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Genome rearrangements activate the Epstein–Barr virus gene whose product disrupts latency

C. ROONEY*, N. TAYLOR†, J. COUNTRYMAN†, H. JENSON*‡, J. KOLMAN†, AND G. MILLER*†‡

Departments of *Epidemiology and Public Health, †Molecular Biophysics and Biochemistry, and ‡Pediatrics, Yale University School of Medicine, New Haven, CT 06510

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ABSTRACT **A defective Epstein–Barr virus (EBV) containing a deleted and rearranged genome (het DNA) causes latent EBV to replicate. This activity maps to the 2.7-kilobase-pair WZhet fragment. The BZLF1 open reading frame, present within WZhet as well as in the standard viral *Bam*HI Z fragment, encodes the protein ZEBRA, which induces viral replication. Using gene transfers into Burkitt lymphoma cells, we now demonstrate that rearranged sequences juxtaposed to BZLF1 in het DNA facilitate expression of ZEBRA protein. Two stretches of EBV sequences within a palindromic region of het DNA contain positive regulatory elements. One set, derived from the viral large internal repeat, is newly positioned upstream of BZLF1; the second set is downstream of BZLF1 in het DNA. The capacity of defective HR-1 viruses to disrupt latency of the standard EBV genome is due to abnormal regulation of the BZLF1 gene as a result of genomic rearrangements.**

Many viruses establish persistent lifelong infections within their hosts. One mode of persistence, latency, requires selective repression of viral gene expression. Mechanisms are required to overcome this inhibition to replicate progeny virus particles that can spread within the host and infect other individuals. This report concerns the transition between the latent and replicative life cycles of Epstein–Barr virus (EBV) as studied in B lymphocytes. During latency, EBV expresses a limited number of genes (currently estimated at 10), while during replication there is expression of many viral genes leading to production of infectious virus (1–11).

Previous work identified an EBV gene product that switches the virus from latency into replication (12–15). This polypeptide, encoded within the BZLF1 open reading frame, is called ZEBRA (*Bam*HI Z, EBV replication activator) (16). Introducing BZLF1 sequences, cloned in vectors with strong heterologous promoters, activates EBV replication in latently infected cells. While all plasmids containing BZLF1 driven by heterologous promoters are able to activate replication, EBVs differ in their capacity to switch between latency and replication (17–19). Standard EBVs remain latent in most lymphoid cells, although the replicative cycle can be induced by stimuli such as phorbol esters or butyrate (20, 21). Certain defective EBVs with deleted and rearranged genomes do not establish latency. When such viruses are added to cells in which the standard EBV is latent, they transactivate the latent genome, causing it to replicate (17, 18).

Two mechanisms could explain biologic differences between standard and defective viruses. Point mutations between ZEBRA polypeptides from standard and defective viruses might account for different levels of activity of the protein (13). More plausibly, the ZEBRA gene may be aberrantly regulated in the defective virus because of rearrangements in the het genome (22–24). The present experiments were designed to investigate the effect of these genome rearrangements on expression of the BZLF1 product and disruption of latency.

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MATERIALS AND METHODS

Cell Lines. Gene transfers were made into the EBV genome-negative Burkitt lymphoma (BL) cell line IARC/BL41 (25) or into an EBV-converted variant of BL41 derived by superinfection with EBV from HR-1 cell clone HH514-16, which lacks defective DNA (BL41/CL 16).

Plasmids. EBV DNA fragments originally derived from het DNA were cloned into pSV2neo, pBR322, or pACYC184. Plasmids expressing ZEBRA included pSV2neo WZhet and pACYC184 *Eco*RI het 16. A set of BAL-31 deletion mutants invaded pSV2neo WZhet from the 5′ end (13). Subfragments of *Eco*RI het 16 were prepared (see Fig. 2 legend).

Transfection. Stationary-phase cells were exposed for 30 min at 37°C to 1–5 μg of DNA in RPMI medium containing 100 μg of DEAE-dextran per ml. Thereafter, cells were washed and resuspended in conditioned growth medium with or without phorbol 12-myristate 13-acetate (PMA) at 4 ng/ml for 48 hr.

Polypeptide Detection. ZEBRA was detected by Western blotting with monospecific antibodies raised in rabbits immunized with a TrpE-BZLF1 fusion protein (N.T., J.C., C.R., D. Katz, and G.M., unpublished data). Replicative polypeptides were detected with human WC antiserum (26).

RESULTS

Rearranged Sequences from *Bam*HI W Enhance ZEBRA Expression After Transfection of BL Cells. Countryman *et al.* had shown that rearranged sequences in WZhet did not contribute to the structural gene for the ZEBRA protein nor were they obligatory for activating EBV replication in a somatic cell hybrid (D98/HR-1) (13). These conclusions were based on the use of deletion mutants in which sequences were progressively removed from the *Bam*HI W region of pSV2neo WZhet (Fig. 1 *A* and *B*).

To determine whether rearranged sequences from *Bam*HI W affected expression of ZEBRA in B cells the same mutants were transfected into BL41/CL16 cells. Expression of ZEBRA was monitored 48 hr after transfection by using monospecific anti-BZLF1 antibody (Fig. 1*C*). After transfection of BL41/CL16 cells with the deletion mutants, expression of ZEBRA was unaffected by removal of 240 base pairs (bp) of *Bam*HI W sequences (mutant 67) but diminished in mutant 22 in which 855 bp of *Bam*HI W sequences were removed. Expression was so reduced in mutants 81 and 80 (990 and 1210 bp removed) that ZEBRA could not be detected unless transfections were performed in the presence of PMA, which enhances transcription from the simian virus 40 (SV40)

Abbreviations: EBV, Epstein–Barr virus; PMA, phorbol 12-myristate 13-acetate; SV40, simian virus 40.

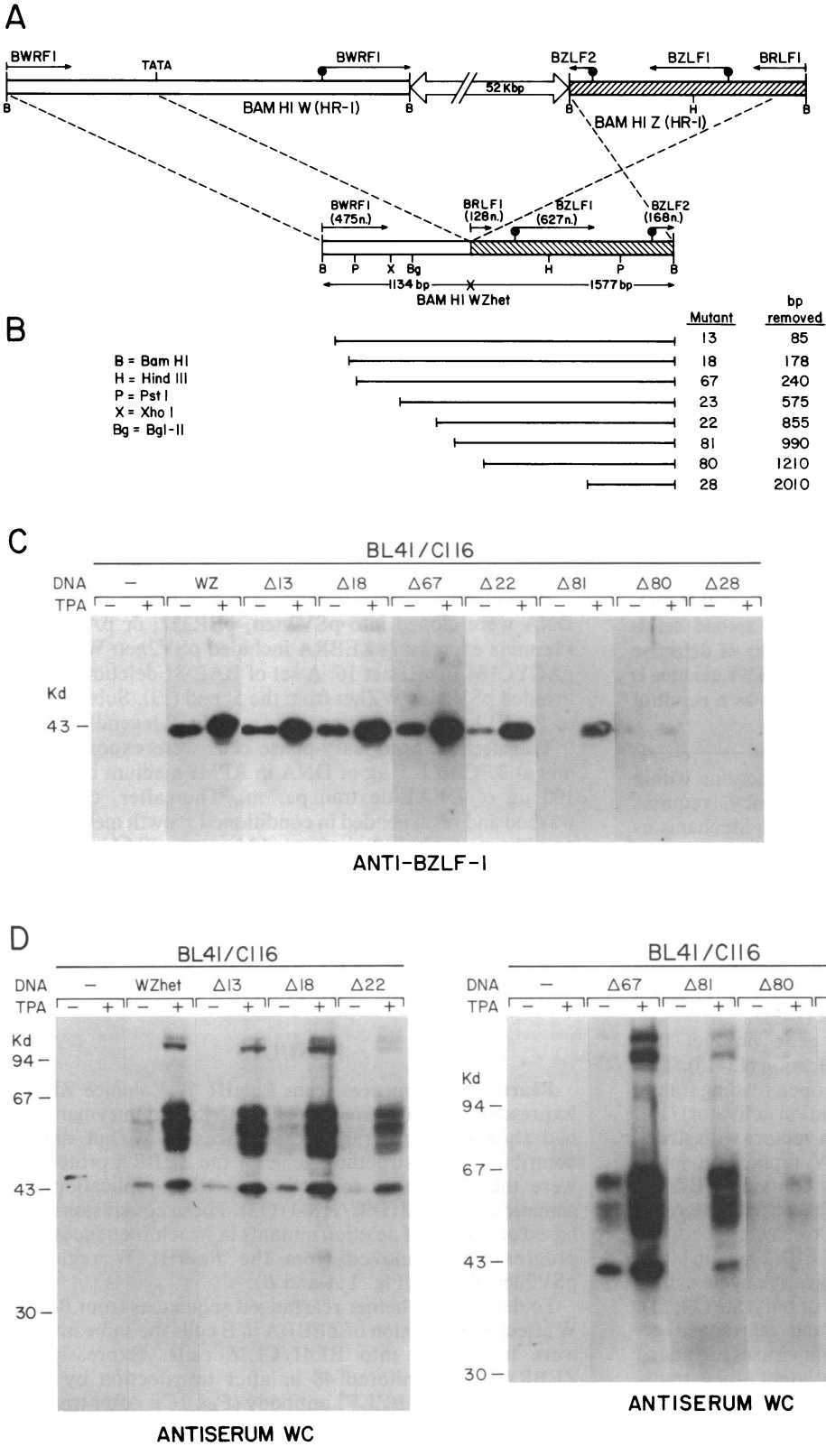

FIG. 1. Expression of ZEBRA and disruption of latency by WZhet and by deletion mutants that remove *Bam*HI W sequences from WZhet. (*A*) Map of WZhet based on its nucleotide sequence (27). (*B*) Position of deletion mutants that progressively remove *Bam*HI W sequences from the left end of WZhet. (*C*) Effect of deletion of *Bam*HI W sequences on expression of ZEBRA 48 hr after transfection of BL41/CL16 cells treated (+) or untreated (−) with PMA (TPA). Immunoblots reacted with rabbit antiserum raised to a TrpE–BZLF1 fusion protein. (*D*) Induction of expression of EBV replicative polypeptides 48 hr after transfection of BL41/CL16 cells with WZhet and with deletion mutants of WZhet. Cells were treated (+) or unexposed (−) to PMA. Immunoblots reacted with human antiserum WC, which recognizes many replicative polypeptides.

promoter/enhancer system (28). Even in the presence of PMA, ZEBRA expression from mutant 80 was only weakly detected. Mutant 28, which invades the BZLF1 open reading frame, did not express the ZEBRA polypeptide.

Mock-transferred BL41/CL16 cells did not express replicative polypeptides spontaneously or after treatment with PMA (Fig. 1*D*). Disruption of latency by the deletion mutants correlated with the level of ZEBRA expression (compare Fig. 1 *C* and *D*). Mutants such as 13, 18, and 67, which expressed ZEBRA at the same level as WZhet, also disrupted latency with the same efficiency; mutants 22, 81, and 80 showed a diminished capacity to disrupt latency. Increased levels of ZEBRA expression seen in the presence of PMA was accompanied by increased induction of replicative polypeptides.

These findings represent the first direct evidence that the rearrangement of sequences in WZhet provided signals that enhanced the expression of the BZLF1 product and its

biologic effect of disrupting latency in B-lymphoid cells. The signals appear to be located between bp 240 and bp 1143 of *Bam*HI W (the point of recombination with *Bam*HI Z in WZhet).

Expression of ZEBRA in the Absence of the SV40 Promoter/Enhancer System. We postulated that a heterologous promoter/enhancer system might not be needed if more of the genome rearrangements in het DNA were included together with the BZLF1 open reading frame. We tested this hypothesis by carrying out transfections with plasmids containing only rearranged EBV DNA (Fig. 2).

Expression of ZEBRA was studied after transfection of B cells with the entire *Eco*RI het 16 palindrome (Fig. 3). Recipient cells were BL41, which lacked an EBV genome, or BL41/CL16, which carried a latent EBV genome (Fig. 3). In both types of cells, the *Eco*RI het 16 palindrome cloned on pACYC184 expressed ZEBRA at similar levels as did WZhet driven by the SV40 promoter/enhancer. Expression of ZEBRA was greater in stably converted BL41/CL16 cells with a latent EBV genome than in EBV genome-negative BL41 cells (compare Fig. 3 *A* and *B*). Expression of ZEBRA could not be detected in BL41 cells transfected with pBR322 WZhet or with pSV2neo carrying *Bam*HI Z (Fig. 3*A*). In BL41 cells, ZEBRA expression from WZhet was thus dependent on both the heterologous SV40 promoter and the *Bam*HI W sequences. However, the requirement for the heterologous promoter could be overcome by using the entire *Eco*RI het 16 palindrome of which WZhet is a subfragment.

That efficient expression of ZEBRA from *Eco*RI het 16 did not require a heterologous promoter was further demonstrated by experiments in which the EBV DNA inserts were excised from their vectors (Figs. 3*B* and 5*A*). Expression of ZEBRA from pSV2neo *Bam*HI Z and pSV2neo WZhet mutant 80 was abolished when the inserts were excised, and that from pSV2neo WZhet was considerably reduced (Fig. 3*B*). In contrast, expression of ZEBRA was unaffected or even slightly enhanced by excision of *Eco*RI het 16 from its vector.

ZEBRA Expression from Cloned Subfragments Encompassing the Center of the Palindrome. We considered the hypothesis that 1.2 kbp of sequences within the center of the palindrome of defective DNA contributed to the higher levels of ZEBRA expression by *Eco*RI het 16 than by WZhet. Therefore, expression of ZEBRA from *Eco*RI het 16 was compared with that from the plasmid pMM277, which contained the entire IR1 region of *Eco*RI het 16 linked to one ZEBRA gene (see Fig. 2). In BL41 cells pMM277 produced a weak, but definite, signal of ZEBRA expression, which was seen in the presence of PMA (Fig. 4 *B* and *C*; data not shown). Since ZEBRA was never expressed from pBR WZhet in BL41 cells (Fig. 3; data not shown), these results suggested that plasmids containing the central palindromic *Bam*HI W sequences contained additional signals that increased ZEBRA production. Expression from pMM445, which contains two copies of WZhet as well as the central portion of the palindrome, was slightly stronger than that from pMM277, which contains only one copy (data not shown). However, neither plasmid was as active as *Eco*RI het 16 (Fig. 4).

To determine whether the higher level of expression of ZEBRA observed after transfection with *Eco*RI het 16 by comparison to pMM277 was the result of production of additional polypeptides synthesized by the larger of the two fragments of het DNA, immunoblots of BL41 cells transfected with *Eco*RI het 16 were probed with polyvalent human antisera (WC and Marshall) that are highly reactive to many EBV replicative products (6). The predominant polypeptide

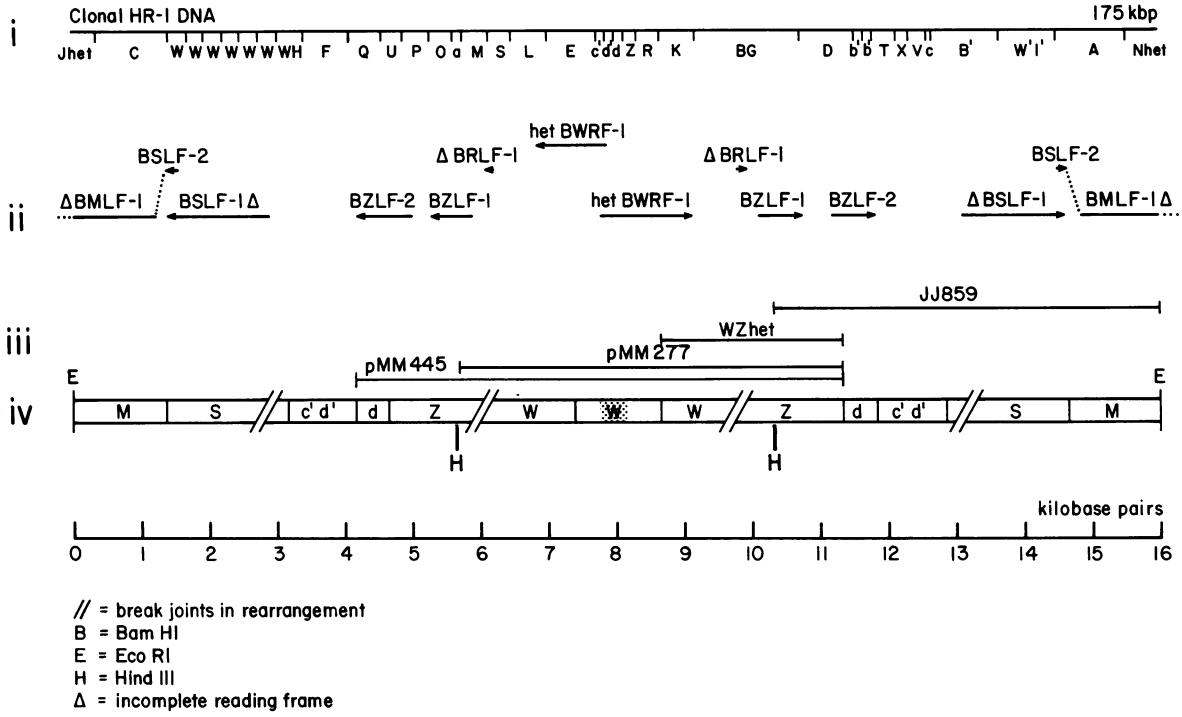

FIG. 2. Map of the 16-kbp palindrome, designated *Eco*RI het 16, present in HR-1 clone 5-defective virus. (*i*) A *Bam*HI restriction endonuclease map of the standard HR-1 genome (29). (*ii*) Location of open reading frames found in *Eco*RI het 16 (Cambridge nomenclature). BRLF1 is truncated at its amino terminus due to the recombination between *Bam*HI Z and *Bam*HI W (see Fig. 1*A*). Dotted line indicates the MS-early antigen, which is composed of one exon from BSLF2 and another from BMLF1 (30, 31). (*iii*) Four plasmids that contain subfragments of *Eco*RI het 16. pJJ859 is the rightward 5.8-kbp *Hin*dIII *Bam*HI subfragment cloned in pSV2neo (23). pWZhet is the 2.7 kbp *Bam*HI subfragment. pMM277, which is 5.6 kbp, contains one complete WZhet, the 1.2-kbp *Bam*HI WZhet fragment in the center of the palindrome, and the 1.7-kbp *Bam*HI/*Hin*dIII subfragment from a second WZhet cloned in pBR322. pMM445, cloned into pACYC184 as a 7.1-kbp partial *Bam*HI digestion product, contains two copies of WZhet, the middle *Bam*HI WZhet plus one copy of *Bam*HI d (0.5 kbp). (*iv*) *Bam*HI restriction map of *Eco*RI het 16. Stippled area indicates 341 bp of unique sequences from the *Bam*HI W fragment of the standard HR-1 genome, which form the center of the palindrome (24). Slash markers represent points of recombination between noncontiguous regions of the standard HR-1 genome.

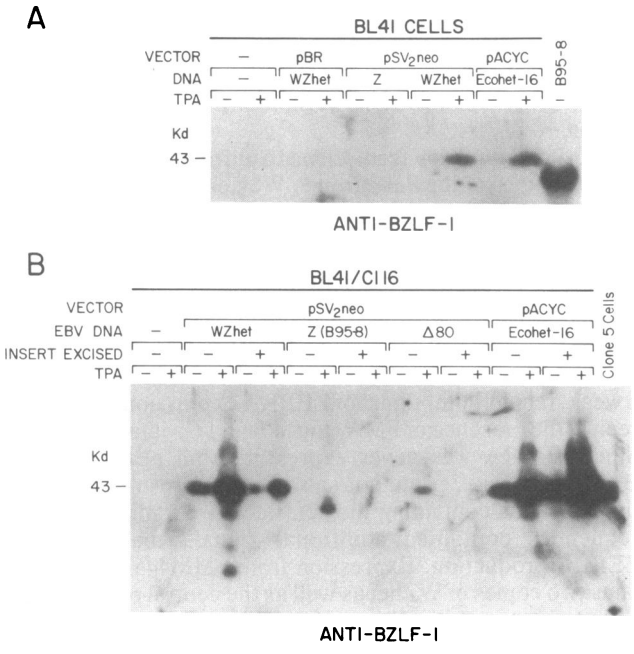

FIG. 3. Expression of ZEBRA after transfection of BL cells with *Eco*RI het 16 in the absence of a heterologous eukaryotic promoter. Immunoblots reacted with rabbit antibodies raised to the TrpE–BZLF1 fusion protein. Transfections were done in the presence (+) or absence (−) of phorbol ester PMA (TPA). (*A*) Recipient cells were IARC/BL41 cells, which do not contain an EBV genome. (*B*) Recipient cells were BL41 cells, which had been stably converted to latent EBV infection after superinfection with HR-1 clone 16 virus (BL41/clone 16). In *B*, expression before (−) or after (+) the EBV inserts were excised from pSV2neo with *Bam*HI (WZhet, *Bam*HI Z, and 80) or from pACYC184 with *Eco*RI.

identified after transfection of *Eco*RI het 16 was ZEBRA (Fig. 4 *C* and *D*).

Comparison of ZEBRA Expression by *Eco*RI het 16 and pMM277 in Cells with an Endogenous EBV Genome. In BL41/CL16 cells consistently higher levels of ZEBRA were made by *Eco*RI het 16 than by pMM277 (Fig. 5*A*). When the EBV inserts in both plasmids were excised, ZEBRA expression was unaffected. The results show that promoter/enhancer systems functional in B cells are found on both fragments of defective DNA but are more active in the larger palindrome.

*Eco*RI het 16 contains additional open reading frames, BSLF1, BSLF2, and BMLF1 (truncated at its carboxyl end), which are not present in pMM277 or pMM445 (see Fig. 2). Some of these sequences encode trans-activators (30, 31). We looked for trans-activating effects of the downstream sequences within *Eco*RI het 16 by cotransfecting pMM277 with a pSV2neo plasmid (pJJ859) that contains these additional sequences (see Fig. 2). When these plasmids were cotransfected into BL41/CL16 cells (Fig. 5*B*), no enhancement of ZEBRA expression or latency disruption was observed.

DISCUSSION

A number of findings help to explain the capacity of HR-1 virus containing het DNA to disrupt latency. Rearrangements of EBV genomic sequences in het DNA allow the expression of the ZEBRA gene product, which induces virus replication. Rearrangements that are important involve sequences from IR1 (*Bam*HI W), which have been repositioned upstream of BZLF1 (Fig. 1; refs. 3 and 5–7) as well as sequences from *Bam*HI S and M, which are now in closer proximity downstream of BZLF1 than they are in the standard genome (Fig. 2). Furthermore, a 16-kbp palindrome of het DNA reproduced the latency disrupting effects of defective HR-1 virus

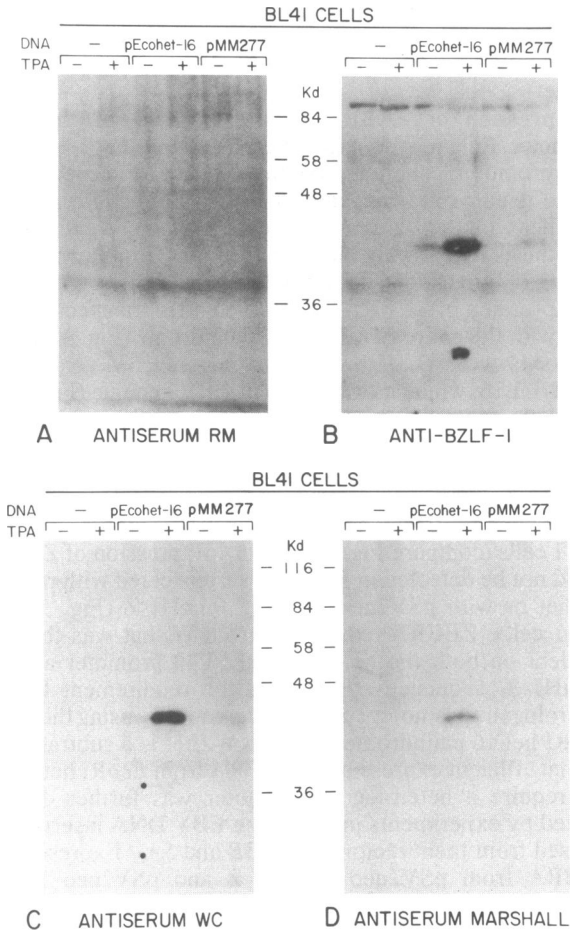

FIG. 4. Comparison of polypeptides expressed in BL41 cells transfected with *Eco*RI het 16 or with cloned subfragment pMM277 (see Fig. 2). Transfections in the presence (+) or absence (−) of PMA (TPA). Four replicate immunoblots reacted with rabbit anti-BZLF1 (*B*) or human antisera RM (*A*), WC (*C*) or Marshall (*D*). The ZEBRA polypeptide was recognized by human sera WC and Marshall, which have antibody to early antigens (EA), but not by RM, which does not have anti-EA. Dots in *C* indicate polypeptides that were weakly recognized by WC serum.

without requiring any promoter/enhancer system derived from another virus (Fig. 3; data not shown). In addition, the level of ZEBRA product was shown to correlate with the extent of expression of induced replicative polypeptides (Fig. 1). The effects of EBV regulatory regions on expression of EBV genes were studied in human BL cells, which are a natural host for the virus.

Effects of Translocated IR1 Sequences on ZEBRA Expression. Sequences from IR1 were partly responsible for the high levels of ZEBRA expression by defective viral DNA. Deletion of IR1 sequences reduced the ability of transfected plasmids with the SV40 promoter to express ZEBRA (Figs. 1 and 3). Plasmids that contained the central palindromic portion of IR1 plus WZhet could function in both BL41 and BL41/CL16 cells independent of any heterologous promoter/enhancer (Figs. 4 and 5).

Regions of IR1 that are thought to be important in control of expression of latent genes in standard EBV are partially or totally excluded from het DNA. A promoter in IR1 that functions in transcription of latent messages is partially deleted at the point of recombination in WZhet. Two exons, W1 and W2, which are found in the leaders of EBV nuclear antigen mRNAs and encode a latent nuclear antigen are also excluded from het DNA. None of the cDNAs for latent transcripts analyzed so far contains those IR1 sequences that are present in *Eco*RI het 16 (32–34).

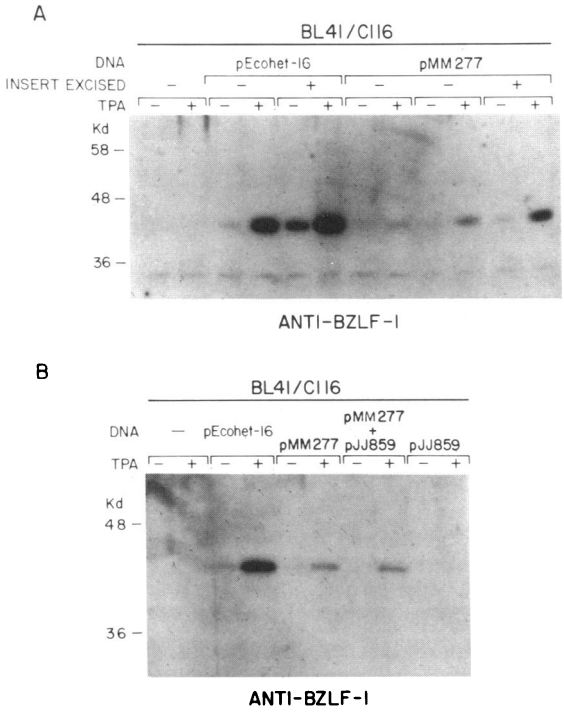

FIG. 5. Comparison of ZEBRA expression by *Eco*RI het 16 and its cloned subfragments after transfection into BL41/CL16 cells in the presence (+) or absence (−) of PMA (TPA). Immunoblots reacted with anti-BZLF1 antiserum. (*A*) pEcoRI het 16 and pMM277 (Fig. 2) compared. Plasmids digested with *Eco*RI (*Eco*RI het 16) or *Pvu* I and *Sal* I (pMM277) to excise the het DNA insert. Two transfections with pMM277 that had not been digested with restriction enzyme; in one, plasmid was "mock-digested" by incubation in enzyme buffer before transfection. (*B*) pEcoRI het 16, pMM277, and pJJ859 (Fig. 2) compared. In part of this experiment, pMM277 and pJJ859 were transfected singly or together.

Experiments with the deletion mutants and with partial digests of *Eco*RI het 16 suggest that there may be more than one functional domain in IR1. Possible candidates for enhancer elements are the sequence 5′-GGGACTTT-3′ found at bp 458 and the sequence 5′-GGACTTT-3′ located at bp 820. These are similar to the consensus 5′-RGGGACTTTCC-3′ (R = purine), which is present in the immunoglobulin κ-chain enhancer and in several viral enhancers including cytomegalovirus and human immunodeficiency virus. This motif forms a binding site for the NF-KB transcription factor (35).

Other Signals in *Eco*RI het 16 That Affect ZEBRA Expression. Expression of ZEBRA from *Eco*RI het 16 was much more efficient than from plasmids lacking downstream sequences from *Bam*HI M and S (Figs. 4 and 5). This increased ZEBRA expression is unlikely to result from additional trans-active products encoded within *Eco*RI het 16 since levels of ZEBRA expression were not altered by supplying sequences downstream of BZLF1 in trans (Fig. 5). The hypothesis that additional regulatory signals in *Eco*RI het 16 are cis-active is more attractive. Potential cis-active signals downstream of BZLF1 probably reside in the promoter region of the BSLF2–BMLF1 open reading frames, which contain a binding site for the PMA-inducible transcription factor AP-1 (30, 36).

Significance of These Findings. Some of the regulatory sequences identified may contribute to the B-lymphocyte specificity of EBV. Preliminary experiments indicate that EBV genes linked to IR1 are expressed in human B lymphocytes and not in other cells.

The findings help to explain the divergent biology of the defective HR-1 virus. They show that genome rearrangements in het DNA have brought strong cis-active constitutive and PMA-inducible regulatory elements together with a viral gene whose product disrupts latency. In the context of the unrearranged genome this gene is silent. The observations raise the possibility that genome rearrangements may be a more widespread mechanism used by viruses to switch from a latent to a replicative life cycle. In the case of EBV, the switch is likely to represent an escape from controls imposed by the host B cell. The activation of ZEBRA expression through genomic rearrangements would be analogous to other recombinational events that occur in lymphocytes, such as immunoglobulin gene rearrangements and translocations that activate oncogenes.

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