American Journal of Pathology, Vol. 147, No. 4, October 1995

Detection of Heterogeneous Epstein-Barr Virus Gene Expression Patterns within Individual Post-Transplantation Lymphoproliferative Disorders

Joost J. Oudejans,* Mehdi Jiwa,* Adriaan J. C. van den Brule,* Friedrich A. Grässer,† Anja Horstman,* Wim Vos,* Philip M. Kluin,‡ Paul van der Valk,* Jan M. M. Walboomers,* and Chris J. L. M. Meijer*

*From the Department of Pathology, * Free University Hospital, Amsterdam, The Netherlands; Institut für Medizinische Mikrobiologie und Hygiene,† Universitätskliniken des Saarlandes, Homburg, Germany; and the Department of Pathology,‡ University Hospital Leiden, Leiden, The Netherlands*

Using RT-PCR analysis of Epstein-Barr virus (EBV) latent gene transcription in EBV-harboring cell lines (JY and RAJI) and in post-transplantation lymphoproliferative disorders (PT-LPDs), we detected transcription of all tested latent genes (EBNA1, EBNA2, LMP1, LMP2A, and BARF0) in all cases, suggesting the presence of similar EBV expression patterns in both PT-LPDs and cell lines. In addition, the detection of immediate early (ZEBRA) and early gene (BHRF1) transcripts in cell lines and PT-LPDs indicates that activation of the virus lytic cycle occurs. To investigate EBV expression patterns at the single-cell level, a combination of immunohistochemistry and RNA in situ hybridization (including double-staining procedures) was used. In the JY and RAJI cell lines, the latency type 3 expression pattern was detected in 80 to 90% of the cells as shown by the co-expression of EBNA2 and LMP1. In contrast, in the three PT-LPDs that could be analyzed by double staining, cells expressing both EBNA2 and LMP1 were rarely detected. A mixture of at least three different cell populations were identified: (1) cells exclusively expressing EBER1/2 and EBNA1 (latency type 1); (2) cells expressing EBER1/2, EBNA1, and LMP1 (latency type 2); and (3) cells expressing EBER1/2, EBNA1, and EBNA2 in the absence of LMP1. Activation of the lytic cycle was observed in a small minority of cells, as demonstrated by detection of ZEBRA and EA-D in all cases and GP350/220 in two cases. Thus, in contrast to EBV-transformed cell lines, the observed EBV gene expression pattern in PT-LPDs reflects a mixture of multiple EBV-harboring subpopulations expressing different subsets of EBV-encoded proteins. These data indicate that the operational definitions of EBV latencies in vitro cannot easily be applied to PT-LPDs but that a continuum of different latency expression patterns can be detected at the single cell level in these lymphomas with, in a small minority of cells, progression to the virus lytic cycle. (Am J Pathol 1995, 147:923–933)

Diffuse B-cell lymphoproliferations are a well known, often fatal complication in patients with a severe immunodeficiency. These lymphomas are frequently associated with the Epstein-Barr virus (EBV).[1] In acquired immune deficiency syndrome (AIDS)-related Burkitt's lymphoma, EBV is detected in approximately 35% of cases (comparable to the situation in nonimmunocompromised patients in nonendemic areas), whereas in AIDS-related immunoblastic polymorphous large cell B-cell lymphomas with plasmacytoid differentiation, the virus is detected in nearly all cases.[2,3] Post-transplantation lymphoproliferative disorders (PT-LPDs) are nearly always associated with EBV.[4–7] Usually, these PT-LPDs are rapidly developing tumors of monoclonal composition.[6,7] The

Supported by a grant from the Dutch Cancer Society (VU 94–749).

Accepted for publication July 5, 1995.

Address reprint requests to Dr. J. J. Oudejans, Department of Pathology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.

presence of EBV in morphologically malignant cells was demonstrated by a RNA *in situ* hybridization assay (RISH) using the abundantly transcribed noncoding EBER1/2 RNAs.[2,8]

In vitro studies have identified several EBV-encoded proteins that might be responsible for the pathogenic effect of EBV. Of the latent proteins, EBNA2 and LMP1 are essential for the transforming potential of EBV,[9,10] whereas expression of other EBV-encoded proteins might give a growth advantage because of their functional homology to human proteins (BCRF1, homologous to interleukin-10[11] and BHRF1, homologous to *bcl-2*[12]). To determine the possible role of these proteins in the development of lymphomas, several groups have investigated EBV latent gene expression *in vitro* and *in vivo*.

With nonmorphological methods, EBV latent gene expression was primarily investigated in certain EBV-positive cell lines.[13] Three types of latency could be identified. In Burkitt's lymphoma-derived cell lines, expression is detected of one EBV nuclear antigen (EBNA1), two abundant noncoding RNAs (EBER1 and EBER2),[14,15] and certain rightward reading transcripts derived from the EBV *Bam*HI A fragment (BARF0[15]; latency type 1). In EBV-transformed lymphoblastoid cell lines as well as in some cultured EBV-harboring Burkitt cell lines like RAJI, expression was detected of at least all six EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, -B and -C, and EBNA4), the three latent membrane proteins (LMP1, LMP2A, and 2B), EBER1/2, and BARF0[16,17] (latency type 3). This latency type 3 pattern was also detected in B-cell lymphomas arising in immunocompromised patients.[4,5,18–20] In latency type 2, frequently detected in EBV-associated malignancies (nasopharyngeal carcinoma,[21] Hodgkin's disease,[22] and certain T-cell lymphomas[23]), expression is restricted to EBNA1, EBER1/2, BARF0, LMP1, and LMP2A and/or LMP2B. This latency type 2 pattern could be transiently detected in a Burkitt's lymphoma-derived cell line, normally expressing the latency type 1 pattern, when stimulated to enter the lytic cycle.[24] Thus, individual cells can drift from one latency pattern to another.

The different EBV expression patterns are associated with different promoter uses. In latency type 3, all six EBNAs can be expressed from highly spliced transcripts that are generated by one of two promoters located on the *Bam*HI fragments C and W located near the lefthand end of the genome and referred to as C or W promoter.[9] In latency types 1 and 2, selective expression of EBNA1 without expression of the other EBNAs is a result of activation of a separate EBNA1 promoter located on the *Bam*HI F fragment, referred to as F promoter.[21,22,25,26]

As nonmorphological methods like Western blotting revealed the expression of all known EBV latent genes in PT-LPDs,[18] similar to the EBV latent gene expression observed in lymphoblastoid cell lines, these lymphomas are thought to represent the *in vivo* counterpart of lymphoblastoid cell lines. Moreover, expression of this latency type 3 expression pattern was confirmed by the detection of both EBNA2 and LMP1 in tissue sections of B-cell lymphomas arising in immunocompromised patients.[2,4,5,19,20]

The aim of the present study was to investigate whether, in EBV-associated PT-LPDs, all EBV-harboring cells show the same EBV expression pattern or whether heterogeneous EBV expression patterns are present at the single-cell level. The results showed that in EBV-positive cell lines latency type 3 was found at the single-cell level in nearly all cells, intermingled with sporadic LMP1-negative, ZEBRA-positive cells, whereas in EBV-associated PT-LPDs a heterogeneous EBV expression pattern was observed with a mixture of cells expressing either latency type 1 (EBER1/2 and EBNA1) or latency type 2 (EBER1/2, EBNA1, and LMP1) or cells expressing lytic genes, whereas cells expressing latency type 3 (as shown by co-expression of EBER1/2, EBNA1, EBNA2, and LMP1) were rarely found.

Materials and Methods

Materials

PT-LPDs

Characteristics are summarized in Table 1. Lymphoma samples were derived from biopsy (cases 1 and 2) or autopsy specimens (cases 3, 4, and 5) of patients, all children, who were subjected to an allogeneic bone marrow transplantation after having been treated for acute lymphocytic leukaemia in three cases and for chronic myeloid leukaemia in one case (see Table 1). The fifth patient received a bone marrow transplant to treat a combined immunodeficiency and thrombocytopenia in the Wiskott-Aldrich syndrome (case 4). These patients subsequently developed a diffuse B-cell lymphoma within 2 to 12 months. In all cases except case 2, localizations of the lymphoma were found in nodal as well as in extranodal tissues.

Table 1. *Patient Material*

Case	Age/ sex	Indications for bone marrow transplantation	Histology	Growth pattern	Sites		CD30-positive cells*	Clonality
					Nodal	Extranodal		
1 (12253)	8/M	CML	Polymorphous Large cell	Diffuse	Neck	Tonsil, lung	10%	Polyclonal
2 (839)	10/F	ALL	Polymorphous Large cell	Diffuse	Neck	None	20%	Monoclonal†
3 (302)	15/F	ALL	Polymorphous Large cell	Diffuse	Generalized	Generalized, except bone marrow	30%	Monoclonal
4 (91–1)	4/M	Wiscott-Aldrich syndrome	Polymorphous Large cell	Diffuse	Generalized	Generalized	10%	Monoclonal
5 (165)	9/F	ALL	Polymorphous Large cell	Diffuse	Generalized	Liver, stomach, bone marrow	30%	Oligoclonal

ALL, acute lymphocytic leukemia; CML, chronic myeloid leukemia.
*The number of CD30-positive cells was estimated on frozen tissue sections.
†Clonality was determined by Southern blot analysis.

Cell Lines

Cell lines included in this study were the EBV-transformed lymphoblastoid cell line JY, the EBV-positive Burkitt's lymphoma-derived cell line RAJI, and the HH514C16 cell line, which harbors the P3HR1 EBV strain. This strain lacks the EBNA2 open reading frame and the last common EBNA-specific exon, because of a deletion spanning the *Bam*HI Y and part of the *Bam*HI H fragment[9] (see Figure 1). This cell line served as a negative control for EBNA2 analysis by immunohistochemistry and for reverse transcription polymerase chain reaction (RT-PCR) analysis of EBNA2, Y3/U/K-spliced EBNA1, and Y2/HF-spliced BHRF1 transcripts. The EBV-negative Burkitt's lymphoma-derived cell line BJAB was used in all experiments as a source of EBV-negative control material.

Methods

Preparation of Clinical Samples and Cell Lines

For RT-PCR and immunohistochemistry, 40 5-μm sections were cut from snap-frozen material. In case 1, the amount and quality of the frozen tissue available was limited. In this case, sections were cut for RT-PCR analysis and for hematoxylin and eosin (H&E) staining. To obtain suitable RNA, 10 sections were homogenized with micropestles (Eppendorf, E. Merck Nederland BV, Amsterdam, The Netherlands) in a RNAzol B buffer according to the manufacturer's protocol (Cinna/Biotecx, Houston, TX). For the cell lines, approximately 5×10^6 cells were pelleted and RNA was isolated by using the same RNAzol B protocol. RNA was stored in 100% ethanol at −80°C until further processing. The remaining 30 sections were mounted on poly-L-lysine-coated slides, fixed, and used for immunohistochemistry and H&E staining. To confirm that the sections used for RT-PCR contained lymphoma, the first and the last section of the frozen material used for RT-PCR were H&E stained and examined by light microscopy.

RT-PCR Analysis

RT-PCR analysis was performed with intron-flanking primers. Differentially spliced transcripts were iden-

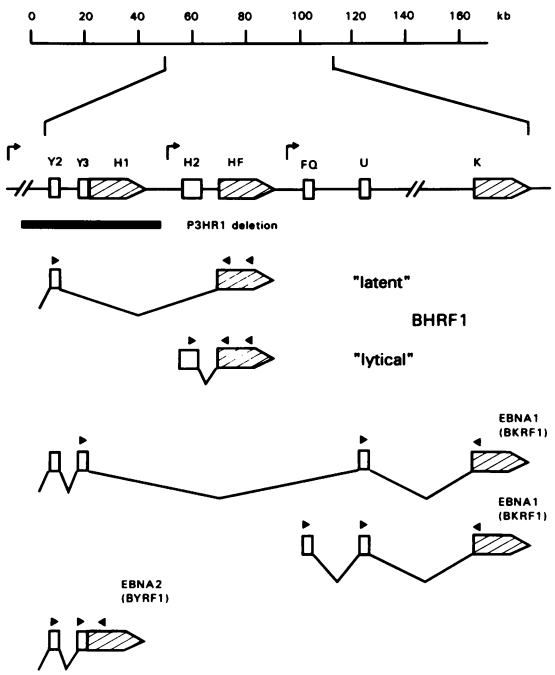


Figure 1. *Schematic presentation of the EBV genome, presenting the different tested BHRF1, EBNA1, and EBNA2 transcripts. The* shaded arrows *represent the exons containing the different open reading frames. The C or W, F, and "lytic" BHRF1 promoter are represented by the* black arrows. *The intron-flanking primers used for amplification by RT-PCR and the internal oligomers used as probe are represented by* black triangles *in relation to the specific exons. The direction of the triangles correlates with sense or antisense orientation of the oligomer.*

Table 2. *Sequences of PCR Primers and Oligo Probes*

Transcript	Oligo*	Sequence (5′–3′)	B95.8 genomic coordinates	Amplimer length
EBNA1	Y3[†](s)	TGGCGTGTGACGTGGTGTAA	48397–48416	Y3/U/K spliced, 265 bp
	Q(s)	GTGCGCTACCGGATGGCG	62440–62457	Q/U/K spliced, 236 bp
	K(as)	CATTTCCAGGTCCTGTACCT	107986–107967	
	U(probe, s)	AGAGAGTAGTCTCAGGGCAT	67544–67563	
EBNA2	Y2[‡](s)	TACGCATTAGAGACCACTTTGAGCC	47897–47921	195 bp
	H1(as)	AAGCGCGGGTGCTTAGAAGG	48478–48459	
BHRF1	H2(s)	GTCAAGGTTTCGTCTGTGTG	53830–53849	H2/HF spliced, 211 bp
	H3(as)	TTCTCTTGCTGCTAGCTCCA	54480–54461	Y2/HF spliced, 248 bp
	probe(as)	ATGCACACGACTGTCCCGTATACAC	54435–54411	
LMP1	1(s)	TGTACATCGTTATGAGTGAC	169356–169337	240 bp
	2(as)	ATACCTAAGA/CAAGTAAGCA	168956–168965/ 169042–169052	
	3(probe, as)	ACAATGCCTGTCCGTGCAAA	169081–169100	
LMP2A	A1(s)	ATGACTCATCTCAACACATA	166874–166893	280 bp
	AB2(as)	CATGTTAGGCAAATTGCAAA	380–361	
	probe(as)	ATCCAGTATGCCTGCCTGTA	81–62	
BARFO	A3(s)	AGAGACCAGGCTGCTAAACA	157154–157173	240 bp
	A4(as)	AACCAGCTTTCCTTTCCGAG	159194–159175	
	probe(s)	AAGACGTTGGAGGCACGCTG	157359–157378	
ZEBRA	Z1(s)	CGCACACGGAAACCACAACAGC	102684–102663	227 bp
	Z2(as)	CGGCGGATAATGGAGTCAACATCC	102250–102273	
	probe(as)	GCTTGGGCACATCTGCTTCAACAGG	102300–102276	

*The sense (s) or antisense (as) orientation is indicated in brackets.
[†]The Y3 primer was also used as a probe for detection of EBNA2 transcripts.
[‡]The Y2 primer was also used in combination with the H3 primer to detect Y2/HF-spliced BHRF1 transcripts.

tified by specific primer combinations and subsequent hybridization with internal oligo probes. The primer sequences and oligo probes used for analysis of EBNA1, LMP1, BARF0, and H2/HF-spliced BHRF1 transcripts have been published elsewhere.[21,22,27] For analysis of EBNA2, ZEBRA, and Y2/HF-spliced BHRF1 transcripts,[28] primers were designated by using the PCR plan software (PCgene release 6.7, IntelliGenetics, Mountain View, CA). Primers and probes are listed in Table 2. For reverse transcriptase reactions, an amount of RNA was used, equivalent of one 5-μm section or of 1×10^5 cells for biopsies and cell lines, respectively. After centrifugation, pellets were resuspended in 5 μl of distilled water. Reverse transcriptase reactions were performed in a final volume of 20 μl containing 25 pmol of one to four of each EBV antisense primer specific for the different genes. The conditions used for RT-PCR analysis and Southern blot hybridization were described previously.[29] To exclude false positive signals caused by DNA amplification, simultaneous reactions were performed with omission of the reverse transcriptase.

Immunohistochemistry

CD30 expression was demonstrated with the BerH2 antibody (DAKO, Copenhagen, Denmark). To detect EBV-specific proteins, monoclonal antibodies against LMP1 (CS1–4, DAKO and S12, Organon Teknika, Oss, The Netherlands[30]), EBNA2 (pE2, DAKO), Early-D (DuPont, Wilmington, DE), ZEBRA (DAKO), and GP350/220 (Organon Teknika) were used. The antibodies were visualized with an avidin-biotin-horseradish peroxidase complex and diaminobenzidine/H_2O_2 staining method, as previously described by us.[30]

EBNA1 expression was detected in paraffin-embedded tissues with a mixture of two recently generated anti-EBNA1-specific rat monoclonal antibodies, 1H4–1 and 2B4–1.[31] To increase sensitivity, a few adjustments were made. Before incubation with the anti-EBNA1 antibody, tissues were boiled for 15 minutes in a citrate buffer (0.1 mol/L, pH 6.0). Incubation with the antibodies was done overnight at room temperature with a 1:50 diluted antibody (final concentration, 44 μg/ml). Detection of the antibodies was performed with a biotinylated goat anti-rat antibody, followed by incubation with an avidin-biotin-horseradish peroxidase complex. The peroxidase was visualized by incubation for 3 minutes in 0.2 mg/ml diaminobenzidine, 0.002% H_2O_2, and 0.07% $NiCl_2$ in 50 mmol/L Tris-HCl, pH 7.6, followed by silver enhancement of the diaminobenzidine-nickel precipitate as described previously.[32]

Combination RISH and Immunohistochemistry

After performing the EBER1/2 RISH assay,[32] remaining peroxidase activity was inactivated with 0.3%

Table 3. *Analysis of EBV Gene Expression in PT-LPDs and Cell Lines*

	Percentage of cells positive at protein level by immunohistochemistry*						At mRNA level by RT-PCR†			
							EBNA1		BHRF1	
	EBNA1	EBNA2	LMP1	ZEBRA	EA-D	GP350/220	C/Wp	Fp	C/Wp	Hp
Lymphomas										
1	ND	ND	5	ND	ND	ND	+	−	+	+
2	<5	10	20	<5	<5	0	+	+	+	−
3	>90	10	20	<5	<5	0	+	+	+	−
4	>90	10	<5	<5	<5	10	+	+	+	+
5	50	20	20	10	<5	10	+	+	+	+
Cell lines										
JY	100	>90	80–90	<5	<5	<5	+	+	+	+
RAJI	100	>90	80–90	0	0	0	+	+	+	+
HH514C16	100	0	<5	<5	<5	0	−	+	−	+
BJAB	0	0	0	0	0	0	−	−	−	−

*For EBNA1, the number of positive cells was estimated on paraffin-embedded tissue sections. For EBNA2, LMP1, ZEBRA, EA-D, and GP350/220, the number of positive cells was estimated on frozen tissue sections, except in case 1, in which the number of positive cells was estimated on paraffin-embedded tissue sections. ND, not done.

†Results for EBNA2, LMP1, LMP2A, ZEBRA, and BARFO are not indicated. All post-transplant lymphomas and EBV-positive cell lines were positive for all tested transcripts, except EBNA2, which was not detected in the HH514C16 cell line.

H_2O_2/methanol and fixed with 4% paraformaldehyde for 10 minutes, followed by the above described immunohistochemical detection method.

Double-Staining Procedure with EBV-Specific Antibodies

Two different strategies were used. The anti-EBNA2 and anti-ZEBRA antibodies are both of the IgG1 class, whereas the anti-LMP1 antibody S12 is an IgG2a class antibody, allowing simultaneous incubation with both EBNA2 and LMP1 or ZEBRA and LMP1. The second incubation was performed with a mixture of anti-IgG1-alkaline phosphatase and anti-IgG2a-horseradish peroxidase or anti-IgG1-horseradish peroxidase and anti-IgG2a-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). The antibodies were visualized first by the diaminobenzidine/H_2O_2 method and second by adding fast blue.

As both EBNA2 and ZEBRA are IgG1 mouse antibodies, a different method had to be used. Slides were incubated first with EBNA2, followed by anti-IgG1-AP and visualized with naphthol AS-MX phosphate/fast blue BB/0.2 mol/L Tris-HCl, pH 8.5 (Sigma Chemical Co., St. Louis, MO). Endogenous alkaline phosphatase activity was first blocked by adding 1 mmol/L levamisol (Sigma) to the reaction mixture. Subsequently, slides were incubated with anti-ZEBRA, followed by a biotin-labeled anti-IgG1 antibody. After incubation with streptavidin-horseradish peroxidase, antibodies were visualized with aminoethylcarbazole (Sigma). An identical procedure was performed *vice versa*, with first anti-ZEBRA and secondly anti-EBNA2. Apart from the EBV-negative

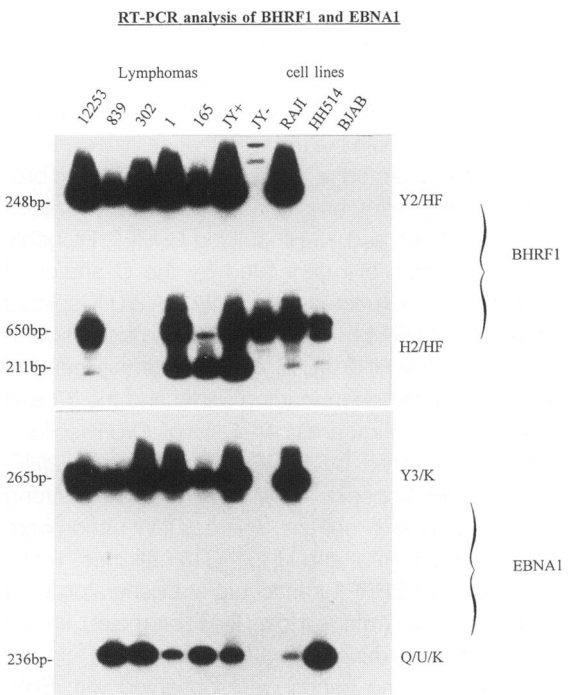


Figure 2. *RT-PCR analysis of BHRF1- and EBNA1-specific transcripts. Results are shown of Southern blot analysis with internal oligonucleotide probes for BHRF1-specific transcripts* (panels 1 *and* 2) *and EBNA1-specific transcripts* (panels 3 *and* 4). *Amplification of Y2/HF-spliced BHRF1 transcripts generates a 248-bp fragment* (panel 1) *and a 211-bp fragment is detected when H2/HF-spliced transcripts are present* (panel 2). *The 650-bp band detected in some cases with the H2/H3 primer set is probably a result of co-amplification of DNA, as identical bands are detected when the reverse transcriptase is omitted (JY− lane). Amplification of Y3/U/K-spliced EBNA1 generates a 265-bp fragment* (panel 3) *and a 236-bp fragment is detected when Q/U/K-spliced EBNA1 transcripts are present* (panel 4). *As control for specific RNA amplification, in every panel JY RNA was analyzed by PCR while no reverse transcriptase was included (JY− lane).*

Table 4. *Percentages of Cells in PT-LPDs and Cell Lines Displaying the Different EBV Expression Patterns*

EBV genes expressed	PT-LPDs,* mean (range)	JY	RAJI	HH514C16	EBV expression pattern resembling
EBER1/2, EBNA1	60 (5–90)	<10	<10	>90	Latency 1
EBER1/2, EBNA1, LMP1	10 (5–20)	0	0	<5	Latency 2
EBER1/2, EBNA1, EBNA2 and LMP1	<1	80–90	80–90	0	Latency 3
EBER1/2, EBNA1, EBNA2 and ZEBRA[2]	<5	<5	0	0	Lytic
EBER1/2, EBNA1, EBNA2	5 (5–15)	<10	<10	0	

*Given are the means of percentages of positive cells in three PT-LPDs (cases 2, 3, and 5). The means do not add up to 100% because EBNA1 staining was not observed in 100% of EBER1/2-positive cells in all cases.

cell line BJAB, negative controls consisted of simultaneous processed slides with omission of the first and second specific antibody, respectively. Semiquantative estimation of positive staining cells was scored as follows: less than 5%, 10%, 20%, 30%, and so forth.

Results

Cell Lines

Analysis of EBV Expression at mRNA Level

Results are summarized in Table 3. In the JY, RAJI, and HH514C16 cell lines, strong RT-PCR signals were detected analyzing BARF0, LMP1, LMP2A, and ZEBRA transcription. In both the JY and RAJI cell lines, strong bands were detected at the gel level by ethidium bromide staining after amplification for Y3/U/K-spliced EBNA1, EBNA2, and Y2/HF-spliced BHRF1. Probably these transcripts are generated by the C and/or W promoter.[9] As expected, no EBNA2, Y3/U/K-spliced EBNA1 or Y2/HF-spliced BHRF1-specific signals were detected in the HH514C16 cell line, as this cell line lacks the EBNA2 open reading frame and the Y2 and Y3 exons (see Figure 1). Analysis of Q/U/K-spliced EBNA1, generated by the F promoter and H2/HF-spliced BHRF1-specific transcripts revealed a strong signal only in the JY cell line, whereas faint bands were detected in the RAJI and HH514C16 cell lines. These H2/HF-spliced BHRF1 transcripts are generated by a unique promoter (referred to as the H promoter), known to be activated during the EBV lytic cycle.[27] In the BJAB cell line, no bands were observed with EBV-transcript-specific primers. The hybridization results obtained for BHRF1 and EBNA1 analysis are shown in Figure 2.

Analysis of EBV Expression at the Protein Level

Results are summarized in Tables 3 and 4. In both the JY and RAJI cell lines, co-expression of EBNA1, EBNA2, and LMP1 was observed in the large majority (80 to 90%) of cells (Figure 3A). In the JY cell line, expression of the lytic proteins ZEBRA, EA-D, and GP350/220 was observed in less than 5% of the cells. Double-staining procedures demonstrated that ZEBRA-positive cells were never LMP1 positive (Figure 3B) but frequently, if not always, EBNA2 positive (data not shown). Although with RT-PCR a faint ZEBRA-positive band was observed, no expression of ZEBRA, EA-D, or GP350/220 was detected by immunohistochemistry in the RAJI cell line. In the HH514C16 cell line, all cells were shown to express EBNA1. As expected, EBNA2 was never detected in the HH514C16 cell line, because of the deletion spanning the EBNA2 open reading frame (Figure 1). Expression of ZEBRA, EA-D, and LMP1 was restricted to less than 5% of the cells. Expression of GP350/220 was not found. The BJAB cell line remained negative for all EBV-specific antibodies tested.

PT-LPDs

Histology

Tumor characteristics are summarized in Table 1. All five PT-LPDs consisted of a diffuse polymorphous

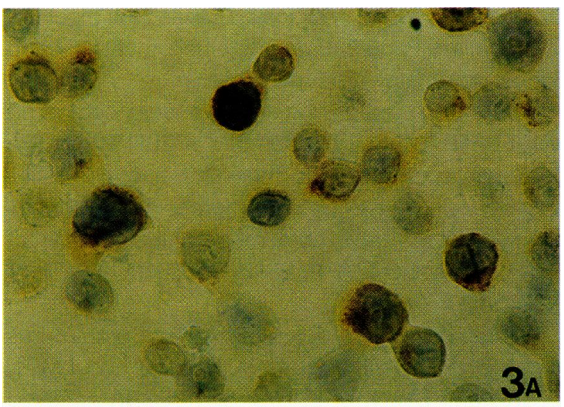
Figure 3. *JY cell line.* A: *Double staining for EBNA2 and LMP1 (S12). Blue nuclear staining indicates expression of EBNA2. Brown staining indicates LMP1 expression. Double-positive staining is detected in 80 to 90% of the cells. Original magnification, ×600.* B: *Double staining for ZEBRA and LMP1 (S12). Blue nuclear staining indicates expression of ZEBRA. Brown staining indicates LMP1 expression. Double-positive cells are not observed. Original magnification, ×600.*

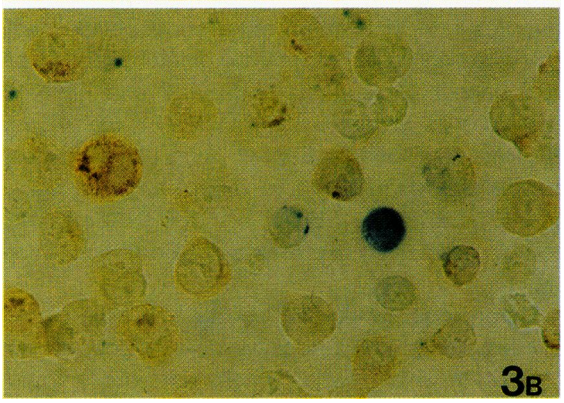
Figure 4. *PT-LPD case 5; EBNA2/LMP1 (S12) double staining. Cells positive for both EBNA2 (brown nuclear staining) and LMP1 (blue cytoplasmic staining) are not observed. Original magnification, ×600.*

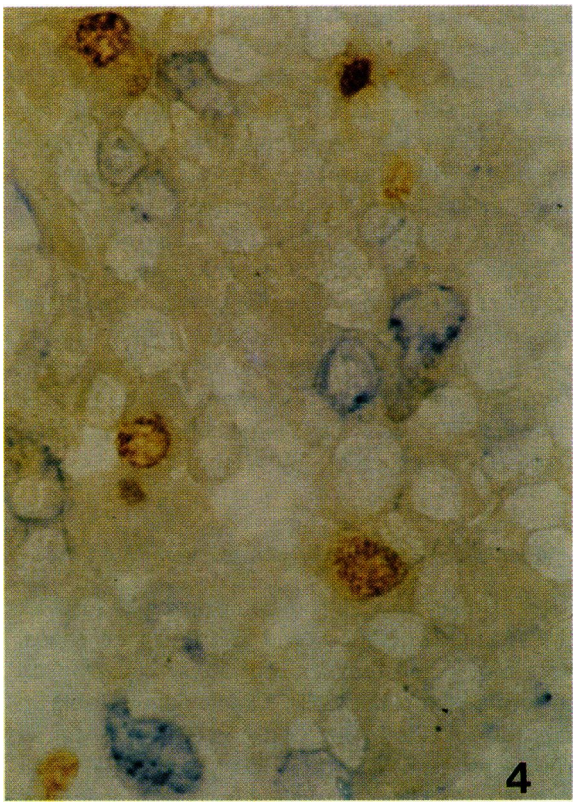
Figure 5. *a) PT-LPD case 2.* A: *CD30-positive staining is observed in large anaplastic cells. Original magnification, ×600.* B: *In similar cells, expression of LMP1 was detected. Hematoxylin counterstaining; original magnification, ×600.*

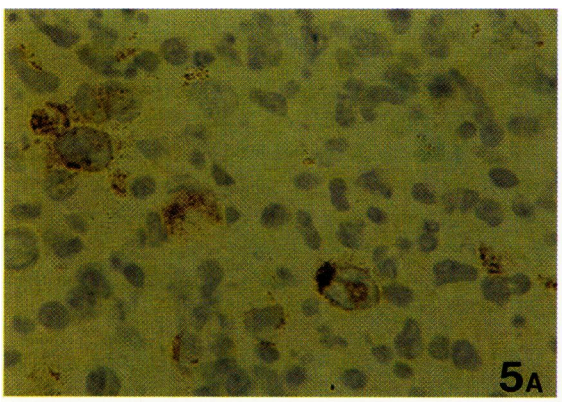
Figure 6. *PT-LPD case 5; EBNA2/ZEBRA double staining. Brown staining indicates EBNA2 expression. Blue staining indicates ZEBRA expression. Some of the EBNA2-positive cells also express ZEBRA* (arrowheads). *Original magnification, ×400.*

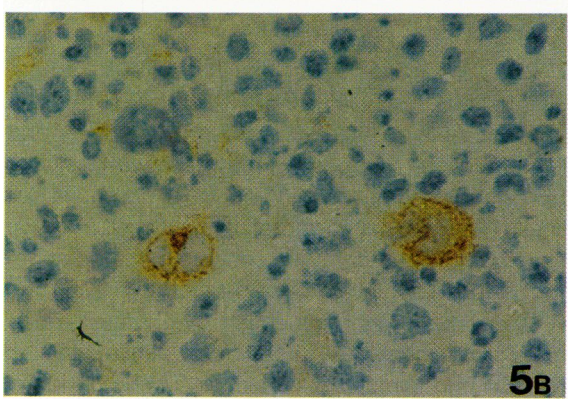
Figure 7. *PT-LPD case 3. A diffuse positive staining for EBNA1 is detected. Hematoxylin counterstaining; original magnification, ×600.*


3A

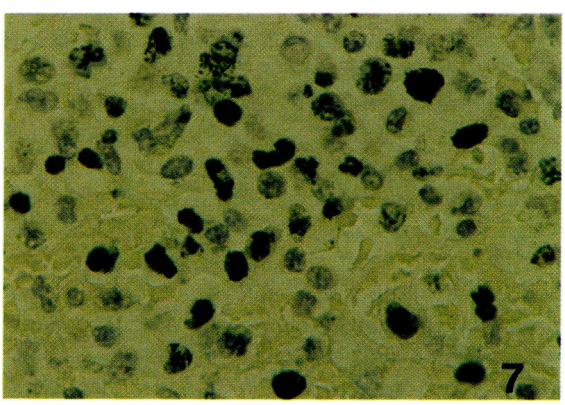

3B


4


5A


5B


6


7

population of centroblast- and immunoblast-like cells, and smaller plasmacytoid cells with a variable number of CD30-positive anaplastic cells (see Figure 5A). These PT-LPDs are difficult to classify according to the Kiel classification (and the Working Formulation) because of their marked polymorphism, which is usually not seen in B-cell lymphomas arising in immunocompetent patients.[33] Three of the five PT-LPDs were monoclonal proliferations as proven by immunohistochemical detection of a restricted immunoglobulin light chain expression in two cases and by Southern blot analysis in the third case. With the EBER1/2 RISH assay, all cases stained positive in at least 70% of small and large cells, indicating the ubiquitous presence of EBV in PT-LPDs.

Analysis of EBV Expression at the mRNA Level

RT-PCR analysis revealed transcription of EBNA2, LMP1, LMP2A, BARF0, and ZEBRA in all cases. Moreover, dual promoter usage was detected by analyzing EBNA1 and BHRF1 transcription (Table 3). In all tested cases, Y3/U/K-spliced EBNA1 as well as Y2/HF-spliced BHRF1-specific transcripts were detected, suggesting that both genes were transcribed by the C or W promoter. In four cases, Q/U/K-spliced EBNA1, generated by the F promoter, and in three cases, H2/HF-spliced BHRF1-specific transcripts, generated by the H promoter, were also found. Thus, by RT-PCR analysis, transcription of all investigated latent as well as immediate early and early genes was detected.

Analysis of EBV Expression at the Protein Level

By immunohistochemistry, EBNA1, EBNA2, ZEBRA, EA-D, and LMP1 staining was found in all tested cases. GP350/220 expression was detected in only two of four tested cases (Table 3).

In two cases, diffuse positive staining for EBNA1 was found, comparable to the EBER1/2 staining pattern (Table 3; cases 3 and 4, Figure 7). In one case, focal expression of EBNA1 was detected in approximately 50% of EBER1/2-positive cells (Table 3; case 5). In the fourth case tested, only scattered EBNA1-positive cells were observed (Table 3, case 2). In all tested cases, the number of EBNA2-positive cells was found to be between 10 and 20% of the neoplastic cells. Expression of ZEBRA was usually restricted to less than 5% of the cells, except in case 5 in which approximately 10% of the cells were positive. LMP1 expression ranged from less than 5% to approximately 20% of the neoplastic cells. EA-D expression was found in all tested cases in at most 5% of the cells. GP350/220 was detected in cases 4 and 5 in approximately 10% of the cells.

When these morphological data were compared with EBER1/2 RISH, the number of EBNA1-positive cells was comparable to the number of EBER1/2-positive cells in two cases, whereas the EBER1/2-positive cells always outnumbered the number of cells expressing LMP1, EBNA2, ZEBRA, EA-D, or GP350/220.

In three cases tested, double-staining procedures for either EBER1/2 and LMP1, EBNA2 and LMP1, ZEBRA and LMP1, or EBNA2 and ZEBRA revealed the presence of at least three subpopulations of EBV-harboring cells, expressing different subsets of EBV genes (Table 4): (1) EBER1/2- and EBNA1-positive cells without staining for LMP1, EBNA2, or ZEBRA; (2) EBER1/2-, EBNA1-, and LMP1-positive but EBNA2- (Figure 4) and ZEBRA-negative cells; and (3) EBER1/2-, EBNA1-, and EBNA2-positive cells sometimes co-expressing ZEBRA (Figure 6) or LMP1.

Morphologically the LMP1-positive cells were usually larger than LMP1-negative cells, except in one case (4) in which few large cells were present and LMP1 was detected only in a highly restricted number of small cells. Although for technical reasons double stainings with anti-EBNA1 could not be performed, the expression of EBNA1 in the large majority of neoplastic cells in two cases suggests that EBNA1 is expressed in all cells, including the LMP1- or EBNA2-positive cells.

Thus, in the three tested PT-LPDs, cells expressing the latency type 3 pattern were rarely found. However, it appeared that cells expressing a restricted latency pattern, resembling type 1 or 2 latency were intermingled with cells expressing immediate early and/or late EBV antigens.

Discussion

Using RT-PCR, we have shown that EBNA1, EBNA2, LMP1, LMP2A, BARF0, BHRF1, and ZEBRA were transcribed in all tested PT-LPDs and in the JY and RAJI cell lines, suggesting the presence of similar EBV expression patterns in both PT-LPDs and in cell lines. However, using morphological methods, we have demonstrated that, in contrast to the homogeneous EBV expression pattern found in the JY and RAJI cell lines, heterogeneous EBV expression patterns are present in PT-LPDs. Using double staining of RISH and immunohistochemistry, we could demonstrate cells expressing either latency type 1 or

latency type 2 as well as cells expressing EBV lytic genes within individual cases. Thus, in contrast to EBV-positive cell lines, cells expressing latency type 3 were rarely observed. Therefore, the results obtained by RT-PCR, in fact, reflect a mixture of cells expressing either type 1 or type 2 latency and cells expressing EBV lytic genes. These data are in agreement with previous reports showing that, in lymphomas expressing the latency type 3 pattern, not all morphologically identifiable tumor cells express either EBNA2 or LMP1 and that activation of the virus lytic cycle frequently occurs in a small minority of cells.[2,5,20,34]

RT-PCR analysis revealed dual promoter usage in both cell lines and PT-LPDs. It was shown that, in the RAJI cell line, only EBNA1 and BHRF1 transcripts derived from the C or W promoter were present, whereas EBNA1- and BHRF1-specific transcripts generated by both the C or W promoter and the F and H promoter, respectively, were detected in the JY cell line, also expressing lytic genes in a small minority of cells. This is in agreement with a previous report demonstrating activation of the F promoter just after expression of the immediate early protein, ZEBRA.[27] The fact that this dual promoter usage for EBNA1 and BHRF1 was also observed in the five tested PT-LPDs suggests that, analogous to the JY cell line, part of the EBV-harboring cells are strictly latent, with EBNA1 and BHRF1 generated by the C or W promoter, and part of the cells have entered the lytic cycle with EBNA1 and BHRF1 generated by the F and H promoter, respectively. Activation of the C promoter in all five and W promoter in three PT-LPDs was confirmed by RT-PCR analysis with primers from appropriate C and W exons[35] (data not shown). However, this does not exclude the possibility that EBNA1 and EBNA2 as well as BHRF1 can also be generated by other, yet unidentified promoters.

In cell lines, double-staining procedures revealed the presence of a latency type 3 expression pattern in nearly all cells, intermingled with sporadic LMP1-negative, ZEBRA-positive cells. In contrast, in the PT-LPDs, EBNA2-expressing cells were rarely LMP1 positive but were sometimes found to co-express ZEBRA. As ZEBRA is the first protein to be expressed in lytic cells,[36] co-expression of EBNA2 and ZEBRA could indicate that EBNA2 expression may have been present at the time ZEBRA expression was turned on or that EBNA2 is also expressed during the EBV lytic cycle. This latter possibility would be consistent with *in vitro* data demonstrating the expression of EBNA2 early in the virus lytic cycle.[9] Interestingly, co-expression of LMP1 and ZEBRA was never observed in EBV-harboring cell lines or in PT-LPDs, indicating that LMP1 is not consistently expressed during the EBV lytic cycle.

In the majority of EBER1/2-positive cells in the PT-LPDs, no expression of either EBNA2 or LMP1 was detected. These immunohistochemical data suggest that these cells display a latency type 1 expression pattern. Consequently, strong Q/U/K-spliced EBNA1-specific RT-PCR signals would be expected because, in latency type 1, EBNA1 is generated by the F promoter.[21,22,25] However, stronger signals were obtained when C or W promoter-derived transcripts (EBNA1- as well as BHRF1- and EBNA2-specific transcripts) were analyzed, suggesting a latency type 3 pattern in the majority of cells. This discrepancy might be explained by two reports showing that, in latently EBV-infected normal B lymphocytes, the C promoter is activated with expression of EBNA1 but without expression of EBNA2.[35,37] Thus, a latency-type-1-like expression pattern can also be associated with C promoter usage. To determine whether in these cells EBV gene expression is indeed restricted to latency type 1 or whether EBNA2 expression is restricted to a level below the sensitivity of the antibodies used requires additional investigation.

In lymphomas induced by injection of EBV-positive lymphoblastoid cell line cells in SCID mice, a model that has been shown to be remarkably similar to human PT-LPDs,[38,39] a similar restricted expression of EBNA2 and LMP1 was observed.[39] Reduced expression of viral antigens was related to plasmacytoid differentiation and withdrawal from the cell cycle.[39] Analogous to these data, the smaller, only EBER1/2- and EBNA1-positive cells in our group of PT-LPDs could reflect resting, terminally differentiated EBV-harboring cells.

The latency type 2 expression pattern was mainly detected in large anaplastic cells (Figure 5B), also expressing the CD30 activation marker (Figure 5A). Comparable large anaplastic CD30-positive cells (Reed-Sternberg and Hodgkin cells) present in EBV-positive cases of Hodgkin's disease display a similar EBV latency type 2 expression pattern.[22,40] Interestingly, LMP1-positive, EBNA2-negative cells could also be transiently induced *in vitro* in Burkitt's lymphoma-derived cell lines, normally expressing latency type 1, that were stimulated to enter the lytic cycle.[24] Thus, the observed heterogeneous EBV expression patterns detected in our group of PT-LPDs could also reflect a dynamic process in which cells drift between different latency stages with eventual progression to the lytic cycle. Alternatively, the heterogeneous expression patterns could be explained by the co-existence of several EBV clones. In our

group of PT-LPDs this could not be excluded, as we did not perform analysis of EBV clonality. However, this seems unlikely as it was described recently that in the majority of PT-LPDs EBV involvement is monoclonal.[6,7]

We conclude that, in contrast to EBV-transformed lymphoblastoid cell lines and Burkitt's lymphoma cell lines expressing type 3 latency, the EBV expression patterns detected by RT-PCR in PT-LPDs is a mixture of multiple EBV-harboring subpopulations, expressing different subsets of EBV-encoded proteins. These data indicate that the operational definitions of EBV latencies *in vitro* cannot easily be applied to PT-LPDs but that a continuum of different EBV latency expression patterns can be detected at the single-cell level in these lymphomas, with progression to the virus lytic cycle in a minority of cells.

References

1. Thomas JA, Allday MJ, Crawford DH: Epstein-Barr virus-associated lymphoproliferative disorders in immunocompromised individuals. Adv Cancer Res 1991, 57:329–380
2. Hamilton-Dutoit SJ, Rea D, Raphael M, Sandvej K, Delecluse HJ, Gisselbrecht C, Marelle L, van-Krieken HJ, Pallesen G: Epstein-Barr virus-latent gene expression and tumor cell phenotype in acquired immunodeficiency syndrome-related non-Hodgkin's lymphoma: correlation of lymphoma phenotype with three distinct patterns of viral latency. Am J Pathol 1993, 143:1072–1085
3. Knowles DM, Dalla-Favera R: AIDS-associated malignant lymphoma. Textbook of AIDS Medicine. Edited by S Broder, T Merigan, D Bolognesi. Baltimore, Williams and Wilkins, 1994, pp 431–464
4. Young L, Alfieri C, Hennessy K, Evans H, O'Hara C, Anderson KC, Ritz J, Shapiro RS, Rickinson AB, Kieff E, Cohen JI: Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. N Engl J Med 1992, 321: 1080–1085
5. Rea D, Fourcade C, Leblond V, Rowe M, Joab I, Edelman L, Bitker M, Gandjbakhch I, Suberbielle C, Farcet J, Raphael M: Patterns of Epstein-Barr virus latent and replicative gene expression in Epstein-Barr virus B cell lymphoproliferative disorders after organ transplantation. Transplantation 1994, 58:317–324
6. Kaplan MA, Ferry JA, Harris NL, Jacobson JO: Clonal analysis of posttransplant lymphoproliferative disorders, using both episomal Epstein-Barr virus and immunoglobulin genes as markers. Am J Clin Pathol 1994, 101:590–596
7. Knowles DM, Cesarman E, Chadburn A, Frizzera G, Chen J, Rose EA, Michler RE: Correlative morphologic and molecular genetic analysis demonstrates three distinct categories of posttransplantation lymphoproliferative disorders. Blood 1995, 85:552–565
8. Howe JG, Steitz, JA: Localization of Epstein-Barr virus-encoded small RNAs by *in situ* hybridization. Proc Natl Acad Sci USA 1986, 83:9006–9010
9. Middleton T, Gahn TA, Martin JM, Sugden B: Immortalizing genes of Epstein-Barr virus. Adv Virus Res 1991, 40:19–55
10. Kaye KM, Izumi KM, Kieff E: Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc Natl Acad Sci USA 1993, 90:9150–9154
11. Miyazaki I, Cheung RK, Dosch HM: Viral interleukin-10 is critical for the induction of B-cell growth transformation by Epstein-Barr virus. J Exp Med 1993, 178:439–447
12. Henderson S, Huen D, Rowe M, Dawson C, Johnson G, Rickinson A: Epstein-Barr virus-coded BHRF1 protein, a viral homologue of *bcl*-2, protects human B-cells from programmed cell death. Proc Natl Acad Sci USA 1993, 90:8479–8483
13. Miller G: Epstein-Barr virus: biology, pathogenesis, and medical aspects. Virology. Edited by BN Fields, DM Knipe. New York, Raven Press, 1990, pp 1921–1958
14. Rowe M, Rowe DT, Gregory CD, Young LS, Farrell PJ, Rupani H, Rickinson AB: Differences in B-cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma. EMBO J 1987, 6:2743–2751
15. Brooks LA, Lear AL, Young LS, Rickinson AB: Transcripts from the Epstein-Barr virus *Bam*HI A fragment are detectable in all three forms of virus latency. J Virol 1993, 67:3182–3190
16. Speck SH, Strominger JL: Transcription of Epstein-Barr virus in latently infected, growth-transformed lymphocytes. Adv Viral Oncol 1989, 8:133–150
17. Gregory CD, Rowe M, Rickinson AB: Different Epstein-Barr-virus (EBV) B-cell interactions in phenotypically distinct clones of a Burkitt lymphoma cell line. J Gen Virol 1990, 71:1481–1495
18. Gratama JW, Zutter MM, Minarovits J, Oosterveer MA, Thomas ED, Klein G, Ernberg I: Expression of Epstein-Barr virus-encoded growth-transformation-associated proteins in lymphoproliferations of bone-marrow transplant recipients. Int J Cancer 1991, 47:188–192
19. Thomas JA, Hotchin, NA, Allday MJ, Amiot P, Rose M, Jacoub M, Crawford DH: Immunohistology of Epstein-Barr virus-associated antigens in B-cell disorders from immunocompromised individuals. Transplantation 1990, 49:944–953
20. Rea D, Delecluse HJ, Hamilton-Dutoit SJ, Marelle L, Joab I, Edelman L, Finet JF, Raphael M: Epstein-Barr virus latent and replicative gene expression in posttransplant lymphoproliferative disorders and AIDS-related non-Hodgkin's lymphomas. Ann Oncol 1994, 5(suppl 1):S113-S116
21. Brooks L, Yao QY, Rickinson AB, Young LS: Epstein-Barr virus latent gene transcription in nasopharyngeal

carcinoma cells: co-expression of EBNA1, LMP1, and LMP2 transcripts. J Virol 1992, 66:2689–2697
22. Deacon EM, Pallesen G, Nieboditek G, Crocker J, Brooks L, Rickinson AB, Young LS: Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. J Exp Med 1993, 177: 339–349
23. Chen C, Sadler RH, Walling DM, Su IH, Hsieh H, Raab-Traub N: Epstein-Barr virus (EBV) gene expression in EBV-positive peripheral T-cell lymphomas. J Virol 1993, 67:6303–6308
24. Rowe M, Lear AL, Croom-Carter D, Davies AH, Rickinson AB: Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. J Virol 1992, 66:122–131
25. Sample J, Brooks L, Sample C, Young L, Rowe M, Gregory C, Rickinson AB, Kieff E: Restricted Epstein-Barr virus protein expression in Burkitt lymphoma is due to a different Epstein-Barr nuclear antigen 1 transcriptional initiation site. Proc Natl Acad Sci USA 1991, 88:6343–6347
26. Schaefer BC, Woisetschlaeger M, Strominger JL, Speck S: Exclusive expression of Epstein-Barr virus nuclear antigen 1 in Burkitt lymphoma arises from a third promoter, distinct from the promoter used in latently infected lymphocytes. Proc Natl Acad Sci USA 1991, 88:6550–6554
27. Lear AL, Rowe M, Kurilla MG, Lee S, Henderson S, Kieff E, Rickinson AB: The Epstein-Barr virus (EBV) nuclear antigen-1 *Bam*HI F promoter is activated on entry of EBV-transformed B cells into the lytic cycle. J Virol 1992, 66:7461–7468
28. Austin PJ, Flemington E, Yandawa CN, Strominger JL, Speck SH: Complex transcription of the Epstein-Barr virus *Bam*HI fragment H rightward open reading frame 1 (BHRF1) in latently and lytically infected B lymphocytes. Proc Natl Acad Sci USA 1988, 85:3678–3682
29. Oudejans JJ, van den Brule AJC, Jiwa NM, de Bruin PC, Ossenkoppele GJ, van der Valk P, Walboomers JMM, Meijer CJLM: BHRF1, the Epstein-Barr virus (EBV) homologue of the *bcl-2* (proto-)oncogene is transcribed in EBV associated B-cell lymphomas and in reactive lymphocytes. Blood 1995, 86:1893–1902
30. Jiwa NM, Oudejans JJ, Dukers DF, Vos W, Horstman A, van der Valk P, Middeldorp JM, Kluin PH, Walboomers JMM, Meijer CJLM: Immunohistochemical demonstration of different latent membrane protein-1 epitopes of Epstein-Barr virus in lymphoproliferative diseases. J Clin Pathol, 1995, 48:438–442
31. Grässer FA, Murray PG, Kremmer E, Klein K, Remberger K, Feiden W, Reynolds G, Niedobitek G, Young LS, Mueller-Lantzsch N: Monoclonal antibodies directed against the Epstein-Barr virus encoded nuclear antigen 1 (EBNA1): immunohistologic detection of EBNA1 in the malignant cells of Hodgkin's disease. Blood 1994, 84:3792–3798
32. Jiwa NM, Kanavaros P, De Bruin PC, van der Valk P, Horstman A, Vos W, Mullink H, Walboomers JMM, Meijer CJLM: Presence of Epstein-Barr virus harbouring small and intermediate-sized cells in Hodgkin's disease: is there a relationship with Reed-Sternberg cells? J Pathol 1993, 170:129–136
33. Hamilton-Dutoit SJ, Pallesen G, Franzmann MB, Karkov J, Black F, Skinhøj P, Pedersen C: AIDS-related lymphoma: histopathology, immunophenotype, and association with Epstein-Barr virus as demonstrated by *in situ* nucleic acid hybridization. Am J Pathol 1991, 138:149–163
34. Pallesen GP, Hamilton-Dutoit SJ, Rowe M, Lisse I, Ralfkiaer E, Sandvej K, Young LS: Expression of Epstein-Barr virus replicative proteins in AIDS-related non-Hodgkin's lymphoma cells. J Pathol 1991, 165: 289–299
35. Qu L, Rowe D: Epstein-Barr virus latent gene expression in uncultured peripheral blood lymphocytes. J Virol 1992, 66:3715–3724
36. Rooney CM, Rowe DT, Ragot T, Farrel PJ: The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J Virol 1989, 63:3109–3116
37. Chen F, Zou J, di Renzo L, Winberg G, Hu L, Klein E, Klein G, Ernberg I: A subpopulation of normal B cells latently infected with Epstein-Barr virus resembling Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP-1. J Virol 1995, 69:3752–3758
38. Pisa P, Cannon MJ, Pisa EK, Cooper NR, Fox RI: Epstein-Barr virus induced lymphoproliferative tumors in severe combined immunodeficient mice are oligoclonal. Blood 1992, 79:173–179
39. Rochford R, Hobbs MV, Garnier J, Cooper NR, Cannon M: Plasmacytoid differentiation of Epstein-Barr virus-transformed B-cells *in vivo* is associated with reduced expression of viral latent genes. Proc Natl Acad Sci USA 1993, 90:352–356
40. Joske DJL, Emery-Goodman A, Bachmann E, Bachmann F, Odermatt B, Knecht H: Epstein-Barr virus burden in Hodgkin's disease is related to membrane protein gene expression but not to active viral replication. Blood 1992, 80:2610–2613