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# Expression of the BZLF1 Latency-Disrupting Gene Differs in Standard and Defective Epstein-Barr Viruses

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**Previous experiments using gene transfer of plasmids with heterologous promoters identified an Epstein-Barr virus (EBV) gene (BZLF1) whose product (ZEBRA) switches the virus from a latent to a replicative state. We have now studied expression of ZEBRA in lymphoid cells harboring either standard virus or a mixture of standard and defective (heterogeneous [het]) viruses. A high-titer rabbit antiserum to a TrpE-BZLF1 fusion protein was used to identify ZEBRA expressed from standard and het EBV DNA. These ZEBRA proteins could be distinguished from each other on the basis of their electrophoretic mobilities. ZEBRA could not be detected in cells latently infected with standard EBV. However, within 6 h after induction of replication by sodium butyrate, ZEBRA appeared and persisted long thereafter. Synthesis of ZEBRA was insensitive to phosphonoacetic acid or acycloguanosine, behavior characteristic of an early replicative protein. ZEBRA was constitutively expressed in cells containing both defective and standard EBV genomes. ZEBRA was made predominantly from the het genome but also from the standard genome. Control of BZLF1 expression appears to occur at the transcriptional level. No BZLF1-specific transcript was detected in cells containing only standard latent EBV. BZLF1 transcripts could be detected in these cells if virus replication was induced by treatment with butyrate. Cells bearing both standard and het genomes did not require addition of an exogenous inducing agent to transcribe the BZLF1 gene. The experiments suggest that regulation of transcription of the BZLF1 gene is a pivotal event in the control of EBV replication.**

Epstein-Barr virus (EBV) is maintained in immortal B lymphocytes predominantly in the latent state. Expression of lytic cycle products occurs spontaneously in a small proportion of EBV-infected lymphocytes; however, virus replication can be induced in many more cells by such agents as phorbol esters, butyrate, and serum factors (2, 21, 31). Virus from the P3HR-1 subline of the Jijoye Burkitt's lymphoma (BL) also has the capacity to induce EBV replication (13, 25).

The P3HR-1 cell line contains two forms of EBV DNA, a standard genome and a defective (heterogeneous [het]) genome which is extensively deleted and rearranged (7, 14). Experiments with cellular subclones of the P3HR-1 cell line implicated the het DNA in activation of replication of the standard EBV genome. HR-1 clone 16 (HH514-16), which is representative of the majority of HR-1 subclones, contains the standard genome but has lost het DNA and with it the capacity to synthesize viral replicative products spontaneously. HR-1 clone 5 (HH543-5), a rare clone which contains hypermolar levels of het DNA as well as standard EBV DNA, spontaneously enters virus replication (26). Clone 5 cells release a mixture of standard and het viruses which activate virus replication when added to cells bearing a latent genome.

The capacity to activate replication has been mapped to the BZLF1 open reading frame, which encodes a protein termed ZEBRA (Z EB replication activator) or EB1 (5, 10). The protein itself is responsible for activation of early gene expression (9). Although the BZLF1 open reading frame is present in both standard and het genomes, in the latter the reading frame is inverted relative to its position in the standard genome. In the het genome, BZLF1 has new upstream and downstream regions as the result of intramolecular recombinations (15, 17). This altered genome configuration suggests that the BZLF1 gene might be aberrantly regulated in het DNA.

Previous experiments which defined the function of the BZLF1 gene and its product relied exclusively on gene transfers of plasmids in which expression of the BZLF1 gene was driven by strong heterologous promoters (5, 9, 12, 30). The purpose of the experiments described here was to study the pattern of ZEBRA expression during the life cycle of standard and defective EBVs in BL cells, a natural target.

(These findings were presented in preliminary form at the 12th International Herpesvirus Workshop, Philadelphia, Pa., 13 August 1987.)

## MATERIALS AND METHODS

**Cells.** A prototype HR-1 cellular subclone which lacks het DNA is HH514-16 (clone 16). Clone HH543-5 (clone 5) contains het DNA in addition to the standard HR-1 genome. EBV genome-negative BL lines IARC/BL30 and IARC/BL2 were stably converted to carriage of B95-8 or HR-1 clone 16 strains of EBV (4). These lines, designated BL30/CL16, BL30/B95-8, BL2/CL16, and BL2/B95-8, expressed EBV nuclear antigen type 1 in every nucleus and maintained EBV in a tightly latent state (27). Expression of the BZLF1 gene was studied in these converted BL cell lines 48 h after infection with clone 5 virus in the presence or absence of TPA (4 ng/ml). Lymphoid cell lines were grown in RPMI 1640 with 8% fetal calf serum, penicillin, amphotericin B (Fungizone), and streptomycin. COS-1 cells, which were used to examine recombinant plasmids for expression of

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proteins, were grown in Dulbecco modified Eagle medium with 8% fetal bovine serum.

**Chemical induction.** HR-1 cells were induced with sodium butyrate (3 mM) or TPA (20 ng/ml). In some experiments, induction was accompanied by inhibition of viral DNA replication with 100 μg of phosphonoacetic acid (PAA) per ml or 100 μM acycloguanosine (ACV).

**Transfection.** COS-1 cells were transfected by the DEAE-dextran method, followed by chloroquine treatment (22). Cells were exposed to 10 μg of plasmid DNA 3 days after subculture at a 1:5 split ratio. Cells were seeded in 100-mm dishes for analysis of proteins or 60-mm dishes with cover slips for immunofluorescence.

**Plasmids.** pSV2neo plasmids containing either WZhet (the het *Bam*HI fragment from defective EBV containing the BZLF1 open reading frame) or the standard *Bam*HI Z fragment from EBV FF41 or HR-1 have been described previously (9). A 679-base-pair (bp) *Nae*I-*Pvu*II fragment, encompassing the unspliced BZLF1 open reading frame, was isolated from WZhet. The overhanging end produced by *Pvu*II digestion was filled in with the Klenow fragment of DNA polymerase and deoxynucleotide triphosphates. The fragment was inserted into the unique *Sma*I site of the TrpE bacterial expression vector pATH 11, which was a gift from T. J. Koerner via M. Carlson.

**Induction of the TrpE-BZLF1 expression plasmid.** *Escherichia coli* HB101 containing the TrpE-BZLF1 plasmid was grown overnight in LB. The cells were then diluted 1:50 in M9 medium containing 1% Casamino Acids (Difco Laboratories, Detroit, Mich.) and 50 μg of ampicillin per ml. Bacteria were grown to an optical density at 600 nm of 0.2; indoleacrylic acid (Sigma Chemical Co., St. Louis, Mo.) was then added to a final concentration of 10 μg/ml. Growth was continued with vigorous aeration for 4 h. An insoluble protein fraction was prepared as described previously (18). The fusion protein was visualized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, followed by staining with Coomassie brilliant blue.

**Immunizations.** The TrpE-BZLF1 fusion protein from 50 ml of cell culture was recovered in the insoluble protein fraction and electrophoresed on a 10% preparative SDS-polyacrylamide gel. Gel strips containing the fusion protein were ground and emulsified in complete Freund adjuvant. Two female rabbits were immunized with the protein by subcutaneous injection. Each rabbit was injected with approximately 25 mg of protein and was boosted every 2 weeks with an equivalent amount of protein in phosphate-buffered saline. Serum was collected 7 days after each boost.

**Antigen and polypeptide detection.** To detect ZEBRA by anti-immunoglobulin immunofluorescence, cells were incubated with a 1:30 dilution of rabbit antiserum to BZLF1 and then with a 1:50 dilution of rhodamine-conjugated anti-rabbit immunoglobulin. Extracts for immunoblotting were prepared by suspending $2 \times 10^6$ cells in 50 μl of SDS sample buffer. Extracts were sonicated for 15 s and boiled for 5 min before electrophoresis on 10% SDS-polyacrylamide gels. Some cell extracts were denatured in sample buffer containing 8 M urea and electrophoresed in a 10% polyacrylamide gel containing 4 M urea. Procedures used for immunoblotting have been described elsewhere (9). Filters were incubated with the ZEBRA-specific rabbit antibody or with a polyvalent human serum, RM, which detects latent EBV proteins as well as a p21 late viral protein (28).

**Northern (RNA) blots.** Total cellular RNA was harvested from uninduced cells and at 24 and 48 h after induction by the guanidium isothiocyanate method (6). RNA was electrophoresed on 2.2% HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-formaldehyde gels and transferred to nytran (23). Probes were a 1.0-kbp *Hin*dIII-*Bam*HI subfragment of WZhet or the 5′ *Bam*HI-*Hin*dIII fragment of WZhet mutant 80 (9), radiolabeled by the random primer method (11). Blots were hybridized according to the method of Church and Gilbert (8).

## RESULTS

**Properties of the BZLF1 fusion protein.** A BZLF1 fusion protein was obtained by cloning the 679-bp *Nae*I-*Pvu*II subfragment of WZhet in frame with the *trpE* coding sequences contained in the pATH 11 vector. The BZLF1 portion of the fusion protein made in bacteria would not be identical to that made in eucaryotic cells if eucaryotic ZEBRA is expressed exclusively from spliced mRNAs (3). The EBV BZLF1 insert should encode 218 amino acids: 9 amino acids upstream of the two methionines at the 5′ end of the BZLF1 open reading frame, 167 amino acids from exon 1 of BZLF1, and 42 amino acids encoded in the first intron. If the BZLF1 gene is not spliced in eucaryotic cells, the 218-amino-acid insert included in the bacterial fusion protein would encompass the entire 209-amino-acid BZLF1 open reading frame.

The calculated molecular size of the protein encoded by the EBV insert is 23.3 kilodaltons (kDa) (16). The pATH 11 vector contains sequences coding for a 36-kDa TrpE polypeptide. The observed electrophoretic mobility of the TrpE-BZLF1 fusion protein, 72 kDa, was about 13 kDa larger than calculated. The ZEBRA polypeptide from eucaryotic cells also migrated about 13 kDa slower than expected (16). The synthesis of fusion protein was highly inducible by addition of indoleacrylic acid to HB101 cells transformed with the pATH 11-BZLF1 construct. After treatment of the bacterial cell lysate with DNase I, the fusion protein accounted for about 50% of the total protein in the insoluble pellet (Fig. 1).

**Detection of polymorphic ZEBRA polypeptides by rabbit antisera raised to the TrpE-BZLF1 fusion protein.** Initial experiments evaluated the specificity of the antisera obtained from rabbits immunized with the TrpE-BZLF1 fusion protein. Postimmune rabbit sera recognized a nuclear antigen in COS-1 cells transfected with pSV2neo-WZhet and in HR-1 clone 16 cells treated with TPA (Fig. 2). Preimmune rabbit sera did not react with COS-1 cells or HR-1 cells by immunofluorescence or by immunoblotting (not shown). The postimmunization rabbit sera did not detect antigens in untransfected COS-1 cells or in uninduced clone 16 cells in an immunofluorescence assay (not shown).

It was known from immunoblot analysis using polyvalent human sera, as well as a weakly reactive rabbit antiserum raised to a LacZ-BZLF1 fusion protein, that ZEBRA polypeptides from various EBV strains have different electrophoretic mobilities (9). By means of chimeric mutants and DNA sequence analysis, it was previously found that three-point mutations at amino acid positions 25, 51, and 73 in BZLF1 account for the 3-kDa difference in electrophoretic mobilities of HR-1 het ZEBRA (39 kDa) and standard HR-1 ZEBRA (36 kDa) (9, 16). There is also a reproducible difference in electrophoretic mobility of about 1 to 2 kDa between ZEBRA from standard HR-1 and EBV FF41. This mobility difference between FF41 ZEBRA and HR-1 ZEBRA disappeared when the proteins were further denatured in 8 M urea; however, even after denaturation in urea, HR-1 het ZEBRA migrated about 3 kDa slower than did standard HR-1 ZEBRA (D. A. Katz, unpublished data).

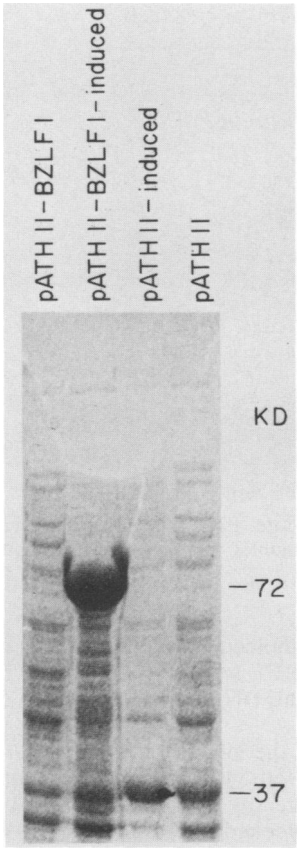


FIG. 1. Appearance of the TrpE-BZLF1 fusion protein on a polyacrylamide gel stained with Coomassie brilliant blue.

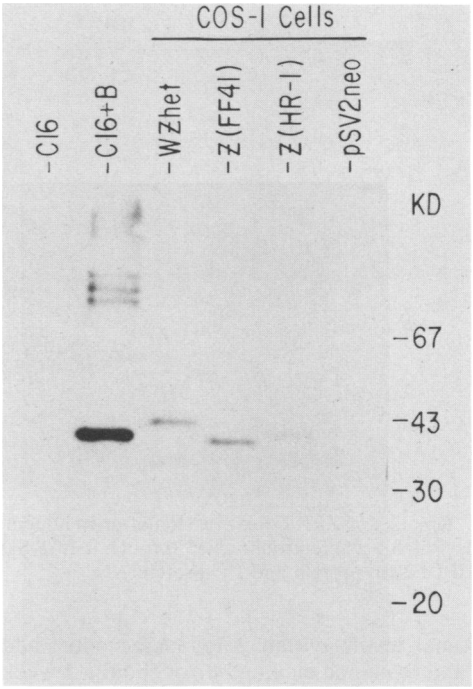


FIG. 3. Reactivity of rabbit antiserum to the TrpE-BZLF1 fusion protein by immunoblotting. Extracts were prepared from HR-1 clone 16 cells which were untreated (C16) or treated with butyrate for 3 days (C16+B). COS-1 cells were transfected with pSV2neo plasmids containing WZhet, the *Bam*HI Z fragment from EBV FF41 and HR-1, or no insert. The immunoblot was probed with a 1:100 dilution of rabbit antiserum to BZLF1.

The rabbit antiserum to TrpE-BZLF1 recognized the polymorphic ZEBRA proteins made after transfection of COS-1 cells with the BZLF1 gene from various EBV strains (Fig. 3). Furthermore, using this serum, we showed that the ZEBRA polypeptide made in lymphoid cells from intact virus comigrated with the polypeptide made when the encoding region from that virus was transfected into COS-1 cells. For example, the ZEBRA polypeptide made in butyrate-induced clone 16 cells comigrated with ZEBRA expressed in COS-1 cells transfected with pSV2neo–*Bam*HI-Z (HR-1) (Fig. 3). Three higher-molecular-size bands of 70 to 90 kDa were also detected with antibody to BZLF1 on immunoblots of extracts prepared from induced clone 16 cells. These may be products of BRLF1, whose mRNA seems to be colinear with that of BZLF1 at the 3′ end (3, 29).

**Kinetics of ZEBRA expression in cells containing only a standard EBV genome.** The antiserum was used in a time course study to determine whether ZEBRA was an early or late viral protein. ZEBRA could be detected within 6 h after addition of butyrate to clone 16 cells. Expression reached

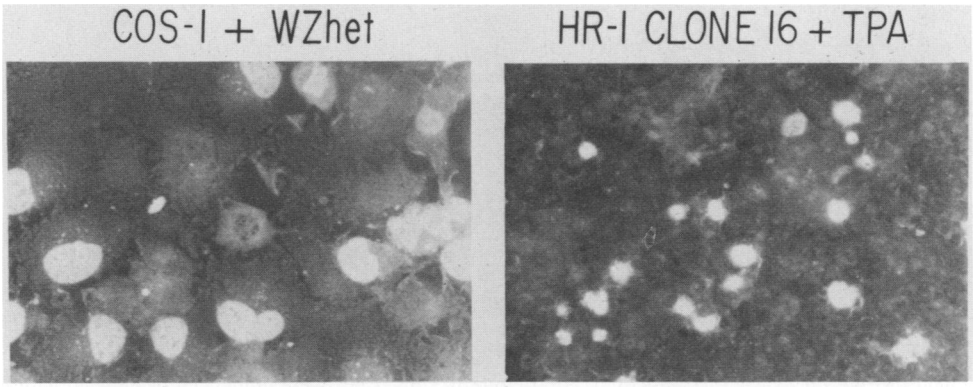


FIG. 2. Reactivity of rabbit antiserum to the TrpE-BZLF1 fusion protein by immunofluorescence. COS-1 cells had been transfected 3 days previously with pSV2neo-WZhet. HR-1 clone 16 cells were treated with TPA for 3 days. The rabbit antiserum was used at a 1:30 dilution; rhodamine-conjugated anti-rabbit immunoglobulin was used at a 1:50 dilution.

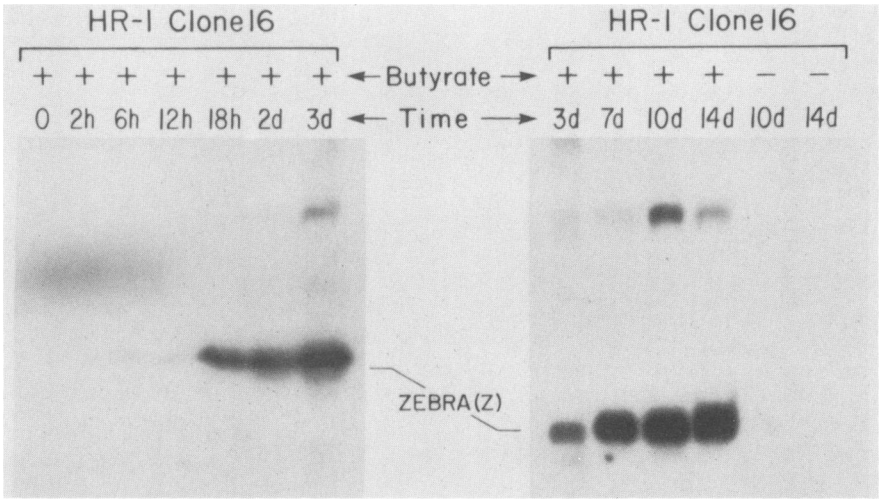


FIG. 4. Kinetics of ZEBRA expression after treatment of HR-1 clone 16 cells with butyrate. Each time point represents an extract of 2 × $10^6$ cells which was electrophoresed through a 10% SDS-polyacrylamide gel. The immunoblot was probed with rabbit antiserum to the TrpE-BZLF1 fusion protein and $I^{125}$-protein A.

near-maximal levels within 2 to 3 days after addition of butyrate and remained elevated throughout a 2-week period. However, cells cultured for 2 weeks in the absence of an inducing agent failed to express detectable levels of ZEBRA. The higher-molecular-weight immunoreactive proteins were first detected about 3 days after butyrate treatment (Fig. 4).

Additional kinetic experiments were performed in which clone 16 cells were induced with butyrate in the presence or absence of inhibitors of EBV DNA synthesis, PAA or ACV. Treatment with these inhibitors did not alter ZEBRA expression. The higher-molecular-weight proteins reactive with the antibody to BZLF1 were also made in the presence of inhibitors of viral DNA synthesis (Fig. 5 and data not shown).

The efficacy of the inhibitors was monitored in two ways. Appearance of a late viral protein, p21, induced in clone 16 cells and recognized by the RM antiserum 3 and 5 days after induction, was blocked by PAA (Fig. 5). When ACV was used, it was found that the drug had blocked viral DNA replication. This finding was assessed in parallel extracts by

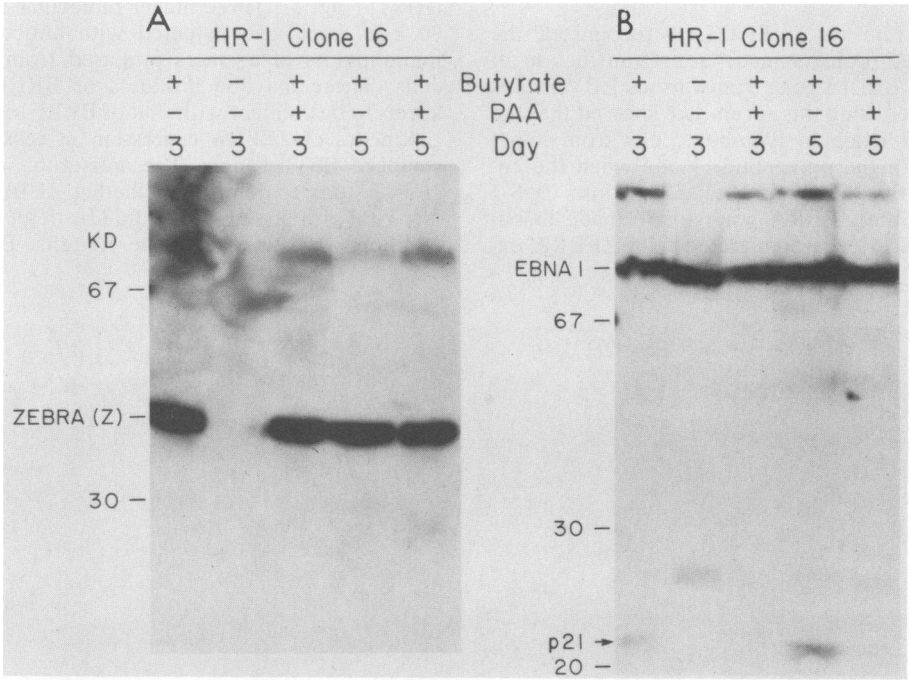


FIG. 5. Lack of effect of PAA on ZEBRA expression in HR-1 clone 16 cells. Cells were uninduced or induced with butyrate. A portion of induced cells was also treated with PAA. Cell extracts were analyzed after 3 or 5 days on duplicate immunoblots with rabbit anti-BZLF1 serum (A) or polyvalent human antiserum RM, which recognizes a p21 late protein (B). Note that ZEBRA expression was not affected by PAA but p21 synthesis was inhibited.

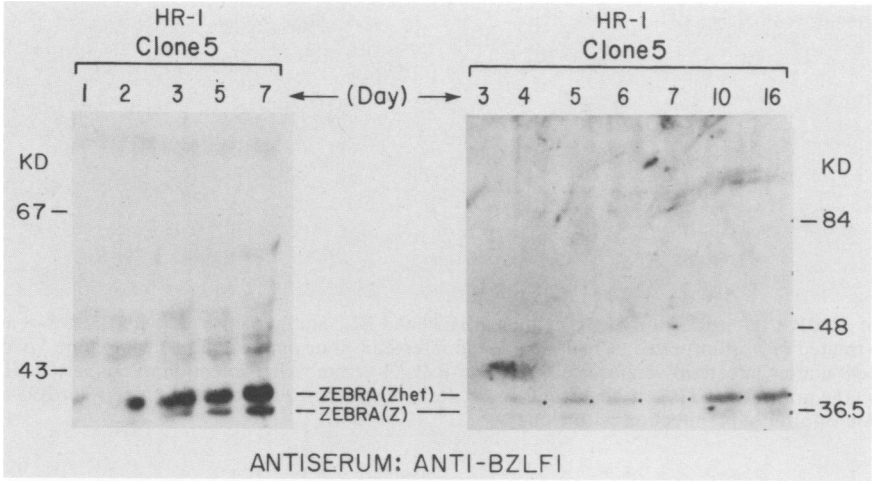


FIG. 6. Constitutive expression of ZEBRA in HR-1 clone 5 cells. Duplicate experiments are illustrated. Extracts were prepared at intervals after splitting the cells at a ratio of 1:4 (left) or 1:5 (right). Note that there was a delay in ZEBRA expression after the cells were split. Beginning at 2 to 4 days after subculture, ZEBRA was expressed without addition of an inducing agent. The het variant was more abundant than the standard variant.

the appearance of linear EBV DNA 48 to 72 h after addition of butyrate and the failure of linear DNA to appear when ACV was present (not shown) (B. Z. Katz, unpublished data). As expected, neither ACV nor PAA affected expression of latent products, EBV nuclear antigens, recognized by the RM antiserum. These experiments demonstrated that in clone 16 cells, ZEBRA was not expressed during latency and behaved as an early replicative protein after chemical induction.

**Constitutive expression of ZEBRA in cells bearing both standard and defective EBV genomes.** It was of interest to compare ZEBRA expression in clone 16 cells with expression in clone 5 cells which spontaneously undergo viral replication. The polymorphisms of ZEBRA encoded by WZhet and standard *Bam*HI-Z (Fig. 3) were used to monitor expression of the two ZEBRA variants in clone 5 cells which harbor both standard and defective genomes. Clone 5 cells expressed ZEBRA spontaneously, without addition of inducing agents (Fig. 6). There was generally a lag in ZEBRA expression after subculture into fresh medium, but ZEBRA was detected by 2 to 4 days after subculture and persisted thereafter. The het variant was predominant and was detected before standard ZEBRA.

The effects of two inducing agents, TPA and butyrate, were tested in clone 5 cells (Fig. 7). At 3 and 4 days after subculture, het ZEBRA was made spontaneously in the absence of an inducer. In the presence of butyrate, both standard and het ZEBRA were activated. In many experiments (not shown), butyrate preferentially activated expression of standard ZEBRA. TPA seemed to induce het ZEBRA to a greater extent than standard ZEBRA. These experiments emphasized the separate controls on expression of ZEBRA from the two genomes.

**Exogenous infection with virus stocks containing both standard and defective genomes.** Virus stocks prepared from clone 5 cells, containing a mixture of standard and defective EBV genomes, were inoculated into EBV genome-negative BL cells that had been stably converted by HR-1 (clone 16) and B95-8 virus strains (4, 29). The converted lines were used as targets in order to compare expression of the endogenous standard virus with that of the superinfecting het and standard virus mixture.

All four EBV-converted BL cell lines maintained the virus in a tightly latent state. In these lines, neither TPA nor butyrate stimulated expression of ZEBRA from the endogenous EBV genome (Fig. 8 and data not shown). The same viruses (HR-1 clone 16 and B95-8), however, could be induced in other cell backgrounds. When the EBV-converted cells were superinfected by clone 5 virus stocks, het ZEBRA was markedly induced by TPA treatment (Fig. 8). Het ZEBRA expression in these four BL lines was not accompanied by synthesis of standard ZEBRA, as it was in clone 5 cells. Nonetheless, expression of the het ZEBRA

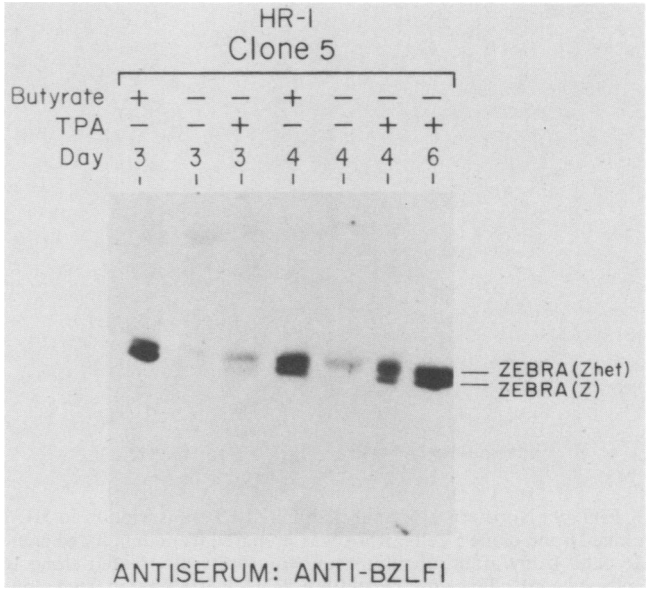


FIG. 7. Effects of inducing agents on expression of the two ZEBRA variants in HR-1 clone 5 cells. Uninduced cells expressed mainly the het variant; after addition of TPA or butyrate both the het and standard ZEBRAs were expressed. TPA had a greater effect on the het variant. Butyrate preferentially stimulated the standard variant.

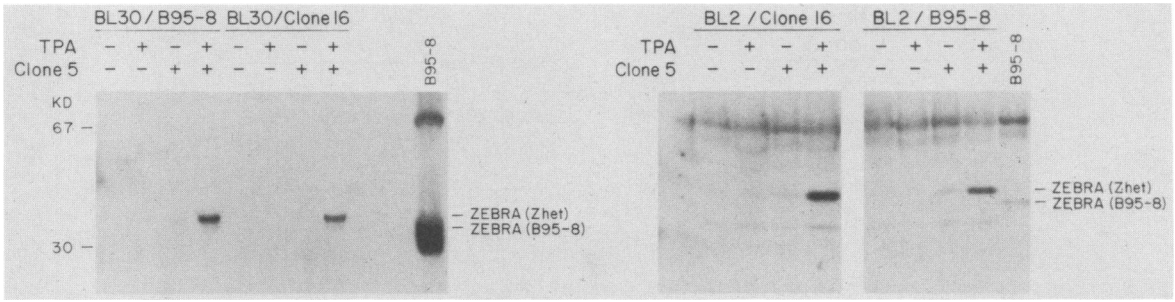


FIG. 8. Expression of ZEBRA after infection of BL cell lines BL30 and BL2 stably converted with the B95-8 and HR-1 clone 16 strains of EBV. The cells were treated (+) or untreated (−) with TPA and infected (+) or uninfected (−) with clone 5 virus stocks. Western blots (immunoblots) prepared 48 h after infection were reacted with anti-BZLF1 serum. After infection of converted BL30 or BL2 cells, the het ZEBRA was expressed in the presence of TPA. In these cells, however, the standard ZEBRA from clone 16 or B95-8 virus was not expressed either after TPA induction or after superinfection with clone 5.

protein was associated with the production of a large number of early antigens (not shown).

**Transcription of BZLF1 in HR-1 cells bearing standard and defective EBV genomes.** Northern blot analysis was performed to determine whether the observed differences in standard and het ZEBRA expression were due to regulation at the transcriptional level. RNA from clone 16 and clone 5 cells was probed with a 523-bp fragment representing 264 bp upstream of BZLF and 259 bp of the first coding exon. Two prominent mRNAs of 3.4 and 0.9 kb were detected 1 and 2 days after butyrate treatment (Fig. 9). In clone 5 cells, the same two mRNAs were expressed spontaneously without addition of an inducing agent. In uninduced clone 16 cells, no transcripts were identified with this probe. The experiments indicated that differences in ZEBRA expression by clone 16 and clone 5 cells were likely the result of differences in transcriptional control.

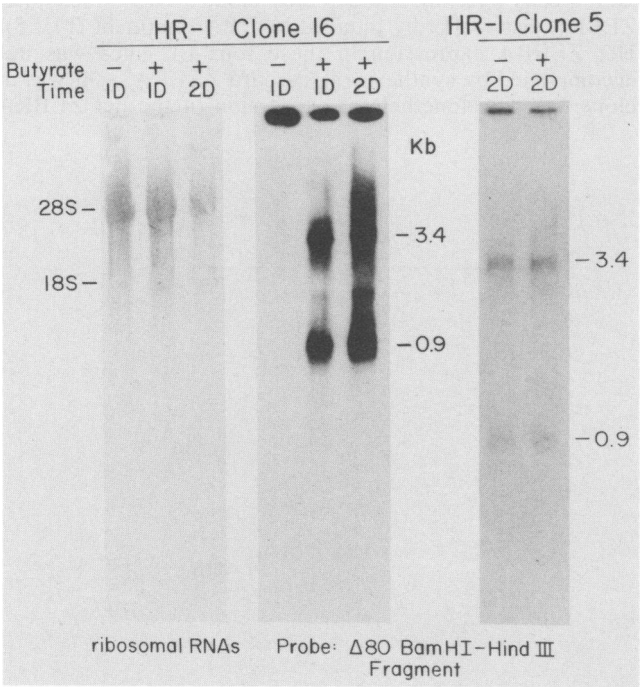


FIG. 9. Northern blot analysis of BZLF1 transcription in HR-1 clone 16 and clone 5 cells. RNA was prepared from uninduced clone 16 cells 1 day after subculture and from butyrate-treated clone 16 cells at 1 and 2 days after subculture. RNA was prepared from clone 5 cells 2 days after subculture in the absence or presence of butyrate. Both blots were probed with a 523-bp subfragment of mutant 80 of WZhet (9). After hybridization, the filter containing RNA from clone 16 cells was stained with methylene blue to demonstrate that the levels of rRNA from the uninduced and induced cells were similar.

## DISCUSSION

Since the BZLF1 gene product can activate the expression of many EBV genes in transfection experiments, it has been assumed that this gene plays a pivotal role in the switch between latency and replication in the context of the intact virus (5, 10, 12, 30). However, little is known about the regulation of this gene in natural infection. There have been no reports describing the effects of different variables on expression of ZEBRA protein in lymphoid cells. Our experiments define at least three sets of variables which affect ZEBRA expression: presence of defective virus, cell background, and addition of chemical inducers such as sodium butyrate and TPA.

**Defective virus.** The presence of defective virus was associated with constitutive ZEBRA expression. The defective genomes are easily lost from clone 5 cells (24). Those clone 5 cells which contained the defective virus expressed ZEBRA, whereas those that had lost het DNA no longer made the ZEBRA polypeptide (not shown). Parental HR-1 cells, which contain several different families of defective EBV DNA molecules, express many different polypeptides which are reactive with the anti-BZLF1 serum. ZEBRA is synthesized spontaneously unless all of the defective populations have been lost from the cells (J. Kolman, unpublished data).

Seibl et al. (29) observed a 40-kDa BZLF1-specific protein in hybrid-selected translations with RNAs from induced HR-1 cells. They also immunoprecipitated a 35-kDa BZLF1 protein from metabolically labeled cells. Although they concluded that the 35-kDa protein was processed from a 40-kDa precursor, their studies could not distinguish between expression of products from the standard and defective HR-1 EBV genomes (29). However, our system enabled us to distinguish between the role of standard and defective viruses in ZEBRA expression. Furthermore, Seibl et al. did not evaluate the effects of inducers on ZEBRA expressed from either standard or defective EBV.

The polymorphisms of the ZEBRA polypeptide provided a powerful tool with which to assess ZEBRA expression from

the defective and standard genomes. In the absence of inducers, most of the ZEBRA in clone 5 cells was derived from the defective genome (Fig. 6 and 7). When virus stocks containing both types of genomes were used to infect EBV-converted BL cells, het ZEBRA was exclusively expressed (Fig. 8). These results suggest that aberrant controls on ZEBRA expression in the defective genome are responsible for the high level of expression. In related experiments using gene transfer into BL cells, ZEBRA was expressed at high levels from plasmids containing the rearranged *Eco*RI het 16-kbp palindromic fragment; ZEBRA was not made when it was introduced on a standard *Bam*HI Z fragment (27). Thus, the acquisition of new positive regulatory signals as the results of the genome rearrangements is likely to explain the high level of spontaneous het ZEBRA expression in clone 5 cells.

Some of the ZEBRA in clone 5 cells bearing het DNA is derived from the BZLF1 open reading frame in the standard genome. This finding suggests that an autoregulatory loop may exist; het ZEBRA may activate the expression of standard ZEBRA, either directly or by the action of other EBV products whose expression is stimulated by ZEBRA.

**Cell background.** Cell background provides an important influence on ZEBRA expression. Upon de novo infection of primary B cells with virus stocks, with or without defective genomes, ZEBRA synthesis is not detected (not shown). In EBV-converted BL lines there is tight regulation of standard ZEBRA expression; inducing agents such as TPA or butyrate are incapable of activating ZEBRA expression. Preferential expression of het ZEBRA in these cells may be due to a gene dosage effect or may be the result of the juxtaposition of a TPA-responsive element in the het virus. Nonetheless, a high level of het ZEBRA expression in a BL cell background does not activate expression of standard ZEBRA, as it does in other cells (Fig. 6 and 7). In cell lines in which EBV is less tightly latent, such as clone 16 (Fig. 3), Raji, or in vitro EBV-infected immortalized cord blood B cells such as X50-7, there is no spontaneous synthesis of ZEBRA, but its synthesis can be activated by chemical inducers and het DNA. The B95-8 and FF41 marmoset cell lines, which spontaneously replicate EBV without the requirement for defective DNA or an inducing chemical, also spontaneously synthesize ZEBRA (Fig. 8). Thus, the tendency of an EBV-containing cell line to exhibit a tightly latent or a productive phenotype correlates with stringent versus loose control of ZEBRA expression. The nature of these host cell-specific controls can only be conjectured. They may represent positive or negative regulatory elements.

**Chemical induction.** It seems likely that the pathways by which chemicals such as TPA and butyrate activate EBV early gene expression intersect with those which control ZEBRA gene expression. This hypothesis can be tested, since inducing agents vary in their effects on cell lines carrying different EBV genomes. For example, TPA is a much more potent inducing stimulus of early antigen synthesis in Raji cells than is butyrate, and it was found that ZEBRA expression is activated to a greater extent in Raji cells by TPA than by butyrate (not shown). However, these experiments cannot distinguish which replicative genes are TPA inducible. For example, TPA may activate EBV early replicative genes, whose products in turn may activate more ZEBRA.

The inducing drugs affect standard and defective EBV in different ways. The standard EBV genome in clone 16 is more efficiently induced to express ZEBRA by butyrate than by TPA (Fig. 7). The defective genome in clone 5, however, is more strongly affected by TPA. The different effects of chemical inducers on het versus standard ZEBRA are transient (Fig. 7). A likely explanation is that the het and standard ZEBRA proteins are capable of stimulating each other. This hypothesis is supported by the finding that spontaneous expression of het ZEBRA protein is eventually accompanied by expression of the standard ZEBRA protein.

Rooney et al. (27) have shown through gene transfers that in the genetic environment of het DNA, het ZEBRA expression is markedly induced by TPA. A likely site for the TPA-responsive element in the defective DNA is the promoter element for the MS-EA gene (BMLF1), which is positioned downstream of BZLF1 in het DNA. This promoter contains a binding site for the AP-1 transcription factor, which is TPA inducible (1, 20).

**Nature of the control.** Our experiments so far indicate that the major controls on ZEBRA expression are at the transcriptional level. Chemical induction of cells containing only standard virus such as clone 16, Raji, and B95-8 (not shown) is invariably associated with marked increases in BZLF1 mRNA (Fig. 9). However, in clone 5 cells which contain a defective virus, BZLF1-specific transcripts are made constitutively and are not always increased by butyrate treatment. The level of ZEBRA mRNAs in these cells varies with culture conditions such as temperature, cell density, and interval since feeding (not shown). These variables probably reflect cellular controls on the het virus.

Our results help clarify previously published results on *Bam*HI-Z transcripts. Biggin et al. detected two transcripts of 2.8 and 1.0 kb from BRLF1 and BZLF1, respectively, in HR-1 superinfected Raji cells treated with protein synthesis inhibitors and suggested that these immediate-early transcripts are derived from the defective genome (3). However, under the conditions of their experiment, they could not ascertain whether the transcripts were expressed from the Raji genome, the HR-1 standard genome, or the HR-1 defective genome. Laux et al. showed that these two transcripts could be detected in Raji cells after induction with TPA (19). However, in contrast to transcripts made after superinfection, those induced by TPA were not detected in the presence of cycloheximide. Laux et al. suggested that the mechanisms of activation of replication by superinfection and by TPA are different.

Our results show that in clone 5 cells, which, like HR-1, contain a defective virus, two transcripts of similar size are made constitutively. In clone 16 cells, which, like Raji cells, contain only a standard virus, no BZLF1-homologous transcripts are made in the absence of an inducing agent. On the basis of the results of all three laboratories, we hypothesize that in cells containing a defective virus, transcripts are made constitutively and may not require new protein synthesis, whereas in the standard virus, new protein synthesis may be needed to activate transcription of BZLF1.

Future work will entail a search for differences in the control mechanisms on BZLF1 in the standard and defective viruses. Control may involve repressors encoded by the virus or the cell as well as transcription factors which are activated by inducing agents.

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