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The Switch Between EBV Latency and Replication

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This paper reviews experiments done in the author's laboratory which led to the discovery of an Epstein-Barr virus (EBV) gene, BZLF1, whose product, ZEBRA, switches the virus from latency to the replicative phase of its life cycle. Recent experiments are summarized which explore the effects of EBV genome rearrangements and cell background on the expression of ZEBRA, and which investigate the viral targets of ZEBRA action.

BIOLOGIC PROPERTIES OF EBV

There are two central biologic phenomena which virologists interested in the pathogenesis of Epstein-Barr virus (EBV) hope to explore at a molecular level. The first is the immortalization of B lymphocytes. The virus changes a resting B cell into a continuously proliferating cell *in vitro* and similar events accompany *in vivo* infection. The central question is: Which viral and cellular genes participate in this process? Among the crucial viral products are a group of ten genes which are expressed in latent infection. These include six nuclear products (EBNAs), two small RNAs (EBERs), a latent membrane protein (LMP) and an mRNA (TP) which represents a second putative membrane protein [1–6].

Once the cell is immortalized, it usually maintains the EBV genome in a latent state, expressing these ten products. The virus's life cycle can, however, be switched between latency and replication by a variety of inducing stimuli. Following induction, as many as 100 viral genes are expressed. Inducing agents include the phorbol ester TPA, butyrate, anti-immunoglobulin, and serum factors. A potent inducing stimulus is virus from the HR-1 strain of EBV (Fig. 1).

The HR-1 strain is an unusual mutant EBV. It has two remarkable properties. The first is that the virus is unable to immortalize B lymphocytes [7]. This defect correlates with a large genomic deletion which affects the structural gene for EBNA 2 as well as two downstream exons of a protein we call EBNA 4 and others term leader protein (LP) [8–10]. Recent studies by Rooney and colleagues indicate that this deletion also impairs expression of EBNA 1 and EBERs after HR-1 virus infects primary lymphocytes [11]; that is, the deletion in HR-1 virus affects expression of other genes which are not deleted but apparently dependent for their synthesis on the deleted gene product(s). The second remarkable property of HR-1 virus is that when it is added to cells with a latent EBV, HR-1 activates the expression of early replicative proteins [12].

Abbreviations: EBV: Epstein-Barr virus het DNA: heterogeneous DNA LMP: latent membrane protein LP: leader protein ZEBRA: Z EB replication activator

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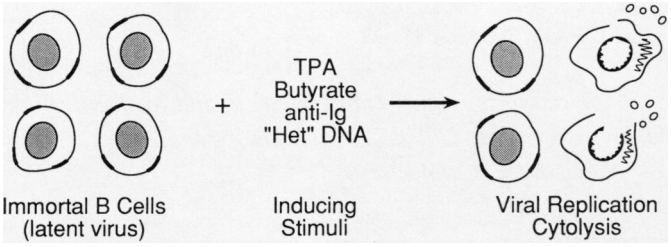


FIG. 1. Latency to replication switch. Immortal B lymphocytes expressing only the latent products can be induced to express replicative products, and ultimately mature virus, by a variety of inducing stimuli.

Which viral and cellular gene products control the switch between latency and replication?

We wished to identify the viral genomic alterations which could account for these remarkable biologic properties. A potentially confusing finding was that HR-1 viral DNA contained submolar and hypermolar restriction fragments which were not present in preparations of viral DNA from other strains. We called this het (for heterogeneous) DNA. The approach we took was to examine clones of the HR-1 cell line for the presence of het DNA. We found that nearly all of the HR-1 cell clones had lost the het DNA which was retained in only one of 200 HR-1 subclones which were examined [13,14]. Of more interest, there was a marked difference in the biologic characteristics of HR-1 cell clones which lacked het DNA and the rare one which retained it. Retention of het DNA was associated with the spontaneous expression of viral replicative products. In the majority of clones which lacked het DNA, the virus remained latent.

An additional experiment demonstrated that HR-1 virus stocks containing het DNA were able to transactivate immortalizing EBV when added to a cell line with latent virus [15]. Virus could be recovered from some het (−) cell clones by treatment with chemical inducing agents such as phorbol ester. When added in multiplicities equivalent to that of het+ virus, however, virus without het DNA was found to be incapable of activating replication of the endogenous virus. The virus which was recovered from latently infected cells following addition of HR-1 het+ virus was shown to be the endogenous virus latent in the cells and not a recombinant, by use of restriction fragment length polymorphisms which distinguished the endogenous from the superinfecting virus.

CHARACTERISTICS OF het DNA

Het DNA represents a defective EBV genome. It is maintained in the HR-1 culture by spreading extracellularly from cell to cell rather than by partitioning vertically as does the standard EBV plasmid [16]. Het DNA is extensively deleted and rearranged. There have been several intramolecular recombinations which join non-contiguous portions of the genome, and in several instances the intramolecular recombinations are accompanied by inversion of sequences.

A crucial portion of het DNA is arranged as a 16 Kbp palindrome [17]. This palindrome is perfect except for the central 341 bp, which is derived from the viral large internal repeat; however, this central region is also capable of forming a stem and loop structure [18]. The net result of these rearrangements in het DNA is to truncate some open reading frames and to bring other open reading frames next to new upstream and downstream sequences.

Identification and Characterization of the Active Component of het DNA

The experiment which led to identification of the region of het DNA which disrupts latency consisted first of molecular cloning all of the het DNA as individual *Bam*HI fragments into the pSV2neo vector. These recombinant plasmids were introduced individually into monolayer cells (D98/HR-1) which contained a latent EBV genome. From these experiments one 2.7 Kbp fragment, designated WZhet, was found by Countryman to activate expression of many viral replicative polypeptides [19]. The effect of introducing WZhet was similar to treating the cells with an inducing chemical, such as TPA.

The DNA sequence of WZhet was determined by Jenson [20]. It represents a recombination and inversion of two regions of EBV DNA which are usually more than 50 Kbp apart on the standard EBV genome. Only one intact open reading frame is present: BZLF1. Three other open reading frames, BWRF1, BZLF2, and BRLF1 are truncated in the active WZhet fragment. Therefore, while it was likely that the BZLF1 open reading frame encoded the responsible product, the contributions of the other partial open reading frames needed to be evaluated.

Countryman found that the product of the BZLF1 gene present in WZhet had a slower electrophoretic mobility than the product from the standard *Bam*HI Z fragment. This result suggested that perhaps a product active in disrupting latency was formed by exon fusion or shuffle. When deletion mutants were constructed which removed all of the sequences from *Bam*HI W, however, the protein was unaffected [21]. Thus the rearrangement did not contribute to the structure of the protein. Instead anomalous electrophoretic mobility was due to one or more of five point mutations present in WZhet, as contrasted with standard *Bam*HI Z [20].

By placing site-specific mutations in BZLF1 and then introducing the plasmids into cells with latent EBV it was shown that the *protein* encoded by BZLF1 was responsible for disrupting latency. Those mutations which impaired the ability of the reading frame to make protein also abolished the capacity of the plasmid to disrupt latency. Since the responsible protein was encoded within *Bam*HI Z, we called it ZEBRA (*Z EB R*eplication *A*ctivator).

Regulation of ZEBRA Differs in HR-1 Cells With and Without het DNA (Fig. 2)

Recent experiments by Taylor have shown that the presence of het DNA affects control of ZEBRA expression. In cells lacking het DNA (such as clone 16), neither BZLF1 mRNA nor ZEBRA protein is made spontaneously. When clone 16 cells are treated with chemical inducers, such as sodium butyrate, however, ZEBRA transcription is stimulated and ZEBRA polypeptide is made [22].

By contrast, cells containing het DNA (such as clone 5) synthesize BZLF1 mRNA and ZEBRA protein spontaneously. These results suggest that regulation of transcription of BZLF1 differs in the standard and het genome and that this regulation is the pivotal event in the switch between latency and replication.

Genome Rearrangements in het DNA Activate ZEBRA (Fig. 3)

Cliona Rooney explored those regions of the palindromic het DNA which are responsible for activating expression of the BZLF1 gene [23]. Her experimental approach was to construct various plasmids containing BZLF1 and to introduce them using gene transfer techniques into cultured EBV genome-negative Burkitt lymphoma cells. The expression of ZEBRA was monitored with a specific antiserum to the protein. If the standard *Bam*HI Z fragment was used, without the addition of a

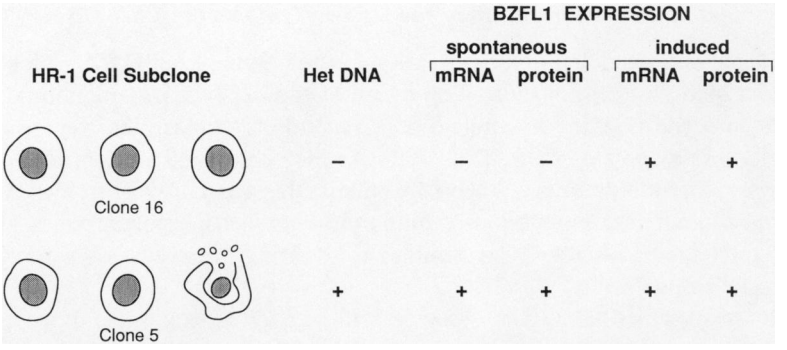
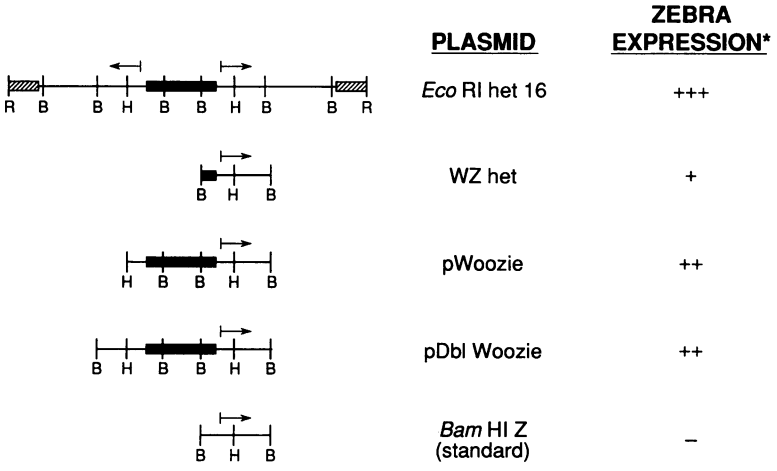


FIG. 2. Differences in the regulation of BZLF1 in HR-1 cell clones with or without het DNA. Clone 16 cells express only latent products, represented by shaded nuclei. A subpopulation of clone 5 cells produce virus. BZLF1 expression was studied in untreated cells or after induction with TPA or butyrate. mRNA was estimated by Northern blotting and ZEBRA protein was detected on Western blots probed with monospecific antiserum to BZLF1.

heterologous promoter, no ZEBRA could be identified. The entire *Eco*RI het 16 palindrome produced a high level of ZEBRA expression. A much weaker signal was seen with the WZhet subfragment; if more of the rearranged upstream sequences were included, such as in the plasmids "pWoozie" or "pDbl Woozie" (Fig. 3), the signal increased, but not to the level seen with *Eco*RI het 16. This finding indicated that sequences downstream of BZLF1 in *Eco*RI het 16 were important. In related ex-



* After Transfection into Burkitt Lymphoma Cells

FIG. 3. Genome rearrangements in het DNA activate ZEBRA. *Eco*RI het 16 and various other plasmids were cloned in pACYC184. In the diagrams of the plasmids, the bold lines indicate rearranged sequences from the large internal repeat (*Bam*HI W); the hatched lines indicate rearranged sequences from *Bam*HI S; the letters refer to restriction endonuclease recognition sites (**B,** *Bam*HI; **R,** *Eco*RI; **H,** *Hind* III). They were transfected into EBV genome negative Burkitt lymphoma cells by the DEAE Dextran method. ZEBRA expression was scored after 48 hours by probing Western blots with the monospecific antiserum to BZLF1.

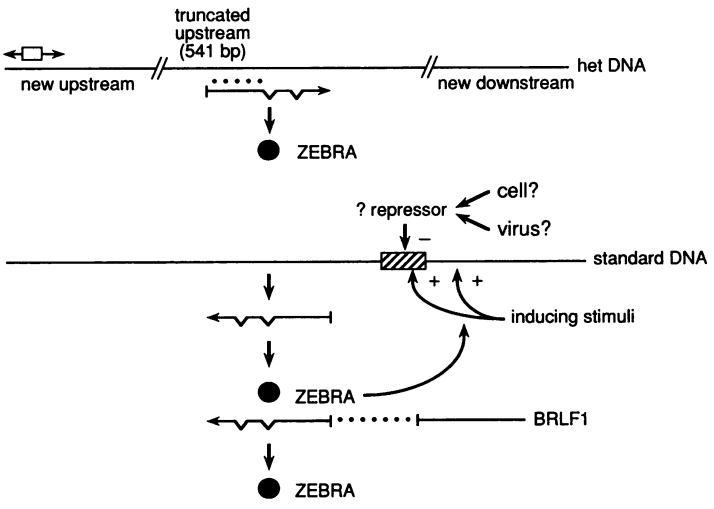


FIG. 4. Regulation of BZLF1 expression differs in het DNA and standard EBV DNA. In het DNA, there are novel sequences upstream and downstream of BZLF1 due to intramolecular recombinations. The BZLF1 gene is transcribed spontaneously, and the ZEBRA protein is made. het ZEBRA has five point mutations (*dots*) in comparison to standard ZEBRA. In the standard genome, BZLF1 transcription is repressed, but inducing stimuli release transcription of at least two mRNAs which contain BZLF1. The larger mRNA also contains BRLF1 [12]. ZEBRA positively activates its own expression.

periments, the effect of progressive deletions of the rearranged upstream *Bam*HI W sequences was examined when pSV2neo WZhet was transfected into BL cells carrying a latent EBV genome. A region of about 900 bp of *Bam*HI W sequences appears to be important for ZEBRA expression and for disruption of latency. Thus the genome rearrangements of het DNA have brought novel upstream and downstream sequences next to the BZLF1 open reading frame. The exact functional role played by these rearrangments is not yet clear. Experiments so far indicate that they do not provide *trans* active products, but are likely to be *cis* active signals which affect BZFL1 transcription. It is likely that the downstream elements function as "enhancers."

A summary of models for the regulation of BZLF1 transcription is found in Fig. 4. In het DNA, BZLF1 is constitutively transcribed, presumably as the result of new positive *cis* active elements which are newly positioned upstream and downstream of the gene; however, the intramolecular recombinations in het DNA have also truncated the normal region upstream of BZLF1 to 541 bp. If this region in standard virus contains *negative* regulatory elements, then they have been removed in the recombination event.

In the standard genome there is suppression of BZLF1 transcription, presumably as the result of repressors encoded by the cell or by the virus or as the result of a lack of positive regulatory elements. Chemical inducers turn on BZLF1 transcription, perhaps by activating transcription factors or by removing or inactivating repressors. The control point(s) of regulation are not yet clear. They may be directly upstream of BZLF1 or possibly upstream of BRLF1 which is a gene co-linear with BZLF1. ZEBRA itself may play an autostimulatory role in regulating its own transcription.

TARGETS OF THE BZLF1 GENE

A central question is whether BZLF1 activates the expression of many EBV genes or only one or a few. If the latter is the case, does it activate certain EBV genes directly, e.g., by binding to DNA, or indirectly, perhaps by interaction with cellular transcription factors?

Our general approach to this question has been to attempt to identify those genes which are activated by ZEBRA. In our first such experiment BZLF1, carried on a vector with a selectable marker (G418 resistance) was stably introduced into X50-7 cells with a latent genome, using the electroporation technique. Some clones of X50-7 cells which became resistant to the antibiotic also expressed early and late viral replicative antigens [24]. One colony was subcloned; about half of the subclones expressed early antigens, some expressed membrane antigens, and a few went on to produce mature virus. One subclone yielded high titers of transforming virus. Three clones which expressed replicative antigens also retained an integrated WZhet fragment. From these experiments, we concluded that in some instances WZhet was capable of driving the entire EBV replicative cycle to completion. A corollary of this experiment was that in some cells both early and late viral genes are activated by BZLF1 expression. Thus many viral genes are activated as the result of ZEBRA expression.

There are several drawbacks to this set of experiments: *(1)* it was difficult to identify and analyze the single copy of integrated WZhet DNA; *(2)* only one cell line, X50-7, was studied; in this cell line ZEBRA activated a cascade leading to synthesis of virus; *(3)* direct evidence that ZEBRA expression was correlated with the disruption of latency was lacking.

In a recent series of experiments, we prepared BZLF1 plasmids which were carried on vectors with the EBV plasmid origin (oriP) [25]. These were introduced stably into three different cell lines using G418 selection. The extent of expression of EBV was monitored by testing the BZLF1 transformants for virus and by assaying them with a panel of monoclonal antibodies to early and late EBV gene products. The remarkable finding from this series of experiments was that each cell line responded to a different extent to the introduction of the BZLF1 gene (Fig. 5). X50-7 cells made early and late products and released transforming virus, as they had in earlier experiments when WZhet was introduced without oriP. Since the introduced BZLF1 gene was derived from the het DNA, it was marked with the polymorphic protein marker. Therefore it could be shown that cells which responded produced ZEBRA from the endogenous genome as well as from the plasmid which was introduced. This finding indicated that ZEBRA is autostimulatory. X50-7.17, a single cell subclone of the X50-7 line, produced late viral antigens representing the viral envelope but never released any infectious virus. By contrast Raji cells made only early antigens.

These experiments led to several conclusions. Early genes are the proximal targets of BZLF1 action. The ZEBRA product does not directly influence late genes in Raji cells and, by inference, probably not in X50-7 cells. Additional cell or viral factors must be required beyond the point of ZEBRA action to drive the viral replicative cycle into viral DNA synthesis, late gene expression, and virion production.

CELL BACKGROUND

It has been known for many years that the cell background plays a crucial role in the regulation of EBV expression [26]. Host cell species and even age of the donor of the

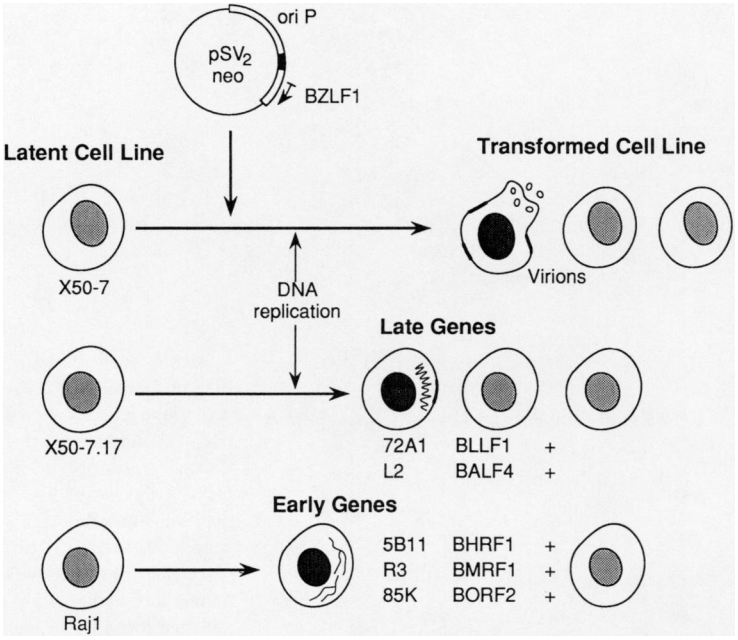


FIG. 5. Stable activation of EBV early genes by oriP/BZLF1 plasmids. A series of cell lines which were resistant to G418 were derived after transfection with pSV_2neo/oriP/BZLF1 plasmids. The resultant transformed cells were scored for expression of EBV replicative functions with a panel of monoclonal antibodies and were tested for release of virus which could immortalize lymphocytes. Only X50-7 cells released virions. A clone of X50-7 cells made late antigens. Raji cells expressed only early genes. The length of the arrows indicates extent of progression along the pathway of replicative gene expression.

target cell play a role in the extent to which the virus switches from latency into replication. This cell control is likely to act at the level of control of ZEBRA. For example, the same virus strain, B95-8, has been used to infect three different sorts of cells: EBV genome negative Burkitt lymphoma cells, human umbilical cord lymphocytes, and marmoset cells. These three cell types differ in their regulation of EBV from tightly latent (the Burkitt lymphoma cells) to inducible (the umbilical cord cells) to productive (the marmoset cells). The controls correlate with ZEBRA expression, which is either tightly restricted, inducible, or spontaneously active (Table 1). Thus the cells' control on ZEBRA expression is likely to account for different levels of EBV replication.

TABLE 1
Cell Controls on ZEBRA Expression by Standard EBV

		Expression of Endogenous ZEBRA Inducible by		
Behavior	Cells/Virus	Spontaneous	TPA	het(+) Virus
Tightly latent	BL41/B95-8	−	−	−
Latent/Inducible	X50-7/B95-8	−	+/−	+
Productive	B95-8/B95-8	+	+	+

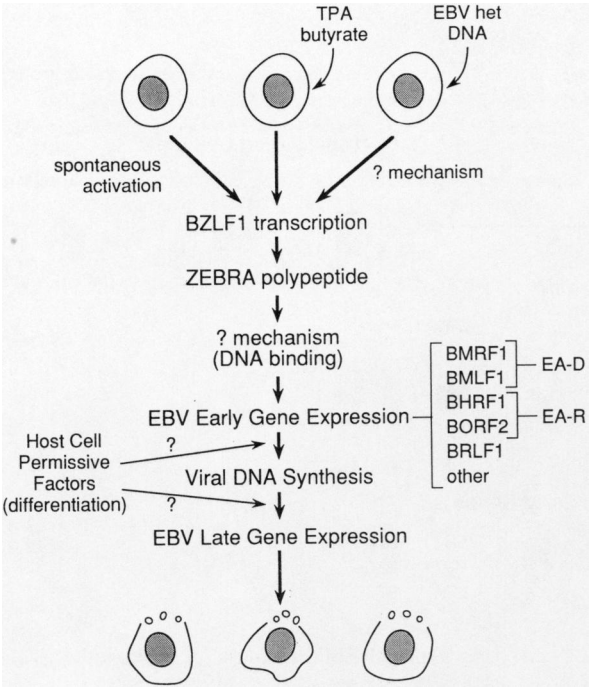


FIG. 6. Proposed pathway for activation of latent EBV. Both spontaneous and chemically induced activation of replication are thought to induce BZLF1 transcription, but the mechanism is unknown. The ZEBRA polypeptide activates EBV early gene expression, perhaps by DNA binding and transcriptional activation. Additional host cell and viral factors are required for entry into viral DNA synthesis, EBV late gene expression, and virus maturation.

SUMMARY

It now seems likely that control of transcription of the BZLF1 gene mediates the switch from latency to replication which occurs during spontaneous activation (marmoset cells), chemical-induced activation, or activation by HR-1 virus with het DNA (Fig. 6). The ZEBRA polypeptide then activates a number of EBV early genes by a mechanism which is unknown but presumably involves DNA binding. Additional viral and cellular gene products are needed to permit the virus to enter lytic viral DNA synthesis and to produce late genes and viral particles. It is likely that host cell permissivity factors are crucial in these later steps. These permissivity factors may well be closely related to cell differentiation, since it is known that EBV is latent in lymphocytes but replicates in epithelial cells.

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