Variable Expression of Latent Membrane Protein in Nasopharyngeal Carcinoma Can Be Related to Methylation Status of the Epstein-Barr Virus BNLF-1 5'-Flanking Region

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Seven virus-coded proteins, the nuclear proteins EBNA-1 to EBNA-6 and the latent membrane protein (LMP), are regularly expressed in Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines. In nasopharyngeal carcinoma (NPC), only EBNA-1 is regularly expressed; LMP is detected in about 65% of the tumors. In Burkitt's lymphoma tumors only EBNA-1 is expressed. We have recently shown that the methylation patterns of the EBV genome varied between these cell types. In virally transformed lymphoblastoid cell lines of normal origin, the EBV DNA is completely unmethylated. In contrast, in the Burkitt's lymphoma-derived cell line Rael and in a nude mouse-passaged NPC tumor, C15, there was an extensive methylation of CpG pairs. The methylation extended into the coding regions of the two expressed genes, EBNA-1 (in both tumor types) and LMP (in C15). Two presumptive control regions were exempted from this overall methylation: the *oriP* that contains both an origin of DNA replication and an EBNA-1-dependent enhancer and the 5'-flanking region of the BNLF-1 open reading frame that codes for LMP. The latter was only exempted in the LMP expressing NPC. We have now investigated the relation between expression of LMP and methylation of DNA in the 5'-flanking 1 kb region of BNLF-1, coding for LMP. LMP was methylated in 3 of 12 NPC biopsies that did not express LMP but was partially or totally unmethylated in the remaining 9 that expressed the protein. The three BNLF-1 exons were highly methylated in all the tumors. The *oriP* region was unmethylated in all the tumors, as in the previously studied Rael cell line and nude mouse-passaged NPC. Also, the *Bam*HI W enhancer region involved in the expression of EBNA nuclear proteins was methylated. None of the biopsies expressed EBNA-2. Our data show that the EBV genomes are highly methylated in NPC tumors. The strong reverse correlation between the methylation of the putative control region of the LMP gene and the expression of LMP suggests that methylation has a role in the regulation of this gene.

Seven virus-coded proteins are regularly expressed in Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines (LCLs): the nuclear proteins EBNA-1 to EBNA-6 and the latent membrane protein (LMP) (8, 9, 18–21, 24, 30, 32).

In nasopharyngeal carcinoma (NPC) only EBNA-1 and LMP were detected (14, 43), and in Burkitt's lymphoma (BL) tumors and phenotypically representative cell lines only EBNA-1 was detected (13, 35, 36). So far, EBNA-1 has been reported in 75 NPC biopsies, whereas LMP was only detected in 45 of 80 (56%) NPC biopsies. The biopsies showed similar expression patterns, irrespective of geographical origin (North and East Africa, China, Malaysia, or the United Kingdom), although more of the Chinese NPC biopsies (71%) than those of East or North African origin (25 to 62% in the two published studies) expressed LMP. EBNA-2 to EBNA-6 could not be detected in any of the biopsies.

We have recently shown that the methylation of the EBV genomes differs among the three EBV-carrying cellular

prototypes. In EBV-transformed B-cell lines of normal origin (LCLs), the viral genomes are unmethylated (12). In the BL-derived cell line Rael and in the nude mouse-passaged NPC C15 tumor, CpG pairs of the EBV genomes were extensively methylated. The exons of expressed genes, EBNA-1 and LMP, were methylated as well, but two potential control regions were unmethylated: the *oriP* that contains one origin of DNA replication and an EBNA-1-dependent and EBNA-regulating enhancer (31, 38) and the 5'-flanking region of BNLF-1 that codes for the LMP (in the NPC, which expressed LMP). Rescue of the virus from BL and from NPC into LCLs has led to demethylation of the viral DNA in all derived lines.

DNA methylation can participate in the control of eukaryotic genes (6, 10). The cause-effect relationship is controversial (6, 11, 23). Recent studies have shown that the methylation of specific CpG pairs within the DNA-binding motifs of transcription factors can prevent their binding and downregulate the transcription of an adjacent gene (2, 28, 41). Hence, the methylation of specific sites can directly influence gene expression and hypomethylation of 5' promoter-enhancer regions is often correlated with expression, whereas methylation of structural genes is not (6).

The cell phenotype-dependent differences in EBV DNA methylation and the apparent correlation with viral gene expression suggest that methylation is involved in viral gene control in latently infected cells (12). This suggestion was tested in the present paper by focusing on the fact that only about 56% of NPCs express LMP (14). We have investigated

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the methylation of CpGs in BNLF-1 and in 5'-flanking regions. The putative EBNA-controlling region in *oriP* and the enhancer in the internal repeat 1 have been studied in parallel. We have found a strong correlation between LMP expression and lack of methylation of the 5'-flanking region of BNLF-1, extending 1 kb upstream of the promoter. This correlation is in line with the recently discovered regulating elements in this region involved in LMP control (12a, 17). The correlation between LMP expression and the methylation status of the LMP regulatory sequence suggests a modulating role of methylation or, alternatively, that the methylation status depends on the activity of the LMP regulatory sequence.

MATERIALS AND METHODS

Cell lines and tissue culture. Rael is a BL-derived cell line (27) with a stable type I BL phenotype, expressing only EBNA-1 but not EBNA-2 to -6 or LMP. The CBC- and NAD-C15-STO cell lines were established by transfer of the virus carried by the NPC designated C15, serially propagated in nude mice, to normal B cells (12). All lines were grown in RPMI 1640 medium (GIBCO Biocult, Long Island, N.Y.), supplemented with 100 U of V penicillin per ml, 100 μ g of streptomycin per ml, and 10% fetal calf serum. The cells were fed every 3 to 4 days and cultured in incubators with 5% CO₂ atmosphere and high humidity.

Biopsies. NPC biopsies were done by local surgery in the Shanghai area. The tumors were stored frozen (-70°C) until prepared for DNA or protein blotting. CAO is a Chinese EBV DNA-positive NPC tumor passaged in nude mice (20 passages [5]). C15, C17, and C18 are nude mouse-passaged, EBV DNA-positive African NPC tumors, analyzed after 15 to 50 passages (4). The tissues were prepared for immunoblotting as described earlier (14).

Immunoblotting. Immunoblotting was performed as described earlier (13, 14). A previously characterized serum sample from a healthy EBV-seropositive donor was used as the antibody source for detection of EBNA-1 (VCA, 1:320; EAD, <1:10; EAR, 1:320; EBNA, 1:320; and EBNA-2, 1:80). Alternatively, we also used the sera of a CLL patient PG (VCA, 1:1,280; EA(D), 1:640; EA(R), 1:20; EBNA-1, 1:320; and EBNA-2, 1:80). EBNA-1 was detected by a human serum that has been affinity purified on an EBNA-1-specific polypeptide (no. 107) (14). LMP was detected by the S-12 monoclonal antibody (MAb) provided by D. Thorley-Lawson (Department of Pathology, Tufts University School of Medicine, Boston, Mass.). The human serum sample was used at a 1:20 dilution and the S-12 MAb was used at a 1:1,000 dilution of ascitic fluid. Alternatively, a pool of MAbs against LMP, kindly supplied by Martin Rowe (CRC Laboratories, Birmingham, United Kingdom; CS.1 to CS.4 [34]), was used at a 1:1,000 dilution. LMP could be detected in a control sample prepared from B95-8 cells when an immunoblotting lane was loaded with material corresponding to 5×10^3 cells. LMP could not be detected in samples with lower numbers of cells. Normally the lanes are loaded with material from 10^5 to 10^6 cells; LMP would thus be detected if it were expressed in 0.5% of the cells (given a level of expression per cell similar to that of B95-8 cells). This gives an indication of the level of sensitivity needed to detect LMP in NPC biopsies, although accurate cell numbers in biopsies cannot be determined.

Analysis of DNA methylation. Eukaryotic and viral DNA are predominantly methylated in CpG pairs. Methylation was analyzed by digesting high-molecular-weight cellular

FIG. 1. DNA probes used to study methylation by Southern blotting. Horizontal black lines (■) between boxes with numbers (e.g., 7032) show probes used in this study. In several cases smaller probes (●) were used as an alternative. Small vertical bars indicate restriction sites detected by the isoschizomer *MspI* or *HpaII* (in the latter case only when the CpG was unmethylated). Major fragments resulting from complete digestion with these enzymes are indicated by size (in base pairs). Identified enhancers and EBNA-1-binding regions have been indicated within the probes (□). The major latent promoters (▶) and the early promoter for the latent membrane protein (▷) are indicated. *oriP* and the exons for EBNA-1, EBNA-2, and LMP are indicated beneath the probes (■). (a) *oriP* region in the *BamHI* C fragment. In particular the 916-bp fragment within *oriP*, cleaved out by *MspI* (●) was used. (b) *BamHI* W fragment, with the *AvaI* fragment used as a probe to detect the W enhancer (●). The figure shows the first of a series of identical *BamHI* W fragments. (c) Probe used to detect the 3' part of the EBNA-2-coding exon. (d) *BamHI* K fragment, with a smaller *MspI-MspI* probe within the repeat region of BKRF-1 (●). (e) Probe recognizing the LT 3 region, obtained by digestion of the *EcoRI* D_{het} fragment with *XhoI*. A, B, and C show the three exons for LMP. (f) Two probes for analysis of the 5'-flanking sequence of LT 3, either as an *XhoI-EcoRI* fragment or obtained by digestion with *BglI* (●). The maps and coordinates given are based on the B95-8 sequence and on molecular weight markers included in our blots (*HindIII*-cleaved lambda DNA and a 1-kb ladder (Bethesda Research Laboratories)).

and viral DNAs with either *HpaII* (specificity, CCGG) or *HhaI* (specificity, GCGC). For each 1 μ g of DNA, 10 U of enzyme was added to complete digestion. The *HpaII* digests were controlled by parallel digestion with *MspI*, an isoschizomer which is not inhibited by methylation. The restriction enzymes were obtained from Amersham (Amersham, England). Digestion was performed overnight or for longer times in the enzyme-specific buffers provided by the manufacturer.

FIG. 2. Immunoblots showing expression of EBV proteins in NPC biopsies. (A) Detection of LMP with S-12 MAbs. Biopsies 3, 5, and 10 were negative, while expression was weak but positive in 7, 12, and 13. (B) Detection of EBNA-1 by a monospecific human EBNA-1 serum, obtained by affinity purification with an EBNA-1 polypeptide. (C) Detection of EBNA-1 with a polyvalent EBNA-positive serum (PG). Ramos was used as a negative control, and B95-8 was used as a positive control. The positions of standard molecular weight markers as well as the positions of EBNA-1 and LMP are indicated.

The digested DNAs were separated on a 1.5% agarose gel (Bethesda Research Laboratories, Inc., Bethesda, Md.) 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. 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-1.5 M NaCl, pH 7.5. Transfer to nitrocellulose (Hybond N; Amersham) was done in 20× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA

[pH 7.7]) overnight. Prehybridization was performed with 6× SSPE-1% sodium dodecyl sulfate (SDS)-100 µg of herring sperm DNA (Boehringer Mannheim) per ml-10× Denhardt solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin) at 42°C overnight.

The probes were labeled with [³²P]dCTP (Amersham) by random primer oligolabeling with the Klenow fragment of *Escherichia coli* DNA polymerase (16). Hybridization with specific probes was performed at 42°C for 16 to 24 h in 6× SSPE-100 µg of herring sperm DNA-50% formamide-1%

TABLE 1. Designations and EBV protein expression in biopsies and cell lines used in this study

Cell (origin) ^a	Expression			Reference
	EBNA-1	EBNA-2 to -6	LMP	
1 (Ch NPC)	+	-	+	
2 (Ch NPC)	+	-	+	
3 (Ch NPC)	+	-	-	
4 (Ch NPC)	+	-	+	
5 (Ch NPC)	+	-	-	
6 (Ch NPC)	+	-	+	
7 (Ch NPC)	+	-	+	
8 (Ch NPC)	+	-	+	
9 (Ch NPC)	+	-	+	
10 (Ch NPC)	+	-	-	
11 (Ch NPC)	+	-	+	
12 (Ch NPC)	+	-	+	
13 (Ch NPC)	+	-	+	
C15 (NuMo NPC)	+	-	+	4
CAO (NuMo NPC)	+	-	+	5
B95-8 (Marmoset LCL)	+	+	+	
Rael (BL)	+	-	-	29
CBM1-Ral-STO (LCL)	+	+	+	12
CBC-SEB-CHI-3 (LCL)	+	+	+	
CBC-SEB-CHI-7 (LCL)	+	+	+	

^a Ch, Chinese; NuMo, nude mouse passaged; B95-8, marmoset B cells transformed by infectious mononucleosis-derived EBV; CBC, cord blood-derived cells. Rael and CBM1-Ral-STO carry the same strain of EBV.

dextran sulfate containing the radioactive probe. Subsequently the filters were washed in 2× SSPE–0.1% SDS for 15 min at room temperature, two times in 0.2× SSPE–0.1% SDS for 30 min 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 (12).

Probes. The following probes were used (Fig. 1): the *Bam*HI K fragment; the pMBamH₂ fragment (cloned from the M-ABA cell line, kindly provided by Georg Bornkamm), which covers the 3' part of the EBNA-2A exon BYRF-1 (coordinates 48848 to 49964); a *Msp*I-*Msp*I digest from the *Bam*HI C fragment (coordinates 8189 to 9105), covering part of *oriP*; a *Msp*I-*Msp*I fragment from the *Bam*HI K fragment, covering part of the EBNA-1 exon BKRF-1 (coordinates 108161 to 108924); an *Xho*I-*Xho*I fragment from the *Eco*RI D_{het} fragment covering most of the LMP exons (coordinates 167484 to 169424); an *Xho*I-*Eco*RI fragment (coordinates 169424 to 172281) and a *Bgl*II-*Bgl*II fragment (coordinates 169449 to 170290), both covering the promoter and 5'-flanking sequences of LMP; and an *Ava*I-*Ava*I fragment covering the *Bam*HI W enhancer (coordinates 13827 to 14460).

RESULTS

Expression of EBV-encoded proteins. Thirteen Chinese NPC biopsies from the Shanghai area were included in this study. They were EBV DNA positive, as shown by Southern blotting (for details, see below). Expression of the EBNA's and LMP was assayed by immunoblotting by using the polyvalent human serum, PG (Fig. 2C), the affinity-purified

FIG. 3. Methylation pattern of the BNLF-1 exons in NPC biopsies. Biopsy and control cell line DNAs cleaved with *Hpa*II (H) or *Msp*I (M) and probed with an *Xho*I-*Xho*I probe (Fig. 1e). Biopsies 3, 5, and 10 were LMP negative. As controls, B95-8 (predominantly un-methylated), Rael (methylated), and two EBV-transformed LCLs (CBC-SEB-CHI-3 and -7, un-methylated) were used. The positions of the molecular weight marker fragments are indicated. The easily identified major B95-8 fragments can also be used as references (357, 494, and 818 bp).

FIG. 4. Methylation pattern of the 5' BNLf-1-flanking region. Same blot shown Fig. 3, stripped and reprobed with a *BglI-BglI* probe (smaller subfragment in Fig. 1f), covering the promoter and approximately 800 bp upstream of the LMP exons. Sample DNAs cleaved with *HpaII* and *MspI*. Biopsies 3, 5, and 10 and the control cell line Rael did not express LMP, while all the other samples did. The positions of the DNA molecular weight marker fragments as well as the positions of three minor B95-8 fragments (in parentheses: 177, 146, and 107 bp; they are easy to identify together with the larger 506-bp fragment) are indicated.

FIG. 5. Schematic drawing of the BNLf-1 region and upstream sequences in two NPC biopsies (one expressing LMP [b; no. 12] and one not expressing LMP [c; no. 10]). *MspI* and *HpaII* cleavage sites are indicated (●). For reference, the B95-8 map with cleavage sites, LMP exons, and the promoter is provided (a).

human serum specific for EBNA-1 (Fig. 2B), and the S-12 MAb for LMP (Fig. 2A). The data on protein expression are summarized in Table 1 and are related to those of earlier studies summarized in Table 2. All EBV DNA-positive biopsies expressed EBNA-1, as shown by both the polyvalent and the monospecific reagent. Ten of the thirteen (77%) EBV DNA-positive biopsies were LMP positive (no. 1, 2, 4, 6, 7, 8, 9, 11, 12, and 13). Of these, no. 7, 12, and 13 gave relatively weaker signals. Nevertheless, inspection of these lanes in reflected light and by transillumination showed that they were clearly positive, whereas no signal at all could be detected in no. 3, 5, and 10. Biopsy 7 had a slightly higher LMP molecular weight than the others. A small variation in LMP size is common. C15 and CAO have been shown in earlier studies to be LMP positive (4, 14).

Methylation of the BNLf-1 reading frames (LMP coding) and the 5'-flanking region. Twelve of the biopsies were selected for DNA methylation studies. Two nude mouse-passaged NPC tumors, C15 (4) and CAO (5), were also included. As controls we have included two LCLs established from Chinese wild-type EBV (CBC-SEB-CHI-3 and -7) that carry unmethylated genomes: the type I BL cell line Rael that contains highly methylated EBV DNA and the virus-producing B95-8 line. The materials and their EBV antigen expression are listed in Table 1. The biopsy DNA was cleaved in parallel with *HpaII* (methylation sensitive) and *MspI* (methylation-insensitive isoschizomer) and hybridized with the *XhoI* probe (Fig. 1), covering the promoter

FIG. 6. Methylation of the *oriP* region in NPC biopsies. Same blot shown in Fig. 3 and 4, stripped and reprobed with the *oriP* probe (the 916-bp subfragment depicted in Fig. 1a). The position of the *MspI*-*MspI* 916-bp fragment in the control cell line B95-8 is indicated. Sample DNAs were cleaved with *HpaII* and *MspI*.

and all three coding exons of BNLF-1 and extending over most of the 3'-untranslated region of the LMP-coding transcript. The B95-8 DNA showed a largely unmethylated pattern (Fig. 3, lane B95-8 H) with four major fragments corresponding to the expected lengths of 818, 494, 357, and 253 bp from the sequence data which was comparable with the fragments obtained with *MspI*. There was also one large fragment of around 2.5 kb, suggesting that some genomes in B95-8 cells were completely methylated over the whole region covered by the probe. Rael cells, in contrast, contained only four large fragments (2.5, 3.0, and 4.0 kb and a larger one). This reflects a high degree of methylation, extending to CpG sites outside the region covered by the probe. The four smaller fragments were only seen in the *MspI* (M) lane, confirming the absence of any detectable hypomethylation. The Rael counterpart of the 494-bp B95-8 fragment was smaller, about 400 bp. The LCLs CBC-SEB-CHI-3 and -7 carried totally unmethylated viral DNA in the whole region. An 818-bp fragment was consistently present, but there was some size variation in the three smaller fragments. CBC-SEB-CHI-3 had a Rael-like fragment size distribution, while CBC-SEB-CHI-7 showed fragment sizes intermediate between those of Rael and B95-8 cells. The size variation of the 357- and 293-bp fragments may conceivably influence the size of the LMP, since both fragments are located within the major BNLF-1 coding exon of LMP.

In contrast to B95-8 cells and the two cord blood-derived cells, all the NPC tumors showed extensive EBV DNA methylation. Nude mouse-passaged CAO and biopsy tumors 4, 5, and 10 contained one major unmethylated 357-bp fragment, corresponding to the BNLF-1 coding exon. CAO and tumor 4 expressed LMP, while tumors 5 and 10 did not. The unmethylated fragment gave a weak signal in three cases, suggesting that it was only present in a fraction of the EBV genomes. In biopsy tumor 10 the fragment was equimolar with the other fragments. These four tumors and the remaining seven showed the presence of much larger fragments, which were the result of incomplete cleavage due to methylation protection of restriction sites. The resulting fragments varied between 0.7 and 5 kb. Most of them were in the size order of 1.5 to 4 kb. They all gave strong signals. This suggests that the *HpaII* restriction sites are methylated and that methylation extends from a few hundred base pairs to 1 kb outside the probed region, in most cases. At least 10 of the tumors showed traces of uncleaved DNA. These fragments have been derived from genomes that were meth-

ylated over several tens of kilobases outside the BNLF-1 region. This is also supported by the lack of any incomplete cleavage with *MspI*. Moreover, staining the gels with ethidium bromide before blotting showed that good digestion occurred (data not shown). All NPC biopsies contained the 818-bp fragment after *MspI* cleavage; the smaller fragments were of various sizes, as in the cell lines. The 357-bp fragment was not detectable in eight of the tumors; only two small fragments could be seen. Seven tumors contained the 400-bp fragment associated with Rael cells and lacked the 494-bp fragment associated with the B95-8 virus.

The LMP-coding exons varied slightly in size between different biopsies. They were highly methylated in the whole coding region, both in LMP-positive and LMP-negative tumors. In contrast, a good correlation was found between the methylation status of the 5'-flanking region of the LMP gene and its expression. The flanking region was completely unmethylated in seven tumors (tumors 6, 12, 2, 4, 13, 8, and C15), all of which expressed LMP (Fig. 4). The flanking region was partially methylated in CAO, which expressed LMP at a low level. The region was extensively methylated in tumors 3, 5, and 10, which did not express LMP. The smallest fragment that could be obtained from these tumors after digestion with *HpaII* was 2.5 kb. Similar tests on the various control cell lines further corroborated the close relationship between methylation of the 5'-flanking region and lack of expression, or vice versa. The two human cord blood-derived LCLs and the B95-8 line expressed LMP, whereas the Rael line did not. The LMP-flanking region was unmethylated in the former three but highly methylated in Rael, as expected.

The 5'-flanking region of the LMP gene showed considerable microheterogeneity between the different virus isolates. The large, 506 bp B95-8 fragment had 400-bp counterparts in nine of the tumors and in the cord blood-derived cells. It was larger in biopsy 8 and of the same size in Rael. The resolution of the smaller fragments was not sufficient to detect minor variations. The 175-bp fragment was about the same size in all viral DNAs examined.

Three of three LMP-negative biopsies showed extensive methylation of the whole 800-bp region immediately 5' of the BNLF-1 promoter and extending outside into the LMP gene. The same region was completely unmethylated in six of seven LMP-positive tumors. The seventh (CAO) was partially methylated around the large 400-bp fragment but

FIG. 7. Methylation of the *Bam*HI K fragment and EBNA-1 exon in NPC biopsies. Southern blot showing NPC biopsies and control cell lines probed with the whole *Bam*HI K fragment (Fig. 1d). Sample DNAs cleaved with *Hpa*II (H) or *Msp*I (M). The positions of molecular weight marker fragments are indicated, and the major B95-8 fragments can easily be identified (1,132, 774, and 529 bp). All the NPC samples show extensive methylation of all fragments. They all express EBNA-1. The control cell lines CBC-SEB-CHI-3 and -7 gave very weak signals with this exposure, although the same amount of cellular DNA was loaded in all lanes. The *Bam*HI K fragment was unmethylated in these samples.

yielded one or two smaller fragments, because of a lack of methylation of the surrounding restriction sites.

Figure 5 is a schematic restriction map of the relevant BNLF-1 region and its 5'-flanking sequence in an LMP-positive (tumor 12) and an LMP-negative (tumor 10) biopsy. The dashed lines indicate the size variations of these fragments compared with those of B95-8 cells.

Lack of methylation in the *oriP* region. A 916-bp probe was used to analyze methylation in the *oriP* region. The probe covers the middle part of the region, with the EBNA-1 enhancer just upstream and the EBNA-1-binding dyad symmetry motif in the 3' end (31, 38).

The same unmethylated 916-bp fragment was detected in 11 tumors and in the controls (Fig. 6). In our previous study (12) the same region was unmethylated in all BL cells and LCLs examined and represented the only known area exempted from high overall EBV DNA methylation in the type I BL line Rael.

The exon of EBNA-1 showed a high degree of methylation in all the NPC tumors (Fig. 7).

Methylation of the *Bam*HI W enhancer. Recently, a B-cell-

specific enhancer in the *Bam*HI W fragments that can influence the expression of EBNA-2 was described (33). It is contained within an *Ava*I fragment (Fig. 1). We screened the DNA from nine NPC tumors and controls with this fragment as a labeled probe after *Msp*I and *Hpa*II cleavage (Fig. 8). All were EBNA-2 negative by immunoblotting. All were methylated in this region, although not as extensively as in the LMP region. Thus, a whole ladder of fragments, ranging from 0.9 to 4 kb, was generated after *Hpa*II cleavage. The single 558-bp fragment found in unmethylated DNA was not detected in any of them, however. After *Msp*I cleavage, this fragment was detected in all tumors. It was slightly larger than that in B95-8 cells, around 600 bp. As shown earlier, the EBNA-2 exon was methylated in NPC tumors (12). The *Bam*HI W enhancer region was unmethylated in the LCLs that expressed EBNA-2 (Fig. 8).

DISCUSSION

We have found an overall difference in EBV DNA methylation of NPC tumors and EBV-transformed LCLs of

FIG. 8. Methylation of the *Bam*HI W enhancer. The same blot as in Fig. 3, 4, and 6, stripped and reprobed with an *Ava*I-*Ava*I probe covering the *Bam*HI W enhancer (Fig. 1b). The DNA samples were cleaved with *Hpa*II or *Msp*I. The control cell lines B95-8, CBC-SEB-CHI-3, and -7 expressed EBNA-2 to -6, while all the other samples (NPC biopsies) expressed only EBNA-1. The 558-bp fragment could easily be identified in the control cell line B95-8, and it is slightly larger in all the other samples.

normal B-cell origin. EBV genomes are extensively methylated in the former and unmethylated in the latter. We have found a similar difference previously between the type I BL line Rael and the derived LCLs (12).

In addition to this overall difference, there is a superimposed, expression-related difference in the 5'-flanking regions which is known or suspected to contain regulatory sequences (Table 2).

It is known that methylation of 5'-flanking regulatory sequences may influence gene expression, whereas methylation of the coding exons has no such effect as a rule (3, 11, 25, 26). The recent findings of single, specific methylation sites within the DNA binding motif of several proteins involved in transcriptional regulation show a potential direct role of methylation in gene control (2, 26, 37, 41). A search for specific critical CpG methylations within the relevant promoter and enhancer regions in the EBV genome will obviously be of great interest. Our data suggest that meth-

ylation of regulatory regions influences EBNA and LMP control.

The promoter for LMP-coding transcripts has been mapped to the EDL-1 site (coordinate 169546). We have found a complete correlation between LMP expression and lack of methylation of the promoter region and some 800 bp upstream of it. This is particularly remarkable since the surrounding EBV DNA is extensively methylated in NPC tumors, including the three LMP-encoding exons. The same region was methylated in all tumors that failed to express LMP. This suggests that methylation participates in the regulation of LMP expression. Direct studies on LMP transcriptional regulation will be necessary to establish any direct causal relationship. The region that showed an expression-correlated methylation pattern coincides with the recently described LMP regulatory sequence (17).

Promoters for EBNA transcripts have been found in two regions so far, *Bam*HI-C (BCR 2, P₁₁₃₀₅) and *Bam*HI-Ws

TABLE 2. DNA methylation and gene expression patterns in coding and control regions

Cell type	Methylation ^a						Protein expression		
	<i>oriP</i>	EBNA-1 exon	<i>Bam</i> HI W enhancer	EBNA-2 exon	BNLF-1 5' sequence	LMP exon	EBNA-1	EBNA-2	LMP
NPC (LMP ⁺)	-	+	+	+	-	+	+	-	+
NPC (LMP ⁻)	-	+	+	+	+	+	+	-	-
C15	-	+	+	+	-	+	+	-	+
C15 LCL	-	-	-	-	-	-	+	+	+
Rael	-	+	+	+	+	+	+	-	-
CBM1-Ral-STO	-	-	-	-	-	-	+	+	+

^a *oriP*, Origin of latent DNA replication; EBNA-1 exon, BKRF-1 exon for EBNA-1 protein; *Bam*HI W enhancer, enhancer 5' of Wp in internal repeat 1; EBNA-2 exon, BYRF-1 coding exon for EBNA-2; BNLF-1 5' sequence, LMP control region; LMP exon, three LMP-coding exons. +, Methylation; -, no methylation.

TABLE 3. NPC biopsy protein expression^a

No. of biopsies	No. expressing:		Reference
	EBNA-1	LMP	
24	24	9	43
56 ^b	56	36	14
13	13	10	This paper

^a Protein expression was tested by immunoblotting with polyvalent and/or monospecific reagents of EBNA-1 and LMP. EBNA-2 to -6 were always negative.

^b Only EBNA-1- and/or EBV DNA-positive biopsies are included.

(P_{14352+nx3073}, $n = 4$ to 11). In the producer line B95-8, several different EBNA transcripts start at the same promoter, P₁₁₃₀₅, whereas EBNA-2 transcripts in an LCL, IB 4, were found to initiate at one of the *Bam*HI W promoters. An enhancer that can transactivate after EBNA-1 binding is localized in *oriP* 3 kb upstream of the *Bam*HI C promoter (31). The *oriP* enhancer may be involved in regulation of transcripts from the *Bam*HI C promoter (38).

The *oriP* region has been unmethylated in all EBV-carrying tissues studied so far, including BL and NPC. This may fit well with the fact that EBNA-1 is the most regularly expressed EBV protein, is found in all EBV DNA-carrying cells studied so far, and is unaffected by the cell phenotype-dependent expression and regulation.

Recently, it was demonstrated that in a selection of BL-derived cell lines and in vitro-transformed LCLs, either the *Bam*HI W or the *Bam*HI C promoter was used irrespective of cell phenotype (42). This study, however, did not include any type I BL cell lines or NPC tumor biopsies. The EBNA-1 transcription initiation remains to be identified in these cell types. Hitt et al. (22) detected no transcripts from the *Bam*HI W region when screening their cDNA library derived from the nude mouse-passaged NPC C15, suggesting that EBNA expression in this NPC is controlled in a different way and without the extensive splicing of the *Bam*HI W regions that has been seen in B-cell lines (Raji, B95-8, IB 4, and JY).

The lack of methylation of *oriP* may also be secondary to its involvement in DNA replication or may even result from the protection by DNA-binding regulatory proteins, including EBNA-1. Recently, an enhancer that is 50 to 400 bp upstream of the *Bam*HI W promoter (33) and that functions in B-cell lines but not in HeLa cells has also been demonstrated. This enhancer is likely to be important for regulation of EBNA expression. The *Bam*HI W promoter-enhancer was hypomethylated in the EBNA-2-expressing LCLs but methylated in all the nonexpressing NPCs.

Our study has further confirmed the specific pattern of EBV protein expression in NPC: EBNA-1 expression in all tumors that are EBV DNA positive, and LMP expression only in a large fraction. This fraction has been repeatedly higher in Chinese NPCs than in NPC materials from other high or medium-high endemic regions. Earlier we detected LMP in 71% of Chinese tumors (12), and now we have detected LMP in 10 of 13 (77%) tumors in two consecutive, randomly chosen groups (Table 3). This compares with 25 to 62% in earlier investigated tumors from North Africa and East Africa (14, 43).

LMP can transform established strains of rodent fibroblasts (1, 40). Fåhræus et al. (15) have recently found that expression of LMP in an EBV-negative epithelial cell line (RHEK-1) changes the morphology of the transfected cells

and inhibits keratinization. Dawson et al. (7) demonstrated simultaneously that LMP inhibits differentiation in a human keratinocyte cell line. NPC cells are low differentiated or anaplastic. Whether the expression of LMP can be related to the level of differentiation and to the clinical course of the tumor remains a question.

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