JOURNAL OF VIROLOGY, Aug. 2005, p. 10709–10717 Vol. 79, No. 16
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.16.10709–10717.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Epstein-Barr Virus Nuclear Antigen 2 (EBNA2) Gene Deletion Is Consistently Linked with EBNA3A, -3B, and -3C Expression in Burkitt's Lymphoma Cells and with Increased Resistance to Apoptosis

Gemma L. Kelly,[1] Anne E. Milner,[1] Rosemary J. Tierney,[1] Debbie S. G. Croom-Carter,[1] Markus Altmann,[2] Wolfgang Hammerschmidt,[2] Andrew I. Bell,[1] and Alan B. Rickinson[1]*

Cancer Research UK Institute for Cancer Studies, The University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom,[1] and Department of Gene Vectors, GSF-National Research Center for Environment and Health, Marchioninistrasse 25, Munich D-81377, Germany[2]

Received 27 January 2005/Accepted 4 May 2005

Most Epstein-Barr virus (EBV)-positive Burkitt's lymphomas (BLs) carry a wild-type EBV genome and express EBV nuclear antigen 1 (EBNA1) selectively from the BamHI Q promoter (latency I). Recently we identified a distinct subset of BLs carrying both wild-type and EBNA2 gene-deleted (transformation-defective) viral genomes. The cells displayed an atypical "BamHI W promoter (Wp)-restricted" form of latency where Wp (rather than Qp) was active and EBNA1, -3A, -3B, -3C, and -LP were expressed in the absence of EBNA2 or latent membrane proteins 1 and 2. Here we present data strongly supporting the view that the EBNA2-deleted genome is transcriptionally active in these cells and the wild-type genome is silent. Single-cell cloning of three parental Wp-restricted BL lines generated clones carrying either both viral genomes or the EBNA2-deleted genome only, never clones with the wild-type genome only. All rescued clones displayed the Wp-restricted form of latency characteristic of the parent line and retained the original parent cell phenotype. Interestingly, Wp-restricted parent lines and derived clones were markedly more resistant to inducers of apoptosis than standard latency I BL lines. Furthermore, in vitro infection of EBV-negative BL lines with an EBNA2 gene-deleted virus generated EBV-positive converts with Wp-restricted latency and a similarly marked apoptosis resistance. We postulate that, in the subset of BLs displaying Wp-restricted latency, infection of a tumor progenitor cell with an EBNA2 gene-deleted virus has provided that cell with a survival advantage through broadening antigen expression to include the EBNA3 proteins.

Epstein-Barr virus (EBV), a human B-lymphotropic herpesvirus with cell growth-transforming ability, is linked to three different B-cell malignancies, endemic Burkitt's lymphoma (BL), Hodgkin's disease, and posttransplant lymphoproliferative disease (PTLD) (36). We know relatively little about the role played by the virus in these different tumor contexts, except for those oligoclonal or polyclonal PTLD lesions which arise in the setting of profound T-cell impairment, classically in early posttransplant patients. These lesions express the same spectrum of EBV latent-cycle proteins (53) as do B cells transformed by EBV to permanent lymphoblastoid cell lines (LCLs) in vitro (19). This latency III form of infection is characterized by BamHI C promoter (Cp) and to a lesser extent BamHI W promoter (Wp) activity, leading to expression of EBV nuclear antigen EBNA1, -2, -3A, -3B, -3C, and -LP, and by activation of EBNA2-responsive promoters elsewhere in the viral genome, leading to expression of latent membrane proteins LMP1 and LMP2. The LCL-like nature of viral gene expression in PTLDs strongly suggests that EBV is the principal, perhaps the sole, driving force behind these lymphoproliferations.

The situation is quite different in endemic BL, where EBV's role clearly complements that of the principal cellular genetic change, namely, activation of the c-*myc* oncogene by translocation to an immunoglobulin (Ig) gene locus (26). BL tumor cells carry a transformation-competent wild-type EBV genome but display a latency I form of infection in which the nuclear antigen EBNA1 is selectively expressed from the BamHI Q promoter while all latency III-associated promoters are silent (39, 42, 43). The highly restricted nature of EBV gene expression in BL raises questions as to what extent, if at all, the virus contributes to the malignant cell phenotype; most interest in this regard has focused on the potential role of either the EBNA1 protein or the noncoding EBER RNAs in mediating partial protection from the strong proapoptotic effects associated with c-*myc*-driven cell growth (23, 24, 40, 41).

In recent work we identified a subset of BL tumors which did not display the typical latency I form of infection. Instead the tumor cells expressed five nuclear antigens, namely, EBNA1, -3A, -3B, and -3C and a truncated (W_1W_2 repeat domain only) EBNA-LP, in the absence of EBNA2 and of the LMPs (18). This was associated with transcription exclusively from Wp and was hence termed "Wp-restricted latency." Interestingly, all three BL cell lines of this type carried a transformation-com-

* Corresponding author. Mailing address: Cancer Research UK Institute for Cancer Studies, The University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom. Phone: 441214144492. Fax: 441214144486. E-mail: a.b.rickinson@bham.ac.uk.

petent wild-type genome and also a transformation-defective genome in which a deletion had removed the EBNA2 gene and the Y_1Y_2 unique exons of EBNA-LP. These findings raised a number of questions. What is the origin of the EBNA2-deleted virus? What is the virus genome content of individual tumor cells? Are Wp transcripts derived from both or just from one of these genomes? Is the presence of an EBNA2-deleted genome consistently linked to Wp restriction? Does the broadened pattern of viral antigen expression in Wp-restricted latency alter the cell phenotype in ways that might be important in tumor pathogenesis?

Here we have addressed these questions by establishing single-cell clones from three Wp-restricted BL lines, Sal-, Oku-, and Ava-BL, and determining their viral genome contents and patterns of latent gene expression. In addition, we asked whether in vitro infection of EBV-negative BL cell lines with a recombinant EBNA2-deleted virus strain established a Wp-restricted form of latency. Finally, we compared BL lines showing Wp-restricted latency with classical latency I BL lines for their susceptibility to apoptosis.

MATERIALS AND METHODS

Maintenance of cell lines and single-cell seeding. The Wp-restricted BL parent lines Sal-BL, Oku-BL, and Ava-BL are described elsewhere (18). Two long-established BL cell lines with EBNA2 gene-deleted (but not wild-type) genomes are Daudi-BL (17, 21) and P3HR1-BL (6, 16, 35); we used a subclone of P3HR1, HH514 cl. 16, specifically chosen because it does not carry any detectable heterogeneous (het) DNA (15). Standard latency I BL lines included Rael-BL, Sav-BL, BL37, Ezem-BL, Mutu-BL, Dant-BL, Silv-BL, Chep-BL, and Jada-BL, as described in earlier work (14, 37). EBV-negative BL cell lines BL2, BL31, and BL41 are described elsewhere (7). All BL lines were maintained in RPMI 1640 medium (Invitrogen) containing 10% (vol/vol) selected fetal calf serum and 2 mM glutamine (standard medium) and further supplemented with 1 mM pyruvate, 50 μM alpha-thioglycerol, and 20 nM bathocupronine disulfonic acid. The LCL panel included the Wp-using X50-7 LCL, the Cp-using B95.8 virus-transformed LCL PS, and LCLs made with virus rescued from the Sal-BL and Oku-BL lines as described previously (18). All LCLs were maintained in standard medium.

In cloning experiments, BL cells were seeded at 0.5 to 1 cell per 0.3-ml U-bottomed well containing 2×10^3 fibroblast feeders. The wells were checked microscopically within a few hours of seeding to eliminate any wells with more than one visible BL cell. The cultures were maintained for up to 4 weeks with regular refeeding before growing cells were harvested and expanded off fibroblasts. Note that for 2 weeks before the original limiting-dilution seeding and throughout the whole cloning procedure, cells were maintained in culture medium supplemented with 200 μM acyclovir to prevent lytic virus production and possible reinfection of cells within the culture. Outgrowth efficiencies were recorded as the observed number of growing wells, expressed as a percentage of the expected number had all seeded cells grown.

Screening for wild-type and EBNA2-deleted genomes. DNA was extracted from 5×10^6 cells by using a DNEasy tissue kit (QIAGEN) according to the manufacturer's instructions. Cells were then screened for EBV genome content by PCR amplification of 400 ng of extracted DNA. To detect the wild-type genome, primers within the EBNA2 gene were used, namely, E2C (5′-AGGG ATGCCTGGACACAAGA-3′) (B95.8 coordinates 48810 to 48829) and E2A (5′-TTGTGACAGAGGTGACAAAA-3′) (B95.8 coordinates 49058 to 49039), and the reaction was cycled 40 times at 95°C for 30 s, 50°C for 90 s, and 72°C for 120 s. Note that a mix of deaza-deoxynucleoside triphosphates containing 200 μM each of dATP, dCTP, and dTTP; 50 μM dGTP, and 150 μM 7-deaza-dGTP was required to amplify this repetition-rich region. Following gel electrophoresis, the samples were transferred to a nitrocellulose membrane by Southern blotting and probed with a ^{32}P-end-labeled radioactive E2 probe (5′-TCCAGCCACAT GTCCCCCCTCTACGCCCGACA-3′) (B95.8 coordinates 48997 to 49028).

To detect the EBNA2-deleted genome, forward primers within the BamHI W fragment were used in conjunction with reverse primers in the BamHI H fragment. For Sal-BL, primers 5′-TCCTCTCCAACCTTCGCTCC-3′ (B95.8 coordinates 13251 to 13270) and 5′-GCCTTCGCTGGCTTCTAACATC-3′ (B95.8 coordinates 54792 to 54771) were used, and the reaction mixture was subjected to 35 cycles of 95°C for 60 s, 57°C for 60 s, and 72°C for 300 s, followed by Southern blotting and hybridization with a radiolabeled WO probe (5′-CGCC AGGAGTCCACACAAAT-3′) (B95.8 coordinates 14391 to 14410). For Oku-BL and Ava-BL, primers 5′-GGGCCAGAGGTAAGTGGACTTT-3′ (B95.8 coordinates 14611 to 14632) and 5′-CCCACCTGGTGACACACCTTA A-3′ (B95.8 coordinates 52938 to 52917) were used and the reaction was cycled 35 times at 95°C for 60 s, 55°C for 60 s, and 72°C for 120 s. Following Southern blotting, the membrane was hybridized with a radiolabeled H probe (5′-GTGT CATTTTAGCCCGTTGG-3′) (B95.8 coordinates 52891 to 52910).

Identification of wild-type and EBNA2-deleted strains. To determine the identities of the resident virus strains in selected lines, we PCR amplified and sequenced across regions in the EBNA1 (codons 471 to 502) and LMP1 (codons 106 to 151) open reading frames. Full details of these procedures are given elsewhere (4, 14). Sequences were compared to the prototype B95.8 sequence (3).

Quantitative RT-PCR assays of EBV gene expression. Total RNA was extracted from 5×10^6 cells by using a Nucleospin RNA extraction kit (Macherery Nagel) according to the manufacturer's instructions. Approximately 400 ng RNA was transcribed into cDNA by using a mix of primers specific for various EBV transcripts as described previously (4). Quantitative reverse transcription-PCR (RT-PCR) assays to detect the Wp-initiated, Cp-initiated, EBNA2, and LMP1 mRNAs and BamHI Q-U-K-spliced EBNA1 latent transcripts and the BamHI FQ-U-K spliced lytic transcript were used (4). Levels of transcription in test cells are expressed relative to that seen in an appropriate positive control cell line, which was assigned an arbitrary value of 1. Control cell lines used include the Wp-using X50-7 LCL for Wp-initiated, EBNA2, and LMP1 transcripts; a standard B95.8 virus-transformed PS-LCL for Cp-initiated transcripts; the latency I line Rael-BL for Qp-initiated EBNA1 transcripts; and the Sal-LCL (containing 5 to 10% lytic cells) for Fp-initiated transcripts.

Western blot analysis of EBV protein expression. Blotting was carried out using monoclonal antibodies (MAbs) 1H4 (anti-EBNA1) (12), PE2 (anti-EBNA2) (53), JF186 (anti-EBNA-LP) (11), E3CA10 (anti EBNA3C type1) (27), and CS1-4 (anti-LMP1) (38).

In vitro infection with EBNA2 gene-deleted recombinant EBV. An EBNA2 gene-deleted EBV genome was made by homologous recombination between the p2089 EBV plasmid (8) and a shuttle vector (p2914) containing a fragment of the EBV genome (B95.8 coordinates 45016 to 53221) with an internal EBNA2 deletion (B95.8 coordinates 48043 to 50280). The resulting p2491 EBV plasmid was stably introduced into HEK293 cells, and virus stocks were generated after transient transfection of expression plasmids carrying *BZLF1* and *BALF4* as described previously (9, 30). The EBV-negative cell lines BL2, BL31, and BL41 were exposed to this virus overnight at a multiplicity of infection of 50, and in each case EBV-converted lines were produced by selection in hygromycin at 100 μg/ml.

Apoptosis assays. Approximately 3×10^4 BL cells were seeded into wells of a flat-bottomed 96-well plate and treated with either a final concentration of 1 μg/ml ionomycin (Sigma) or 10 μg/ml anti-IgM antibody (ICN Flow) at 37°C. Following 48 h of ionomycin treatment or 72 h of anti-IgM treatment, cells were harvested, washed in 1× phosphate-buffered saline, and resuspended in 0.5 ml saline (prewarmed to 37°C). Syto 16 (Molecular Probes Europe, Leiden, The Netherlands) was added to the cells at a final concentration of 25 nM and incubated at room temperature for 1 h, at which time 2.5 μg/ml propidium iodide (Sigma) was added and the cells analyzed immediately on a flow cytometer as described previously (29). A two-dimensional dot plot of Syto 16 fluorescence (*y* axis) versus propidium iodide fluorescence (*x* axis) was generated. Syto 16 is taken up only by viable cells, and propidium iodide enters only cells whose membranes have become permeabilized; therefore, this technique distinguishes between viable cells (Syto 16 positive, propidium iodide negative), apoptotic cells (Syto 16 negative, propidium iodide negative), and necrotic cells (Syto 16 negative, propidium iodide positive) (44). Data for 10,000 cells were collected for each cell line.

RESULTS

Single-cell cloning of Wp-restricted BL lines. Figure 1 presents the different programs of EBV latent gene expression seen in classical latency I BL lines, where EBNA1 mRNA is selectively expressed from Qp; in Wp-restricted BL lines, where EBNA1, -3A, -3B, -3C, and -LP mRNAs are expressed from Wp; and in standard latency III LCLs, where all six EBNA mRNAs are transcribed principally from Cp (and to a

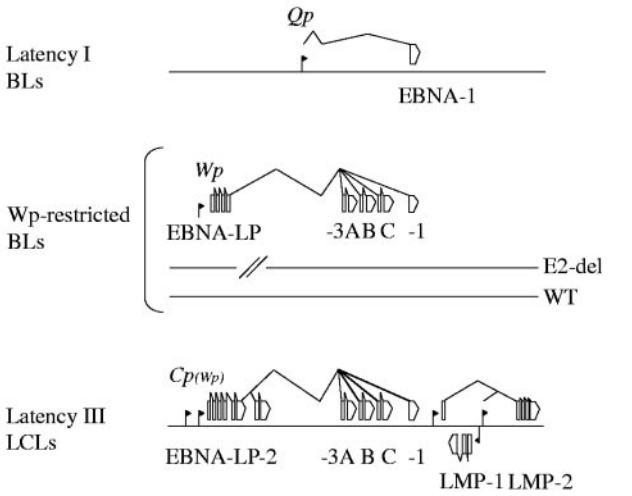
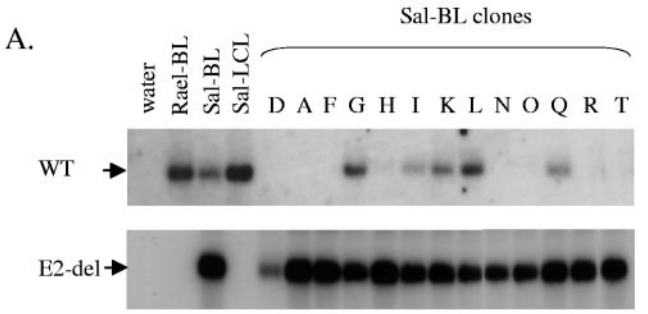


FIG. 1. Diagrammatic representation of the different patterns of EBV latent gene expression in BLs and LCLs. Conventional latency I BLs express EBNA1 only from the BamHI Q promoter. Atypical Wp-restricted BLs carry both wild-type and EBNA2-deleted genomes and express EBNA1, -3A, -3B, -3C, and -LP from the BamHI W promoter. Latency III LCLs express EBNA1, -2, -3A, -3B, -3C, and -LP predominantly from the BamHI C promoter and LMP1 and -2 from separate promoters in the BamHI N fragment.



B.

	Del +WT	WT only	Del only
Sal-BL	33/54	0/54	21/54
Oku-BL	10/47	0/47	37/47
Ava-BL	10/26	0/26	16/26

FIG. 2. A. DNA PCR analysis of EBNA2-positive wild-type (WT) (upper panel) and EBNA2-deleted mutant (Del) (lower panel) EBV genomes in Rael-BL, Sal-BL, Sal-LCL, and 13 Sal-BL clones. B. Summary of EBV genome contents in Sal-BL, Oku-BL, and Ava-BL clones; results are expressed as a fraction of the total number of clones analyzed for that cell line.

lesser extent from Wp) and the LMP1 and -2 mRNAs are transcribed from their separate promoters. As shown, the distinguishing characteristic of Wp-restricted BL lines is the presence of two EBV genomes, one a transformation-competent wild-type genome and the other an EBNA2 deletion mutant (18). The immediate objective of cell cloning experiments was to determine the distribution of these different genomes at the level of individual tumor cells and to look for correlations between genome content and patterns of virus latency.

In the first set of experiments three Wp-restricted BL lines, Sal-, Oku-, and Ava-BL, were seeded at 0.5 to 1 cell per well on a fibroblast feeder layer. Outgrowth efficiencies were in the range of 20 to 50%, which is fully consistent with growth being derived from a single cell. All clones were then screened by PCR amplification for the presence of wild-type genomes by using primers within the EBNA2 gene and for the presence of the EBNA2-deleted genomes by using primers from the BamHI W and H fragments immediately flanking the deletion. Figure 2A shows results from a representative set of Sal-BL clones screened alongside a typical latency I BL line (Rael-BL) carrying only a wild-type genome, the Sal-BL parent line itself carrying both wild-type and EBNA2-deleted genomes, and the Sal-LCL generated by transforming normal B cells with the wild-type genome rescued from Sal-BL. All 13 Sal-BL clones in Fig. 2A carried the EBNA2-deleted genome as detected by amplification of a BamHI W/H fusion fragment of the correct size, whereas only six of these clones contained a wild-type genome as detected by an amplifiable EBNA2 sequence. The overall results of these experiments are summarized in Fig. 2B. In all we screened 54 Sal-BL clones, 47 Oku-BL clones, and 26 Ava-BL clones. In each case, individual clones carried either both wild-type and EBNA2-deleted genomes or the EBNA2-deleted genome only. We never detected clones carrying only a wild-type genome.

Sequence analysis of wild-type and EBNA2-deleted genomes. Access to clones of Sal-, Oku-, and Ava-BL containing EBNA2-deleted genomes only thus allowed those genomes to be compared with the corresponding wild-type genomes that had been rescued from Sal-, Oku-, and Ava-BL parent lines by B-cell transformation to LCLs. In each case, we sequenced the EBNA2-deleted and wild-type genomes across regions of the EBNA1 and LMP1 genes previously shown to be polymorphic between different virus isolates, namely, EBNA1 codons 471 to 502 (14) and LMP1 codons 106 to 151 (4, 10).

As summarized in Table 1, at both the EBNA1 and LMP1 loci, virus strains from these three African BL patients shared many of the same sequence changes relative to the Caucasian B95.8 prototype. However in addition to these genetic markers of geographic origin, there were individual sequence changes in either EBNA1 or LMP1 that discriminated between the Sal-, Oku-, and Ava-BL isolates. Importantly, however, for each patient the EBNA2-deleted and wild-type genomes had identical sequences. This strongly suggests that in each case the EBNA2-deleted virus originated from the coresident wild-type virus strain and not from an independent source.

EBV gene expression in single-cell clones from Wp-restricted BL lines. For each of the three parent lines, we then studied between 3 and 6 clones that were positive for the EBNA2-deleted genome only and an equal number that were positive for both genomes. Viral transcription was analyzed using a set of recently developed quantitative RT-PCR assays designed to discriminate between different forms of EBV latency (4). One set of assays detected typical latency III-associated transcripts, namely, mRNAs initiated from Wp or from Cp as well as the individual EBNA2 and LMP1 mRNAs. An-

TABLE 1. Sequence analysis across EBNA1 and LMP1 polymorphic regions

Parameter	Sequence at indicated gene											
	EBNA1							LMP1				
Codon no.	471	476	487	492	499	500	502	106	126	129	150	151
Codon in B95.8	CAA	CCG	GCT	AGT	GAC	GAA	ACT	TTC	TTA	ATG	GAC	CTC
Amino acid	Q	P	A	S	D	E	T	F	L	M	D	L
Codon (amino acid) in[a]:												
Sal WT	gAA (E)	CaG (Q)	ctT (L)	tGT (C)	Gag (E)	Gat (D)	AaT (N)	TaC (Y)		Att (I)	agC (S)	aTC (I)
Sal Del	gAA (E)	CaG (Q)	ctT (L)	tGT (C)	Gag (E)	Gat (D)	AaT (N)	TaC (Y)		Att (I)	agC (S)	aTC (I)
Oku WT	gAA (E)	CaG (Q)	ctT (L)	tGT (C)	Gag (E)	Gat (D)	AaT (N)	TaC (Y)	TTt (F)	Att (I)	agC (S)	aTC (I)
Oku Del	gAA (E)	CaG (Q)	ctT (L)	tGT (C)	Gag (E)	Gat (D)	AaT (N)	TaC (Y)	TTt (F)	Att (I)	agC (S)	aTC (I)
Ava WT		CaG (Q)	aCT (T)	tGT (C)	Gat (D)			TaC (Y)	TTt (F)	Att (I)	agC (S)	aTC (I)
Ava Del		CaG (Q)	aCT (T)	tGT (C)	Gat (D)			TaC (Y)	TTt (F)	Att (I)	agC (S)	aTC (I)

[a] WT, wild-type virus; Del, EBNA2-deleted virus. Lowercase letters in codons indicate changes from the B95.8 sequence.

other assay detected the latency I-associated (BamHI Q-U-K-spliced) EBNA1 mRNA expressed from Qp; because this also detects an early lytic-cycle transcript from the upstream BamHI F promoter Fp, it was always used in conjunction with an assay specifically detecting the BamHI FQ-U-K-spliced RNA. Levels of transcription in test samples are expressed relative to that seen in an appropriate positive control standard cell line (assigned a value of 1). The relevant standard lines were the X50-7 LCL for Wp-initiated transcripts and for the EBNA2 and LMP1 mRNAs, the B95.8-transformed PS-LCL for Cp transcripts, Rael-BL for Qp transcripts, and the Sal-LCL (containing 5 to 10% cells in the lytic cycle) for Fp transcripts.

Similar patterns of results were obtained on all three parent BL cell backgrounds, and these are illustrated in Fig. 3 by representative data from four Sal-BL and four Oku-BL clones; these include clones carrying both wild-type and EBNA2-deleted genomes (Sal-BL clones Q and L and Oku-BL clones 4 and 16) and clones carrying only the EBNA2-deleted genome (Sal-BL clones N and O and Oku-BL clones 1 and 11). Irrespective of genome content, all clones showed patterns of transcription like that of the relevant parent BL line that was assayed in parallel. There was strong expression from Wp (at levels up to 12-fold higher than that seen in the Wp-using standard LCL X50-7) in the absence of any significant Cp activity or of any significant levels of EBNA2 and LMP1 mRNAs. Although some clones showed amplification of BamHI Q-U-K-spliced transcripts, this was not indicative of Qp usage since the same clones always gave detectable FQ-U-K-spliced RNA signals, reflecting the presence of some lytically infected cells within the culture. Note that Sal- and Oku-LCLs were also included as internal controls in these same assays and showed a typical latency III profile with dominance of Cp over Wp activity, expression of the EBNA2 and LMP1 mRNAs, and again (in the case of the Sal-LCL) some FQ-U-K-spliced transcripts from a subpopulation of lytically infected cells.

These same clones were then screened for EBV antigen expression by Western blotting using MAbs specific for EBNA1, EBNA-LP, EBNA2, EBNA3C, and LMP1. Figure 4 shows the relevant immunoblots. Sal-BL clones, whether carrying both genomes or the EBNA2-deleted genome only, gave protein profiles identical to that of the Sal-BL parent line, with expression of EBNA1, a ladder of (W_1W_2 repeat domain only) EBNA-LP species, and EBNA3C in the absence of any detectable EBNA2 or LMP1. All Oku-BL clones, again irrespective of EBV genome content, expressed EBNA1 and EBNA3C but in this case lacked detectable expression of an EBNA-LP species in addition to the absence of EBNA2 and LMP1. This reflects the fact that the Oku-BL parent line itself showed only low-level EBNA-LP expression (Fig. 4) and at the single-cell level was heterogeneous for EBNA-LP staining (data not shown). Clearly the above patterns are quite distinct from that seen in a reference LCL, in this case X50-7, where EBNA1, EBNA-LP, EBNA2, EBNA3C, and LMP1 are all constitutively expressed.

Wp-restricted latency in the Daudi- and P3HR1-BL cell lines. The above results from Sal-, Oku-, and Ava-BL cell clones prompted us to examine EBV gene expression in two other long-established BL cell lines known to carry EBNA2-deleted virus genomes only. These are Daudi-BL (21), whose EBNA2 gene-deleted status was identified long after its establishment in culture (17), and P3HR1-BL, which was originally derived in vitro by subcloning of a parent BL line, Jijoye (6, 16, 35). Importantly, we found that both of these lines show patterns of expression somewhat similar to Wp-restricted latency. Quantitative RT-PCR analyses detected high levels of Wp-initiated transcripts with no or very low Cp transcription, no EBNA2 mRNA, and either trace levels of or low LMP1 transcription. Furthermore, by immunoblotting, both lines showed detectable expression of EBNA1 and EBNA3C proteins in the absence of EBNA2, a pattern again typical of our Wp-restricted BL lines; LMP1 protein was undetectable in Daudi-BL but was occasionally present at trace levels in P3HR1-BL (data not shown).

Apoptosis assays on Wp-restricted BL lines and clones. In our earlier work, we found no obvious difference in cell morphology or cell growth between Wp-restricted BL lines and classical latency I BL lines when they were maintained under optimal culture conditions. Here we were interested to determine whether the broadening of latent-cycle antigen expression to include EBNA3A, -3B, -3C, and (in some cases) -LP in Wp-restricted cells might alter the cellular response to stress signals. Classical latency I lines are known to be susceptible to apoptosis induced by ligation of surface IgM (13). At 72 h postinduction, cultures were dually stained with Syto 16 to

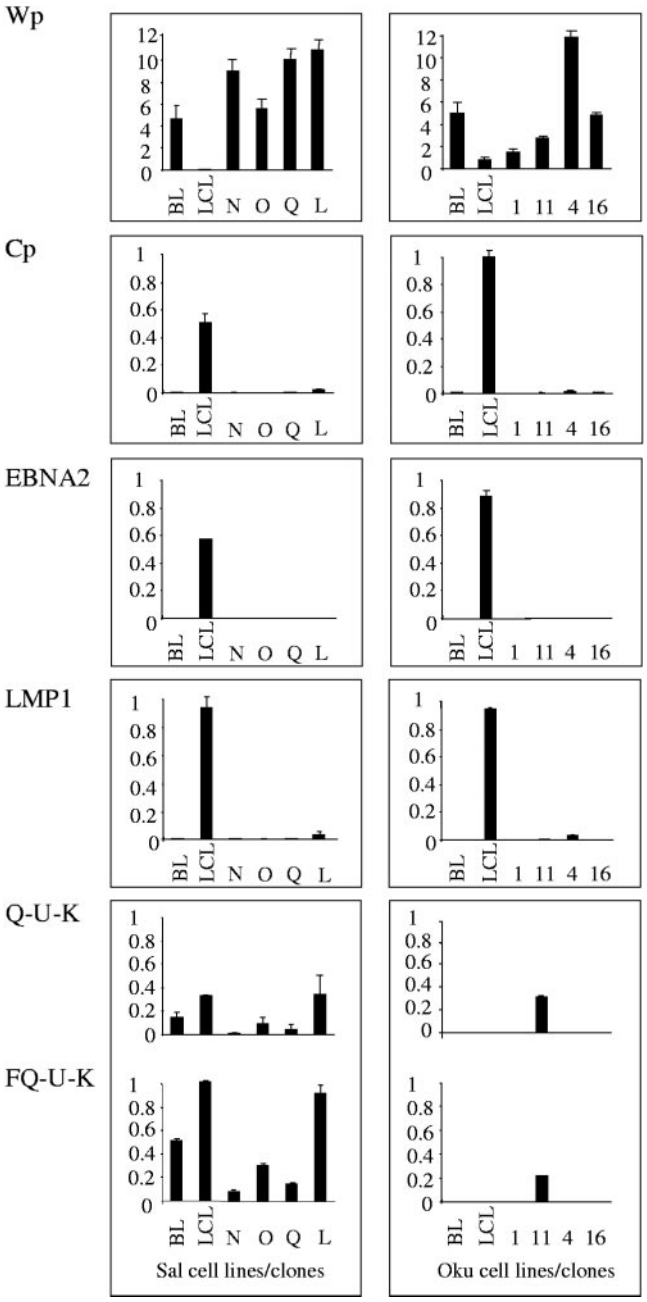


FIG. 3. Quantitative RT-PCR analyses of EBV gene expression in Sal-BL, Sal-LCL, Oku-BL, Oku-LCL, clones with EBNA2-deleted genomes only (Sal-BL clones N and O and Oku-BL clones 1 and 11), and clones with both wild-type and EBNA2-deleted genomes (Sal-BL clones Q and L and Oku-BL clones 4 and 16). Assays to detect Wp-initiated, Cp-initiated, EBNA2, LMP1, and BamHI Q-U-K-spliced EBNA1 latent transcripts and the BamHI FQ-U-K-spliced lytic transcript were used. Levels of transcription are expressed relative to that seen in an appropriate positive control cell line, which was assigned an arbitrary value of 1. Error bars indicate standard deviations.

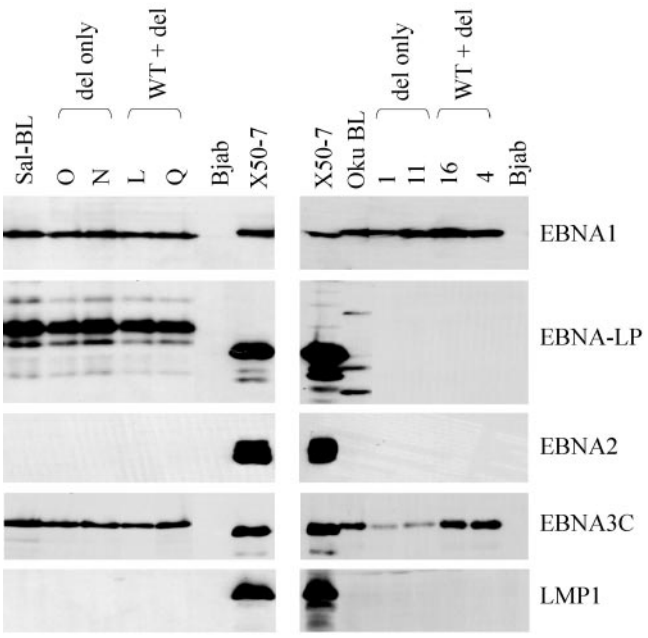


FIG. 4. Western blot analysis for expression of EBV latent antigens EBNA1, -LP, -2, and -3C and LMP1 in Sal-BL, Oku-BL, and derived cell clones (as described for Fig. 3); the EBV-negative B-cell lymphoma line BJAB was used as a negative control, and the X50-7 LCL was used as a positive control for blotting.

distinguish viable cells from dead cells and with propidium iodide (a dye which selectively enters necrotic cells) to distinguish necrotic cell death from apoptosis. Figure 5A shows typical FACS profiles of Syto 16 (*y* axis) and propidium iodide (*x* axis) staining for the standard latency I Sav-BL and the Wp-restricted Oku-BL lines with and without surface IgM ligation. In untreated cultures, around 85% of the cells in both lines were viable and appear in the top left quadrant of the profile. However, following exposure to the anti-IgM antibody, fewer than 10% of the Sav-BL cells remained viable and the great majority of dead cells were apoptotic (bottom left quadrant). By contrast, 75% of the Oku-BL cells remained viable and only 15% of cells were apoptotic. Throughout such assays we found that the number of BL cells dying by necrosis (bottom right quadrant) was always less than 20%, both for latency I lines and for Wp-restricted lines.

In all we screened eight latency I BL lines in these assays alongside the Wp-restricted BL lines, Sal-BL, Oku-BL, Ava-BL, Daudi-BL, and P3HR1-BL. Note that all of these lines were first shown to have equivalent levels of surface IgM expression, with the single exception of P3HR1-BL, which was surface IgM positive but with weaker staining.

Figure 5B summarizes the results of the anti-IgM induction assays, giving the mean levels of apoptosis induction at 72 h seen for each line in three or four replicate experiments of this type. There was a clear difference between the latency I cell lines as a group, where, depending upon the line in question, between 20 and 80% of cells were induced into apoptosis, and the Wp-restricted lines, where the range was 2 to 15% (with Daudi-BL reproducibly giving the highest value). These differences were not unique to anti-IgM-induced apoptosis but were also observed following exposure to the calcium ionophore ionomycin, which is also frequently used as an inducer of apoptosis in such cell systems (13) (data not shown). We then went on to repeat these assays using the same Sal-BL and Oku-BL clones as used for Fig. 3 and 4. The results of the

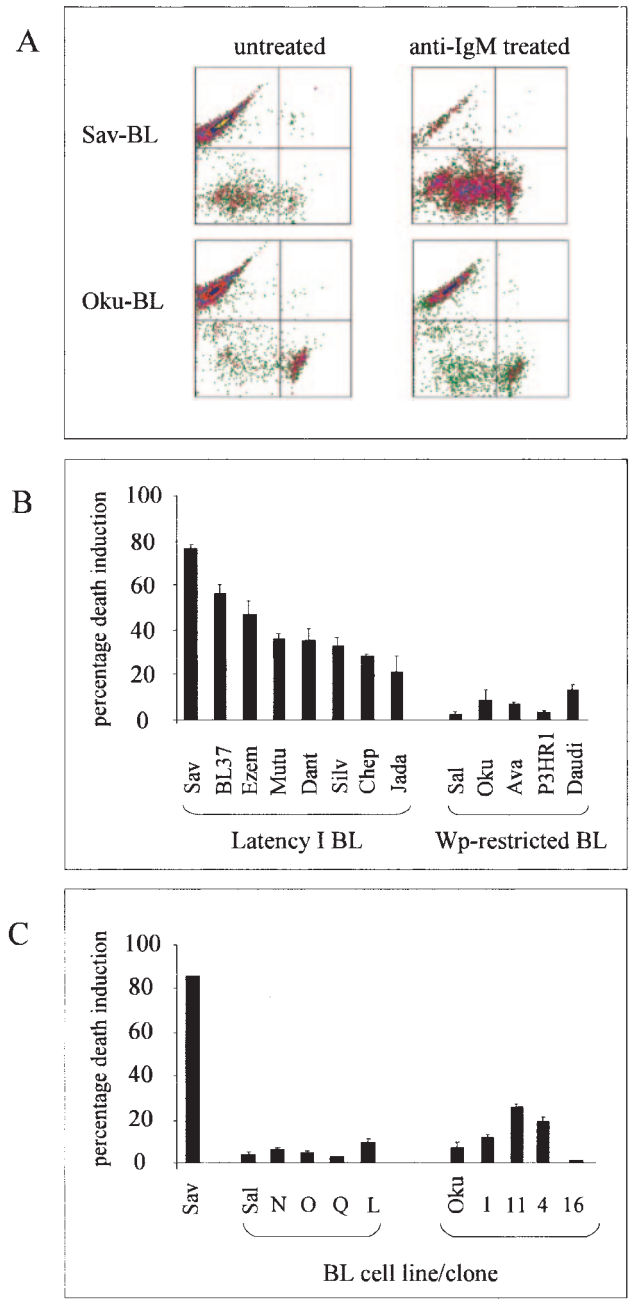


FIG. 5. Analysis of anti-IgM induced apoptosis in latency I and Wp-restricted BLs. A. FACS profiles of Syto 16 fluorescence (*y* axis) versus propidium iodide fluorescence (*x* axis) in the latency I Sav-BL and the Wp-restricted Oku-BL with (right panels) and without (left panels) 72 h of anti-IgM treatment. Cells in the top left quadrant represent viable cells, cells in the bottom left quadrant are apoptotic, and cells in the bottom right quadrant are necrotic. B and C. Summary of percent death induction following 72 h of anti-IgM treatment in a panel of latency I and Wp-restricted BLs (B) and in the latency I Sav-BL line (C) versus the same Sal-BL and Oku-BL parent lines and clones as in Fig. 3. Error bars indicate standard deviations.

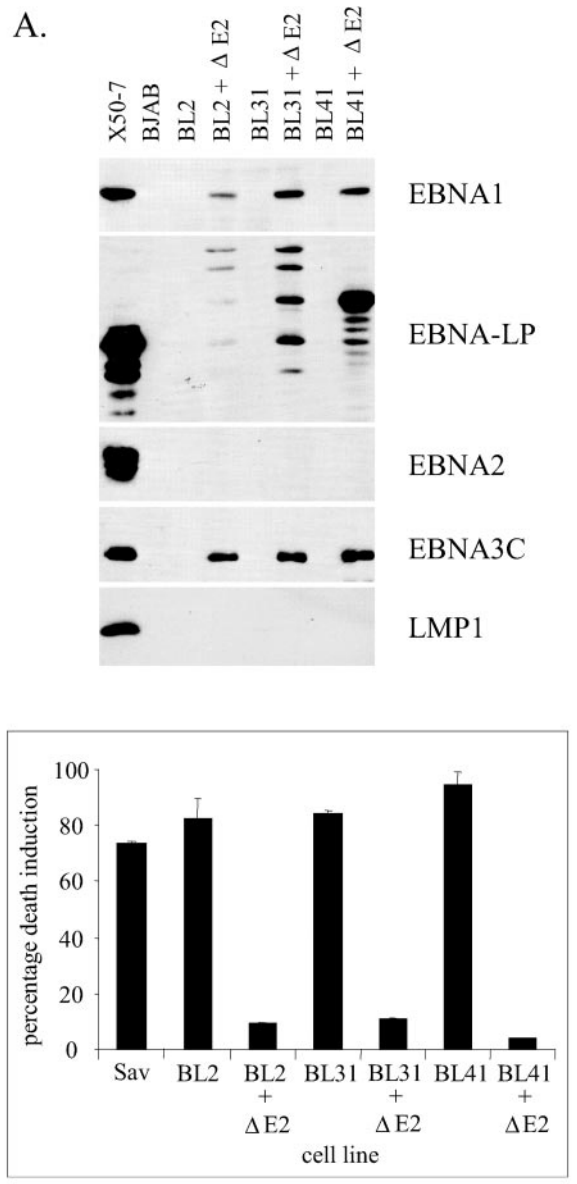


FIG. 6. Characterization of EBV-negative BL cell lines and EBV-positive converts infected with an EBNA2 gene-deleted recombinant EBV. A. Western blot analysis for expression of EBNA1, -LP, -2, and -3C and LMP1; the EBV-negative B-cell lymphoma line BJAB was used as a negative control, and the X50-7 LCL was used as a positive control for blotting. B. Results of apoptosis assays 48 h after ionomycin treatment; Sav-BL was used as a reference latency I BL line. Error bars indicate standard deviations.

anti-IgM-induction assays with these clones and their parent lines are shown in Fig. 5C, in each case comparing them with the latency I Sav-BL line. The assays confirmed that the observed Sal-BL and Oku-BL clones were again much more resistant to anti-IgM-induced apoptosis than latency I BL cells. These differences were also apparent in assays using ionomycin as the apoptosis trigger (data not shown).

In vitro infection of BL cells with recombinant EBNA2 gene-deleted EBV. To further study the association between EBNA2 gene deletion, Wp-restricted latency, and apoptosis resistance, we infected three EBV-negative BL cell lines (BL2, BL31, and BL41) with a recombinant hygromycin-resistant EBV strain from which the EBNA2 open reading frame had been deleted but in which the full EBNA-LP open reading frame remained

intact. Virus-positive converts of all three lines were selected in hygromycin and analyzed for EBV gene expression. As shown in Fig. 6A, all three converts expressed EBNA1 and EBNA3 proteins in the absence of EBNA2 and of LMP1; a ladder of full-length EBNA-LP species was also expressed in these converts, albeit at variable levels. This pattern of protein expression clearly resembled Wp-restricted latency, and indeed quantitative RT-PCR analyses confirmed strong transcription from Wp (and also some Cp transcripts) in the absence of any detectable Qp activity in these cells (data not shown). These converts were then tested alongside their EBV-negative parent BL lines in apoptosis assays. Figure 6B shows representative results from such experiments. The EBV-negative parent lines were as sensitive as the EBV-positive latency I Sav-BL cells to ionomycin treatment (and to anti-IgM [data not shown]), whereas the Wp-restricted converts were reproducibly as resistant to both triggers as the Wp-restricted Sal-BL, Oku-BL, and Ava-BL lines.

DISCUSSION

An earlier study had identified 3 of 15 early-passage endemic BL cell lines which diverged from the classical latency I form of infection and instead expressed Wp-initiated transcripts encoding EBNA1, -3A, -3B, and -3C and a truncated EBNA-LP (18). This atypical transcription pattern was not an artifact of in vitro passage, since in each case Wp was found to be active in the original tumor biopsy sample. Importantly, Southern blotting and PCR amplification showed that all three Wp-using BLs were also unique in carrying both an EBNA2-deleted genome and a wild-type EBV genome. The present study resolves a number of the issues raised by these findings.

First, we sought to determine the EBV genome content at the single-cell level in these BL lines. In all three cases clones derived from the early-passage cultures of the parental line all carried the EBNA2-deleted genome, either alone or with a coresident wild-type genome; no clones with only a wild-type genome were ever established. Furthermore, all clones resembled the parental line in cell growth phenotype and displayed the same Wp-restricted latency. Here we used real-time PCR assays, recently developed for a range of EBV transcripts (4), in order to characterize this pattern of latent gene transcription in quantitative as well as qualitative terms. This revealed that Wp-restricted latency is associated with very high levels of Wp activity, ranging from 2- to 12-fold higher than that seen in the X50-7 LCL control. It is worth noting that X50-7 is itself an unusual LCL which uses Wp exclusively; most standard LCLs (in which Cp is the dominant EBNA promoter) show much lower levels of Wp transcription. The contrast between Wp usage in the Sal-, Oku-, and Ava-BLs and that seen in standard LCLs is therefore even more striking.

When viral antigen expression was examined, all the Sal-, Oku-, and Ava-BL clones resembled the parent lines in expressing EBNA1, -3A, -3B, and -3C in the absence of EBNA2 and the LMPs. Interestingly, however, there were differences between clones derived from Sal-BL and Oku-BL with respect to EBNA-LP protein status. All Sal-BL clones and the Sal-BL parent line consistently expressed high levels of EBNA–LP with a similar ladder of species reflecting the different numbers of W_1W_2 repeat exons used to encode the protein. By contrast, most Oku-BL clones (including all those illustrated in Fig. 4) lacked EBNA-LP expression; this is in accordance with the fact that EBNA–LP levels in the Oku-BL parent line itself are lower and many cells in that line were negative when stained with the JF186 antibody. We could not determine the status of Ava-BL in this context, since the Ava EBV strain has a sequence change in the W_1 repeat exon that abrogates recognition by this MAb (11).

The overall results from clonal analysis therefore strongly support the view that, for all three Wp-restricted tumors, the EBNA2-deleted genome was present in every tumor cell. At least some of these cells also carried a wild-type genome, but this was clearly not essential for continued cell growth. The fact that all clones, with or without wild-type genomes, displayed Wp-restricted latency further supports the view that in the original tumor viral transcripts are coming exclusively from the EBNA2-deleted genome. This immediately would explain the atypical pattern of EBNA expression, since, because of the deletion, the transcriptionally active genome cannot encode EBNA2 or the unique exons of EBNA-LP. The inference is that the wild-type genome is a silent passenger that is retained through the action of EBNA1 (the genome maintenance protein) expressed from the coresident EBNA2-deleted genome. It is known that EBV episomes can be lost from latently infected cells if there is no selective advantage to their retention (20, 49, 52). We infer this to be the reason underlying the loss of the wild-type virus genome from some cells in the tumor. Indeed, we have noticed that long-term in vitro passage of the parental lines themselves is associated with a progressive loss of wild-type genome number but stable retention of EBNA2-deleted genomes (data not shown). It will be interesting to see whether silencing of the wild-type genome in these tumor cells is associated with some form of selective epigenetic modification.

We further reasoned that if Wp restriction is a defining feature of infection with an EBNA2-deleted EBV, then we would see similar transcription in Daudi-BL and P3HR1-BL, both of which are long-term endemic BL-derived lines which are known to carry EBNA2-deleted (but not wild-type) genomes (6, 16, 17, 21, 35). This proved to be the case, emphasizing the potential parallels between these long-established lines and the ones described in our recent report (18). More importantly, in vitro infection of EBV-negative BL cell lines with a recombinant EBNA2-deleted EBV strain reproducibly generated EBV-converted lines with a Wp-restricted form of latency, expressing EBNA1, -3A, -3B, -3C, and -LP in the absence of EBNA2 or the LMPs. These findings further support the view that the existence of Wp-restricted latency in endemic BL tumors is a direct consequence of the presence of an EBNA2 gene-deleted virus.

To try to understand the origin of the EBNA2-deleted genome in the Sal-BL, Oku-BL, and Ava-BL lines, we took advantage of the fact that in each case the EBNA2-deleted genome had been isolated in BL subclones and the wild-type genome had been rescued in an in vitro-transformed LCL. Sequencing across two polymorphic regions of the genome, in EBNA1 and LMP1, was able to distinguish between the Sal, Oku, and Ava virus strains but in each case the EBNA2-deleted and wild-type virus pairs were identical. This strongly supports the view that for each tumor the coresident EBNA2-

deleted and wild-type genomes are of the same lineage and do not represent independent virus strains. This was important to show because recent results strongly suggest that most EBV-infected individuals carry more than one EBV strain (47). How the EBNA2 deletion arose is not clear, but the fact that EBNA2 gene rearrangements have been detected regularly in oral hairy leukoplakia lesions (51) and occasionally in healthy donor throat washings (48) suggests that the present EBNA2-deleted genomes probably arose at sites of virus replication in vivo. Although such a transformation-defective virus might be thought to be incapable of establishing itself in the long-lived memory B-cell pool (2), it may still be able to do so if it coinfects a target B cell at the same time as a transformation-competent wild-type virus. There may therefore be circumstances in which dually infected cells form part of the B-cell pool from which BL arises.

Exactly when the EBNA2 deletion mutant was acquired during the development of the Sal, Oku, and Ava tumors remains a matter of speculation, but a more tractable question is why such a rare product of recombination should be so frequently associated with endemic BL. Studies on Akata-BL, a latency I BL cell line which carries a wild-type EBV genome but which is unusual in readily generating EBV-negative subclones in vitro (45), have suggested that latency I functions, in particular the EBERs, confer enhanced resistance to apoptosis (23, 24, 40, 41). Such an effect could offer a particular advantage during the pathogenesis of BL, since the cardinal feature of this tumor, high-level c-*myc* expression, drives cell growth but also sensitizes the cells to apoptotic signals (28). Indeed, BL itself has a high apoptotic rate within the tumor population in vivo, albeit more than matched by the very high rate of cell proliferation (5). We were interested to see whether the broadening of EBV gene expression in Wp-restricted tumors conferred even further resistance to apoptosis. Experiments comparing the responses of latency I and Wp-restricted BL lines to two different apoptotic triggers strongly suggested that Wp-restricted latency did bring a marked additional advantage. This was consistently observed whether the parental BL lines themselves or their derived clones were tested and was independent of the presence or absence of the wild-type genome. Furthermore, the same marked effect was apparent where a Wp-restricted type of infection was established in EBV-negative BL lines by using a recombinant EBNA2 gene-deleted virus. In principle any of four viral proteins, EBNA3A, -3B, -3C, and -LP, could be mediators of enhanced survival. However, the fact that Oku-BL clones lacking detectable EBNA-LP expression still retained that advantage leads us to argue that one or more of the EBNA3 proteins are responsible for the effect. It is known from the best-studied member of this family, EBNA3C, that the EBNA3 proteins are likely to have multiple effects on cell cycle progression (1, 22, 25, 32, 33, 50), and in addition at least one of these proteins, EBNA3B, has been associated with increased BL cell survival in transfection assays (46).

The most important point is that in the pathogenesis of endemic BL, the growth-promoting effect of deregulated c-*myc* expression needs to be complemented by EBV functions, yet it seems that latent EBV infection can be tolerated by the tumor clone only if EBNA2 is switched off. This was reflected in an in vitro reconstruction of c-*myc*/EBV complementation, where high c-*myc* expression appeared to be incompatible with the expression either of EBNA2 itself or of an EBNA2-regulated gene (31, 34). One way to retain EBV without EBNA2 is through classical latency I infection, with Qp-mediated expression of EBNA1 guaranteeing maintenance of the viral genome (39, 42, 43). Another way, which confers an even greater survival advantage over that usually provided by latency I, is through Wp-restricted latency, but arguably this is possible only where the preneoplastic clone happens to contain an EBNA2-deleted virus genome.

ACKNOWLEDGMENT

This work was supported by Cancer Research UK.

REFERENCES

1. **Allday, M. J., and P. J. Farrell.** 1994. Epstein-Barr virus nuclear antigen EBNA3C/6 expression maintains the level of latent membrane protein 1 in G_1-arrested cells. J. Virol. **68:**3491–3498.
2. **Babcock, G. J., L. L. Decker, M. Volk, and D. A. Thorley-Lawson.** 1998. EBV persistence in memory B cells in vivo. Immunity **9:**395–404.
3. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, and C. Seguin.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature **310:**207–211.
4. **Bell, A., K. Groves, G. Kelly, D. Croom-Carter, E. Hui, A. Chan, and A. Rickinson.** Analysis of EBV latent gene expression in endemic Burkitt lymphoma and nasopharyngeal carcinoma tumour cells using quantitative real time PCR assays. Submitted for publication.
5. **Berard, C. W.** 1985. Morphological definition of Burkitt's tumour: historical review and present status. IARC Sci. Pub. **60:**31–35.
6. **Bornkamm, G. W., J. Hudewentz, U. K. Freese, and U. Zimber.** 1982. Deletion of the nontransforming Epstein-Barr virus strain P3HR-1 causes fusion of the large internal repeat to the DSL region. J. Virol. **43:**952–968.
7. **Calender, A., M. Billaud, J. P. Aubry, J. Banchereau, M. Vuillaume, and G. M. Lenoir.** 1987. Epstein-Barr virus (EBV) induces expression of B-cell activation markers on in vitro infection of EBV-negative B-lymphoma cells. Proc. Natl. Acad. Sci. USA **84:**8060–8064.
8. **Delecluse, H. J., T. Hilsendegen, D. Pich, R. Zeidler, and W. Hammerschmidt.** 1998. Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. Proc. Natl. Acad. Sci. USA **95:**8245–8250.
9. **Dirmeier, U., B. Neuhierl, E. Kilger, G. Reisbach, M. L. Sandberg, and W. Hammerschmidt.** 2003. Latent membrane protein 1 is critical for efficient growth transformation of human B cells by Epstein-Barr virus. Cancer Res. **63:**2982–2989.
10. **Edwards, R. H., D. Sitki-Green, D. T. Moore, and N. Raab-Traub.** 2004. Potential selection of LMP1 variants in nasopharyngeal carcinoma. J. Virol. **78:**868–881.
11. **Finke, J., M. Rowe, B. Kallin, I. Ernberg, A. Rosen, J. Dillner, and G. Klein.** 1987. Monoclonal and polyclonal antibodies against Epstein-Barr virus nuclear antigen 5 (EBNA-5) detect multiple protein species in Burkitt's lymphoma and lymphoblastoid cell lines. J. Virol. **61:**3870–3878.
12. **Grasser, F. A., P. G. Murray, E. Kremmer, K. Klein, K. Remberger, W. Feiden, G. Reynolds, G. Niedobitek, L. S. Young, and N. Mueller-Lantzsch.** 1994. 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 **84:**3792–3798.
13. **Gregory, C. D., C. Dive, S. Henderson, C. A. Smith, G. T. Williams, J. Gordon, and A. B. Rickinson.** 1991. Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. Nature **349:**612–614.
14. **Habeshaw, G., Q. Y. Yao, A. I. Bell, D. Morton, and A. B. Rickinson.** 1999. Epstein-Barr virus nuclear antigen 1 sequences in endemic and sporadic Burkitt's lymphoma reflect virus strains prevalent in different geographic areas. J. Virol. **73:**965–975.
15. **Heston, L., M. Rabson, N. Brown, and G. Miller.** 1982. New Epstein-Barr virus variants from cellular subclones of P3J-HR-1 Burkitt lymphoma. Nature **295:**160–163.
16. **Hinuma, Y., and J. T. Grace.** 1968. Cloning of Burkitt lymphoma cells cultured *in vitro*. Cancer **22:**1089–1095.
17. **Jones, M. D., L. Foster, T. Sheedy, and B. E. Griffin.** 1984. The EB virus genome in Daudi Burkitt's lymphoma cells has a deletion similar to that observed in a non-transforming strain (P3HR-1) of the virus. EMBO J. **3:**813–821.
18. **Kelly, G., A. Bell, and A. Rickinson.** 2002. Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. Nat. Med. **8:**1098–1104.

19. **Kieff, E., and A. Rickinson.** 2001. Epstein-Barr virus and its replication, p. 2511–2573. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. II. Lippincott, Williams & Raven, Philadelphia, Pa.
20. **Kirchmaier, A. L., and B. Sugden.** 1995. Plasmid maintenance of derivatives of *oriP* of Epstein-Barr virus. J. Virol. **69:**1280–1283.
21. **Klein, E., G. Klein, J. S. Nadkarni, J. J. Nadkarni, H. Wigzell, and P. Clifford.** 1968. Surface IgM-kappa specificity on a Burkitt lymphoma cell *in vivo* and in derived culture lines. Cancer Res. **28:**1300–1310.
22. **Knight, J. S., N. Sharma, D. E. Kalman, and E. S. Robertson.** 2004. A cyclin-binding motif within the amino-terminal homology domain of EBNA3C binds cyclin A and modulates cyclin A-dependent kinase activity in Epstein-Barr virus-infected cells. J. Virol. **78:**12857–12867.
23. **Komano, J., S. Maruo, K. Kurozumi, T. Oda, and K. Takada.** 1999. Oncogenic role of Epstein-Barr virus-encoded RNAs in Burkitt's lymphoma cell line Akata. J. Virol. **73:**9827–9831.
24. **Komano, J., M. Sugiura, and K. Takada.** 1998. Epstein-Barr virus contributes to the malignant phenotype and to apoptosis resistance in Burkitt's lymphoma cell line Akata. J. Virol. **72:**9150–9156.
25. **Krauer, K. G., A. Burgess, M. Buck, J. Flanagan, T. B. Sculley, and B. Gabrielli.** 2004. The EBNA-3 gene family proteins disrupt the G2/M checkpoint. Oncogene **23:**1342–1353.
26. **Magrath, I.** 1990. The pathogenesis of Burkitt's lymphoma. Adv. Cancer Res. **55:**133–270.
27. **Maunders, M. J., L. Petti, and M. Rowe.** 1994. Precipitation of the Epstein-Barr virus protein EBNA-2 by an EBNA-3c-specific monoclonal antibody. J. Gen. Virol. **75:**769–778.
28. **Milner, A. E., R. J. Grand, C. M. Waters, and C. D. Gregory.** 1993. Apoptosis in Burkitt lymphoma cells is driven by c-myc. Oncogene **8:**3385–3391.
29. **Milner, A. E., D. H. Palmer, E. A. Hodgkin, A. G. Eliopoulos, P. G. Knox, C. J. Poole, D. J. Kerr, and L. S. Young.** 2002. Induction of apoptosis by chemotherapeutic drugs: the role of FADD in activation of caspase-8 and synergy with death receptor ligands in ovarian carcinoma cells. Cell Death Differ. **9:**287–300.
30. **Neuhierl, B., R. Feederle, W. Hammerschmidt, and H. J. Delecluse.** 2002. Glycoprotein gp110 of Epstein-Barr virus determines viral tropism and efficiency of infection. Proc. Natl. Acad. Sci. USA **99:**15036–15041.
31. **Pajic, A., M. S. Staege, D. Dudziak, M. Schuhmacher, D. Spitkovsky, G. Eissner, M. Brielmeier, A. Polack, and G. W. Bornkamm.** 2001. Antagonistic effects of c-myc and Epstein-Barr virus latent genes on the phenotype of human B cells. Int. J. Cancer **93:**810–816.
32. **Parker, G. A., T. Crook, M. Bain, E. A. Sara, P. J. Farrell, and M. J. Allday.** 1996. Epstein-Barr virus nuclear antigen (EBNA) 3C is an immortalizing oncoprotein with similar properties to adenovirus E1A and papillomavirus E7. Oncogene **13:**2541–2549.
33. **Parker, G. A., R. Touitou, and M. J. Allday.** 2000. Epstein-Barr virus EBNA3C can disrupt multiple cell cycle checkpoints and induce nuclear division divorced from cytokinesis. Oncogene **19:**700–709.
34. **Polack, A., K. Hortnagel, A. Pajic, B. Christoph, B. Baier, M. Falk, J. Mautner, C. Geltinger, G. W. Bornkamm, and B. Kempkes.** 1996. c-myc activation renders proliferation of Epstein-Barr virus (EBV)-transformed cells independent of EBV nuclear antigen 2 and latent membrane protein 1. Proc. Natl. Acad. Sci. USA **93:**10411–10416.
35. **Rabson, M., L. Gradoville, L. Heston, and G. Miller.** 1982. Nonimmortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye. J. Virol. **44:**834–844.
36. **Rickinson, A. B., and E. Kieff.** 2001. Epstein-Barr virus, p. 2575–2627. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. II. Lippincott, Williams & Raven, Philadelphia, Pa.
37. **Rooney, C. M., C. D. Gregory, M. Rowe, S. Finerty, C. Edwards, H. Rupani, and A. B. Rickinson.** 1986. Endemic Burkitt's lymphoma: phenotypic analysis of tumor biopsy cells and of derived tumor cell lines. J. Natl. Cancer Inst. **77:**681–687.
38. **Rowe, M., H. S. Evans, L. S. Young, K. Hennessy, E. Kieff, and A. B. Rickinson.** 1987. Monoclonal antibodies to the latent membrane protein of Epstein-Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells. J. Gen. Virol. **68:**1575–1586.
39. **Rowe, M., D. T. Rowe, C. D. Gregory, L. S. Young, P. J. Farrell, H. Rupani, and A. B. Rickinson.** 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J. **6:**2743–2751.
40. **Ruf, I. K., P. W. Rhyne, C. Yang, J. L. Cleveland, and J. T. Sample.** 2000. Epstein-Barr virus small RNAs potentiate tumorigenicity of Burkitt lymphoma cells independently of an effect on apoptosis. J. Virol. **74:**10223–10228.
41. **Ruf, I. K., P. W. Rhyne, H. Yang, C. M. Borza, L. M. Hutt-Fletcher, J. L. Cleveland, and J. T. Sample.** 1999. Epstein-Barr virus regulates c-MYC, apoptosis, and tumorigenicity in Burkitt lymphoma. Mol. Cell. Biol. **19:**1651–1660.
42. **Sample, J., L. Brooks, C. Sample, L. Young, M. Rowe, C. Gregory, A. Rickinson, and E. Kieff.** 1991. 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 **88:**6343–6347.
43. **Schaefer, B. C., M. Woisetschlaeger, J. L. Strominger, and S. H. Speck.** 1991. Exclusive expression of Epstein-Barr virus nuclear antigen 1 in Burkitt lymphoma arises from a third promoter, distinct from the promoters used in latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **88:**6550–6554.
44. **Schuurhuis, G. J., J. W. Oberink, P. M. Brontje, and H. J. Broxterman.** 1997. The PGP specific combination Syto(R)16/PSC833 detects early apoptosis in CD34 positive progenitor cells. Exp. Haematol. **25:**754.
45. **Shimizu, N., A. Tanabe-Tochikura, Y. Kuroiwa, and K. Takada.** 1994. Isolation of Epstein-Barr virus (EBV)-negative cell clones from the EBV-positive Burkitt's lymphoma (BL) line Akata: malignant phenotypes of BL cells are dependent on EBV. J. Virol. **68:**6069–6073.
46. **Silins, S. L., and T. B. Sculley.** 1995. Burkitt's lymphoma cells are resistant to programmed cell death in the presence of the Epstein-Barr virus latent antigen EBNA-4. Int. J. Cancer **60:**65–72.
47. **Sitki-Green, D., M. Covington, and N. Raab-Traub.** 2003. Compartmentalization and transmission of multiple Epstein-Barr virus strains in asymptomatic carriers. J. Virol. **77:**1840–1847.
48. **Sixbey, J. W., P. Shirley, M. Sloas, N. Raab-Traub, and V. Israele.** 1991. A transformation-incompetent, nuclear antigen 2-deleted Epstein-Barr virus associated with replicative infection. J. Infect. Dis. **163:**1008–1015.
49. **Sugden, B., and N. Warren.** 1988. Plasmid origin of replication of Epstein-Barr virus, *oriP*, does not limit replication in *cis*. Mol. Cell. Biol. **5:**85–94.
50. **Wade, M., and M. J. Allday.** 2000. Epstein-Barr virus suppresses a $G(_2)$/M checkpoint activated by genotoxins. Mol. Cell. Biol. **20:**1344–1360.
51. **Walling, D. M., S. N. Edmiston, J. W. Sixbey, M. Abdel-Hamid, L. Resnick, and N. Raab-Traub.** 1992. Coinfection with multiple strains of the Epstein-Barr virus in human immunodeficiency virus-associated hairy leukoplakia. Proc. Natl. Acad. Sci. USA **89:**6560–6564.
52. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81:**3806–3810.
53. **Young, L., C. Alfieri, K. Hennessy, H. Evans, C. O'Hara, K. C. Anderson, J. Ritz, R. S. Shapiro, A. Rickinson, and E. Kieff.** 1989. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. N. Engl. J. Med. **321:**1080–1085.