

## BZLF1 Activation of the Methylated Form of the *BRLF1* Immediate-Early Promoter Is Regulated by BZLF1 Residue 186

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**The Epstein-Barr virus (EBV) genome is highly methylated in latently infected cells. We recently reported that the EBV immediate-early (IE) protein BZLF1 (Z) preferentially binds to and activates transcription from the methylated form of the *BRLF1* IE gene promoter (Rp). We now report that serine residue 186 in the Z DNA-binding domain plays an important role in the ability of Z to bind to and activate methylated Rp. A Z mutant containing an alanine residue at position 186 [Z(S186A)] was significantly defective in binding to methylated, as well as unmethylated, ZREs (Z-responsive elements) in Rp and was unable to activate lytic EBV gene transcription from the methylated or demethylated form of the viral genome. A Z mutant containing threonine at residue 186 [Z(S186T)] bound only to the methylated form of the ZRE-2 site in Rp and induced lytic EBV gene transcription from the methylated, but not demethylated, form of the viral genome. The defect in both of these mutants was primarily due to an inability to activate the Rp in the context of the viral genome. Finally, a Z mutant containing an aspartic acid at position 186 [Z(S186D)] did not bind to either the consensus AP-1 site or to the methylated or unmethylated Rp ZRE-2 site and did not induce lytic gene transcription. These results indicate that replacement of serine with threonine at residue 186 in the Z DNA-binding domain differentially affects its ability to reactivate the unmethylated, versus methylated, viral genome.**

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is associated with B-cell lymphomas, nasopharyngeal carcinoma, gastric carcinomas, and other malignancies (44, 56). EBV primarily infects two cell types, epithelial cells and B cells (44, 50). In normal oral epithelial cells, the virus exists in a lytic state (34, 37, 44, 51), whereas EBV infection of B cells usually results in a latent type of infection (34, 44). However, in a small percentage of B cells, the virus periodically reactivates and replicates in the lytic manner. This reactivation is initiated by expression of the two immediate-early genes, *BZLF1* and *BRLF1* (8, 13, 15, 33, 46, 47, 51, 55), which encode the transcriptional activators Z and R, respectively.

Z is a bZip protein homologous to the cellular proteins c-Fos and c-Jun and binds directly to AP-1 and AP-1-like motifs (known as Z-responsive elements [ZREs]) present in many lytic viral promoters (12, 19, 21). Z expression results in activation of lytic EBV gene expression in essentially all EBV-positive cell lines. Z initially activates expression of the *BRLF1* immediate-early promoter (Rp) by binding directly to three somewhat atypical ZRE sites in Rp, referred to as ZRE-1, ZRE-2, and the recently identified ZRE-3 (6, 41). Z and R together then activate the expression of the entire complement of lytic EBV genes, thus inducing lytic EBV infection.

The EBV genome in latently infected cells is extensively

methylated (18, 39). Methylation likely suppresses the expression of lytic viral genes, thus helping to maintain latent infection. In addition, methylation of viral promoters driving LMP-1 and EBNA-2 expression is associated with the most stringent form of viral latency (type I) (2, 18, 28, 30, 38, 52), in which LMP-1 and EBNA-2 are not expressed. It has been proposed that DNA methylation causes transcriptional repression by multiple different mechanisms, including modification of the histone acetylation state and prevention of transcription factor binding to DNA (5, 7, 9, 10, 14, 16, 26, 29, 31, 32, 35, 36, 40, 48, 49, 54). However, we have recently shown that Z unexpectedly preferentially binds to and activates transcription from the methylated form of Rp (6). In addition, Z preferentially activates lytic EBV gene transcription from the methylated form of the viral genome in latently infected cells (6).

Two of the three known ZRE sites in Rp (ZRE-2 and ZRE-3) contain CpG motifs and hence could be methylated in latently infected cells. We recently showed that Z preferentially binds to the methylated form of the Rp ZRE-2 site (TGAGCGA) and binds only to the methylated form of the Rp ZRE-3 site (TTCGCGA) (6). The Rp ZRE-1 site (TGAGCCA) cannot be methylated. The crystal structure of a Fos-Jun heterodimer bound to a consensus AP-1 site indicates that the residues in the basic DNA-binding domain of c-Fos and c-Jun which make direct contact with DNA are analogous to Z residues 182, 185, 186, 189, and 190 and that Z residue 186 would directly contact the methylated cytosine in the Rp ZRE-2 motif (TGAGCGA) (25). Interestingly, the residues that directly contact DNA are identical in the c-Fos, c-Jun, and Z proteins, with the exception of the Z serine residue at position 186,

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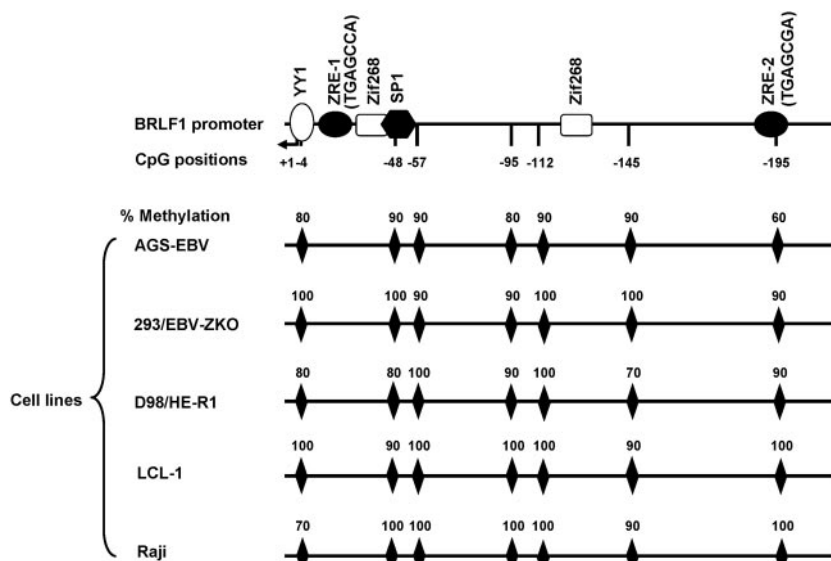


FIG. 1. The *BRLF1* IE promoter (Rp) is highly methylated in latently infected cells. The methylation status of CpG sites in Rp in 5 EBV-positive cell lines (indicated on the left) was determined by sodium bisulfite treatment followed by PCR and sequencing. The position of each CpG site, represented by a diamond, with respect to the R transcription start site (+1) is indicated at the top. The percentage of clones with methylated CpG at each position is indicated on top of the diamond representing that CpG for each cell line analyzed; 10 clones for each cell were sequenced.

reaction mixture and incubated at 4°C for 10 min before the addition of labeled probe (10,000 cpm). For competition EMSAs, a 10-fold, 50-fold, or 250-fold excess of cold competitor DNA was also added to the reaction mixture prior to the addition of labeled probe. The reaction mixtures were incubated further for 30 min at 4°C after the addition of labeled probe. The reaction mixtures were then loaded onto a 4% polyacrylamide gel and run in 0.5× Tris-borate-EDTA buffer at room temperature. The binding was quantitated using a phosphorimager (Molecular Dynamics) or NIH Image (National Institutes of Health).

**Expression and purification of GST-Z protein.** Saturated bacterial culture (250  $\mu$ l) was diluted in 10 ml of LB plus ampicillin and incubated at 37°C to an  $A_{600}$  of 0.6. Protein expression was induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and incubation at 30°C for 2 h. Bacteria were pelleted, resuspended in 500  $\mu$ l of 1× phosphate-buffered saline (PBS), and then sonicated on ice. Fifty microliters of 1× PBS plus 10% Triton X-100 was added, and the mixture was then centrifuged for 10 min at 13,000 rpm at 4°C. A mixture of 100  $\mu$ l of extract, 50  $\mu$ l of 50% slurry of glutathione-agarose beads, and 500  $\mu$ l of buffer C (50 mM Tris [pH 7.5], 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1% NP-40, 10% glycerol, 1× Complete protease inhibitors [Roche]) was incubated at 4°C for 2 h on an orbital mixer (Adams Nutator). Beads were pelleted, and the supernatant was removed, resuspended in 500  $\mu$ l of buffer C, and then incubated for 5 min at 4°C on a nutator. Beads were repelleted and washed three times with buffer C. The bead pellet was then resuspended in 100  $\mu$ l of 50 mM Tris (pH 7.5) and 20 mM reduced glutathione and incubated on a nutator for 5 min at room temperature. Beads were pelleted for 1 min at 13,000 rpm, and the supernatant was removed to a microcentrifuge tube. This step was repeated. The combined supernatant was dialyzed with a 3,500-molecular weight cutoff Slide-A-Lyzer mini dialysis unit (Pierce) against 50 mM Tris (pH 8.0) and 10% glycerol for 2 h at 4°C.

**CAT assays.** CAT vectors were mock methylated or methylated in vitro using SssI CpG methylase (New England Biolabs), and methylation was confirmed by complete resistance to HpaII cutting. Methylated plasmids were subsequently cleaned by phenol-chloroform extraction and ethanol precipitation prior to transfection into cells. Cell extracts were prepared 2 days posttransfection and incubated at 37°C with [<sup>14</sup>C]chloramphenicol in the presence of acetyl coenzyme A. The percent acetylation was determined by thin-layer chromatography followed by phosphorimager screening (Molecular Dynamics).

**DNA transfection.** DNA (1  $\mu$ g of the CAT construct plasmids and 4  $\mu$ g of the protein expression plasmids) was transfected into DG75 cells resuspended in RPMI 1640 medium (Gibco BRL) by electroporation with a Zapper electroporation unit (Medical Electronics Shop, University of Wisconsin) at 1,500 V. Lipofectamine 2000 (Gibco BRL) was used to transfect DNA into EBV-positive 293 cells (or 293T cells) according to the manufacturer's instructions. A DNA/Lipofectamine 2000 ratio of 1:2 (mass/volume) was used.

**Viral genome methylation influence on disruption of viral latency.** 293 cells latently infected with a Z-deleted EBV genome (293/EBV-ZKO) were treated for 5 days in the presence or absence of 5-aza-2'-deoxycytidine (5-aza-2'-dc, 1  $\mu$ M; Sigma). 5-Aza-2'-dc was removed during the lipofection procedure and then replaced after 6 h. Expression of lytic EBV proteins was quantitated by immunoblot analysis 2 days after transfection.

**Immunoblot analysis.** Cell extracts harvested as described for the CAT assays or cell extracts made using a buffer containing 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris (pH 8.0), 50 mM sodium fluoride, 50 mM  $\beta$ -glycerophosphate, 2 mM sodium vanadate, and 1× Complete protease inhibitor (Roche) were electrophoresed on a 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis denaturing gel. The proteins were transferred onto a nitrocellulose membrane (Protran), blocked in 1× PBS-5% milk-0.1% Tween 20, and incubated with primary antibody for 1 h at room temperature using anti-EBV BZLF1 (Z, 1:100; Argene), anti-BRLF1 (R, 1:100; Argene), anti-EBV EA-D (BMRF1, 1:100; Capricorn), or anti- $\beta$ -actin (1:5,000; Sigma) antibody. The membrane was washed with 1× PBS-0.1% Tween 20, incubated with secondary antibody (goat anti-mouse  $\kappa$ -horseradish peroxidase, 1:10,000; Southern Biotechnology) at room temperature for 1 h, and washed. The results were visualized with the ECL chemiluminescent kit (Amersham) according to the manufacturer's instructions.

## RESULTS

**The *BRLF1* promoter is highly methylated in cells with latent EBV infection.** To determine the extent of *BRLF1* promoter (Rp) methylation in latently infected cells, we examined its methylation status in five different EBV-infected cell lines using sodium bisulfite/PCR analysis. The cell lines examined included two epithelial cell lines (EBV-positive gastric carcinoma [AGS] cells and EBV-positive 293 cells), a fusion line created with HeLa cells and the Burkitt lymphoma P3HR1 line (D98/HE-R1 cells), a lymphoblastoid cell line (LCL-1), and a Burkitt lymphoma line (Raji). Following bisulfite treatment of the DNA, the *BRLF1* promoter sequence was cloned and 10 independent clones were sequenced for each cell line.

The Rp sequence was highly methylated in each of the cell lines analyzed, whether epithelial or B-cell derived (Fig. 1). The CpG motif contained within the Rp ZRE-2 site (TGAG



CGA) was almost completely methylated in every cell line examined, with the exception of the AGS-EBV cells, in which this site was methylated in 60% of the clones sequenced. Interestingly, the AGS-EBV cells have a small amount of detectable constitutive *BRLF1* gene expression, whereas the other cell lines are essentially completely latent (data not shown), suggesting that activation of Rp by cellular transcription factors may preferentially occur when the promoter is less methylated. The methylation status of the Rp ZRE-3 site was also examined in EBV-positive 293 cells; the ZRE-3 site was fully methylated in this cell line (data not shown). As Z efficiently induces *BRLF1* expression in 293 cells (as well as the other lines examined), these results indicate that Z activates *BRLF1* transcription in latently infected cells even when the Rp ZRE-2 and ZRE-3 sites are methylated.

**Z residue 186 regulates binding to the methylated and unmethylated forms of the Rp ZREs.** We recently showed that wild-type Z preferentially binds to and activates transcription from the methylated form of Rp (6). To determine whether Z residue 186 regulates binding to methylated ZRE sites, we performed EMSAs to compare the ability of in vitro-translated wild-type Z, Z(S186A), and Z(S186T) to bind to labeled oligonucleotide probes containing the ZRE-1, ZRE-2, or ZRE-3 sites in Rp or the consensus AP-1 site from the *BMRF1* early viral promoter. The ZRE-2 and ZRE-3 probes were synthesized so that they contained either unmethylated or methylated cytosine at the CpG motifs.

The in vitro-translated Z(S186A) mutant was impaired in comparison to wild-type Z for binding to the ZRE-1 site (Fig. 2A), the unmethylated as well as methylated forms of the ZRE-2 site (Fig. 2A), and the methylated ZRE-3 site (Fig. 2B), although as previously reported (1, 24), it bound at least as well as wild-type Z to the consensus AP-1 site (Fig. 2B). The in vitro-translated Z(S186T) mutant was partially compromised for binding to the Rp ZRE-1 site (Fig. 2A), the consensus AP-1 site (Fig. 2B), and the methylated Rp ZRE-3 site (Fig. 2B). Most interestingly, however, while binding of the Z(S186T) mutant to the unmethylated form of the Rp ZRE-2 site was severely impaired (only 2% binding compared to the wild-type protein), it bound almost as well as the wild-type Z to the methylated form of Rp ZRE-2 (Fig. 2A). Competition analysis using the methylated versus unmethylated forms of ZRE-2 as cold competitor DNA confirmed that the Z(S186T) mutant bound almost exclusively to the methylated form of ZRE-2 (Fig. 3). These results indicate that Z residue 186 regulates binding to both methylated and unmethylated ZRE sites in the *BRLF1* promoter.

EMSA was also performed using whole-cell extracts of DG75 cells transfected with expression vectors for wild-type Z, Z(S186A), or Z(S186T) (Fig. 4A). When transfected into DG75 cells, the Z(S186A) mutant was even more impaired in its binding (relative to wild-type Z) than when expressed as an in vitro-translated protein. In DG75 cells, Z(S186A) bound extremely poorly to the unmethylated and methylated forms of all three Rp ZRE sites (Fig. 4A), although binding to the AP-1 site was observed (data not shown). In the case of the Z(S186T) mutant, although its expression level was much higher than wild-type Z in DG75 cells (Fig. 4B), Z(S186T) binding to the unmethylated form of ZRE-2 was almost undetectable, while this mutant clearly bound to the methylated

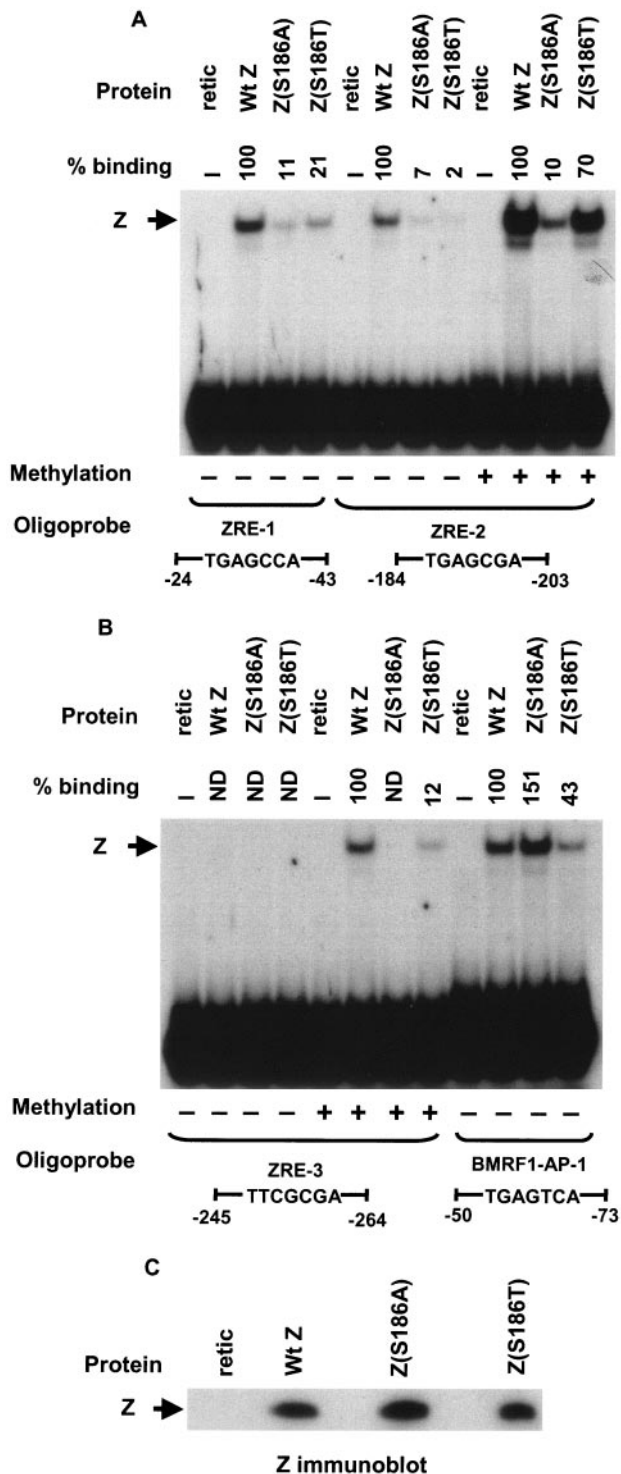


FIG. 2. Mutation of Z residue 186 affects binding to the three Rp ZRE sites. Binding of in vitro-translated wild-type Z (Wt Z), Z(S186A), and Z(S186T) to oligonucleotide probes containing the Rp ZRE-1 or ZRE-2 sites (A) or the Rp ZRE-3 site or the AP-1 site in the early EBV *BMRF1* gene promoter (BMRF1-AP-1) (B) was compared. The sequences and methylation status of each probe are indicated. Rabbit reticulocyte lysate (retic) was used as a negative control for each probe. The binding abilities of mutant Z proteins to each probe were measured relative to wild-type Z, which was set at 100% for each probe. ND, nondetectable binding; +, present; -, absent. (C) The expression level of each in vitro-translated Z protein was analyzed by immunoblotting.

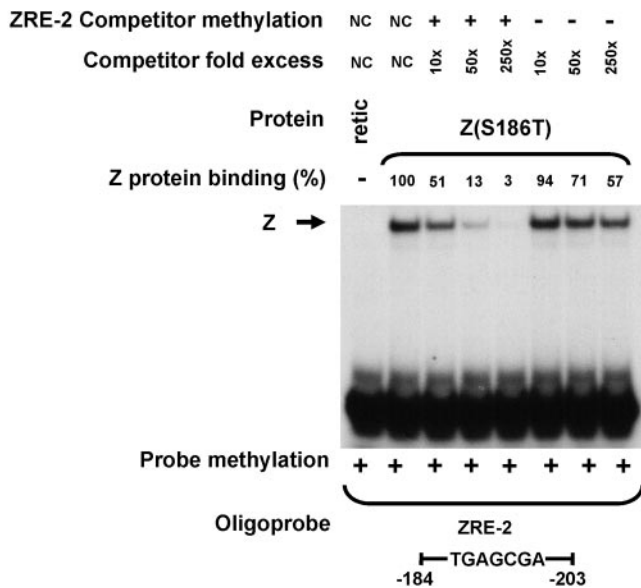


FIG. 3. Z(S186T) binds almost exclusively to the methylated form of the Rp ZRE-2 site. Increasing amounts of methylated versus unmethylated ZRE-2 cold competitor oligoprobe were used to compete binding of Z(S186T) to a methylated  $^{32}$ P-labeled ZRE-2 probe. Z binding complexes were quantitated, and the percent Z binding (relative to the amount in the absence of specific competitor DNA, set at 100%) is indicated. +, present; -, absent; retic, rabbit reticulocyte lysate; NC, no competitor.

form of this site (Fig. 4A). When the expression levels of wild-type Z and Z(S186T) in DG75 cells were normalized, the two proteins bound to methylated Rp ZRE-2 with similar efficiency (Fig. 4C). The partial defects of in vitro-translated Z(S186T) binding to the ZRE-1 (Fig. 2A) and ZRE-3 (Fig. 2B) sites were not apparent when this mutant was expressed in DG75 cells at a high level (Fig. 4A).

**Z residue 186 plays a critical role in activating methylated Rp.** To determine whether Z serine residue 186 is required for activation of methylated Rp, we compared the ability of wild-type Z, the Z(S186A) mutant, and the Z(S186T) mutant to activate the methylated or unmethylated Rp-CAT construct in DG75 (Fig. 5A) or 293T (Fig. 5B) cells. Prior to transfection, the CAT vectors were methylated (or mock methylated) with SssI. Methylation was confirmed by resistance to HpaII cutting (data not shown). The mock-methylated or methylated Rp-CAT construct was cotransfected with a control vector or vectors expressing either wild-type Z or one of the Z mutants.

We have previously shown that all three Rp ZRE sites are important for activation of the unmethylated *BRLF1* promoter by wild-type Z, whereas only the ZRE-2 and ZRE-3 sites (which can be methylated) are required for activation of the methylated promoter (6). Consistent with its inability to bind to either the methylated ZRE-2 site or the methylated ZRE-3 site in vivo (Fig. 4A), the Z(S186A) mutant was unable to activate the methylated form of Rp-CAT in either DG75 cells (Fig. 5A) or 293T cells (Fig. 5B). Somewhat surprisingly, given its poor binding in vivo to each of the unmethylated Rp ZRE sites (Fig. 4A), the Z(S186A) mutant could activate the unmethylated form of Rp-CAT in DG75 cells (Fig. 5A). This result suggests that Z(S186A) may activate the unmethylated

*BRLF1* promoter in plasmid-based reporter gene assays through a mechanism not involving direct DNA binding.

The Z(S186T) mutant, which binds to the methylated but not unmethylated form of ZRE-2 and is partially defective for binding to the ZRE-1 and ZRE-3 sites, was defective for activation of the unmethylated form of Rp-CAT (in DG75 cells) (Fig. 5A) and partially defective for activation of the methylated form of Rp-CAT in DG75 (Fig. 5A) and 293T cells (Fig. 5B). These results suggest that Z residue 186 is important for activating methylated Rp in vivo.

**Z residue 186 regulates the ability of Z to induce lytic EBV gene transcription in latently infected cells with a methylated viral genome.** To determine whether the methylation status of the latent EBV genome differentially affects the ability of wild-type Z, Z(S186A), and Z(S186T) to disrupt viral latency, 293 cells latently infected with *BZLF1*-deleted EBV (293/EBV-ZKO) (20) were treated with the DNA demethylating agent 5-aza-2'-dc prior to transfection with a control vector or expression vectors for wild-type Z, Z(S186A), or Z(S186T). The sensitivity of the Rp region to cutting by the methylation-sensitive HpaII restriction enzyme was examined by Southern blot analysis before and after 5-aza-2'-dc treatment; the results confirmed that the Rp region of the EBV genome was efficiently demethylated following treatment (6). Cellular extracts were analyzed 2 days posttransfection for the expression of Z, R, BMRF1 (an early viral protein), or  $\beta$ -actin by immunoblotting.

As previously reported (6), prior demethylation of the viral genome significantly reduced, but did not completely abolish, the ability of wild-type Z to activate transcription of either the immediate-early (IE) *BRLF1* gene or the early *BMRF1* gene (Fig. 6A). In contrast, the Z(S186T) mutant, which binds only to the methylated form of the Rp ZRE-2 motif, only induced lytic EBV gene transcription from the methylated viral genome (Fig. 6A). Furthermore, the defect in the Z(S186T) mutant was limited to its inability to activate the Rp, since the defect in activating *BMRF1* transcription from the unmethylated viral genome could be rescued by cotransfection with an R expression vector (Fig. 6B). The Z(S186A) mutant, as expected, could not induce lytic gene transcription from either the methylated or demethylated viral genome (Fig. 7), although in both cases this defect was rescued by cotransfection with the R expression vector (Fig. 7 and data not shown). These results suggest that the serine residue at position 186 in Z plays an important role in inducing lytic viral infection from both the unmethylated and methylated viral genomes.

**Phosphorylation of the serine residue at position 186 is not required for the ability of Z to bind preferentially to the methylated Rp ZRE-2.** Serine residue 186 is phosphorylated by protein kinase C (PKC) in vitro (4) and thus could potentially be phosphorylated in vivo (as well as in reticulocyte extracts). Previous results by another group showed that in vitro phosphorylation of Z protein by PKC decreased its binding to unmethylated ZRE sites (4). To examine whether Z phosphorylated at serine 186 behaves differently than the unphosphorylated protein in regard to its binding to methylated versus unmethylated ZRE motifs, we performed several different experiments. First, we compared the binding of a bacterially expressed and purified protein containing Z linked in frame to GST (GST-Z) to the methylated versus unmethylated forms of

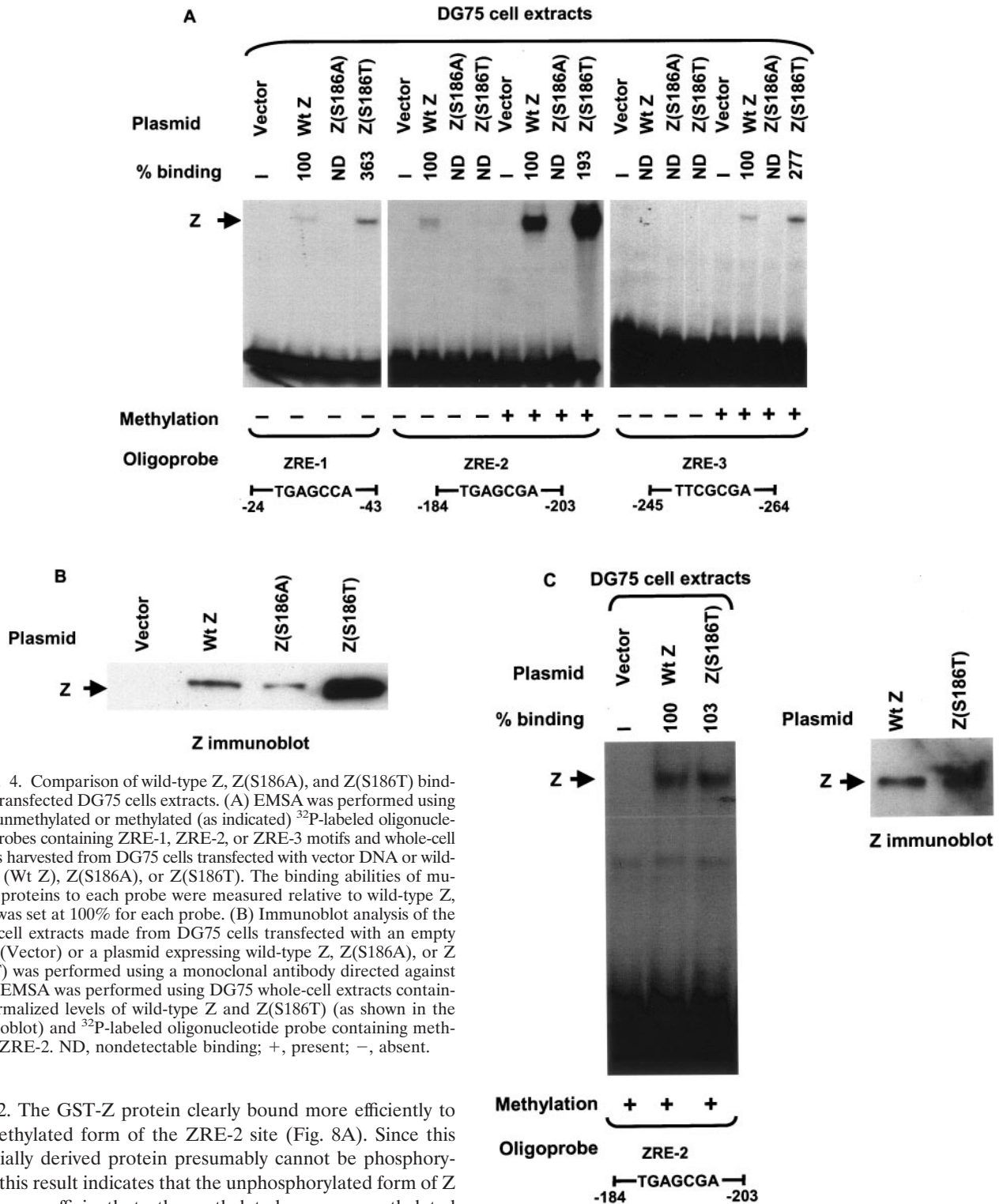


FIG. 4. Comparison of wild-type Z, Z(S186A), and Z(S186T) binding in transfected DG75 cells extracts. (A) EMSA was performed using either unmethylated or methylated (as indicated) <sup>32</sup>P-labeled oligonucleotide probes containing ZRE-1, ZRE-2, or ZRE-3 motifs and whole-cell extracts harvested from DG75 cells transfected with vector DNA or wild-type Z (Wt Z), Z(S186A), or Z(S186T). The binding abilities of mutant Z proteins to each probe were measured relative to wild-type Z, which was set at 100% for each probe. (B) Immunoblot analysis of the whole-cell extracts made from DG75 cells transfected with an empty vector (Vector) or a plasmid expressing wild-type Z, Z(S186A), or Z(S186T) was performed using a monoclonal antibody directed against Z. (C) EMSA was performed using DG75 whole-cell extracts containing normalized levels of wild-type Z and Z(S186T) (as shown in the immunoblot) and <sup>32</sup>P-labeled oligonucleotide probe containing methylated ZRE-2. ND, nondetectable binding; +, present; -, absent.

ZRE-2. The GST-Z protein clearly bound more efficiently to the methylated form of the ZRE-2 site (Fig. 8A). Since this bacterially derived protein presumably cannot be phosphorylated, this result indicates that the unphosphorylated form of Z binds more efficiently to the methylated versus unmethylated form of the Rp ZRE-2 site.

Second, we constructed a Z mutant, Z(S186D), in which the serine residue at position 186 was switched to an aspartic acid residue to mimic the phosphorylated form of Z. Interestingly, the in vitro-translated Z(S186D) mutant did not bind detectably to either the AP-1 site in the *BMRFI* promoter or the methylated or unmethylated forms of the Rp ZRE-2 site

(Fig. 8B). Consistent with its inability to bind to DNA, the Z(S186D) mutant was also unable to induce R and *BMRFI* expression in 293/EBV-ZKO cells (Fig. 8C). This suggests that phosphorylation of Z at position 186 is certainly not required for its ability to bind preferentially to the methylated Rp ZRE-2 site and may instead globally inhibit Z binding to multiple different motifs.



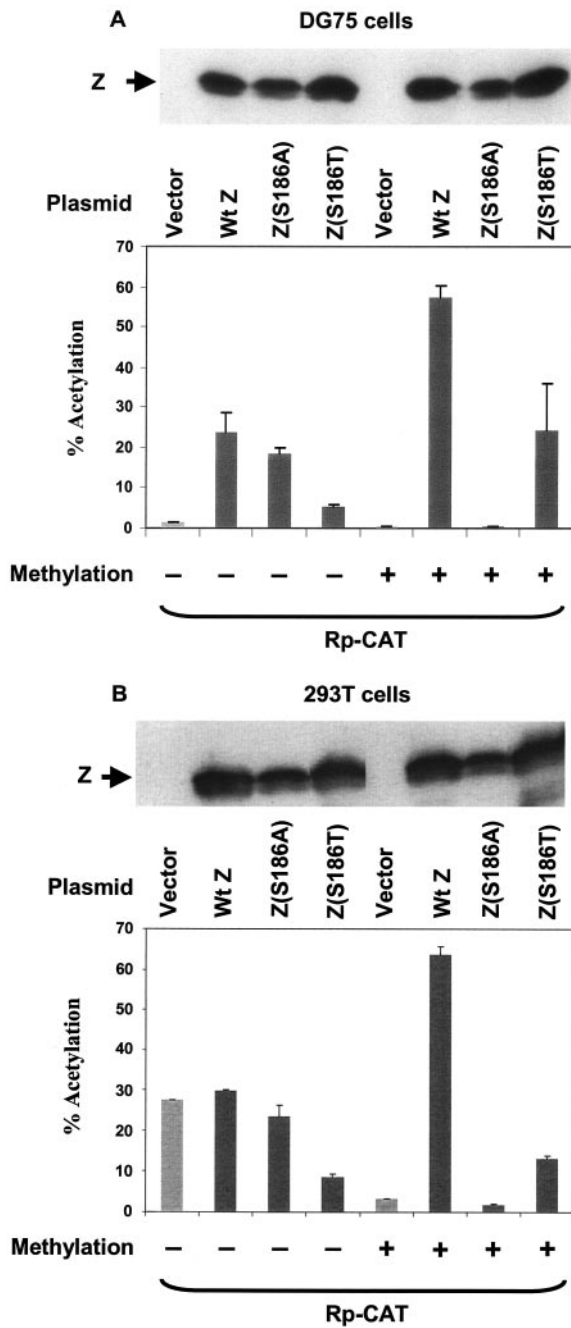


FIG. 5. Mutation of Z residue 186 affects its ability to activate methylated Rp in CAT reporter gene assays. A vector containing the *CAT* gene linked to the *BRLF1* promoter (Rp-CAT), either methylated or mock-methylated in vitro, was transfected into DG75 cells (A) or 293T cells (B) in combination with either empty vector (vector) or vector expressing wild-type Z (Wt Z), Z(S186A), or Z(S186T), as indicated. CAT activity was measured as the percentage of acetylation. The methylation status of each promoter construct is indicated. +, present; -, absent.

**DISCUSSION**

DNA methylation plays an important role in regulation of both viral and cellular gene expression. Most models regarding the effect of DNA methylation on gene transcription propose that gene methylation leads to gene silencing either by pre-

venting the binding of transcription factors to promoters or by imposing a restrictive chromatin state. Nevertheless, we recently demonstrated that the EBV Z protein, which mediates the switch between latent and lytic EBV infection, actually requires its immediate downstream target promoter, Rp, to be methylated to activate this promoter efficiently (6). We iden-

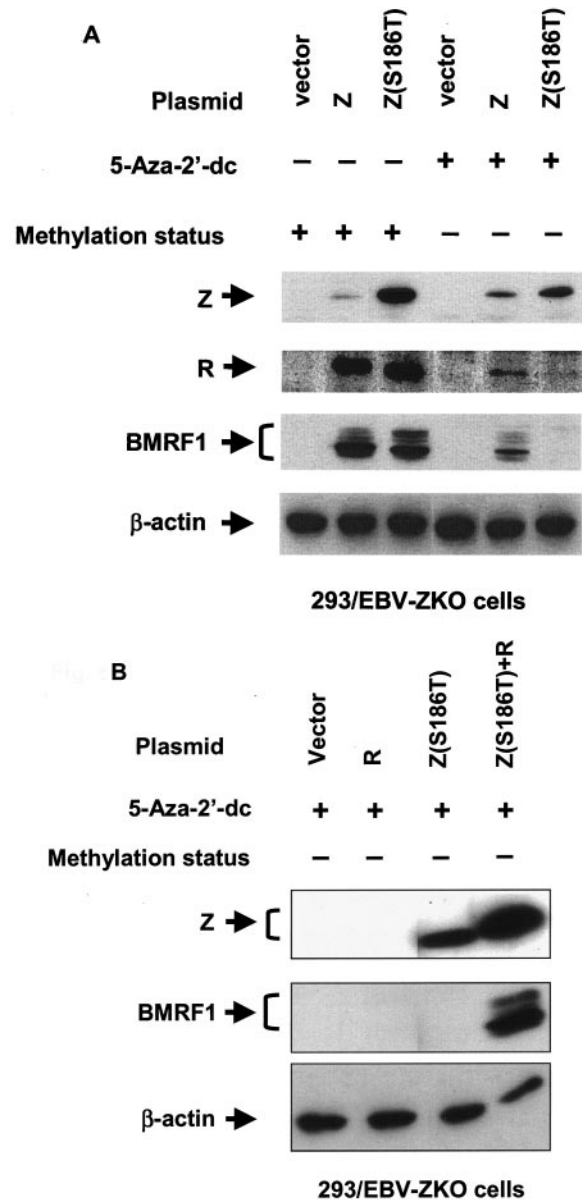


FIG. 6. Changing Z residue 186 from serine to threonine affects the ability of Z to induce lytic EBV infection in a methylation-dependent manner. (A) 293/EBV-ZKO cells (20) were treated with 5-aza-2'-dc or not treated, as shown, prior to transfection with either empty vector (Vector) or a vector expressing wild-type Z (Wt Z) or Z(S186T). The methylation status of the EBV genome after the 5-aza-2'-dc treatment is indicated. Immunoblots were performed on cell extracts 2 days after transfection with monoclonal antibody directed against Z, BMRF1 (an early lytic EBV protein), R, or β-actin, as indicated. (B) 293/EBV-ZKO cells pretreated with 5-aza-2'-dc were transfected with empty vector or a vector expressing R, Z(S186T), or both R and Z(S186T). The methylation status of the EBV genome is indicated. Immunoblotting was performed for Z, BMRF1, or β-actin, as indicated. +, present; -, absent.

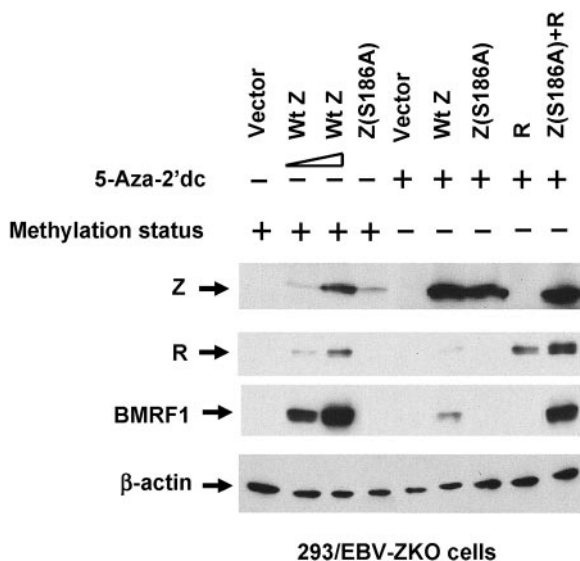


FIG. 7. The Z(S186A) mutant is defective for inducing lytic EBV gene expression regardless of the methylation state of the EBV genome. 293/EBV-ZKO cells (untreated or pretreated with 5-aza-2'-dc, as indicated) were transfected with empty vector (vector) or a vector expressing wild-type Z (Wt Z, 0.5 μg in lane 2 and 1 μg in lane 3), Z(S186A), R, or both Z(S186A) and R, and immunoblots were performed for Z, R, BMRF1, or β-actin, as indicated. The methylation status of the EBV genome is indicated. +, present; -, absent.

tified two ZRE sites in Rp (ZRE-2 and ZRE-3) that can be methylated through a CpG motif and showed that Z binding to these atypical ZRE sites, as well as Z activation of Rp, was surprisingly enhanced by methylation. Most importantly, Z induced lytic EBV gene transcription in latently infected cells more efficiently when the viral genome was methylated than when it was demethylated. In contrast, the ability of the R IE protein to disrupt viral latency in 293 cells latently infected with *BRLF1*-deleted EBV (293/EBV-RKO) cells was enhanced by prior demethylation of the viral genome (6).

In this study, we have examined the effect of serine residue 186 in the Z DNA-binding domain on the ability of Z to efficiently bind to and activate the methylated versus unmethylated forms of Rp. As the crystal structure of a Fos-Jun heterodimer binding to a consensus AP-1 site suggested that Z residue 186 would directly contact the methylated cytosine in the Rp ZRE-2 motif (TGAGCGA) (25), we hypothesized that this residue might be important for mediating Z activation of methylated Rp. Our results indicate that a Z mutant in which residue 186 is switched from a serine to threonine, Z(S186T), binds to the methylated form but not to the unmethylated form of the Rp ZRE-2 site in vitro and induces lytic EBV transcription from the methylated viral genome but not from the demethylated viral genome in vivo. The inability of this mutant to activate early lytic EBV gene transcription from the demethylated viral genome was rescued by R, suggesting that the Z(S186T) defect primarily reflects its inability to induce transcription from unmethylated Rp. In contrast, the Z(S186A) mutant, although able to bind efficiently to the consensus AP-1 site, could not bind efficiently to any of the three Rp ZRE sites in either the methylated or unmethylated form. Consistent with this binding defect, the Z(S186A) mutant could not acti-

vate transcription from either the methylated or demethylated form of the EBV viral genome.

The previous description of the Z(S186T) mutant as being close to wild-type Z in its ability to induce lytic EBV infection from the intact viral genome in Raji cells (24) is not particularly surprising given our finding here that Rp is highly methylated in Raji cells (as well as the other cell lines examined). Another group recently examined the methylation state of the *BRLF1* and *BZLF1* promoters in a series of nasopharyngeal carcinomas (11). Similar to our results here, Rp was found to be highly methylated in latently infected tumors. Whether the Z(S186T) mutant would have a more obvious defect in situations such as primary lytic EBV infection in which the viral genome does not become methylated is as yet unknown.

Serine and threonine are very similar polar amino acids containing a hydroxyl group. This might explain the fact that Z(S186T) retains the ability to bind to and activate methylated Rp and induce lytic EBV infection from the methylated viral genome. On the other hand, the replacement of serine 186 with alanine, a nonpolar amino acid, significantly decreases Z binding to all three Rp ZRE sites, regardless of the ZRE site methylation status, and abolishes the ability of Z to induce lytic gene expression from the intact viral genome regardless of the viral methylation state.

One surprising aspect of our results was the lack of correlation between the ability of the Z(S186A) mutant to activate the unmethylated Rp-CAT vector versus its ability to activate *BRLF1* transcription from the unmethylated viral genome. The inability of this mutant to activate the *BRLF1* transcription from the intact viral genome (in either the methylated or unmethylated state) is consistent with its inability to bind significantly to any of the three Rp ZRE sites in vivo. The unresolved issue, however, is how this mutant activates the unmethylated Rp-CAT vector in reporter gene assays. It is possible that very weak binding of this mutant is sufficient to activate the unmethylated promoter in the context of naked DNA but not in the chromatin context of the intact viral genome. Alternatively, we favor the hypothesis that Z(S186A) activates the unmethylated Rp-CAT construct through an indirect mechanism not requiring direct protein binding to the Rp ZRE sites. Consistent with this, another Z mutant that is globally defective in DNA binding (due to an alteration of residues 178, 179, and 180) was reported to activate certain promoters (including the *BZLF1* and *BRLF1* promoters) through an indirect mechanism (22). However, this alternative mechanism for Z transcriptional effects is clearly not sufficient to activate lytic gene transcription in the context of the intact viral genome.

Phosphorylation is a common mechanism for regulating the function of proteins, and Z can be phosphorylated in vitro at serine 186 by PKC (4). We speculated that phosphorylation of Z residue 186 in vivo (which could occur on either a serine or threonine) may be required for Z binding to methylated Rp ZRE sites, although PKC-mediated phosphorylation of GST-Z was previously reported by another group to inhibit its binding in vitro to unmethylated ZRE sites (4). However, our results did not support this hypothesis. Bacterially expressed and purified GST-fused Z protein (GST-Z), which is not phosphorylated, also bound preferentially to the methylated form of the Rp ZRE-2 motif. Furthermore, a mutant form of Z in which serine 186 was replaced with aspartic acid to mimic a phos-



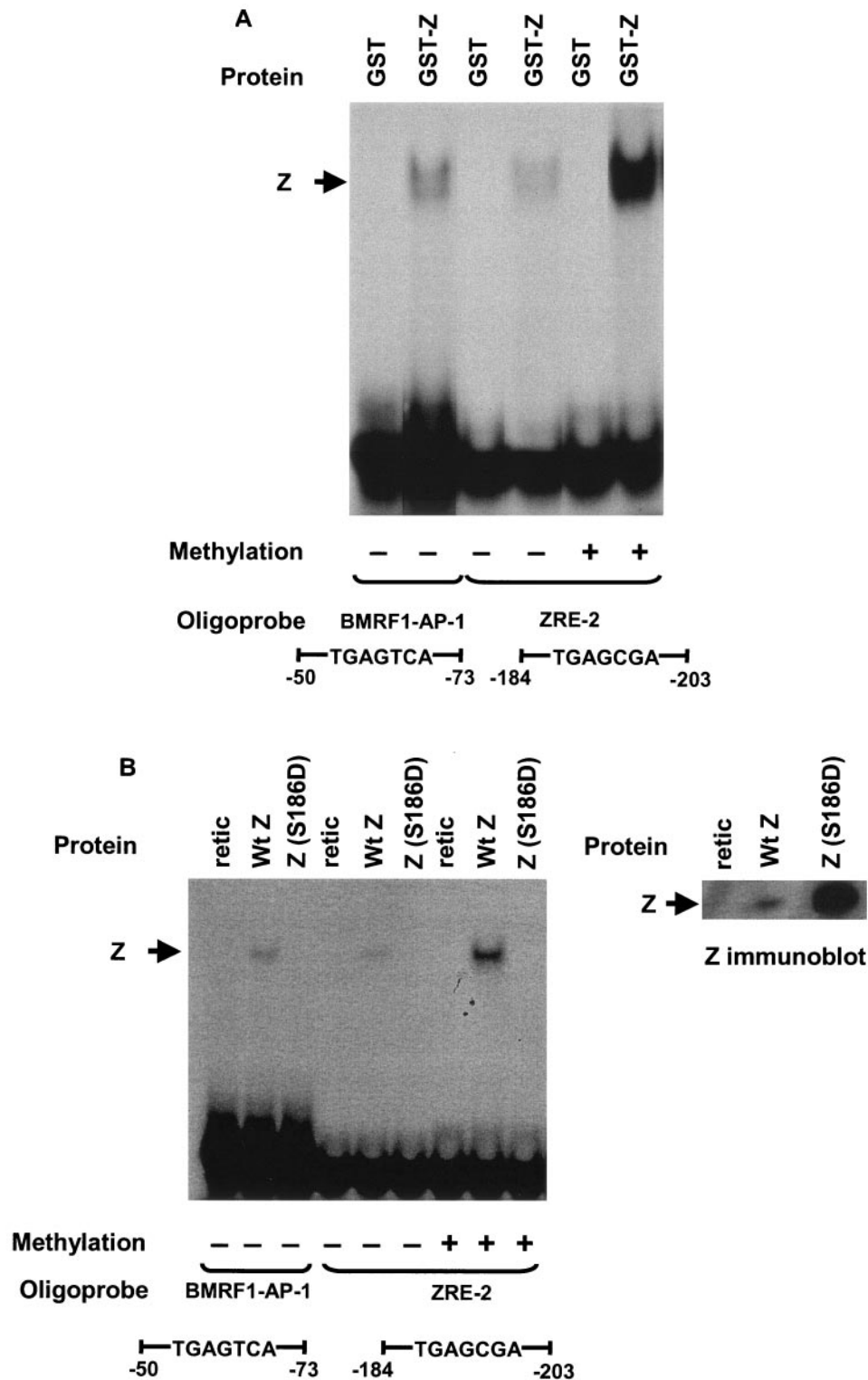


FIG. 8. Phosphorylation of Z serine residue 186 is not required for Z binding to methylated Rp ZRE-2. (A) EMSA was performed using bacterially expressed purified GST-Z and  $^{32}$ P-labeled oligonucleotide probes containing the AP-1 site in the *BMRF1* promoter and either unmethylated or methylated (as indicated)  $^{32}$ P-labeled oligonucleotide probes containing ZRE-2. (B) EMSA was performed using in vitro-translated wild-type Z (Wt Z) and Z(S186D) (as shown) and  $^{32}$ P-labeled oligonucleotide probes containing the AP-1 site in the *BMRF1* promoter and either unmethylated or methylated (as indicated)  $^{32}$ P-labeled oligonucleotide probes containing ZRE-2. (C) 293/EBV-ZKO cells (20) were transfected with either control vector (Vector) or a vector expressing wild-type Z (Wt Z) or Z(S186D). Immunoblots were performed on cell extracts 2 days after transfection with monoclonal antibody directed against Z, R, BMRF1, or  $\beta$ -actin, as indicated. +, present; -, absent; retic, rabbit reticulocyte lysate.

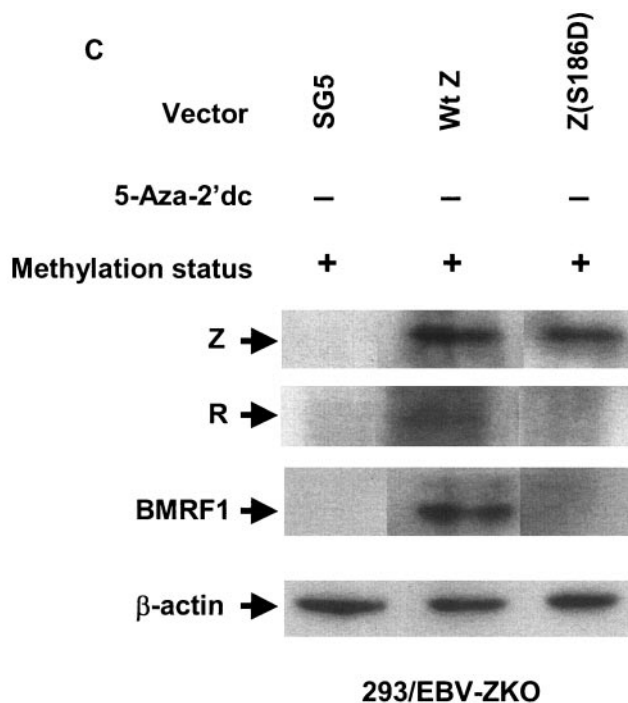


FIG. 8—Continued.

phorylated residue did not bind detectably to either the AP-1 site in the *BMRF1* promoter or the unmethylated or methylated form of the Rp ZRE-2 site. Consistent with its DNA-binding defect, Z(S186D) was also unable to induce R and BMRF1 expression in 293/EBV-ZKO cells. Thus, phosphorylation of Z at position 186 is certainly not a prerequisite for its ability to bind preferentially to the methylated form of Rp ZRE-2 and may instead inhibit Z DNA binding globally. Furthermore, the majority of evidence suggests that Z is not constitutively phosphorylated at position 186 in vivo, nor is PKC required for Z disruption of viral latency (17). As there is some evidence suggesting that Z binds to DNA sites using more than one protein conformation (17), another potential explanation for the binding effects of the Z(S186A) and Z(S186T) mutations is that different Z conformations are required for binding to the methylated versus unmethylated forms of the Rp ZRE-2 site and that only wild-type Z can assume the different conformations required to bind to both forms of this site.

Methylation of the EBV genome may protect the host cell by shutting off transcription of potentially transforming latency genes (in particular, LMP1) expressed in type II and III latency and converting the virus to the most stringent, nontransforming, form of latency, type I (42, 45). Nevertheless, EBV genome methylation also promotes persistence of EBV by inhibiting expression of viral latency proteins that are recognized by cytotoxic T cells (42, 45). In addition, methylation of the EBV genome presumably inhibits the well-known ability of unmethylated CpG motifs to activate Toll-like receptor 9 (3). Interestingly, LMP1 was recently shown to activate expression of cellular methyl transferases (53), suggesting that EBV actually promotes its own genome methylation. Although methylation may be beneficial to the virus in terms of promoting viral latency and evading the host immune system, if EBV could not

efficiently convert back to the lytic form of infection from a methylated viral genome under appropriate circumstances, it would not have successfully infected the great majority of the human population. The ability of the EBV lytic switch protein Z to activate the methylated form of the IE *BRLF1* promoter likely contributes to the long-term success of this virus as both a latent and lytic pathogen.

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