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Rescue of the Epstein–Barr Virus BZLF1 Mutant, Z(S186A), Early Gene Activation Defect by the BRLF1 Gene Product

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Expression of the Epstein–Barr virus (EBV) immediate-early protein, BZLF1 (Z), is sufficient to disrupt viral latency. Z transcriptionally activates the EBV early genes by binding to upstream Z-responsive elements (ZREs). Recently, a serine-to-alanine mutation of Z residue 186 (within the basic DNA binding domain) was shown to inhibit the ability of Z to induce lytic infection in latently infected cells, although the Z(S186A) mutant could still bind several known ZREs and activated an early EBV promoter (BMRF1) in transient reporter gene assays (Francis, A. L., Gradoville, L., and Miller, G. (1997). *J. Virol.* 71, 3054–3061). We now show that a specific deficiency in the ability to bind to ZRE elements in the immediate-early BRLF1 promoter may account for the inability of Z(S186A) to activate BRLF1 expression. Furthermore, we demonstrate that the ability of Z(S186A) to induce early BMRF1 and BHRF1 gene expression is rescued by cotransfection with a BRLF1 expression vector. However, the Z(S186A)/BRLF1 (R) combination cannot induce full lytic replication, suggesting that Z(S186A) may also be deficient in a replication-specific function. These results suggest that in the context of the intact viral genome, both Z and R expression are required for activation of early gene transcription in latently infected cells.

INTRODUCTION

Epstein–Barr virus (EBV) is a human herpesvirus that infects ~90% of the world's population. EBV is the causative agent of infectious mononucleosis and has been found to be associated with several forms of cancer, including lymphomas and nasopharyngeal carcinoma (Rickinson and Kieff, 1996; Zur Hausen *et al.,* 1970). EBV primarily infects two cell types, epithelial cells and B cells (Rickinson and Kieff, 1996). In oral epithelial cells, the virus readily replicates and exists in a lytic state (Kieff, 1996; Li *et al.,* 1992; Rickinson and Kieff, 1996; Sixby *et al.,* 1984). EBV infection of B cells usually results in a latent type of infection (Kieff, 1996; Rickinson and Kieff, 1996). However, in a small percentage of B cells, the virus can become reactivated and replicate in a lytic manner. This reactivation is initiated by expression of the immediate-early genes, BZLF1 and BRLF1 (Chevallier-Greco *et al.,* 1986; Countryman and Miller, 1985; Kenney *et al.,* 1989b; Rooney *et al.,* 1988; Rooney *et al.,* 1989; Takada *et al.,* 1986; Zalani *et al.,* 1996).

The BZLF1 (Z) and BRLF1 (R) proteins both have specific functional domains (Fig. 1) for transactivation, DNA binding, and dimerization (protein–protein interactions) (Cox *et al.,* 1990; Flemington *et al.,* 1992; Gruffat *et al.,* 1990A; Hardwick *et al.,* 1992; Kouzarides *et al.,* 1991; Lieberman *et al.,* 1989; Lieberman and Berk, 1990; Manet *et al.,* 1991; Packham *et al.,* 1990; Quinlivan *et al.,* 1993; Urier *et al.,* 1989). Z is a member of the bZIP family of proteins and has homology within the DNA binding domain to the c-Fos and c-Jun proteins (Chang *et al.,* 1990; Farrell *et al.,* 1989; Flemington and Speck, 1991). Z functions as a transcriptional activator of other EBV early gene promoters (Holley-Guthrie *et al.,* 1990; Kenney *et al.,* 1989a; Lieberman *et al.,* 1989; Quinlivan *et al.,* 1993). Z binds to Z-responsive elements (ZREs), which are present in many of the EBV early gene promoters (Chang *et al.,* 1990; Farrell *et al.,* 1989; Lieberman *et al.,* 1989; Quinlivan *et al.,* 1993; Schepers *et al.,* 1993), as well as in the promoters of the two immediate-early genes (BRLF1 and BZLF1) (Flemington and Speck, 1990; Packham *et al.,* 1990). Z has also been shown to activate its own promoter by a nonbinding, indirect mechanism (Flemington *et al.,* 1994), although it has not been shown whether this type of activation is important in the context of the intact viral genome. Z functions together with R to activate the early EBV promoters (Cox *et al.,* 1990; Giot *et al.,* 1991; Holley-Guthrie *et al.,* 1990; Quinlivan *et al.,* 1993). In B cells, expression of Z, but not R, is sufficient to disrupt latency, suggesting that during the disruption of viral latency in B cells, Z is the initial viral gene expressed and that Z subsequently activates R expression (Chevallier-Greco *et al.,* 1986; Countryman and Miller, 1985; Takada *et al.,* 1986; Zalani *et al.,* 1996). In epithelial cells, however, expression of either Z or R is sufficient to disrupt viral latency (Zalani *et al.,* 1996). Z activity is also neces-

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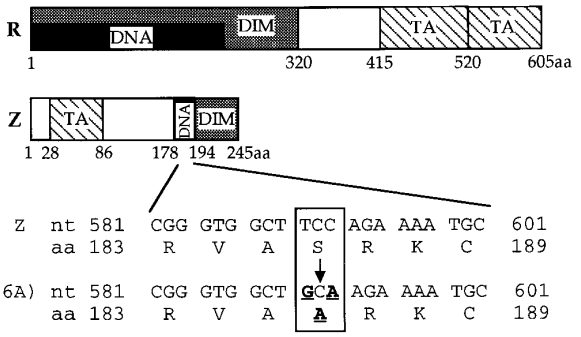


FIG. 1 Functional domains of the R and Z proteins. The R and Z domains for DNA binding (DNA), protein-protein dimerization (DIM), and transactivation (TA) are presented. The Z(S186A) mutation is shown below the Z protein.

sary for replication of the viral lytic origin of replication, ori-Lyt (Fixman *et al.,* 1992, 1995; Schepers *et al.,* 1993).

Recently, an interesting EBV Z mutation has been described (Francis *et al.,* 1997) in which amino acid 186 is changed from a serine to an alanine [Z(S186A); Fig. 1]. This mutation, located in the basic DNA binding region of the protein, abolishes the protein's ability to disrupt viral latency, as measured by the inability of Z(S186A) to induce expression of the EBV immediate-early BRLF1 or early BMRF1 genes from the endogenous viral genome (Francis *et al.,* 1997). However, this mutated form of Z retains its ability to bind to two known ZREs in the BMRF1 promoter and was shown to transcriptionally activate the BMRF1 promoter in reporter gene assays (Francis *et al.,* 1997).

In contrast to the previous report (Francis *et al.,* 1997), we now demonstrate that the Z(S186A) mutation changes the binding specificity of the protein. Although Z(S186A) binds efficiently to the ZRE sites in the BMRF1 promoter (and activates this promoter in transient reporter gene assays), it binds inefficiently to the ZRE sites in the immediate-early BRLF1 promoter and does not activate BRLF1 expression in latently infected cells. Furthermore, we now demonstrate that the ability of Z(S186A) to activate BMRF1 expression in latently infected cells can be rescued by cotransfection with the BRLF1 gene product. Therefore, although Z alone is sufficient to activate many early EBV promoters in transient reporter assays, both Z and R expression are required for early EBV gene expression within the context of the intact viral genome.

RESULTS

Z(S186A) cannot bind to the ZREs of the R promoter

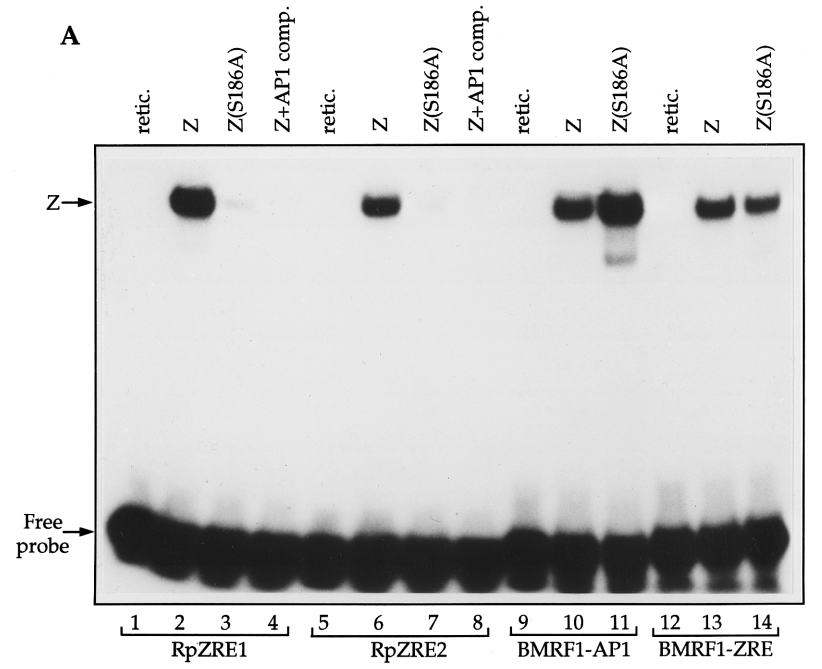
The BRLF1 promoter contains two ZREs that bind Z (Packham *et al.,* 1990), although the importance of these sites in the disruption of viral latency has not been established. Although it was previously shown (Francis *et al.,* 1997) that Z(S186A) can bind to the ZRE core motifs present in the BRLF1 promoter, the previously reported experiments used oligonucleotide probes not containing the authentic BRLF1 promoter ZRE flanking sequences. We performed an electromobility shift assay (EMSA) to determine whether Z(S186A) binds efficiently to the two BRLF1 ZRE sites containing genuine flanking sequences (Fig. 2A). Although Z(S186A) bound as well as or better than wild-type Z to the consensus AP1 and ZRE sites in the BMRF1 promoter, and only slightly less efficiently than wild-type Z to the ZRE sites in the EBV polymerase promoter (Fig. 2B, lanes 10–12), Z(S186A) could not bind efficiently to either ZRE in the BRLF1 promoter. In the BZLF1 promoter, which contains two ZRE sites (ZIIIA and ZIIIB), Z(S186A) bound to the ZIIIB site but did not bind to the ZIIIA element (Fig. 2B). Therefore, Z(S186A) is deficient in its binding to certain ZRE sites contained within the BRLF1 and BZLF1 promoters.

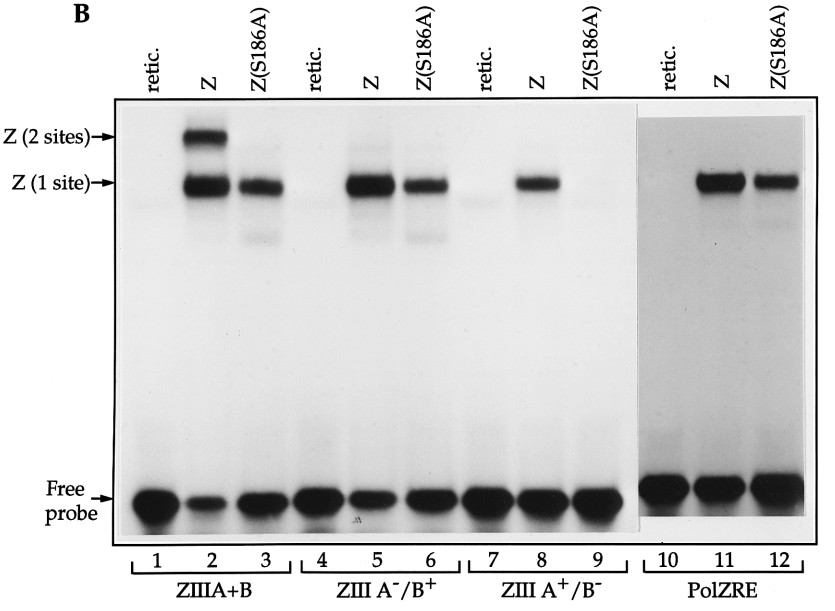
The Z(S186A) mutation is a conversion of amino acid 186 from serine to alanine. Because serine 186 is a possible target for phosphorylation, serine 186 may require phosphorylation (which could occur in reticulocyte lysates) for Z to bind to the ZREs of the BRLF1 promoter. To study the potential effect of Z phosphorylation, we inserted the 186 mutation into a bacterial GST-Z fusion protein. Fig. 3A shows that bacterially produced wild-type Z and Z(S186A) behaved identically to the proteins produced by *in vitro* translation in their relative binding affinities. Because phosphorylation of Z is unlikely to occur in bacteria, it does not appear that Z(S186A) phosphorylation is required for binding to the BRLF1 promoter.

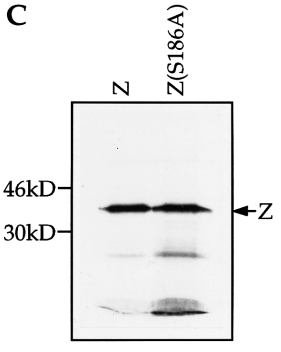
Z(S186A) cannot activate the R promoter in the endogenous genome

BRLF1 expression may be necessary for the disruption of latency because the Z and R proteins cooperate to activate certain EBV early gene promoters in some transient reporter gene assay systems (Cox *et al.,*

FIG. 2. Z(S186A) cannot bind efficiently to the ZREs of the R promoter. (A) An EMSA was performed with ^{32}P-labeled ZRE probes (RpZRE1 and RpZRE2, from the R promoter; BMRF1-AP1 and BMRF1-ZRE, from the BMRF1 promoter) and *in vitro* translated Z and Z(S186A) proteins. Rabbit reticulocyte lysate (retic.) was used as a control for each probe. An unlabeled AP1 competitor was used to specify the Z binding complexes (lanes 4 and 8). (B) An EMSA was performed with ^{32}P-labeled ZRE probes (ZIIIA+B, from the Z promoter; PolZRE, from the EBV polymerase promoter) and *in vitro* translated Z and Z(S186A) proteins. ZIII A^-/B^+ contains a mutant ZIIIA element and a wild-type ZIIIB element. ZIII A^+/B^- contains a wild-type ZIIIA element and a mutant ZIIIB element. (C) Equal amounts of *in vitro* translated Z and Z(S186A) proteins were used for all of the EMSAs. (D) The sequences of each of the ZRE probes are presented. The core binding sequences are underlined. The ZIIIA+B oligo contains two ZRE sites, in opposite orientations, and we have shown each separately.







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RpZRE1	-26	TCTTTTATGAGCCATTGGCA	-45
RpZRE2	-184	AAGCTTATGAGCGATTTTAT	-203
BMRF1-AP1	-73	GATGACCTTTGAGTCAGGTGGCTA	-50
BMRF1-ZRE	-119	TAATTTCTTTGAGCAAGAGAGTTC	-96
PolZRE	-48	GCGCTGCATGAGCAAAACCAGGAG	-25
ZIIIA	-134	ATGCATGAGCCACAGGCATTGCTAATGTACCT	-103
ZIIIB	-103	AGGTACATTAGCAATGCCTGTGGCTCATGCAT	-134

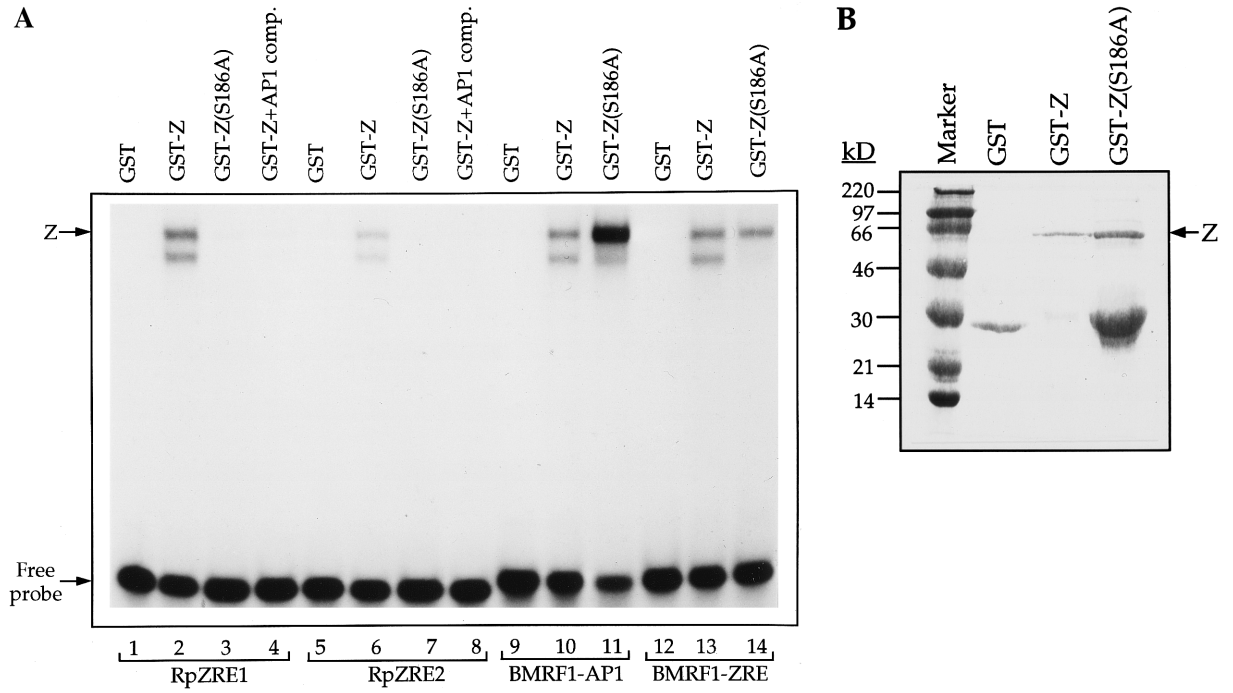

FIG. 3. Binding of bacterially produced Z and Z(S186A) proteins to the ZREs of the R promoter. (A) An EMSA was performed in an identical manner to that in Fig. 2A, with ^{32}P-labeled ZRE probes (RpZRE1 and RpZRE2, from the R promoter; BMRF1-AP1 and BMRF1-ZRE, from the BMRF1 promoter), except that bacterially produced GST, GST-Z, and GST-Z(S186A) proteins were used. An unlabeled AP1 competitor was used to specify the Z binding complexes (lanes 4 and 8). (B) Coomassie-stained gel showing the relative amounts of GST, GST-Z, and GST-Z(S186A) proteins used in the EMSA.

1990; Gruffat *et al.,* 1990A; Holley-Guthrie *et al.,* 1990). Wild-type Z has been shown to activate BRLF1 expression from the endogenous genome (Kolman *et al.,* 1996; Le Roux *et al.,* 1996; Zalani *et al.,* 1996), but it is not known whether Z binding to the BRLF1 promoter is required for activation of BRLF1 in the context of the intact viral genome. To examine the effect of Z(S186A) on BRLF1 expression in latently infected cells, Raji or D98/HE-R-1 cells were transfected with wild-type Z or Z(S186A), and the level of BRLF1 expression was monitored by immunoblot analysis. As shown in Fig. 4, wild-type Z activated BRLF1 expression in both B cells and epithelial cells, whereas Z(S186A) produced no detectable BRLF1 expression in either cell type. Equal amounts of Z and Z(S186A) proteins were produced from the transfected plasmids as judged by immunoblot analysis with an anti-Z antibody (data not shown). Similar to Z(S186A), the Z311 mutant could not activate BRLF1 expression in latently infected cells (data not shown). Thus Z-induced activation of BRLF1 from the

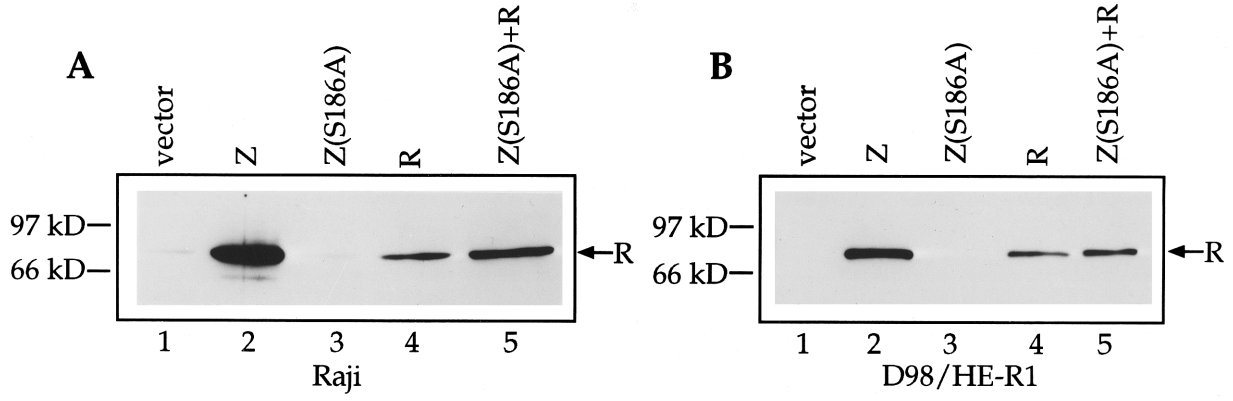

FIG. 4. Z(S186A) does not induce R protein expression. (A) Raji cells were transfected with a total of 10 μg of vector control DNA and/or the Z and R expression vectors as shown. Immunoblot analysis was performed using a monoclonal antibody directed against the R protein [anti-R (1:10) from Alain Sergeant]. (B) D98/HE-R1 cells were transfected with a total of 10 μg of vector control DNA or the Z and R expression vectors as shown, and immunoblot analysis was performed as described in A.

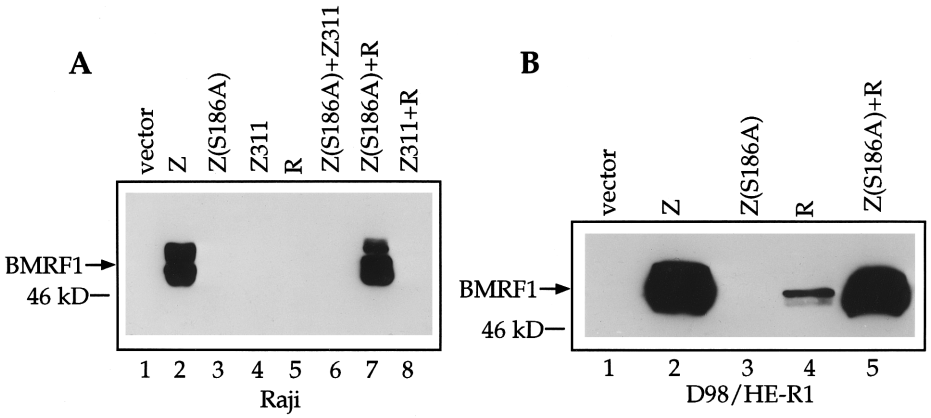

FIG. 5. The R protein can restore the ability of Z(S186A) to induce BMRF1 expression. (A) Raji cells were transfected with a total of 10 μg of either vector control DNA and/or the various Z and R expression vectors as indicated. Immunoblot analysis was performed using a monoclonal antibody directed against the BMRF1 protein [anti-EBV, EAD (1:40), from Capricorn]. (B) D98/HE-R1 cells were transfected with a total of 10 μg of either vector control DNA or the various Z and R expression vectors as indicated, and immunoblot analysis was performed as described in A.

endogenous viral genome likely requires direct binding to the BRLF1 promoter.

The addition of BRLF1 (R) can bypass the Z(S186A) mutation and lead to the expression of BMRF1

The inability of Z(S186A) to activate BRLF1 transcription suggests that the addition of exogenous R might rescue the ability of Z(S186A) to disrupt viral latency. Therefore, we examined the ability of wild-type Z, Z(S186A), and Z311, in the presence or absence of co-transfected R, to induce early EBV gene expression in a variety of latently infected cell types (Fig. 5). The expression of wild-type Z alone induced BMRF1 expression in B cells (Raji), as expected. As previously shown, neither the expression of Z(S186A) alone nor R alone in Raji cells induced BMRF1 expression. However, the combination of Z(S186A) plus R induced BMRF1 expression in Raji cells as efficiently as wild-type Z. Thus the presence of R is able to rescue the ability of Z(S186A) to activate BMRF1 expression. Equivalent results were obtained using the latently infected EBV-positive B cell lines Jijoye and B95–8 (data not shown). Similar results were also obtained in the D98/HE-R-1 cell line (Fig. 5B), except that in this cell type R alone induced a certain level of BMRF1 expression (as previously reported in Zalani *et al.,* 1996). Similar to Z(S186A), the Z311 mutant, which cannot bind directly to DNA, did not activate BMRF1 expression in either B cells or epithelial cells. However, in contrast to Z(S186A), Z311 could not be rescued by the addition of R (Fig. 5A). Therefore, direct Z binding to early EBV promoters is likely required for their activation in the endogenous genome.

Z(S186A) plus R can activate another early protein, BHRF1

To test whether the combination of Z(S186A) plus R can activate other early gene promoters in latently infected cells, we performed immunofluorescence on transfected cells, examining BHRF1 expression. As a control, we also examined BMRF1-expressing cells from the same transfection. As shown in Table 1, transfection of Raji cells with wild-type Z induced a significantly higher level of BHRF1 expression (6.4%) than the vector-transfected cells (0.6%). Similarly, the combination of Z(S186A) plus R-transfected cells had a significantly higher level of BHRF1 expression (4.1%) than the vector-transfected cells, whereas Z(S186A) or R alone had no effect. Thus the Z(S186A)-plus-R combination activates a variety of early EBV proteins in latently infected B cells.

Z(S186A) can transcriptionally activate several EBV early and immediate-early promoters in transient reporter gene assays

Although it is clear that Z(S186A) cannot activate the BMRF1 promoter in the context of the intact viral genome, a previous study demonstrated that Z(S186A) activates the BMRF1 promoter in transient reporter gene assays (Francis *et al.,* 1997). To extend this analysis, we

TABLE 1

BHRF1 and BMRF1 Expression in Transfected Raji Cells

Transfected plasmid(s)	BHRF1 expression (%)	BMRF1 expression (%)
Vector	0.6	1.0
Z	6.4	15.6
Z(S186A)	0.3	0.7
R	0.7	1.0
Z(S186A) + R	4.1	21.3

Note. Raji cells were transfected with vector control DNA or the various expression vectors as indicated. The percentage of cells positive for BHRF1 or BMRF1 expression was determined with BHRF1 or BMRF1 antibody staining and FACS analysis.

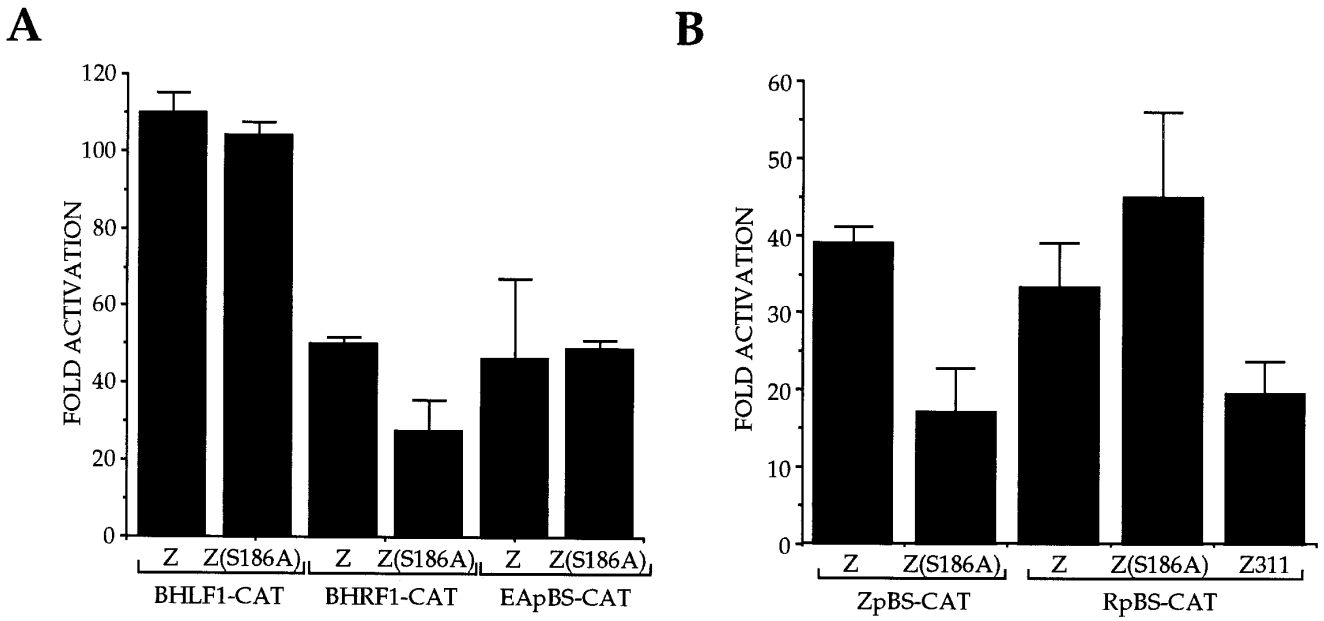

FIG. 6. Z(S186A) can transactivate EBV early and immediate-early gene promoters in reporter gene assays. (A) DG75 cells were transfected with 5 μg of each promoter construct (BHLF1-CAT, BHRF1-CAT, or EApBS-CAT) and 5 μg of either vector control DNA, Z expression plasmid, or Z(S186A) expression plasmid. CAT assays were performed as described. The average fold-activation, relative to vector alone, is presented. (B) DG75 cells were transfected with 5 μg of reporter construct (ZpBS-CAT or RpBS-CAT) and 5 μg of either vector control DNA, Z expression plasmid, Z(S186A) expression plasmid, or Z311 expression plasmid.

assessed the ability of Z(S186A) to activate a variety of early EBV gene targets promoters (BHLF1, BHRF1, and BMRF1) in transient reporter gene assays. As shown in Fig. 6A, Z(S186A) significantly activated all three of these early EBV promoters.

We next examined the effect of Z(S186A) versus wild-type Z on the activity of the EBV immediate-early promoters, BZLF1 and BRLF1, in transient reporter gene assays. As shown in Fig. 6B, both the wild-type Z and Z(S186A) significantly activated the BZLF1 promoter in transient reporter gene assays (although the wild-type Z was more efficient). Similarly, Z(S186A) activated the BRLF1 promoter at least as efficiently as the wild-type Z in transient reporter gene assays. However, because a mutant form of Z (Z311), which is unable to bind directly to DNA, also significantly activated (15- to 20-fold) the BRLF1 promoter in these assays, we conclude that Z can activate both EBV immediate-early promoters through indirect (non-DNA binding) mechanisms in transient transfection assays. These results suggest (in contrast to the results obtained in the intact viral genome) that Z-induced activation of the BZLF1 and BRLF1 promoters in transient reporter gene assays does not require direct binding of Z.

Z(S186A) plus R cannot fully disrupt latency

To compare the ability of wild-type Z versus Z(S186A) plus R to induce full lytic viral replication in latently infected B cells, we performed Southern blot analysis to distinguish latent (episomal) versus lytic (linear) EBV genomes, as described previously (Sato *et al.*, 1990). As shown in Fig. 7, in Z-transfected Jijoye cells, the linear form of the virus is induced, as expected. However,

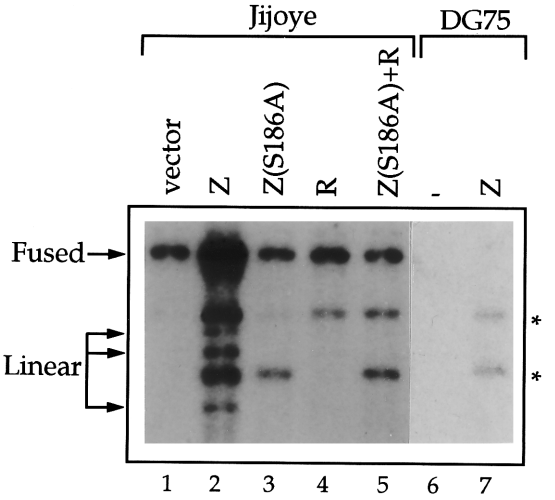

FIG. 7. The combination of Z(S186A) plus R cannot induce lytic viral replication. Jijoye and DG75 cells were transfected with 10 μg of vector control DNA or the Z and R expression vectors as indicated. The viral DNA was isolated 72 h posttransfection, cut with *Bam*HI, and analyzed by Southern blot using a ^{32}P-labeled riboprobe spanning the 19-kb *Xho*I sequence of EBV. This assay distinguishes between latent virus (fused termini only) and lytic virus (an increased number of fused termini plus linear forms). The position of the replication linear intermediates is indicated (* indicates the position of transfected plasmids also detected by the probe).

TABLE 2

VCA Expression in Transfected B95-8 Cells

Transfected plasmid(s)	VCA expression (%)
Vector	1.0
Z	14.1
Z(S186A)	1.9
R	1.4
Z(S186A) + R	1.1

Note. B95-8 cells were transfected with vector control DNA or the various expression vectors as indicated. The percentage of cells positive for VCA expression was determined with VCA antibody staining and FACS analysis.

neither Z(S186A) alone nor Z(S186A) plus R-transfected cells exhibited the replicative form of the virus, indicating that the viral genome did not replicate in these cells. The extra bands that are present in lanes 3–5 of Fig. 7 correspond to plasmid sequences that were also detected by the probe and were observed in DG75 (EBV-negative) cells transfected with Z.

To confirm the above result, we performed immunofluorescence on transfected B95–8 cells, testing for viral capsid antigen (VCA) expression. VCA is an EBV late protein that is expressed only after all of the early proteins have been expressed and the genome has been lytically replicated. As shown in Table 2, B95–8 cells transfected with wild-type Z had a significantly higher level of VCA expression (14.1%) than the vector-transfected cells (1.0%). However, neither Z(S186A) alone nor the Z(S186A)-plus-R combination induced significantly more VCA expression than the vector alone. Therefore, the combination of Z(S186A) plus R, although sufficient to activate at least two EBV early promoters (BMRF1 and BHRF1), does not result in late protein expression and cannot fully disrupt latency.

DISCUSSION

Expression of the EBV immediate-early protein Z activates the transcription of early EBV promoters and is sufficient for disruption of EBV latency. Although the other EBV immediate-early protein, R, can function synergistically with Z to activate certain EBV early promoters in reporter gene assays (Cox *et al.,* 1990; Holley-Guthrie *et al.,* 1990; Quinlivan *et al.,* 1993), expression of BRLF1 alone is not sufficient to disrupt viral latency in B cell lines (Zalani *et al.,* 1996), and R is not absolutely essential for ori-Lyt replication in plasmid-based assays (Fixman *et al.,* 1992, 1995). Therefore (in the absence of a mutant virus missing the BRLF1 gene product), it has remained uncertain whether BRLF1 expression is essential for Z-induced disruption of viral latency in B cells or merely increases its efficiency.

In this report, we demonstrate that the Z(S186A) mutant is impaired in its ability to bind (and activate) the BRLF1 promoter from the endogenous viral genome in latently infected cells. However, because the Z(S186A) mutant retains its ability to bind (and activate) at least a subset of EBV early promoters, the addition of R protein to Z(S186A) restores the ability of this mutant to activate expression of EBV early genes (e.g., BMRF1 and BHRF1) from the endogenous viral genome. Therefore, in the context of the intact viral genome, activation of the BRLF1 promoter is the first essential step in Z-mediated disruption of viral latency, and early EBV transcription clearly requires both BZLF1 and BRLF1 expression.

The findings reported here indicate that the results obtained using transient reporter gene assays do not always accurately predict the effects (and mechanisms) of the Z and R proteins in the context of the intact EBV genome. For example, although both the Z(S186A) and Z311 (neither of which bind directly to the BRLF1 promoter) mutants can activate the BRLF1 promoter in transient reporter gene assays (through as-yet-undefined indirect mechanisms), we show here that neither of these mutants can activate BRLF1 expression from the endogenous viral genome. Francis *et al.* (1997) have likewise found that Z(S186A) does not induce BRLF1 transcription in Raji cells. Thus it is almost certain that direct Z binding is required for activation of BRLF1 in the intact virus (unless both the Z311 and Z(S186A) mutants have serendipitously altered another function of BZLF1).

Although we (and others) have previously used reporter gene assays to predict that certain early EBV promoters (including BMRF1 and BHRF1) are synergistically activated by the Z-plus-R combination (Cox et al., 1990; Holley-Guthrie *et al.,* 1990; Quinlivan *et al.,* 1993), this effect was very cell type dependent and was not observed at all in certain epithelial cells (Gruffat *et al.,* 1990B; Holley-Guthrie *et al.,* 1990; Quinlivan *et al.,* 1993). In contrast, in the context of the intact virus, Z(S186A) activation of the early EBV promoters clearly requires the R helper function in both B cells and epithelial cells. Although the requirement for R during disruption of latency in the intact genome may simply reflect its known function as a transcriptional transactivator, it remains possible that R has other essential roles in the intact genome that are not measured in plasmid-based assays. At least two R binding sites (in the BMLF1 and BHRF1 promoters) have been shown to have an R-dependent enhancer function (Chevrier *et al.,* 1989; Cox *et al.,* 1990; Gruffat *et al.,* 1990a; Kenney *et al.,* 1989A), suggesting that R binding in the intact viral genome could potentially alter DNA structure. For example, if the early promoters in the latent EBV genome are normally tightly wound in chromatin such that Z cannot easily bind to upstream ZRE sites, R binding to early EBV enhancers might be required to alter chromatin structure before Z binding could occur. Alternatively, R could activate the function of

cellular proteins required for Z-induced activation of early gene promoters in the intact genome.

The Z(S186A) mutant cannot bind efficiently to either of the BRLF1 ZRE sites *in vitro,* correlating with its inability to activate BRLF1 expression *in vivo.* When comparing the sequences of various ZRE sites in the BMRF1, BZLF1, polymerase, and BRLF1 promoters (Fig. 2C), it is immediately apparent that the Z(S186A) mutant is unable to bind to either of the two ZRE sites (RpZRE1 and ZpZIIIA) containing the core sequence TGAGCCA. The other site not bound by Z(S186A), RpZRE2 (TGAGCGA), is the only site that contains a G at the sixth nucleotide position. Interestingly, however, Z(S186A) binds even more efficiently than wild-type Z to the consensus AP1 binding site (TGAGTCA). The basic DNA binding domain in the Z(S186A) protein more closely resembles that of the c-Jun and c-Fos proteins than wild-type Z, perhaps explaining why Z(S186A) binds even more efficiently than wild-type Z to the consensus AP1 site, yet (like c-Jun and C-Fos) cannot bind to at least some ZRE sites.

Although it has been speculated that the inability of Z(S186A) to disrupt viral latency reflects the loss of serine 186 phosphorylation (Francis *et al.,* 1997), it is clear from our results (comparing bacterial and *in vitro* translated proteins) that phosphorylation of serine 186 is not required for binding to the BRLF1 ZRE sites. Therefore, this particular aspect of the *in vivo* Z(S186A) phenotype (the inability to activate the BRLF1 promoter) is unlikely to reflect decreased Z phosphorylation. It is possible that wild-type Z can adopt more than one conformation and that although one conformation favors binding to the BRLF1 ZRE sites, another conformation favors binding to early promoter ZREs. In this case, the Z(S186A) mutant may have lost the ability to adopt the conformation that binds to the BRLF1 ZREs. The ability of Z to assume subtly different conformations *in vivo* could act to regulate its binding affinity for the different classes of promoters (immediate-early versus early versus delayed-early), thereby ensuring that Z initially activates the BRLF1 immediate-early promoter and then preferentially binds to early promoters when adequate levels of the R protein have been synthesized.

Even though the combination of Z(S186A) plus R can activate a variety of early genes from the endogenous viral genome, Z(S186A) plus R does not induce full lytic viral replication of the virus in B cells. It is possible that this lack of lytic replication reflects the inability of Z(S186A) to bind (and activate) an essential early replication gene promoter, similar to its inability to bind the BRLF1 promoter. We have shown that Z(S186A) binds to at least two of the six known essential replication proteins (BMRF1 and polymerase) but have not tested the promoters of the remaining four genes known to be required for ori-Lyt replication (Fixman *et al.,* 1992, 1995). Alternatively, the Z(S186A) mutant may be specifically defective in its replicative functions, as has been reported for other Z mutants (Sarinsky *et al.,* 1996). It remains possible, for example, that phosphorylation of serine 186, although not required for transcriptional activation of early EBV promoters, is essential for ori-Lyt replication.

The Z(S186A) mutant demonstrates that Z-induced activation of BRLF1 is essential for disruption of viral latency in B cells and should prove a useful tool for mapping the domains in transfected R protein required for this function. The Z(S186A) mutation also demonstrates that Z recognizes various ZRE sites through different mechanisms, an aspect of Z binding that is potentially exploited by the virus to regulate Z binding in a temporal fashion.

MATERIALS AND METHODS

Cell lines

Raji and Jijoye are EBV-positive Burkitt's lymphoma cell lines. DG75 is an EBV-negative Burkitt's lymphoma cell line. B95–8 is a productively infected, EBV-positive, marmoset B cell line. The epithelial D98/HE-R-1 cell line was formed by fusion of a HeLa cell subclone (D98) with the EBV-positive Burkitt's lymphoma cell line P3HR/1 (Glaser and O'Neill, 1972). All lymphoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Epithelial cell lines were maintained in Dulbecco's modified Eagle's medium H supplemented with 10% fetal calf serum.

EBV plasmids

EApBS-CAT contains the early EBV BMRF1 promoter sequences from −331 to +1 linked to the chloramphenicol acetyltransferase (CAT) gene (Quinlivan *et al.,* 1993). BHRF1-CAT contains a 1020-bp *Nae*I–*Hin*cII fragment of the early EBV BHRF1 promoter (EBV positions 52800–53819) linked to the CAT gene (Gutsch *et al.,* 1994). BHLF1-CAT contains the early EBV BHLF1 promoter sequences from −1031 to +165 linked to the CAT gene (Zhang *et al.,* 1996). ZpBS-CAT was constructed by removing the BZLF1 promoter (sequences from −552 to +12) linked to the CAT gene (an *Xba*I–*Bam*HI fragment) from Z-CAT (Kenney *et al.,* 1989B) and inserting it into the pBS vector. RpBS-CAT contains the immediate-early BRLF1 promoter sequences from −962 to +5 linked to the CAT gene (Zalani *et al.,* 1992).

The Z expression vector contains the BZLF1 gene (genomic clone) downstream of the early SV40 promoter (gift of Erik Flemington) (Flemington *et al.,* 1992). The Z(S186A) expression vector was derived from the aforementioned Z expression vector but has a point mutation that changes amino acid 186 (in the DNA binding domain) from serine to alanine (gift of Erik Flemington). The R expression vector contains the BRLF1 genomic clone downstream of the CMV immediate-early promoter in the

pHD1013 vector (Holley-Guthrie *et al.,* 1990). The Z311 expression vector contains a mutated form of the BZLF1 cDNA (in which amino acid 185 is altered from alanine to lysine) downstream of the CMV immediate-early promoter, in the pHD1013 vector (Giot *et al.,* 1991; Kenney *et al.,* 1992). The Z311 mutation abolishes DNA binding activity (Giot *et al.,* 1991; Kenney *et al.,* 1992).

The Z cDNA plasmid contains the BZLF1 cDNA in the pSP64 vector (a gift from Paul Farrell) (Farrell *et al.,* 1989). The Z(S186A) cDNA plasmid contains the mutated BZLF1 cDNA (amino acid 186 altered from serine to alanine) in the pBlueScript KS^+ vector (gift of Erik Flemington). pGEX-3X-Z(S186A) was constructed by swapping the 3′ half of the Z(S186A) cDNA (an *Nhe*I–*Eco*RI fragment) with the 3′ half of the Z cDNA (an *Nhe*I–*Eco*RI fragment) in the pGEX-3X-Z vector (Quinlivan *et al.,* 1993).

DNA purification

Plasmid DNA was purified through QIAGEN columns as described by the manufacturer (QIAGEN). Viral DNA was purified as follows: cells were resuspended in 0.4 ml of lysis buffer (10 mM Tris–HCl, pH 7.9, 10 mM EDTA, 0.6% SDS, 1 M sodium chloride), incubated for 20 min at room temperature, and stored overnight at 4°C. The extract was centrifuged for 30 min at 4°C. The supernatant (containing viral DNA and RNA) was incubated with RNase for 2 h at 37°C. The pellet (containing protein, SDS, and cellular DNA) was saved for protein analysis.

DNA transfection

DNA (5–10 μg) was transfected into cells by electroporation with a Zapper electroporation unit (Medical Electronics Shop, University of Wisconsin) at 1500 V as described previously (Tonneguzzo *et al.,* 1986). All cells were resuspended in RPMI 1640 medium before electroporation.

CAT assays

Cell extracts were prepared 48 h posttransfection and incubated at 37°C with [^{14}C]chloramphenical in the presence of acetyl coenzyme A as described previously (Gorman *et al.,* 1982). The percent acetylation of chloramphenical was quantified by thin layer chromatography followed by PhosphorImager screening (Molecular Dynamics).

Immunoblot analysis

Raji and D98/HE-R-1 cells were transfected with a total of 10 μg of DNA. Immunoblot analysis was performed for the detection of the EBV BMRF1 and BRLF1 proteins follows: briefly, 30 μg of protein was loaded onto each lane, and SDS–PAGE was performed. The proteins were transferred overnight onto nitrocellulose (Protran), blocked in 1× PBS/5% milk/0.1% Tween 20, and incubated in primary antibody for 1 h at room temperature [anti-EBV, EA-D (1:40), from Capricorn; anti-R (1:10), from Alain Sergeant]. The membrane was washed in PBS/0.1% Tween 20, incubated in secondary antibody for 1 h at room temperature [goat anti-mouse κ-horseradish peroxidase (GAM-κ-HRP; 1:2000), from Southern Biotechnology] and washed, and the results visualized with the ECL chemiluminescent kit (Amersham) according to the manufacturer's instructions.

Z protein expression

The Z cDNA plasmid was linearized with *Eco*RI and transcribed with SP6 polymerase, using the Riboprobe System II kit from Promega. The resulting RNA was translated in the presence of [^{35}S]methionine using the rabbit reticulocyte lysate system (Promega). The Z(S186A) cDNA plasmid was linearized with *Sac*I, transcribed with T3 polymerase, and translated as for Z. Protein quantities were normalized by running aliquots of each protein on an SDS–polyacrylamide gel, followed by autoradiography.

Glutathione-*S*-transferase (GST), GST-Z, and GST-Z(S186A) proteins were induced, sonicated, and centrifuged. Protein quantities were normalized by affinity purifying aliquots of the proteins, followed by SDS–PAGE and Coomassie staining. Crude protein was used for EMSAs.

EMSAs

EMSAs were performed as described previously (Garner and Revzin, 1981). The synthetic double-stranded oligonucleotides used in the binding reactions were 5′-end labeled with ^{32}P using the Klenow reaction. RpZRE1 (5′-GATCTCTTTTATGAGCCATTGGCA-3′) spans −26 to −45, relative to the RNA start site, of the BRLF1 promoter (Packham *et al.,* 1990). RpZRE2 (5′-GATCAAGCTTATGAGCGATTTTAT-3′) spans −184 to −203, relative to the RNA start site, of the BRLF1 promoter (Packham *et al.,* 1990). AP1 (5′-GATCGATGACCTTTGAGTCAGGTGGCTA-3′) spans −73 to −50, relative to the RNA start site, of the BMRF1 promoter (Quinlivan *et al.,* 1993). ZRE (5′-GATCTAATTTCTTTGAGCAAGAGAGTTCC-3′) spans −119 to −96, relative to the RNA start site, of the BMRF1 promoter (Quinlivan *et al.,* 1993). PolZRE (5′-GATCGCGCTGCATGAGCAAAACCAGGAG-3′) spans −48 to −25, relative to the RNA start site, of the EBV polymerase promoter (Furnari *et al.,* 1992). ZIIIA+B (5′-GATCATGCATGAGCCACAGGCATTGCTAATGTACCT-3′) spans −134 to −103, relative to the RNA start site, of the BZLF1 promoter (Flemington and Speck, 1990). ZIII A^-/B^+ (5′-GATCATGCAGAATTCGCAGGCATTGCTAATGTACCT-3′) contains a mutant ZIIIA site; ZIII A^+/B^- (5′-GATCATGCATGAGCCACAGGCAGCAGCTGTGTACCT-3′) contains a mutant ZIIIB site. The *in vitro*

translated proteins and GST proteins were prepared as described above.

The binding reactions were conducted in a buffer consisting of 100 mM potassium chloride, 20 mM HEPES (pH 7.3), 10% glycerol, 0.2 mM EDTA, 4 mM dithiothriotol, and protease inhibitors with 4 μg of poly(dI-dC)/poly(dI-dC) (Pharmacia). Five microliters of *in vitro* translated protein or 5 μg of GST protein was added to each reaction and incubated at room temperature for 10 min before the addition of labeled probe (20,000 cpm). After addition of the probe, the reactions were incubated 30 min at room temperature and then loaded onto a 4% polyacrylamide gel and run in 0.5$\times$ Tris–borate–EDTA (TBE) at room temperature.

EBV termini analysis

Jijoye and DG75 cells were transfected with 10 μg of DNA. Viral DNA was prepared at 72 h posttransfection. Twenty micrograms of DNA was cut with *Bam*HI, run on an 0.8% agarose gel, and transferred to Hybond-N. The blot was probed with a ^{32}P-UTP-labeled riboprobe spanning the EBV sequences in the 1.9-kb *Xho*I fragment (a gift from Nancy Raab-Traub) (Sato *et al.,* 1990).

Fluorescence activated cell sorter (FACS) analysis

B95–8 or Raji cells were transfected with 10 μg of DNA. Cells were harvested 48–72 h posttransfection. Cells were washed twice with PBS, fixed with 60% acetone for 10 min on ice, washed three times in PBS–0.5% bovine serum albumin (BSA), and incubated with the primary antibody [VCA (1:10), from Virotech; BMRF1 (1:30), from Capricorn; EA-R (1:50), from Chemicon] for 1 h at room temperature. The cells were washed three times and then incubated in the secondary antibody [GAM-FITC (1:100), from Sigma] for 1 h at room temperature. The cells were washed three times and resuspended in 0.5 ml of PBS. The percentage of immunofluorescent cells was determined with a FACS machine (Becton-Dickinson).

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