61,882 - ND	64,415 - Me	62,730 - Un[b]	61,857 - Me[c]		60,899 - Me
61,924 - Un[a]		62,743 - Un[b]	62,385 - Un		61,185 - Me
62,015 - Me		62,935 - Me	62,430 - ND		62,106 - Un
62,385 - Un[d]		62,947 - Me	62,444 - Un		62,284 - Un
62,430 - Un			62,575 - Un		62,583 - Un
62,587 - Un[b]					62,720 - Un
62,589 - Un[b]					62,944 - Me
					63,205 - Me
					63,301 - Un
					63,543 - Me

FIG. 7. Analysis of methylation in the type I BL cell lines Akata, Rael, and Mutu I. Me, cytosine of CpG within enzyme recognition site is methylated; Un, cytosine of CpG within enzyme recognition site is unmethylated; ND, methylation status could not be unambiguously determined; a, this *Bst*UI site is either methylated or not present in Rael; b, at least one of these two closely spaced *Bst*UI and *Hae*II sites is unmethylated; c, these *Hae*II and *Hha*I sites examine the same CpG residues; d, these *Bst*UI and *Hha*I sites examine distinct CpG residues.

blocked by CpG methylation, was indicated by the consistent observation of submolar fragments of the expected sizes (based on the B95.8 sequence) in the Akata and Mutu I digested samples (data not shown). These submolar fragments most likely represent rapidly replicating viral DNA from the small population of cells which had entered the lytic cycle, as is characteristic of these two cell lines (68).

In order to address the question of whether or not the CpG methylation pattern characteristic of type I BL EBV genomes is a uniform finding in type I latency, the CpG methylation pattern of two EBV-positive in vitro-established, non-BL-derived cell lines was determined. NPC-KT and D98HR1 (described above and in Materials and Methods), two epithelium-like cell lines which we have recently shown exhibit a restricted pattern of EBNA gene expression (i.e., Qp-driven EBNA1 expression) (70), were examined. Notably, other EBV-positive epithelial lines have been reported to adopt a type I latency phenotype (23, 43), raising the question of the mechanism for this behavior. By using the bisulfite-PCR sequencing technique (see Materials and Methods), the precise methylation pattern at Cp was determined for DNA isolated from NPC-KT and D98HR1 and was compared to those in the type I latent BL cell line Rael and the type III latent cell lines JC5 and P3HR1. In the bisulfite-PCR method, genomic DNA is first treated with bisulfite to selectively convert unmethylated cytosine residues to uracils, and this modified template is PCR amplified (note that only one strand of the template is amplified in this method). Any cytosines present in the PCR product thus represent methylated cytosines (in the original template), since the unmethylated cytosines are converted to uracils and thus amplified as thymines.

As shown in Fig. 9, both D98HR1 and NPC-KT, like the type I BL cell line Rael, were almost always methylated at every CpG dinucleotide examined in the Cp promoter, extending from the EBNA2-dependent enhancer to beyond the transcription initiation site. Methylation at the CpG located at 10,985 bp has previously been shown to dramatically inhibit transcription from Cp (58). One clone examined in the NPC-KT cell line was completely unmethylated. However, since NPC-KT has demonstrated significant spontaneous lytic transcription in our analyses (70) and is known to efficiently support the EBV lytic cycle (66), this clone most likely represents viral genomic DNA produced by lytic replication, which would not be expected to be CpG methylated. In contrast to the three Qp-using cell lines examined, the type III latent cell lines JC5 and P3HR1 were nearly completely unmethylated at Cp, as expected (Fig. 9).

To provide further evidence that the silencing of the type III latency EBNA gene promoters is the direct effect of methylation of the EBV genome, the D98HR1 cell line was treated

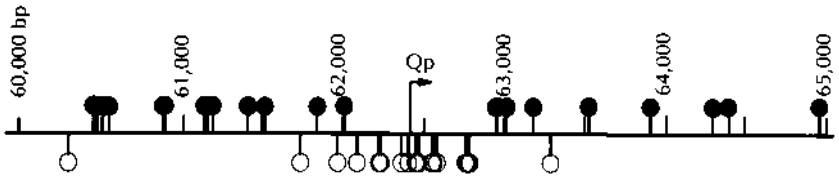
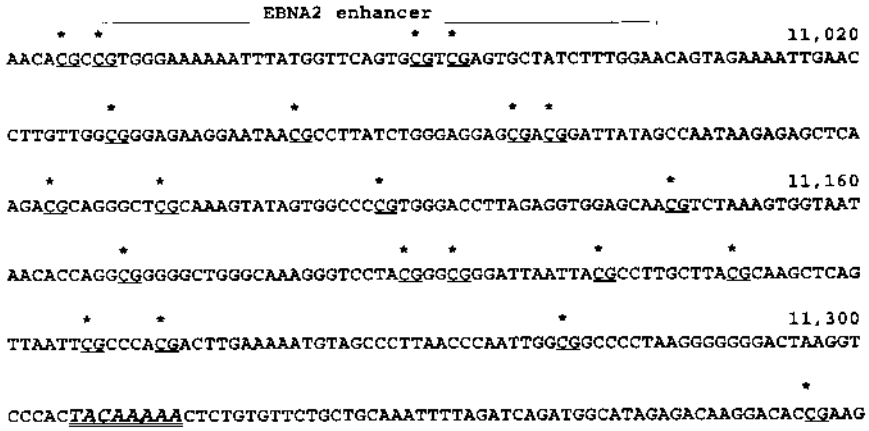


FIG. 8. Qp is hypomethylated in type I latently infected BL cell lines. A schematic representation of the data presented in Fig. 7 is shown, with the positions of methylated (filled circles) and unmethylated (open circles) cytosines indicated. The EBV genome coordinates are given above the genome, and the Qp transcription initiation site is also shown.



EBV genome coordinate	Rael	NPC-KT	D98HR1	JC5	P3HR1
	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4	1 2 3 4
10,956	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
10,959	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
10,985	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
10,988	X X 0 X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,030	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,045	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,063	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,066	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,095	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,104	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,122	X X X X	0 X X X	X 0 X X	0 0 0 0	0 0 0 0
11,146	X X X 0	0 0 X X	X 0 X 0	0 0 0 0	0 0 0 0
11,171	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,194	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,198	0 X 0 X	0 X X 0	X X X X	0 0 0 0	0 0 0 0
11,210	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,221	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0
11,238	X X X X	0 X X X	X X X X	X 0 0 0	0 0 0 0
11,294	X X X X	0 0 X X	X X X X	X 0 0 0	0 0 0 0
11,276	X X X X	0 X X X	X X X 0	0 0 0 0	0 0 0 0
11,385	X X X X	0 X X X	X X X X	0 0 0 0	0 0 0 0

FIG. 9. Methylation of Cp in cell lines exhibiting restricted EBV latency. Shown in the upper panel is the region upstream of the Cp transcription initiation site which was characterized for the presence of methylated cytosine residues. CpG dinucleotides are indicated by an underline and an asterisk above the cytosine residue. Also shown are the EBNA2-dependent enhancer (overbar) (28), the Cp TATA box (double underlined), and the EBV genomic coordinates. The lower panel gives the results obtained with four independent clones derived by the bisulfite PCR approach (7, 56), as described in Materials and Methods. The sequences of the PCR primers used in this analysis are given in Table 1. Rael, NPC-KT, and D98HR1 are type I or II latently infected cell lines, while JC5 is a type III latently infected LCL and P3HR1 is a type III latently infected BL cell line. X, methylated cytosine; 0, unmethylated cytosine.

with the demethylating agent 5-azacytidine, which has previously been shown to be capable of inducing transcription from the type III EBNA gene promoters, Cp and Wp, in type I BL cell lines (1, 45, 67). The RT-PCR experiment reported in Fig. 10B demonstrates that although no Wp activity was detected in the mock-treated cells, Wp-initiated transcripts were easily detected at 18, 49, and 73 h after induction with 5-azacytidine. The ladder of PCR products is due to the presence in Wp-initiated transcripts of multiple copies of the W2 exon to which the downstream PCR primers hybridize, as has been previously observed (71). Wp-initiated transcripts were not detected in the type I BL line Rael, but a strong signal (showing a banding pattern nearly identical to that of the 5-azacytidine-induced D98HR1 samples) was generated by amplification of the cDNA derived from the polyclonal LCL AW (data not shown). As a control, the same cDNAs were examined by PCR for the presence of Qp-initiated EBNA1 transcripts (Q/U/K spliced). Q/U/K-spliced transcripts were detected in Rael and all the D98HR1 samples, as shown in Fig. 10B, but not in the LCL AW (data not shown). In other work (70), Q/U/K-spliced EBNA1 transcripts, but not Cp- or Wp-initiated Y3/U/K-spliced EBNA1 transcripts, were detected in the NPC-KT cell line. The data in Fig. 10 and 11A and B thus collectively demonstrates that D98HR1 and NPC-KT are Qp-using type I (or type II) latent cell lines, and, like the type I BL lines, they do not use the type III latency promoters Cp and Wp because of CpG methylation at these loci. Additionally, the observation that D98HR1 is heavily methylated at Cp (even though its EBV-positive parent, P3HR1, is mostly unmethylated) strongly suggests that selective pressures, other than in vivo immune se-

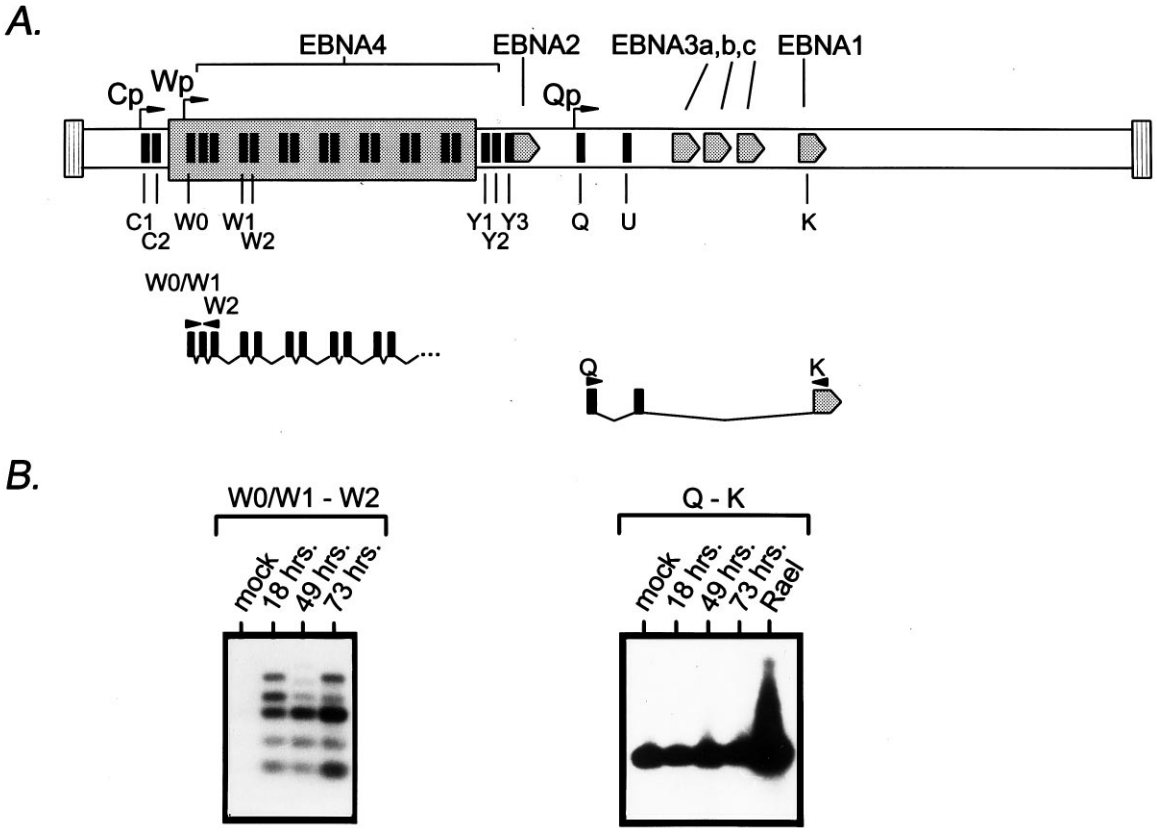


FIG. 10. Induction of type III latency EBNA gene promoter usage in D98HR1 cells treated with 5-azacytidine. (A) Schematic illustration of the EBV genome, with the positions of the EBNA gene promoters active in types I and II latency and type III latency and the positions of the EBNA gene coding sequences indicated. The PCR primers employed to detected Wp-initiated (W0/W1 and W2 PCR primers) and Qp-initiated transcripts (Q and K PCR primers) are shown, and their sequences are given in Table 1. (B) Time course of 5-azacytidine induction of Wp-initiated transcripts in D98HR1 cells. RNA was harvested from D98HR1 cells at the indicated times after addition of 5-azacytidine to the culture medium. First-strand cDNA synthesis and subsequent amplification with the indicated primers were carried out as described in Materials and Methods. The left panel demonstrates the induction of Wp-initiated transcripts. Note that the ladder of PCR products observed is anticipated, since Wp-initiated transcripts contain multiple copies of the W2 exon to which the downstream W2 PCR primer can hybridize. The right panel shows the presence of Qp-initiated transcripts in both mock-treated and 5-azacytidine-treated D98HR1 cells. RNA prepared from the type I latently infected Rael cell line is shown as a positive control.

lection against type III latency-associated antigens, can give rise to a restricted latency phenotype via the methylation of type III latency promoters. Finally, the demonstration that 5-azacytidine activates Wp in D98HR1 provides evidence that both type III EBNA gene promoters (Cp and Wp) are silenced by host cell methylation in this cell line and is consistent with our previous observations for other type I latently infected cell lines (67).

DISCUSSION

In this report we have addressed the principal factors likely to be responsible for regulating the choice between the restricted (types I and II) and immortalizing (type III) programs of EBV latency. Perhaps surprisingly, the choice between type I and II latency and type III latency appears to depend entirely upon whether or not the type III latency EBNA gene promoters, Cp and Wp, become heavily methylated. Since Qp serves as a housekeeping promoter for EBNA1 expression, EBNA1 protein should be produced regardless of the cell type infected. Collectively, the findings presented in Fig. 2 and 3, as well as the results of Puglielli et al. (55), strongly argue that Cp is not a B-cell-specific promoter but rather requires only transactivation by EBNA1 for activity. Thus, unless Cp is inactivated by methylation early in infection, Cp and the type III latency program will be activated by Qp-generated EBNA1 protein. The obligatory conclusion, therefore, is that hypomethylation of Cp (and Wp) results in type III latency, whereas hypermethylation of Cp (and Wp) results in type I or II latency, since Qp is apparently protected from CpG methylation (Fig. 8 and 11).

The protection of housekeeping promoters such as Qp from methylation is not without precedent. In fact, evidence indicates that in normal eukaryotic cells, all CpG islands associated with housekeeping promoters are hypomethylated (3, 38). Studies of the mouse *aprt* gene (4, 44) have indicated that the housekeeping promoter for this gene remains unmethylated during the widespread CpG methylation of the developing embryonic genome. These investigators found that protection from methylation was dependent upon multiple Sp1 sites present in the *aprt* gene promoter. Although there are potential Sp1 sites (i.e., GC boxes) upstream of the Qp transcription initiation site, experiments have not yet been performed to determine whether these Qp sequences actually bind Sp1 and whether they are required for protecting the promoter from methylation.

One model to explain the methylation of the type III EBNA gene promoters in type I BL cells postulates that cytotoxic T lymphocyte (CTL)-driven immune selection results in outgrowth of rare BL tumor cells which do not express the highly immunogenic EBV nuclear antigens (such as EBNAs 3a and

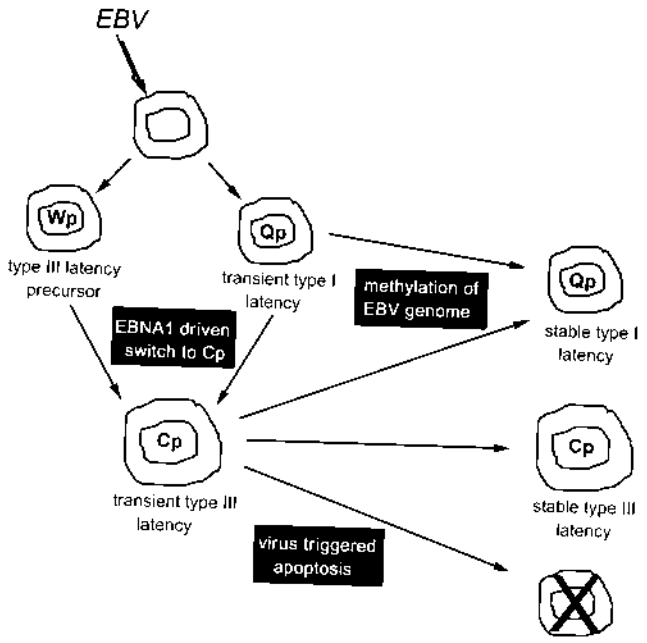


FIG. 11. Proposed model for events leading to stable type I (also type II) or type III latency. EBV infection of a particular cell can lead either to initial Wp activity, as observed during infection of primary B cells (82, 71), or to transcription from Qp. Expression of EBNA1 from either Qp or Wp will lead to activation of transcription from Cp (data presented in this paper and references 71 and 55). At this point, there are three possibilities: (i) a stable type III latent infection may be established, (ii) expression of specific viral antigens may trigger cell death, or (iii) subsequent methylation of the viral genome by host cell methyltransferase may lead to inactivation of Cp, followed by upregulation of transcription initiation from Qp and the establishment of a stable type I or type II latent infection. It is possible that in some cell types methylation of the viral genome occurs rapidly and leads directly to a stable type I or type II latent infection.

3c; see reference 51). CpG methylation has been shown to be deregulated in a wide variety of tumors (reviewed in reference 38) and, in particular, there is evidence that inappropriate inactivation of the tumor suppressor p16 is the result of hypermethylation of the p16 promoter region. This methylation-induced defect may play a crucial role in a variety of tumors (46). In the case of EBV and African BL, it is possible that a hyperactive CpG methylase during the genesis of a BL tumor cell causes inactivation of Cp via methylation, thereby leading to cessation of EBNA production. Attenuation of EBNA1 protein levels would then lead to derepression of Qp and a switch to an EBNA1-only phenotype. Such a cell could proliferate independently of the EBV growth transforming antigens due to c-*myc* translocation (and perhaps other essential mutations) and would obtain a tremendous growth advantage over type III latent tumor cells, since BL cells expressing EBNA1 only cannot be eliminated by the anti-EBV CTL response (59). Even if the EBV genome methylation is normally unstable (as may be evidenced by the rapid drift of explanted BL tumor cells to a type III phenotype in vitro; see reference 61), constant CTL-driven selective pressure would be expected to result in maintenance of Cp-methylated genomes in vivo.

However, EBV genomes may normally be heavily methylated in the wild-type precursors of BL tumor cells (see below), and BL would thus represent the maintenance of the preexisting methylation status rather than inappropriate de novo methylation, as in the tumors having hypermethylated p16 promoters. Moreover, there is still no formal proof that EBV contributes to the tumorigenic phenotype of African BL, although at least one report has suggested that as yet undefined EBV-encoded functions are essential for specific tumorigenic properties of BL cells (74).

Additionally, two observations argue that mechanisms other than immune selection can give rise to the types I and II latency pattern of EBV genome methylation. First, the studies of Li et al. (43) demonstrated that epithelial cell lines which stably express the EBV receptor (CR2/CD21) exhibit a type I latent phenotype when infected with EBV in vitro. Also, Wang et al. (80) found that when EBV-negative BL cells were infected with a recombinant EBV clone which harbors a selectable marker, the resulting cell lines frequently expressed only EBNA1. Since no immune selection was operative in either of the above studies, the available evidence argues that some as yet undefined nonimmune selection can result in the preferential generation of type I latent EBV-positive cells. In the context of the model presented in this report, a preference for type I latency indicates that one of the following is true: (i) heavy methylation of the EBV genome occurs frequently during the early stages of infection of certain cell types, or (ii) methylation of the viral genome is a relatively rare event, but a selection against cells using Cp (i.e., cells having genomes hypomethylated at Cp) is operative, perhaps due to toxicity of one or more type III latency-associated EBNA gene products in some cell types (Fig. 11). These possibilities may be distinguished by monitoring EBV latent gene expression and EBV genome methylation over an extended time course following EBV infection of an appropriate cell type, such as a CR2-positive epithelial cell line.

Circumstantial evidence related to the genome composition of EBV may indicate that CpG methylation is a consistent feature of the latent viral genome in vivo. Specifically, analyses of observed versus predicted frequencies of occurrence of the CpG dinucleotide in various viral genomes have shown that EBV and all other analyzed gammaherpesviruses are similar to vertebrate genomes in that they show a striking deficiency of CpG dinucleotides, whereas other large DNA viruses (including alpha- and betaherpesviruses) show the predicted genomic frequency of CpG (24, 32, 33). Also, like vertebrates, the gammaherpesviruses have an excess of CpA and TpG dinucleotides, which are presumed to be a hallmark of spontaneous deamination of 5MeCpG, yielding TpG and its complement, CpA. Thus, these findings have been interpreted as evidence that the persistent latent form of the genome of EBV and other gammaherpesviruses in vivo is heavily methylated and that this methylation is responsible, over an evolutionary timescale, for the depletion of CpG bases and the appearance of excess TpG and CpA sequences (24). Importantly, it is clear that other poorly defined mechanisms can also result in suppression of CpG in viral genomes (32, 33). However, the observations of Miyashita et al. (50), who found that EBV in healthy carriers resides primarily or exclusively in resting B cells, indicate that the type III (immortalizing) latency program is inactive and thus suggests that Cp and Wp are methylated in these infected cells.

In summary, the data presented here demonstrates a direct relationship between the establishment of restricted viral latency and methylation of the viral genome. In addition, a mechanistic framework in which to understand this relationship is provided. The ability of EBNA1 to activate the type III latency EBNA gene promoter Cp clearly dictates that, since EBNA1 is expressed in all known forms of viral latency, inactivation of Cp is required for the establishment of type I and II latency. Thus, EBV has evolved not simply to coexist with host-cell-determined CpG methylation but to utilize this DNA modification system to dictate a choice between very different

latent life cycle programs. If vertebrate CpG methylation occurs as a host defense response in an attempt to neutralize invading viral DNA, as some have speculated (2, 3), then at least some DNA viruses which can establish a latent, lifelong infection in these hosts have likely evolved mechanisms to incorporate the methylation-induced changes in their genomes as a functional event in their latent life cycles.

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