Unique Epstein–Barr virus (EBV) latent gene expression, EBNA promoter usage and EBNA promoter methylation status in chronic active EBV infection

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Chronic active Epstein–Barr virus infection (CAEBV) has been considered to be a non-neoplastic T-cell lymphoproliferative disease associated with Epstein–Barr virus (EBV) infection. In EBV-associated diseases, the cell phenotype-dependent differences in EBV latent gene expression may reflect the strategy of the virus in relation to latent infection. We previously reported that EBV latent gene expression was restricted; EBV nuclear antigen 1 (EBNA1) transcripts were consistently detected in all spleen samples from five CAEBV patients, but EBNA2 transcripts were detected in only one sample. EBV latent gene expression is controlled by distinct usage of three EBNA promoters (Cp, Wp and Qp). In this study, we examined the EBNA promoter usage by RT-PCR and the methylation status in the Cp and Wp regions using bisulfite PCR analysis in spleen samples from CAEBV patients. EBNA1 transcripts were unexpectedly initiated not from Qp but from Cp in all samples in spite of the restricted form of latency. Furthermore, while Cp was active, Cp was heavily methylated, indicating that CAEBV has unique EBV latent gene expression, EBNA promoter usage and EBNA promoter methylation status, in part due to unique splicing of Cp-initiated transcripts and an activation mechanism in hypermethylated Cp.

INTRODUCTION

Epstein–Barr virus (EBV) is a human herpesvirus that persists for the lifetime of the infected host and is associated with an array of conditions that range from asymptomatic infection and infectious mononucleosis (IM) to lethal lymphoid and epithelial malignancies, such as lymphoproliferative disease (LPD), nasopharyngeal carcinoma (NPC), Burkitt’s lymphoma (BL), Hodgkin’s disease (HD) and B-cell lymphoma in the immunocompromised host (Griffin, 2000). EBV has been recognized as a B-lymphotropic virus. However, recent evidence indicates that EBV can also infect T cells (Jones et al., 1988; Kikuta et al., 1988). EBV may play a role in the development of non-neoplastic T-cell LPD, including chronic active EBV infection (CAEBV) (Kikuta et al., 1988, 1989; Ohga et al., 1999) and EBV-associated haemophagocytic syndrome (EBV-AHS) (Kawaguchi et al., 1993; Su et al., 1994). CAEBV is characterized by IM-like symptoms, such as fever, lymphadenopathy, splenomegaly and hepatitis, which persist over a period of months to several years. Laboratory findings include anaemia, thrombocytopenia, leukopenia and hypergammaglobulinaemia with extremely high titres of antibodies against EBV lytic-cycle proteins (Rickinson, 1986; Schooley et al., 1986). In EBV-positive neoplastic T-cell lymphoma, viral transcripts consisting of EBV nuclear antigen 1 (EBNA1), latent membrane protein 1 (LMP1) and LMP2A/2B have been detected by RT-PCR (Chen et al., 1993). This expression pattern is characteristic of latency II EBV infection. We previously reported that EBNA1 transcripts were consistently detected in all samples from five CAEBV patients but that EBNA2 transcripts were detected in only one spleen sample (Yoshioka et al., 2001). These results indicated that the restricted form of EBV latency might play a pathogenic role in the development of CAEBV. However, the exact nature of CAEBV, neoplastic or non-neoplastic LPD, is still not known.

It has been reported that several mechanisms, including methylation and action of viral latent proteins and cellular factors, operate to regulate the promoters and control latent gene expression. Methylation of the cytosine residue of CpG dinucleotides (CpG) in eukaryote DNA is an important mechanism of promoter regulation and genetic imprinting (Singal & Ginder, 1999). EBV latent gene expression is controlled by distinct usage of three promoters (Cp, Wp and Qp). Promoter activity has been shown to be inversely correlated with the methylation status of promoters, although Qp is a remarkable exception (Robertson et al., 1995, 1996; Robertson & Ambinder, 1997a, b; Falk...
et al., 1998; Salamon et al., 2001; Tierney et al., 2000b). When Cp is hypomethylated, it permits expression of all EBNAs during latency III. On the other hand, Qp gives rise to a selective expression of EBNA1 during latency I, while latency II is characterized by expression of EBNA1, LMP1 and LMP2 with transcripts initiated from Qp and LMP promoters (Brooks et al., 1992; Deacon et al., 1993). Cp was hypermethylated and inactivated in BL and HD (Robertson et al., 1996; Tierney et al., 2000a). Multiple usage of Cp, Wp and Qp was found in EBV-infected cells of patients with IM (Tierney et al., 1994; Laytragoon-Lewin et al., 1997; Niedobitek et al., 1997). Wp was hypermethylated and Cp was completely unmethylated in most patients with IM (Tierney et al., 2000a). Promoter usage in healthy carriers is controversial (Tierney et al., 1994; Chen et al., 1995). Robertson et al. (1997b) reported that about half of Cp was methylated in healthy carriers. Paulson & Speck (1999) reported that Wp was hypermethylated but that Cp was only sparsely to moderately methylated in healthy carriers. The purpose of this study was to determine the EBNA promoter usage and to characterize the methylation status of the Cp and Wp regions in CAEBV.

**METHODS**

**Tissue samples and cells.** Spleen samples were obtained from five patients with CAEBV after obtaining informed consent from the patients’ parents. The diagnosis of CAEBV was based on clinical and virological evidence. The results of the serological study, cell types and clonality of EBV-infected cells are summarized in Table 1. The AKATA cell line and B95-8 EBV-immortalized reference cell line (R-LCL) were used as positive controls for Cp-initiated transcripts and Cp/Wp-initiated transcripts by RT-PCR, respectively. The BJAB cell line was used as a negative control for all RT-PCR analyses.

Genomic DNA was extracted from frozen tissue samples by standard methods as previously reported (Yoshioka et al., 2001). Total RNA was extracted from frozen spleen samples and cell lines using an RNAzol B (TEL-TEST) according to the manufacturer’s protocol.

**RT-PCR.** Five spleen samples were examined for the presence of viral RNA transcripts by RT-PCR. The conditions for RT-PCR were described previously (Yoshioka et al., 2001). The primer pairs were designed in order to characterize the promoter usage of EBNA1 transcripts (Fig. 1, Table 2). Cp- and Wp-initiated transcripts have BamHI-C1/C2/W1/W2- and BamHI-W0/W1/W2-spliced structures, respectively. Therefore, the primer pair C1/C2-5’ and W2-3’ was used for Cp-initiated transcripts, and the primer pair W0/W1-5’ and W2-3’ was used for Wp-initiated transcripts. Since Qp-initiated transcripts have a BamHI-Q/U/K-spliced structure, the primer pair Q-5’ and K-3’ was used for the first amplification and the primer pair U-5’ and K-3’ was used for the second amplification for Qp-initiated transcripts. To evaluate the sensitivity of the RT-PCR, serial 10-fold dilutions of cDNA from the positive control for each RT-PCR analysis were subjected to PCR.

**Southern blot hybridization.** Aliquots (10 μl each) of the PCR-amplified product were subjected to electrophoresis through a 1-0% agarose gel and transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia). Hybridization and washing were then performed according to the manufacturer’s protocol. The W1/W2- and U/K-spliced PCR-amplified products from the R-LCL were cloned into the pCR2.1-TA cloning vector (Invitrogen), and the cloned fragments were labelled by ECL random prime labelling and detection systems (Amersham Pharmacia) and used as probes for Cp/Wp- and Qp-initiated transcripts, respectively. The W1/W2-spliced products were amplified by the PCR using the primer pair W1-5’ (5’-GAGACCGAAGTGAAGGCCCT-3’) and W2-3’ (5’-GAGACCGAAGTGAAGGCCCT-3’; coordinates 14561–14580) and W2-3’. Similarly, PCR-amplified products of β-actin were used as a probe for β-actin.

**Bisulfite PCR analysis of EBV genome methylation.** Before bisulfite PCR analysis, untreated DNA was sequenced to confirm the sequences of the CpG sites in the samples. Although several point mutations were detected in promoter regions, the primers used in this study were not affected by the mutations (data not shown). The methylation status of CpG was determined by methylation-specific bisulfite PCR analysis (Frommer et al., 1992; Herman et al., 1996; Kubota et al., 1997) in five spleen samples. In this method, the bisulfite treatment converts unmethylated cytosine to uracil but does not affect the methylated cytosine. PCR primers that anneal selectively to unmethylated DNA after bisulfite conversion were designed. Briefly, 10 μg aliquots of DNA were denatured in NaOH (0.2 mol l\(^{-1}\)) for 10 min at 37°C. Each sample was resuspended in 520 μl of freshly prepared sodium bisulfite (3-0 mol l\(^{-1}\); Sigma) and 30 μl of hydroquinone (10 mmol l\(^{-1}\); pH 5; Sigma), overlaid with mineral oil and incubated for 20 h at 50°C. After incubation, the bisulfite-modified DNA was purified and desalted with a Wizard DNA Clean-up system (Promega), desfoninated in NaOH (0.3 mol l\(^{-1}\)),

**Table 1. EBV virological study in patients with chronic active EBV infection**

For reference see Yoshioka et al. (2001). Abbreviations: VCA, viral capsid antigen; EA, early antigen; EBNA, EBV nuclear antigen; ND, not determined.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)/sex</th>
<th>VCA-IgG</th>
<th>VCA-IgM</th>
<th>EA-IgG</th>
<th>EBNA</th>
<th>Clonality*/EBV-infected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt-1</td>
<td>5/F</td>
<td>320</td>
<td>&lt;10</td>
<td>40</td>
<td>&lt;10</td>
<td>ND/ND</td>
</tr>
<tr>
<td>Pt-2</td>
<td>22/M</td>
<td>560</td>
<td>40</td>
<td>160</td>
<td>640</td>
<td>+/B</td>
</tr>
<tr>
<td>Pt-3</td>
<td>2/M</td>
<td>10240</td>
<td>&lt;10</td>
<td>2560</td>
<td>160</td>
<td>+/CD4</td>
</tr>
<tr>
<td>Pt-4</td>
<td>5/F</td>
<td>5120</td>
<td>&lt;10</td>
<td>5120</td>
<td>10</td>
<td>ND/CD2</td>
</tr>
<tr>
<td>Pt-5</td>
<td>6/F</td>
<td>20480</td>
<td>&lt;10</td>
<td>5120</td>
<td>40</td>
<td>+/ND</td>
</tr>
</tbody>
</table>

*Clonality of EBV-infected cells was determined by terminal repeat analysis.
precipitated with ammonium acetate (Wako), ethanol and 1-2 μg of carrier glycogen (Boehringer Mannheim), and resuspended in 50 μl of sterile deionized water. For each sample, 2 μl aliquots of bisulfite-modified DNA were amplified with primers specific for the regulatory regions of Cp and Wp as shown in Table 3.

The PCR-amplified products were then cloned into the pCR2.1-TA cloning vector. Four to 10 colonies were analysed for each DNA sample. Each clone analysed was generated from an independent bisulfite PCR reaction and therefore reflects amplification of independent viral genomes. Plasmid DNA was extracted and sequenced using a BigDye Dye terminator cycle sequencing ready reaction kit (Perkin Elmer Applied Biosystems) with an ABI PRISM 310 Genetic Analyser (Perkin Elmer Applied Biosystems).

http://vir.sgmjournals.org 1135

Fig. 1. RT-PCR analysis of EBNA promoter usage in patients with chronic active EBV infection. Results of Southern blot hybridization are shown together with the exons (boxes), primers (arrows), probe sites (closed box) and sites of transcriptional promoter (flags) for each mRNA. Lane 1, pt-1; lane 2, pt-2; lane 3, pt-3; lane 4, pt-4; lane 5, pt-5; lane 6, negative control; lane 7, positive control.

Table 2. Primer sequences used for RT-PCR analysis

<table>
<thead>
<tr>
<th>Promoter usage</th>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Coordinates*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp</td>
<td>C1/C2-5'</td>
<td>CATCTAAACCGAATGAAAGAA</td>
<td>11470–11479/11626–11635</td>
<td>Tierney et al. (1994)</td>
</tr>
<tr>
<td>Wp</td>
<td>W0/W1-5'</td>
<td>GTACCACAAATCCTAG</td>
<td>14399–14410/14554–14558</td>
<td>Tierney et al. (1994)</td>
</tr>
<tr>
<td>Cp and Wp</td>
<td>W2-3'</td>
<td>CACCTGAGGTGACCGCTTA</td>
<td>14832–14813</td>
<td>Tierney et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Q-5'</td>
<td>GTGCCGCTACCCGATGCG</td>
<td>62440–62457</td>
<td>Tierney et al. (1994)</td>
</tr>
<tr>
<td>Qp</td>
<td>U-5'</td>
<td>GATGAGCGTTTGGAGAGCTGATCTGCA</td>
<td>67510–67539</td>
<td>Imai et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>K-3'</td>
<td>CATTTCCAGTTCTACCTCCAGTT</td>
<td>107986–107967</td>
<td>Brooks et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>β-actin-5'</td>
<td>CACTGTTACTAGAATAGAAG</td>
<td>107986–107967</td>
<td>Busson et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>β-actin-3'</td>
<td>CCTGTTACTAGAATAGAAG</td>
<td>107986–107967</td>
<td>Busson et al. (1992)</td>
</tr>
</tbody>
</table>

*The coordinates are given with reference to the B95-8 genomic sequence (Baer et al., 1984).
RESULTS

RT-PCR

U/K-spliced EBNA1 transcripts were consistently detected in all samples from the five CAEBV patients as described previously (Yoshioka et al., 2001). In this study, C1/C2/W2-initiated transcripts were detected in all samples, but none of the samples expressed Wp- or Qp-initiated transcripts (Fig. 1). These results showed that EBNA1 transcription was initiated from Cp in all samples. Cp-, Wp- and Qp-initiated transcripts could be detected down to dilutions of $10^{-5}$, $10^{-4}$ and $10^{-4}$ of cDNA from the positive controls, respectively. EBNA gene transcripts, promoter usage and sensitivities are summarized in Table 4.

Bisulfite analysis of EBV genome methylation

Fig. 2 shows the methylation status of the regulatory regions upstream of the EBNA gene promoters, Cp and Wp, in samples from the five CAEBV patients.

DISCUSSION

Expression of different panels of latent gene products is controlled by use of three different EBNA promoters (Cp, Wp and Qp). Cp and Wp permit the full spectrum of latent gene expression, as has been shown in IM or EBV-immortalized lymphoblastoid cell lines (LCLs) in vitro (Tierney et al., 1994; Kieff, 1996). In contrast, Qp usage has

<table>
<thead>
<tr>
<th>Table 3. Primer sequences used for bisulfite PCR analysis</th>
</tr>
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<tbody>
<tr>
<td>Primer* Sequence (5′–3′) Coordinates†</td>
</tr>
<tr>
<td>Cp-5′ outer ATATCCCCATTTAAAAACCC 10843–10861</td>
</tr>
<tr>
<td>Cp-3′ outer GTTAAAGGGCTTATATGTT 11425–11406</td>
</tr>
<tr>
<td>Cp-5′ inner CATACACCTAAACCAACC 10898–10916</td>
</tr>
<tr>
<td>Cp-3′ inner ATGGGGGGTTTTGGGTTTT 11385–11367</td>
</tr>
<tr>
<td>Wp-5′ outer CCCCCAATTTATCAATA 13796–13816</td>
</tr>
<tr>
<td>Wp-3′ outer TGGAGTGTGGGTATATAG 14660–14641</td>
</tr>
<tr>
<td>Wp-5′ inner CCTATCACCACCTACCA 13918–13936</td>
</tr>
<tr>
<td>Wp-3′ inner GGGGAAGGTATGAAATTGGGT 14486–14469</td>
</tr>
</tbody>
</table>

*Primer sequences obtained from Paulson et al. (1999).
†The coordinates are given with reference to the B95-8 genomic sequence (Baer et al., 1984).

<table>
<thead>
<tr>
<th>Table 4. Summary of EBV transcripts in chronic active EBV infection and sensitivities of RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Tissue   EBNA1* EBNA2* Cp-initiated transcripts Wp-initiated transcripts Qp-initiated transcripts</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Pt-1 Spleen     +    –    +    –    –    –</td>
</tr>
<tr>
<td>Pt-2 Spleen     +    +    +    –    –    –</td>
</tr>
<tr>
<td>Pt-3 Spleen     +    –    +    +    –    –</td>
</tr>
<tr>
<td>Pt-4 Spleen     +    –    +    –    –    –</td>
</tr>
<tr>
<td>Pt-5 Spleen     +    –    +    –    –    –</td>
</tr>
<tr>
<td>PC R-LCL        +    +    +    –    NT</td>
</tr>
<tr>
<td>PC AKATA        NT   NT   NT   +    NT</td>
</tr>
<tr>
<td>NC BJAB         –    –    –    –    –    –</td>
</tr>
<tr>
<td>Sensitivity†…  $10^{-4}$ $10^{-4}$ $10^{-5}$ $10^{-4}$ $10^{-4}$</td>
</tr>
</tbody>
</table>

*Transcripts and sensitivities of EBV latent genes were reported by Yoshioka et al. (2001).
†Maximum dilution of cDNA in which a positive signal could be detected in each RT-PCR.
Fig. 2. Methylation status of the EBNA1 gene promoters, Cp and Wp, in patients with chronic active EBV infection. The genome coordinate for each CpG site is given with reference to the B95-8 genomic sequence (Baer et al., 1984).

(A) CpG methylation in the region upstream of Cp. The locations of the CCAAT box, the TATAA box, transcription start site and the C1 exon are shown. The transcription factors (CBF1 and CBF2) of Cp and the relative positions of the CpG site are also indicated. (B) CpG methylation in the region upstream of Wp. The locations of the CCAAT box, the TATAA box, transcription start site, the W0 exon and the W1 exon are shown. The transcription factors (YY1, BSAP, CREB, RFX) of Wp and the relative positions of the CpG site are also indicated.
been reported in EBV-positive malignancies, such as BL, NPC and HD, which show restricted forms of latent gene expression (Brooks et al., 1992; Tao et al., 1998). We previously reported that EBV latent gene expression was restricted in CAEBV, indicating that Qp, but not Cp/Wp, might be used in most cases of CAEBV (Yoshioka et al., 2001). However, Cp-initiated transcripts exclusively were detected in all samples, but none of the samples expressed Wp- or Qp-initiated transcripts. An EBV-positive T-cell lymphoma is a clonal expansion of a single EBV-infected cell with a pattern of gene expression of latency II, which may indicate Qp usage (Pallesen et al., 1993; Chiang et al., 1996). Cp usage in CAEBV was apparently distinct from promoter usage of neoplastic T-cell lymphoma. We examined the sensitivity of each RT-PCR analysis in order to determine whether the RT-PCR technique is sensitive enough for definitive analysis of EBNA promoter usage. Cp-, Wp- and Qp-initiated transcripts could be detected down to a dilution of at least $10^{-4}$ of cDNA from the positive controls, as shown in Table 3. This indicated that the RT-PCR assay could detect Qp-initiated transcripts, if any are present. Furthermore, genomic DNA around the primer regions was sequenced in all the samples. Although several point mutations were detected around the primer regions, there were no mutations that would influence the annealing of PCR in the primer regions (data not shown). Moreover, PCR analysis was performed using another primer pair, reported by Zetterberg et al. (1999) to detect Q/U/K-spliced EBNA1 transcripts, but no Qp-initiated transcripts were detected (data not shown).

During latent EBV infection of human B lymphocytes, six EBNA Genes (EBNA1, EBNA2, 3A, 3B, 3C and LP) are expressed from a single complex transcriptional unit that spans nearly 100 kb of the viral genome by means of alternative splicing and alternative polyadenylation sites (Kieff, 1996). The first EBNA genes expressed are EBNA-LP and EBNA2, by initiation from Wp. Subsequently, transcription of EBNA2 activates Cp, LMP and a number of cellular promoters for growth response genes, and dominant promoter usage switches from Wp to Cp (Alfieri et al., 1991; Tierney et al., 2000a). Generally Cp-initiated EBNA1 cDNA has a Y1/Y2/Y3/U/K-spliced structure downstream of W1/W2 repeats. However, Tierney et al. (1994) reported that C1/C2/W2-initiated transcripts were detected in all 14 IM patient blood samples, whereas Cp-initiated, Y3/U/K-spliced EBNA1 transcripts were detected in only half of the samples. We detected C1/C2/W2-initiated transcripts in all five samples but could not detect Y3/U/K-spliced EBNA1 transcripts in all samples except for that from patient 4 (data not shown). Furthermore, several unique Cp-initiated transcripts that skipped one or more exons downstream of a Cp-initiated site have been reported (Bodescot & Perricaudet, 1986; Qu & Rowe, 1992, 1995). The mechanisms regulating alternative splicing and polyadenylation site usage within the EBNA transcription unit remain largely unknown. Although Cp permits the full spectrum of EBNA gene expression, EBV latent gene expression is restricted in CAEBV. Therefore, we speculate that Cp-initiated transcripts have unique splicing patterns and preferentially induce EBNA1 transcripts in most cases of CAEBV.

Methylation of CpG is an important mechanism of promoter regulation and EBV latent gene expression. EBNA promoter activity has been shown to be inversely correlated with the methylation status of the promoters Cp (Robertson et al., 1995, 1996; Robertson & Ambinder, 1997a, b; Salamon et al., 2001), Wp (Tierney et al., 2000b) and LMP1 (Falk et al., 1998). Methylation may inhibit transcription by a number of mechanisms, either by direct blocking of the binding of transcription factors to DNA or through methylation of methyl-CpG-binding proteins, which recruit histone deacetylases to the DNA, leading to the remodelling of chromatin into an inactive configuration (Singal & Ginder, 1999). Wp is exclusively utilized during the initial stage of EBV infection in primary B lymphocytes, followed by a switch to Cp usage (Alfieri et al., 1991; Tierney et al., 2000a). Hypermethylation in the Wp region indicated that the promoter of EBNA might have already switched from Wp to Cp or Qp in CAEBV, which is consistent with the results of transcriptional analysis. The mechanism of Wp down-regulation in CAEBV might be the same as that in EBV-infected B cells, and hypermethylation of Wp might be sufficient to prevent transcription from Wp in CAEBV. Qp is a TATA-less promoter at the centre of a hypomethylated island (Schafer et al., 1995). Qp was found to be completely unmethylated in samples from patients (data not shown). Qp might be inactivated by several factors, such as EBNA1, E2F, interferon response factors (IRFs)-1, -2 and -7 and high-mobility group protein (HMG)-1 (Y)-containing complex, under the conditions of Cp activation in CAEBV (Sung et al., 1994; Schafer et al., 1997; Chen et al., 1999).

Cp activity is the major difference between latency III and the other latency forms. Several mechanisms that may regulate Cp activity have been described. First, Cp can be activated by binding of EBNA1 to oriP, an enhancer 3 kb upstream of Cp (Puglielli et al., 1996). Second, glucocorticoid response elements (GREs) about 900 bp upstream of Cp respond to glucocorticoids and activate Cp (Evans et al., 1996). Third, the EBNA2 response region in the Cp region is critical for Cp activity. The EBNA2 response region contains the binding sites for the cellular DNA-binding proteins CBF1 and CBF2. The methylation of CpG sequences in the CBF2-binding domain inhibits Cp activity, and CBF2 binding is crucial for EBNA2-mediated activation of Cp (Robertson et al., 1995, 1996). Finally, several cellular factors that control the transcription activity of Cp have been identified. NF-Y transcription factor interacts with the CCAAT box in the −71 to −63 region of Cp. Members of the C/EBP transcription factor family interact with the C/EBP consensus sequence in the −119 to −112 region of Cp. Sp1 and Egr-1 interact with a GC-rich sequence in the −99 to −91 region of Cp. NF-Y,
members of the C/EBP, and Sp1 are positive regulators of Cp activity, and Egr-1 is a negative regulator of Cp activity (Nilsson et al., 2001).

There was a discrepancy between Cp usage and the hypermethylation status of the Cp region in CAEBV. The level of methylation in the Cp region in CAEBV was, surprisingly, as high as that in BL (Robertson et al., 1996; Tierney et al., 2000a). We cannot rule out the possibility that we could not detect cells with unmethylated Cp by bisulfite PCR analysis because the population size of such cells was very small. However, since Cp is regulated in a very complex manner as mentioned above, it could be concluded that the hypermethylation status of the Cp region might be insufficient to prevent transcription from Cp. This finding is consistent with the genetic analysis of Cp function by Evans et al. (1996). Furthermore, CAEBV is considered to be a T-cell LPD and only occasionally a B-cell LPD. Patient 2 was a rare case of B-cell LPD, but the methylation status was the same as that of T-cell LPD, indicating that hypermethylation in the Cp region occurs regardless of the phenotypic difference in EBV-infected cells.

In this study, we found unique EBV latent gene expression, EBNA promoter usage and methylation status of EBNA promoters in CAEBV. The unique relationships, seemingly contradictory, might offer the key for understanding the pathogenesis of CAEBV.

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