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# Polymorphic Proteins Encoded within BZLF1 of Defective and Standard Epstein-Barr Viruses Disrupt Latency

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**These experiments identify an Epstein-Barr virus-encoded gene product, called ZEBRA (*Bam*HI fragment Z Epstein-Barr replication activator) protein, which activates a switch between the latent and replicative life cycle of the virus. Our previous work had shown that the 2.7-kilobase-pair WZhet piece of rearranged Epstein-Barr virus DNA from a defective virus activated replication when introduced into cells with a latent genome, but it was not clear whether a protein product was required for the phenomenon. We now use deletional, site-directed, and chimeric mutagenesis, together with gene transfer, to show that a 43-kilodalton protein, encoded in the BZLF1 open reading frame of het DNA, is responsible for this process. The rearrangement in defective DNA does not contribute to the structural gene for the protein. Similar proteins with variable electrophoretic mobility (37 to 39 kilodaltons) were encoded by *Bam*HI Z fragments from standard, nondefective Epstein-Barr virus genomes. Plasmids expressing the ZEBRA proteins from B95-8 and HR-1 viruses were less efficient at activating replication in D98/HR-1 cells than those which contained the ZEBRA gene from the defective virus. It is not yet known whether these functional differences are due to variations in expression of the plasmids or to intrinsic differences in the activity of these polymorphic polypeptides.**

The life cycle of the Epstein-Barr herpesvirus (EBV) in immortalized human B lymphocytes is divided into two phases called latency and replication. During latency the viral genome is circular and largely extrachromosomal. Only certain viral genes are expressed in latency: those encoding two small RNAs (EBERs), a family of at least four nuclear products (EBNAs), and a latent membrane protein (6, 11–13, 19, 25, 29). Latent EBV genomes replicate coordinately with the cell genome and are equally partitioned to daughter cells. The viral genome contains an origin of plasmid maintenance (*oriP*), which is thought to play a role in genome partitioning (34). During the phase of viral replication a large number (more than 50) of new mature viral transcripts and as many viral polypeptides appear (14). These are conventionally classified as early antigens (preceding viral DNA replication) and late antigens, including capsid and envelope antigens. Linear viral DNA is encapsidated into viral particles.

The switch between latency and replication can be activated by diverse stimuli including phorbol ester tumor promoters, sodium butyrate, anti-immunoglobulin, and a factor in serum (2, 18, 35). The pathway by which these signals activate viral replication is unknown.

A class of defective EBV virions is also able to disrupt latency and activate replication of latent EBV (22). These defective virions contain an extensively deleted and rearranged EBV genome, called heterogeneous (het) DNA. To identify the viral genes on het DNA which are responsible for disrupting latency, cloned het DNA fragments have been introduced into cells with a latent EBV genome. One DNA clone, termed *Bam*HI WZhet or *Bam*HI het 2.7, is capable of activating the expression of many viral replicative polypeptides within 24 h after transfection into a monolayer somatic cell hybrid line, D98/HR-1, with a latent genome (5). Up to 1% of transfected D98/HR-1 cells express replicative antigens, whereas less than 0.01% of the cells spontaneously synthesize these products. The same DNA clone, when stably transferred into the X50-7 lymphoblastoid cell line, under drug selection also markedly enhanced expression of the viral replicative cycle (10). Some clones of X50-7 cells which have received and express WZhet make viral replicative products in 10% of the cells, whereas less than 0.01% of parental X50-7 cells express replicative products. Furthermore X50-7 cells containing WZhet release infectious virus. Thus WZhet is competent to drive the EBV replicative cycle to completion with the synthesis of mature virions.

The active 2,700-base-pair (bp) WZhet fragment is extensively rearranged. It represents a fusion and inversion of two portions of EBV DNA which are separated by more than 50 kbp on the standard EBV genome. This rearranged DNA fragment contains only one complete open reading frame. Transfers of DNA which have been resected in this open reading frame do not activate viral replication in D98/HR-1 cells (5). Such experiments favor the idea that WZhet does not act by some property of the DNA itself, for example, by binding a repressor, but instead must make a product. However this point has not been proved by experiment.

The purpose of the present experiments was to define the product responsible for disruption of latency. We wished to compare the product made by standard and defective virus and to learn whether the rearrangement of DNA in WZhet contributed to coding sequences of the product.

(This work was presented in preliminary form at the 11th annual Herpesvirus Workshop, Leeds, England, August 1986.)

## MATERIALS AND METHODS

**Viral DNA fragments.** Identification and cloning of the rearranged 2.7-kbp WZhet fragment was as described previously (5). The standard *Bam*HI Z fragments of viral DNA from EBV strains B95-8, FF41, and P3J-HR-1 were cloned into pSV2neo by using conventional techniques (28). WZhet

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and standard *Bam*HI-Z from the HR-1 strain were sequenced by the dideoxy chain-termination method (26).

**Mutant plasmids.** Deletion mutants which invaded WZhet DNA from the 5' end were constructed by the *Bal* 31 exonuclease technique as described previously (24). WZhet in pBR322 was linearized with *Eco*RI and digested for various lengths of time with *Bal* 31, and *Bam*HI linkers were ligated onto the ends. The constructs were digested with *Bam*HI and ligated into pSV2neo. The sizes of the deletions were determined by electrophoresis on polyacrylamide gels after digestion with *Sma*I and a variety of other restriction endonucleases.

Two site-specific mutants were generated at the *Hin*dIII and *Nhe*I sites in BZLF1. The *Hin*dIII site was removed by filling in the 3' recessed ends left by *Hin*dIII digestion by using the Klenow fragment of DNA polymerase I and deoxynucleotides, followed by religation. The *Nhe*I site was eliminated by removing the 5' overhang with mung bean nuclease followed by religation. WZhet fragments containing these mutations had lost the respective restriction endonuclease recognition site but were not otherwise altered in electrophoretic mobility.

A chimeric plasmid pSV2neo-WZhet-psi 1 was constructed to contain 5' sequences of WZhet (*Bam*HI to *Hin*dIII) and 3' sequences from standard (HR-1) *Bam*HI-Z (*Hin*dIII to *Bam*HI). The 1.7-kbp *Bam*HI-*Hin*dIII subfragment of WZhet was subcloned from pSVod into pSV2neo. This plasmid is designated pCJ1.7. A *Hin*dIII fragment encompassing the carboxy end of standard HR-1 BZLF1 and adjacent sequences of pSV2neo was then cloned into pCJ1.7.

A second chimeric plasmid, pSV2neo-WZhet-psi 2, was constructed to contain 5' sequences of standard (HR-1) *Bam*HI-Z (*Bam*HI to the second *Hin*dIII site) and 3' sequences from WZhet (*Hin*dIII to *Bam*HI). pSV2neo-Z (HR-1) was cleaved completely with *Eco*RI and only partially with *Hin*dIII (partial digestion was monitored by agarose gel electrophoresis). When partial digestion was optimized, pSVOd-1.0 (5), which had also been digested with *Eco*RI and *Hin*dIII was added, and the mixture was ligated. After identification of DNA clones containing the proper insert, the insert was recloned into the *Bam*HI site of pSV2neo (see Fig. 8A).

To construct pSV2neo-BZLF1, mutant 81 was digested with *Bam*HI and *Nco*I. The *Nco*I site is at the first base of the BZLF2 open reading frame. The ends were flushed by using the Klenow fragment of DNA polymerase I and nucleoside triphosphates. The 1,556-bp fragment containing the BZLF1 open reading frame and surrounding sequence was excised from a 5% polyacrylamide gel. This fragment was ligated into pSV2neo which had been cut at the *Bam*HI site, whose ends were flushed as above and treated with calf intestine phosphatase (Boehringer Mannheim Biochemicals).

**Cells.** D98/HR-1 cells, a gift from R. Glaser (Ohio State University), are hybrids between adenine phosphoribosyltransferase-negative D98 cells and the P3J-HR-1 Burkitt lymphoma line (7). These cells have a very low rate of spontaneous expression of the EBV replicative cycle (<1 in $10^6$ cells produce viral replicative antigens) but do express EBNA 1 in every cell. They were maintained in minimal essential medium containing 10% fetal bovine serum and MAGGT (0.6 μM methotrexate, 50 μM adenosine, 50 μM guanosine, 0.1 mM glycine, 16 μM thymidine) (23). D98/HR-1 cells were maintained by weekly splits at a 20:1 cell ratio and by a 5:1 split when used for transfection.

COS-1 cells were used to examine chimeric plasmids for expression of proteins (8). The cells were grown in Dulbecco modified minimal essential medium with 5% fetal bovine serum. They were maintained by weekly 20:1 cell splits and seeded at 5:1 cell splits into 100-mm petri dishes before transfection.

**Transfection.** D98/HR-1 cells and COS-1 cells were seeded into 100-mm petri dishes 3 days before transfection. D98/HR-1 cells were transfected with 8 μg of cloned DNA by the calcium method (9). Transfers of 10 μg of DNA into COS-1 cells was by the DEAE-dextran method followed by treatment with chloroquine (21).

**Immunoblots.** At 48 to 72 h after transfection, cells from a 100-mm dish were scraped into 0.5 ml of sodium dodecyl sulfate sample buffer. Extracts were sonicated for 15 s and boiled for 5 min. Approximately one third of each total extract was electrophoresed on a sodium dodecyl sulfate–10% polyacrylamide gel and transferred to a nitrocellulose filter (31). A skim milk solution (Blotto) was used to block the filter before incubation with antibody (17).

**Antisera.** Polyclonal human antiserum SC with high titers to EBV replicative proteins, including ZEBRA, came from a patient with putative chronic EBV infection. The serum was the gift of J. Jones, Denver, Colorado. A human antiserum, RM, from a healthy individual convalescent from mononucleosis was used to identify the latent EBV nuclear antigens (25). A monospecific antiserum (Alexander) was prepared in a rabbit to a fusion protein between β-galactosidase and the last 330 nucleotides of BZLF1 (27).

## RESULTS

**Structure of the WZhet fragment (Fig. 1).** On the basis of nucleotide sequence, the rearranged EBV DNA fragment which activates the switch from the latent to the replicative life cycle of the virus is 2,711 bp in length. (H. Jenson and G. Miller, submitted for publication). It contains 1,134 bp from *Bam*HI-W, the first large internal repeat in the genome, and 1,577 bp from *Bam*HI-Z, a region which is more than 50 kbp away in the standard EBV genome. WZhet DNA contains only a single complete open reading frame (BZLF1 in the Cambridge terminology), whose orientation in the rearranged het DNA is opposite to that in the standard EBV genome (1). Portions of three other open reading frames are represented in the active WZhet fragment; these are designated BWRF1 (in the *Bam*HI-W region) and BRLF1 and BZLF2 (in the *Bam*HI-Z portion). The junction between sequences derived from *Bam*HI-W and those from *Bam*HI-Z is at the first T in the sequence TATAAA which is thought to function as a TATA box for latent mRNAs (15). Thus this TATA box is removed. Furthermore two exons of a multiply spliced latent nuclear protein encoded within *Bam*HI-W are not found in WZhet (3).

One underlying purpose of our experiments was to learn whether this rearrangement of DNA affected the structure of a product responsible for activating EBV replication, for example, by fusing exons which were not contiguous on the genome. Accordingly we examined a series of deletion mutants which removed progressively more of the sequences from *Bam*HI-W. The location of these deletions is shown in Fig. 1B.

**Identification of the products from WZhet and from standard *Bam*HI Z fragments (Fig. 2).** To identify protein products encoded by the rearranged WZhet fragment and by the components of standard EBV DNA from which WZhet was derived, *Bam*HI WZhet, *Bam*HI-W, and *Bam*HI-Z from

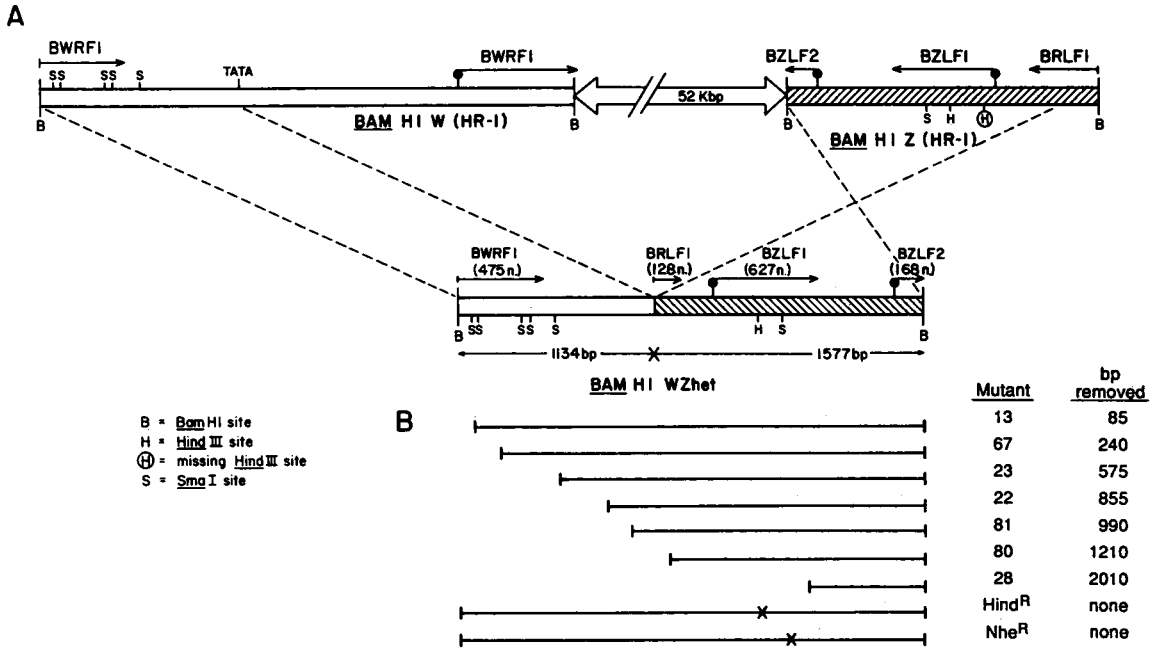


FIG. 1. (A) Diagram of the structure of the *Bam*HI WZhet fragment, based on its nucleotide sequence (Jenson and Miller, submitted). Letters such as BWRF1 and BZLF1 refer to open reading frames in the Cambridge terminology, e.g., BZLF1 is *Bam*HI-Z leftward frame one (1). Numbers in parentheses (n) are base pairs from each reading frame remaining in WZhet. (B) The location and extent of deletions and site-directed mutations used in this study.

several EBV strains were cloned in pSV2neo and introduced into COS-1 cells. A single prominent protein detected by immunoblotting was expressed from WZhet and the standard *Bam*HI Z fragment from three EBV strains. No protein was detected after transfer of pSV2neo-*Bam*HI-W into COS-1 cells (data not shown). This result suggested that the protein made by WZhet was likely to be encoded by sequences derived from *Bam*HI-Z. However, reproducible differences in electrophoretic mobility among these proteins were observed. WZhet caused the expression of a protein of about 43 kilodaltons (kDa). The protein from the *Bam*HI-Z DNA fragment of EBV strain HR-1 (from which WZhet is derived) migrated at about 39 kDa, and those expressed from comparable DNA fragments of two immortalizing viruses, B95-8 and FF41, ran at about 37 kDa. A chimeric plasmid, WZhet-psi 1, which contained the leftmost *Bam*HI-*Hin*dIII subfragment (and thus the amino terminus of BZLF1) of WZhet and the leftmost *Bam*HI-*Hin*dIII subfragment (and corresponding carboxy end of BZLF1) of standard (HR-1) *Bam*HI-Z (see Fig. 1 and Fig. 8) was constructed. WZhet-psi 1 produced a protein with electrophoretic mobility identical to that of the protein encoded by WZhet. The ZEBRA protein encoded by the second chimeric plasmid WZhet-psi 2 (see Fig. 8), which contained the amino terminus of standard HR-1 BZLF1 and the carboxy terminus of het BZLF1, migrated at about 42 kDa (data not shown; J. Countryman, Ph.D. Thesis, Yale University, New Haven, Conn., 1987).

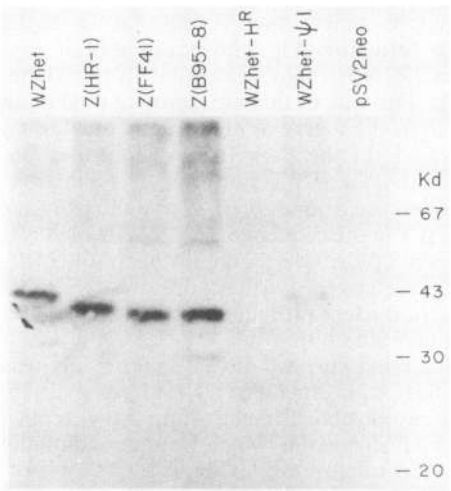


FIG. 2. Electrophoretic mobility of ZEBRA proteins. Shown is an immunoblot of proteins extracted from COS-1 cells transfected with various pSV2neo plasmids containing WZhet, standard *Bam*HI-Z from strains HR-1, FF41, and B95-8, WZhet with the *Hin*dIII site filled in (WZhet-H$^R$), and the chimeric plasmid (WZhet-psi 1). The immunoblot was reacted with a 1:250 dilution of a polyvalent human antiserum, SC. Note the variation in electrophoretic mobility of the ZEBRA proteins.

**The product from WZhet and standard *Bam*HI-Z is encoded by BZLF1.** We obtained four types of direct evidence which proved that the 43-kDa protein expressed by WZhet was encoded by BZLF1. Restriction endonuclease site-directed frameshift mutations were produced in BZLF1—one at the *Hin*dIII site (260 bp into the open reading frame) and another at the *Nhe*I site (420 bp into BZLF1). Neither of these mutations, both of which would be expected to put the coding region out of frame, was able to express protein in COS-1 cells (Fig. 2, lane 5; data not shown).

We examined deletion mutants which removed progressively more DNA from the *Bam*HI-W region of the WZhet fragment for their capacity to express the product in COS-1 cells (Fig. 3). A mutant such as number 81, which removed

all but 147 bp of *Bam*HI-W, still expressed a 43-kDa protein in COS-1 cells. Mutant number 80, which removed all of the *Bam*HI W sequences as well as 73 bp from BRLF1, also expressed a 43-kDa protein, although in several trials less of the protein appeared to be made in COS-1 cells. These experiments showed that the differences in electrophoretic mobility between the 43-kDa WZhet protein and the 39-kDa standard Z (HR-1) protein were not due to fusion of exons which were derived from *Bam*HI-W. A deletion mutant (number 28 in Fig. 3) which invaded BZLF1 did not make any proteins in COS-1 cells detectable by immunoblotting.

A plasmid which entirely eliminated the BZLF2 reading frame from mutant 81 by resection at the *Nco*I site also encoded the 43-kDa protein (data not shown; Countryman, Ph.D. thesis). Together these mutants narrowed the coding region for the 43-kDa ZEBRA protein to 1,335 bp within *Bam*HI-Z, which contains only the BZLF1 open reading frame.

Antibodies raised in a rabbit to the carboxy end of BZLF1 expressed in bacteria as a *lac* fusion protein, detected the protein made in COS-1 cells (Fig. 4). This antibody detected the same difference in electrophoretic mobility between WZhet and the standard Z (HR-1) protein which was seen with polyvalent antibodies.

These lines of evidence together indicate that the polymorphic protein seen after gene transfer into COS-1 cells is encoded entirely in sequences derived from a 1.3-kbp subfragment of *Bam*HI-Z.

**Disruption of latency by plasmids containing standard *Bam*HI-Z sequences.** One assay for disruption of latency is to measure on immunoblots the abundance of a number of EBV replicative polypeptides in D98/HR-1 cells which have been transfected with different plasmids (Fig. 5). D98/HR-1 cells usually have a low background of cells spontaneously induced into the viral replicative cycle (<1 in $10^6$). This background accounts for the EBV polypeptides seen in D98/HR-1 cells which received pSV2neo. After induction of replication by phorbol ester (phorbol 12-myristate 13-acetate), there was an overall increase in both the abundance and number of viral replicative polypeptides. Induction by WZhet and the chimeric WZhet-psi 1 appeared, on inspection, to be slightly less efficient than induction by phorbol 12-myristate 13-acetate (Fig. 5). Standard *Bam*HI-Z fragments from three EBV strains, HR-1, FF41, and B95-8, induced appearance of replicative polypeptides at a lower level than WZhet or WZhet-psi 1. Densitometer tracings of the autoradiograph at the position of two replicative

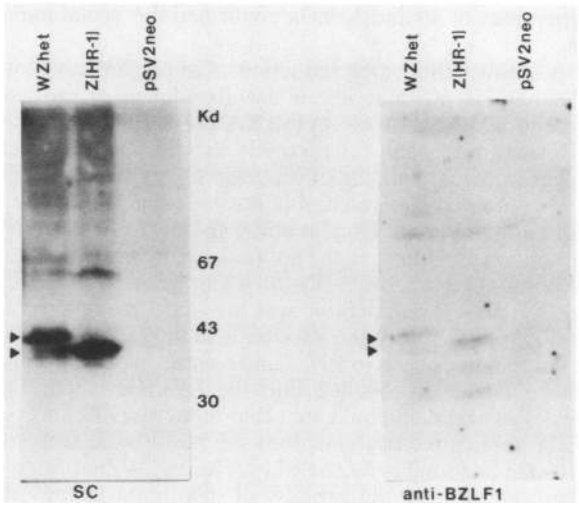


FIG. 4. Detection of ZEBRA proteins with human antiserum and a rabbit antiserum raised to a fusion protein containing the carboxy end of BZLF1. pSV2neo plasmids containing WZhet or standard *Bam*HI-Z were transfected into COS-1 cells. Immunoblots of COS-1 cell extracts were reacted with a 1:250 human antiserum SC or with a 1:50 dilution of the rabbit anti-BZLF1. Note that both antisera detect the same difference in electrophoretic mobility of the proteins.

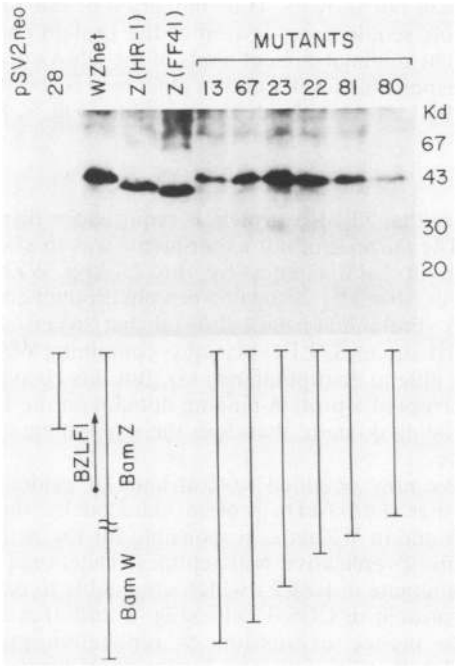


FIG. 3. ZEBRA proteins made in COS-1 cells by WZhet and deletion mutants which invade WZhet from the left end. Note that mutant 80, which eliminates all of the sequences from *Bam*HI-W, still makes a ZEBRA protein with the electrophoretic mobility of WZhet ZEBRA protein. Mutant 28, which invades BZLF1, makes no protein.

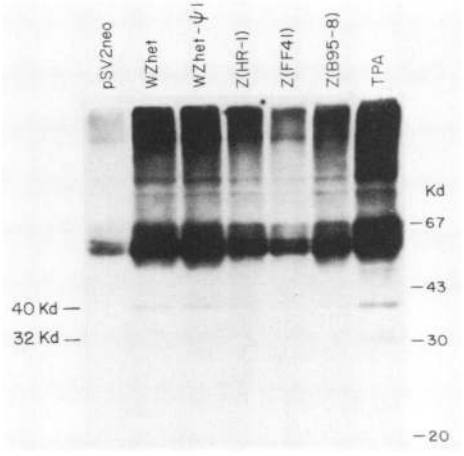


FIG. 5. Induction of expression of EBV replicative proteins after treatment of D98/HR-1 cells with TPA or after transfection with pSV2neo alone or containing WZhet, standard *Bam*HI-Z from strains HR-1, FF41, or B95-8, or the chimeric mutant WZhet-psi 1. An immunoblot of D98/HR-1 cell extracts was reacted with a 1:250 dilution of human antiserum SC.

polypeptides of 40 and 32 kDa confirmed the visual impressions.

It is known that after induction of the EBV replicative cycle there is no increase in the abundance of the latent polypeptide EBNA 1 or its mRNA (32). This served as a control to show that cell extracts in which we measured changes in the abundance of replicative polypeptides (Fig. 5 and 6) contained equivalent amounts of total protein. When an immunoblot containing identical amounts of cell extracts (Fig. 5) was probed with an antiserum which contains antibodies directed primarily to latent gene products, the level of EBNA 1 polypeptide was the same in all lanes (data not shown). Therefore the variations in induction of replicative polypeptides seen in Fig. 5 and 6 were due to differences in the plasmids introduced into the D98/HR-1 cells. This series of experiments indicated that induction of expression of EBV replicative proteins does not require the rearrangement such as found in WZhet; however the WZhet fragment is more efficient at the process of disrupting latency than standard *Bam*HI-Z.

**Effects of deletional, site-directed, and chimeric mutations on induction of D98/HR-1 cells.** We compared induction by pSV2neo plasmids containing WZhet, standard HR-1 Z, and various deletional and frameshift mutations of WZhet (Fig. 6 and 7). Once again induction of D98/HR-1 cells by WZhet was more efficient than induction by Z (HR-1). Deletions which removed *Bam*HI-W sequences from the WZhet fragment were still able to induce replicative proteins. Mutant 28, which invaded BZLF1 and failed to express protein, was unable to disrupt latency. Mutant 80, which removed all of the *Bam*HI-W sequences, and mutant 81, which left only 144 bp of *Bam*HI-W, both induced replication; however the level of induction by these two mutants was somewhat lower than that brought about by other deletion mutants which retained more *Bam*HI-W sequences.

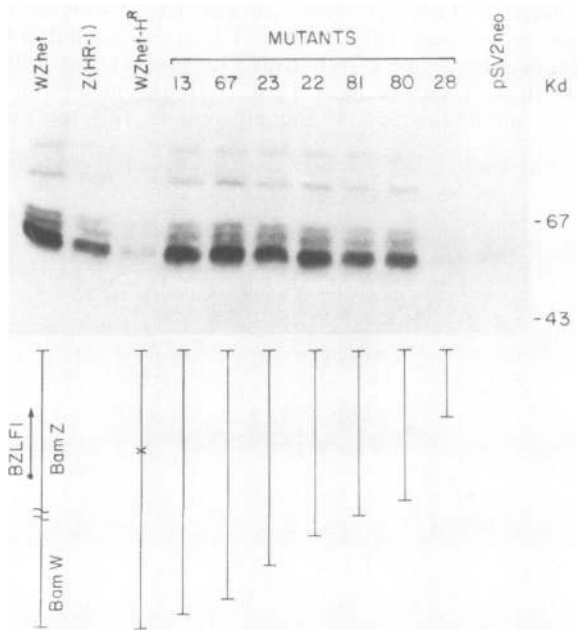


FIG. 6. Induction of expression of EBV replicative polypeptides in D98/HR-1 cells by WZhet, standard HR-1 *Bam*HI-Z, WZhet with the *Hin*dIII fill-in (WZhet-H$^R$), and various deletion mutants from the left end. Note that neither WZhet-H nor mutant 28, both of which alter BZLF1, induces expression of EBV replicative polypeptides.

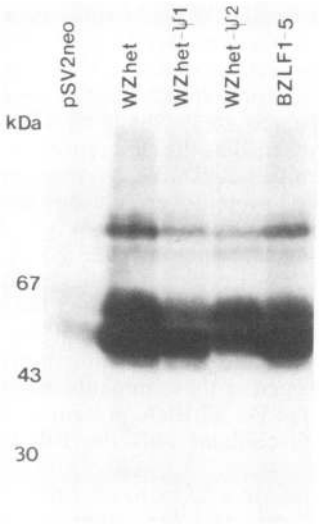


FIG. 7. Induction of replicative antigens by WZhet, two chimeric mutants, and BZLF1; immunoblot of replicative antigens induced in D98/HR-1 cells after transfection with pSV2neo or pSV2neo containing WZhet, the two chimeric mutants (WZhet-psi 1 and WZhet-psi 2), or the BZLF1 open reading frame (BZLF1-5). The polyvalent human antiserum SC was used to detect replicative antigens.

The two site-directed mutations at the *Hin*dIII and *Nhe*I sites in BZLF1, which destroyed the ability of the plasmids to express protein in COS-1 cells (Fig. 3), also eliminated the capacity of these plasmids to induce replicative polypeptides (Fig. 6; data not shown). Thus induction of the EBV replicative cycle required expression of the protein encoded by BZLF1. The minimal current limits of the DNA encoding the protein responsible for disruption of latency is about 1.3 kbp. Mutants BZLF1-5 and 80 define this limit (Fig. 7, 8, and 9).

## DISCUSSION

**Evidence that ZEBRA protein is required for disruption of latency.** The purpose of our experiments was to ask whether disruption of EBV latency by the 2.7-kbp WZhet DNA fragment occurred by virtue of a protein product encoded by this DNA. Preliminary data showed that, when cleaved at the *Hin*dIII site in BZLF1, plasmids containing WZhet were no longer able to disrupt latency (5). But this cleavage could have interrupted a protein-binding domain on the DNA or a protein-coding domain; therefore the experiments were not conclusive.

We have now obtained several lines of evidence which indicate that the 43-kDa protein encoded by the BZLF1 reading frame in WZhet is responsible for the activation of expression of replicative polypeptides. Deletional and site-directed mutants in BZLF1 which are unable to express the ZEBRA protein in COS-1 cells (Fig. 2 and 3) are likewise unable to induce expression of replicative proteins in D98/HR-1 cells (Fig. 5, 6, and 7). Standard viruses without defective genomes encode ZEBRA proteins which vary from that encoded by WZhet both in electrophoretic mobility (Fig. 2) and in their capacity to disrupt latency (Fig. 5 and 6). Thus there appears to be a family of ZEBRA proteins which vary in their electrophoretic mobility and in their biologic

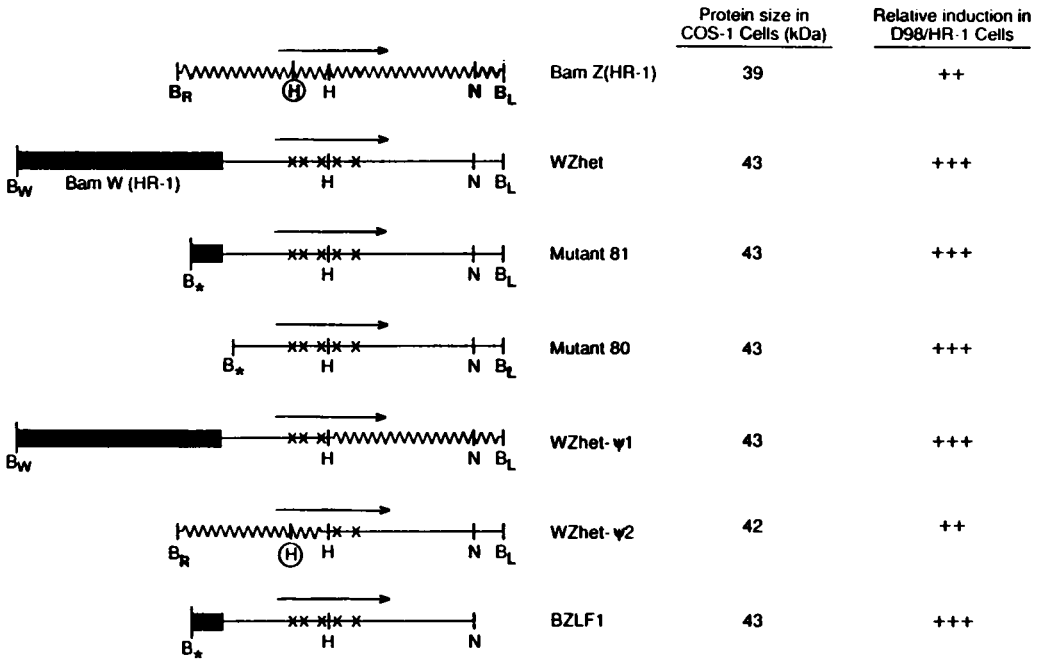


FIG. 8. Structure of WZhet and mutant DNA; comparison of WZhet DNA with standard HR-1 *Bam*HI-Z, two deletion mutants (81 and 80), two chimeric mutants (WZhet-psi 1 and WZhet-psi 2), and BZLF1. For each DNA the electrophoretic mobility of ZEBRA in COS-1 cells and the relative induction efficiency in D98/HR-1 cells is noted. Symbols and abbreviations: +++, maximum induction efficiency; 0, the least induction, as by pSV2neo alone; $B_R$, right-hand *Bam*HI site in standard *Bam*HI-Z; $B_L$, left-hand *Bam*HI site in standard *Bam*HI-Z; H, *Hin*dIII site; Ⓗ, *Hin*dIII site missing; $B_W$, left-hand *Bam*HI site in *Bam*HI-W; $B_*$, *Bam*HI site created by *Bam*HI linkers; N, *Nco*I site; ▭, *Bam*HI-W sequence; ——, WZhet sequence; ∿∿∿, *Bam*HI-Z sequence; X, location of amino acid differences which distinguish BZLF1 (WZhet) from BZLF1 (HR-1).

activity. This might account for differences among EBVs in their tendency to enter the replicative life cycle spontaneously or in their capacity to disrupt latency when added to cells with a latent genome.

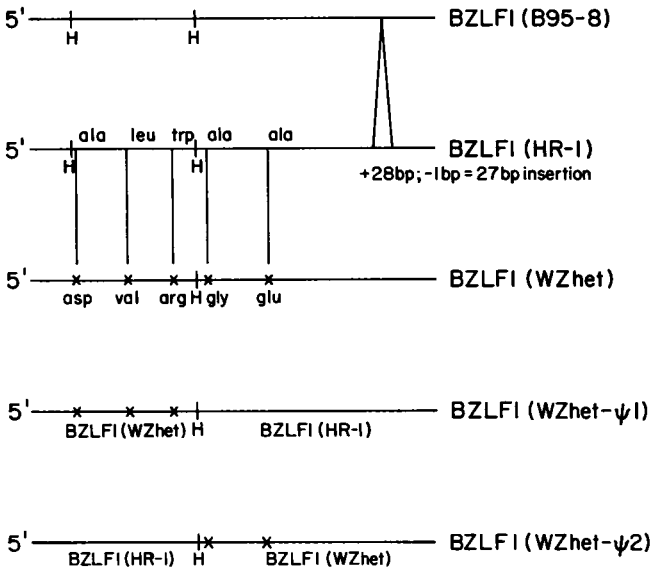


FIG. 9. Diagrammatic comparison of BZLF1 from standard *Bam*HI-Z, WZhet, and the chimeric mutants. BZLF1 (HR-1) contains an additional 28 bp later followed by a 1-bp deletion, by comparison to BZLF1 (B95-8). The relative locations of the five amino acid changes which distinguish BZLF1 (HR-1) from BZLF1 (WZhet) are shown, as are their positions in the chimeric mutants.

In related experiments we have studied ZEBRA expression during constitutive expression of the viral replicative life cycle in cell lines which spontaneously make virus and after induction of replication by TPA or butyrate (N. Taylor, J. Countryman, C. Rooney, D. Katz, and G. Miller, manuscript in preparation). Using a potent antiserum to ZEBRA we have detected ZEBRA expression whenever the viral replicative cycle is activated. Thus ZEBRA expression does not require defective virus, but is an invariant marker for switch of the virus from latency to replication.

**Differences in electrophoretic mobility of ZEBRA proteins.** According to DNA sequence data which will be presented in detail elsewhere (Jenson and Miller, submitted), the BZLF1 from B95-8 virus (the prototype transforming strain which was completely sequenced [1]) differs considerably from that in HR-1 virus, the nonimmortalizing EBV strain which was the source of the defective virus. The reading frame is 27 bp longer in HR-1, and there is a frameshift which could result in the translation of a protein which differs in its carboxy end (Fig. 8 and 9; data not shown). In addition there are 27 scattered bp changes of which 15 result in an amino acid change. This might explain the reproducible difference seen in the electrophoretic mobility between ZEBRAs from B95-8 and HR-1 (Fig. 2 and 3).

However there is also a reproducible 4-kDa difference in electrophoretic mobility between ZEBRA made from the HR-1 defective virus and that made by the HR-1 standard virus (Fig. 2, 3, and 4). Although this difference is only illustrated in this report in COS-1 cells transfected with pSV2neo-Z (HR-1) and pSV2neo-WZhet (Fig. 2 and 3), the same difference in size is seen in HR-1 cells with and without defective virus (Taylor et al., in preparation). We initially thought that this difference in electrophoretic mobility might

be the consequence of a contribution of *Bam*HI-W DNA to the coding sequences for the protein. However deletional mutagenesis (Fig. 3) disproved this hypothesis. In comparing sequences of BZLF1 between HR-1 and WZhet, there are only five amino acid changes and no deletions or insertions (Fig. 8; H. Jenson, manuscript in preparation). These amino acid alterations may directly affect electrophoretic mobility, or they may affect posttranslational modification of the protein, if it occurs. Three of these five amino acid changes are present in the chimeric WZhet-psi 1, which has the electrophoretic mobility of WZhet.

The BZLF1 reading frame is 627 bp long in WZhet, assuming that it is used in an unspliced form. This reading frame would encode a protein of about 23 kDa, yet the protein migrates at 43 kDa. We have shown that only 1.3 kbp of DNA is required to encode this 43-kDa protein and that no other identified reading frames besides the 627-bp BZLF1 contribute to it. Therefore the anomalous migration of ZEBRA might be due to posttranslational modification or might result from its high proline content (28 of 209 amino acids in the WZhet BZLF1).

**Related work by others.** Takada et al. have shown that the *Bam*HI Z fragment from B95-8 virus, when cloned downstream of powerful transcription signals in an avian retrovirus long terminal repeat and introduced into Raji cells, will activate transcription of the *Bam*HI-H and -F regions of the genome (30). Chevallier-Greco et al. confirmed and extended this finding (4). Plasmids containing BZLF1 from HR-1 driven by the early simian virus 40 promoter and enhancer, caused induction of expression of many transcripts in Raji cells. Plasmids containing the *Bam*HI-M and -S region, which encodes a promiscuous transactivator (20, 33), were unable to activate transcription in Raji cells. Similarly we have not observed disruption of latency in either D98/HR-1 or X50-7 cells by the MB'het fragment, which contains the coding region for this transactivator (20, 33). Using a reporter plasmid of the EBV DR promoter linked to chloramphenicol acetyltransferase, Chevallier-Greco found that the BZLF1 function interacted with the *Bam*HI-M and -S functions in genome-negative BJAB cells. Neither Takada et al. nor Chevallier-Greco et al. attempted to identify the protein product responsible for disruption of latency, which we do in our study. Studies of the latter group, however, which also included a deletion mutant in BZLF1 and vectors lacking enhancers or promoters, indicated that a protein was likely to be responsible for transcription.

Seibl et al. (27) studied the products of the *Bam*HI-Z region by immunoprecipitation of proteins made by in vitro translation of hybrid selected mRNAs and also those made in vivo. They found variations in electrophoretic mobility of proteins translated in vitro from B95-8 and HR-1 mRNAs hybrid selected with plasmids containing BZLF1. However, Seibl et al. were unable to distinguish the proteins made in HR-1 cells from the defective virus as contrasted with those encoded by the standard HR-1 virus. Our studies demonstrate this reproducible difference in electrophoretic mobility, which is an extremely useful marker (Fig. 2, 3, and 4). Seibl et al. did not conduct any functional studies which related *Bam*HI-Z products to the disruption of latency.

What is novel in our report is the correlation between structural and functional studies. By using a variety of mutants we were able to show that expression of the ZEBRA proteins is needed for disruption of latency in D98/HR-1 cells.

**Role of the rearranged sequences in WZhet.** Our experiments show that the sequences from *Bam*HI-W do not play a role in encoding the WZhet protein (Fig. 3). Furthermore standard *Bam*HI Z fragments, with no *Bam*HI-W attached, are able to activate expression of replicative proteins in D98/HR-1 cells, albeit at a lower efficiency than WZhet (Fig. 5 and 6). Thus activation of latency does not absolutely require *Bam*HI W sequences. How does one reconcile this with previous biologic experiments, which showed that defective EBV is a potent agent able to induce EBV replication, whereas standard EBVs are considerably less efficient at the process (22)?

As shown in this report, the ZEBRA protein encoded by WZhet is more efficient at the induction of replication than comparable proteins from standard viruses. Furthermore, in the defective virus there is a large, 56-kbp palindrome which contains two copies of the WZhet fragment (16). Thus defective virus might also be more active by virtue of increased copy number of the ZEBRA gene. The most likely explanations, however, relate to differences in regulatory signals.

Our experiments do not directly address questions about the control of expression of ZEBRA. In the plasmid pSV2neo, transcription is probably being driven by the simian virus 40 promoter. One obvious potential role for the sequences derived from *Bam*HI W in the defective virus and possibly in plasmids as well is to provide crucial *cis*-active sequences which enhance the expression of ZEBRA. The lower levels of induced viral replicative proteins seen (Fig. 6) with mutants 81 and 80 which remove considerable portions of the *Bam*HI-W sequences is consistent with this hypothesis. Recent experiments indicate that more ZEBRA protein is expressed after transfection of Burkitt lymphoma cells when the BZLF1 reading frame is linked to *Bam*HI-W (C. Rooney et al., submitted for publication). More must be learned of the nature of the regulation of ZEBRA expression in the standard and defective viruses. Clearly sequences other than those from *Bam*HI-W are upstream of the gene in the standard genome and undoubtedly provide control signals (Fig. 1). Defective genomes may lack negative regulatory signals usually present in standard virus or may contain novel positive-regulatory elements in the upstream region of the ZEBRA gene. The deciphering of these signals will be needed to learn why ZEBRA expression is usually suppressed during EBV latency.

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