Epstein-Barr Virus and Hodgkin's Disease: Transcriptional Analysis of Virus Latency in the Malignant Cells

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Summary

Epstein-Barr virus (EBV) is associated with a number of different human tumors and appears to play different pathogenetic roles in each case. Thus, immunoblastic B cell lymphomas of the immunosuppressed display the full pattern of EBV latent gene expression (expressing Epstein-Barr nuclear antigen [EBNA]1, 2, 3A, 3B, 3C, and -LP, and latent membrane protein [LMP]1, 2A, and 2B), just as do B lymphoblastoid cell lines transformed by the virus in vitro. In contrast, those EBV-associated tumors with a more complex, multistep pathogenesis show more restricted patterns of viral gene expression, limited in Burkitt's lymphoma to EBNA1 only and in nasopharyngeal carcinoma (NPC) to EBNA1 and LMP1, 2A, and 2B. Recent evidence has implicated EBV in the pathogenesis of another lymphoid tumor, Hodgkin's disease (HD), where the malignant Hodgkin's and Reed-Sternberg (HRS) cells are EBV genome positive in up to 50% of cases. Here we extend preliminary results on viral gene expression in HRS cells by adopting polymerase chain reaction–based and in situ hybridization assays capable of detecting specific EBV latent transcripts diagnostic of the different possible forms of EBV latency. We show that the transcriptional program of the virus in HRS cells is similar to that seen in NPC in several respects: (*a*) selective expression of EBNA1 mRNA from the BamHI F promoter; (*b*) downregulation of the BamHI C and W promoters and their associated EBNA mRNAs; (*c*) expression of LMP1 and, in most cases, LMP2A and 2B transcripts; and (*d*) expression of the "rightward-running" BamHI A transcripts once thought to be unique to NPC. This form of latency, consistently detected in EBV-positive HD irrespective of histological subtype, implies an active role for the virus in the pathogenesis of HD and also suggests that the tumor may remain sensitive to at least certain facets of the EBV-induced cytotoxic T cell response.

There is now strong evidence implicating EBV, a herpes virus widespread in human communities, in the pathogenesis of at least three human tumors. These are the immunoblastic B cell lymphomas to which immunosuppressed patients are especially prone, another tumor of B cell origin (endemic Burkitt's lymphoma [BL][1]) and an epithelial malignancy (nasopharyngeal carcinoma [NPC]) (1). All carry the EBV genome in episomal form in the malignant cells and are positive for EBERs, small noncoding nuclear viral RNAs now recognized as the most consistent indicator of latent EBV infection (2–4). However, the contribution made by the virus to tumor development appears to be different in each of these situations, and this is to some extent reflected in the different patterns of viral gene expression observed in the tumor cells.

The least complex case involves immunoblastic B cell lymphoma, where tumor cell growth appears to be directly EBV driven in a manner analogous to that of the lymphoblastoid cell lines (LCLs) that arise when EBV infects normal resting B cells in vitro (5). Both tumor- and in vitro transformed cells show the same pattern of virus latent gene expression

[1] *Abbreviations used in this paper:* APAAP, alkaline phosphatase anti-alkaline phosphatase; BL, Burkitt's lymphoma; Cp, C promoter; EBNA, Epstein-Barr nuclear antigen; Fp, F promoter; HD, Hodgkin's disease; HRS, Hodgkin and Reed-Sternberg; LMP, latent membrane protein; NPC, nasopharyngeal carcinoma; Wp, W promoter.

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encompassing six Epstein-Barr nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and -LP) and three latent membrane proteins (LMP1, 2A, and 2B) (6–8). It is known from studies on LCLs that the different EBNAs are encoded by individual mRNAs generated by differential splicing of the same long primary transcripts expressed from the BamHI C promoter (Cp) or the BamHI W promoter (Wp) (9), while the LMP transcripts are expressed from separate promoters in the BamHI N region of the EBV genome (9, 10). The situation is different in BL, where EBV forms part of a complex multistep pathogenesis and where virus latent gene expression is restricted to EBNA1 only (11, 12). Recent work has shown that this is achieved through selective expression of a uniquely spliced EBNA1 mRNA from a novel promoter (Fp) in the BamHI F region of the viral genome; Cp, Wp, and the LMP promoters are silent in BL cells (13, 14). Yet a third form of latency is displayed by NPC cells, where Fp-driven EBNA1 transcripts are detected (again leading to expression of EBNA1 in the absence of other EBNAs), as well as most or all the LMP mRNAs (15–17); in addition, NPC cells express a series of spliced rightward-running BamHI A transcripts that may encode other, as yet poorly characterized, latent proteins (18, 19). It remains to be seen whether this particular form of latency, where the virus appears to encode some but not all of its growth transformation–associated proteins, is unique to NPC or can on occasions be adopted by the virus in a lymphoid cell environment.

Here we address the question of EBV latent gene expression in another malignancy, Hodgkin's disease (HD), whose association with the virus has only recently been fully realized and where the role played by EBV in tumor development remains to be determined. HD is an unusual tumor in which the malignant population of Hodgkin and Reed-Sternberg (HRS) cells constitutes only a minority of the tumor mass, the different histological subtypes of HD being distinguished by the nature of the normal cell infiltrate; the HRS cells themselves are probably of lymphoid origin but often cannot be assigned unequivocally to either T or B cell lineages by conventional markers (20, 21). Epidemiological studies first raised the possibility of a link between EBV and HD (22–24), but direct evidence came with the detection of EBV DNA in tumor biopsies in 19–50% of HD cases and with the localization of the EBV genome and of EBERs to HRS cells by in situ hybridization (25–28). Recently we and others observed by immunohistological staining that at least one viral protein, LMP1, was detectable in these same cells apparently in the absence of a second latent protein, EBNA2 (29, 30). This raised the possibility of a form of viral latency in HD similar to that found in NPC. Given the limitations of immunohistochemical methods for the analysis of EBV latent protein expression and the paucity of tumor cells in HD, it was clear that a proper resolution of this question would require a different experimental approach. In the present study we use a combination of PCR-based and in situ hybridization assays specific for defined viral mRNAs to characterize in detail the resident pattern of EBV latent gene transcription in virus-positive HD. The consistency with which one form of viral latency was observed has implications both for the possible role of the virus in HD pathogenesis and for the possible susceptibility of the tumor to EBV-specific immune T cell control.

Materials and Methods

Hodgkin Lymphoma Tissue. Lymph node biopsy specimens from 23 HD patients were obtained, snap frozen, and stored at −80°C. 40 sections were subsequently cut from each biopsy specimen, 10 fixed in acetone and stored at −20°C (for use in immunohistochemical staining), and the remaining 30 fixed in paraformaldehyde, washed in PBS, dehydrated through graded ethanols, and stored at −80°C (for use in in situ hybridization). The remaining biopsy tissue was halved with half the tissue being used in immunoblotting and the other half being used in the RNA PCR.

Control Cell Cultures and Tumor Tissue. For immunohistochemistry and RNA in situ hybridization, cytospins of cell lines of known EBV status were prepared and used as controls. These included the EBV-transformed LCL X50-7 and the EBV-positive BL cell line Akata. Also used was the NPC cell line C15, kindly provided by Dr. P. Busson (Institut Gustave Roussy, Villejuif, France) and passaged in SCID mice (31). Reference cell lines used as positive controls in the RNA PCR included C15, X50-7, and B95.8 (an EBV-transformed marmoset LCL) with the EBV-negative BL cell line, BL41, serving as a negative control. In the immunoblotting, X50-7 and C15 were used as positive controls, and BJAB (an EBV-negative BL cell line) and the transplantable line NOR (an EBV-negative SCID mouse–passaged nasopharyngeal carcinoma [17]) were used as negative controls.

Immunoblotting. Protein samples from SCID mouse–passaged tumors, B cell lines, and HD biopsies were separated by discontinuous PAGE, blotted onto nitrocellulose membranes as previously described (15), and probed with the mAb specific for LMP (clone CS1-4 [32]) or with polyclonal human sera reactive against EBNA proteins (15).

Immunohistology. Acetone-fixed frozen sections of HD biopsies were stained using the alkaline phosphatase anti–alkaline phosphatase (APAAP) technique as previously described (33) using the mAb CS1-4 at a dilution of 1:50. Cytospins of reference cell lines Akata BL and X50-7 LCL were stained using the same technique.

In Situ Hybridization. For the preparation of RNA probes, cDNA fragments were subcloned into the plasmid pBluescript KS containing promoters for T7 and T3 polymerases. The pBSW plasmid contains the BamHI W fragment of EBV subcloned at the BamHI site. The plasmids pBSJJJ1 and pBSJJJ2 contain EBER1- and EBER2-specific fragments, respectively. These fragments were derived from plasmids pJJJ1 and pJJJ2 (34), kindly provided by Dr. J. Arrand (Paterson Institute for Cancer Research, Manchester, UK), and subcloned into the BamHI and EcoRI, and EcoRI and HindIII sites, respectively. After linearization with the appropriate restriction enzyme, ^{35}S-labeled antisense (complementary to the mRNA) or sense (anticomplementary, negative control) run-off transcripts were generated using either T3 or T7 RNA polymerases (Bethesda Research Laboratories, Gaithersburg, MD) as described previously (35). The length of the RNA probes was adjusted to ~100–200 bases by controlled alkaline hydrolysis. For in situ hybridization experiments using the pBSJJJ1 and pBSJJJ2 probes, the antisense probes were mixed to increase sensitivity, as were the sense probes.

RNA-RNA hybridization was performed as described previously (35). Fixation and treatment of sections with HCl and pronase followed by postfixation in 4% paraformaldehyde/PBS, acetylation, and dehydration was as previously described (35). Hybridization mixture (25 μl) containing 50% deionized formamide, 2× SSC,

10% dextran sulphate, 0.2 mg/ml yeast tRNA, and 2–5 × 10^5 cpm of labeled probe was applied per section. Hybridization was performed at 50°C for 12 h. Excess probe was removed by washing in 50% formamide, 1× SSC at 52°C for 4 h, followed by a digestion with 20 μg/ml RNase A for 30 min at 37°C. Slides were rinsed again in 2× SSC, dehydrated, dipped into Ilford G5 emulsion, exposed, developed, and counterstained as previously described (35). In some experiments, HD sections and cytospin preparations were treated with RNase-free DNase I (Bethesda Research Laboratories) in concentrations of up to 40 μg/ml at 37°C for 30 min before hybridization to the RNA probes.

Extraction of RNA for PCR Analysis. RNA was extracted from HD biopsies, the transplantable tumor cell line C15, and the reference B cell lines using the RNAzol B method according to the manufacturer's protocol (Cinna/Biotecx, Houston, TX). Frozen specimens were pulverized in a small glass homogenizer before extraction with RNAzol B.

Amplification and Analysis of RNA Transcripts by PCR. RNA samples were treated and analyzed as described previously (17). Essentially the samples were heated for 2 min at 90°C, rapidly cooled on ice, reverse transcription PCR was performed in a one-tube reaction for 60 min at 42°C, the samples were heated to 94°C for 7 min and cooled to 70°C, and *Taq* DNA polymerase was added. Samples were subjected to 40 rounds of amplification in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Amplification conditions were as follows: denaturation for 30 s at 94°C, primer annealing for 90 s at 45°C, and extension for 240 s at 70°C. For the specific amplification of LMP1 mRNAs, a higher annealing temperature of 50°C and an extension temperature of 72°C for 120 s were used. The sequences and coordinates of EBV-PCR primers utilized to detect LMP1, LMP2, and EBNA1 transcripts in this study are as previously described (17) and are based on the published B95.8 genomic sequence (36). Two sets of primers based on the splice pattern of the 18.8 cDNA were used to detect the BARF0 transcript (18). The first primer pair spans the large first intron of the 18.8 cDNA and the second primer pair spans the second and third introns. The sequences of the primers and probes used to detect the BARF0 transcript are described in the legend to Fig. 7.

Amplified samples were analyzed by electrophoresis through 3% Nusieve agarose gels and then by Southern transfer onto Hybond N^+ nylon membranes (Amersham Corp., Arlington Heights, IL). Detection of EBNA1, LMP1, LMP2A, LMP2B, and BARF0 mRNA products was achieved by hybridization to end-labeled oligonucleotide probes, the sequences of which are as previously described (17, 37); the sequences of the BARF0 probes are described in the legend to Fig. 7. Oligonucleotide probes were hybridized at 42°C overnight in the presence of 5× SSC (1× SSC is 0.1 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, 0.5% SDS, and 100 μg/ml of calf thymus DNA in 50% formamide.

As an additional control to check for amplifiable mRNA in each individual HD biopsy extract, amplifications were performed by using PCR primers specific for human CD45 mRNA. These primers (5′-GGAACTGACACGCAGACATT-3′ and 5′-CTCAGAGTGGTTGTTTCAGA-3′) span an intron in the CD45 gene and generate a 260-bp amplified product that is visible on ethidium bromide–stained agarose gels.

Results

Histological Analysis of LMP1 and EBER Expression in HD Biopsies. 16 HD biopsies were selected as EBV-positive cases on the basis of immunostaining for LMP1 and of in situ hybridization for the EBER RNAs. The majority of these HD cases were of the mixed cellularity histological subtype in accordance with the previously reported preferential association of EBV with this form of the disease (29). However, EBV-positive cases of nodular sclerosing and lymphocyte predominant subtypes were also included. In all 16 cases the LMP1 staining was restricted to the HRS cells with characteristic strong membrane and cytoplasmic reactivity (Fig. 1 *A*, Table 1). These same cases showed EBER expression in the tumor cells by in situ hybridization (Fig. 1 *B*, Table 1). The cell-to-cell variation in the intensity of the autoradiographic signal among HRS cells was similar to that observed in LCLs, BL cell lines, and NPC tumors. As a further check for specificity, no signal was observed with the control sense EBER probes on these HD cases or on EBV-positive controls. Seven cases of HD that were negative for both LMP1 and EBER expression, again including a range of histological subtypes of the tumor, were used as EBV-negative control specimens in these and subsequent analyses.

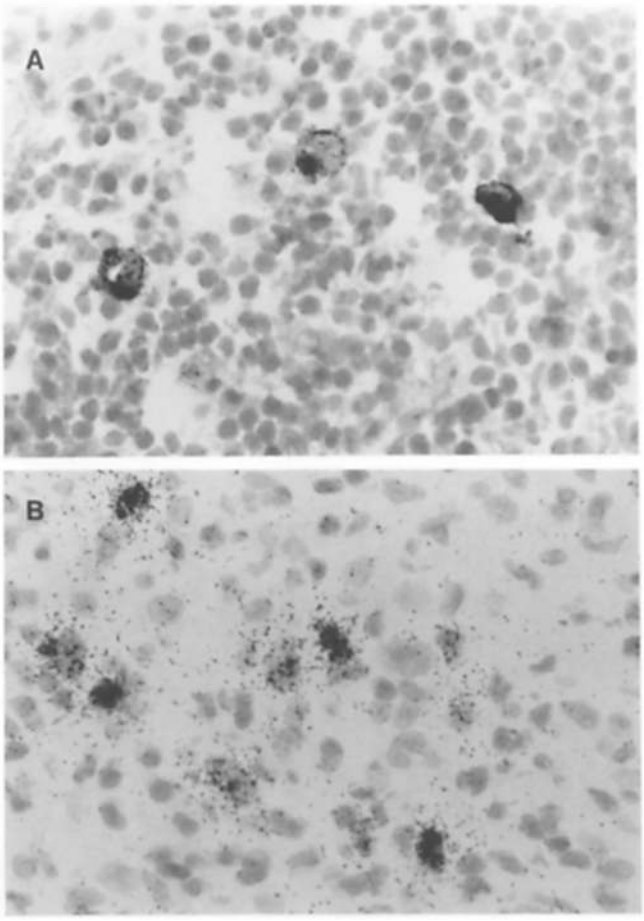

Figure 1. Detection of EBV in the malignant HRS cells of HD. (*A*) Immunohistological staining of an EBV-positive HD biopsy, HD8, with the mAb CS1-4 demonstrates the expression of LMP1 in the tumor cells (APAAP, hematoxylin counterstaining, ×340). (*B*) In situ RNA-RNA hybridization of an EBV-positive HD biopsy, HD8, reveals strong nuclear labeling in the tumor cells with EBER-specific antisense probes (3-d exposure, H+E counterstaining, ×340). No signal was detected with the control sense probes.

Table 1. *EBV Latent Gene Expression in Hodgkin's Disease Biopsies*

HD biopsy (subtype)	In situ hybridization		LMP1 staining	PCR detection of spliced transcript								
				Cp, Wp	EBNA1		LMP1		LMP2		BamHIA	
	EBER	BamW			Y/U/K	Q/U/K	2.8	3.7	A	B	A1/2	A3/4
HD1 (ns)	+	−	+	−	−	+	+	+	+	+	+	+
HD2 (mc)	+	−	+	−	−	+	+	+	−	−	+	+
HD3 (mc)	+	−	+	−	−	+	+	+	−	+	+	+
HD4 (ns)	+	−	+	−	−	+	+	+	+	+	+	+
HD5 (ns)	+	−	+	−	−	−	+	−	−	−	+	+
HD6 (ns)	+	−	+	−	−	+	+	+	+	−	+	+
HD7 (mc)	+	−	+	−	−	+	+	+	−	−	+	+
HD8 (mc)	+	−	+	−	−	−	−	−	−	−	+	+
HD9 (mc)	+	−	+	−	−	−	+	+	+	+	+	+
HD10 (mc)	+	−	+	+	+	+	+	+	+	+	+	+
HD11 (mc)	+	−	+	−	−	+	+	+	+	+	+	+
HD12 (lp)	+	−	+	−	−	+	+	+	+	+	−	−
Latency I: BL	+	−	−	−	−	+	−	−	−	−	ND	ND
Latency II: NPC	+	−	+	−	−	+	+	+	+	+	+	+
Latency III: LCL	+	+	+	+	+	−	+	+	+	+	+	+

mc, mixed cellularity; ns, nodular sclerosing; lp, lymphocyte predominant.

Analysis of LMP1 Expression at the Protein and mRNA Level. We first sought independent evidence that the immunohistochemical staining obtained above and in earlier work using the LMP1-specific mAb CS1-4 did indeed reflect expression of the authentic LMP1 protein. Immunoblot analysis for LMP1 in extracts of HD biopsies revealed expression of the appropriately sized full-length protein; generally, the amount of protein detectable correlated with the prevalence of HRS cells in the biopsy as assessed by LMP1 and CD30 immunostaining (Fig. 2). As a control EBV-negative HD cases with similar numbers of HRS cells as assessed by CD30 immunostaining were LMP1 negative by immunoblotting. The variable size of the LMP1 band in each EBV-positive HD biopsy is consistent with the presence of a different EBV isolate in each of these cases. The ability to detect LMP1 in total biopsy extracts reinforces the impression that LMP1 levels in HRS cells are unusually high. Similar immunoblotting analysis on these same biopsy extracts with polyspecific human sera reactive against the EBNA proteins gave uniformly negative results (data not shown).

RNA prepared from snap-frozen biopsies of 12 EBV-positive and 5 EBV-negative HD specimens was analyzed for LMP1 mRNA by PCR after reverse transcription to cDNA using a 5′ (2.8) primer situated in the first exon and a 3′ primer spanning the exon 2-3 boundary (Fig. 3). These primers, which we have previously used to examine LMP1 transcripts in NPC biopsies, yielded the predicted 381-bp product in 11 of the 12 EBV-positive HD biopsies; no signal was detected from the EBV-negative HD biopsies even after long overexposures of the autoradiographs (Fig. 3). RNA from the transplantable NPC line C15 was used as a positive control for LMP1 mRNA while that from the EBV-negative BL41 cell line served as a negative control. Since we have previously used PCR to show that fresh NPC biopsies also express the longer 3.7-kb

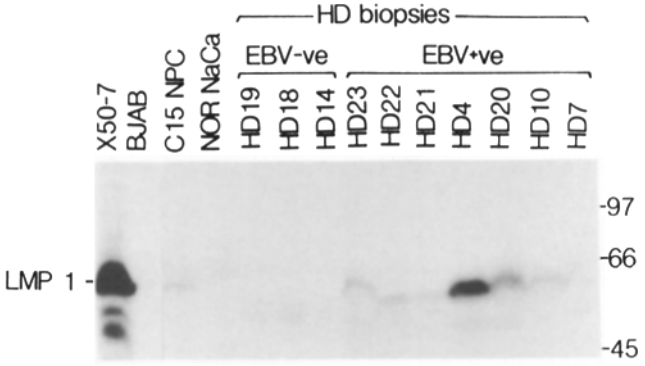

Figure 2. Immunoblot of LMP1 protein in extracts from EBV-positive and -negative HD biopsies. Control extracts were provided by the EBV-transformed LCL X50/7, the EBV-negative BL cell line BJAB, the transplantable EBV-positive NPC line C15, and the transplantable EBV-negative pharyngeal carcinoma line NOR NaCa. The immunoblot was probed with the anti-LMP1 mAb CS1-4.

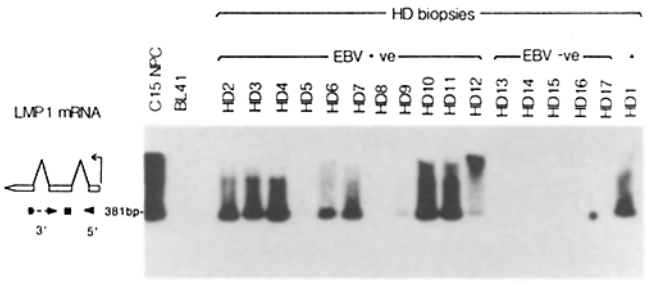

Figure 3. PCR analysis of LMP1 mRNA in 12 EBV-positive (HD1 to HD12) and 5 EBV-negative (HD13 to HD17) HD biopsies. The EBV-negative BL cell line BL41 and the transplantable EBV-positive NPC line C15 served as controls. Total RNA from the biopsies and cell lines was analyzed by reverse transcription and PCR amplification by using a 5′ (2.8 [◄]) primer and a 3′ primer spanning the exon 2-3 splice junction (▪-►). The 381-bp LMP1-specific amplified products were detected with an oligonucleotide probe from exon 2 (■).

LMP1 mRNA (containing additional untranslated upstream sequences), first identified in the C15 NPC line, LMP1 transcription in HD was further examined using the 3′ primer described above in combination with the 5′ (3.7) LMP1 primer derived from a sequence upstream of the 2.8-kb mRNA start site. This primer combination yielded the predicted 3.7-kb mRNA–specific 460-bp product in 10 of the 12 EBV-positive HD biopsies; again, no signal was detected from the EBV-negative HD biopsies (data not shown). As the presence of amplifiable mRNA in each of the HD extracts was verified by using primer combinations specific for the cellular CD45 mRNA (data not shown), the inability to detect both the 2.8- and 3.7-kb LMP1 mRNAs in biopsy HD8 is likely to be due to the low number of HRS cells in this particular biopsy.

Analysis of LMP2A and LMP2B Expression at the mRNA Level. RNA from the same series of HD biopsies was examined for LMP2A and LMP2B transcripts by PCR using a common 3′ primer to exon 3 in combination with LMP2A- and LMP2B-specific 5′ primers from the unique first exons of the two mRNAs (Fig. 4). These primers, which we have previously used to examine LMP2 transcription in NPC biopsies, yielded the predicted 280-bp LMP2A product or the predicted 324-bp LMP2B product in 8 of the 12 EBV-positive HD biopsies; no signal was detected from the EBV-negative HD biopsies even after long overexposures of the autoradiographs (Fig. 4). In two cases, HD3 and HD6, only one form of LMP2 was detected while the remaining biopsies were positive for both LMP2A and LMP2B.

Analysis of EBNA1 Expression at the mRNA Level. We postulated that, even though immunoblotting could not detect EBNAs in total HD biopsy extracts, the latent EBV infection in HRS cells (like all other known forms of EBV latency) would be associated with expression of at least one nuclear antigen, the virus genome maintenance protein EBNA1. Identification of an EBNA1 transcript would not only constitute strong evidence that this was the case, but also the splice structure of the transcript would help to characterize the resident form of virus latency. Thus, RNA from the HD biopsies was examined for EBNA1 transcribed from either: (*a*) the Fp using a 3′ primer within the BamHI K EBNA1 open reading frame (ORF) in combination with a 5′ primer from the BamHI Q exon, or (*b*) the Cp/Wp using the same 3′ primer in combination with a 5′ primer from the BamHI Y3 exon. These primers, which we have previously used to examine EBNA1 transcription in LCL, BL, and NPC cells, yielded the predicted 236-bp Q/U/K spliced product diagnostic of Fp-driven EBNA1 transcription in the control C15 NPC line and in 9 of the 12 EBV-positive HD

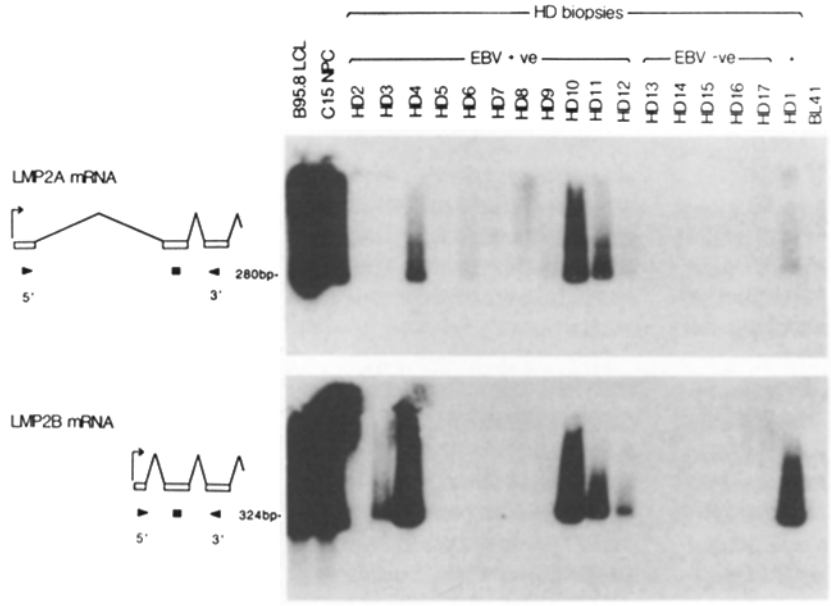

Figure 4. PCR analysis of LMP2 mRNAs in 12 EBV-positive (HD1 to HD12) and 5 EBV-negative (HD13 to HD17) HD biopsies using LMP2A-specific (*top*) and LMP2B-specific (*bottom*) primer combinations. The EBV-negative BL cell line BL41, the EBV-positive B95.8 LCL, and the transplantable EBV-positive NPC line C15 served as controls. Total RNA from the biopsies and cell lines was analyzed by reverse transcription and PCR amplification by using a 3′ primer (◄) from the common exon 3 of LMP2 in combination with a 5′ primer (►) from the unique first exon of either LMP2A (*top*) or LMP2B (*bottom*). The 280-bp LMP2A-specific and 324-bp LMP2B-specific amplified products were detected with an oligonucleotide probe from the common exon 2 (■).

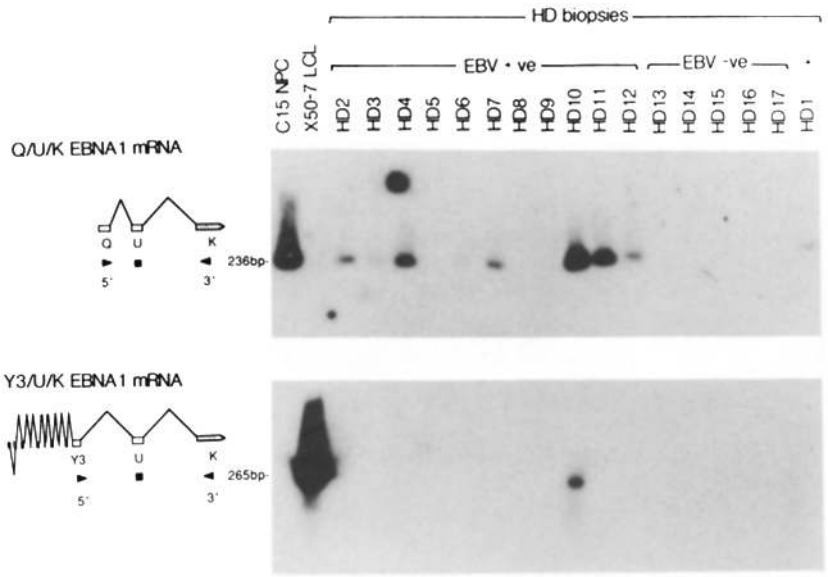

Figure 5. PCR analysis of EBNA1 mRNAs in 12 EBV-positive (HD1 to HD12) and 5 EBV-negative (HD13 to HD17) HD biopsies. The EBV-positive B95.8 LCL and the transplantable EBV-positive NPC line C15 served as controls. Total RNA from the biopsies and cell lines was analyzed by reverse transcription and PCR amplification by using a 3′ primer (◄) from the EBNA1-coding (*shaded*) BamHI K exon in combination with a 5′ primer (►) either from the BamHI Q exon (*top*) or from the BamHI Y3 exon (*bottom*). Amplified products were detected with an oligonucleotide probe from the common BamHI U exon (■).

biopsies; no signal was detected in the X50/7 LCL or from the EBV-negative HD biopsies even after long overexposures of the autoradiographs (Fig. 5). In contrast, the 265-bp Y3/U/K spliced product diagnostic of Cp/Wp-driven EBNA1 transcription was readily detected in the X50/7 LCL but in only one of the EBV-positive HD biopsies: HD10, which is also positive for Fp transcription (Fig. 5).

Analysis of Cp/Wp mRNA Transcription. To confirm the lack of Cp/Wp activity in EBV-positive HD suggested by the EBNA1 mRNA analysis, we used two further assays to examine more directly transcription from these promoters. First, we used in situ hybridization with a BamHI W riboprobe specific for "rightward-running" transcripts containing BamHI W sequences. While the control X50/7 LCL gave a strong cytoplasmic signal with this probe, no positive cells were observed in any of the EBV-positive HD samples, including HD10, which by PCR was positive for Cp/Wp-driven EBNA1 transcripts (Fig. 6). Second, using PCR primers from appropriate W exons (37), we were able to confirm that the majority of the EBV-positive HD cases were negative for Cp/Wp activity (data not shown); however, RNA extracted from HD10 did contain BamHI W mRNA–specific transcripts in accord with the earlier finding of Cp/Wp-driven EBNA1 mRNA in this particular sample.

Analysis of BamHI A Transcription. The detection of abundant transcription over the BamHI A region of the EBV genome in NPC prompted us to examine these transcripts in EBV-positive HD. We developed two sets of oligonucleotide primers that were specific for the large BamHI A–encoded 18.8 mRNA first isolated from a cDNA library made to the nude mouse–passaged C15 NPC tumor line (Fig. 7). These PCR primers spanned the introns of this transcript and gave the products of predicted size on RNA extracted from the C15 line; no signals were ever detected from RNA extracted from EBV-negative B cell lines (Fig. 7). A further conformation of the specificity of the primers spanning the first intron of the 18.8 mRNA (the A1/A2 primers) is their inability to detect BamHI A–specific mRNA in the B95.8 cell line due to a deletion of the first exon. However, the A3/A4 primers spanning the second and third introns confirmed that the 3′ end of the 18.8-like mRNA is correctly spliced in B95.8 cells. Both sets of primers detected the correctly spliced BamHI A transcript in all EBV-positive HD biopsies except HD12; no signal was detected from the EBV-negative HD biopsies even after long overexposures of the autoradiographs (Fig. 7).

Table 1 presents a summary of all the data obtained from the 12 EBV-positive HD biopsies by in situ hybridization for EBERs and for BamHI W–containing transcripts, by immunohistochemical staining for LMP1 and by PCR amplification for EBNA1, LMP, and BamHI A mRNAs. Below the HD data are shown the different patterns of results displayed by the three well-established forms of viral latency in the same assays. Note that the forms of latency characteristic of BL, NPC, and LCL cells are now referred to as latency I, II, and III respectively.

Discussion

While much of the early work suggesting an association of EBV with HD was circumstantial, more direct evidence comes from recent studies in which EBV genomes and the EBV-encoded EBERs have been detected in the malignant HRS cells of up to 50% HD cases (25–28). To investigate the expression of EBV latent proteins in HD, we originally used mAbs to LMP1 and EBNA2 in an immunohistochemical study; this revealed LMP1, but no detectable EBNA2, expression in the HRS cells of 48% of cases with a predominance of positivity in the mixed cellularity histological subtype of the disease (29). This result was subsequently confirmed by other groups (30, 38, 39) and suggested that the pattern of EBV gene expression in HD was similar to that observed in NPC (15, 16). Subsequent studies of EBV latent protein

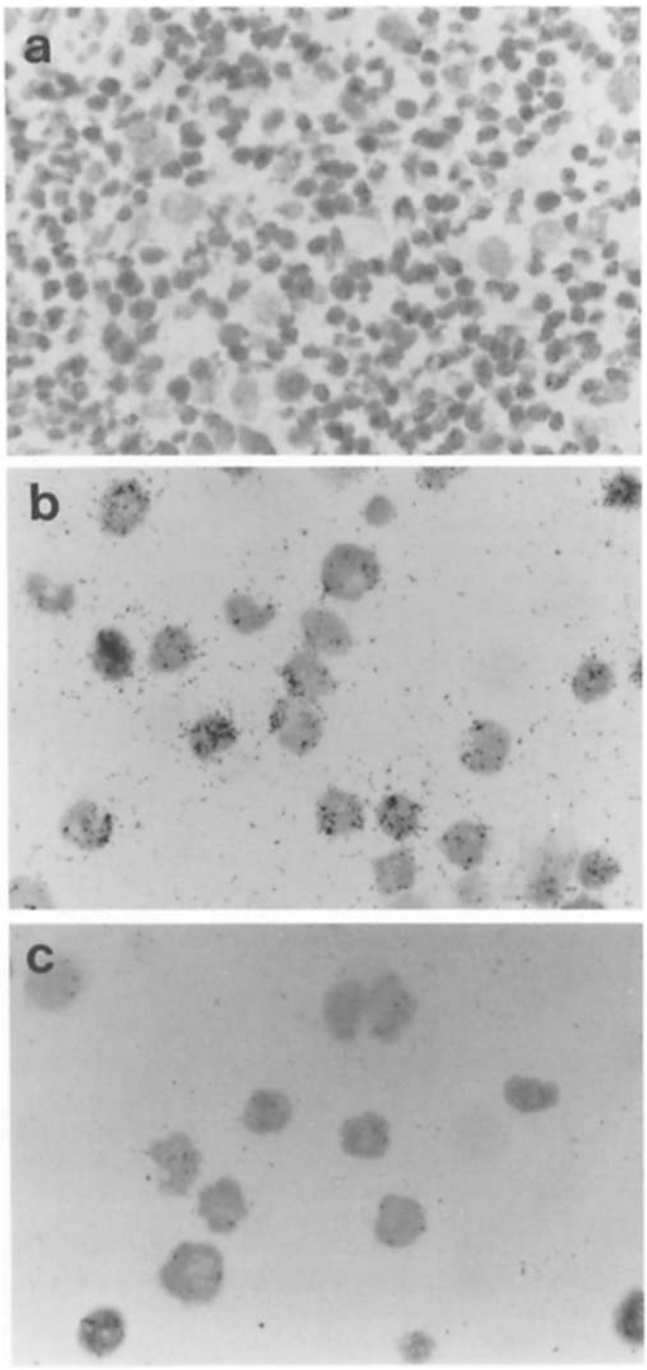

Figure 6. In situ RNA-RNA hybridization of an EBV-positive HD biopsy, HD1, with a BamHI W-specific antisense probe (*a*, 7-d exposure, H+E counterstaining, ×340) confirms absence of Cp/Wp promoter activity, whereas the same probe shows an intense cytoplasmic mRNA signal in X50/7 LCL cells (*b*, 5-d exposure, H+E counterstaining, ×340). No corresponding signal is seen in X50/7 cells with the control sense probe (*c*, 5-d exposure, H+E counterstaining, ×340).

expression in HD have been hampered by the limited range of available reagents for immunohistochemical staining and by the relative paucity of malignant HRS cells in HD biopsies, which prevents their analysis by the standard immunoblotting approach. Interestingly, in the present study we were able to detect LMP1 at the protein level by immunoblotting in those EBV-positive HD cases with sufficient numbers of HRS cells. This testifies to the high-level expression of LMP1 in HRS cells already apparent by immunohistochemistry. High-level expression of LMP1 from inducible heterologous promoters has been shown to be extremely toxic in a variety of different cell types (40). That HRS cells can sustain such levels of LMP1 is itself interesting, and its continued expression argues for an important role of this oncogenic protein in the pathogenesis of HD (41). The pleiotropic nature of LMP1's effects in a variety of cell systems, notably the multiple phenotypic changes induced in B cell lines and the inhibition of epithelial cell differentiation, likewise suggests that this protein may contribute to the malignant transformation of HRS cells (42, 43).

In this study we have further characterized the pattern of EBV latent gene expression in EBV-positive HD by reverse transcription PCR amplification of specific viral mRNAs using primer-probe combinations previously validated and used in the analysis of EBV transcription in NPC biopsies (17). This approach is ideally suited to the analysis of HD biopsies where the content of malignant HRS cells is small. Analysis of LMP1 transcription in HD biopsies consistently detected transcripts with the same splicing pattern as originally identified in the 2.8-kb LMP1 mRNA expressed in LCL cells (44); furthermore, use of an alternative 5′ primer indicated that at least some, and possibly all, of these transcripts represent the longer 3.7-kb LMP1 mRNA initiating from a promoter upstream of the 2.8-kb mRNA start site (N.B., the 5′ [2.8] primer, would amplify both 2.8- and 3.7-kb transcripts). This LMP1-specific 3.7-kb mRNA was originally described in the C15 NPC line and has also been observed in fresh biopsies of NPC (19). Thus, LMP1 transcription in EBV-positive HD resembles that observed in NPC. Although EBNA2 has been shown to transactivate the 2.8-kb LMP1 promoter and is indeed required for LMP1 expression in EBV-infected B cells (45–47), the presence of the 3.7-kb and possibly also the 2.8-kb LMP1 transcript in HD and NPC cells in the absence of EBNA2 expression suggests that the relevant promoter(s) are no longer EBNA2 dependent in these particular cell environments.

The lack of specific antibodies to LMP2 protein prevents immunohistological analysis of HRS cells with respect to LMP2 status (N.B. LMP2A and 2B are antigenically related since all of the LMP2B sequence is contained within LMP2A [48]). However, we have recently used PCR to examine the expression of LMP2A and LMP2B mRNA in NPC biopsies (17). In the present study we have demonstrated that both LMP2A and LMP2B transcripts are frequently expressed in EBV-positive HD. The inability to detect LMP2 mRNA in four of the HD biopsies is probably due to the low number of HRS cells in those cases. Thus, in HD5 and HD8 neither LMP2 mRNA nor EBNA1 mRNA is detectable even though these cases are positive for LMP1 protein and EBERs at the histological level (see Table 1). The regularity of LMP2 expression in HD again indicates that both the LMP2A and LMP2B promoters (the latter located in the same region as the 2.8-kb LMP1 promoter [10]) can function in the absence

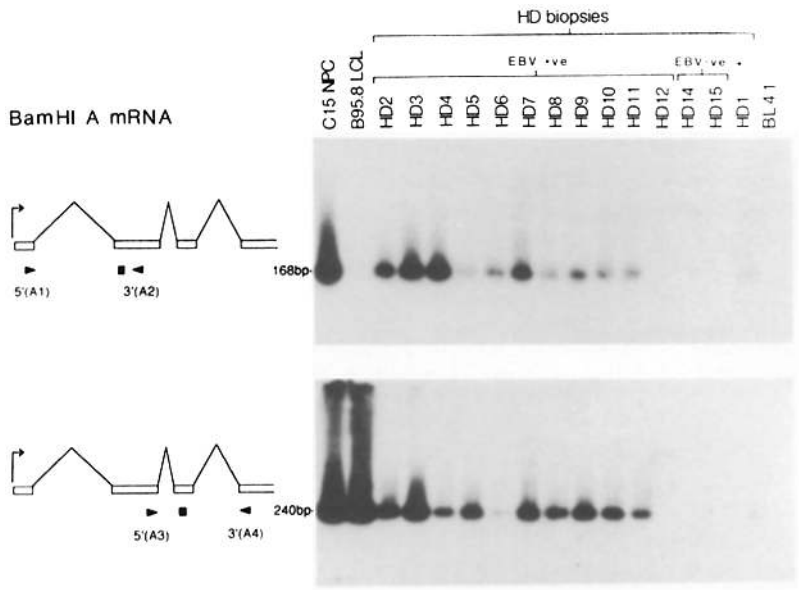

Figure 7. PCR analysis of BamHI A transcripts in 12 EBV-positive (HD1 to HD12) and 5 EBV-negative (HD13 to HD17) HD biopsies. The EBV-positive B95.8 LCL and the transplantable EBV-positive NPC line C15 served as controls. Total RNA from the biopsies and cell lines was analyzed by reverse transcription and PCR amplification using two sets of primers. The first primer pair (A1/A2) span the large first intron of the "rightward-running" 18.8 cDNA, and the second primer pair (A3/A4) span the second and third introns (18). The A1 primer (5'-ATG-GCCGGAGCTCGTCGACG-3') lies within a 11,835-bp fragment present in the Raji BL (as well as most other) virus strains but is deleted from B95.8 between nucleotides 152012 and 152013 of the B95.8 sequence (60). Thus, amplification with the A1 primer in combination with the A2 primer (5'-CCTTCGATATCGAGTGTCTG-3') gives the predicted 168-bp amplified product detected with the end-labeled A1/A2 oligonucleotide probe (5'-ACC-AGAGGACGCAGGATATC-3') in RNA extracted from the C15 NPC line and in the EBV-positive HD samples but not from B95.8. The combination of primer A3 (5'-AGAGACCAGGCTGCTAAACA-3') with primer A4 (5'-AACCAGCTTTCCTTTCCGAG-3') yielded the predicted 240-bp product detected with the end-labeled A3/A4 probe (5'-AAGACGTTGGAGGCACGCTG-3').

of EBNA2, even though both are EBNA2 dependent in LCL cells (48). Thus, it is likely that LMP2 proteins are regularly expressed in EBV-positive HD biopsies as in NPC (17, 49), again raising the possibility of an effector role for these proteins in the development of HD. Furthermore, antibodies to LMP2 are frequently detected in NPC patients but not in tumor-free controls (51); similar serological studies in HD patients will be of interest.

A particularly interesting aspect of the current study is the finding that EBV-positive HD biopsies consistently express EBNA1 mRNA with the same Q/U/K splice structure as has been recently identified in NPC biopsies as well as in BL cell lines that retain the original BL phenotype (13, 14, 17, 36). Our recent work shows that this splice pattern is diagnostic of a distinct EBNA1 mRNA transcript driven from a novel promoter Fp close to the BamHI F/Q boundary. Thus, it would appear that the Fp is responsible for EBNA1 transcription in HRS cells as opposed to the Cp/Wp promoters, which are active in LCLs (9). This was further confirmed in HD by the lack of Cp/Wp activity as assessed by both PCR and in situ hybridization. The Cp/Wp activity detected by PCR in one of the EBV-positive HD biopsies, HD10, is likely to reflect the presence of EBV-infected reactive lymphoid cells, a phenomenon that we and others have previously observed in a number of EBV-positive and -negative HD cases (50–52). Our inability to detect Cp/Wp activity by in situ hybridization in the reactive lymphoid component of HD10 may be due to sampling differences such that these few EBV-positive cells were present in the tumor portion from which RNA was extracted but not in the thin sections used for the in situ studies. The overall result of EBNA1 transcription analysis strongly suggests that the EBV genome maintenance protein EBNA 1 is expressed in HD as in both NPC and BL without concomitant expression of the other EBNAs by virtue of the selective use of the Fp promoter. Studies on the regulation of Fp activity will help define the cellular factors responsible for the selective use of Fp in different cell types.

The identification of novel latent transcripts running through the BamHI A region of the EBV genome in the opposite orientation to conventional BamHI A transcripts of the virus lytic cycle came from work on a nude mouse–passaged NPC cell line C15 (18, 19). Such "rightward-running" transcripts appear, from limited analyses published to date, to be a consistent feature of NPC (53). Using PCR primers spanning introns in the largest BamHI A–specific cDNA isolated from the C15 NPC line, we have recently detected transcripts with this splice structure in 10 fresh NPC biopsies (Brooks et al., manuscript in preparation). Given the similarities between NPC and HD with regard to EBV gene expression, we analyzed EBV-positive HD cases for such mRNAs and found easily detectable expression of the appropriately spliced transcript in all but one case. While a putative BamHI A–encoded protein product has been identified (53), the lack of a suitable antibody reagent precludes analysis of the expression of this protein in biopsies of NPC and HD. Whatever the function of the protein encoded by the BamHI A transcript, the expression of this mRNA in EBV-positive HD clearly indicates that it is not preferentially expressed in epithelial cells as has previously been suggested (53). Indeed our recent findings show that latently infected B cell lines also contain the same transcript (Brooks et al., manuscript in preparation).

The finding of a consistent pattern of EBV latent gene transcription in EBV-positive HD further supports a role for the virus in the pathogenesis of this malignancy. This particular form of infection (with coexpression of EBNA1, LMP1, 2A, and 2B, and BamHI A transcripts) is identical to that ob-

served in NPC and is likely to be similar to that in certain other malignancies of lymphoid origin whose association with EBV is now becoming better documented. Thus, EBV-positive cases of AIDS-related lymphoma, CD30-positive anaplastic large cell lymphoma, and peripheral T cell lymphoma have been described where LMP1 is often, but not always, expressed in the absence of EBNA2 (54–56). There are now two in vitro model systems in which this form of latency can be induced, in one case by broadening the highly restricted viral latent gene expression seen in BL cells (57), and in another case by repressing some of the viral genes expressed in LCLs (37). Interestingly, the latter example involved cell fusion of an EBV-transformed B cell with another parent cell of non-B cell origin; it is at least formally possible that HRS cells arise by such a fusion event in vivo.

Another important aspect of the present work is its implications for the possibility of CTL control over EBV-positive HD. Recent analyses indicate that, while many EBV-specific CTL responses are directed towards target proteins such as EBNA3A, 3B, or 3C, which are downregulated in HD cells, at least two of the viral proteins that are expressed, namely LMP1 and 2, can provide target peptides when presented in the context of at least some common HLA class I antigen types (58, 59). If EBV-positive HD cells are indeed sensitive to such responses (and one has to remember that the primary role of EBV-specific CTLs is to survey viral infection of the B cell pool), then it may be that those HLA class I antigens selectively presenting LMP1- or LMP2-derived epitopes will prove to be protective against the EBV-positive but not the EBV-negative form of HD. Now that target epitopes from LMP1 and LMP2 are being defined at the peptide level, it will be possible to determine whether these sequences are conserved in HD-derived EBV isolates and/or whether peptide-specific responses can be elicited from the T cell memory of appropriate HD patients.

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