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Functional and Physical Interactions between the Epstein-Barr Virus (EBV) Proteins BZLF1 and BMRF1: Effects on EBV Transcription and Lytic Replication

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The Epstein-Barr virus (EBV) proteins BZLF1 and BMRF1 are both essential for lytic EBV replication. BZLF1 is a transcriptional activator which binds directly to the lytic origin of replication (oriLyt) and plays a critical role in the disruption of viral latency. The BMRF1 protein is required for viral polymerase processivity. Here we demonstrate that the BMRF1 gene product functions as a transcriptional activator and has direct (as well as indirect) interactions with the BZLF1 gene product. The BMRF1 gene product activates an essential oriLyt promoter, BHLF1, but does not activate two other early EBV promoters (BMRF1 and BHRF1). Direct interaction between the BMRF1 and BZLF1 gene products requires the first 45 amino acids of BMRF1 and the bZip domain of BZLF1. The effect of the BZLF1-BMRF1 interaction on early EBV transcription is complex and is promoter specific. The oriLyt BHLF1 promoter is activated by either the BZLF1 or BMRF1 gene product alone and is further activated by the combination of the BZLF1 and BMRF1 gene products. Enhanced activation of BHLF1 transcription by the BMRF1-BZLF1 combination does not require direct interaction between these proteins. In contrast, BZLF1-induced activation of the BMRF1 promoter is inhibited in the presence of the BMRF1 gene product. A point mutation in the BZLF1 protein (amino acid 200), which prevents in vitro interaction with the BMRF1 protein but which does not reduce BZLF1 transactivator function, allows the BZLF1 protein to activate the BMRF1 promoter equally well in the presence or absence of the BMRF1 gene product. Therefore, direct interaction between the BZLF1 and BMRF1 proteins may inhibit BZLF1-induced transcription of the BMRF1 promoter. BZLF1 mutated at amino acid 200 is as efficient as wild-type BZLF1 in promoting replication of an oriLyt plasmid. However, this mutation reduces the ability of BZLF1 to induce lytic replication of the endogenous viral genome in D98/HE-R-1 cells. Our results indicate that functional and physical interactions between the BMRF1 and BZLF1 proteins may modulate the efficiency of lytic EBV infection. The BMRF1 gene product clearly has a transcriptional, as well as replicative, role during lytic EBV infection.

Epstein-Barr virus (EBV) is a human herpesvirus which is closely associated with some human malignancies, including Burkitt's lymphoma and nasopharyngeal carcinoma (45, 75). In the latent form of EBV infection, the virus replicates as an episome using the oriP origin of replication in conjunction with the viral protein EBNA-1 (37). With the onset of lytic infection, the virus converts to a linear form and uses a different origin of replication, oriLyt (32). Lytic EBV replication requires the gene products of six viral genes: BALF5 (the catalytic component of the viral polymerase), BMRF1 (the polymerase processivity factor), BALF2 (single-stranded DNA binding protein), BSLF1 (primase), BBLF4 (helicase), and BBLF2/3 (helicase-primase-associated protein) (20, 21). In addition, the immediate-early gene product BZLF1 plays an essential role in lytic EBV replication (21, 54).

Overexpression of BZLF1 in EBV-infected cells is sufficient to initiate conversion of latent infection into productive infection (8, 12, 52, 60). The BZLF1 protein, a member of the bZip family, is a transcriptional activator which binds directly as a homodimer to Ap1-like BZLF1-responsive elements (ZREs) (4, 9, 19, 23, 43, 65). The BZLF1 protein activates expression of early viral proteins, including essential replicative proteins (3, 5, 7, 13, 33, 35, 36, 50).

However, recent studies suggest that in addition to its transcriptional function, BZLF1 plays a direct role in oriLyt replication. The minimal oriLyt contains the transcriptional control regions for two EBV early genes, BHLF1 and BHRF1 (32). There are seven BZLF1 binding motifs (ZREs) within oriLyt, and mutation of these sites completely abolishes oriLyt replication (54). Although these binding sites are important for transcriptional activation of the BHLF1 and BHRF1 promoters, BZLF1 binding to oriLyt appears to be required for oriLyt replication in a manner independent of its transcriptional activator role. Replacement of the oriLyt ZREs with alternative transcriptional activator binding motifs does not rescue oriLyt replication, although transcription can be restored (54). Furthermore, even when the six essential viral replicative proteins are expressed under the control of strong heterologous promoters, BZLF1 is still required for oriLyt-mediated replication (21). Although BZLF1 is essential for oriLyt replication, the mechanism(s) by which BZLF1 promotes replication are not fully defined and seem to be separable from transcriptional activation. Interestingly, the BMRF1 and BZLF1 gene products have been shown to colocalize within intranuclear viral replication compartments (61).

In this report, we demonstrate that the BMRF1 gene prod-

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uct, in addition to its role as the polymerase processivity factor (6, 10, 11, 14, 38, 39, 42, 62–64), activates expression of an essential oriLyt promoter, BHLF1. We also find that the BMRF1 gene product interacts directly with the BZLF1 gene product in vitro as well as in vivo. The BMRF1-BZLF1 interaction is mediated through the first 45 amino acids of BMRF1 and the bZip domain of BZLF1 and produces promoter-specific effects. The early BHLF1 promoter is activated by either the BMRF1 or BZLF1 gene products alone and is further activated by the combination of the BZLF1 and BMRF1 gene products. However, the BMRF1 gene product inhibits BZLF1-induced activation of its own promoter.

A BZLF1 protein mutated at amino acid 200 cannot interact in vitro with the BMRF1 gene product. This mutant BZLF1 protein is similar to wild-type BZLF1 as a transcriptional activator and has equivalent function in plasmid-based oriLyt replication assays. Nevertheless, mutation of amino acid 200 reduces the ability of BZLF1 to activate lytic infection of the endogenous viral genome in D98/HE-R-1 cells. The direct and indirect interactions between BZLF1 and BMRF1 may therefore modulate the efficiency of lytic EBV replication.

MATERIALS AND METHODS

Cell lines. HeLa is a cervical epithelial cell line. The latently infected D98/HE-R-1 cell line was formed by fusion of a HeLa subclone (D98) with the EBV-positive Burkitt's lymphoma P3HR-1 (27). Epithelial cells were maintained in Dulbecco modified Eagle medium H supplemented with 10% fetal calf serum. The B95-8 cell line (an EBV-infected marmoset B-cell line) and the Akata cell line (an EBV-positive human Burkitt lymphoma line) (59) were maintained in RPMI 1640 medium with 10% fetal calf serum.

Plasmids. The construction of the BZLF1 expression plasmid pCMV-Z has been described previously (50). pCMV-Z contains the BZLF1 cDNA (a gift from Paul Farrell) in the pGEM2-based vector pHD1013 (a gift from E. S. Huang) such that the BZLF1 immediate-early gene is under the control of the strong cytomegalovirus immediate-early promoter. The BMRF1 expression plasmid contains the 1,341-bp *Bcl*I-*Bgl*II fragment (from the *Bam*HI M fragment of EBV) (1) subcloned in the *Bam*HI and *Bgl*II sites of the pSG5 vector (Stratagene) such that BMRF1 gene expression is driven by the simian virus 40 (SV40) early promoter. The BMRF1 in vitro translation plasmid contains the identical *Bcl*I-*Bgl*II fragment inserted into the pBS SK+ phagemid vector (Stratagene). The promoter plasmids BMRF1-CAT and BHRF1-CAT have been described previously (31, 50) and contain the early EBV promoters BMRF1 and BHRF1 linked to the chloramphenicol acetyltransferase (CAT) gene in the Bluescript SK+ phagemid vector (Stratagene). The BHLF1-CAT plasmid contains the early BHLF1 promoter (amino acids −1031 to +165 relative to the mRNA start site) linked to CAT in the Bluescript SK+ vector. The plasmids for in vitro translation of wild-type BZLF1 protein and the double point mutants of the BZLF1 dimerization domain (a gift from Erik Flemington and Sam Speck) have been described elsewhere (24, 31). Single point mutations of the BZLF1 bZip domain were made with the CLONTECH transformer site-directed mutagenesis kit according to the manufacturer's instructions. All point mutants were sequenced. BZLF1Δ200 contains a tyrosine to glutamic acid mutation, and BZLF1Δ225 contains a leucine to glutamic acid mutation. The oriLytΔKpn plasmid (used in plasmid replication assays) was made by ligating the EBV *Sst*II-*Hin*cII fragment (EBV sequences 52,623 to 53,819) into the *Hin*cII site of the pBS+ plasmid (Stratagene). The nonessential *Kpn*I-*Kpn*I fragment of oriLyt (EBV sequences 52,944 to 53,207) was subsequently deleted. The plasmid pRZSphHind contains the *Hin*dIII-*SpH*I fragment (spanning the EBV sequences from −235 to +115 relative to the BZLF1 mRNA start site) inserted into the *Hin*cII site of the pBS+ plasmid (Stratagene). This plasmid was used to generate a riboprobe for RNase protection assays.

Bacterial proteins. The construction of the glutathione *S*-transferase (GST)-Z fusion protein has been previously described (31, 73). GST-Z contains the entire BZLF1 open reading frame inserted in frame into the pGEX-3X vector (Pharmacia) downstream of the GST protein. In-frame deletions of GST-Z were constructed and named to reflect the deleted amino acids (see Fig. 3A). The GST-BMRF1 protein contains the first 303 residues of BMRF1 cloned in frame in the pGEX-3X vector, downstream of the GST protein. In-frame deletions of GST-BMRF1 were also constructed and named to reflect the amino acids missing (see Fig. 2B).

DNA transfections. Plasmid DNA was purified by using a QIAGEN Maxi kit as specified by the manufacturer. DNA was transfected by electroporation, using 10 μg of DNA and 10^7 cells per condition. Cells were shocked at 1,500 V with a Zapper electroporation unit (Medical Electronics Shop, University of Wisconsin, Madison, Wis.). Epithelial cells were harvested and suspended into RPMI 1640 medium before electroporation.

CAT assays. Cell extracts were prepared 48 h after transfection and incubated at 37°C with [^{14}C]chloramphenicol in the presence of acetyl coenzyme A as described previously (28). The percent acetylation of chloramphenicol was quantitated by thin-layer chromatography followed by scintillation counting or AMBIS scanning (AMBIS, Inc.).

EBV terminus analysis. To distinguish latent versus productive EBV infection, Southern blot analysis was used to determine the state of the EBV termini (fused versus unfused). Total cellular DNA was analyzed 3 days after transfection with the BZLF1 expression plasmid by cutting it with the *Bam*HI enzyme and probing with a ^{32}P-labelled riboprobe spanning the EBV sequences in the 1.9-kb *Xho*I fragment. As previously described (51), this probe can be used to determine the relative amount of EBV DNA containing fused versus unfused viral termini. Unfused termini are only observed in the linear form of the virus and are diagnostic of lytic infection.

oriLyt plasmid replication assays. oriLyt plasmid replication assays were performed as previously described (54, 55). The oriLytDKpn plasmid was transfected into D98/HE-R-1 cells with either pHD1013 vector DNA, the wild-type BZLF1 expression plasmid, or the mutant BZLF1 expression plasmid. Total cellular DNA was harvested 3 days after transfection, cut with the *Bam*HI and *Dpn*I restriction enzymes, separated on an agarose gel, and analyzed by the Southern blot technique using a ^{32}P-labelled single-stranded RNA probe spanning oriLyt. The efficiency of replication was determined by quantitating the amount of the replicated (*Dpn*-resistant) band in each condition, with the input DNA band serving as a control for transfection efficiency.

Coimmunoprecipitation and immunoblot analysis. Coimmunoprecipitation experiments were performed 3 days after induction of B95-8 cells with TPA (12-*O*-tetradecanoylphorbol-13-acetate) and sodium butyrate. Cells were rinsed with cold phosphate-buffered saline (PBS), pelleted, frozen in dry ice, thawed, and resuspended in 0.4 ml of lysis buffer (50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40 [NP-40] [pH 8.0]) supplemented with the protease inhibitors 1 mM phenylmethylsulfonyl fluoride and 1 μM pepstatin (Boehringer-Mannheim). The cell lysate was then sonicated six times and pelleted again, and equal amounts of protein supernatant (approximately 100 μg) were incubated overnight at 4°C with 10 μl of either a BMRF1-specific monoclonal antibody (previously known as R3 [49]; Capricorn) or an Rb-specific monoclonal antibody (Santa Cruz Biotechnology). Extracts were then immunoprecipitated with protein A-Sepharose 4B beads (Sigma) and washed five times at 4°C in buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.7), 75 mM KCl, 0.1 mM EDTA, 25 mM $MgCl_2$, 10 mM dithiothreitol, and 0.15% NP-40. The immunoprecipitated proteins were separated on a sodium dodecyl sulfate (SDS)–8% gel, transferred to nitrocellulose paper, and analyzed by immunoblot analysis using the BZLF1-specific antibody BZ.1 (directed against the dimerization domain) (71). Immunoblot analysis was performed in PBS with 5% powdered milk, using a 1:10 dilution of BZ.1 monoclonal antibody, followed by washing three times in room temperature with 3% powdered milk–PBS and then addition of the secondary antibody (1:2,000 dilution of goat anti-mouse kappa chain horseradish peroxidase-conjugated serum [Southern Biotechnology Associates]). Immunoblotting was done by using enhanced chemiluminescence performed as recommended by the manufacturer (Amersham Life Sciences).

GST-fusion protein affinity chromatography. The interaction of the ^{35}S-labelled in vitro-translated BZLF1 and BMRF1 proteins with the GST-BMRF1 and GST-BZLF1 fusion proteins was analyzed by affinity chromatography as previously described (57, 66). Bacterial cultures (50 ml) containing the various pGEX vectors were induced with 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG) and then pelleted and resuspended in 5 ml of PBS, sonicated, and cleared of bacterial debris by centrifugation at 12,000 × *g*. For each condition, 100 μl of lysate was then incubated in 1 ml of PBS for 20 min at room temperature with 10 μl of 50% glutathione-agarose beads (Sigma) and the GST proteins were purified by three cycles of centrifugation and washing in 1-ml aliquots of PBS, followed by one wash in 1-ml aliquots of buffer containing 20 mm HEPES (pH 7.7), 25 mM NaCl, 0.1 mM EDTA, 25 mM $MgCl_2$, 1 mM dithiothreitol, and 0.15% NP-40. The purified GST protein was then resuspended in 1 ml of the final wash buffer with 15 μl of ^{35}S-labelled, in vitro-translated protein for 1 h at room temperature. Bead complexes were washed five times in the above buffer, centrifuged, resuspended in Laemmli sample buffer, and then analyzed by electrophoresis on SDS–8% polyacrylamide gels, followed by autoradiography. In parallel experiments, the purified GST fusion proteins were analyzed by electrophoresis on SDS–8% polyacrylamide gels to show that each of these proteins was intact and present in equivalent amounts.

Electromobility shift assays. Electromobility shift assays were performed as previously described (26). Whole cell extracts were prepared by washing the cells twice with ice-cold PBS and then resuspending the cell pellet in 0.4 ml of lysis buffer (50 mM Tris [pH 7.9], 150 mM NaCl, 0.5% NP-40 [pH 8.0], 0.5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μM of pepstatin). The cell lysate was then sonicated six times and pelleted again. Equal amounts of protein supernatant (approximately 10 μg) were incubated with either 10,000 cpm of a ^{32}P-end-labelled probe containing the four BZLF1 binding motifs from the BHLF1 promoter (the *Sac*II-*Kpn*I fragment from EBV sequences [1] 52,623 to 52,944) or a ^{32}P-end-labelled oligonucleotide probe (5′GATCATGTGCAAGCTATGTGCAATG3′ and 5′GATCCATTGCACATAGCTTGCACAT3′) containing two consensus ZRE sites. Binding reactions

were carried out at room temperature for 15 min (prior to adding the labelled probe) in a buffer consisting of 50 mM Tris (pH 7.9), 250 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EDTA, and 5 mM dithiothreitol. Poly(dI-dC)-poly(dI-dC) (4 μg) was added as a nonspecific competitor DNA. After addition of the labelled probe, the reaction was incubated an additional 15 min at room temperature, loaded onto a 4% polyacrylamide gel–1% Tris glycine gel, and run in 0.5× Tris glycine buffer (0.25 M Tris, 0.19 M glycine) at room temperature. The gel was dried and subjected to autoradiography.

RNase protection assays. Total cellular RNA was isolated by using the Trizol reagent (GIBCO-BRL) 24 h after transfection of D98/HE-R-1 cells with the pHD1013 vector or the wild-type or mutant BZLF1 expression vectors or 4 h after treatment of Akata cells with TPA (30 ng/ml). RNase protection was performed by using the RNase protection assay system (Promega) as described by the manufacturer. The plasmid pRZSphHind was cut with *Dra*I (position −34 relative to the BZLF1 mRNA start site) and transcribed with T3 RNA polymerase in the presence of ^{32}P-labelled UTP to yield a 156-bp antisense riboprobe spanning the Zp initiation site. Fifteen micrograms of total RNA was hybridized with 20,000 cpm of riboprobe at 50°C for 16 h and then digested with 3 U of RNase I at room temperature for 1 h. Samples were denatured and fragments were resolved by electrophoresis through an 8 M urea–polyacrylamide gel. Hybridization to Zp- and Rp-initiated transcripts yields protected species of 115 and 148 bp, respectively. The transfected Z vector yields a protected fragment of 80 bp.

RESULTS

The BMRF1 gene product specifically activates the oriLyt promoter BHLF1. In addition to its role as a replicative protein, the BMRF1 gene product was reported by one group to activate the SV40 early promoter (47). However, two other groups observed no BMRF1 transactivator function (6, 67), and no EBV promoter has ever been shown to be activated by the BMRF1 gene product. To examine the potential transcriptional role of the BMRF1 protein during lytic replication, cotransfection studies were performed in HeLa cells, using reporter gene constructs driven by three different early EBV promoters: the BMRF1 promoter, the BHRF1 promoter, and the BHLF1 promoter. The BMRF1 promoter drives expression of the BMRF1 gene product, whereas the BHRF1 and BHLF1 promoters are the two promoters contained within oriLyt. Each of these promoters has been previously shown to be directly bound (and activated) by the BZLF1 gene product (4, 50, 54).

As shown in Fig. 1, the BMRF1 gene product in HeLa cells had no significant effect on the constitutive activity of its own promoter (BMRF1) or that of the BHRF1 promoter. However, the BMRF1 gene product produced a 20-fold activation in the constitutive expression of the BHLF1 promoter. Similar results were obtained in an EBV-negative B-cell line (data not shown). Thus, in addition to its direct replicative function, the BMRF1 gene product can activate a promoter (BHLF1) which has previously been shown to be an essential element of oriLyt (55).

The BZLF1 protein interacts directly with the BMRF1 gene product through the BZLF1 bZip dimerization domain. The previously reported colocalization of the BMRF1 and BZLF1 proteins within intranuclear viral replication compartments (61) raised the possibility that these proteins may interact directly. To confirm that the BZLF1 and BMRF1 proteins directly interact and to map the domains in the BZLF1 and BMRF1 proteins required for direct interaction, we performed in vitro affinity chromatography assays with GST fusion proteins (66). The BZLF1 and BMRF1 proteins (in vitro translated and labelled with ^{35}S) were both retained by the GST-Z protein (containing the entire 245-amino-acid BZLF1 open reading frame fused in frame to GST) (Fig. 2A) but not by the control GST protein alone. In contrast, the catalytic component of the viral polymerase (BALF5) did not interact directly with GST-Z.

In-frame deletions of the BMRF1 in vitro translation vector

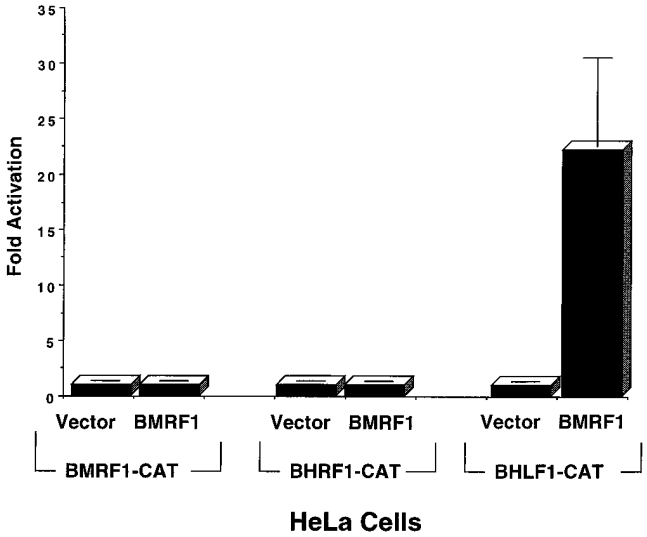

FIG. 1. The BMRF1 gene product activates the BHLF1 oriLyt promoter. Five micrograms of the BMRF1-CAT reporter construct (containing the early EBV BMRF1 promoter), the BHRF1-CAT reporter construct (containing the BHRF1 early EBV promoter), or the BHLF1-CAT reporter construct (containing the early EBV BHLF1 promoter) was transfected into HeLa cells with either 5 μg of pSG5 vector DNA (Vector) or 5 μg of the BMRF1 expression vector (BMRF1). The percent acetylation of ^{14}C-labelled chloramphenicol in each condition was measured as previously described (28). Results are expressed as the amount of CAT activity induced by the BMRF1 expression vector relative to the amount induced by the vector alone.

and the GST-BMRF1 fusion protein were constructed to map the domain(s) of BMRF1 required for interaction with BZLF1 (Fig. 2B). The results of these experiments indicate that interaction between the BMRF1 and BZLF1 gene products is mediated through the amino-terminal half of BMRF1 and requires the first 45 amino acids of BMRF1 (Fig. 2C to E). It is clear that the BMRF1-BZLF1 interaction does not require BMRF1 DNA binding function, since a mutant form of BMRF1 unable to bind DNA (BMRF1Δ124-217) still interacts with BZLF1. Furthermore, a BMRF1 mutant missing the first 45 amino acids is unable to interact with BZLF1 in vitro, although this mutant can bind DNA efficiently in vitro (17a). Thus, the region of BMRF1 required for interaction with Z appears to be separable from its DNA binding domain. In contrast, the polymerase processivity function of BMRF1 clearly requires its DNA binding function (39).

To map the domain(s) of BZLF1 required for interaction with BMRF1, we created a series of mutations within the GST-Z and in vitro-translated BZLF1 proteins (Fig. 3A) and analyzed the ability of such mutants to homodimerize and interact with BMRF1. Preliminary mapping indicated that the bZip dimerization domain of BZLF1 is sufficient for interaction with BMRF1 (Fig. 3B). In vitro-translated proteins, each containing two point mutations within the BZLF1 dimerization domain (gifts from Erik Flemington), were then tested for the ability to interact with the GST-BMRF1 fusion protein (Fig. 3C). A BZLF1 protein containing double point mutations within amino acids 200 and 225 could homodimerize (although less efficiently than wild-type BZLF1) but no longer interacted efficiently with BMRF1. A BZLF1 protein containing point mutations within amino acids 197 and 200 also shared this phenotype but was not further studied since this particular mutant has previously been shown to bind poorly to ZREs (56).

We next constructed single point mutations in amino acids

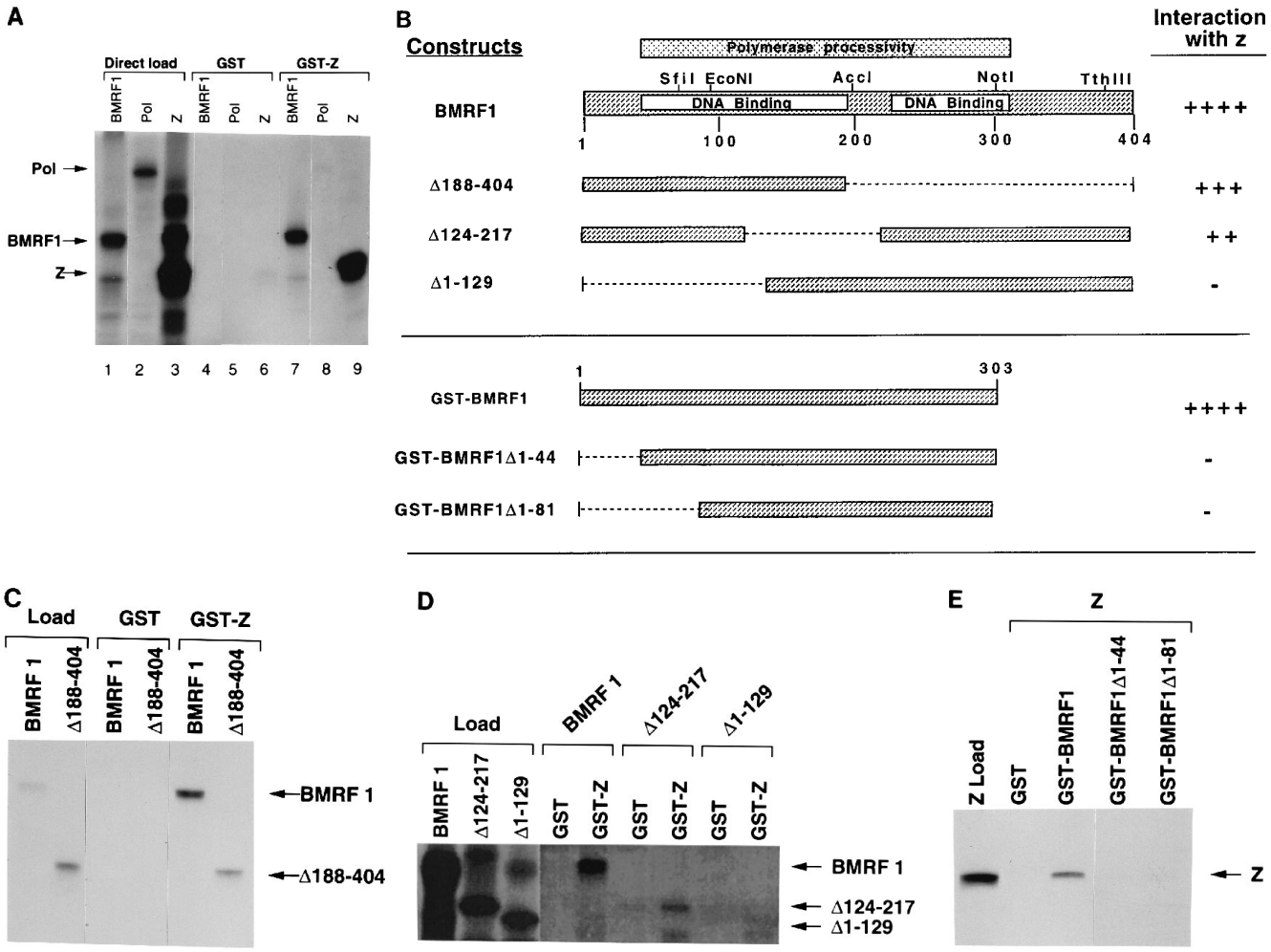

FIG. 2. The BZLF1 and BMRF1 proteins interact in vitro. (A) The entire BZLF1 open reading frame was inserted in frame into the pGEX-3X vector (Pharmacia) downstream of the GST protein to create the GST-Z fusion protein. The BMRF1, viral polymerase (BALF5), and BZLF1 proteins were in vitro translated, ^{35}S-labelled, and tested for their ability to interact with the GST-Z protein (or the GST protein alone) using affinity chromatography assays (66). Lanes: 1 to 3, input ^{35}S-labelled, in vitro-translated BMRF1, viral polymerase (Pol), and BZLF1 proteins (Z); 4 to 6, retention of each protein by the GST protein; 7 to 9, retention of each protein by the GST-Z fusion protein. The size of each full-length in vitro-translated protein is indicated by a labelled arrow. The direct load contains 3 μl of the in vitro-translated proteins, whereas 10 μl of each in vitro-translated protein was used in the affinity chromatography assays. Approximately 30% of the labelled BMRF1 and BZLF1 proteins is retained by the GST-Z protein. (B through E) In-frame deletions of the BMRF1 protein (removing the amino acids indicated) (C and D) were ^{35}S-labelled, in vitro translated, and evaluated for the ability to interact with GST-Z in affinity chromatography assays as shown in panel A. GST-BMRF1 fusion proteins (containing the first 303 amino acids of BMRF1 fused to GST or missing the amino acids indicated) were also constructed and evaluated for their ability to retain in vitro-translated, ^{35}S-labelled BZLF1 protein (panel E). The results are summarized on the right side of panel B. The BMRF1 residues between 1 and 193 and between 238 to 302 have been previously shown to be required for DNA binding (39).

200 and 225 and tested each of these mutants for the ability to homodimerize and interact with BMRF1 (Fig. 3D). The BZLF1Δ200 protein (containing a tyrosine to glutamic acid switch at residue 200) could homodimerize as efficiently as the wild-type BZLF1 but was unable to interact efficiently with BMRF1 at room temperature. This mutant protein was thus used in subsequent studies to examine the functional role(s) of the BZLF1-BMRF1 direct interaction. The BZLF1 protein mutated at amino acid 225 could still interact with BMRF1 although with slightly reduced affinity.

The BZLF1 and BMRF1 proteins can be coimmunoprecipitated in vivo. Coimmunoprecipitation studies were performed to determine if the BZLF1 and BMRF1 gene products can interact in vivo in EBV-infected cells. EBV-infected marmoset B cells (B95-8) were treated for 48 h with TPA and sodium butyrate as previously described (44, 74) to induce lytic EBV infection. Cell extracts were immunoprecipitated with monoclonal antibodies directed against the BZLF1, BMRF1, or the retinoblastoma (Rb) protein. The immunoprecipitated proteins were then probed by immunoblot using the BZLF1-specific antibody.

As expected, in B95-8 cells, BZLF1 was immunoprecipitated by the BZLF1-specific antibody and not immunoprecipitated by the Rb antibody (Fig. 4). A portion of BZLF1 (approximately one-quarter of the BZLF1 protein precipitated by the BZLF1-specific antibody) was also immunoprecipitated with the BMRF1-specific antibody in the productively infected B95-8 cells (which express BMRF1 protein). BZLF1 protein overexpressed in EBV-negative HeLa cells was not immunoprecipitated by the BMRF1 antibody (Fig. 4), excluding the possibility that this antibody nonspecifically cross-reacts with BZLF1. These results suggest that the BZLF1 and BMRF1 proteins can interact directly in productively infected cells.

We were unable to show an interaction between the BZLF1

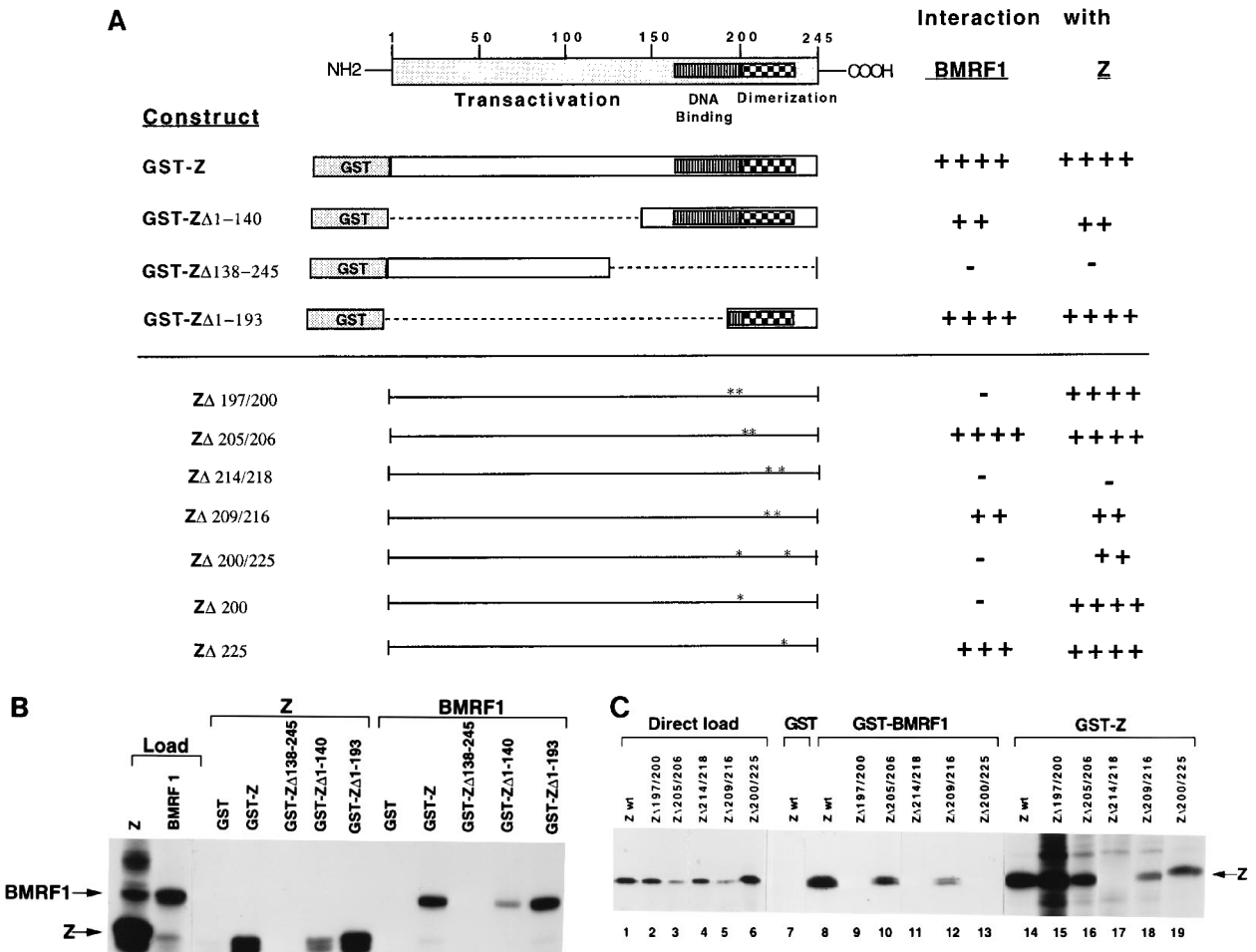

FIG. 3. The bZip dimerization domain of BZLF1 is required for interaction with the BMRF1 protein. In-frame deletions of the GST-Z protein or point mutants of BZLF1 (derived from the in vitro-translation vector) were constructed (A). Constructs are named to reflect the amino acid(s) deleted or mutated. Each mutant protein was tested for the ability to interact with the BZLF1 and BMRF1 proteins with affinity chromatography assays (B, C, and D). Results are summarized on the right of panel A. Approximately 30% of the total labelled BZLF1 protein was retained by GST-BMRF1.

and BMRF1 proteins when the experiment was performed in the reverse direction (using BZLF1 antibody to immunoprecipitate and BMRF1 antibody to probe the immunoblot) (data not shown). However, this negative result is not unexpected, since the BZLF1 antibody recognizes an epitope within the bZip dimerization domain (71) which was found to be the domain also required for interaction between the BZLF1 and BMRF1 proteins. The epitope recognized by the BZ.1 antibody may thus be obscured by the interaction between the BZLF1 and BMRF1 gene products.

The BMRF1 gene product interacts with BZLF1 to regulate early EBV promoters in a promoter-specific fashion. The previous results suggested that the BMRF1 gene product, like BZLF1, functions as a transcriptional activator and, furthermore, that these two proteins can directly interact. Given that both of these proteins are expressed during lytic EBV infection, we next determined if the BMRF1-BZLF1 combination produces synergistic (or antagonistic) effects on the activity of early EBV promoters. We compared the effect of the BZLF1 gene product alone, the BMRF1 gene product alone, and the combination of the two proteins together on three different early EBV promoters (BMRF1, BHRF1, and BHLF1). Each of these promoters is activated by the BZLF1 protein alone, although (as shown in Fig. 1) only the BZLF1 promoter is activated by the BMRF1 gene product alone.

As shown in Fig. 5A, cotransfection of BMRF1 and BZLF1 did not alter the expression of either gene product, as detected by immunoblot. The effect of the BMRF1 gene product on BZLF1-induced activation of early EBV promoters was found to be complex and promoter specific. The BHRF1 promoter was not activated by the BMRF1 gene product alone, and cotransfection of BMRF1 protein did not alter the level of

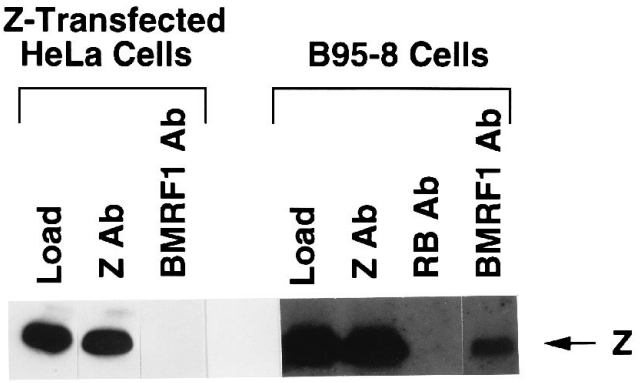

FIG. 4. The BZLF1 and BMRF1 proteins can be coimmunoprecipitated in vivo. The EBV-positive marmoset B-cell line, B95-8, was treated for 48 h with TPA and sodium butyrate as previously described (44, 74) to induce lytic viral infection. Cell extracts were prepared and immunoprecipitated with either a BZLF1-specific monoclonal antibody (BZ.1), a BMRF1-specific monoclonal antibody (Capricorn), or a monoclonal antibody directed against the cellular retinoblastoma tumor suppressor protein, Rb. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose filter, and analyzed by immunoblot for the presence of coimmunoprecipitated BZLF1 protein using the BZ.1 antibody. BZLF1 is coimmunoprecipitated with the BMRF1 antibody, but not the Rb antibody, from B95-8 cell extracts. The BMRF1 antibody does not immunoprecipitate BZLF1 transfected into the EBV-negative HeLa cell line, indicating that this antibody does not cross-react with BZLF1.

BZLF1-induced activation (Fig. 5B). In the case of the BMRF1 promoter, the BMRF1 gene product alone produced no effect, but the presence of cotransfected BMRF1 protein significantly inhibited the activation produced by BZLF1 alone (Fig. 5C).

The BHLF1 promoter was consistently activated by either the BMRF1 gene product alone or the BZLF1 gene product alone (Fig. 5D). Furthermore, activation of the BHLF1 promoter was consistently greater with the combination of the BZLF1 and BMRF1 gene products than with either protein alone. Thus, the BHLF1 promoter element, which overlaps a known essential domain in oriLyt (55), is specifically activated by the BMRF1 gene product alone and is further activated by the combination of the BZLF1 and BMRF1 gene products together.

BMRF1 decreases BZLF1 binding to ZREs. The BZLF1 protein binds to a specific Ap1-like motif present in most early EBV promoters, including the BMRF1, BHLF1, and BHRF1 promoters. The BMRF1 protein has potent nonspecific DNA binding activity but is not known to bind to a specific DNA motif (39). To determine if the BMRF1 protein can alter BZLF1 binding to DNA, the amount of specific BZLF1 binding to a synthetic ^{32}P-labelled oligonucleotide probe containing two ZREs was compared using protein extracts from cells transfected with BZLF1 alone, BMRF1 alone, or a combination of the BZLF1 and BMRF1 gene products (Fig. 6). Under our electromobility shift assay conditions, cells transfected with BMRF1 alone did not have an observable protein binding to the ZRE probe (presumably because BMRF1 binding activity was completely inhibited by the unlabelled, nonspecific competitor DNA). The extracts from cells transfected with the BZLF1 protein alone, as expected, contained a binding protein (BZLF1) which was specifically inhibited by the Ap1 binding motif. In the presence of cotransfected BMRF1 protein, BZLF1 binding to the ZRE probe was consistently decreased (by an average of 75%). No new binding complex (as might be expected if the BMRF1-BZLF1 heterodimer binds DNA) was observed. Similar results were obtained using in vitro-translated BZLF1 and BMRF1 proteins (data not shown). A probe containing the four ZRE sites in the BHLF1 promoter also produced similar results (data not shown), indicating that decreased BZLF1 binding in the presence of BMRF1 is not probe specific. As shown in Fig. 5A, the presence of the cotransfected BMRF1 gene product did not alter the level of transfected BZLF1 in these extracts.

These results are consistent with the hypothesis that the BMRF1-BZLF1 complex is less efficient in binding to ZREs. We cannot completely exclude the possibility that the BZLF1-BMRF1 binding complex is too unstable to be seen under our gel electrophoresis conditions or that the BZLF1-BMRF1 complex specifically binds to alternative DNA motifs. The very potent nonspecific DNA binding ability of BMRF1 may make the ZRE probe unavailable for specific BZLF1 binding in these assays.

Direct interaction between the BZLF1 and BMRF1 proteins in vitro correlates with the ability of the BMRF1 gene product to inhibit its own transcription but is not required for synergistic activation of the BHLF1 promoter. The previous results indicate that the interactions between the BMRF1 and BZLF1 transcription factors produce complex, promoter-specific effects. It is likely that indirect, as well as possibly direct, interactions between the BZLF1 and BMRF1 proteins contribute to these effects. To determine what role, if any, the direct interaction between the BZLF1 and BMRF1 proteins plays in the transcriptional regulation of early EBV promoters, we examined whether mutation of BZLF1 amino acid 200 (a mutation which prevents direct interaction with the BMRF1 protein in vitro) alters the interactions between BZLF1 and BMRF1 in vivo.

As shown in Fig. 7A, the mutant BZLF1Δ200 protein is not intrinsically impaired as a transcriptional activator, although it may be slightly less stable than wild-type BZLF1 in vivo (Fig. 7C). Interestingly, mutation of BZLF1 amino acid 200 was found to alter the interaction between the BZLF1 and BMRF1 proteins in vivo in regard to regulation of the BMRF1 promoter (Fig. 7A). Whereas the ability of wild-type BZLF1 to activate BMRF1 transcription was consistently decreased by the presence of the BMRF1 gene product ($P < 0.5$ in a two-tailed t test), the ability of mutant BZLF1 protein to activate BMRF1 transcription was not significantly inhibited by the BMRF1 gene product. Thus, direct interaction between the BZLF1 and BMRF1 proteins in vitro correlates with the ability of the BMRF1 gene product to inhibit its own transcription in vivo.

In the case of the BHLF1 promoter, which is maximally activated by the combination of the BMRF1 and BZLF1 proteins, the mutant BZLF1 protein, like wild-type BZLF1, could also interact with the BMRF1 gene product to enhance BHLF1 transcription (Fig. 7B). Therefore, the increased BHLF1 promoter activity induced by the BMRF1-BZLF1 combination in vivo did not correlate with the ability of the BZLF1 and BMRF1 proteins to interact in vitro. Since it has previously been shown that BZLF1 can sometimes activate transcription through a nonbinding mechanism (presumably mediated through interactions between BZLF1 and other transcription factors) (22), we also examined whether a nonbinding mutant of BZLF1, Z311 (mutated at residue 185) (35), can cooperate with the BMRF1 gene product to activate the BHLF1 promoter. The Z311 mutant was unable to activate the BHLF1 promoter efficiently by itself (Fig. 7b) and could not cooperate with the BMRF1 gene product to activate BHLF1 transcription. Therefore, activation of the BHLF1 promoter by the BMRF1-BZLF1 combination requires a BZLF1 DNA

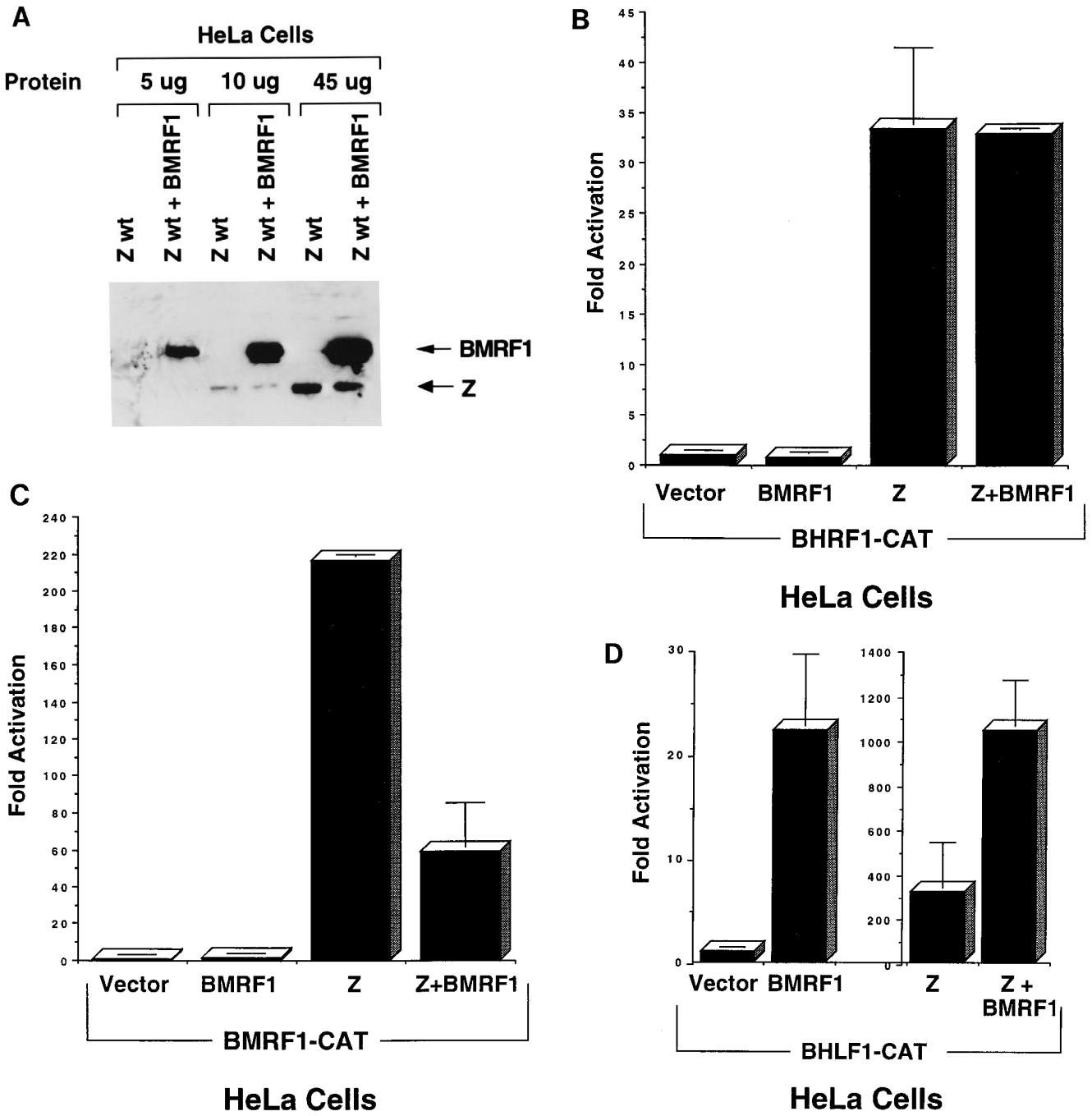

FIG. 5. The BMRF1 gene product modulates BZLF1 transactivator function. (A) The expression level of transfected BZLF1 in the presence and absence of the cotransfected BMRF1 gene product was determined by immunoblot analysis. Cotransfection of BMRF1 does not significantly alter the expression of transfected BZLF1 protein or vice versa. (B) Five micrograms of the BHRF1-CAT reporter construct (containing the BHRF1 early EBV promoter) was transfected into HeLa cells with either 5 μg of pSG5 vector DNA–5 μg of pHD1013 vector DNA (Vector), 5 μg of the BMRF1 expression vector–5 μg of the pHD1013 vector (BMRF1), 5 μg of pSG5 vector DNA–5 μg of the BZLF1 expression vector (Z), or 5 μg of the BMRF1 expression vector–5 μg of the BZLF1 expression vector (Z + BMRF1). The percent acetylation of ^{14}C-labelled chloramphenicol in each condition was measured as previously described (28). Results are expressed as the amount of CAT activity induced by the BMRF1 and BZLF1 expression vectors relative to the amount induced by the vector alone. (C) Five micrograms of the BMRF1-CAT reporter construct (containing the early EBV BMRF1 promoter) was transfected into HeLa cells under the same conditions described for the BHRF1-CAT construct. BZLF1-induced activation of the BMRF1 promoter was significantly inhibited by the presence of the BMRF1 gene product ($P < 0.05$, using a two-tailed t test with 3 degrees of freedom). (D) Five micrograms of the BHLF1-CAT reporter construct, containing the early EBV BHLF1 promoter, was transfected into HeLa cells under the same conditions described for BMRF1-CAT. The fold activation induced by each transactivator (compared with the effect of vector DNA alone) is shown. Note that the scale for the Z and Z + BMRF1 conditions is different from that for the Vector and BMRF1 conditions. The activation of the BHLF1 promoter was significantly greater using the combination of the BZLF1 and BMRF1 gene products versus that for BZLF1 alone ($P < 0.05$ in a two-tailed t-test with 4 degrees of freedom).

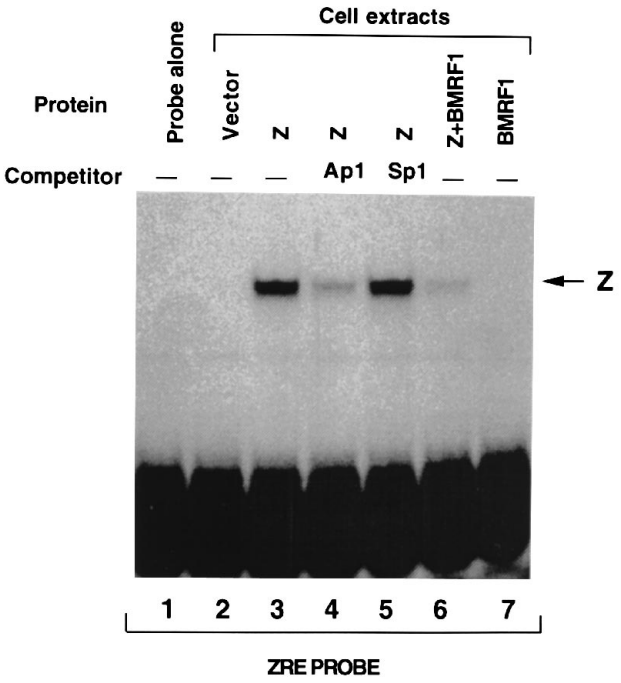

FIG. 6. The BMRF1 gene product decreases BZLF1 binding to ZREs. HeLa cells were transfected with either vector DNA (lane 2), BZLF1 alone (lanes 3 to 5), BZLF1 and BMRF1 (lane 6), or BMRF1 alone (lane 7). Cell extracts were prepared and tested for their ability to bind to a labelled oligonucleotide probe containing two ZRE sites. The specific BZLF1 complex is indicated by the arrow Z. The BMRF1 gene product reduced binding of BZLF1 to the synthetic oligonucleotide probe.

binding function but does not require direct interaction between the BZLF1 and BMRF1 proteins.

The BZLF1Δ200 mutant is impaired in its ability to induce lytic replication of the latent EBV genome. The previous results suggest that direct, as well as indirect, interactions between the BZLF1 and BMRF1 gene products can modulate the ability of BZLF1 to activate various EBV early promoters. However, since the BZLF1 and BMRF1 proteins have replicative as well as transcriptional functions, the BZLF1-BMRF1 interaction might also be important in modulating replication. To further examine this point, we compared the ability of wild-type BZLF1 with that of mutant BZLF1Δ200 to mediate lytic replication in EBV-infected D98/HE-R-1 cells.

Two different types of replication assays were used. The first assay quantitates the ability of transfected BZLF1 to induce replication of a cotransfected plasmid containing the EBV oriLyt element (21, 55). The second assay (the terminus assay) (51, 53) examines the ability of transfected BZLF1 to induce lytic replication of the endogenous genome. The terminus assay distinguishes between the latent virus genome, which contains only fused (episomal) termini, and the lytically replicated genome, which has both an increased number of fused termini (due to concatameric replication intermediates) (53) and unfused termini (due to the replicated linear form).

Although the BZLF1 gene product can activate expression of reporter gene constructs driven by the BZLF1 promoter in transient assays (23), we and others have found that overexpression of the BZLF1 gene product in latently infected cells does not significantly activate expression of the endogenous viral BZLF1 promoter (40, 72). Hence, essentially all of the BZLF1 protein in BZLF1-transfected D98/HE-R-1 cells is derived from the expression vector (rather than the endogenous genome), allowing us to compare the replication efficiencies of the wild-type and mutant proteins. The relative amounts of transfected BZLF1 RNA and BZLF1 RNA derived from the endogenous D98/HE-R-1 genome are shown in Fig. 8A. An RNase protection assay demonstrated that less than 1% of the total BZLF1 RNA is derived from the endogenous genome.

Since it is likely that defects in BZLF1 transcription function could inhibit its replication function, we first demonstrated that the wild-type and mutant BZLF1 proteins have similar transcriptional function in D98/HE-R-1 cells (a latently infected, EBV-positive epithelial cell line). As shown in Fig. 8A, the wild-type and mutant BZLF1 proteins activated the immediate-early BZLF1 and BRLF1 promoters in the endogenous D98/HE-R-1 viral genome with similar efficiencies. Likewise, the wild-type and mutant BZLF1 proteins produced similar activation of two early EBV promoters (BHRF1 and BHLF1) in D98/HE-R-1 cells in transient reporter gene assays (Fig. 8B). The BMRF1 promoter construct was more efficiently activated by the mutant BZLF1Δ200 protein than by wild-type BZLF1 in D98/HE-R-1 cells, presumably because the ability of the wild-type, but not mutant, BZLF1 protein to activate the BMRF1 promoter is inhibited in the presence of BMRF1 protein derived from the endogenous D98/HE-R-1 viral genome (Fig. 7A). Transfection of the BZLF1Δ200 protein consistently induced as much (or more) BMRF1 expression from the endogenous D98/HE-R-1 genome (assessed by Western blot analysis) as did the wild-type BZLF1 protein (Fig. 8C). Thus, in a variety of different transcriptional assays, the mutant BZLF1 functioned as well as (or better than) the wild-type BZLF1 in D98/HE-R-1 cells.

The results of the replication experiments are shown in Fig. 8D and E. In plasmid-based replication assays, the wild-type and mutant BZLF1 proteins induced similar levels of replication (Fig. 8D). However, in the terminus assay (which measures replication from the endogenous viral genome), the amount of EBV replication induced by the BZLF1Δ200 mutant was consistently less than that induced by wild-type BZLF1, in spite of the previous finding that early gene expression induced from the endogenous genome was similar (Fig. 8E). In control experiments, the amount of the cellular actin gene was found to be similar in each condition.

DISCUSSION

In this report we have demonstrated that the EBV replicative protein BMRF1 functions as a specific activator of an essential oriLyt promoter (BHLF1) in addition to its previously defined role as the viral polymerase processivity factor. We have also shown that the BMRF1 protein can physically and functionally interact with the EBV BZLF1 transactivator protein. Our results suggest that the BZLF1 and BMRF1 proteins each have transcriptional, as well as replicative, roles during lytic EBV infection. The direct and indirect interactions between the BZLF1 and BMRF1 proteins may thus modulate both viral transcription and viral replication.

The BZLF1 gene product plays a unique role in EBV biology in that it is the only viral protein currently known to initiate the switch from latent to lytic infection. BZLF1 transactivator function is required for expression of the early viral proteins (BALF5, BMRF1, BALF2, BSLF1, BBLF4, and BBLF2/3) directly involved in mediating oriLyt replication. Transcription factors have been shown to play an important, but auxiliary, role in mediating the replication of other viruses (16, 30, 41, 46, 68, 69). It has been suggested that BZLF1 may play a role in replication apart from its transcriptional function (21, 54).

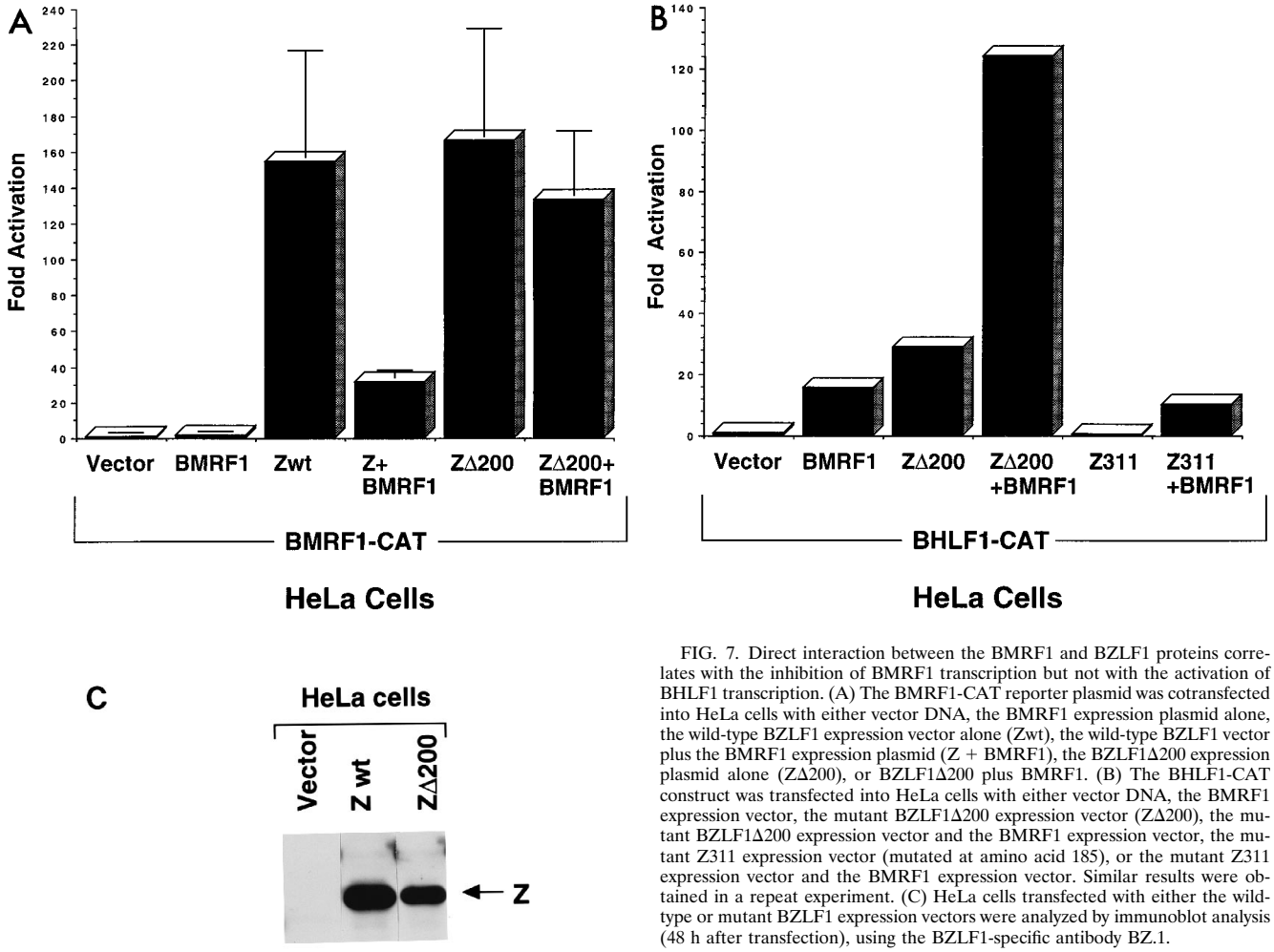

FIG. 7. Direct interaction between the BMRF1 and BZLF1 proteins correlates with the inhibition of BMRF1 transcription but not with the activation of BHLF1 transcription. (A) The BMRF1-CAT reporter plasmid was cotransfected into HeLa cells with either vector DNA, the BMRF1 expression plasmid alone, the wild-type BZLF1 expression vector alone (Zwt), the wild-type BZLF1 vector plus the BMRF1 expression plasmid (Z + BMRF1), the BZLF1Δ200 expression plasmid alone (ZΔ200), or BZLF1Δ200 plus BMRF1. (B) The BHLF1-CAT construct was transfected into HeLa cells with either vector DNA, the BMRF1 expression vector, the mutant BZLF1Δ200 expression vector (ZΔ200), the mutant BZLF1Δ200 expression vector and the BMRF1 expression vector, the mutant Z311 expression vector (mutated at amino acid 185), or the mutant Z311 expression vector and the BMRF1 expression vector. Similar results were obtained in a repeat experiment. (C) HeLa cells transfected with either the wild-type or mutant BZLF1 expression vectors were analyzed by immunoblot analysis (48 h after transfection), using the BZLF1-specific antibody BZ.1.

oriLyt contains numerous BZLF1 binding sites, and BZLF1 binding to these sites appears to be required for replication in a way that is at least partially separable from the BZLF1 transcriptional effect (54). Similar to the proposed functions of other origin-binding proteins (2, 15, 17, 18, 25, 29, 46, 48, 58, 70), BZLF1 binding to oriLyt could potentially be required for stabilization of the replication complex and/or opening up the chromatin structure around the replication initiation site.

The BMRF1 protein is known to be a component of the active viral polymerase (10, 34, 38–40, 42, 62–64) but has not previously been shown to activate lytic EBV transcription. Oguro et al. (47) reported that the BMRF1 gene product can activate expression of the early SV40 promoter. We have likewise found that the BMRF1 gene product activates the SV40 early promoter (data not shown), although the magnitude of this effect is less than what we observe with the BHLF1 promoter. However, two other groups (6, 67) have failed to find that BMRF1 is a transcriptional activator. Most recently, Chen et al. created a fusion protein containing the yeast GAL4 DNA binding domain linked to either the carboxy-terminal 100 amino acids of BMRF1 (a region rich in prolines) or the entire BMRF1 protein (6). These GAL4-BMRF1 fusion proteins did not activate transcription of a reporter gene construct containing GAL4 DNA binding motifs (6). It is possible that these negative results reflect improper folding of the GAL4-BMRF1 fusion proteins. Alternatively, the BMRF1 protein, rather than containing a conventional transactivator domain, may mediate its effects indirectly by regulating the activity of one or more cellular transcription factors. Regardless of the mechanism, our finding that the BMRF1 gene product activates oriLyt transcription was unexpected and suggests that the role of BMRF1 in oriLyt replication is much more complex than previously appreciated.

The physical interaction between BMRF1 and BZLF1 requires the first 45 amino acids of BMRF1 and the bZip domain (specifically amino acid 200) of BZLF1. The amino-terminal 45 amino acids in BMRF1 do not contain a bZip dimerization domain or other known sequence motifs and are not required for DNA binding activity or polymerase processivity function (39). Although defining the precise BMRF1 sequences required for interaction with BZLF1 will require further study, it should be possible to construct BMRF1 mutants which retain polymerase processivity function but which have lost the ability to interact directly with BZLF1.

We have shown here that the BMRF1 and BZLF1 gene products directly and indirectly interact to regulate EBV transcription and possibly EBV replication as well. In regard to transcriptional effects, our data suggest that one result of the direct interaction between the BZLF1 and BMRF1 proteins is negative regulation of the BMRF1 promoter. BZLF1-dependent transcription of the BMRF1 promoter is clearly reduced in the presence of the BMRF1 protein, suggesting a mecha-

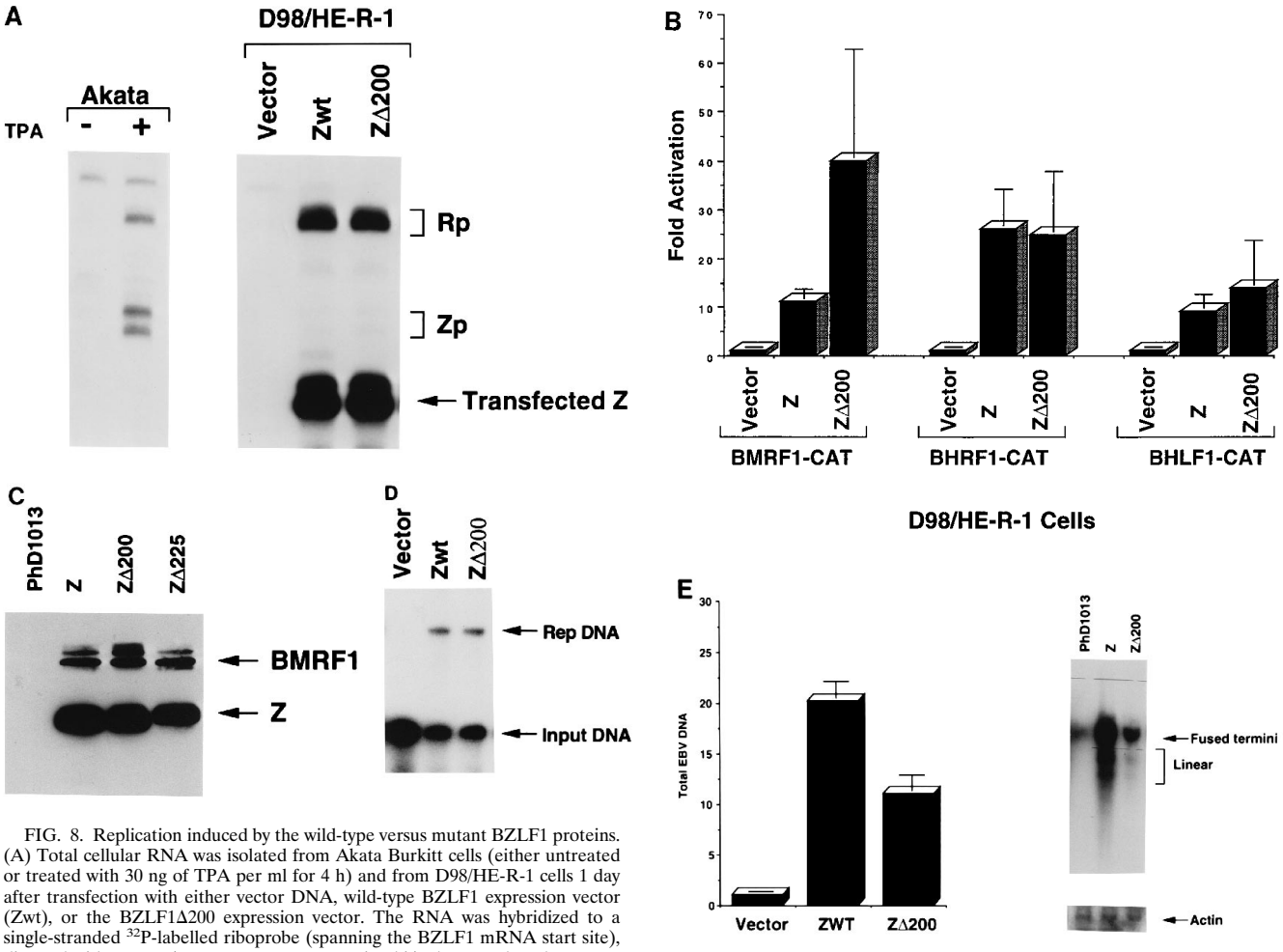

FIG. 8. Replication induced by the wild-type versus mutant BZLF1 proteins. (A) Total cellular RNA was isolated from Akata Burkitt cells (either untreated or treated with 30 ng of TPA per ml for 4 h) and from D98/HE-R-1 cells 1 day after transfection with either vector DNA, wild-type BZLF1 expression vector (Zwt), or the BZLF1Δ200 expression vector. The RNA was hybridized to a single-stranded ^{32}P-labelled riboprobe (spanning the BZLF1 mRNA start site), digested with RNase (Promega RNase protection kit), denatured, and run on a 6% sequencing gel. Hybridization of this probe to Zp (the promoter driving BZLF1 transcription)- and Rp (the promoter driving BRLF1 transcription)-derived messages results in protected fragments of 115 and 148 bp, respectively. The transfected BZLF1 vector yields an 80-bp protected fragment. (B) Five micrograms of the BMRF1-CAT, BHRF1-CAT, or BHLF1-CAT plasmid was transfected into D98/HE-R-1 cells with either 5 μg of pHD1013 vector DNA, 5 μg of the wild-type BZLF1 expression vector, or 5 μg of the mutant BZLF1Δ200 expression vector. The fold activation induced by each BZLF1 construct is shown. (C) D98/HE-R-1 cells were transfected with 5 μg of wild-type BZLF1, BZLF1Δ200, or BZLF1Δ225. The level of BMRF1 expression induced from the endogenous genome by each BZLF1 vector was measured by immunoblot. (D) D98/HE-R-1 cells were transfected with 5 μg of the oriLytΔKpn plasmid–5 μg of pHD1013 vector DNA, 5 μg of the oriLytΔKpn plasmid–5 μg of the wild-type BZLF1 expression vector, or 5 μg of the oriLytΔKpn plasmid–5 μg of the BZLF1Δ200 expression vector. DNA was analyzed 3 days after transfection by cutting with the restriction enzymes *Bam*HI and *Dpn*I and probing with a ^{32}P-labelled riboprobe spanning the EBV oriLyt sequences. The positions of the *Dpn*I-cut (Input) and *Dpn*I-resistant (replicated [Rep]) oriLytΔKpnI plasmid are shown. The wild-type and mutant BZLF1 proteins had similar replication efficiencies in two separate experiments with this plasmid-based replication assay. (E) D98/HE-R-1 cells were transfected with 5 μg of pHD1013 vector DNA, 5 μg of the wild-type BZLF1 expression vector, or 5 μg of the BZLF1Δ200 expression vector. DNA was analyzed 3 days after transfection by cutting with the restriction enzyme *Bam*HI and probing with a ^{32}P-labelled riboprobe spanning the EBV sequences in the 1.9-kb *Xho*I (termini) fragment. As previously described (51, 53), lytic replication results in increased amounts of fused viral termini (resulting from the replicated concatameric intermediates) as well as unfused termini (seen only in the replicated linear form). A representative experiment is shown on the right. The blot was reprobed with a ^{32}P-labelled probe species for the cellular actin gene, which confirmed that equal amounts of DNA were present in each condition. Quantitation of the results (from three experiments) is shown on the left.

nism whereby the BMRF1 gene product can negatively regulate its own expression. In contrast, a BZLF1 protein mutated at residue 200 (which is unable to directly interact with the BMRF1 protein) activated BMRF1 transcription equally well in the presence or absence of the BMRF1 protein. Thus, the ability of BMRF1 to inhibit its own transcription seems to require its direct interaction with BZLF1.

Our DNA binding studies suggest that the BZLF1-BMRF1 complex may be incapable of binding to ZRE sites, potentially providing a mechanism by which expression of the BMRF1 gene product inhibits BMRF1 promoter activity. Nevertheless, BZLF1-induced transcription of at least two other early EBV promoters (BHLF1 and BHRF1) continued to be active (or in the case of the BHLF1 promoter, actually increased) in the presence of the BMRF1 gene product, even though BZLF1 binding to these promoters was also found to be decreased. It is possible that the BMRF1-induced reduction in BZLF1 binding activity only has functional significance in those promoters (such as the BMRF1 promoter) which have relatively few upstream ZREs. We have previously shown that deletion of either of the two upstream ZRE sites in the BMRF1 promoter drastically reduces BZLF1-induced transcription (50), suggesting that both ZRE sites need to be simultaneously bound by BZLF1 for activation of this promoter. In contrast, promoters containing a larger number of upstream ZREs may be active when only a portion of the ZRE sites are occupied by BZLF1. The divergent oriLyt promoters (BHRF1 and BHLF1) share seven ZREs (55).

In this study, we have demonstrated that BMRF1, like BZLF1, has a dual role as both a transcriptional activator and an essential replicative protein. The linkage between the transcriptional activation of oriLyt and the assembly of the EBV replication machinery over oriLyt is complex and probably

differs greatly from the activation of alphaherpesvirus replication origins. An unresolved issue is whether the BZLF1-BMRF1 interaction plays a role in oriLyt replication apart from its role in transcriptional regulation. Mutational analysis of the BZLF1 and BMRF1 genes within the context of the intact viral genome should help to clarify this issue.

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