Dual EBNA1 Promoter Usage by Epstein-Barr Virus in Human B-Cell Lines Expressing Unique Intermediate Cellular Phenotypes

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The use of different viral promoters for the expression of the EBNA1 gene product appears to be a critical step in the regulation of Epstein-Barr virus latent gene expression and may reflect the extent of differentiation of B-cell hosts. Low-passage Burkitt lymphoma cell lines resemble immature B cells in that they express CD10 (CALLA) and do not express B-cell activation antigens. In these cells, transcription from a promoter located in the *Bam*HI F fragment of the viral genome results in the exclusive expression of EBNA1, referred to as the latency I pattern of viral gene expression. In contrast, high-passage Burkitt lymphoma cells and lymphoblastoid cell lines resemble activated B cells in that they do not express CD10 but do express activation antigens such as CD23. In these cells, the use of two promoters located in the *Bam*HI W and C fragments of the viral genome leads to the expression of all six EBNA gene products (latency III). We have found that four human B-cell lines, DB, LBW2, LBW14, and Josh 7, stably express a pattern of B-cell differentiation antigens intermediate between those found in latency I and latency III cell lines and characterized by the coexpression of CD10 and CD23. The pattern of EBNA1 promoter usage in these cell lines was examined to determine whether their intermediate cellular phenotype was reflected in their patterns of viral gene expression. DB, LBW2, and LBW14 utilize both the *Bam*HI F promoter region and *Bam*HI W promoter region to transcribe the EBNA1 gene. This stable pattern of mixed promoter usage for the expression of the EBNA gene products in B cells has not previously been described. In addition, these three B-cell lines expressed lower levels of the viral latent gene product EBNA2 than those typically observed in latency III cells. The lower levels of activation of viral and cellular promoters known to be regulated by EBNA2 also correlated with the reduced levels of EBNA2 expression in these cells. These included the viral LMP1 and LMP2A promoters and the cellular CD23B promoter. The fourth B-cell line, Josh 7, expressed EBNA1 mRNAs derived from both the *Bam*HI W promoter and *Bam*HI C promoter, similar to latency III cells. The intermediate cellular phenotype in Josh 7 cells appeared to be due, in part, to a deficiency in the expression of viral LMP1. These results show that the B-cell lines express unique intermediate patterns of expression of viral and cellular gene products and suggest that a continuum of latency states may exist for Epstein-Barr virus-infected B lymphocytes.

The ability of Epstein-Barr virus (EBV) to immortalize primary human B lymphocytes and its strong association with several human malignancies have stimulated intensive study on the nature of the EBV-host cell interaction. Two patterns of latent EBV infection in human B cells which reflect differences in both the pattern of viral gene expression and the B-cell phenotype have been described. Freshly isolated EBV-positive Burkitt lymphoma (BL) tumors express surface markers, such as CD10, that are typical of immature B cells. In culture, these tumor cells are generally spherical and grow as a single cell carpet. These cells express a single viral nuclear antigen, EBNA1, which typifies the latency I pattern of viral gene expression.

EBV-induced immortalization of primary human B cells leads to the outgrowth of lymphoblastoid cell lines (LCLs) which display a latency III pattern, characterized by the expression of all nine viral latent gene products. These include the nuclear antigens EBNA1, -2, -3a, -3b, and -3c, the leader protein EBNA4, and latent membrane protein 1 (LMP1), LMP2A, and LMP2B (reviewed in reference 18). Often, BL

cell lines evolve to a latency III pattern of gene expression during passage in culture (37). Viral latent gene expression induces changes in B-cell phenotype which are consistent with the phenotype of activated B cells. Specifically, EBNA2 and LMP1 act to upregulate B-cell activation antigens, such as CD23, and cellular adhesion molecules, like ICAM-1 and LFA-1 (48, 51). Furthermore, the expression of CD10 is significantly downregulated (51). Unlike BL cell lines, LCLs are usually pleiomorphic and generally grow in large clumps rather than single-cell suspensions.

EBV exhibits a dual cellular tropism, infecting epithelial cells in addition to lymphoid cells. EBV-infected epithelial cells, epitomized by nasopharyngeal carcinoma (NPC) tumor cells, display a pattern of viral gene expression designated latency II. This is characterized by the restricted expression of EBNA1 in the absence of EBNA2 to -4 and the variable expression of LMP1 and LMP2 (2).

A key point in the regulation of viral latent gene products appears to be the promoter used in transcription of the EBNA1 gene. In latency I BL cell lines and latency II NPC cell lines, EBNA1 gene transcription occurs from a promoter located in the *Bam*HI F fragment of the viral genome. The activation of this promoter alone leads to the exclusive expression of EBNA1 (38, 44). In contrast, in LCLs and latency III BLs, transcription from promoters in the *Bam*HI W or C

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TABLE 1. Characteristics of the round B-cell lines

Cell line	Source ^a	EBV gene expression			Immuno- globulin ^b	Surface antigen expression ^c					Karyotype
		EBV type ^d	EBER ^e	BZLF1 ^e		CD3	CD5	CD19	CD20	Class II	
DB	Normal PBL	1	+	±	μV _H P LC ⁻	-	-	+	+	+	46, XY, -4, -8, -19, t(9;14) t(4;19), + iso(8q), + iso(8q)
LBW2	CVID 1 PBL	1	+	±	μV _H P LC ⁻	-	-	+	+	+	46, XY, -4, -8, -8, -19, t(9;14) t(4;19), + iso(8q), + iso(8q)
LBW14	CVID 2 PBL	1	+	±	μV _H P LC ⁻	-	-	+	+	+	46, XY, -4, -8, -8, -19, t(9;14) t(4;19), + iso(8q)
Josh 7	XLA BML	1	+	±	Ig ⁻	-	-	+	+	+	48, XY, -4, -8, +7, t(4;?), t(9;14), (9;17), +der(21), +iso (8q)

^a BML, bone marrow lymphocytes; CVID, combined variable immunodeficiency; XLA, X-linked agammaglobulinemia.

^b μV_HP, truncated immunoglobulin M μ-chain expression (1); LC⁻, immunoglobulin light chain negative; Ig⁻, immunoglobulin negative.

^c Determined by flow cytometry. Class II, major histocompatibility complex class II.

^d Determined by PCR.

^e Determined by Northern analysis.

fragments results in the expression of EBNA1 to -4 through differential splicing (45). The activities of the W and C promoters are often mutually exclusive (55). Transcription from the W promoter, utilized during the initial stages of EBV infection of primary B cells, is downregulated in most established LCLs in favor of the dominant C promoter (56). EBNA2 has been strongly implicated in the activation of the C promoter (34, 47, 54). In addition, EBNA2 has been shown to activate the viral LMP1 and LMP2 promoters as well as the cellular promoter for CD23 (49, 51, 52, 58).

We have characterized several unique EBV-harboring human B-cell lines generated in vitro from the peripheral blood lymphocytes (PBLs) or bone marrow of individuals without apparent lymphoid malignancy. Unlike typical LCLs, these cells are spherical in shape and have been referred to as round B-cell lines (8). The generation of these B-cell lines correlated with the use of cells from patients with some degree of B-cell cytopenia, although one round B-cell line was isolated from a healthy immunocompetent individual. These cell lines are more similar to BL cell lines than to LCLs because they grow as single cells or small aggregates and express CD10. They do, however, express moderate levels of CD23. Because of their unique B-cell phenotype, we analyzed these B-cell lines for their pattern of viral latent gene expression. In this report, we show that they express unusual patterns of EBV latent gene expression which are intermediate between latency I and latency III. Furthermore, the round B-cell lines display a novel pattern of promoter use for the expression of the EBNA1 gene, characterized by stable activation of both the F and W latent viral promoters. This dual promoter usage has been observed previously only as a transient feature of B-cell hosts infected with EBV. These results provide evidence that a continuum of latency states exists for EBV-infected human B lymphocytes that may be related to their stage of differentiation and activation.

MATERIALS AND METHODS

Cell lines and culture conditions. The round B-cell lines used in this study have been stably maintained as clonal populations for at least 12 years. LBW2 and LBW14 were derived by culturing PBLs from separate individuals with combined variable immunodeficiency in the presence of an unspecified strain of EBV (12, 13). DB was generated under identical conditions with PBLs from a healthy individual (13).

The Josh 7 cell line was isolated in a separate laboratory (8); it arose spontaneously in a culture of bone marrow lymphocytes from a patient with X-linked agammaglobulinemia without the addition of exogenous EBV. Unlike typical LCLs, these cells are spherical and grow in culture at a high rate as single cells or small aggregates. Additional characteristics of these cell lines that have been previously determined in published (1, 30) or unpublished experiments are summarized in Table 1. The presence of the B-lineage-associated cell surface markers CD19 and CD20 and the existence of gene rearrangements for both immunoglobulin heavy- and light-chain loci attest to their B-lymphoid origin (Table 1) (30). In addition, it has been established that each of the round B-cell lines are clonally distinct by class II major histocompatibility complex (DRw) haplotype, cytogenetic analysis, and restriction fragment length polymorphism analysis of two polymorphic loci (1, 13, 30). Initial karyotype analysis of these four distinct B-cell lines has shown that they possess several novel cytogenetic abnormalities in common (Table 1; performed by Nagesh Rao, Wake Forest University Medical Genetics Unit).

While the round B-cell lines were known to be EBNA positive by immunofluorescent staining (8, 12), the nature of the infecting virus was ill defined, especially in the case of Josh 7 in which exogenous virus was not used in its generation. Two basic strains of EBV, types 1 and 2, exist worldwide. These two EBV strains contain significant sequence variations in their EBNA2 and EBNA3 genes, with EBV type 1 possessing a much higher efficiency for the immortalization of primary human B cells (the B95.8 virus is prototypical of the EBV type 1 strain) (4, 43). A PCR method described by Sixbey et al. (42), which distinguishes strain-specific differences in EBNA2 gene structure, was used to assign the EBV strain carried by each round B-cell line. All the round B-cell lines were found to carry only EBV type 1 EBNA2 gene sequences (Table 1). Furthermore, Southern analysis of the round B-cell lines with two separate probes specific for the B95.8 virus and the terminal regions of the viral genome has shown that these cell lines are clonally infected with a type 1 virus distinct from the B95.8 strain (data not shown) (31, 32). Additional experiments have shown that the round B-cell lines, like all the other EBV-infected cell lines described to date, express the viral transcripts which encode the EBER1 and EBER2 gene products (Table 1).

The LCLs used as controls in this work are typical latently infected LCLs and express EBV and cellular gene products in a manner consistent with published observations. LBW4, LBW25, and LBW26 were derived by EBV infection of juvenile bone marrow lymphocytes (LBW4) (17) or healthy adult PBLs (LBW25 and LBW26) (12). The LCL GMO2292 was obtained from the NIGMS Human Genetic Mutant Cell Repository and carries an HGPRT mutation. Other human B-cell lines used in this work were the EBNA2-deficient African BL cell line Daudi (ATCC CCL 213) and the EBV-negative B-lymphoma cell line BJAB (a gift of P. Cresswell, Duke University).

All cells were maintained, unless otherwise stated, in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, and 100 U of penicillin-streptomycin per ml.

Immunofluorescent staining. Cell samples analyzed by either direct or indirect immunofluorescent staining were prepared as described by Gathings et al. (9). Briefly, 10^6 cells were washed twice in cold wash buffer containing 5% fetal bovine serum and 0.1% sodium azide and incubated on ice for 30 min with individual monoclonal antibodies at a final concentration of 100 μ g/ml. Antibodies against CD11a (LFA-1) and CD54 (ICAM-1) were obtained from AMAC Inc., Westbrook, Maine. Antibodies against CD23 or phycoerythrin-conjugated CD23 and against CD10 (CALLA) or fluorescein isothiocyanate-conjugated CD10 were obtained from Becton Dickinson, San Jose, Calif. For indirect immunofluorescent labeling experiments, similar incubations were performed with the addition of a phycoerythrin-conjugated secondary anti-mouse immunoglobulin G antibody (SBA, Inc., Birmingham, Ala.) at a final concentration of 50 μ g/ml. Stained cells were fixed in 1% paraformaldehyde and analyzed with a FACStar flow cytometer, and the results were expressed as mean fluorescence intensity on a linear scale or as contour plots representing fluorescence intensity on a logarithmic scale.

Isolation of nucleic acids. For the isolation of RNA, cells were grown to a maximum of 7×10^5 /ml and total cellular RNA was prepared with RNazol B (Tel-Test, Inc., Friendswood, Tex.) according to the manufacturer's instructions. Poly(A)⁺ RNA was enriched by affinity chromatography on oligo(dT)-cellulose (22). For the isolation of genomic DNA, approximately 5×10^7 cells were harvested and high-molecular-weight DNA was prepared as described by Perry et al. (29).

Northern (RNA) and Southern blot analysis. Northern analysis, using 5 μ g of poly(A)⁺ RNA per lane, was performed as described by Nelson et al. (26) except that nylon membranes (MSI, Westboro, Mass.) were used and heparin (Sigma, St. Louis, Mo.) was substituted for Denhardt's solution in hybridization buffers at 50 μ g/ml. Southern blotting was done according to the methods described by Schuler et al. (41), with 1% nonfat dry milk used instead of heparin in the hybridization buffer.

The cDNA probes for EBNA2, LMP2, and EBERs were a kind gift from J. Sample (St. Jude's Children's Research Hospital) (39). The LMP1 probe was a 2.9-kb *MluI*-*Bam*HI genomic fragment spanning the LMP1 coding region and isolated from the plasmid pEcoDhet (50). The *Bam*HI B' plasmid was obtained from S. Speck (Harvard Medical School). The terminal probe (Xho 1.9) and the *Bam*HI H cDNA probe were gifts from N. Raab-Traub (University of North Carolina at Chapel Hill) (32). The human β -actin cDNA probe was a gift from R. Perry's laboratory (Fox Chase Cancer Center). The BZLF1 cDNA probe pK5Z has been described by Rooney et al. (35). Radiolabeled probes were generated by random primed second-strand synthesis (6). Oligonucleotide

probes were 5' end labeled by using T4 polynucleotide kinase (Promega, Madison, Wis.) with [γ -³²P]ATP (Amersham, Arlington Heights, Ill.) (33).

Western immunoblot analysis. EBNA2 and LMP1 proteins were detected by immunoblotting with a modification of the method described by Maniatis et al. (22). Briefly, 50 μ g of protein from each cellular lysate was separated on 7.5% polyacrylamide gels and transferred by electroblotting (1 h at 100 V and 4°C) to nitrocellulose membranes (Bio-Rad, Hercules, Calif.) in 25 mM Tris-HCl-0.195 M glycine-20% methanol buffer. Membranes were preincubated in TBS-5% milk (50 mM Tris-HCl, 150 mM NaCl, 5% nonfat dry milk) for 2 h and then incubated overnight at 4°C with the primary antibody at a concentration of 2 μ g/ml in Tris-buffered saline (TBS)-5% milk. Membranes were then washed extensively in TBS-0.05% Tween 20 and incubated with a goat anti-mouse immunoglobulin G (whole-molecule) antibody conjugated to alkaline phosphatase (Sigma) (working dilution, 1:7,000) in TBS-Tween for 2 h. Membranes were developed by using the Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium enzyme substrate system for alkaline phosphatase (Promega). The PE2 (EBNA2) monoclonal antibody was obtained from Vector Laboratories Inc. (Burlingame, Calif.), and the S12 (LMP1) monoclonal antibody was a gift from D. Thorley-Lawson (Tufts University) (23).

PCR analysis. Two micrograms of poly(A)⁺ RNA per reaction was used to synthesize cDNA by reverse transcription (10) with 100 pmol of a 3' primer. Specific cDNAs were subsequently amplified by PCR (7). Amplification products were analyzed by electrophoresis on 2% Nusieve agarose gels (FMC, Rockland, Maine), blotted onto nylon membranes, and probed with 5'-end-labeled internal oligonucleotide sequences specific for particular areas of interest. The primer and internal probe sequences for the EBNA1 F promoter, the combined C and W (C/W) region promoter, and the LMP2 transcript PCR assays were exactly the same as those stated by Kerr et al. (16). The primer sequences for BHRF1 transcripts were precisely those reported by Lear et al. (20). The sequences for the CD23 primers and internal probe were obtained from Yokota et al. (57).

RESULTS

Round B-cell expression of surface markers regulated by latent EBV infection. Four human B-cell lines, LBW2, LBW14, DB, and Josh 7, were established by EBV infection of B cells from peripheral blood or bone marrow yet have a round morphology distinct from that of typical EBV-infected LCLs (48). Instead, the morphology and growth characteristics of the round B-cell lines are similar to those of latency I BLs and EBV-negative B-cell lymphoma cell lines (37). To determine the cellular phenotype of these cell lines, we analyzed the expression of B-cell surface markers affected by latent EBV infection by indirect immunofluorescence and flow cytometry (Fig. 1). All four round B-cell lines express high levels of CD10 (CALLA), which is an early B-cell marker expressed in latency I BL cell lines but not in latency III BLs or LCLs (Fig. 1A). The levels of CD10 expression in the round B-cell lines are similar to that in the EBV-negative cell line BJAB. The EBV-positive cell line Daudi, in which the expression of the latency III phenotype is prevented by the deletion of the gene encoding EBNA2, also expresses significant levels of CD10 (15). In contrast, typical LCLs such as LBW4 and LBW26 express little or no CD10 (Fig. 1A). Unlike CD10, the B-cell activation antigen CD23 is expressed at high levels in LCLs (e.g., LBW4 and LBW26) and not expressed in EBV-negative BJAB cells or

FIG. 1. Expression of cell surface molecules which are regulated by latent EBV infection. Each of the indicated cell lines was labeled by indirect immunofluorescent labeling for the expression of CD10 (A), CD23 (B), CD54 (ICAM-1) (C), and CD11a (LFA-1) (D). The labeled cell lines were analyzed by flow cytometry. Cell surface expression is represented as mean fluorescence intensity on a linear scale.

EBNA2-negative Daudi cells (Fig. 1B). The expression of CD23 in the round B-cell lines, however, is intermediate between that of LCLs and that of BJAB or Daudi cells.

The levels of expression of the cellular adhesion molecules ICAM-1 and LFA-1, as well as the level of homotypic adhesion, are often increased following EBV infection of B cells (51). Figure 1C shows that the round B-cell lines expressed levels of ICAM-1 comparable to those observed for the BJAB and Daudi cell lines. The LCL LBW4 expressed typically high levels of ICAM-1, whereas the LBW26 cell line is somewhat unusual in that it expressed only low levels of this adhesion molecule. Figure 1D shows the results obtained for the expression of LFA-1. As in the case of ICAM-1, the round B-cell lines expressed levels of LFA-1 similar to the levels expressed in the BJAB and Daudi cell lines. Both LBW4 and LBW26 expressed high levels of LFA-1, typical of LCLs. The results of these experiments indicate that the expression of CD10, ICAM-1, and LFA-1 in round B-cell lines is comparable to those of the EBV-negative cell line BJAB and the EBNA2-deleted cell line Daudi, yet the expression of the activation antigen CD23 is intermediate between the levels expressed by BJAB and Daudi cells and those expressed by the LCLs LBW4 and LBW26.

The data in Fig. 1 suggest that the round B-cell lines represent an intermediate stage of B-cell activation. Alternatively, the round B-cell lines may experience a drift in the B-cell phenotype, as observed with some BL cell lines (37). In

that case, some cells may express CD10 alone while others express CD23 solely. To address this possibility, the round B-cell lines were analyzed by double-label immunofluorescence for coexpression of CD10 and CD23. In addition, the round B-cell line LBW14 was subcloned and two separate subclones were selected and analyzed for coexpression of CD10 and CD23. Figure 2A and B show that the DB and LBW14 round B-cell populations consistently coexpress the B-cell markers CD10 and CD23. All CD23-positive cells also express CD10. Analysis of the round B-cell lines LBW2 and Josh 7 also showed a similar pattern of coexpression of these cell markers (data not shown). Figure 2C and D show that there was no difference in the pattern of expression of CD10 and CD23 by the two subclones of LBW14 compared with that of the parental cell line, indicating that the expression of these markers is not due to phenotypic drift in the population.

Promoter usage for the expression of the EBNA1 gene in round B-cell lines. The observation that four B-cell lines, DB, LBW2, LBW14, and Josh 7, display a B-cell phenotype intermediate between that of BL cell lines and typical EBV-infected LCLs suggests that they may have a novel program of viral latency gene expression. The promoter usage for the expression of the EBNA1 gene in these cell lines was determined since this appears to be a key point at which the expression of viral latency gene products is regulated. In latency I BL cell lines, the transcription of the EBNA1 gene occurs from a

FIG. 2. Coexpression of CD10 and CD23 in the round B-cell lines. The round B-cell lines DB (A) and LBW14 (B) and two subclones of LBW14, P1 (C) and P2 (D), were labeled by direct double immunofluorescent labeling for the expression of CD10 and CD23. The labeled cell lines were analyzed by flow cytometry. Cell surface coexpression of these markers is depicted as fluorescence intensity shown as contour plots on a logarithmic scale.

promoter located in the *Bam*HI F fragment of the viral genome. The activation of this promoter alone leads to the exclusive expression of EBNA1 (38). In contrast, in LCLs and latency III BLs, transcription from promoters located within the *Bam*HI W fragment or C fragment of the viral genome results in the expression of EBNA1 to -4 through differential splicing (45).

Poly(A)⁺ RNAs from the four round B-cell lines were analyzed for the presence of EBNA1 transcripts by reverse transcription and PCR amplification with a 3' primer derived from the EBNA1 coding region (16). EBNA1 transcripts originating from the C/W promoter region were amplified with a 5' primer sequence which hybridizes to the *Bam*HI Y3 exon and generates an amplification product of 265 bp. Transcripts

expressed from the F promoter were amplified with a primer sequence from the *Bam*HI Q exon which gives rise to a 236-bp amplification product (16). As seen in Fig. 3, DB, LBW2, and LBW14 cell lines generate EBNA1 transcripts from both the F and C/W promoter regions, with F usage clearly predominating in DB and LBW2 samples. Utilization of both of these promoter regions has been observed previously only as a transient feature of B-cell hosts infected with EBV (16, 20, 36). The BL cell line Daudi also expressed a transcript derived from the F promoter. However, an amplification product is not generated by the Y3 exon primer since this cell line contains a deletion in the EBNA2 coding region which encompasses the Y3 exon (15). While the growth characteristics and surface marker expression of the Josh 7 cell line are similar to those of the other round B-cell lines, it differs by utilizing the C/W promoter region for the transcription of the EBNA gene products, with markedly reduced usage of the F promoter. This is comparable to the pattern of promoter usage seen in the control LCLs LBW25 and LBW26.

Because the round B-cell lines produce EBNA1 transcripts that include the Y3 region, a separate experiment was done to distinguish between transcripts derived from the W promoter and those derived from the C promoter (Fig. 4). Transcripts expressed from the W promoter were amplified with a 5' primer sequence from the W0 exon which gives rise to a 210-bp amplification product (16). Transcripts generated from the C promoter were detected with a 5' primer sequence which hybridizes to the C1 exon and amplifies a 240-bp product (16). Reverse transcription and amplification of poly(A)⁺ RNAs from DB, LBW2, and LBW14 cells gave low but detectable amounts of PCR products from W promoter-derived transcripts. The Josh 7 cell line utilized the W promoter to a degree similar to that of the LCL controls (Fig. 4). C promoter-derived transcripts were also detected in the Josh 7 cell line and the LCLs LBW25 and LBW26. However, even after extensive autoradiographic exposure, no C promoter-specific PCR products were seen with RNAs from the DB, LBW2, or LBW14 cell line. The Daudi cell line did not yield detectable C promoter-derived transcripts but did express transcripts derived from the W promoter, as described previously (55). These transcripts were not detected in the experiment shown in Fig. 3 because of the deletion of the Y3 primer sequence explained above. Thus, the Daudi cell line resembles the round B-cell lines in its use of both the F and W promoters, although the results in Fig. 4 indicate that transcripts from the W promoter are expressed at much higher levels in Daudi cells

FIG. 3. Analysis of EBNA1 transcripts originating either from the F promoter or from C/W promoters in the round B-cell lines. PCR products were generated from cDNA with 2 μ g of poly(A)⁺ RNA from each of the cell lines shown, a 3' primer from the EBNA1 coding region, and the indicated 5' primer. EBNA1 transcripts originating from the C/W promoter region were amplified with a 5' primer sequence which hybridizes to the *Bam*HI Y3 exon and generates an amplification product of 265 bp. Transcripts expressed from the F promoter were amplified with a 5' primer sequence from the *Bam*HI Q exon which gives rise to a 236-bp amplification product. Two LCLs (LBW25 and LBW26) and the EBNA2-negative BL cell line Daudi were included as controls. PCR products were separated on an agarose gel and probed with an internal oligonucleotide capable of hybridizing to both products.

FIG. 4. Analysis of EBNA transcripts originating either from the W promoter (Wp) or from the C promoter (Cp) in the round B-cell lines. PCR products with Wp- versus Cp-specific 5' primers were generated from cDNA by using 2 μ g of poly(A)⁺ RNA from each of the indicated cell lines. Southern analysis was performed with an internal oligonucleotide probe capable of hybridizing to transcripts arising from either promoter. Transcription from the Wp yields a PCR product of 210 bp, and transcription from the Cp gives a 240-bp product as indicated. Two LCLs (LBW25 and LBW26) and the EBNA2-negative BL cell line Daudi were included as controls.

than in the round B-cell lines. Unlike the round B-cell lines, however, Daudi possesses a deletion in the EBNA2 coding region which prevents the activation of the C promoter. Therefore, the round B-cell lines DB, LBW2, and LBW14 demonstrate a unique pattern of dual F and W promoter usage for the expression of the EBNA gene products.

Lytic-cycle gene expression in round B-cell lines. Recently, Lear et al. (20) reported that dual W/C and F promoter usage could be demonstrated in LCLs in which viral lytic-cycle replication occurs either spontaneously or as a result of induction. In this case, the activation of the F promoter is an early event which occurs during the switch from the latency III state to the lytic cycle (20). The use of the F promoter in the DB, LBW2, and LBW14 cell samples, therefore, could be due to the presence of a subpopulation of cells which are undergoing a lytic EBV infection. To address this possibility, the round B-cell lines were examined for the expression of BZLF1 transcripts, a viral transcription factor involved in the activation of the lytic cycle (27, 35). Figure 5A shows that BZLF1 mRNA was only marginally detectable in the four round B-cell lines. Furthermore, this level of BZLF1 expression was considerably lower than the levels observed in the typical LCLs LBW26 and GMO2292, which do not show any F promoter activity (Fig. 3 and data not shown).

In order to provide further evidence that the F promoter expression observed in the round B-cell lines was not due to spontaneous entry into the lytic cycle, a separate PCR experiment (20) was done to analyze the expression of the spliced 1.7-kb BHRF1 mRNA, an abundant immediate-early lytic-cycle transcript (28). Poly(A)⁺ RNA was reverse transcribed and then amplified with primers specific for the *Bam*HI H3 and *Bam*HI H2 exons, respectively. A 213-bp product specific for this transcript was detected on a Southern blot with a radiolabeled *Bam*HI H cDNA probe. Figure 5B shows that the round B-cell lines LBW14, LBW2 and Josh 7 express little, if any, of this BHRF1 transcript. The DB cell line expresses BHRF1 transcripts at a level similar to those of the LCLs LBW26 and GMO2292. Consistent with the data obtained for the expression of BZLF1 (Fig. 5A), there does not appear to be a correlation between F promoter use in the round B-cell lines and the entry of these cells into the lytic cycle.

Southern analysis of the structure of the EBNA2 gene. A deletion or rearrangement in the EBNA2 gene in the round

FIG. 5. Expression of lytic-cycle transcripts in the round B-cell lines. (A) Northern analysis of BZLF1 mRNA in the round B-cell lines. Five micrograms of poly(A)⁺ RNA from each of the indicated cell lines was analyzed by Northern blot with a radiolabeled BZLF1 cDNA probe. The steady-state levels of BZLF1 mRNA in the round B-cell lines were significantly lower than the levels observed in the LCLs LBW26 and GMO2292. (B) PCR analysis of class IV BHRF1 transcripts in the round B-cell lines. PCR products with primers specific for class IV BHRF1 transcripts were generated from cDNA by using 2 μ g of poly(A)⁺ RNA from each of the indicated cell lines. Southern analysis was performed with a *Bam*HI H cDNA probe. The expected 213-bp product is indicated. Two LCLs (LBW26 and GMO2292) were included as positive controls. In identical reactions, little, if any, PCR product was detected in the round B-cell lines LBW14, LBW2, and Josh 7.

B-cell lines, like that in Daudi cells described above, could lead to their intermediate phenotype and dual EBNA1 promoter usage. The structure of the EBNA2 gene in the round B-cell lines was therefore determined by Southern analysis. *Bam*HI- and *Pst*I-digested genomic DNAs from the DB, LBW14, and Josh 7 cell lines were analyzed by Southern blot with an EBNA2 cDNA probe. This probe encompasses the entire EBNA2 open reading frame, which corresponds to the *Bam*HI H and Y fragments of the EBV genome, as well as a small amount of the repeated W fragment sequence (39). As shown in Fig. 6, no obvious rearrangement in the *Bam*HI Y and H regions of the EBV genome was observed in the round B-cell lines; both the round B-cell samples and the reference LCLs exhibited a *Bam*HI H and Y restriction fragment pattern (6.4 and 1.7 kb, respectively) typical of EBV type 1 strains. No bands for the Daudi BL line are seen, as it has a 6-kb deletion that spans the *Bam*HI H and Y sequence (15). Because equal amounts of DNA were loaded into all lanes, the stronger signals of the EBNA2 gene fragments in the DB and LBW14 *Bam*HI-digested DNA samples most likely reflect the natural variation in copy number of EBV genomes per cell seen in LCLs (46). These results suggest that the intermediate phenotype of the round B cells is not due to the deletion of the EBNA2 coding region.

EBNA2 gene expression in round B-cell lines. To determine if the intermediate cellular phenotype of the round B-cell lines was due to aberrant EBNA2 expression, the levels of EBNA2 gene expression in these cell lines were determined by Northern analysis of EBNA2 mRNA and Western analysis of

FIG. 6. Southern analysis of the EBNA2 gene structure in the round B-cell lines. Five micrograms of genomic DNA from each of the indicated cell lines was digested with *Bam*HI or *Pst*I and analyzed by Southern blot with a full-length EBNA2 cDNA probe. Confirmation that equal amounts of DNA were loaded into all lanes was done by ethidium bromide staining of the gel prior to transfer. Two LCLs and the BL cell line Daudi, which has a deletion of the EBNA2 gene, were included as positive and negative controls, respectively.

EBNA2 protein. Poly(A)⁺ RNA from each round B-cell line was analyzed for the presence of EBNA2 mRNA by Northern blot (Fig. 7A). The round B-cell lines DB, LBW2, LBW14, and Josh 7 expressed EBNA2 transcripts at far lower steady-state levels than did the LBW25 and LBW26 LCL controls. In addition, the EBNA2-hybridizing transcript was 3.0 kb. This is smaller than the EBNA2 mRNAs expressed in the LBW25 and LBW26 LCLs. This variation in EBNA2 mRNA size between B-cell lines, even between those infected with the same virus stock, has precedents and is due to a structural polymorphism in the repeated *Bam*HI W region of EBV genomes (5). Analysis of the levels of β -actin mRNA indicated that approximately equal amounts of RNA were loaded into all lanes.

The expression of EBNA2 protein in the round B-cell lines was examined by Western blots in which cellular lysates were probed with the PE2 monoclonal antibody to the EBNA2 protein (Fig. 7B). The round B-cell lines DB, LBW2, LBW14, and Josh 7 expressed an EBNA2 protein with the same apparent molecular mass (82 kDa) as that expressed by the LBW25 and LBW26 LCL controls. This indicates that the differences in the sizes of the EBNA2 mRNAs (Fig. 7A) are not due to differences in the size of the EBNA2 coding region. The round B-cell lines expressed approximately three- to fourfold less EBNA2 protein than did the LBW25 and LBW26 LCL controls, with the exception of the Josh 7 cell line, which expressed EBNA2 protein at somewhat higher levels. The quantitative differences between the round B-cell lines and LCLs were confirmed by analyzing serial dilutions of cell extracts by Western blots (data not shown). These results were consistent with the lower levels of EBNA2 transcripts observed in the round B-cell lines. However, the differences between the round B-cell lines and LCLs were more striking when EBNA2 mRNA (Fig. 7A) rather than the differences in EBNA2 protein expression (Fig. 7B) was analyzed.

Expression of viral and cellular genes coregulated by EBNA2. EBNA2 has been shown to play a role in upregulating the expression of several viral and cellular genes. Since the round B-cell lines express lower levels of EBNA2 protein than

FIG. 7. Expression of EBNA2 in the round B-cell lines. (A) Northern analysis of EBNA2 mRNA in the round B-cell lines. Five micrograms of poly(A)⁺ RNA from each of the indicated cell lines was analyzed by Northern blot with a full-length EBNA2 cDNA probe. Approximately equivalent amounts of RNA were loaded into all lanes as shown when the blot was rehybridized with a β -actin cDNA probe (bottom). The expression of a 3.0-kb EBNA2 transcript was barely detectable in the round B-cell lines, in contrast to the higher levels of a 3.8-kb EBNA2 transcript seen in the two LCLs (LBW25 and LBW26). The BL cell line Daudi possesses a deletion in the EBNA2 coding region and serves as a negative control for EBNA2 expression. (B) Western analysis of EBNA2 protein in the round B-cell lines. Fifty micrograms of protein from each of the cell lines shown was analyzed by Western blot with an EBNA2 monoclonal antibody. The 82-kDa EBNA2 protein is indicated.

typical LCLs, the effects of this low level of expression on other EBNA2-responsive genes were investigated. EBNA2 upregulates the expression of the viral LMP1 and LMP2 (49, 53, 58). Therefore, poly(A)⁺ mRNAs from the round B-cell lines were assayed for the presence of LMP1 by Northern analysis. Figure 8A shows that the cell lines LBW14, LBW2, and DB express LMP1 transcripts, though at somewhat lower levels than the LCL LBW4. LMP1 mRNA was present in much smaller amounts in Josh 7 cells than in the other round B-cell lines and was observed in Josh 7 samples only upon a fivefold-longer autoradiographic exposure. The 2.6-kb LMP1 transcript seen in the round B-cell lines was the expected size for B-cell hosts (39). However, a 3.7-kb LMP1 transcript expressed in NPC tumors and in some LCLs was not observed in any of the B-cell lines tested (11).

By Western blot (Fig. 8B) with the anti-LMP1 monoclonal antibody S12 (23), a 56-kDa LMP1 protein was readily detected in the LBW14, LBW2, and DB cell lines while little or no LMP1 protein was detected in the Josh 7 cell line. A significantly higher level of LMP1 protein was detected in the LCL LBW4. This result is consistent with the levels of LMP1 mRNA expressed in the round B-cell lines and suggests that

FIG. 8. Expression of LMP1 in the round B-cell lines. (A) Northern analysis of LMP1 mRNA in the round B-cell lines. Five micrograms of poly(A)⁺ RNA from each cell line indicated was analyzed for the presence of LMP1 transcripts. The 2.6-kb LMP1 transcript is indicated. The LCL cell line LBW4 was included as a positive control for LMP1 expression. Approximately equivalent amounts of RNA were loaded into all lanes as determined when the blot was rehybridized with a β -actin cDNA probe (results not shown). (B) Western analysis of LMP1 expression in the round B-cell lines. Fifty micrograms of protein from each cell line shown was analyzed by Western blot for the presence of LMP1 protein. The 56-kDa LMP1 protein is indicated.

the low level of EBNA2 expression in the round B cells leads to incomplete activation of the LMP1 promoter.

The LMP2 gene lies across the terminal regions of the viral genome, and two distinct LMP2 mRNAs can be generated. LMP2A and LMP2B mRNAs have differing 5' exons because of the use of different promoters, with subsequent alternative splicing of the unique 5' exon to the common exons 2 to 9 in the primary transcript (19, 40). To determine if the round B-cell lines express normally initiated LMP2A or LMP2B transcripts, a separate PCR experiment with primers specific to the 5' exon unique to each message was carried out (16). Figure 9 shows apparently normally initiated LMP2B transcripts in the round B-cell lines DB, LBW2, LBW14, and Josh 7, but little or no mRNA containing the LMP2A 5' exon could be detected. As expected, transcripts of both LMP2A and LMP2B were expressed in the LCL control samples LBW25 and LBW26. In contrast to the round B-cell lines, the EBNA2-deficient cell line Daudi expressed LMP2A transcripts but not LMP2B transcripts.

The EBV latent gene products EBNA2 and LMP1 induce the expression of the B-cell activation antigen CD23 (51, 52). Two species of CD23 mRNA, types A and B, which utilize different promoters and include unique 5' exons have been described. Type A is expressed in all B cells, whereas type B is expressed only in response to activating signals (57). EBNA2 and LMP1 induce the expression of type B CD23 mRNA and also increase the expression of constitutive type A mRNA (51). Since the round B-cell lines express intermediate levels of CD23, a PCR experiment was performed to distinguish the expression of CD23A from that of virally induced CD23B (Fig. 10). Poly(A)⁺ mRNAs from the four round B-cell lines were

FIG. 9. Analysis of LMP2 transcripts in the round B-cell lines. PCR products with LMP2A- versus LMP2B-specific primers were generated by using 2 μ g of poly(A)⁺ RNA from each of the indicated cell lines. Amplification products were separated on an agarose gel, blotted onto a nylon membrane, and probed with an internal oligonucleotide capable of hybridizing to both products. Transcripts originating from the LMP2A promoter give rise to a PCR product of 280 bp, whereas transcripts from the LMP2B promoter give a product of 324 bp, as indicated. The EBNA2-negative cell line Daudi and the LCLs LBW25 and LBW26 were included as controls.

analyzed for the presence of CD23A and CD23B transcripts by reverse transcription with a 3' primer sequence common to both transcripts and then by amplification with 5' primer sequences unique to each CD23 transcript. The type A amplification product is 320 bp, whereas the type B product is 316 bp. Figure 10 shows that only constitutive type A CD23, and not inducible type B, was detected in the round B-cell lines. Both forms of CD23 transcripts were detected in LCLs, as expected. This result is consistent with the lower levels of total CD23 on the surfaces of the round B-cell lines and the lower levels of EBNA2 and LMP1 compared with those of the LCL controls (Fig. 1, 5, and 6).

DISCUSSION

An important step in the regulation of EBV latent gene expression is the use of either the F promoter or the C/W promoter region to express EBNA1 (38, 56). The promoter used for EBNA1 expression determines whether only EBNA1 or all six EBNA genes are expressed. The round B-cell lines DB, LBW2, and LBW14 are unique among previously described B-cell systems in that they utilize both of these promoter regions to transcribe the EBNA1 gene (Fig. 2 and 3). The transcription of EBNA1 in latency I BLs and latency II

FIG. 10. Analysis of CD23 transcripts in the round B-cell lines. Two micrograms of poly(A)⁺ RNA from each of the cell lines shown was used to generate PCR products with CD23A- versus CD23B-specific primers. Amplification products were identified by Southern blot with an internal oligonucleotide probe capable of hybridizing to both products. CD23A transcripts give rise to a PCR product of 320 bp, and CD23B transcripts give a product of 316 bp, as shown. The LCLs LBW25 and LBW26 were included as controls for CD23 expression.

NPC cell lines occurs exclusively from the F promoter (38, 44). Yet, unlike latency I or latency II cell lines, the round B-cell lines show activation of the F promoter as well as limited use of the W promoter. The W promoter is typically used during the early stages of EBV infection of primary B cells prior to switching to the C promoter (56).

The apparent lack of transcripts derived from the C promoter in the DB, LBW2, and LBW14 round B-cell lines implies that they have failed to switch to the C promoter for the expression of the EBNA genes as is normally seen in latent infections of LCLs. Alternatively, it is possible that viral C promoters in the round B-cell lines are not capable of being activated as a result of mutation. This is not likely to be the case, however, since preliminary evidence indicates that the C promoters in the round B-cell lines can be used in response to activating signals (unpublished data). The use of dual promoters in the DB, LBW2, and LBW14 cell lines is accompanied by a pattern of coexpression of the cellular differentiation antigens CD10 and CD23 that is intermediate between those of latency I and latency III B-cell lines (Fig. 1 and 2). Transient dual expression of F and C/W transcripts has been observed previously in the evolution of latency I BL cells and LCLs to a latency III phenotype during passage in culture and in latency III LCLs switching to the lytic cycle (16, 20, 36). Similarly, transient coexpression of CD10 and CD23 has been observed during the evolution of BL cell lines in culture (37). Thus, DB, LBW2, and LBW14 cell lines appear to stably express an intermediate stage in a continuum of B-cell latency states.

In contrast to the other round B-cell lines, Josh 7 cells express very few transcripts from the F promoter and demonstrate approximately equal use of the W and C promoters. While the Josh 7 cell line has a morphology and cell surface phenotype similar to those of the other round B-cell lines, this cell line is more lymphoblastoid-like in its use of viral promoters for EBNA expression. These data suggest that the cellular phenotype of this cell line results from a pattern of viral gene expression that differs from that of the other three round B-cell lines. This idea is further supported by the observation that in contrast to the other round B-cell lines, Josh 7 cells did not express detectable LMP1 gene products (Fig. 6). Thus, the cellular phenotype of the Josh 7 cell line may result from an absence of viral or cellular factors necessary for LMP1 expression.

The BL cell line Daudi is similar to the DB, LBW2, and LBW14 round B-cell lines both in cellular phenotype and in viral promoter usage (Fig. 1, 2, and 3). As shown, Daudi cells utilize both the F and W promoters for the transcription of the EBNA genes (Fig. 2 and 3), although the transcription from the W promoter is much more pronounced in Daudi cells than in the round B-cell lines (Fig. 3). The transcription from the W promoter in Daudi cells has been previously described (55). The deletion of the region of the viral genome encoding the EBNA2 gene in Daudi cells (15) prevents the EBNA2-dependent switch to the C promoter (56). In contrast to Daudi cells, the deletion of the EBNA2 coding region was not detected in the round B-cell lines (Fig. 4). Additionally, both EBNA2 mRNA and protein were expressed, although at significantly lower levels than those commonly observed in LCLs (Fig. 5). These findings suggest that the round B-cell lines and the Daudi BL cell line express their latency phenotypes through different mechanisms. Nonetheless, the similarity of the pattern of gene expression in Daudi cells to that in the three round B-cell lines DB, LBW2, and LBW14 suggests that a critical level of EBNA2 protein is prevented from accumulating in the round B-cell lines, thereby inhibiting the activation

of the C promoter and the expression of the latency III pattern of viral gene products.

The reduced expression of the EBNA2 gene in the round B-cell lines correlated with less transcriptional activity of coregulated viral and cellular promoters such as LMP1, LMP2A, and CD23 (51, 52, 53, 58). The reduced expression of the LMP1 gene may contribute to the unusual cellular phenotype observed in the round B-cell lines, since it has been shown that this protein is specifically involved in the downregulation of CD10 and the upregulation of ICAM-1 and LFA-1 in transfection experiments of EBV-negative BL cell lines (51).

While all the round B-cell lines appear to express normally spliced LMP2B transcripts, LMP2A transcripts could not be detected. LMP2A and LMP2B are not required for *in vitro* transformation of primary human B cells (21). Instead, they appear to be involved in regulating the reactivation of the virus *in vivo* (24). The lack of expression of LMP2A has not previously been observed in either B-cell or NPC cell systems (25). Since this protein has recently been shown to associate with key components of the B-cell signaling pathway and to block the mobilization of calcium following immunoglobulin M receptor ligation (25), the lack of expression of this protein in the round B-cell lines may have contributed to the establishment of their latency phenotype.

The expression of CD23 during latent infection of LCLs has been shown to be activated by both EBNA2 and LMP1. LMP1 specifically upregulates the activity of the CD23B promoter, whereas EBNA2 activates a regulatory element common to the CD23A and CD23B promoters (51, 52). Since the round B-cell lines express reduced levels of both EBNA2 and LMP1, it is not surprising that the round B-cell lines express only CD23 transcripts from the constitutively active CD23A promoter. The intermediate levels of CD23 observed on the cell surfaces of round B-cell lines, therefore, likely reflect the effects of limited EBNA2 and LMP1 expression. Taken together, the low levels of expression of LMP1, LMP2, and CD23 support the idea that the lack of sufficient expression of EBNA2 may be responsible for the cellular phenotype and the pattern of viral genes expressed in the DB, LBW2, and LBW14 cell lines. If so, the question of why these cells express less EBNA2 than typical LCLs does then arise. These cell lines do have other features that distinguish them from typical LCLs, including the expression of truncated forms of immunoglobulin heavy chains (1, 30) and the presence of chromosomal translocations (Table 1). It is possible that some of these features may contribute to a cellular environment that is unfavorable for the expression of EBNA2 at normal levels.

An intermediate pattern of EBV latent gene expression, latency II, in NPC cells and, more recently, in EBV-infected Reed-Sternberg cells and peripheral T-lymphoma cell lines has been described (3, 14, 44). Latency II expression has also been experimentally induced in two B-cell models (16, 36). The defining characteristics of this phenotype include the exclusive expression of EBNA1 from the F promoter and the expression of LMP1 and LMP2 without the expression of EBNA2 or other EBNA proteins. The round B-cell lines DB, LBW2, and LBW14 have a pattern of viral gene expression which is clearly distinct from latency II. For example, these three round B-cell lines utilize both the F and W promoter regions for the transcription of the EBNA genes (Fig. 2 and 3) and EBNA2 is expressed, albeit at a low level (Fig. 5).

It is becoming increasingly apparent that strictly defined latency states may not reflect the dynamic nature of the EBV-host interaction, as evidenced by the differential expression of latent gene products in various B-cell environments (16, 36, 37). Instead, our studies support the idea that a continuum

of latency states may be expressed in EBV-infected B lymphocytes. Therefore, the round B-cell lines represent unique members of this continuum. While the ability of EBV-infected host cells to express different patterns of viral gene expression may be a key feature in the link between infection with this virus and the development of a number of human malignancies, the molecular basis for this relationship is not well understood. The round B-cell lines, which stably express different patterns of viral latency, may provide an important tool through which to further address this question.

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