# The bZip Dimerization Domain of the Epstein–Barr Virus BZLF1 (Z) Protein Mediates

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The Epstein–Barr virus (EBV) immediate-early (IE) protein, BZLF1 (Z), initiates the switch from latent to lytic infection. Z transactivation of an early viral promoter, BMRF1, is relatively inefficient in lymphoid cells (compared with epithelial cells), unless the other EBV IE protein, BRLF1, is also present. Cellular proteins, including the p65 component of NF-κB, have been shown to interact directly with Z *in vitro* through the bZip dimerization domain and inhibit Z-induced transactivation. Here we precisely define a residue within the bZip dimerization domain of Z (amino acid 200) which is required for interaction *in vitro* with the p65 component of NF-κB, but is not essential for Z homodimerization. In lymphoid cells, a Z mutant which has been altered at amino acid 200 (tyrosine to glutamic acid) transactivates both the early BMRF1 promoter and the immediate-early BZLF1 promoter (Zp) four- to fivefold better than wild-type Z. In contrast, mutation of amino acid 200 does not affect Z transactivator function in epithelial cells. The results suggest that Z function is specifically inhibited by a lymphoid-specific protein(s) through amino acid 200 in the bZip dimerization domain. Modulation of Z's activator function may help to regulate the stringency of viral latency in lymphocytes.  © 1997 Academic Press

## INTRODUCTION

Epstein–Barr virus (EBV) is a human herpesvirus which infects B and T lymphocytes and certain epithelial cells (Rickinson and Kieff, 1996). EBV infection is strongly associated with the development of several human malignancies, including Burkitt's lymphoma and nasopharyngeal carcinoma (Rickinson and Kieff, 1996; Zur Hausen *et al.,* 1970). In B lymphocytes, which are immortalized by EBV, as well as in T cells, viral infection is usually latent, with only limited viral gene expression (Kieff, 1996; Rickinson and Kieff, 1996). In contrast, EBV infection in epithelial cells frequently results in viral replication and production of infectious virions (Kieff, 1996; Li *et al.,* 1992; Rickinson and Kieff, 1996; Sixby *et al.,* 1984). Thus, cellular factors must regulate the stringency of viral latency.

*In vitro,* overexpression of the EBV immediate-early gene, BZLF1, is sufficient to initiate the switch from latent to lytic infection (Chevallier-Greco *et al.,* 1986; Countryman and Miller, 1985; Kenney *et al.,* 1989; Rooney *et al.,* 1988, 1989; Takada *et al.,* 1986). Agents which induce BZLF1 transcription, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and anti-immunoglobulin, disrupt viral latency (Flemington *et al.,* 1991B; Laux *et al.,* 1988; Rickinson and Kieff, 1996; Takada *et al.,* 1989; Zur Hausen *et al.,* 1979). The BZLF1 (Z) protein is considered to be a member of the bZip superfamily based on its sequence and structural similarity with other bZip proteins

(Chang *et al.,* 1990; Farrell *et al.,* 1989; Flemington and Speck, 1991; Kouzarides *et al.,* 1991). Z transcriptionally activates early viral promoters by directly binding as a homodimer to upstream Z-response elements (ZREs), which are similar (sometimes identical) to AP1-binding motifs (Chang *et al.,* 1990; Chevallier-Greco *et al.,* 1989; Chi *et al.,* 1995; Cox *et al.,* 1990; Farrell *et al.,* 1989; Flemington and Speck, 1990; Kenney *et al.,* 1989a,b; Lieberman and Berk, 1990; Lieberman *et al.,* 1990; Packham *et al.,* 1990; Quinlivan *et al.,* 1993; Rooney *et al.,* 1989; Urier *et al.,* 1989). Dimerization of Z is essential for its DNA-binding activity, as well as its transactivator function. Although the dimerization domain of Z does not possess typical heptad leucine repeats, dimerization occurs through a coiled-coil type interaction (Chang *et al.,* 1990; Flemington and Speck, 1991; Kouzarides *et al.,* 1991; Lieberman and Berk, 1991).

Given the ability of the BZLF1 gene product to disrupt viral latency, cellular regulation of Z function is likely to play a key role in modulating the stringency of viral latency. We have reported that Z-induced transactivation of the BMRF1 early promoter is cell-type specific (Holley-Guthrie *et al.,* 1990; Quinlivan *et al.,* 1993). Whereas Z alone can maximally activate expression of the BMRF1 early promoter in certain epithelial cell lines (i.e., HeLa and Hep-2 cells), efficient activation of the same promoter requires both Z and another EBV immediate-early protein, BRLF1 (R) (Hardwick *et al.,* 1988), in other cell lines (the Jurkat T-cell line and the EBV-negative Louckes B-cell line) (Holley-Guthrie *et al.,* 1990; Quinlivan *et al.,* 1993).

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Z interacts directly with several different cellular proteins, including the NF-$\kappa$B transcription factor, the p53 tumor suppressor protein, and the RAR and RXR retinoic acid receptors (Gutsch *et al.,* 1994; Sista *et al.,* 1993, 1995; Zhang *et al.,* 1994). Each of these interactions requires the bZip dimerization domain of Z and results in inhibition of Z transactivator function. The inhibition of Z function by NF-$\kappa$B (or other members of the rel protein family) could potentially be mediated in a cell-specific fashion. In most cell types, NF-$\kappa$B is retained in the cytoplasm as an inactive complex with its inhibitory protein, I$\kappa$B (Baeuerle, 1991; Baeuerle and Baltimore, 1988; Beg *et al.,* 1992; Lenardo and Baltimore, 1989). However, in B cells, a certain amount of NF-$\kappa$B is present in the nucleus constitutively as a heterodimer of the p50 and p65 subunits (Lenardo and Baltimore, 1989).

Here we have more precisely mapped the domain(s) of Z required for direct interaction with the p65 component of NF-$\kappa$B and have constructed a Z protein (mutated at amino acid 200) which retains transactivator function, but no longer interacts efficiently with NF-$\kappa$B. The phenotype of this mutant Z protein is cell-type specific and modulated by the presence of the other EBV IE protein, R. In lymphoid cells, the transcriptional activator function of the mutant Z protein is four- to fivefold greater than that of the wild-type protein. In epithelial cells, the mutant and wild-type proteins have similar transactivator function. Our results suggest that Z is negatively regulated in a lymphoid-specific fashion by a cellular protein (potentially NF-$\kappa$B or other members of the rel family) through amino acid 200 within the dimerization domain.

## MATERIALS AND METHODS

### Cell lines

The cell lines used include the EBV-negative Burkitt's lymphoma cell line, DG75, the EBV-positive Burkitt's lymphoma cell line, Raji, the human T-cell line, Jurkat, and the human cervical epithelial cell line, HeLa. Lymphoid cell lines were propagated in RPMI 1640 medium (GIBCO, Gaithersburg, MD) supplemented with 10% fetal bovine serum. The HeLa cell line was maintained in Dulbecco's modified Eagle's medium H supplemented with 10% fetal bovine serum. All cell lines were maintained at 37° in a humidified atmosphere containing 5% $CO_2$.

### Plasmids

The double point mutants of BZLF1 were gifts from Erik Flemington and Sam Speck and have been described previously (Flemington and Speck, 1991). Single amino acid mutants of BZLF1 were made using the Transformer Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA) as specified by the manufacturer's instructions. The final mutants were confirmed by DNA sequencing done at the UNC-CH Automated DNA Sequencing Facility on a Model 373A DNA Sequencer using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Z200E contains a tyrosine to glutamic acid mutation at amino acid 200, and Z225E contains a leucine to glutamic acid mutation at amino acid 225 (Fig. 1B). The mutants (constructed within the BZLF1 *in vitro* translation vector, pSP64) were subsequently cloned into the pHD1013 expression vector (a gift from E. S. Huang), downstream of cytomegalovirus immediate-early promoter (pCMV-Z200E and pCMV-225E), and the PGEX-3X vector (Pharmacia, Alameda, CA), in-frame and downstream of the glutathione *S*-transferase (GST) protein (pGST-Z200E and pGST-Z225E).

The promoter plasmids, BMRF1-CAT, BMRF1-$\Delta$AP1-CAT, BMRF-$\Delta$ZRE-CAT (previously named BMRF1-$\Delta$106), and BMRF1-$\Delta$AP1/ZRE (previously named BMRF1-$\Delta$AP1/106) have been described (Quinlivan *et al.,* 1993). The BMRF1-CAT vector contains the EBV early BMRF1 promoter linked to the chloramphenicol acetyltransferase (CAT) gene in the Bluescript SK(+) phagemid vector (Stratagene). The BMRF1-$\Delta$AP1-CAT, BMRF1-$\Delta$ZRE-CAT, and BMRF1-$\Delta$AP1/ZRE-CAT constructs contain point mutations of the BMRF1 promoter in the AP1 site (located at $-60$ relative to the mRNA start site), an upstream ZRE site (located at $-106$), or both the AP1 and the ZRE sites, respectively (Quinlivan *et al.,* 1993). The Z-CAT plasmid was constructed by insertion of the BZLF1 promoter (103,625–103,181) into the pCAT3M vector, upstream of the CAT gene as described before (Kenney *et al.,* 1989b).

The expression plasmid pCMV-Z has been previously described (Quinlivan *et al.,* 1993) and contains the BZLF1 cDNA in the pHD1013 vector (a gift from E. S. Huang), under the control of cytomegalovirus immediate-early promoter. The pCMV-R plasmid contains the BRLF1 gene linked to the CMV-IE promoter in the pUC18 vector as described before (Quinlivan *et al.,* 1993). The GST-p65 plasmid was created by insertion of the intact p65 coding sequence downstream of the GST protein in the pGEX-1N vector (AMRAD Corp.) as described (Gutsch *et al.,* 1994; Stein *et al.,* 1993).

The plasmid CMV-Z311 (derived from the SV40-driven Z311 vector, a gift from Alain Sergeant) (Giot *et al.,* 1991) contains the Z cDNA, with a site-directed mutation (an alanine to lysine switch at amino acid 185) within the DNA-binding domain, cloned into the pHD1013 vector. This mutation greatly reduces the DNA-binding function of Z without affecting nuclear localization (Giot *et al.,* 1991). The plasmid CMV-Z200E/311 contains both the Z311 mutation (amino acid 185) and the Z200E mutation (amino acid 200).

### DNA preparation and transfection

Plasmid DNA was purified using the Qiagen Maxi kit (Qiagen, Chatsworth, CA) as specified by the manufac-

turer. Transfection of DNA into both lymphoid and epithelial cell lines was accomplished by electroporation (Tonneguzzo *et al.,* 1986). For each condition, $10^7$ cells were shocked at 1500 V, using the Zapper electroporation unit (Medical Electronics Shop, University of Wisconsin). Epithelial cells were harvested and resuspended in RPMI 1640 medium for electroporation.

## CAT assays

Cell extracts were prepared 48 hr after transfection and incubated at 37° with [$^{14}$C]chloramphenicol in the presence of acetyl coenzyme A as described previously (Gorman *et al.,* 1982). The percentage of acetylation of chloramphenicol was quantitated by thin-layer chromatography followed by PhosphorImager screening (Molecular Dynamics).

## Z protein expression

The plasmids used for *in vitro* translation of wild-type Z protein contain the BZLF1 cDNA within the pSP64 (Promega) phagemid vector (a gift from P. Farrell). The plasmids were digested with *Eco*RI restriction enzyme, transcribed by SP6 polymerase (Promega), and then translated in the presence of $^{35}$S-labeled methionine using a rabbit reticulocyte lysate system (Promega).

The construction of the plasmid glutathione *S*-transferase (GST)–Z has been described previously (Gutsch *et al.,* 1994). The GST–Z plasmid contains the full-length BZLF1 cDNA inserted downstream (in-frame) of the GST gene in the pGEX-3X vector (Pharmacia). The bacterial proteins were induced by 1 m*M* isopropylthiogalactopyranoside (IPTG) for 2–3 hr at 37°. Bacteria were pelleted, resuspended in phosphate-buffered saline (PBS), sonicated, and cleared by centrifugation (Smith and Johnson, 1987).

## GST fusion protein affinity chromatography

The interaction of $^{35}$S-labeled *in vitro* translated wild-type or mutant Z proteins with the GST–Z and GST–p65 fusion proteins was analyzed by affinity chromatography as previously described (Artandi and Calame, 1993; Smith and Johnson, 1987). For each condition, 0.3 ml of bacterial culture containing GST fusion proteins was incubated with 50 $\mu$l of 50% glutathione–agarose beads (Sigma) for 30 min at room temperature. The coated beads were washed three times with 1 ml of 1× PBS. The purified beads were then resuspended in 0.5 ml buffer containing 20 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.7), 25 m*M* NaCl, 2.5 m*M* MgCl, 0.1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 0.05% Nonidet-P40 (NP-40) and incubated with 10 $\mu$l of *in vitro* translated proteins. The binding reactions were conducted at room temperature for 1 hr or at 4° overnight. The bead complexes were washed five times in 1 ml of the above buffer and boiled in SDS–loading buffer. The bound *in vitro* translated proteins were separated on a 0.1% SDS–8% polyacrylamide gel and submitted to autoradiography (enhanced with 1 *M* sodium salicylate for $^{35}$S-labeled proteins).

## Electromobility shift assays (EMSAs)

Electromobility shift assays were performed as previously described (Garner and Revzin, 1981). The synthetic double-stranded oligonucleotides used in binding reactions were end-labeled with $^{32}$P. The double ZRE probe (5′GATCATGTGCAAGCTATGTGCAATG3′) contains two ZRE binding sites. Nuclear extracts were made by lysing cells in a buffer containing 10 m*M* Tris (pH 8.0), 60 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, 1 m*M* PMSF, and 0.5% NP-40, followed by centrifugation to separate cytoplasm and nuclear portions. The nuclear portion was then resuspended in 20 m*M* HEPES (pH 7.9), 0.75 m*M* spermidine, 0.2 m*M* EDTA, 2 m*M* DTT, 1 m*M* PMSF, 20% glycerol, and 0.4 *M* NaCl and rotated at 4° for 30 min followed by centrifugation for 10 min. The resultant supernatant was used in electromobility shift assays. The *in vitro* translated proteins and GST proteins were made as described before.

The EMSA binding reactions were conducted in a buffer consisting of 100 m*M* KCl, 20 m*M* HEPES (pH 7.3), 10% glycerol, 0.2 m*M* EDTA, 4 m*M* DTT, 0.5 m*M* PMSF with 2 $\mu$g of poly(dI–dC)·poly(dI–dC) (Pharmacia). Five microliters of *in vitro* translated protein, or 10 $\mu$g of nuclear extract protein, was added to each reaction and incubated at room temperature for 15 min prior to adding the labeled probe. For the competition binding assay, 0, 10, 50, or 100× unlabeled AP1 oligonucleotide (Promega) was added into the preincubation reactions. The labeled probe (20,000 cpm) was then added and further incubated for an additional 15 min at room temperature. The reaction mixture was loaded onto a 5% polyacrylamide gel and run in 0.5× Tris–borate buffer at 4°.

## Coimmunoprecipitation and immunoblotting analysis

To assess the stability of wild-type and mutant Z proteins, DG75 cells were transfected with the pHD1013 vector, the wild-type Z vector, or the Z200E vector. Five hours posttransfection, cells were treated with 50 $\mu$g/ml cyclohexamide. Transfected cell extracts were prepared immediately prior to cyclohexamide treatment (Time 0) and at 24 and 48 hr posttreatment. Protein extracts were separated on an 8% denaturing polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. Transfected Z protein was quantitated using an ECL Western blot kit (Amersham) according to the manufacturer's specifications. The membrane was blocked with 5% nonfat milk and probed with the anti-Z monoclonal antibody BZ.1 [a gift from A. Rickinson (Young *et al.,* 1991)] at a 1:50 dilution. A goat anti-mouse $\kappa$-chain immunoglobulin conjugated with horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham,

AL) was used as the secondary antibody at a 1:2000 dilution. Proteins were detected by luminescence reagent and exposed to X-ray film.

Coimmunoprecipitation was performed on DG75 cells transfected with the Z wild-type or Z200E expression plasmids (alone or cotransfected with the p65 expression vector). The cells were harvested 24 hr after transfection, resuspended in 250 $\mu$l buffer [20 m$M$ N-2-hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid (HEPES; pH 7.7), 25 m$M$ NaCl, 2.5 m$M$ MgCl, 0.1 m$M$ EDTA, 1 m$M$ dithiothreitol (DTT), 0.05% NP-40], followed by sonication and centrifugation to remove cellular debris. Sixty micrograms of protein in each condition was incubated with 15 $\mu$l of polyclonal anti-p65 rabbit antibody (Santa Cruz Biotechnology) or 30 $\mu$l normal rabbit serum in a total volume of 250 $\mu$l of the resuspension buffer. The reaction was rocked at 4° for 1 hr. Forty microliters of 50% protein A–Agarose (Sigma) beads were added and rotated for another hour. The beads were washed three times with the same buffer, resuspended in 20 $\mu$l 2× SDS–PAGE loading buffer, separated on an 8% polyacrylamide gel, and transferred to a nitrocellulose membrane, followed by Western immunoblot detection with the anti-Z monoclonal antibody BZ.1 as described above.

## RESULTS

### The BZLF1 protein interacts with NF-$\kappa$B p65 in a region which is not required for Z dimerization

Previously, we have demonstrated that Z interacts directly with the p65 component of NF-$\kappa$B *in vitro* and that overexpression of p65 *in vivo* inhibits Z transactivator function (Gutsch *et al.*, 1994). The bZip dimerization domain of Z is required for direct interaction with the rel homology domain of p65 (Gutsch *et al.*, 1994). To map precisely the critical amino acid residues of Z required for p65 binding, a series of Z proteins containing point mutations in the bZip dimerization domain was examined for the ability both to homodimerize and to heterodimerize with p65 *in vitro* by affinity chromatography assays (Figs. 1A–1C) (Artandi and Calame, 1993).

As reported before, two Z proteins (Z214R/218R and Z214S/218S) containing mutations within the interface of the coiled-coil helix lost dimerization ability, as well as interaction with p65 (Fig. 1C, Z214R/218R data not shown) (Flemington and Speck, 1991; Gutsch *et al.*, 1994). Two Z proteins (Z205R/206D and Z209R/216E) containing mutations predicted to reside on the back of the helix could homodimerize, as well as interact with GST-p65 (Figs. 1A–1C). These data are consistent with our previous findings that BZLF1 dimerization is required for p65 interaction (Gutsch *et al.*, 1994). However, in contrast to our previous findings (Gutsch *et al.*, 1994), we now find that two mutant Z proteins (Z197K/200S and Z200E/225E), which we had reported to be dimerization incom-

petent, are able to dimerize to Z (although the Z200E/Z225E mutant dimerizes less efficiently than wild-type Z) but no longer bind to p65 (Fig. 1C). We are uncertain why we did not detect dimerization with these mutants in the previous studies, but the present results agree with those of Sista *et al.* (1995).

These results suggest that the ability of Z to interact with p65 *in vitro* can be separated from its homodimerization potential. To map the exact residue(s) required for p65 interaction, we generated single amino acid mutations in Z residues 200 (containing a tyrosine to glutamic acid conversion) and 225 (containing a leucine to glutamic acid change) and tested these proteins for their ability to homodimerize and interact with p65 (Z197/200S was not studied because it is defective in DNA binding) (Sista *et al.*, 1995). As shown in Fig. 1D, each of these mutants could homodimerize as efficiently as wild-type Z (as indicated by the ability to bind to GST–Z-coated agarose beads). Although both wild-type Z and the Z225E mutant could bind efficiently with the GST–p65 fusion protein, the interaction of Z200E with p65 was much weaker, indicating that the Z amino acid 200 contributes significantly to the interaction with p65.

To confirm that the Z200E mutant also interacts less efficiently with p65 *in vivo*, DG75 cells were transfected with the Z wild-type and Z200E expression vectors either alone or in combination with a p65 expression vector (Fig. 1E). Protein extracts were immunoprecipitated with a rabbit polyclonal antibody directed against p65, or normal rabbit serum, and then probed for the presence of coprecipitated Z using immunoblot analysis. A small amount of the wild-type Z was coprecipitated by the p65 antibody in the absence of cotransfected p65 (presumably reflecting the interaction of Z with the endogenous nuclear p65), and a greater amount was coprecipitated in the presence of cotransfected p65. In contrast to wild-type Z, no detectable Z200E was precipitated by the p65 antibody in the absence of cotransfected p65. In the presence of cotransfected p65, Z200E was coprecipitated much less efficiently than wild-type Z. Thus, we have identified a residue within the Z bZip domain, which, while not essential for homodimerization of the protein, is probably involved in its interaction with p65.

### DNA-binding ability of BZLF1 mutants

To determine if the mutant Z200E and Z225E proteins retain DNA binding ability, electromobility shift assays were conducted using *in vitro* translated Z proteins and a $^{32}$P-labeled probe containing two ZRE sites (Fig. 2A). The wild-type and mutant *in vitro* translated Z proteins bound to the ZRE probe with similar efficiencies (Fig. 2A). Similar results were observed using a probe containing an AP1 motif (data not shown).
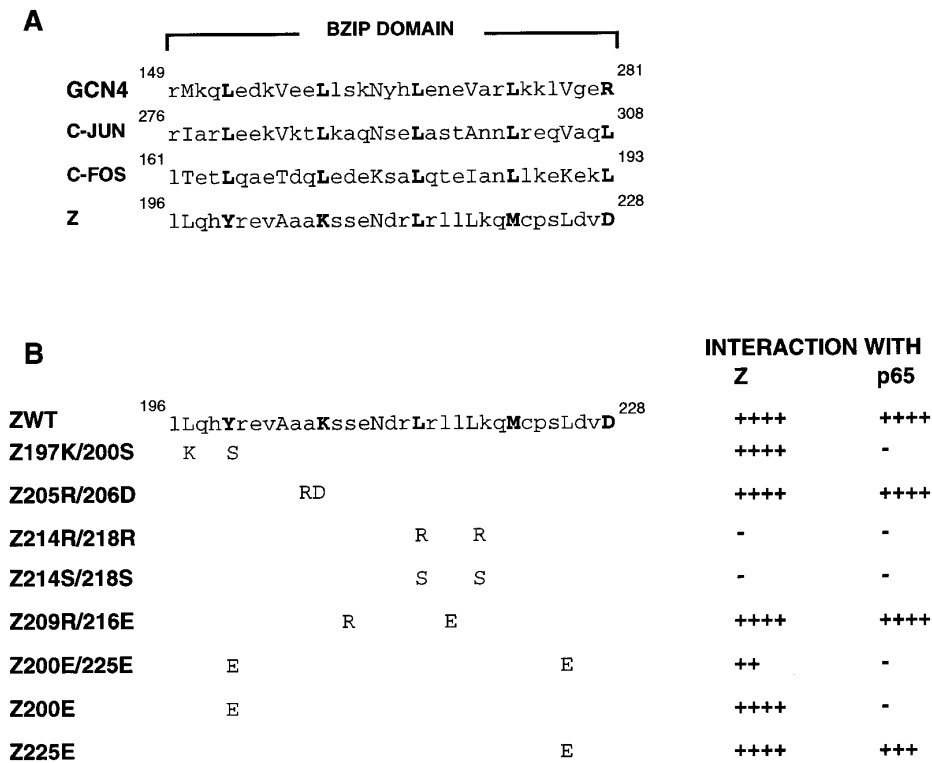
**A**

BZIP DOMAIN

```
         149                                              281
GCN4     rMkqLedkVeeLlskNyhLeneVarLkklVgeR
         276                                              308
C-JUN      rIarLeekVktLkaqNseLastAnnLreqVaqL
         161                                    193
C-FOS    lTetLqaeTdqLedeKsaLqteIanLlkeKekL
         196                              228
Z        lLqhYrevAaaKsseNdrLrllLkqMcpsLdvD
```

**B**

| | | INTERACTION WITH | |
|---|---|---|---|
| | | Z | p65 |
| ZWT | ¹⁹⁶lLqhYrevAaaKsseNdrLrllLkqMcpsLdvD²²⁸ | ++++ | ++++ |
| Z197K/200S | K  S | ++++ | - |
| Z205R/206D | RD | ++++ | ++++ |
| Z214R/218R | R  R | - | - |
| Z214S/218S | S  S | - | - |
| Z209R/216E | R    E | ++++ | ++++ |
| Z200E/225E | E              E | ++ | - |
| Z200E | E | ++++ | - |
| Z225E | E | ++++ | +++ |

FIG. 1. Mapping a region in Z which is required for interaction with p65, but not for dimerization. (A) Amino acid sequence comparisons of the bZip domains in the Z protein and other bZip proteins, GCN4, c-jun, and c-fos. The single-letter amino acid code is used to represent the sequence. The identical and conserved amino acids are indicated by capital letters. The a and d positions of the leucine zipper (which form the interface) are in boldface. (B) A series of Z mutations within the bZip dimerization domain was constructed as shown. Each mutant was tested for the ability to interact with the GST-Z and GST-p65 proteins in affinity chromatography assays as previously described (Artandi and Calame, 1993; Smith and Johnson, 1987). Results are summarized as positive (++++) to negative (−) according to the strength of the dimerized complex in comparison to the wild-type Z. (C) 3 $\mu$l of the in vitro translated $^{35}$S-labeled Z wild-type and various Z mutant proteins were directly loaded onto the gel (lanes 1 to 6). 10 $\mu$l of the in vitro translated proteins were incubated with glutathione agarose beads coated with the GST vector protein (lanes 7 and 14), the GST-Z protein (lanes 8 to 13), or the GST–p65 protein (lanes 15 to 20). The retained in vitro translated proteins are indicated by an arrow. (D) In vitro translated, $^{35}$S-labeled wild-type Z (Zwt), Z200E, and Z225E proteins were directly loaded onto the gel (lanes 1, 6, and 11, respectively), incubated with glutathione agarose beads alone (lanes 2, 7, and 12), incubated with GST protein-coated beads (lanes 3, 8, and 13), incubated with GST–Z protein-coated beads (lanes 4, 9, and 14), or incubated with GST–p65 protein-coated beads (lanes 5, 10, and 15). The retained in vitro translated proteins are indicated by an arrow. 3 $\mu$l of the reticulocyte extracts were directly loaded onto the gel, and 10 $\mu$l of the extracts were used in the affinity chromatography assays. (E) DG75 cells were transfected with different expression plasmids as follows: pHD1013 vector (lanes 1, 5, and 11), Z wild-type (lanes 2, 6, and 12), Z200E (lanes 3, 7, and 13), p65 (lanes 4, 8, and 14), Z wild-type plus p65 (lanes 9 and 15), and Z200E plus p65 (lanes 10 and 16). Cells were harvested 24 hr after transfection and immunoprecipitated with p65-specific rabbit antibody (lanes 11–16) or normal rabbit serum (lanes 5–10). Lanes 1–4 show the cell lysates directly loaded onto the SDS gel without immunoprecipitation. Immunoprecipitated proteins were separated on an SDS gel, transferred to nitrocellulose membranes, and then immunoblotted with the anti-Z monoclonal antibody, BZ.1 (Young et al., 1991). The position of Z protein is indicated.

The in vivo DNA-binding ability of Z200E was also investigated using nuclear extracts from transfected DG75 cells (Fig. 2B) and HeLa cells (Fig. 2C). Although slight differences in the DNA-binding efficiency of the wild-type and mutant Z proteins were observed in the transfected cell extracts from experiment to experiment (presumably reflecting the difficulty of using precisely the same amount of the transfected wild-type and mutant Z proteins), no consistent differences in the efficiency of wild-type versus mutant proteins occurred in either DG75 cells or HeLa cells. We conclude that mutation of amino acids 200 and 225 does not significantly affect Z DNA-binding capability.

## Mutation of Z amino acid 200 enhances transactivator function in a lymphoid-specific fashion

To determine the in vivo phenotype of the Z200E and Z225E mutants, we examined the ability of these proteins to activate an early EBV promoter (BMRF1) in various cell types (Fig. 3A). As we previously reported, wild-type Z cannot efficiently transactivate the BMRF1 promoter in the Jurkat T-cell line unless another viral immediate-early protein, R, is also present (Holley-Guthrie et al., 1990; Quinlivan et al., 1993). Interestingly, transactivation by the Z200E protein was enhanced fivefold in Jurkat cells compared with wild-type Z and was four times stronger
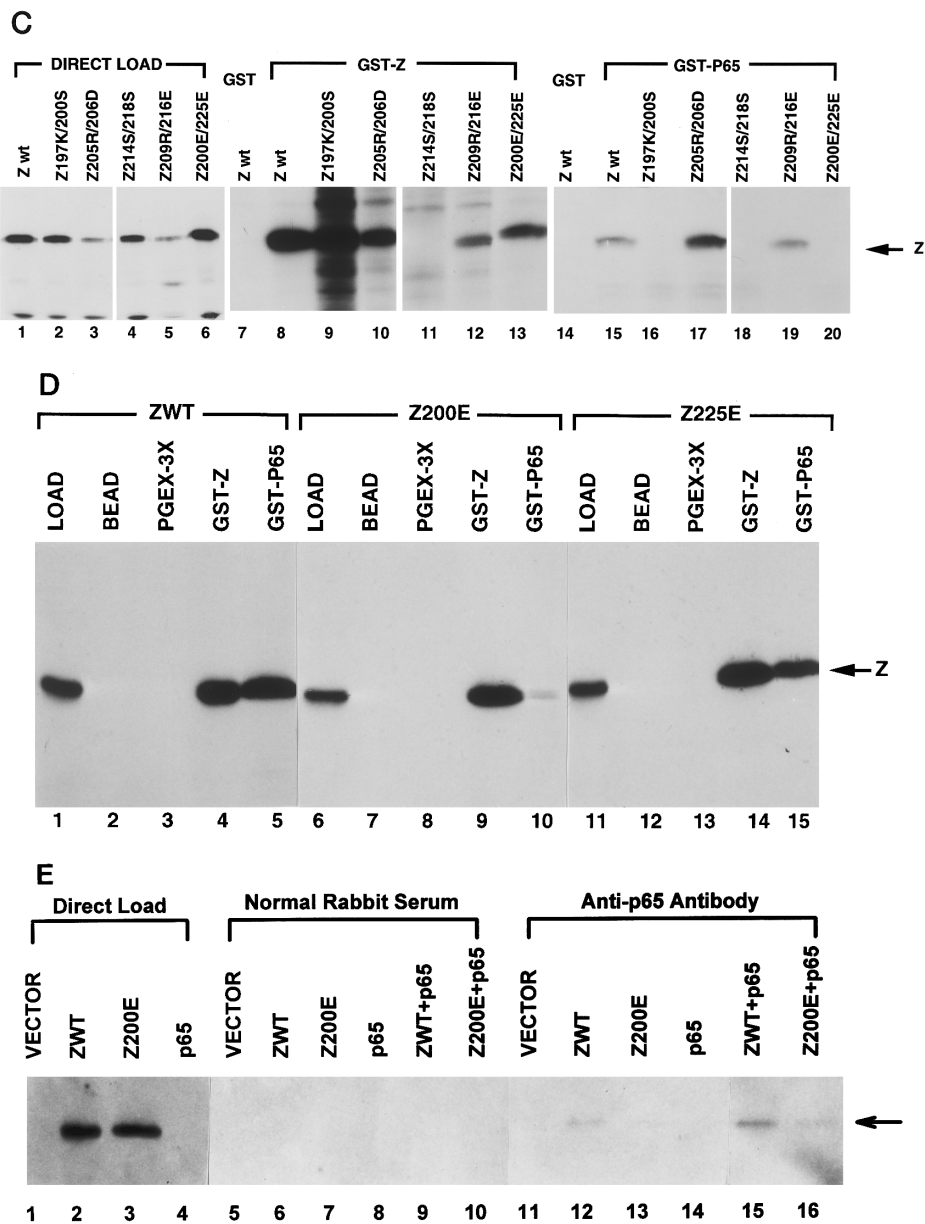
FIG. 1 — *Continued*

than that of wild-type Z in the EBV-negative B-cell line, DG75. In contrast, the transactivator function of Z200E in the epithelial line, HeLa, was similar to that of wild-type Z. The transactivator function of the Z225E mutant was comparable to that of wild-type Z in lymphoid cells, although it was less than wild-type Z in epithelial cells (Fig. 3A). These results suggest that mutation of amino acid 200 within the Z dimerization domain enhances Z transactivator function in a lymphoid-specific fashion.

To confirm that Z200E is less susceptible than wild-type Z to the inhibitory effect of p65 (Gutsch *et al.*, 1994), the ability of wild-type Z versus Z200E to activate the BMRF1 promoter in DG75 cells was compared in the presence and absence of cotransfected p65. In the presence of low doses of p65, wild-type Z transactivator func-

tion was decreased significantly more than that of Z200E (Fig. 3B). However, at higher doses of p65, the wild-type and mutant Z proteins were inhibited to a similar extent (data not shown). The inhibition of Z200E function by higher doses of p65 may reflect either nonspecific toxicity or the fact that this mutant can interact with p65 *in vivo* to a limited extent (Fig. 1E).

The BMRF1 promoter contains both Z- and R-binding sites (Quinlivan *et al.*, 1993). We have shown that Z transactivation of the BMRF1 promoter in lymphoid cells (but not epithelial cells) is significantly enhanced by the presence of the BRLF1 immediate-early gene product (R), even though R alone has little effect on this promoter (Holley-Guthrie *et al.*, 1990; Quinlivan *et al.*, 1993). Thus, R binding to the BMRF1 promoter may
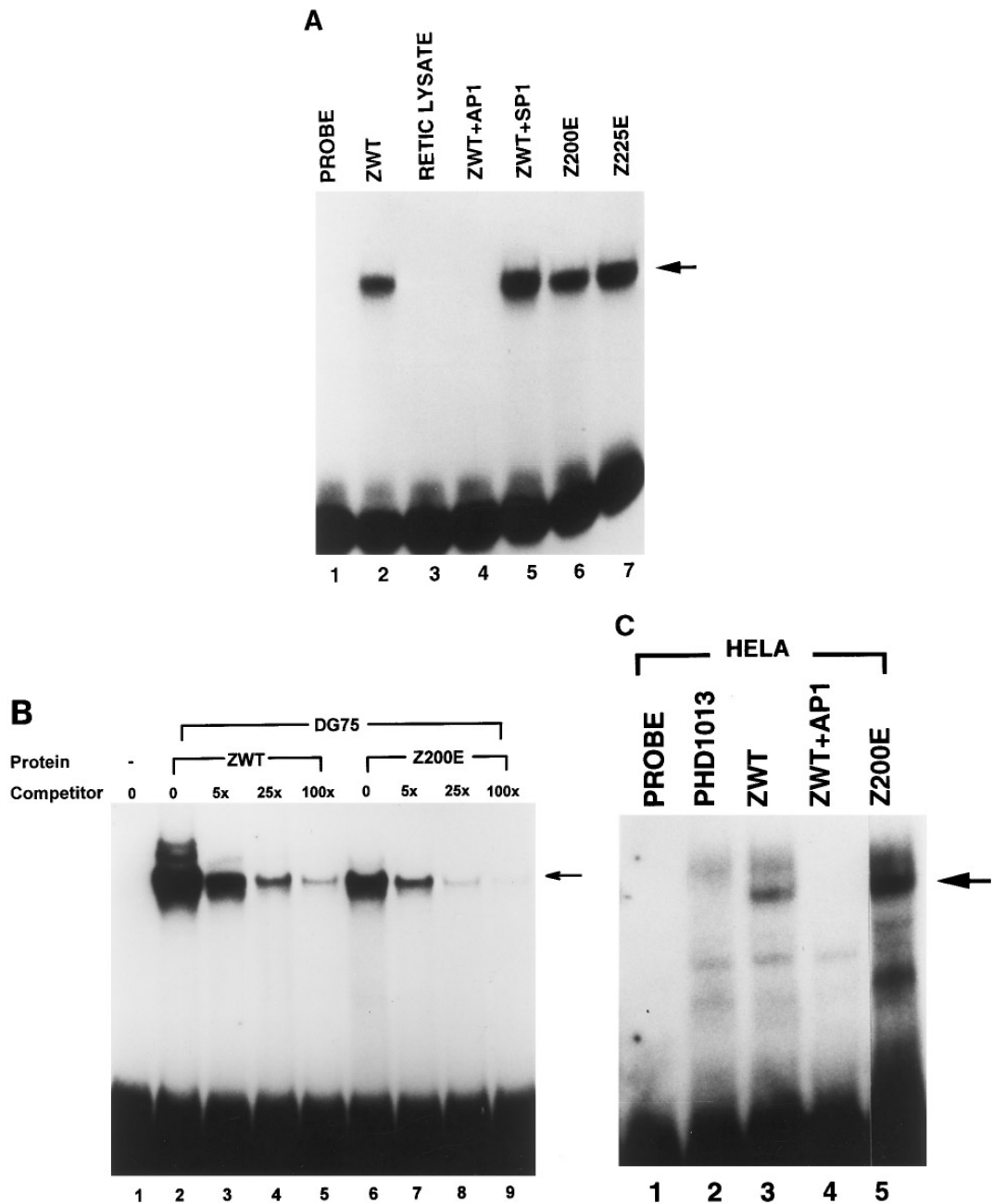
FIG. 2. DNA binding of Z mutants. (A) An electromobility shift assay was performed using proteins *in vitro* translated in reticulocyte lysates. The double ZRE oligonucleotide probe, which contains two consensus ZRE-binding sites, was used as the probe. The wild-type Z-binding complex (lane 2) was specifically competed by competitor DNA containing the AP1 motif (lane 4), but not by competitor DNA containing the Sp1-binding site (lane 5). The Z200E (lane 6) and Z225E (lane 7) proteins bound with efficiency similar to that of the wild-type Z. (B) Nuclear extracts from DG75 cells transfected with the wild-type Z expression vector (lanes 2–5) and the Z200E expression vector (lanes 6–9) were incubated with double ZRE probe in electromobility shift assays. Unlabeled AP1 oligonucleotide was added as a competitor at concentrations of 0, 5, 50, and 100×. Lane 1 is the probe without nuclear extract. The specific Z-binding complex is indicated by the arrow. (C) Nuclear extracts from HeLa cells transfected with the pHD1013 vector (lane 2), the wild-type Z expression vector (lanes 3 and 4), or the Z200E expression vector (lane 5) were incubated with the double ZRE probe in electromobility shift assays. The specific Z-binding complex is indicated by the arrow.

negate the effect(s) of a lymphoid-specific Z inhibitor. The effect of R on the wild-type Z versus the mutant Z200E protein in Jurkat cells is shown in Fig. 3C. R alone had little effect on BMRF1 promoter activity. In the absence of R, mutant Z200E induced much greater transactivation of the BMRF1 promoter than wild-type Z. When R and wild-type Z were cotransfected, transactivation of the BMRF1 promoter was 20-fold greater than that induced by wild-type Z alone. In contrast, cotransfection of Z200E and R together induced only 2.4-fold more transactivation than that observed with Z200E alone. Furthermore, the level of transactivation
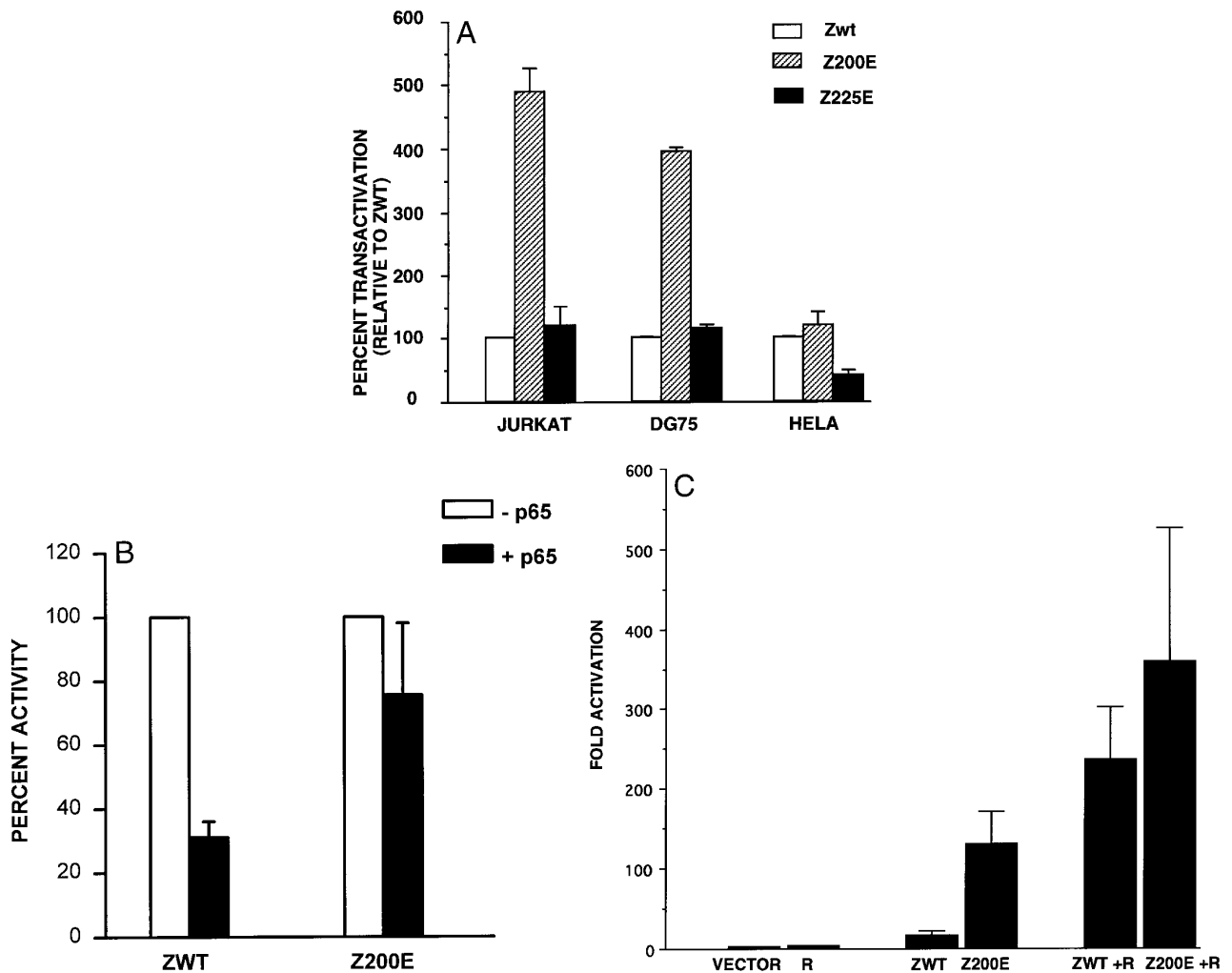
FIG. 3. Transactivator function of wild-type and mutant Z proteins in different cell lines. (A) The BMRF1-CAT (5 $\mu$g) reporter plasmid was cotransfected with the pHD1013 vector or the wild-type versus mutant Z expression plasmids (2.5 $\mu$g each) in Jurkat T cells, DG75 B cells, and HeLa epithelial cells. The percentage acetylation of chloramphenicol under each condition was determined as previously described (Gorman et al., 1982). The fold transactivation of BMRF1 promoter activity induced by each Z expression vector (versus the pHD1013 vector) was calculated. Results are normalized such that the fold activation induced by the wild-type Z vector is set at 100% for each cell line. (B) The BMRF1-CAT (5 $\mu$g) reporter plasmid was cotransfected into Raji cells with the pHD1013 vector, the wild-type Z and mutant Z200E expression vectors alone (1.0 $\mu$g), or the wild-type and mutant Z200E vectors with the p65 expression plasmid (2.5 $\mu$g). The transactivator function of wild-type Z and Z200E in the absence of p65 is normalized as 100% (white bars) and compared to transactivator function in the presence of cotransfected p65 (black bars). (C) 5 $\mu$g of the BMRF1-CAT plasmid was cotransfected into Jurkat cells with various combinations of expression plasmids (5 $\mu$g each) as follows (left to right): pHD1013 vector DNA, the R expression vector, the wild-type Z expression vector, the Z200E expression vector, the R and wild-type Z expression vectors, or the R and mutant Z200E expression vectors. The results are presented as fold activation induced by the various expression vectors (relative to pHD1013 vector DNA).

induced by the Z wild-type/R combination was not significantly different from that induced by the Z200E/R combination. Thus, in the presence of R, the transactivation of the BMRF1 promoter by wild-type Z in lymphoid cells becomes similar to that of Z200E.

A lymphoid-specific increase in Z expression or stability could explain the observed increase in Z200E transactivator function. To examine this possibility, immunoblot analyses were performed on extracts of cells transfected with wild-type, versus mutant, Z proteins (Figs. 4A and 4B). The stability of the wild-type and mutant Z200E proteins was similar in the DG75

cells and HeLa cells. The Z225E mutant also had stability similar to that of the wild-type Z (data not shown).

## Z200E-induced transactivation can occur through both DNA-binding and non-DNA-binding mechanisms

In addition to its usual mechanism of transcriptional activation (which is mediated through direct binding of Z to upstream ZRE/AP1 motifs), Z can also activate transcription of the BZLF1 promoter (Zp) through a mechanism not requiring direct DNA binding (Flemington et al., 1994). This second mechanism of Z-induced transcrip-
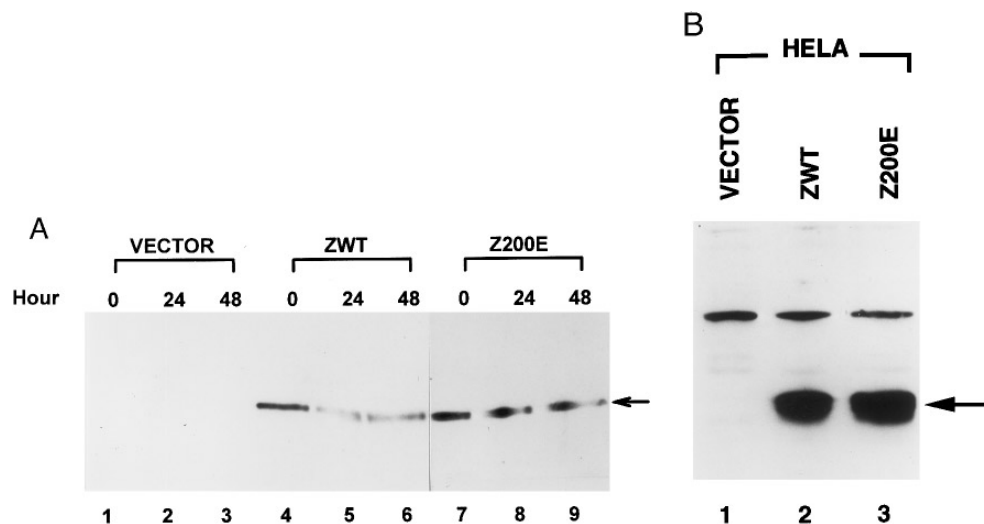
FIG. 4. Protein stability of wild-type Z and mutant Z200E. (A) DG75 cells were transfected with the pHD1013 vector (lanes 1–3), the wild-type Z expression vector (lanes 4–6), or the Z200E expression vector (lanes 7–9). Five hours posttransfection, cells were treated with cyclohexamide (50 $\mu$g/ml). A portion of the transfected cells was harvested immediately prior to adding the cyclohexamide (Time 0) and again at 24 and 48 hr. Cell proteins were separated on an 8% SDS gel, transferred to a nitrocellulose membrane, and immunoblotted using the anti-Z monoclonal antibody, BZ.1. The position of Z is indicated by the arrow. (B) HeLa cells were transfected with the pHD1013 vector (lane 1), the wild-type Z expression vector (lane 2), or the Z200E expression vector (lane 3). Cell lysates were prepared 48 hr after transfection, separated on an SDS–8% polyacrylamide gel, transferred to nitrocellulose membranes, and subjected to immunoblot analysis using the anti-Z monoclonal antibody, BZ.1. The position of Z is indicated by the arrow.

tional activation is presumably mediated through interactions between Z and cellular transcription factors.

The BMRF1 promoter contains two major Z-binding sites (an AP1 site located at −60 and a ZRE site located at −106), both of which are required for efficient activation by wild-type Z (Quinlivan et al., 1993). To determine if the Z200E protein must bind directly to the BZLF1-binding sites in the BMRF1 promoter in order to activate its transcription, we examined the effects of specific deletions of the AP1 site alone, the ZRE site alone, or both sites together. As shown in Fig. 5A, the ability of the mutant Z200E protein to activate the BMRF1 promoter was significantly decreased if either the AP1 site or the ZRE site was abolished. These results suggest that the Z200E protein activates the BMRF1 promoter through a direct binding mechanism. As is the case for wild-type Z, binding of the mutant Z200E protein to both the AP1 site and the ZRE site is required for maximal effect.

To examine if Z200E can transactivate the BZLF1 promoter through a non-DNA-binding mechanism, an additional mutation altering amino acid 185 (a mutation which is known to abrogate Z DNA binding) (Giot et al., 1991) was inserted into the CMV-Z200E plasmid. As was the case with the BMRF1 promoter (Fig. 3A), in the absence of the 185 mutation, the Z200E mutant activated the BZLF1 promoter five times more efficiently than wild-type Z in DG75 cells (Fig. 5B). Mutation of amino acid 185 (plasmid Z311), in the context of the wild-type amino acid 200, has been previously shown to inhibit BZLF1-induced activation of the BMRF1 promoter (Kenney et al., 1992), but as previously described (Flemington et al., 1994) ac-

tually increases transactivation of the BZLF1 promoter (Fig. 5B). Similarly, the CMV-Z200E construct containing an additional mutation of amino acid 185 (pCMV-Z200E/311) transactivates the BZLF1 promoter more efficiently than Z200E (Fig. 5B). As expected, mutation of amino acid 185 inhibits the ability of Z200E to activate the BMRF1 promoter (Fig. 5B).

These data suggest that in DG75 cells, the Z200E mutant is a more efficient transactivator than wild-type Z for either the direct DNA-binding mechanism of transactivation or the indirect mechanism. Although the precise cellular transcription factor(s) mediating the indirect mechanism of transactivation has not been identified, our results indicate that the Z200E mutant is still capable of interacting with this factor(s).

## Wild-type Z and Z200E disrupt viral latency in lymphoid cells with similar efficiency

Expression of wild-type Z in latently infected lymphoid cells is sufficient to activate lytic infection (Chevallier-Greco et al., 1986; Countryman and Miller, 1985; Kenney et al., 1989b; Rooney et al., 1988, 1989; Takada et al., 1986). Although the Z200E mutant has greater transactivator function than the wild-type Z in the absence of R, agents which disrupt viral latency appear to activate Z and R expression simultaneously (Flemington et al., 1991b; Laux et al., 1988). Furthermore, we have shown that transfection of Z into latently infected lymphoid cells results in activation of R expression from the endogenous viral genome (Zalani et al., 1996). Given this ability of Z
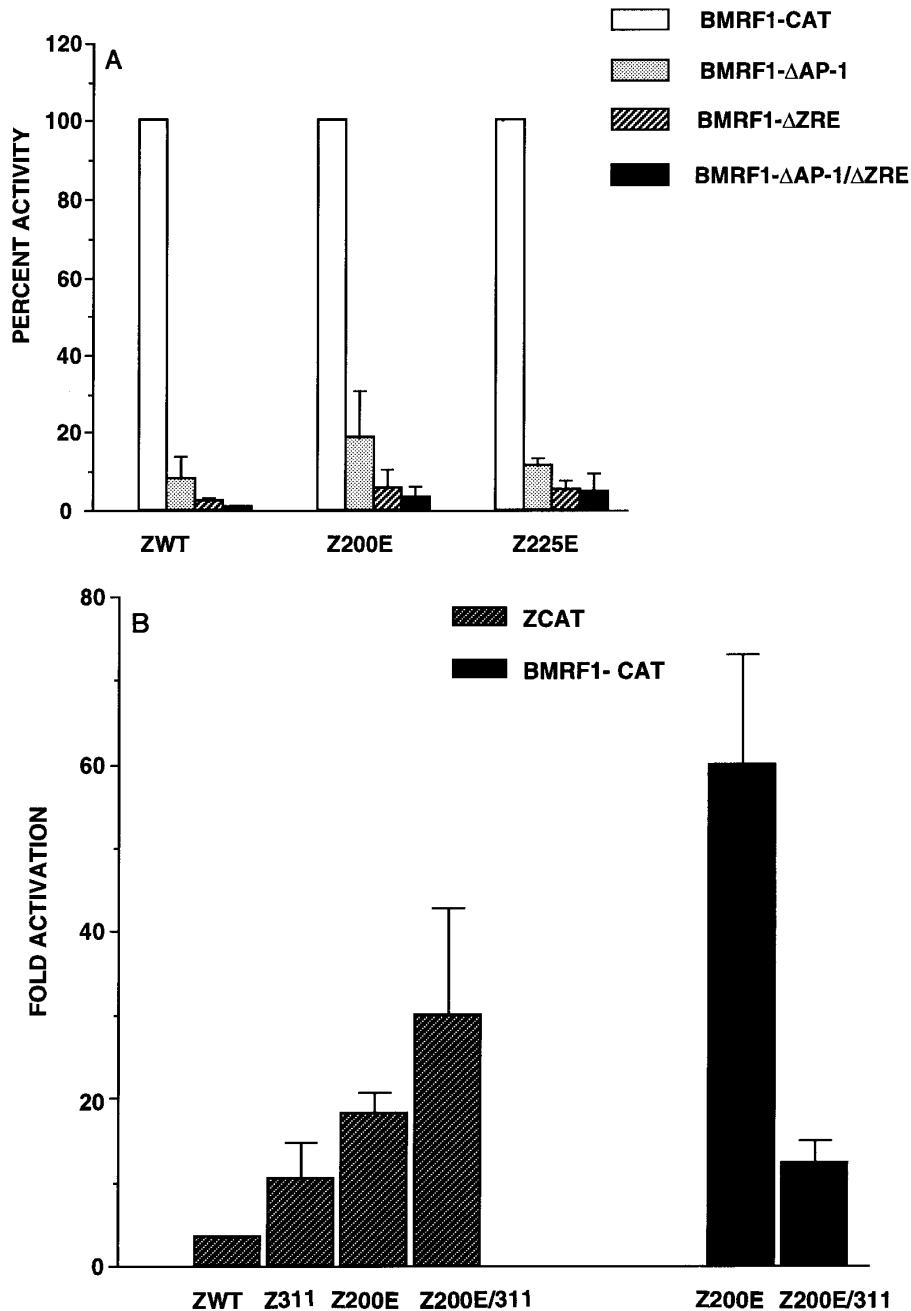
FIG. 5. Z200E can transactivate different promoters by both DNA-binding and non-DNA-binding mechanisms. (A) The wild-type BMRF1-CAT plasmid (open bars), the BMRF1-ΔAP1-CAT construct (in which the BMRF1 promoter AP1 site has been specifically mutated) (stippled bars), the BMRF1-ΔZRE-CAT construct (in which the BMRF1 promoter ZRE site has been specifically mutated) (hatched bars), and the BMRF1-ΔAP1/ZRE-CAT construct (containing mutations in both the AP1 and ZRE sites) (solid bars) were cotransfected with wild-type Z (ZWT), Z200E, or Z225E into DG75 cells. The percentage of acetylation for each condition was determined and the fold activation induced by the various Z expression vectors (in comparison to pHD1013 vector DNA) was calculated. Results are normalized such that the fold activation of the parent BMRF1-CAT construct is set at 100% for each Z expression vector. (B) The Z-CAT plasmid (2.5 μg) (hatched bars), which contains the BZLF1 (Zp) promoter, was cotransfected into DG75 cells with the pHD1013 vector, the wild-type Z vector (ZWT), the mutant Z311 vector (containing a mutated amino acid 185 and a wild-type amino acid 200), the Z200E expression vector (containing a wild-type 185 residue and a mutated amino acid 200), or the Z200E/311 vector (containing mutant 185 and 200 residues) (5 μg each). The BMRF1-CAT plasmid (2.5 μg) (solid bars) was cotransfected with either the Z200E or the Z200E/311 plasmids into DG75 cells. The CAT activity induced by each expression vectors was calculated by fold activation relative to the pHD1013 vector DNA.

to activate endogenous R expression, disruption of viral latency by the wild-type versus Z200E mutants might be anticipated to be similar.

The ability of wild-type Z and Z200E to induce lytic infection in Raji cells (a latently infected Burkitt lymphoma line) was compared (Fig. 6). The wild-type
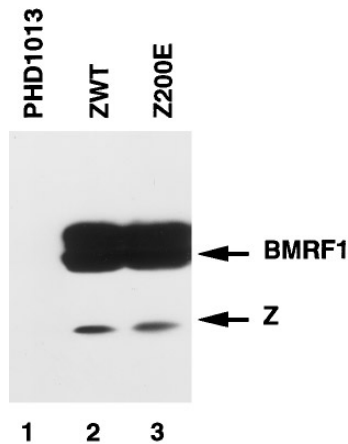
**FIG. 6.** Wild-type Z and mutant Z200E disrupt viral latency with similar efficiency. Latently infected, EBV-positive Raji cells were transfected with 5 $\mu$g of pHD1013 vector DNA (lane 1), wild-type Z vector (lane 2), or Z200E vector (lane 3). The cells were harvested 2 days after transfection and cell lysates were separated by SDS–PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblot using the anti-Z monoclonal antibody, BZ.1 and the anti-BMRF1 antibody (Capricorn Products, Inc.). The positions of the Z and BMRF1 proteins are indicated.

and mutant Z200E expression vectors were transfected into Raji cells, and the amount of lytic viral induction was quantitated by immunoblot analysis using an antibody specific to the early BMRF1 viral protein. As shown in Fig. 6, the wild-type and mutant Z200E expression vectors induced equivalent amounts of the early BMRF1 protein.

## DISCUSSION

EBV infection of B cells and T cells is primarily latent, in contrast to lytic infection in epithelial cells. The immediate-early protein, Z, plays a key role in disruption of viral latency. Z transactivates early viral promoters and initiates the lytic viral cascade. In addition, Z may also play a direct role in lytic replication (Fixman *et al.,* 1995; Schepers *et al.,* 1993). Therefore, cellular regulation of Z is likely to be a crucial factor in determining the stringency of viral latency. In this report, we define a region of Z (amino acid 200 within the bZip dimerization domain) which negatively regulates Z transactivator function in a lymphoid-specific fashion. The data presented here also suggest that amino acid 200 is a critical site through which Z interacts with p65 (and perhaps other members of the rel protein family). Thus, negative regulation of Z in lymphoid cells may be mediated through direct interaction of Z with cellular proteins, including members of the rel family.

The most intriguing aspect of the Z200E phenotype is that it increases Z transactivator function in a lymphoid-specific fashion. This enhanced transactivation by Z200E in lymphoid cells occurs with either the direct DNA-binding mechanism of transactivation or the indirect mecha-

nism. According to the previously proposed coiled-coil model for the bZip domain of Z, amino acid 200 would be located within the hydrophobic interface (position d) of the first heptad repeat of the putative four or five heptad Z coiled-coil helix (Chang *et al.,* 1990; Flemington and Speck, 1991; Kouzarides *et al.,* 1991; Lieberman and Berk, 1990). It is therefore somewhat surprising that replacement of the tyrosine residue normally present at this position with the charged glutamic acid residue still allows efficient homodimerization. Although our data are consistent with the interpretation that p65 interacts directly with amino acid 200 in Z, we cannot exclude the possibility that mutation of amino acid 200 subtly alters Z conformation such that the Z/p65 interaction (mediated through another residue) cannot occur.

In contrast to amino acid 200, mutation of Z amino acid 225 did not significantly alter its interaction with p65. The transactivator function of Z225E was somewhat impaired relative to that of wild-type Z in epithelial cells, but similar to that of wild-type Z in lymphoid cells. Amino acid 225, like amino acid 200, is positioned within the coiled-coil interface (position a) (Flemington and Speck, 1991). Future study will be required to determine if the Z225E phenotype reflects the loss of a direct interaction between Z and a cellular protein.

Although the Z200E mutant was specifically identified in this report for its inability to interact with the p65 component of NF-$\kappa$B, amino acid 200 in Z may be involved in the recognition of other cellular proteins as well, such as the retinoic acid receptors and p53, both of which are known to inhibit Z transactivator function by direct interaction with the dimerization domain of Z. The Z residues required for direct interaction with p65 are similar, but not identical, to those required for interaction with the retinoic acid receptors (Sista *et al.,* 1995). As is the case for p65, the Z proteins containing the bZip double mutations, Z197K/200S and Z200E/225E, are unable to interact directly *in vitro* with the retinoic acid receptors, although each of these mutants can homodimerize (Sista *et al.,* 1995). In contrast to our results, the 205R/206D mutant is unable to interact with the retinoic acid receptors, although this mutant can interact with p65. It has not been determined if the direct interaction between the RARs and Z is primarily mediated through amino acid 200, amino acid 225, or both. The precise Z amino acids required for interaction with p53 have not been identified. The lymphoid-specific phenotype of the Z200E protein, although potentially mediated through decreased interaction with p65, could therefore involve other protein–protein interactions as well. Furthermore, we cannot completely exclude the possibility that the Z200E mutation, rather than freeing Z from a negative regulator, increases interaction with a positively regulating cellular factor (such as TFIID) (Chi *et al.,* 1995; Lieberman and Berk, 1991).

A particularly interesting result was the finding that

cotransfected R significantly enhanced the ability of wild-type Z to transactivate the early BMRF1 promoter in lymphoid cells, while having much less effect on the mutant Z200E protein. Although we have previously shown that direct R binding to the BMRF1 promoter is required for Z/R synergy in lymphoid cells (Quinlivan *et al.,* 1993), the mechanism(s) by which such synergy occurs is not well defined. The Z and R proteins are not known to interact directly. Our data are consistent with a model in which the R protein negates the effect of a lymphoid-specific inhibitor of Z. In this model, the Z200E protein, being less susceptible to the putative inhibitor(s), would not be as dependent upon R for efficient transactivator function in lymphocytes.

Given that Z200E has superior transactivator potential, it might be expected that viruses containing this mutation would have a selective advantage *in vivo.* However, to our knowledge, this particular mutation has not yet been observed in clinical isolates of EBV, suggesting that Z200E must be inferior to wild-type Z *in vivo* during some aspect of the viral life cycle. There may be no selective advantage for the Z200E mutant in the context of the intact viral genome, given that the R protein appears to overcome the lymphoid-specific inhibition of Z function. Furthermore, we have recently shown that Z interacts directly with the viral polymerase processivity factor (the BMRF1 gene product) and that this interaction also requires amino acid 200 (Zhang *et al.,* 1996). In future studies, it will be important to examine the phenotype of Z200E within the context of the intact viral genome.

## ACKNOWLEDGMENTS

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