The Role of Methylation in the Phenotype-dependent Modulation of Epstein–Barr Nuclear Antigen 2 and Latent Membrane Protein Genes in Cells Latently Infected with Epstein–Barr Virus

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SUMMARY

Seven virus-encoded proteins are regularly expressed in Epstein–Barr virus (EBV)-transformed lymphoblastoid (LCL) cell lines: the EBV nuclear antigens EBNA 1 to 6 and the latent membrane protein (LMP). In nasopharyngeal carcinoma (NPC), only EBNA 1 is regularly expressed; LMP is detected in about 50% of the tumours. In Burkitt’s lymphoma (BL) tumours, only EBNA 1 is expressed. Also, in BL-derived cell lines that maintain the phenotypic markers characteristic of the in vivo tumour (group I), only EBNA 1 is expressed. EBV was rescued by induction or cocultivation from one BL cell line with a restricted group I pattern, and from one NPC tumour, into normal B cells. In the resulting LCLs EBNA 1 to 6 and LMP were expressed. We assessed the level of methylation in the genes encoding EBNA 2 and LMP by restriction fragment analysis using the methylation-sensitive enzymes SmaI and HpaII. These genes were extensively methylated in the group I BL line Rael and the nude mouse-passaged C15 NPC tumour, but were demethylated in the derived LCLs. In the LMP-expressing NPC tumour, but were demethylated in the derived LCLs. In the LMP-expressing coding exons were methylated. The EBNA 1 coding exon was methylated in the Rael line and in NPC, in spite of expression. In contrast, CpG pairs in ori P were originally hypomethylated and remained so after their transfer to LCLs. The cell phenotype-dependent pattern of EBV gene methylation correlated with the phenotype-dependent pattern of EBNA and LMP expression. The specific patterns of methylation localized to controlling regions (ori P and 5’ flanking sequences) also suggest a specific role for methylation in the regulation of EBNA and LMP expression.

INTRODUCTION

Seven virus-encoded proteins are regularly expressed in Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs): the EBV nuclear antigens EBNA 1 to 6 and the latent membrane protein (LMP) (Hennessy et al., 1984, 1985, 1986; Hennessy & Kieff, 1985; Dillner et al., 1986a, b; Kallin et al., 1986; Ricksten et al., 1988a; Petti et al., 1988). In nasopharyngeal carcinoma (NPC) tumours only EBNA 1 and LMP have been detected (in about half of the biopsies analysed; Fähræus et al., 1988; Young et al., 1988), and in Burkitt’s lymphoma (BL) tumours and phenotypically representative cell lines, only EBNA 1 has been detected (Rowe et al., 1987a). In BL-derived cell lines cultured in vitro, the pattern of expression varies from the restricted type that only expresses EBNA 1, to a pattern of protein expression similar to that of the LMP (Rowe et al., 1987a). The former cell type has maintained the phenotype of the BL tumour cell (group I), whereas the latter cell type drifted towards the LCL phenotype (group II/III). For this study we have chosen the EBV-carrying BL line Rael, which has maintained a group I phenotype in spite of prolonged culture in vitro (Klein et al., 1972; Masucci et al., 1989).
Treatment of Rael with 1 to 10 μM 5-azacytidine (5-azaC) induced EBNA 2, 3, 4 and 6 and LMP (Masucci et al., 1989). We did not have a suitable reagent to detect EBNA 5 in this cell line. This induction was independent of the more limited induction of early antigen (EA) and viral capsid antigen (VCA). Double fluorescence tests showed that EBNA 2 appears in EA/VCA-negative cells. Some of the known inducers of the EBV lytic cycle, such as 12-O-tetradecanoylphorbol 13-acetate (TPA) and n-butyrate did not induce EBNA 2 or EA/VCA in Rael cells.

5-AzaC is a cytosine analogue with a nitrogen in position 5 of the base ring. When incorporated into DNA instead of cytosine at CpG, methylation is inhibited. Methylation is one of the mechanisms that can control gene expression in eukaryotic cells and in viruses (Cedar, 1988; Doeffer, 1983); it has been suggested that it contributes towards the suppression of the lytic EBV cycle in growth-transformed cells (Szyf et al., 1985).

The EBNA 2 to 6 and LMP inducing effect of 5-azaC has prompted us to investigate whether the phenotype-dependent down-regulation of these proteins in the Rael cell is associated with methylation of the corresponding viral genes. Our study focused on EBNA 2 and LMP.

### METHODS

**Cell lines and tissue culture.** Rael is a BL-derived cell line (Klein et al., 1972). The other cell lines were established in the course of this study (see below). All lines were grown in RPMI 1640 tissue culture medium (Gibco), supplemented with 100 units/ml of penicillin V, 100 μg/ml of streptomycin and 10% foetal calf serum (FCS). The cells were fed every 3 to 4 days, and cultured in incubators with 5% CO2 and high humidity.

**Virus rescue.** EBV was rescued from the BL-derived cell line by induction with 3 mM sodium n-butyrate. After treatment for 3 days, the cells were washed, cultured for 2 more days, and the supernatant was collected and added to mononuclear cells from cord blood, isolated from the interphase of a one-step Ficoll–Isopaque gradient. Virus was successfully transferred from Rael cells resulting in the LCL, CBM1-Ral-STO.

EBV was also rescued from C15, an NPC tumour passed in nude mice (Busson et al., 1988). In this case several procedures were tried: sodium butyrate treatment, TPA treatment and cocultivation with either lymphocytes from cord blood or from an EBV-negative donor (GW). Cocultivation resulted in six LCLs, CBC-C15-STO A, B and C, and NAD-C15-STO A, B and C (Table 1). For Rael and C15 the origin of the virus was confirmed by identical Mr values for EBNA 1 (and LMP in C15) in the parental cells and the derived LCLs.

**Induction with 5-azaC.** The Rael cell line was exposed to 9 μM 5-azaC (Sigma) for 3 days (Masucci et al., 1989). EBNA 2 induction was monitored by anti-complement immunofluorescence with monospecific EBNA 2 reagents (Dillner et al., 1985).

**Immunoblotting.** Immunoblotting was performed as described earlier (Ernberg et al., 1986; Masucci et al., 1989). A previously characterized serum (OR) from a healthy EBV-seropositive donor was used as the source of antibodies for the detection of EBV antigens [VCA 1:320, EA(D) 1:10, EA(R) 1:320, EBNA 1:320, EBNA 2 1:80]. LMP was detected by the S12 monoclonal antibody (MAb) provided by Dr D. Thorley-Lawson (Dept of Pathology, Tufts University School of Medicine, Boston, Mass., U.S.A.). The human serum was used at a 1:20 dilution.

**Analysis of DNA methylation.** Eukaryotic and viral DNA is predominantly methylated in CpG pairs. Methylation was analysed by digesting high Mr cellular plus viral DNA with either Smal (specificity CCCGGG) or HpaII (specificity CCGG). For each μg of DNA 10 units of enzyme was added to complete digestion. In some experiments the DNA was cleaved with both BamHI and Smal. The HpaII digests were compared to parallel digestion with MspI, an isoschizomer which is not inhibited by methylation. The restriction enzymes were obtained from Amersham. Digestion was performed overnight or for longer times in the enzyme-specific buffers provided by the manufacturer.

The digested DNA was separated on a 1.5% agarose gel (Bethesda Research Laboratories) in Tris–borate–EDTA buffer (TBE; 89 mM-Tris base, 89 mM-boric acid, 2 mM-EDTA). Electrophoresis was carried out for 13 h at 50 V and digestion was checked by ethidium bromide staining. Denaturation was carried out in 0.5 M-NaOH with 1.5 M-NaCl for 1 h. The DNA was then neutralized for 1 h in 1 M-Tris-HCl, 1.5 M-NaCl, pH 7.5. Transfer to nitrocellulose (Hybond N, Amersham) was done in 20 X SSPE (0.2 M-NaH2PO4, 3 M-NaCl, 0.02 M-EDTA, pH 7.4) overnight. Prehybridization was performed with 6 X SSPE, 1% SDS, 100 μg/ml herring sperm DNA (Boehringer Mannheim) and 10 X Denhardt’s solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin) at 42 °C overnight.

The probes were labelled with [32P]CTP (Amersham) by random primer oligolabelling with the Klenow fragment of Escherichia coli DNA polymerase (Feinberg & Vogelstein, 1983). Hybridization with specific probes...
was performed at 42 °C for 16 to 24 h in 6 × SSPE, 100 µg herring sperm DNA, 50% formamide, 1% dextran sulphate, and the radioactive probe. Subsequently the filters were washed in 2 × SSPE, 0.1% SDS for 15 min at room temperature, twice in 0.2 × SSPE, 0.1% SDS at 65 °C and finally for 5 min in 0.1 × SSPE. The filters were finally exposed to Fuji medical X-ray film for 4 to 48 h at −90 °C and then developed.

Probes. Cloned EBV DNA fragments were kindly provided by Dr Lars Rymo, Gothenburg, Sweden. The following probes were used (see also Fig. 1): EcoRI–BamHI from the BamHI C fragment (coordinates 7315 to 13215), the BamHI W fragment, the BamHI Y fragment, the BamHI K fragment, and the pMBamH2 fragment (cloned from the M-ABA cell line, kindly provided by Dr Georg Bornkamm), which covers the 3' part of the EBNA 2A exon BYRF1 (coordinates 48848 to 50537). We also used a MspI–MspI digest from the BamHI C fragment (coordinates 8189 to 9105), covering part of ori P, a MspI–MspI fragment from the BamHI K fragment covering part of the EBNA 1 exon BKRF1 (coordinates 108161 to 108924), a MluI–MluI fragment from the EcODhe t fragment covering most of the LMP exons (coordinates 167130 to 169567), a XhoI–EcoRI fragment (coordinates 172284 to 169424) and a BglI–BglI fragment (coordinates 169449 to 170290), both covering the promoter and the 5' flanking sequences of LMP.

**RESULTS**

Expression of EBV-encoded proteins

The BL line Rael and the nude mouse-propagated NPC tumour C15 expressed EBNA 1 but not EBNA 2, 3, 4, 5 or 6. (However we did not have a suitable reagent to detect EBNA 5 in the Rael line or in the derived LCL.) LMP was not expressed in Rael cells but was expressed in C15 at a low level. The derived LCLs that carried the Rael or the C15 virus had the same EBNA 1 sizes as the virus donor line, and they also expressed EBNA 2 to 6 and LMP (Table 1). 5-AzaC induced EBNA 2 to 6 and LMP in the Rael cell line. EA and VCA were also induced at a lower level, and it has been demonstrated by double immunofluorescence that EBNA 2 and EA were induced in different cells (Masucci et al., 1989).

Methylation of the EBNA 2 and LMP coding genes

Utilizing the methylation-sensitive restriction enzymes HpaII and/or Smal we analysed the CpG methylation pattern within the sequences recognized by these enzymes. The probes were selected to detect control regions and coding exons for EBNA 2 and LMP.

For the Rael line extensive methylation occurred in the 3' half of the BamHI C fragment, the BamHI W and Y fragments, and the BYRF1 exon that codes for EBNA 2 (Fig. 2b to e). In the case of BamHI C, only the 3' half was analysed, as it contains the relevant promoters and the ori P region. After cleavage with both BamHI and Smal, the BamHI fragments appeared with no further cleavage by Smal, indicating complete methylation of all the Smal sites. However there was a weak signal from a fragment slightly smaller than 1 kb in BamHI C (Fig. 2b), suggesting two unmethylated sites. This was further investigated with the ori P probe (see below). Potentially there are 18 Smal sites in the region of BamHI C that our probe covers, 17 sites in BamHI W, five in BamHI Y and one covered by the pMBamH2 probe, according to the B95-8 sequence (Baer et al., 1984). We have no indication that the Rael virus differs from B95-8 in any of the sites surrounding larger fragments detected by our gel blot system.

### Table 1. Expression of EBNA and LMP in tumours, tumour-derived cell lines and LCLs

<table>
<thead>
<tr>
<th>Designation</th>
<th>EBNA 1</th>
<th>EBNA 2</th>
<th>EBNA 3</th>
<th>EBNA 4</th>
<th>EBNA 5</th>
<th>EBNA 6</th>
<th>LMP</th>
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<td>Rael</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>ND†</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Rael + 9 µM-5-azaC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND†</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CBM1-Ral-STO</td>
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<td>+</td>
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* Summary of data from this paper, and also from Masucci et al. (1989) and Fähraeus et al. (1988).
† ND, Not determined (there was no suitable reagent to detect EBNA 5 encoded by the Rael virus).
‡ NPC tumour passaged through nude mice in vivo.
Fig. 1. DNA probes used to study methylation by Southern blotting. Horizontal black lines (■) between flags with numbers show the probes that were used in this study. Small vertical bars indicate restriction sites detected by methylation-sensitive enzymes, either Smal (a to d) or HpaII (e to g). Major fragments resulting from complete digestion with these enzymes are indicated on top of the probe line by the size in bp. Two identified enhancers have been indicated within the probe areas (■). The major latent promoters are indicated by small flags (▲). The early promoter for the latent membrane protein is indicated with an open small flag (▲). Ori P and the exons for EBNA 2 and LMP are indicated beneath the probes by shaded boxes (■). (a) the BamHI C probe from EcoRI site 7032 to BamHI site 13215. In some experiments the 916 bp fragment within oriP removed by Mspl/HpaII was used. (b) BamHI W probe, covering the whole BamHI W fragment. The figure shows the first of a series of identical BamHI W fragments. (c) BamHI Y probe, covering the whole BamHI Y fragment. This probe will cross-react partly with BamHI W. (d) pMBamHI, probe, covering the 3' part of the EBNA 2 exon. Note that the probe is smaller than the smallest fragment detected, i.e. 1689 bp after complete Smal cleavage. (e) Probe recognizing the LT 3 region, obtained by cleavage with MluI. (f) Probe for 5' flanking sequence of LT 3, obtained by digestion with XhoI and EcoRI. Sometimes a smaller probe from between the indicated BglI sites (▲) was used. (g) Probe covering the BamHI K fragment. The maps and coordinates given are based on the B95-8 sequence, and on M. markers included in our blots (HindIII-cleaved lambda DNA and 1 kb ladder; Bethesda Research Laboratories). We have found no difference in restriction fragment length between Rael and B95-8 for the larger fragments detected by these probes.
Fig. 2. Analysis of the methylation pattern of the EBNA 2 gene in the Rael line and in CBM1-Ral-STO. (a) Map of the EBNA 2 coding part of the EBV genome, based on the prototype B95-8 sequence (Baer et al., 1984). The BamHI cleavage map is shown. Exons detected in EBNA 2 cDNA clones are designated by (mllm) (Bodescot & Perricaudet, 1986; Wang et al., 1987; Speck & Strominger, 1985; Sample et al., 1986). Pr indicates promoters that may be used for EBNA 2 transcription. E designates enhancers that have been demonstrated (Reisman & Sugden, 1986; Ricksten et al., 1988a). In autoradiographs b to e: R, Rael; 5-A, 5-azacytidine-treated Rael; C, CBM1-Ral-STO. Arrows indicate larger fragments or position of Mr marker. (b) Whole cellular DNA (10 µg) cleaved with BamHI (50 units) + SmaI (100 units) and probed on Southern blots with a 32P-labelled probe covering the EcoRI—BamHI region of the BamHI C fragment (coordinates 7315 to 13215). (c) Cellular DNA (10 µg) cleaved with BamHI and SmaI, and probed with the BamHI W fragment. (d) Whole cellular DNA (10 µg) cleaved with BamHI and SmaI and probed with the BamHI Y fragment. (e) Whole cellular DNA (10 µg) cleaved with BamHI and SmaI and probed with a probe covering the 3' part of BYRF1 (EBNA 2 exon; pMBamH1, probe coordinates 48848 to 50537).
Treatment with 5-azaC increased the occurrence of low Mr fragments in this whole region (coordinates 7315 to 49900) as expected on the basis of the demethylating effect of the drug. CBM1-Ral-STO was hypomethylated in the 3' half of the BamHI C fragment, including the initiation point of transcription (adjacent to promoter BCR2, at coordinate 11305) in several cDNA clones representing EBNA-coding transcripts (Bodescot & Perricaudet, 1986; Speck & Strominger, 1989). In the blot (Fig. 2b) the major Smal fragments (1617, 1335 and 916 bp) are easily identified. The BamHI W region was also hypomethylated in this LCL, as demonstrated by the appearance of the largest Smal fragment (723 bp), and weak signals from several smaller fragments corresponding to a 393 bp fragment and four fragments of 246 to 265 bp (Fig. 2c). In BamHI Y, the major 1.27 kb Sinai fragment is identified (Fig. 2d). Hypomethylation of the single Smal-recognized CpG in the 3' end of the EBNA 2 exon was also detected as a 1.7 kb (1689 bp) fragment by a probe specific for this sequence (pMBamH2) (Fig. 2e).

CBM1-Ral-STO was also hypomethylated in the LMP coding region, as shown by HpaII/MspI cleavage, demonstrating the four largest MspI fragments 357 to 818 bp, and no difference was seen between the effects of the two enzymes (Fig. 3). In contrast, the Rael virus was highly methylated, with the exception of the appearance of a fragment approximately 0.7 kb that may either correspond to the 3' 667 bp fragment extending over the MluI site used for probe trimming, or may be due to partial methylation and joining of fragments within the region (e.g. 293 bp + 57 bp + 357 bp = 707 bp). Methylation in the Rael line also extended to the 5' flanking region of the LMP exons including the promoter (coordinate 169546), as shown with BglI-BglI or XhoI-EcoRI probes, as described in Methods (data not shown).
In contrast, probing part of the ori P region with a specific probe (ori P, Fig. 1 a) yielded the same sized (0-9 kb) fragment after both HpaII and MspI digestion of Rael, in CBM1-Ral-STO and 5-azaC-induced Rael DNA (Fig. 4). This corresponds to the 916 bp fragment obtained after complete digestion, and thus shows that the two CpG sites covered by this probe are non-methylated and are accessible to the methylation-sensitive enzyme in all three cells.

The EBNA 1 exon in the BamHI K fragment represents another region which is active in all the cell types tested. This fragment carries four large open reading frames, one of which codes for EBNA 1. BamHI K probing of HpaII-cleaved DNA generated a pattern with several high M, bands in the Rael line. All fragments except one were larger than the MspI fragments (Fig. 5 a). The MspI pattern would be expected in the case of complete hypomethylation. The Rael DNA generated one 0.7 kb fragment, which is perhaps due to cleavage of unmethylated recognition sites surrounding a 0.7 kb fragment included in the repeat array of EBNA 1. However after stripping and reprobing that same blot with a probe covering only this region, we did not obtain a 0.7 kb signal in the HpaII-digested Rael DNA in spite of overexposure of the blot (Fig. 5 b), suggesting that the fragment detected in Fig. 5 (a) was the result of partial methylation and fusion of several fragments recognized by the BamHI K probe. Compared to Rael, the 5-azaC-treated cells were less methylated, and the CBM1-Ral-STO cells were completely unmethylated in this region.
The nude mouse-passaged NPC tumour C15 showed a similar SmaI cleavage pattern as Rael DNA in the BamHI C–W–Y–H region, but with a slightly less homogeneous methylation of the EBNA 2 exon (Fig. 6a). After transfer of the virus to LCLs (NAD-C15-STO), the EBNA 2 coding region was completely hypomethylated and showed the same cleavage pattern as that of CBM1-Ral-STO, with one 1.7 kb fragment (Fig. 6a). In the BamHI W region the NPC tumour shows a partially methylated pattern, with signals from two 0.2 to 0.3 kb fragments that represent non-methylated fragments (Fig. 6b), whereas the LCL is completely non-methylated.

Using the MluI probe detecting the LMP exons, this region also turned out to be completely methylated in the C15 tumour, whereas it was unmethylated in the LCL (Fig. 7a). The gene is expressed in both cell types. The 5' flanking region was analysed, and showed predominantly hypomethylated DNA (after HpaII digestion) in both the NPC and LCL, with signals from the

Fig. 5. Methylation pattern of the BamHI K region. (a) Whole cellular DNA digested with HpaII/MspI and probed with the BamHI K fragment. Lanes as in Fig. 2 and 3. Larger fragments are indicated by bars. (b) Same blot as in (a), but probed with a HpaII–HpaII digest from the BamHI K region (within the EBNA 1 exon, coordinates 108161 to 108935). The blot was overexposed to reveal any trace of the 774 bp fragment in the Rael HpaII lane. Lanes as in Fig. 2 and 3.
EBV DNA methylation in LCL and tumour cells

Fig. 6. Methylation pattern of the EBNA 2 gene in NPC C15 and LCLs obtained by transferring the virus by cocultivation. (a) Whole cellular DNA digested with SmaI and probed with a probe covering the BamHI H part of BYRF1 (EBNA 2 exon). Lane 1, CBM-1-Rai-STO; lane 2, Rael; lane 3, C15; lane 4, CBC-C15-STO B; lane 5, NAD-C15-STO A; lane 6, NAD-C15-STO B. (b) Whole cellular DNA (lanes as for a) digested with HpaII (H) or MspI (M) and probed with a BamHI W probe. Bars indicate the 558 and 257 bp MspI fragments.

expected 506 and 213 bp fragments. A small fraction of the viral genomes in the NPC tumour was methylated in this region as indicated by hybridization of two large M, fragments, which were actually the same large fragments that were detected with the MluI probe, due to methylation.

DISCUSSION

This comparative study of the three main EBV-carrying cell types revealed a strong correlation between the expression of EBNA 2 and of LMP, and in the pattern of hypo- or demethylation of the corresponding genes and/or their regulatory sequences.

Transfer of EBV from the BL cell line Rael to normal B cells was accompanied by an up-regulation of both genes in the derived LCLs, in parallel with complete demethylation. The NPC C15 tumour expressed LMP at a low level, but did not express EBNA 2. EBNA 2 was up-regulated and the coding gene was demethylated when the EBV genome was transferred to normal B cells. However the LMP coding exons were extensively methylated in the C15 tumour cells expressing LMP, whereas the 5' flanking sequences including the LMP promoter were hypomethylated. In the corresponding LCL the LMP coding exons were unmethylated.

The methylation pattern of the EBNA 1 coding exon BKRF1 correlated with the cell phenotype, and not with expression of EBNA 1 in the Rael line and the Rael virus-derived LCL.
Therefore we have detected two types of methylation pattern. One predominant pattern follows the cell phenotype, with extensive methylation of the parts of the EBV genome that we have analysed in the Rael line and NPC C15, and with total lack of methylation of the same regions in the derived LCLs. Superimposed on this is a gene expression-correlated pattern, involving regulatory regions (ori P enhancer, LMP 5′ flanking sequences, BamHI W promoter –enhancer).

Several studies in other systems have shown that methylation of 5′ flanking regulatory sequences is more critical in gene regulation than is methylation of the coding exons (Kelley et al., 1988; Bonnerot et al., 1988; Keshet et al., 1985; Toniolo et al., 1988). We have analysed the methylation pattern of several such regions. The transcription and transcript processing of the EBNA1 is complex. Analysis of EBNA 1- to 6-encoding cDNA clones has shown extensive splicing (Bodescot et al., 1984, 1987; Bodescot & Perricaudet, 1986; Wang et al., 1987; Sample et al., 1986; Speck & Strominger, 1985, 1989). Promoters were found in two regions, BamHI C (BCR2, P_{11305}) and BamHI Ws (P_{14352+43073}, n = 4 to 11). In the producer line B95-8 several different EBNA transcripts start at the same promoter, P_{11305}, whereas EBNA 2 transcripts in an LCL, IB4, were found to initiate at one of the BamHI W promoters. An enhancer is localized in ori P, 2 kb upstream of the BamHI C promoter, which can trans-activate after EBNA 1 binding (Reisman & Sugden, 1986).

The ori P enhancer may be involved in regulation of transcripts from the BamHI C promoter, although localized 2 kb upstream. The ori P region (Yates et al., 1984) was not methylated in the Rael line or in the derived LCL. The analysis of the region was focused on two putative
methylation sites. This suggests that the region may be constantly accessible or 'open'. There were no suitable CpG sites in the vicinity of the P1305 promoter for a similar analysis.

Recently an enhancer has also been demonstrated 50 to 400 bp upstream of the BamHI W promoter (Ricksten et al., 1988b); this enhancer functions in B cell lines, but not in epithelial cells and is likely to be important for the regulation of EBNA 2 expression. Regulatory elements and promoters for EBNA 1 and EBNA 2 expression is thus localized up to 84 kb upstream of the coding exons (Bodescot et al., 1986). As yet there is no definite information on which promoter/enhancers are involved in EBNA 1 and EBNA 2 expression in the Rael line and the derived LCL. The unmethylated status of the ori P enhancer correlates with EBNA 1 expression. The BamHI W promoter/enhancer was hypomethylated in the cells expressing EBNA 2, but methylated in non-expressers. The promoter for transcripts encoding LMP have been mapped to the EDL 1 site (coordinate 169546). This region was hypomethylated in the otherwise extensively methylated C15 tumour, correlating with the expression of LMP.

The recent findings of specific methylation sites within the contact points of DNA-binding proteins critical for transcriptional regulation (Watt & Molloy, 1988; Saluz et al., 1988) will prompt a search for specific critical CpG methylations within these promoter and enhancer regions. Our data suggest a role for methylation of regulatory regions in EBNA 1, 2 and LMP control.

The induction of EBNA 2 to 6 and LMP by 5-azaC was highly suggestive that methylation is involved in the phenotype-dependent EBV gene expression (Masucci et al., 1989). However the 5-azaC effect does not prove a causal relationship between demethylation and EBNA 2/LMP expression, because 5-azaC has been shown to induce cellular changes both by indirect mechanisms, secondary to the effect of induced proteins, and even in the absence of any methylation (Cedar, 1988). In our study the induction correlated with partial demethylation of the coding genes for EBNA 2 and LMP, which was in line with the EBNA 2 induction in a fraction of the cells, as shown by immunofluorescence (40 to 70%, Masucci et al., 1989). There is probably a contribution from non-methylated genomes from the pool of newly synthesized EBV DNA as a result of induction of the productive virus cycle in some of the cells. We do not take the 5-azaC data as proof of a causative role for demethylation in EBNA 2 and LMP induction. Demethylation may even be secondary to the activation of genes (Cedar, 1988). Too little is known about the control of methylation, its specificity and role in gene control, to permit general conclusions on the cell phenotype-correlated methylation pattern. One possibility is that the methylation of the EBV genome reflects the methylation activity of the host cell. This is supported by the fact that cellular DNA is extensively methylated in the Rael and C15 lines compared to the LCLs (data not shown). In this case, EBV functions that are essential for the maintenance of the virus and its interaction with the host cell, such as ori P, the P1305 and/or perhaps other promoters, would have to be protected from methylation-sensitive down-regulation. This is supported by a recent analysis of CpG pairs, showing that they are under-represented within the EBV genome, compared to the expected frequency based on overall base composition. The frequency of TpG and CpA dinucleotides was higher than expected, suggesting mutational drift due to methylation (Honess et al., 1989). The ori P region and the latent promoter in BamHI C showed pronounced CpG deficiency. This may reflect mutational escape from methylation pressure on the viral genome in a natural host cell (presumably the cell carrying latent EBV).

The methylation in NPC and BL cells may either reflect in vivo selection against virus-carrying (tumour) cells that express certain antigens, or alternatively the viral strategy may favour latent persistence in cells with high intrinsic methylation activity. In the latter case, the tumour cell may reflect the status of the viral genes in the normal precursor cell. The immune surveillance hypothesis would be consistent with the demonstration that EBNA 2, and perhaps LMP, may provide targets for specific cytotoxic T cells.

The second hypothesis implies that the virus persists preferentially in cells that down-regulate potentially immunogenic virus products. Recently we have found that the haematopoetic compartment is probably responsible for long-term viral persistence (Gratama et al., 1988). The normal precursor of the BL cell is a likely candidate for this role. Lewin et al. (1987) showed
recently that small, high density B cells from EBV-positive peripheral blood are capable for direct spontaneous outgrowth of LCLs carrying EBV, although this occurs infrequently. These small, presumably long-lived resting B cells in G0 may be the cells with a high intrinsic methylation activity, carrying latent viral genomes \textit{in vivo} in healthy EBV seropositive individuals.

We conclude that the cell phenotype-dependent, extensive methylation of the EBV genome is modified in some important regulatory regions (oriP, promoters/enhancers) which are correlated with respect to their functional accessibility. We propose that demethylation of regulatory regions is involved in the control of EBNA 2 and LMP genes. We also propose that the methylation of EBV genes may play an important role in the establishment of EBV latency \textit{in vivo}.

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REFERENCES


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