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The Spliced BZLF1 Gene of Epstein-Barr Virus (EBV) Transactivates an Early EBV Promoter and Induces the Virus Productive Cycle

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A spliced cDNA spanning the Epstein-Barr virus BZLF1 gene expresses the BZLF1 protein and is active in inducing the virus productive cycle. A deletion mutant which lacks the N-terminal half of the protein is inactive. Cotransfection experiments in EBV-negative B-lymphocyte cell lines demonstrated that the BZLF1 gene activates the promoter for the BSLF2 + BMLF1 gene in the absence of any other EBV gene product. These results confirmed that the spliced BZLF1 gene is the transactivating gene structure in *Bam*HI-Z.

Epstein-Barr virus (EBV) is a human herpesvirus that infects B lymphocytes and certain epithelial cells (8). In lymphocytes infected with EBV in vitro, latency is normally established; virus is not produced, but the host cell is immortalized and only about 10 of the EBV genes are expressed. In the productive cycle, virus is released and the host cell is eventually killed. About 80 of the approximately 90 viral genes are active in the productive cycle (7). This report concerns the mechanism of the switch from latency to the virus-productive cycle.

The physiological stimulus for activation of the EBV productive cycle in lymphocytes is obscure and may involve the B-cell differentiation state. Various treatments which will activate the EBV productive cycle in lymphocytes in vitro are known. These include addition of chemicals (such as TPA [phorbol-13-myristate], iododeoxyuridine, or butyrate) to the growth medium and superinfection of the EBV-infected cells with certain strains of EBV. The defective EBV (het) particles found in P3HR1 virus preparations are particularly effective in the induction of the productive cycle in a superinfection (21), and it has been demonstrated that transfection of a small het EBV restriction fragment (which is rearranged in the defective virus) is sufficient to induce the productive cycle (6). This fragment, WZhet, contains the BZLF1 reading frame, and mutation of the DNA has shown that this is the important gene for induction (5).

Analysis of the behavior of EBV genes during a P3HR1 superinfection in the presence of inhibitors of protein synthesis (3) has shown that mRNA encoding BZLF1 is expressed as immediate-early RNA, probably from the defective virus, and that this BZLF1 mRNA contains two splices within the BZLF1 reading frame. These splices are also present in the BZLF1 RNA expressed from standard B95-8 EBV genomes in response to TPA induction (3). The BZLF1 gene is also called EB1 (4) and ZEBRA (12).

Cotransfection experiments with delayed-early target promoters fused to a CAT (chloramphenicol acetyltransferase) reporter gene have shown that the BZLF1 gene (4, 6), the BSLF2 + BMLF1 gene (also known as EB2 and MS-EA [4, 18]), and the BRLF1 gene (13) of EBV all encode proteins that act as transactivators of gene expression. On the basis of studies done with the in vitro model systems, it appears that the genes encoded by BZLF1, BSLF2 + BMLF1, and BRLF1 are all involved in EBV reactivation, but their detailed interactions and roles in natural reactivation are still uncertain.

To clarify the role of the BZLF1 protein, we have used a defined cDNA clone and assayed its capacity to transactivate in the absence of other EBV gene products. In this paper, we show that the spliced form of the BZLF1 gene induced productive cycle gene expression, that a deletion mutant of BZLF1 did not induce productive cycle gene expression, and that BZLF1 activated the promoter for BSLF2 + BMLF1 in the absence of any other EBV gene product.

MATERIALS AND METHODS

Plasmid constructions. Plasmids are illustrated in Fig. 1. pUC33-25 (a cDNA clone containing the spliced BZLF1 gene [3]) was linearized at a *Nae*I site just upstream of the BZLF1 reading frame, a *Bam*HI linker (CGGGATCCCG) was added, and the resulting *Bam*HI-*Eco*RI fragment containing BZLF1 was recloned between the *Bam*HI and *Eco*RI sites of pUC 11, giving pUC-BZLF1 (Fig. 1). A *Sma*I site was generated by the addition of the *Bam*HI linker.

The *Bam*HI-*Eco*RI fragment containing BZLF1 from pUC-BZLF1 was cloned between the *Bam*HI and *Eco*RI sites of SP64, giving SP64-BZLF1. The *Sma*I fragment covering the N-terminal half of BZLF1 was deleted from SP64-BZLF1 and replaced with a linker of sequence CATC GATG, giving SP64-SmaΔ. The linker supplied an initiator methionine residue for the translation of SmaΔ. The adenovirus promoter constructs were made by excising the *Bam*HI-*Ssp*I fragment containing BZLF1 from pUC-BZLF1, repairing the ends with Klenow polymerase, and blunt-end cloning it into pMLP-CAT cut with *Hin*dIII and *Nco*I, giving MLP-BZLF1. pMLP-CAT is the *Hin*dIII-to-*Bam*HI CAT fragment of pSV2CAT cloned between the *Hin*dIII and *Bam*HI sites of pMLP10 (15). The CAT sequences were subsequently eliminated during the cloning of MLP-BZLF1. MLP-*Hin*dIIIΔ and MLP-SmaΔ were made from MLP-BZLF1. For MLP-*Hin*dIIIΔ, MLP-BZLF1 was cut with *Hin*dIII and recircularized, which joined the reading frame in phase at the *Hin*dIII site. MLP-SmaΔ was made by cutting MLP-BZLF1 with *Sma*I and recircularizing it, in-

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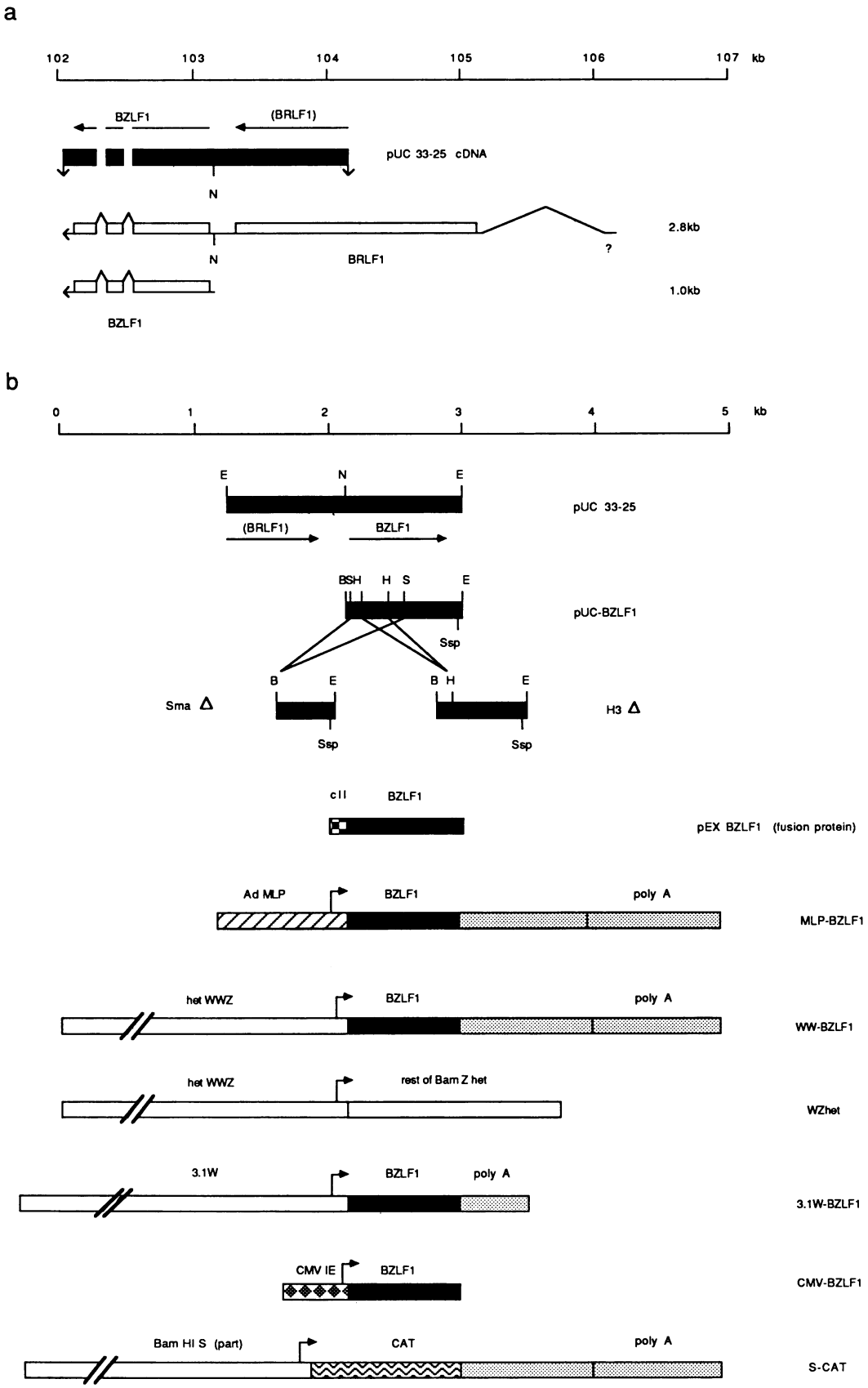
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CMV-BZLF1
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CAT
poly A
S-CAT

FIG. 1. (a) Summary of the structures of the 2.8 and 1.0-kilobase (kb) leftward mRNAs encoded by EBV that contain the BZLF1 gene (3). The spliced RNAs are shown beneath a scale (in kilobases) representing part of the EBV genome; reading frames (□) are indicated. The mapping of the 5′ end of the 2.8-kilobase RNA is incomplete (3). The pUC 33-25 cDNA (a partial cDNA of the 2.8-kilobase RNA) used in this paper is shown (■). The same pUC 33-25 cDNA has been inverted in Fig. 1c. The *Nae*I restriction site in pUC 33-25 is indicated (N). (b) Diagrammatic representation of the BZLF1 parts of the plasmids used in this paper. Only the relevant parts of the plasmids are shown; plasmid vector sequences are omitted. Below an arbitrary scale (in kilobases) the pUC33-25 cDNA (3) which was used to construct pUC-BZLF1 is shown inverted from its orientation in the EBV genome (see panel a). A *Hin*dIII fragment was deleted to make the H3Δ or a *Sma*I fragment was deleted to make the SmaΔ series. These cDNA constructs were cloned in pUC, SP64, the adenovirus major late promoter vector (MLP), pCMV19, pWW, or the *c*II fusion protein vector (pEX) (for details, see Materials and Methods). Restriction sites are abbreviated as follows: E, *Eco*RI; B, *Bam*HI; S, *Sma*I; H, *Hin*dIII; N, *Nae*I; Ssp, *Ssp*I. ■, Sequences derived from pUC 33-25; ▨, adenovirus DNA; ▩, SV40 DNA; ▭, phage λ *c*II; DNA; ▨, CAT gene; ▩, CMV DNA. The structure of the S-CAT reporter plasmid is also shown; the sequence from *Bam*HI-S is 84234 to 87053 of B95-8 EBV (2). (c) Sequence of the B95-8 BZLF1 cDNA and the BZLF1 protein (in the one-letter amino acid code). The sequence of the *Bam*HI-*Eco*RI fragment encoding BZLF1 in pUC-BZLF1 (Fig. 1b) is shown. The numbers 103181 and 102128 relate the cDNA to standard EBV genome numbering (2). The SmaΔ mutant contains the Gly residue at 434 to the C terminus (plus an initiator methionine). The H3Δ mutant lacks the sequence between the *Hin*dIII sites at 105 and 294.

cluding the same oligonucleotide that was used to make SP64-SmaΔ.

WW-BZLF1 was constructed from MLP-BZLF1 by cloning a *Bam*HI-*Nae*I fragment, shown as hetWWZ in Fig. 1b (made from a partial digest of *Eco*RI het 16 [14]), into an *Xho*I site in the MLP element. hetWWZ contained rearranged sequences from *Bam*HI-Z which included the natural EBV promoter for BZLF1. So in WW-BZLF1, the BZLF1 gene was driven by the same promoter as is present in the het-defective EBV (WZhet). In CMV-BZLF1, the BZLF1 gene is transcribed by the cytomegalovirus (CMV) immediate-early promoter. The *Bam*HI-*Eco*RI fragment containing BZLF1 from pUC-BZLF1 was cloned into pCMV19, which contains about 600 base pairs of CMV DNA (including the IE promoter) in pUC19. In 3.1W-BZLF1, the BZLF1 gene is transcribed by the latent cycle promoter in *Bam*HI-W.

The *c*II fusion protein (pEX-BZLF1) was made by cloning the *Bam*HI-*Eco*RI fragment of pUC-BZLF1 into the appropriate pEX vector (D. T. Rowe and J. Clarke, J. Gen. Virol., in press) to obtain the correct phasing of the BZLF1 frame in the bacterial fusion protein.

The S-CAT plasmid was made by inserting the *Bam*HI-*Bgl*II (84234 to 87053) piece of B95-8 EBV into pSVOCAT (10) with the *Bam*HI end next to the CAT gene. The *Hin*dIII site of pSVOCAT had previously been converted to a *Bgl*II site with a linker to accept the S fragment.

293 cell transfections. 293 cells were obtained from P. Gallimore. They were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum and transfected by the calcium phosphate precipitation method (11), typically by using 10 μg of DNA per 75-cm^2 flask. The precipitate was added to the cells for 30 min and then supplemented with medium. The next day, the medium was changed, and 2 days after addition of the precipitate the cells were detached, collected by centrifugation, suspended in 0.5 ml of sodium dodecyl sulfate (SDS) gel sample buffer (17), and sonicated to disperse the DNA. Samples were electrophoresed on 12.5 or 15% gels, transferred to nitrocellulose, and immunoblotted as described previously (23).

The human anti-EBV sera used were CH, which had reciprocal EBV titers of VCA:320, EA:2560, and EBNA:20, and EE, which had reciprocal titers (A. B. Rickinson, personal communication) of VCA:80,000, EA:20,000, and EBNA:64. Both detected the BZLF1 protein. Serum from an EBV-negative donor in our laboratory was used as a control.

Lymphocyte transfections. For the MLP-BZLF1 induction, BL cell lines IARC/BL30 and IARC/BL41 [a gift of G. M. Lenoir, International Agency for Research on Cancer

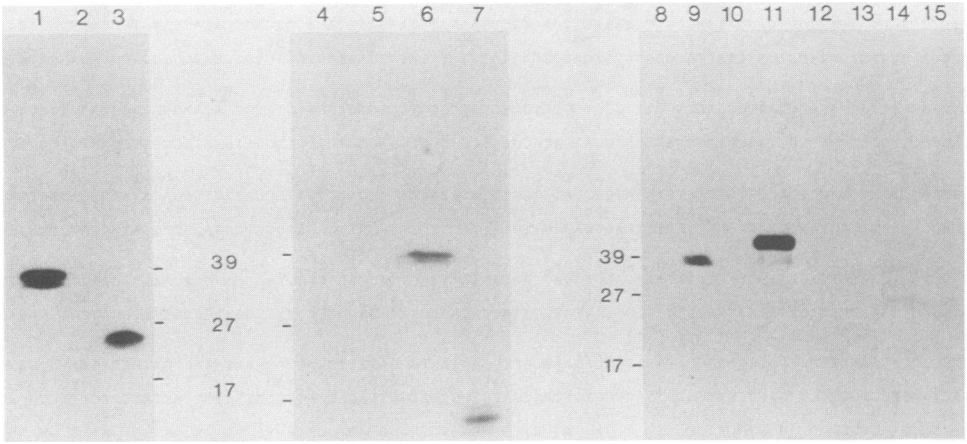

FIG. 2. Immunoblots of BZLF1 proteins made either by transfection into 293 cells or by expression in *E. coli*. The blots were probed with CH (EBV-positive) serum (lanes 1 to 11) or the control serum (lanes 12 to 15). Lanes 1 to 9, 12, and 13 are transient transfections into 293 cells, and lanes 10, 11, 14, and 15 are *E. coli* transformants. Plasmids transfected were (lane 1) MLP-BZLF1, (lane 2) no DNA, (lane 3) MLP-H3Δ, (lane 4) no DNA, (lane 5) pUC18, (lane 6) MLP-BZLF1, (lane 7) MLP-SmaΔ, (lane 8) no DNA, (lane 9) MLP-BZLF1, (lane 10) pEX, (lane 11) pEX-BZLF1, (lane 12) no DNA, (lane 13) MLP-BZLF1, (lane 14) pEX, and (lane 15) pEX-BZLF1. The positions to which size markers (Bio-Rad prestained 39K, 27K, and 17K) migrated are indicated.

(Lyon)] and P3HR-1 clone 16-converted BL30/CL16 and BL41/CL16 were transfected by the DEAE-dextran method. Sixteen hours after the medium was changed and the cells were split 1:1, 8×10^6 cells were washed once in serum-free RPMI 1640 and suspended in 400 μl of RPMI 1640. RPMI 1640 (400 μl) containing 200 μg of DEAE-dextran per ml (molecular weight, 500,000) and 5 μg of DNA was added to the cells, mixed, and placed at 37°C in 5% CO_2-air for 30 min, with mixing done again after 15 min. Cells were washed once and suspended in 10 ml of growth medium (RPMI 1640–penicillin–streptomycin–fungizone–8% fetal calf serum) containing 20% medium conditioned by 18 h of incubation with the same BL cell line. TPA (phorbol-13-myristate) (10 ng/ml) was sometimes included in the medium. Cells were harvested after 48 h for CAT assay or for immunoblotting on 10% gels.

For the inductions in X50-7 (12) and BL41/CL16 cells and the CAT assays, DNA was introduced to the cells by electroporation. A total of 8×10^6 cells in 250 μl of ice-cold complete growth medium were electroporated with 20 μg of DNA in the BioRad gene pulser at 900 V with a capacitance of 250 μF.

CAT assays. Cells were washed twice in phosphate-buffered saline without calcium or magnesium and then suspended at 10^6 cells per 10 μl of 0.25 M Tris chloride (pH 7.5). Cells were freeze-thawed three times in an ethanol-dry ice bath and microcentrifuged for 10 min at 4°C. The supernatants were used for assays which contained 20 μl of cell extract, 50 μl of 0.25 M Tris chloride (pH 7.5), 20 μl of 4 mM acetyl coenzyme A, and 2 μCi of [^{14}C]chloramphenicol. After incubation for 1 h at 37°C, assays were processed as described previously (10).

In vitro transcription and translation. SP6 transcription used template DNAs linearized downstream of the BZLF1 sequences at the *Eco*RI site of SP64 (20). Transcription reactions contained 40 mM Tris chloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine hydrochloride, 0.5 mM each ATP, GTP, CTP, and UTP, 10 mM dithiothreitol, 0.1 mM 7MeGpppG (Boehringer Mannheim Biochemicals), about 50 μg of template DNA per ml, and 500 U of SP6 RNA polymerase (New England BioLabs) per ml. After 60 min at 40°C, RNA was purified by phenol-chloroform extraction and ethanol precipitation. Occasionally, to monitor the transcription, a sample of the reaction mixture was supplemented with [α-^{32}P]UTP, and the radioactive RNA products were analyzed by glyoxalation and agarose gel electrophoresis (19). RNA from the in vitro transcription was translated for 15 to 60 min at 30°C, without removing the template DNA, in mRNA-dependent reticulocyte lysate (Amersham Corp.) with [^{35}S]cysteine as the radioactive label. The lysate was 80% of the translation reaction, and no further salt supplementation was made.

RESULTS

Expression of BZLF1 cDNA and deletion mutants. We previously determined the structures of two overlapping, spliced, 3′ coterminal mRNAs encoding reading frame BRLF1 and a spliced form of the BZLF1 reading frame (3). These mRNAs were studied because they behaved as immediate-early RNAs in superinfection experiments (3). A B95-8 cDNA (pUC33-25) covering all the BZLF1 gene and part of BRLF1 was truncated to contain only BZLF1 sequences. The 5′ end of this BZLF1 cDNA was close to the natural 5′ end of the BZLF1 mRNA, and the sequence of the truncated cDNA (pUC-BZLF1) and the structures of various deletion mutants used in this paper are shown in Fig. 1. The H3Δ mutant of BZLF1 was made by deleting a *Hin*dIII fragment within the N-terminal half of BZLF1, and the SmaΔ mutant is essentially the C-terminal half of BZLF1.

The BZLF1, H3Δ, and SmaΔ constructs were all cloned into a vector containing the adenovirus 2 major late promoter together with 3′ splicing and poly(A) addition signals from simian virus 40. The constructs were arranged to permit expression of the BZLF1 protein or mutants using the adenovirus promoter and the simian virus 40 poly(A) site. The DNAs were transfected into 293 cells, and after 2 days the cells were lysed and the proteins were analyzed by SDS gel electrophoresis and immunoblotting with a human serum with a high titer of antibodies to EBV proteins as a probe. Construct MLP-BZLF1 containing the wild-type BZLF1 gave a strongly reactive band corresponding to the wild-type BZLF1 protein (Fig. 2, lanes 1, 6, and 9). As reported by other workers (6), the protein migrated relative to protein size markers with a molecular weight of about 39,000,

although the protein sequence encoded by the cDNA was only 26,860 daltons. The H3Δ and SmaΔ proteins were expressed at comparable efficiency in 293 transfections and, as expected, were shorter (Fig. 2, lanes 3 and 7). The shorter proteins seen with the deletion mutants confirmed that the protein came from the BZLF1 frame, and the similar yields of the deletion mutant proteins indicated that their stability in cells is not greatly different from that of the full-length BZLF1 protein. The efficient recognition of the SmaΔ protein showed that antigenic epitopes recognized by the CH serum lie within this part of the protein. The predicted molecular weights of BZLF1, H3Δ, and SmaΔ are 26,860, 20,115, and 12,834, respectively, but the proteins migrated anomalously slowly on the gels.

A bacterial fusion protein of the whole BZLF1 gene was also prepared. In pEX-BZLF1, a fusion of 40 amino acids of the phage lambda *c*II gene to the whole of BZLF1 was produced. This protein was recognized by the CH antiserum in immunoblots (Fig. 2, lane 11). The fusion protein was longer than wild-type BZLF1 because of the phage lambda *c*II residues fused to the N terminus. An antiserum from an EBV-seronegative individual showed no reactivity to the BZLF1 protein or the fusion protein (Fig. 2, lanes 12 to 15), although there was a very low level of cross-reactivity with *Escherichia coli* proteins. The anomalously slow migration of BZLF1 is not likely to be a result of a eucaryotic posttranscriptional modification, because the bacterial fusion protein electrophoresed similarly anomalously.

In several different 293 transfection experiments and immunoblots, some splitting of the BZLF1 band into a very close doublet was seen. The nature of this doublet remains to be established, though it has been reported previously in experiments on BZLF1 made in virus-infected cells (5).

In vitro transcription and translation of the BZLF1 protein. The *Bam*HI-*Eco*RI fragments of pUC-BZLF1 and pUC-SmaΔ were cloned into SP64, linearized downstream of the BZLF1 gene by using *Eco*RI, and transcribed with SP6 polymerase. RNA was produced which, when translated in the reticulocyte lysate, yielded proteins identical in size to the bands detected in transfected 293 cells by immunoblotting (Fig. 3, lanes 1, 8, 9, and 11). The main band translated from BZLF1 RNA was the full-length protein, but some fragments were also produced (lane 7). The 60-min time course (lanes 1 to 4) suggested that these are due to specific proteolysis since they are not apparent after only 15 min of translation and tend to accumulate. In the immunoblot (lanes 9 to 11), the protein from 293 cells (lane 9) was detected through the ^{125}I in the protein A, but the in vitro-translated protein (lane 11) was seen principally from the ^{35}S in the in vitro translation. The BZLF1 made by in vitro translation and by transfection into 293 cells also comigrated on SDS gel electrophoresis with the BZLF1 protein in induced B95-8 cells (D.T.R. and P.J.F., unpublished results).

Taken together, the transfection into 293 cells and SP6 transcription-reticulocyte lysate translation experiments demonstrated that the cDNA can efficiently produce a protein with the physical properties expected for the BZLF1 gene product and that the clones for the two deletion mutants express the predicted proteins.

The spliced BZLF1 gene induced the EBV-productive cycle but a deletion mutant did not. To show that the spliced BZLF1 gene is an active inducer of the productive cycle, the plasmid MLP-BZLF1 was transfected into BL41/CL16 cells. This tightly latent line contains HH514-16 HR1 EBV and is not detectably productive even in the presence of TPA (22). The WZhet fragment derived from the het-defective form of

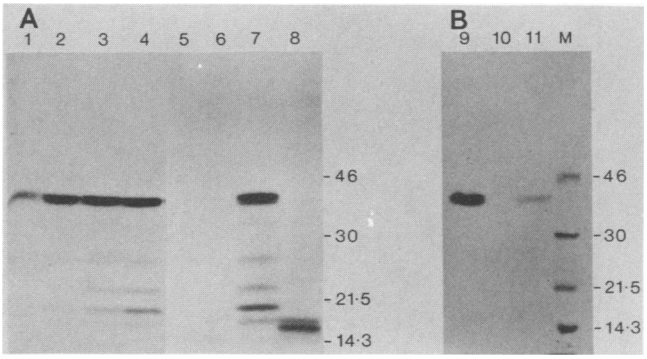

FIG. 3. (A) SP6 transcription and in vitro translation of SP64-BZLF1 constructs. SP64, SP64-BZLF1, or SP64-SmaΔ was linearized and transcribed with SP6, and the resulting RNA was translated in mRNA-dependent reticulocyte lysate with [^{35}S]cysteine. Plasmids transcribed were SP64-BZLF1 (lanes 1 to 4 and 7), no DNA (lane 5), SP64 (lane 6), and SP64-SmaΔ (lane 8). Translation was for 15 (lane 1), 30 (lane 2), 45 (lane 3), and 60 min (lanes 4 to 8). The translation was terminated by addition of 5 volumes of SDS gel sample buffer, and samples were electrophoresed on a 15% polyacrylamide gel (17). The gel was fixed and impregnated with Enlightening (Du Pont Co.); an autoradiograph is shown. (B) Immunoblot of BZLF1 made in 293 cells or by in vitro translation. 293 cells were transfected with MLP-BZLF1 (lane 9) or pUC (lane 10) as a control. Extracts were electrophoresed on a 15% polyacrylamide gel with a 15-min in vitro translation of BZLF1 labeled with [^{35}S]methionine (lane 11). The gel was blotted and developed with CH serum. Lane M contains size markers (kilodaltons are indicated to the right of the lanes).

EBV induced the EBV productive cycle in these cells, and the induction was considerably enhanced by TPA, even though TPA treatment alone did not activate the virus (Fig. 4A, B, and C). The adenovirus MLP-BZLF1 also induced the EBV productive cycle in these cells in the presence of TPA (Fig. 4A, lanes BZLF1). The array of productive cycle proteins induced was visualized by immunoblotting (Fig. 4A) and by immunofluorescence (not shown) using CH serum. Similar results were obtained by transfecting BL30/CL16 cells (not shown). The induction by the MLP-BZLF1 plasmid was weak compared with the induction by WZhet, and this is thought to be mostly due to more efficient expression of BZLF1 from the WZhet promoter in these lymphocyte cell lines (however, see below). We therefore tested constructs with other promoters driving expression of the BZLF1 gene. We transfected the WWhet-BZLF1, CMV-BZLF1, and WW-SmaΔ constructs into X50-7 cells (WW-BZLF1 has the same promoter that is present in WZhet). With these BZLF1 plasmids a much larger induction of EBV productive cycle proteins was observed, and considerable amounts of BZLF1 protein were detected on the Western immunoblots (Fig. 4B).

We have shown elsewhere (9) that BZLF1 is a sequence-specific DNA-binding protein and argued that this is part of the mechanism of action of BZLF1 in the activation of the productive cycle. Many transcription factors can be considered to be composed of two domains, one of which binds DNA and the other of which is involved in interaction with other components of the transcription complex (16). The C-terminal part of BZLF1 (SmaΔ) was sufficient to bind DNA specifically (9), so we tested whether this was able to activate the virus productive cycle (Fig. 4B). No activation was seen with SmaΔ, even though the protein was stably expressed (Fig. 2). In the experiment shown in Fig. 4B, the

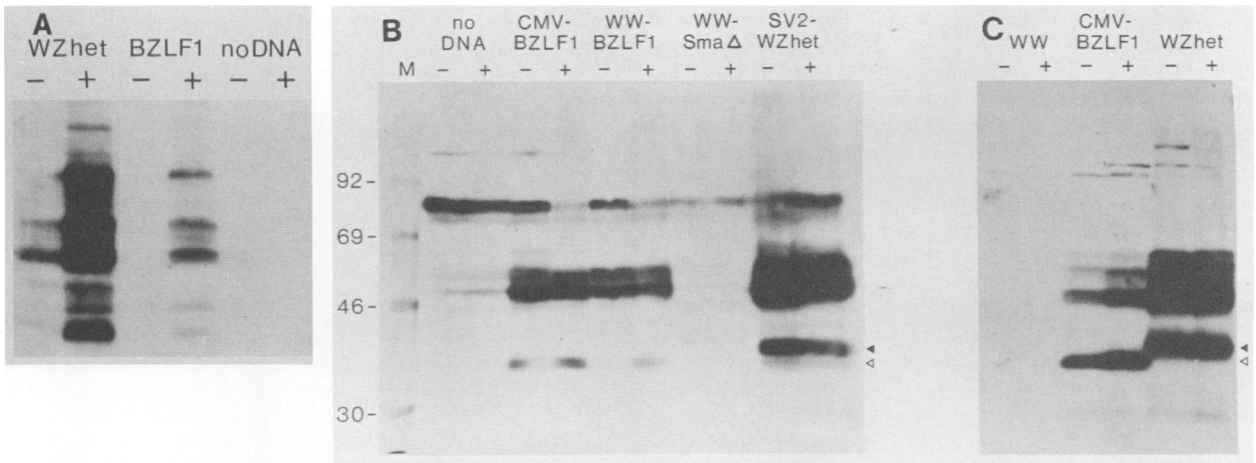

FIG. 4. (A) Transfection of BL41/CL16 cells with BZLF1 constructs and immunoblotting to detect EBV productive cycle proteins. Cells were transfected as indicated with WZhet, MLP-BZLF1, or no DNA by the DEAE-dextran method with (+) or without (−) TPA treatment. The transfected cells were collected, lysed in SDS gel sample buffer, electrophoresed on a 12.5% gel, and immunoblotted. The filter was probed with CH serum. (B) Transfection of X50-7 cells with BZLF1 constructs. X50-7 cells were electroporated as indicated with no DNA, CMV-BZLF1, WW-BZLF1, WW-SmaΔ, or WZhet with (+) or without (−) TPA treatment. The cells were analyzed on a 10% gel as in panel A, but EE serum was used. B95-8 BZLF1 (◁) and het BZLF1 (◀) are indicated. (C) Electroporation of BL41/CL16 cells with BZLF1 constructs. BL41/CL16 cells were electroporated as indicated with pWW (as a control), CMV-BZLF1, or WZhet as described in Materials and Methods. The cells were analyzed as in panel B by using EE serum. pWW is identical to WW-BZLF1 but lacks the BZLF1 sequences.

SmaΔ protein was electrophoresed off the end of the gel. The strong band at about 80K in Fig. 4B is thought to be EBNA-2. The EE serum reacted strongly with EBNA-2 in X50-7 cells, but EBNA-2 was absent in the P3HR1 clone 16 EBV (Fig. 4C).

CMV-BZLF1 and WZhet happened to give similar amounts of BZLF1 in the BL41/clone 16 cells shown in Fig. 4C, but the induction of EBV proteins was greater with WZhet. This indicates that the sequence differences between the het BZLF1 and B95-8 BZLF1 either make the het BZLF1 more active in induction or cause it to react less well with the antibodies in EE serum. The difference in mobility of B95-8 and het BZLF1 protein on SDS gels and the greater activity of het BZLF1 has been reported previously (5, 6).

BZLF1 gene product activated a promoter in *Bam*HI-S. To analyze the role of the protein encoded by BZLF1 in activation, we tested whether it would activate the promoter of another EBV-encoded gene (BSLF2 + BMLF1). The promoter for this gene is close to the left end of *Bam*HI-S. Although interactions have been described previously (4, 18) between the BZLF1 and the BSLF2 + BMLF1 genes, it has not been clear whether other EBV gene products are required to mediate the interaction between the genes. A promoter fusion of part of the *Bam*HI-S sequence of B95-8 EBV to a promoterless CAT gene was transfected into the BL41 EBV-negative Burkitt's lymphoma cell line. Almost no CAT activity was observed. CAT expression was strongly induced when either the CMV-BZLF1 or the 3.1W-BZLF1 DNA construct was cotransfected with the CAT plasmid (Fig. 5). The 3.1W-BZLF1 construct uses another EBV promoter that works well in B lymphocytes to drive expression of BZLF1. This is the *Bam*HI-W promoter that constitutively expresses the EBNA RNA in EBV. The use of EBV-negative BL41 cells in these experiments demonstrated that BZLF1 can transactivate the promoter for BSLF2 + BMLF1 in B lymphocytes in the absence of any other EBV gene product. However, transactivation is much greater in EBV genome-positive BL41/B95-8 cells (not shown), in which secondary cooperation with other EBV gene products may occur.

DISCUSSION

Three transactivators of transcription encoded by BZLF1, BRLF1, and BSLF2 + BMLF1 have been shown to function early in the EBV productive cycle, and it is likely that all three are involved in reactivation from latency. However, the detailed sequence of events which occurs in the transition from latent to productive cycle remains unclear. The transcription of EBV genes occurs in the context of both cellular and viral transcription factors, so it is essential to assay the function of the EBV transactivators in an appropriate cell type, namely, B lymphocytes. To dissect the functions of the individual EBV transactivators, it is desirable to study their activities in the absence of other EBV gene products; subsequently it will be possible to interpret

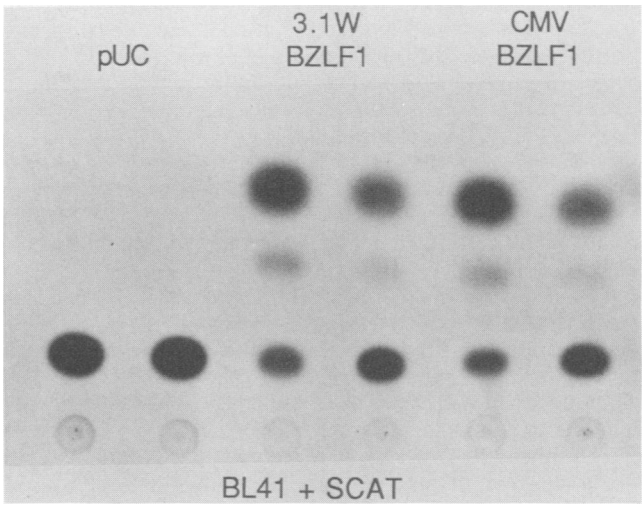

FIG. 5. CAT assay of transactivation by BZLF1. BL41 cells were cotransfected with S-CAT and pUC18 (pUC) as a negative control, 3.1W-BZLF1, or CMV-BZLF1 in duplicate. Details of the transfection protocol and CAT assay are given in Materials and Methods.

their interactions and combined functions. In this paper we have analyzed the function of the BZLF1 gene by using a fully defined cDNA in the correct B-cell type and in the absence of other EBV genes.

We have demonstrated that the spliced mRNA spanning the BZLF1 gene expresses the BZLF1 protein and is active in inducing the productive cycle of EBV in BL41/CL16 cells and in X50-7 cells, at least to the extent of inducing expression of an array of productive cycle proteins. Although TPA alone has no effect on BL41/CL16 cells, the combination of BZLF1 plus TPA usually gave substantially greater induction of the productive cycle than did the BZLF1 construct alone in our transient transfection assay. TPA usually had a similar synergistic effect with BZLF1 expressed from the WZhet plasmid. Our experiments do not at present distinguish whether this is a consequence of TPA increasing the amount of BZLF1 expressed from our constructs or of TPA affecting other factors that cooperate with BZLF1.

It has been reported previously that expression of both BZLF1 and BSLF2 + BMLF1 (EB1 and EB2) are required to activate a delayed-early productive cycle promoter of EBV (4). We have further shown here that BZLF1 can activate the promoter for the expression of BSLF2 + BMLF1 in B lymphocytes in the absence of any other EBV gene product, and we will show elsewhere (C.M.R., manuscript in preparation) that BZLF1 can also down regulate a latent cycle EBV promoter. Although combinations of the EBV transactivators BZLF1, BRLF1, and BSLF2 + BMLF1 are required to fully activate the later stages of the productive cycle (4, 13, 18), our results are consistent with the many lines of evidence that indicate that BZLF1 expression is the first stage in reactivation. Only BZLF1 and BRLF1 behave as immediate-early genes in superinfection experiments (3). BZLF1 is the gene that mediates induction by het EBV (6), and transfection of BZLF1 alone is sufficient to convert a tightly latent lymphoblastoid line to a spontaneously productive line (12). Furthermore, in the synchronous induction of the EBV productive cycle observed in Akata cells in response to cross-linking surface immunoglobulin, BZLF1 RNA accumulates transiently and significantly earlier than other EBV RNAs (24).

The mechanism by which TPA enhances the expression of productive cycle genes remains a complex problem. TPA is known to modulate cellular gene expression, and its action can be mediated through factors that bind at the AP-1 site (1). It is likely that the cellular transcription factor *c-jun* (a component of AP-1) is involved in TPA induction. In the experiments reported here, we have not distinguished the possible effects of TPA on BZLF1 expression from effects of TPA on other EBV genes, perhaps via *c-jun* or other components of the AP-1 factor. Several EBV genes, including for example BMRF1, BSLF2 + BMLF1, and gp350/220, have AP-1 sites near their promoters, so there is considerable scope for AP-1 involvement. We have shown elsewhere that BZLF1 is a sequence-specific DNA-binding protein that binds to the AP-1 site upstream of BSLF2 + BMLF1, and presumably this mediates the activation by BZLF1 (9). Further experiments are required in which BZLF1 can be expressed at a constant high level unaffected by TPA so that effects of TPA on further stages of reactivation can be analyzed independently.

We have found that the promoter used to express BZLF1 is an important factor in analyzing the transactivation. We found that the simian virus 40 promoter works poorly in B lymphocytes unless EBV *Bam*HI-W sequences are present (as is the case in the WZhet plasmid). *Bam*HI-W contains a strong enhancer-like function active in B lymphocytes (22). The CMV immediate-early promoter was a useful strong promoter in the lymphocyte lines we studied. The CMV-BZLF1 plasmid in BL41/CL16 cells directed accumulation of a comparable amount of BZLF1 to that produced by WZhet, but the degree of activation was a fewfold less with the BZLF1 clone. Sequence differences between het and B95-8 BZLF1 may thus result in a small difference in the activities of the two proteins. An alternative possibility we can not exclude is that the het protein reacts less well with the antibodies, so that we underestimated the amount of het BZLF1; the proteins might then have identical activity.

Our preliminary experiments on structural analysis of the BZLF1 protein have shown that deletion of the N-terminal half of the protein abolishes the ability to induce the EBV productive cycle. Mutation on a more subtle scale may allow us to define parts of the protein which are important for particular BZLF1 functions.

The physiological signal for reactivation of EBV in lymphocytes is unknown but probably involves maturation of the lymphocyte. This presumably alters the pattern of cellular transcription factors available to the virus and might result in expression of BZLF1 or the other transactivators. It is attractive to think that the difference between tightly latent and spontaneously productive lymphocyte cell lines might be interpretable in the context of the detailed patterns of cellular transcription factors expressed in those cells. The interaction between the different viral and cellular transactivators is evidently quite complex, but being able to observe a function for BZLF1 in the absence of any other EBV gene product and having the facility to prepare BZLF1 in vitro will allow us to analyze at the biochemical level the detailed function of this critical switch molecule in EBV reactivation.

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LITERATURE CITED

1. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. F. Rahmsdorf, P. Jonat, C. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common cis element recognised by a TPA-modulated trans-acting factor. Cell **49**:729–739.
2. **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, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein Barr virus genome. Nature (London) **310**:207–211.
3. **Biggin, M., M. Bodescot, M. Perricaudet, and P. Farrell.** 1987. Epstein-Barr virus gene expression in P3HR1-superinfected Raji cells. J. Virol. **61**:3120–3132.
4. **Chevallier-Greco, A., E. Manet, P. Chavrier, C. Mosnier, J. Daillie, and A. Sergeant.** 1986. Both Epstein-Barr virus (EBV) encoded transacting factors EB1 and EB2, are required to activate transcription from an EBV early promoter. EMBO J. **5**:3243–3250.
5. **Countryman, J., H. Jenson, R. Seibl, H. Wolf, and G. Miller.** 1987. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. J. Virol. **61**:3672–3679.
6. **Countryman, J., and G. Miller.** 1985. Activation of expression of latent Epstein-Barr herpes virus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. Proc. Natl. Acad. Sci. USA **82**:4085–4089.
7. **Farrell, P. J.** 1987. Epstein-Barr virus, p. 99–107. *In* S. J.

O'Brien (ed.), Genetic maps. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Farrell, P. J.** 1989. Epstein-Barr virus genome. Adv. Viral Oncol. **8**:103–132.
9. **Farrell, P. J., D. T. Rowe, C. M. Rooney, and T. Kouzarides.** 1989. Epstein-Barr virus BZLF1 transactivator specifically binds to a consensus AP-1 site and is related to c-fos. EMBO J. **8**:127–132.
10. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
11. **Graham, F., and A. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
12. **Grogan, E., H. Jenson, J. Countryman, L. Heston, L. Gradoville, and G. Miller.** 1987. Transfection of a rearranged viral DNA fragment, WZhet, stably converts latent Epstein-Barr viral infection to productive infection in lymphoid cells. Proc. Natl. Acad. Sci. USA **84**:1332–1337.
13. **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.
14. **Jenson, H., M. S. Rabson, and G. Miller.** 1986. Palindromic structure and polypeptide expression of 36 kilobase pairs of heterogeneous Epstein-Barr Virus (P3HR1) DNA. J. Virol. **58**:475–486.
15. **Joab, I., D. T. Rowe, J. Bodescot, J.-C. Nicolas, P. J. Farrell, and M. Perricaudet.** 1987. Mapping of the gene coding for Epstein-Barr virus-determined nuclear antigen EBNA3 and its transient overexpression in a human cell line by using an adenovirus expression vector. J. Virol. **61**:3340–3344.
16. **Keegan, L., G. Gill, and M. Ptashne.** 1986. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science **231**:699–704.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lieberman, P. M., P. O'Hare, G. S. Hayward, and S. D. Hayward.** 1986. Promiscuous transactivation of gene expression by an Epstein-Barr virus-encoded early protein. J. Virol. **60**: 140–148.
19. **McMaster, G. K., and G. C. Carmichael.** 1977. Analysis of single stranded and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74**:4835–4838.
20. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
21. **Rabson, M., L. Heston, and G. Miller.** 1983. Identification of a rare Epstein-Barr virus variant that enhances early antigen expression in Raji cells. Proc. Natl. Acad. Sci. USA **80**: 2762–2766.
22. **Rooney, C. M., N. Taylor, N. Countryman, H. Jenson, J. Kolman, and G. Miller.** 1988. Genome rearrangements activate the Epstein-Barr virus gene whose product disrupts latency. Proc. Natl. Acad. Sci. USA **85**:9801–9805.
23. **Rowe, D. T., M. Rowe, G. I. Evan, L. E. Wallace, P. J. Farrell, and A. B. Rickinson.** 1986. Restricted expression of EBV latent genes and T-lymphocyte detected membrane antigen in Burkitt's lymphoma cells. EMBO J. **5**:2599–2607.
24. **Takada, K., and Y. Ono.** 1989. Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. J. Virol. **63**:445–449.