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The Epstein-Barr Virus (EBV) Nuclear Antigen 1 *Bam*HI F Promoter Is Activated on Entry of EBV-Transformed B Cells into the Lytic Cycle

A. L. LEAR,[1] M. ROWE,[1] M. G. KURILLA,[2]† S. LEE,[1] S. HENDERSON,[1] E. KIEFF,[2] AND A. B. RICKINSON[1]*

Department of Cancer Studies, Cancer Research Campaign Laboratories, University of Birmingham, Birmingham B15 2TJ, United Kingdom,[1] and Brigham and Women's Hospital, Boston, Massachusetts 02115[2]

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In Epstein-Barr virus (EBV)-positive Burkitt's lymphoma cell lines exhibiting the latency I form of infection (i.e., EBV nuclear antigen 1 [EBNA1] positive in the absence of other latent proteins), the EBNA1 mRNA has a unique *Bam*HI Q/U/K splice structure and is expressed from a novel promoter, Fp, located near the *Bam*HI FQ boundary. This contrasts with the situation in EBV-transformed lymphoblastoid cell lines (LCLs) exhibiting the latency III form of infection (i.e., positive for all latent proteins), in which transcription from the upstream Cp or Wp promoters is the principal source of EBNA mRNAs. We carried out cDNA amplifications with oligonucleotide primer-probe combinations to determine whether Fp is ever active in an LCL environment. The results clearly showed that some LCLs express a Q/U/K-spliced EBNA1 mRNA in addition to the expected Cp/Wp-initiated transcripts; this seemed inconsistent with the concept of Cp/Wp and Fp as mutually exclusive promoters. Here we show that Fp is indeed silent in latency III cells but is activated at an early stage following the switch from latency III into the virus lytic cycle. Four pieces of evidence support this conclusion: (i) examples of coincident Cp/Wp and Fp usage in LCLs are restricted to those lines in which a small subpopulation of cells have spontaneously entered the lytic cycle; (ii) transcripts initiating from Fp can readily be demonstrated in spontaneously productive lines by S1 nuclease protection; (iii) the presence of Fp-initiated transcripts is not affected by acyclovir blockade of the late lytic cycle; and (iv) infection of latently infected LCLs with a recombinant vaccinia virus encoding the EBV immediate-early protein BZLF1, a transcriptional transactivator which normally initiates the lytic cycle, results in the appearance of the diagnostic Q/U/K-spliced transcripts.

Epstein-Barr virus (EBV), a herpesvirus widespread in human populations, is able to infect at least two lineages of target cell in vivo, stratified pharyngeal epithelium and B lymphocytes. While the infection in epithelium appears to be largely productive (lytic) (16), B cells can harbor the virus as a nonproductive (latent) infection, thereby providing both a means of virus persistence and a reservoir from which infectious virus can be obtained by reactivation (33, 55). It is now apparent that EBV can adopt more than one form of latency in lymphocytes and that these alternative forms of infection are associated with distinct programs of viral transcription and viral promoter usage.

The standard in vitro model for EBV latency is that seen in virus-transformed lymphoblastoid cell lines (LCLs) produced by experimental infection of normal resting B cells (reviewed in references 26 and 50). In such lines, there are two major families of mRNA transcripts. Transcription from the *Bam*HI C promoter (Cp) or from the adjacent *Bam*HI W promoter (Wp) gives rise to mRNAs for each of the six EBV nuclear antigens (EBNAs), 1, 2, 3A, 3B, 3C, and LP (53), while transcription from promoters in *Bam*HI N gives rise to mRNAs for each of the latent membrane proteins (LMPs), 1, 2A, and 2B (22, 28, 44). This pattern of virus gene expression we now refer to as latency III. Note that LCLs either are completely nonproductive of virus particles or else contain a small subpopulation of cells which have switched spontaneously from latency III into lytic cycle. The mechanism of switching is not understood, but the first detectable change in viral gene expression is an activation of the EBV immediate-early genes BZLF1 and BRLF1 (14); the protein products of these genes then initiate the lytic-cycle cascade (2, 9, 18, 38).

Studies of the EBV-associated B-cell malignancy Burkitt's lymphoma (BL) have identified an alternative form of latency (latency I) characterized by selective expression of just one of the latent proteins, EBNA1 (17, 41). In this situation, the Cp/Wp and LMP promoters used in LCL cells are not active; instead, a unique *Bam*HI Q/U/K-spliced EBNA1 mRNA is expressed from an alternative promoter, Fp, near the *Bam*HI F/Q boundary (42, 45). On serial passage, those BL cell lines which retain the cellular phenotype of the original tumor (group I phenotype) continue to display latency I and rarely if ever show any spontaneous activation of individual cells into the lytic cycle. In contrast, other BL cell lines switch on serial passage from the latency I to the latency III transcriptional program and assume a lymphoblastoid (group III) phenotype typical of LCLs; again, such group III BL cell lines are either tightly latent or spontaneously productive in just a small subpopulation of cells (17).

The transcriptional differences between these alternative forms of EBV latency are at present understood only in outline, and many of the controls governing virus promoter usage remain to be elucidated. An immediate issue which we wished to resolve stemmed from recent work using cDNA

* Corresponding author.
† Present address: Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, VA 22908.

TABLE 1. Oligonucleotide sequences

Designation	EBV genome coordinates[a]	Sequence[a]
PCR EBNA1 5′ Q primer	62440–62457	5′-GTGCGCTACCGGATGGCG-3′
PCR EBNA1 5′ Y3 primer	48397–48416	5′-TGGCGTGTGACGTGGTGTAA-3′
PCR EBNA1 3′ K primer	107986–107967	5′-CATTTCCAGGTCCTGTACCT-3′
PCR EBNA1 U probe	67544–67563	5′-AGAGAGTAGTCTCAGGGCAT-3′
PCR BHRF1 5′ H2 primer	53830–53849	5′-GTCAAGGTTTCGTCTGTGTG-3′
PCR BHRF1 3′ H3 primer	54461–54480	5′-TTCTCTTGCTGCTAGCTCCA-3′
S1 mapping C1 probe	11319–11376	5′-CTGGGGGTCTTCGGTGTCCTTGTCTCTATGCCATCTGATCTAAAATTTGCAGCAGAAC-3′
S1 mapping WOW1 probe	14363–14410 14554–14565	5′-GTCTCCCCTAGGATTTGTGTGGACTCCTGGCGCTCTGATGCGACCAGAAATAGCTGCAGG-3′
S1 mapping FQ probe	62221–62279	5′-CTCCGGCGACCTAGTGGTCCCCTCCGGATCCCCCCTCCTCTATCCACCGCCGCCCCGG-3′

[a] From B95.8 genomic sequence (1).

amplification with splice-specific oligonucleotide primers to discriminate between Cp/Wp-driven and Fp-driven EBNA1 mRNAs (3, 42). Clearly, these primers have the potential to discriminate between the two forms of virus latency outlined above, but only if Cp/Wp usage and Fp usage are indeed mutually exclusive in latently infected cells. In this context, we were surprised to find a number of LCLs in which cDNA amplification revealed the presence not only of the expected Cp/Wp-driven transcripts but also of the Fp-driven EBNA1 mRNA (3); such an observation appeared inconsistent with the idea of mutually exclusive promoter usage. In this paper, we show that such examples of dual promoter usage are restricted to spontaneously productive LCLs and that Fp is activated when the cell switches from the latency III state into the lytic cycle.

MATERIALS AND METHODS

Cell lines. The panel of LCLs used in this work included the B95.8 virus producer cell line itself (32), several lines established by B95.8 virus-induced transformation of adult B cells (designated SW/B95, etc.) or of fetal B cells (IB4, FL389), and other lines of either adult or fetal origin carrying other EBV isolates (e.g., X50-7). All LCLs displayed the characteristic group III cellular phenotype (41). In addition, we used different subclones of the EBV-positive BL cell line Mutu, which displayed either a group I or a group III phenotype (17); the EBV-positive virus-producer BL cell line AG876, which showed a group III phenotype (36); and, as controls, the EBV-negative B-lymphoma cell lines Louckes-BL (51) and BJAB (31) and the EBV-negative osteosarcoma cell line TK$^-$-143 (37).

RNA isolation and analysis. Total cellular RNA was extracted from cell lines with RNAzol B (Cinna/Biotecx, Houston, Tex.) in accordance with the manufacturer's instructions.

Analysis of transcripts by PCR. Total RNA (2 μg) was heated for 2 min at 90°C and then rapidly cooled on ice. Reverse transcription using sequence-specific 3′ primers at a concentration of 2 μM was carried out in 1× polymerase chain reaction (PCR) buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg of gelatin per ml) with 200 μM nucleotides, 5 U of avian myeloblastosis virus reverse transcriptase (Pharmacia, Uppsala, Sweden), and 10 U of RNAguard RNase inhibitor (Pharmacia) at 42°C for 60 min. After addition of 5′ primer and 2.5 U of *Taq* polymerase (Boehringer, Mannheim, Germany), amplification was carried out over 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2.5 min. Aliquots of each reaction were analyzed on 2.5% agarose gels run in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) and capillary blotted onto Hybond N+ (Amersham International, Amersham, United Kingdom) membrane, and specific products were detected by using ^{32}P-5′-end-labeled oligonucleotide or ^{32}P universally labeled restriction fragment probes. The actual sequences and EBV genome coordinates of the oligonucleotide primers and probes used in this work are shown in Table 1 and are derived from the B95.8 strain sequence (1); the primer-probe combinations are detailed in the appropriate figure legends.

S1 nuclease protection. ^{32}P-5′-end-labeled oligonucleotide probes were made to map the 5′ ends of EBV latent transcripts originating from promoters in the *Bam*HI C, W, and F regions of the EBV genome. Probe sequences and EBV genome coordinates (1) are shown in Table 1. Total RNA (150 μg) was hybridized to probes overnight and then digested with 100 U of S1 nuclease (BRL, Paisley, Scotland) at 30°C for 30 min. Protected probe fragments were analyzed on 8% polyacrylamide sequencing gels.

Recombinant vaccinia viruses. Vaccinia virus recombinants expressing the immediate-early proteins BZLF1 (Vacc-BZ) and BRLF1 (Vacc-BR) and the early proteins BMLF1 (Vacc-BM) and BHRF1 (Vacc-BH) of the EBV lytic cycle were generated by standard methods of vector insertion into the vaccinia virus thymidine kinase (TK) gene; expression of the introduced EBV sequence (B95.8 strain) was in each case under the control of the vaccinia virus P7.5 early-late promoter (6). The Vacc-BZ recombinant contains the BZLF1 open reading frame cloned as a *Bam*HI-*Eco*RI fragment from the plasmid pUC-BZLF1 (38). The Vacc-BR recombinant contains the BRLF1 open reading frame cloned as a *Hin*dIII-*Sal*I EBV genomic fragment (coordinates 103084 to 105300). The Vacc-BM recombinant contains the BMLF1 exon (coordinates 82085 to 84126) of the BSLF2-BMLF1 coding sequence reconstructed from *Hin*dIII-*Nco*I (82085 to 83783) and *Nco*I-*Pst*I (83783 to 84126) genomic fragments; note that this recombinant encodes a truncated version of the BSLF2-BMLF1 protein lacking N-terminal sequences derived from the small first exon (BSLF2) but containing all of the sequences required for the transactivating function of the full-length protein (29). The Vacc-BH recombinant contains the BHRF1 open reading frame excised from a BHRF1 cDNA (34). The Vacc-TK$^-$ recombinant, free of EBV sequences, was used as a control.

Two tightly latent EBV-transformed LCLs, X50-7 and SG-LCL, were used as recipient cells for infection with the recombinant vaccinia viruses. Aliquots of 10^7 cells were infected at a multiplicity of infection of 5:1 for up to 12 h before being harvested and washed in cold phosphate-

buffered saline. Between 5×10^6 and 8×10^6 cells were taken for preparation of RNA, and the remaining cells were used for analysis of recombinant vaccinia virus protein expression by Western blotting (immunoblotting) of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis-separated cell lysates and by immunofluorescence staining of cell smears.

Analysis of EBV protein expression. Western blot analysis of endogenous and recombinant vaccinia virus-encoded EBV protein expression was performed as described elsewhere (17). Briefly, cell pellets were lysed in SDS-gel sample buffer, sonicated, boiled for 2 min, electrophoresed on a discontinuous SDS-acrylamide gel, and transferred to nitrocellulose filters. Samples were routinely separated on discontinuous Laemmli gels with 7.5% acrylamide resolving gels except that samples destined to be probed for the 17-kDa BHRF1 protein were resolved on a 10% acrylamide gel. The nitrocellulose filters were blocked with skim milk before being probed overnight with either polyspecific human sera or murine monoclonal antibodies. Reactive antibodies from the human sera were detected by incubating the filters with ^{125}I-protein A, while specific binding of monoclonal antibodies was detected with ^{125}I-protein A after the filters were first incubated with rabbit antibodies to mouse immunoglobulin G. For the detection of EBNAs 1, 2, 3A, 3B, and 3C, three selected human sera (PB, RS22, and AMo) were pooled and used diluted 1:100. For the simultaneous detection of several EBV lytic-cycle proteins, serum EE was used diluted 1:400. Under these conditions, serum EE shows weak reactivity with only one of the EBV latent proteins, EBNA2, but has unusually strong reactivity against several lytic-cycle proteins, including the immediate-early proteins BZLF1 and BRLF1 and the two major components of the early antigen EA(D) complex, BSLF2-BMLF1 and BMRF1. Note that serum EE does not detect the BHRF1 protein, a component of the early-antigen EA(R) complex; expression of BHRF1 was therefore determined by using the monoclonal antibody 5B11 (34).

Analysis of EBV lytic-cycle protein expression was also performed by indirect immunofluorescence staining of acetone-fixed cell smears with monoclonal antibodies as described elsewhere (40). The monoclonal antibodies used were BZ.1, which is reactive with BZLF1 (57); R3, which is reactive with BMRF1 (35); 5B11, which is reactive with BHRF1 (34); R63, which is reactive with BORF2, another component of the EA(R) complex (15); 72A1, which is reactive with the late membrane antigen gp340 (20); and V3, which is reactive with the late viral capsid antigen (VCA) (52).

RESULTS

EBNA1 mRNA analysis in a panel of LCLs. In initial experiments, a wide range of LCLs were screened for the presence of EBNA1 mRNAs with the *Bam*HI Q/U/K splicing pattern diagnostic of Fp usage; the same LCLs were simultaneously checked to confirm that the standard Cp/Wp-driven EBNA1 mRNAs with the *Bam*HI Y3/U/K splicing pattern were consistently present. All analysis included the following control cell lines: EBV-negative Loucke BL cells and latently infected subclones of the EBV-positive Mutu BL cell line which had either retained Fp usage on serial passage in vitro (group I clones) or switched to Cp/Wp usage (group III clones). Figure 1 shows representative results from 10 of the LCLs thus analyzed. In some LCLs, no Fp usage was ever detected (IB4, X50.7, FL389, SG/B95), whereas in others, a Q/U/K-spliced transcript was regularly amplified (AR/B95, CMc/B95, DH/B95, JT/B95, B95.8). All LCLs were originally derived by in vitro transformation and contained the expected Y3/U/K-spliced EBNA1 transcripts. Extension of this work to LCLs transformed with a range of virus isolates other than B95.8 gave a similar heterogeneity with respect to Fp usage (data not shown).

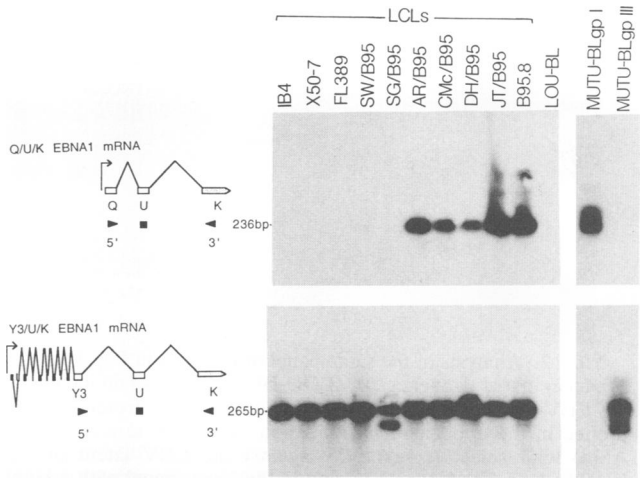

FIG. 1. PCR analysis of EBNA1 transcripts in a representative panel of LCLs analyzed in parallel with three reference cell lines, the EBV-negative sporadic BL cell line Loucke (LOU-BL), and phenotypically different nonproductive subclones of the EBV-positive endemic cell line Mutu exhibiting either latency I (Mutu-BLgp I) or latency III (Mutu-BLgp III). (Upper panel) Detection of EBNA1 mRNA with the Q/U/K-splicing pattern. Following reverse transcription with the EBNA1 3′ K primer (Table 1) from within the EBNA1 open reading frame and PCR amplification after addition of the EBNA1 5′ Q primer from within the noncoding Q exon of Fp-initiated transcripts, specific products of 236 bp were detected by Southern blotting with the EBNA1 U probe from the noncoding U exon. (Lower panel) Detection of EBNA1 mRNA with the Y3/U/K splicing pattern. Following reverse transcription with the EBNA1 3′ K primer described above and PCR amplification after addition of the EBNA1 5′ Y3 primer from within the noncoding Y3 exon of Cp/Wp-initiated transcripts, specific products of 265 bp were detected by Southern blotting with the EBNA1 U probe; note that analysis of the SG/B95 cell line reproducibly gave a doublet of bands with the Y3/U/K primer-probe combination.

Producer status of LCLs showing Fp usage. We noted that two of the LCLs lacking detectable Fp usage were the reference fetal cord blood cell lines IB4 and X50-7, already known not to show any spontaneous entry of cells into lytic cycle (27, 39). We therefore screened the entire panel of LCLs used as described above for virus producer status and found a complete concordance between the presence of a subpopulation of lytically infected cells and the detectability of an Fp-driven EBNA1 transcript. Figure 2A illustrates the pattern of results obtained when protein extracts of the 10 LCLs screened in Fig. 1 were analyzed for latent-cycle antigens (EBNAs 1, 2, 3A, B, and C; Fig. 1, upper panel) and lytic-cycle antigens (lower panel); note that the principal lytic-cycle components detected by immunoblotting with polyvalent human serum are the immediate-early protein BZLF1, visualized at 39 kDa (57), and the two early-antigen EA(D) components BSLF2-BMRF1, at 60 kDa (8), and BMRF1, at 50 to 55 kDa (35). Detectable expression of lytic-cycle products was clearly restricted to the five LCLs already identified as containing Fp-driven transcripts. In

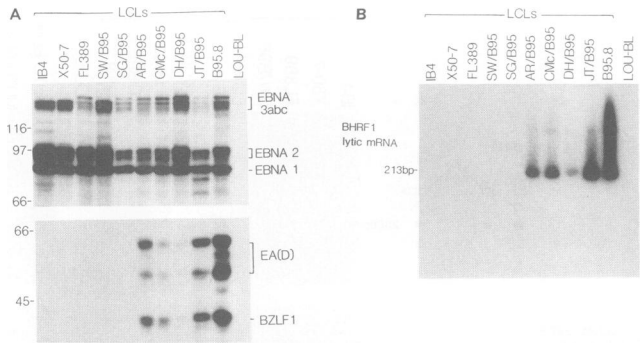

FIG. 2. Analysis of the same panel of LCLs as in Fig. 1 for virus producer status; Loucke-BL (LOU-BL) cells are again included as an EBV-negative control. (A) Immunoblot of protein extracts probed in the upper panel with a pool of human sera (PB, RS22, AMo) with selective reactivity against the EBV latent proteins EBNAs 1, 2, 3A, 3B, and 3C and in the lower panel with a human serum (EE) with high-titer antibodies to a range of lytic-cycle antigens, including the immediate-early protein BZLF1 (39 kDa) and the BSLF2-BMLF1 (60 kDa) and BMRF1 (50 to 55 kDa) components of the EA(D) complex. Positions of molecular size markers (in kilodaltons) are indicated on the left of each gel. (B) Detection of BHRF1 lytic-cycle mRNA with the class IV BHRF1 cDNA splice structure (34). Following reverse transcription with the BHRF1 3′ H3 primer and PCR amplification after addition of the BHRF1 5′ H2 primer, specific products of 213 bp were detected by Southern blotting with a restriction fragment probe of EBV genomic sequences (coordinates 52386 to 56081) incorporating the BHRF1 open reading frame.

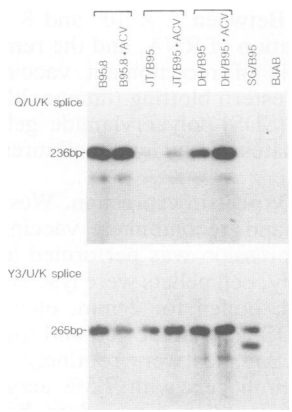

FIG. 3. PCR analysis of EBNA1 transcripts in three spontaneously productive LCLs (B95.8, JT/B95, and DH/B95) grown in normal medium or for 14 days in the presence of 200 μM ACV. Reference lines include latently infected LCL SG/B95 and EBV-negative B-lymphoma cell line BJAB. Assay for detection of the Fp-initiated Q/U/K-spliced transcript (upper panel) and the Cp/Wp-initiated Y3/U/K-spliced transcripts (lower panel) were as described in the legend to Fig. 1.

order to provide an independent index of virus producer status based on mRNA rather than protein analysis, we developed a PCR-based assay specific for the spliced 1.7-kb BHRF1 mRNA, an abundant transcript of the early lytic cycle (see class IV BHRF1 cDNA structure discussed in reference 34). As shown in Fig. 2B, this transcript was again restricted to the subset of LCLs which had been identified by immunoblotting as productive (cf. Fig. 2A) and which contained Fp-initiated transcripts (cf. Fig. 1).

Fp usage in ACV-treated producer cell lines. We next selected a number of LCLs for treatment with the nucleoside analog acyclovir (ACV), which is a proven inhibitor of EBV DNA replication that blocks progression from the early to the late phase of the lytic cycle (30). Figure 3 shows the results of PCR analysis of EBNA1 transcripts in three spontaneously productive cell lines (B95.8, JT/B95, and DH/B95) after maintenance for 14 days either in normal medium or in the presence of an inhibitory concentration (200 μM) of ACV. In each case, prolonged ACV treatment did not reduce the detectability of Q/U/K-spliced transcripts. At the same time, immunofluorescence staining of these ACV-treated LCLs for early- and late-lytic-cycle antigens confirmed the blockade of late lytic cycle.

Further experiments sought direct evidence that PCR detection of Q/U/K-spliced mRNAs in such lines was indeed associated with the presence of Fp-initiated transcripts. Two cell lines (B95.8 and AG876) with relatively high proportions of cells in lytic cycle (6 to 18%) and with easily detectable Q/U/K-spliced transcripts were cultured for 14 days in control and ACV-containing media as described above, and then total RNA was extracted for S1 nuclease protection analysis of radiolabeled oligonucleotide probes spanning the Cp, Wp, and Fp transcription start sites (C1, WOW1, and FQ probes, respectively). In addition to the expected protection of a 40-nucleotide (nt) fragment of the C1 probe by RNA from the Cp-using B95.8 cell line (53), there was also clear protection of 50- and 44- to 47-nt fragments of the FQ probe; this exactly mirrors the pattern of FQ protection already seen with EBNA1 mRNA from group I BL cells (42). The Wp-using AG876 cell line likewise showed protection of 50- and 44- to 47-nt fragments of the FQ probe, in this case along with the expected protection of a 40-nt fragment of the WOW1 probe (54). Again, ACV treatment had no effect on the detectability of Fp-initiated transcripts (Fig. 4).

BZLF1-mediated lytic-cycle entry activates Fp usage. The final set of experiments sought independent evidence for a link between lytic-cycle entry and Fp usage. It is known that the EBV immediate-early gene product BZLF1 is capable of activating the lytic cycle (9, 38), and we therefore first used the recombinant vaccinia virus Vacc-BZ to express BZLF1 in two nonproductive LCLs: X50-7, which is known to express EBNA transcripts from Wp (53), and SG/B95, which (in common with most established LCLs) expresses EBNA transcripts from Cp.

To confirm vaccinia virus-mediated expression of BZLF1, protein extracts from these two cell lines and from EBV-negative cell line 143 were prepared 12 h following infection with Vacc-BZ or with Vacc-TK$^-$ as a control. Figure 5A shows the results obtained when such extracts were immunoblotted and probed with the polyvalent human serum EE. It is clear that vaccinia virus-expressed BZLF1 is detectable in both LCLs as well as in 143 cells as a series of bands, i.e., a major band with an apparent molecular weight of 39,000 and two minor bands at higher apparent molecular weights (presumed to be multimers). Within the time frame of this experiment, we could find no evidence that Vacc-BZ infection of SG/B95 cells had induced any other antigens of the EBV lytic cycle, whether assayed by immunoblotting (Fig. 5A) or by monoclonal antibody staining for individual proteins of the early-antigen complex, namely, BMRF1, BHRF1, and BORF2 (data not shown). In the Vacc-BZ-infected X50-7 cells, however, we did observe induced expression of another protein of the early-antigen complex, namely, BSLF2-BMLF1, visualized as a major 60-kDa band

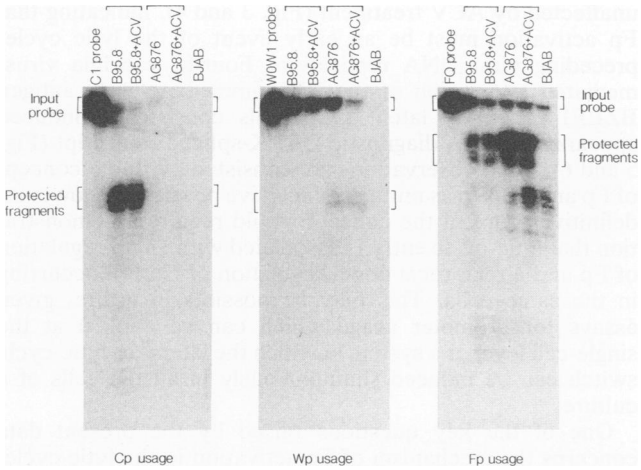

FIG. 4. S1 nuclease protection analysis of EBNA transcripts initiating from Cp, Wp, or Fp in the spontaneously productive cell lines B95.8 and AG876 grown either in normal medium or for 15 days in the presence of 200 μM ACV. The EBV-negative BJAB cell line is included as a negative control. The 5′-end-labeled oligonucleotide probes spanning the Cp, Wp, and Fp transcription start sites (58-nt C1, 60-nt WOW1, and 60-nt FQ probes, respectively; Table 1) were annealed to total cellular RNA, digested with S1 nuclease, and analyzed on 8% polyacrylamide sequencing gels. (Left panel) Specific protection of a 40-nt fragment of the C1 probe indicates Cp usage in B95.8 cells. (Middle panel) Specific protection of a 40-nt fragment of the WOW1 probe indicates Wp usage by AG876 cells. (Right panel) Specific protection of 50- and 44- to 47-nt fragments of the FQ probe indicates Fp usage in B95.8 and AG876 cells. Note that nonspecific protection of a 40- to 42-nt fragment of the FQ probe was observed with both AG876 and BJAB RNA preparations. Immunofluorescence staining of these B95.8 and AG876 cell cultures for lytic-cycle antigens gave the following: AG876, 18.1% BZLF1 positive and 15.0% VCA positive; AG876 plus ACV, 13.5% BZLF1 positive and <0.2% VCA positive; B95.8, 6.0% BZLF1 positive and 6.2% VCA positive; B95.8 and ACV, 4.4% BZLF1 positive and 0.3% VCA positive.

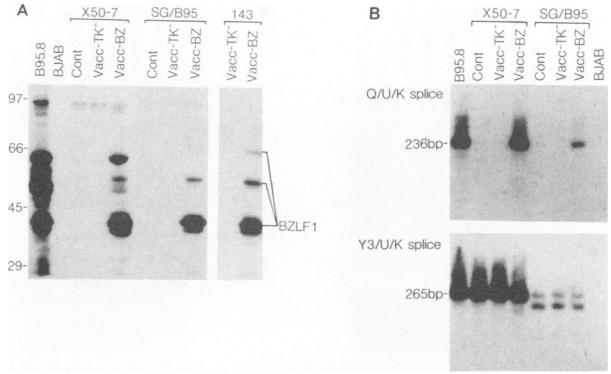

FIG. 5. Induction of Q/U/K-spliced EBNA1 transcripts in latently infected LCLs 12 h after infection with the BZLF1-expressing vaccinia virus recombinant Vacc-BZ. (A) Expression of EBV lytic-cycle proteins in control, Vacc-TK⁻-infected, and Vacc-BZ-infected X50-7 and SG-B95 cells as revealed by immunoblotting of protein extracts with human serum EE as in Fig. 2. Control cells in the analysis included the spontaneously productive B95.8 cell line, the EBV-negative B-cell line BJAB, and Vacc-TK⁻-infected and Vacc-BZ-infected EBV-negative 143 cells. Molecular size markers (in kilodaltons) are on the left. Note that Vacc-BZ-expressed BZLF1 is detectable as a major band at 39,000 (often resolvable as a doublet on shorter autoradiographic exposures) and as less-abundant higher-molecular-weight species presumed to be multimers; the same pattern of bands was also detected when the BZLF1-specific monoclonal antibody BZ1 was used (data not shown). No other EBV lytic-cycle antigens are detectable by immunoblotting in Vacc-BZ-infected SG/B95 cells. However, Vacc-BZ-infected X50-7 cells were induced to express the BSLF2-BMLF1 protein detectable as a major band at 60 kDa and as smaller minor species (8). There was no detectable induction of the 98-kDa BRLF1 protein. The faint doublets seen in all X50-7 tracks in this region of the gel stem from weak reactivity of serum EE against the X50-7 EBNA2 protein. (B) Expression of Q/U/K- and Y3/U/K-spliced EBNA1 transcripts in RNA preparations made in parallel with the protein extracts used for panel A and screened as for Fig. 1.

and smaller minor species (8) in immunoblots (Fig. 5A). Monoclonal antibody staining of the same cells for other early-antigen components remained negative (data not shown).

Analysis of RNA preparations made at the same time from the panel of control and recombinant vaccinia virus-infected LCL cells described above clearly showed that a Q/U/K-spliced EBNA1 transcript was induced as a result of Vacc-BZ infection. Figure 5B shows the results of one of several successive experiments of this kind; in each experiment, the Q/U/K-spliced PCR product was detectable after 12 h in Vacc-BZ-infected cells. When analyzed at regular intervals postinfection, the Q/U/K-spliced transcripts first appeared between 6 and 12 h postinfection in contrast to BZLF1 protein, which was first detectable at 2 h postinfection.

In further studies using X50-7 target cells, we asked whether vaccinia virus-mediated expression of other immediate-early or early proteins of the EBV lytic cycle could mimic the effect of BZLF1. The recombinants tested alongside Vacc-BZ included Vacc-BR, which encodes the 98-kDa immediate-early protein BRLF1; Vacc-BM, which encodes slightly truncated forms (designated BMLF1) of the 60-kDa early-antigen component BSLF2-BMLF1; and Vacc-BH, which encodes the 17-kDa early-antigen component BHRF1. Figure 6A shows immunoblots of protein extracts made from cells 12 h after infection with each of these individual recombinants or with Vacc-TK⁻ as a control. In the upper panel, these blots were probed with the polyvalent human serum EE (reactive against all the lytic-cycle antigens named above except BHRF1), and in the lower panel, they were probed with the BHRF1-specific monoclonal antibody 5B11. It is clear that each vaccinia virus recombinant expresses the relevant EBV lytic-cycle protein in X50-7 cells at levels in excess of those seen in extracts of the spontaneously productive B95.8 cell line. However, when RNA preparations made in parallel from these same vaccinia virus-infected cells were screened by PCR analysis, activation of Q/U/K-spliced EBNA1 transcripts was observed only with the Vacc-BZ recombinant (Fig. 6B).

DISCUSSION

Studies of a variety of cell culture and tumor systems have helped identify three forms of EBV latency. In latency 1, exemplified by BL cell lines retaining the group I cell phenotype, EBNA1 is expressed in the absence of all other latent proteins (17, 41). In latency II, exemplified by the EBV-positive epithelial tumor nasopharyngeal carcinoma, EBNA1 is expressed along with the LMPs (3, 5, 10, 48, 56). It is now clear that in both of these forms of latency, Cp and Wp are suppressed and the Fp-initiated Q/U/K-spliced tran-

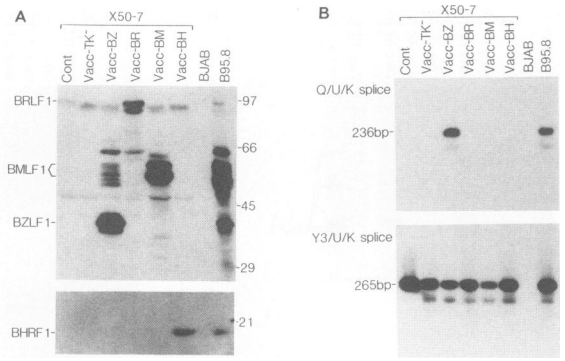

FIG. 6. Screening of vaccinia virus recombinants encoding different EBV lytic-cycle genes for their abilities to induce Q/U/K-spliced EBNA1 transcripts within 12 h of infection of X50-7 cells. (A) Expression of EBV lytic-cycle proteins in X50-7 cells without (Cont) or with infection by the Vacc-TK⁻, Vacc-BZ (BZLF1-encoding), Vacc-BR (BRLF1-encoding), Vacc-BM (BMLF1-encoding), and Vacc-BH (BHRF1-encoding) recombinants. Protein extracts of the spontaneously productive B95.8 cell line and the EBV-negative BJAB cell line are included as controls as for Fig. 5A. Immunoblots of protein extracts were probed with human serum EE (upper panel) and BHRF1-specific monoclonal antibody 5B11 (lower panel). Molecular size markers (in kilodaltons) are on the right. Vacc-BZ infection leads to expression of the BZLF1 protein (major band at 39 kDa) and, as already noted in the legend to Fig. 5A, the BSLF2-BMLF1 protein (major band at 60 kDa plus lower-molecular-weight species). Vacc-BR infection leads to expression of the 98-kDa BRLF1 protein (appearing as a doublet, the lower band of which is superimposed over the weakly detected EBNA2 signal) and the 60-kDa BSLF2-BMLF1 protein. Vacc-BM infection leads to expression of truncated forms of the BSLF2-BMLF1 protein, here designated BMLF1, visualized in the 50- to 58-kDa region of the gel. Vacc-BH infection leads to expression of the 17-kDa BHRF1 protein. (B) Expression of Q/U/K- and Y3/U/K-spliced EBNA1 transcripts in RNA preparations made in parallel with the protein extracts described above and screened as for Fig. 1.

script is used to encode EBNA1 (3, 19, 42, 45, 49). This is in direct contrast to the situation in latency III, exemplified by LCLs, where all six EBNAs and the LMPs are expressed and where Cp and/or Wp is constitutively active (26, 50). The central question which prompted the present work was the status of latency III cells with respect to Fp usage. Thus, in some of the LCLs included for reference in earlier studies, there was no detectable Fp activity, as would be expected if Fp and Cp/Wp were mutually exclusive promoters; in other LCLs, however, we had been able to amplify Q/U/K-spliced transcripts presumably initiated at Fp (3, 42).

This report presents four pieces of evidence indicating that Fp is indeed not active in latency III but becomes activated at an early stage following the switch from latency III into the lytic cycle. First, when a large panel of independently established LCLs was screened, there was absolute concordance between the detectability of Q/U/K-spliced transcripts and the presence within the LCL of a lytically infected subpopulation of cells (Fig. 1 and 2). Second, when RNAs from the prototype virus-producing cell lines B95.8 and AG876, each with a relatively large fraction of cells in the lytic cycle, were used, S1 nuclease protection clearly showed that transcripts were being initiated at exactly the same sites within Fp (Fig. 4) as had been identified earlier in assays with RNAs from BL cells exhibiting latency I (42). Third, Fp usage, whether analyzed by PCR amplification of Q/U/K-spliced transcripts or by S1 nuclease protection, was unaffected by ACV treatment (Fig. 3 and 4), indicating that Fp activation must be an early event of the lytic cycle, preceding viral DNA replication. Fourth, vaccinia virus-mediated expression of the EBV immediate-early antigen BZLF1 in tightly latent LCL cells consistently induced appearance of the diagnostic Q/U/K-spliced transcript (Fig. 5 and 6). Such observations are consistent with the concept of Fp and Cp/Wp as mutually exclusive promoters; however, definitive proof of the concept would require a demonstration that lytic-cycle entry is associated with an up-regulation of Fp and a reciprocal down-regulation of Cp/Wp occurring in the same cells. This may be possible in future, given assays for promoter usage which can be applied at the single-cell level or a system in which the latent- to lytic-cycle switch can be induced simultaneously in all the cells of a culture.

One of the key questions raised by the present data concerns the mechanism of Fp activation in the lytic cycle. In this context, it is important to note that BZLF1 is a transcriptional transactivator which can induce the expression of a variety of early-lytic-cycle genes (4, 7, 12, 14, 21, 23, 47). The other immediate-early protein, BRLF1, also has a transactivating function for many of the same genes (18, 21, 23), but, when the proteins are expressed de novo in latently infected cells, only BZLF1 has been observed to initiate the complete lytic-cycle cascade (9, 38). One possibility, therefore, is that the pathway leading from Vacc-BZ infection to Fp activation involves the BZLF1-induced expression of a "downstream" EBV lytic-cycle gene. Though not conclusive on this point, our findings tend to argue against the involvement of a downstream viral intermediate. Thus, we observed Fp activation in SG/B95 cells at a time postinfection when, by immunoblotting with a polyvalent human serum or by monoclonal antibody staining, we could not detect the expression of any lytic-cycle antigen other than BZLF1 itself (Fig. 5). While these methods of screening for induced antigens are by no means exhaustive, it is nevertheless telling that two of the primary targets of BZLF1 induction, namely, the BRLF1 and BSLF2-BMLF1 proteins (23, 47), could not be detected within the time frame of the experiment. For Vacc-BZ-infected SG/B95 cells, therefore, we infer that Fp is being activated in circumstances when lytic-cycle antigen expression is limited to BZLF1. The situation is less clear-cut with respect to Vacc-BZ infection of X50-7 target cells, since here, at least one downstream EBV lytic gene was induced, namely, BSLF2-BMLF1, a component of early antigen which itself has some transcriptional regulatory activity in reporter gene assays (25, 29). However, this appears unlikely to be an intermediary of BZLF1-induced Fp activation on two counts: first, the vaccinia virus recombinant encoding BRLF1 also induced BSLF2-BMLF1 expression (see 60-kDa protein in Vacc-BM track, Fig. 6A, upper panel) but did not activate Fp; second, the vaccinia virus recombinant directly encoding BMLF1 (a truncated version of BSLF2-BMLF1 which retains the protein's transcriptional regulatory activity [29]) likewise had no effect on Fp usage.

A picture is therefore emerging in which BZLF1, the first viral gene product of the lytic cycle and a viral homolog of the *fos-jun* family of cellular transcriptional transactivators (11), can influence not just lytic gene expression but also the activities of promoters governing EBNA transcription. In this context, experiments with reporter gene constructs have already shown that BZLF1 can down-regulate Cp and Wp activities (24), and the present work raises the possibility of BZLF1 simultaneously up-regulating transcription from Fp.

How these regulatory effects are achieved will become clear only when more is known about the multiple viral and cellular controls governing Cp, Wp, and Fp activities. Interestingly, however, a recent study (46) has proposed a mechanism for BZLF1 down-regulation of Cp which involves a cellular intermediate, the BZLF1-induced c-Fos protein (13). The time lag of some hours which we observed between vaccinia virus-mediated BZLF1 expression and subsequent Fp activation would likewise be consistent with an effect mediated through a BZLF1-induced cellular (or viral) protein. On the other hand, it is possible that the activation of Fp represents a secondary effect of Cp/Wp suppression, i.e., via the removal of transcriptional interference from the long Cp/Wp-initiated transcripts which normally run through the Fp region.

Fp was first identified as a latent-cycle promoter active in those forms of latency, I and II, in which EBNA1 is expressed in the absence of the other EBNAs. It is now clear, however, that Fp is activated as a lytic-cycle promoter when latency III cells switch into the lytic cycle; by inference, Fp might also be expected to remain active when latency I or latency II cells switch directly into the lytic cycle (40). Interestingly, in S1 nuclease protection assays of the kind illustrated in Fig. 4, we found it much easier to detect Fp-initiated transcripts in the spontaneously productive B95.8 and AG876 cell lines than in group I BL cells (28a), indicating a marked difference in the abundance of these transcripts in the latent versus lytic situations. Recent work on Fp activity in latent infection suggests an important negative autoregulatory role for the EBNA1 protein itself that is mediated via EBNA1 binding to consensus sequences in *Bam*HI Q downstream of the transcription start site (43). One possibility is that this usually strong autoregulatory loop is relaxed in lytic cycle, allowing increased Fp transcription. The importance to the virus of this activation of Fp in lytic cycle remains to be determined; there may yet be a role for EBNA1 or for some other protein encoded by an Fp-initiated transcript in the virus replicative cycle.

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REFERENCES

1. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. G. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Séguin, P. S. Tuffnel, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
2. **Biggin, M., M. Bodescot, M. Perricaudet, and P. J. Farrell.** 1987. Epstein-Barr virus gene expression in P3HR1-superinfected Raji cells. J. Virol. **61**:3120–3132.
3. **Brooks, L., Q. Y. Yao, A. B. Rickinson, and L. S. Young.** 1992. Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. J. Virol. **66**:2689–2697.
4. **Buisson, M., E. Manet, M.-C. Trescol-Biemont, H. Gruffat, B. Durand, and A. Sergeant.** 1989. The Epstein-Barr virus (EBV) early protein EB2 is a post-transcriptional activator expressed under the control of EBV transcription factors EB1 and R. J. Virol. **63**:5276–5284.
5. **Busson, P., R. McCoy, R. Sadler, K. Gilligan, T. Tursz, and N. Raab-Traub.** 1992. Consistent transcription of the Epstein-Barr virus LMP2 gene in nasopharyngeal carcinoma. J. Virol. **66**: 3257–3262.
6. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5**:3403–3409.
7. **Chavrier, P., H. Gruffat, A. Chevallier-Greco, M. Buisson, and A. Sergeant.** 1989. The Epstein-Barr virus (EBV) early promoter DR contains a *cis*-acting element responsive to the EBV transactivator EB1 and an enhancer with constitutive and inducible activities. J. Virol. **63**:607–614.
8. **Cho, M.-S., K.-T. Jeang, and S. D. Hayward.** 1985. Localization of the coding region for an Epstein-Barr virus early antigen and inducible expression of this 60-kilodalton nuclear protein in transfected fibroblast lines. J. Virol. **56**:852–859.
9. **Countryman, J., and G. Miller.** 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc. Natl. Acad. Sci. USA **82**:4085–4089.
10. **Fahraeus, R., H. L. Fu, I. Ernberg, J. Finke, M. Rowe, G. Klein, K. Falk, E. Nilsson, M. Yadav, P. Busson, T. Tursz, and B. Kallin.** 1988. Expression of Epstein-Barr virus-encoded proteins in nasopharyngeal carcinoma. Int. J. Cancer **42**:329–338.
11. **Farrell, P. J., D. T. Rowe, C. M. Rooney, and T. Kouzarides.** 1989. Epstein-Barr virus BZLF1 transactivator specifically binds to a consensus AP1 site and is related to c-fos. EMBO J. **8**:127–132.
12. **Flemington, E., and S. H. Speck.** 1990. Autoregulation of Epstein-Barr virus putative lytic switch gene BZLF1. J. Virol. **64**:1227–1232.
13. **Flemington, E., and S. H. Speck.** 1990. Epstein-Barr virus BZLF1 *trans* activator induces the promoter of a cellular cognate gene, c-*fos*. J. Virol. **64**:4549–4552.
14. **Flemington, E. K., A. E. Goldfield, and S. H. Speck.** 1991. Efficient transcription of the Epstein-Barr virus immediate-early BZLF1 and BRLF1 genes requires protein synthesis. J. Virol. **65**:7073–7077.
15. **Goldschmidts, W., J. Luka, and G. R. Pearson.** 1987. A restricted component of the Epstein-Barr virus early antigen complex is structurally related to ribonucleotide reductase. Virology **157**:220–226.
16. **Greenspan, J. S., D. Greenspan, E. T. Lennette, D. I. Abrams, M. A. Conant, V. Petersen, and U. K. Freese.** 1985. Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion. N. Engl. J. Med. **313**:1564–1571.
17. **Gregory, C. D., M. Rowe, and A. B. Rickinson.** 1990. Different Epstein-Barr-virus (EBV) B cell interactions in phenotypically distinct clones of a Burkitt lymphoma cell line. J. Gen. Virol. **71**:1481–1495.
18. **Hardwick, J. M., P. M. Lieberman, and S. D. Hayward.** 1988. A new Epstein-Barr virus transactivator, R, induces expression of a cytoplasmic early antigen. J. Virol. **62**:2274–2284.
19. **Hitt, M. M., M. J. Allday, T. Hara, L. Karran, M. D. Jones, P. Busson, T. Tursz, I. Ernberg, and B. E. Griffin.** 1989. EBV gene expression in an NPC-related tumour. EMBO J. **8**:2639–2651.
20. **Hoffman, G. J., S. E. Lazarowitz, and S. D. Hayward.** 1980. Monoclonal antibody against a 250,000-dalton glycoprotein of Epstein-Barr virus identifies a membrane antigen and a neutralizing antigen. Proc. Natl. Acad. Sci. USA **77**:2979–2983.
21. **Holley-Guthrie, E. A., E. B. Quinlivan, E.-C. Mar, and S. Kenney.** 1990. The Epstein-Barr virus (EBV) BMRF1 promoter for early antigen (EA-D) is regulated by the EBV transactivators, BRLF1 and BZLF1, in a cell-specific manner. J. Virol. **64**:3753–3759.
22. **Hudson, G. S., P. J. Farrell, and B. G. Barrell.** 1985. Two related but differentially expressed potential membrane proteins encoded by the *Eco*RI Dhet region of Epstein-Barr virus B95-8. J. Virol. **53**:528–535.
23. **Kenney, S., E. Holley-Guthrie, E.-C. Mar, and M. Smith.** 1989.

The Epstein-Barr virus BMLF1 promoter contains an enhancer element that is responsive to the BZLF1 and BRLF1 transactivators. J. Virol. **63**:3878–3883.

24. **Kenney, S., J. Kamine, E. Holley-Guthrie, J. C. Lin, E.-C. Mar, and J. Pagano.** 1989. The Epstein-Barr virus (EBV) BZLF1 immediate early gene product differentially affects latent versus productive EBV promoters. J. Virol. **63**:1729–1736.
25. **Kenney, S., J. Kamine, E. Holley-Guthrie, E.-C. Mar, J. C. Lin, D. Markovitz, and J. Pagano.** 1989. The Epstein-Barr virus immediate-early gene product, BMLF1, acts in *trans* by a posttranscriptional mechanism which is reporter gene dependent. J. Virol. **63**:3870–3877.
26. **Kieff, E., and D. Liebowitz.** 1990. Epstein-Barr virus and its replication, p. 1889–1920. *In* B. N. Fields, D. M. Knipe, et al. (ed.), Virology. Raven Press, New York.
27. **King, W., A. L. Thomas-Powell, N. Raab-Traub, M. Hawke, and E. Kieff.** 1980. Epstein-Barr virus RNA. V. Viral RNA in a restringently infected, growth-transformed cell line. J. Virol. **36**:506–518.
28. **Laux, G., M. Perricaudet, and P. J. Farrell.** 1988. A spliced Epstein-Barr virus gene expressed in immortalized lymphocytes is created by circulation of the linear viral genome. EMBO J. **7**:769–774.

28a. **Lear, A.** Unpublished data.

29. **Lieberman, P. M., P. O'Hare, G. S. Hayward, and S. D. Hayward.** 1986. Promiscuous *trans* activation of gene expression by an Epstein-Barr virus-encoded early nuclear protein. J. Virol. **60**:140–148.
30. **Lin, J.-C., M. C. Smith, and J. S. Pagano.** 1984. Prolonged inhibitory effects of 9-(1,3-dihydroxy-2-propoxymethyl)guanine against replication of Epstein-Barr virus. J. Virol. **50**:50–55.
31. **Menezes, J., W. Leibold, G. Klein, and G. Clements.** 1975. Establishment and characterisation of an EBV-negative B-lymphoma line, BJAB, from an exceptional EBV-genome-negative African Burkitt lymphoma. Biomedicine **22**:276–284.
32. **Miller, G., and M. Lipman.** 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc. Natl. Acad. Sci. USA **70**:190–194.
33. **Nilsson, K., G. Klein, W. Henle, and G. Henle.** 1971. The establishment of lymphoblastoid cell lines from adult and from foetal human lymphoid tissue and its dependence on EBV. Int. J. Cancer **8**:443–450.
34. **Pearson, G. R., J. Luka, L. Petti, J. Sample, M. Birkenbach, D. Braun, and E. Kieff.** 1987. Identification of an Epstein-Barr virus early gene encoding a second component of the restricted early antigen complex. Virology **160**:151–161.
35. **Pearson, G. R., B. Vroman, B. Chase, T. Sculley, M. Hummel, and E. Kieff.** 1983. Identification of polypeptide components of the Epstein-Barr virus early antigen complex using monoclonal antibodies. J. Virol. **47**:193–201.
36. **Pizzo, P., I. Magrath, S. Chattopadhyay, R. Biggar, and P. Gerber.** 1978. A new tumor derived transforming strain of Epstein-Barr virus. Nature (London) **272**:629–631.
37. **Rhim, J. S., H. Y. Cho, and R. J. Huebner.** 1975. Non-producer human cells induced by murine sarcoma virus. Int. J. Cancer **15**:23–29.
38. **Rooney, C. M., D. T. Rowe, T. Ragot, and P. J. Farrell.** 1989. The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J. Virol. **63**:3109–3116.
39. **Rowe, D. T., P. J. Farrell, and G. Miller.** 1987. Novel nuclear antigens recognized by human sera in lymphocytes latently infected by Epstein-Barr virus. Virology **156**:153–162.
40. **Rowe, M., A. L. Lear, D. Croom-Carter, A. H. Davies, and A. B. Rickinson.** 1992. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. J. Virol. **66**:122–131.
41. **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.
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. **Sample, J., E. B. D. Henson, and C. Sample.** 1992. The Epstein-Barr virus nuclear protein 1 promoter active in type I latency is autoregulated. J. Virol. **66**:4654–4661.
44. **Sample, J., D. Liebowitz, and E. Kieff.** 1989. Two related Epstein-Barr virus membrane proteins are encoded by separate genes. J. Virol. **63**:933–937.
45. **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.
46. **Sinclair, A. J., M. Brimmell, and P. J. Farrell.** 1992. Reciprocal antagonism of steroid hormones and BZLF1 in switch between Epstein-Barr virus latent and productive cycle gene expression. J. Virol. **66**:70–77.
47. **Sinclair, A. J., M. Brimmell, F. Shanahan, and P. J. Farrell.** 1991. Pathways of activation of the Epstein-Barr virus productive cycle. J. Virol. **65**:2237–2244.
48. **Smith, P. R., and B. E. Griffin.** 1991. Differential expression of Epstein-Barr viral transcripts for two proteins (TP1 and LMP) in lymphocyte and epithelial cells. Nucleic Acids Res. **19**:2435–2440.
49. **Smith, P. R., and B. E. Griffin.** 1992. Transcription of the Epstein-Barr virus gene EBNA1 from different promoters in nasopharyngeal carcinoma and B lymphoblastoid cells. J. Virol. **66**:706–714.
50. **Speck, S. H., and J. L. Strominger.** 1989. Transcription of Epstein-Barr virus in latently infected growth transformed lymphocytes. Adv. Viral Oncol. **8**:133–150.
51. **van Santen, V., A. Cheung, and E. Kieff.** 1981. Epstein-Barr (EBV) RNA. VII. Viral cytoplasmic RNA in a restringently infected cell line transformed *in vitro* by EBV. Proc. Natl. Acad. Sci. USA **78**:1930–1934.
52. **Vroman, B., J. Luka, M. Rodriguez, and G. R. Pearson.** 1985. Characterization of a major protein with a molecular weight of 160,000 associated with the viral capsid of Epstein-Barr virus. J. Virol. **52**:107–113.
53. **Woisetschlaeger, M., J. L. Strominger, and S. H. Speck.** 1989. Mutually exclusive use of viral promoters in Epstein-Barr virus latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **86**:6498–6502.
54. **Woisetschlaeger, M., C. N. Yandava, L. A. Furmanski, J. L. Strominger, and S. H. Speck.** 1990. Promoter switching in Epstein-Barr virus during the initial stages of infection of B lymphocytes. Proc. Natl. Acad. Sci. USA **87**:1725–1729.
55. **Yao, Q. Y., P. Ogan, M. Rowe, M. Wood, and A. B. Rickinson.** 1989. Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. Int. J. Cancer **43**:67–71.
56. **Young, L. S., C. W. Dawson, D. Clark, H. Rupani, P. Busson, T. Tursz, A. Johnson, and A. B. Rickinson.** 1988. Epstein-Barr virus gene expression in nasopharyngeal carcinoma. J. Gen. Virol. **69**:1051–1065.
57. **Young, L. S., R. Lau, M. Rowe, G. Niedobitek, G. Packham, F. Shanahan, D. T. Rowe, D. Greenspan, J. S. Greenspan, A. B. Rickinson, and P. J. Farrell.** 1991. Differentiation-associated expression of the Epstein-Barr virus BZLF1 transactivator protein in oral hairy leukoplakia. J. Virol. **65**:2868–2874.