

# Latency of Epstein–Barr virus is disrupted by gain-of-function mutant cellular AP-1 proteins that preferentially bind methylated DNA

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**ZEBRA** Replication Activator (ZEBRA), a viral basic zipper protein that initiates the Epstein–Barr viral lytic cycle, binds to DNA and activates transcription through heptamer ZEBRA response elements (ZREs) related to AP-1 sites. A component of the biologic action of ZEBRA is attributable to binding methylated CpGs in ZREs present in the promoters of viral lytic cycle genes. Residue S186 of ZEBRA, Z(S186), which is absolutely required for disruption of latency, participates in the recognition of methylated DNA. We find that mutant cellular AP-1 proteins, Jun(A266S) and Fos(A151S), with alanine-to-serine substitutions homologous to Z(S186), exhibit altered DNA-binding affinity and preferentially bind methylated ZREs. These mutant AP-1 proteins acquire functions of ZEBRA; they activate expression of many viral early lytic cycle gene transcripts in cells harboring latent EBV but are selectively defective in activating expression of some viral proteins and are unable to promote viral DNA replication. Transcriptional activation by mutant c-Jun and c-Fos that have acquired the capacity to bind methylated CpG challenges the paradigm that DNA methylation represses gene expression.

ZEBRA Replication Activator (ZEBRA) is related to cellular DNA-binding transcription factors, c-Fos and c-Jun of the AP-1 family, with a basic zipper (bZIP) structural motif (1–7). The DNA-binding specificity of ZEBRA and cellular AP-1 proteins overlap (8, 9). An early hypothesis was that ZEBRA represented a mutated cellular AP-1 protein that was “captured” by a virus, in the manner of the oncogenes of RNA tumor viruses (10). While exploring this hypothesis, we showed that a chimeric protein in which the DNA recognition domain of c-Fos was substituted for that of ZEBRA did not disrupt latency (11).

The crystal structure of the bZIP region of c-Fos/c-Jun heterodimer bound to an AP-1 site (12, 13) showed that five amino acids made hydrogen bonds or van der Waals interactions with bases in the AP-1 site in DNA (Fig. 1). Four of these five amino acids were positionally conserved in the basic domain of ZEBRA. The exceptions were c-Fos (A151) and the homologous c-Jun (A266); the residue at this position in ZEBRA is S186 (Fig. 1). We studied the phenotype of a missense mutant Z(S186A) in which S186 in ZEBRA was changed to an alanine to resemble c-Fos/c-Jun (14). The Z(S186A) mutant was a potent transcriptional activator of plasmid-based reporters bearing the promoters of EBV lytic cycle genes such as *BRLF1* and *BMRFL1*; however, it was unable to drive expression of these genes from the latent viral genome. The primary defect of the Z(S186A) mutant was traced to its inability to activate expression of the R transactivator (Rta) protein from the latent virus. The capacity of Z(S186A) to activate early viral lytic cycle genes, such as *BMRFL1*, a synergistic target, could be rescued by overexpression of Rta (15, 16).

The finding that the Z(S186A) mutant was a potent activator of transcription of plasmid reporters containing promoters of viral lytic cycle genes, but was unable to activate expression of lytic genes from the latent viral genome, pointed to an important function of Z(S186) in targeting a viral genome with epigenetic

modifications of DNA or chromatin. Because the latent EBV genome is extensively methylated at CpGs (17, 18), a plausible explanation for the defect in Z(S186A) came with the seminal discovery that ZEBRA binds preferentially to methylated viral DNA (19). ZEBRA binds preferentially to two methylated ZREs in Rp, the promoter of the *BRLF1* gene, designated ZRE-2 and ZRE-3; binding of Z(S186A) to methylated ZRE-2 and methylated ZRE-3 was reduced or abolished (20).

Important biologic questions about the relationship between ZEBRA and cellular AP-1 proteins, and, in particular, the unique importance of residue S186 of ZEBRA, were left unanswered in a crystal structure of the bZIP portion of ZEBRA bound to DNA (5). The structure was solved with a ZEBRA mutant in which S186 was changed to alanine. The structure shows ZEBRA binding to an AP-1 site, whereas the biologic activity of ZEBRA is dependent on binding to ZREs not to AP-1 sites. Moreover, the DNA sequence in the solved structure was not methylated.

Here, we extend the comparison between ZEBRA and cellular AP-1 proteins: we studied the phenotype of cellular AP-1 proteins containing reciprocal alanine-to-serine substitutions in the basic domain by analyzing the capacity of these mutants to activate the EBV lytic cascade. We investigated whether the mutations were accompanied by changes in DNA-binding affinity and by the acquisition of the capacity to bind methylated DNA. Our experiments demonstrate that single alanine-to-serine changes in the two AP-1 proteins produce a profound gain-of-function associated with the capacity to drive viral lytic gene expression and to bind preferentially to methylated DNA.

## Results

**Mutants Jun (A266S) and Fos(A151S) Substitute for ZEBRA to Initiate the EBV Lytic Cycle.** When viewed against the extensive conservation of basic and nonbasic residues in the regions of bZIP proteins that contact DNA, the serine at position 186 of ZEBRA is unusual (Fig. 1). The corresponding position is alanine in four of five cellular bZIP proteins and valine in CCAAT/enhancer-binding protein alpha (C/EBP $\alpha$ ). We investigated the capacity of missense point mutants in the basic domain of the AP-1 proteins, in which the amino acids of c-Jun and c-Fos corresponding in position to S186 of ZEBRA were converted from alanine to serine, to promote synthesis of *BRLF1* mRNA from a latent EBV genome (Fig. 2A). Plasmids encoding wild-type (wt) ZEBRA, c-Jun, and c-Fos and their corresponding mutants were transfected into 293 cells carrying an EBV bacmid in which the EBV gene *BZLF1*, encoding ZEBRA, was insertionally inactivated (BZKO cells) (21) (Fig. S1).

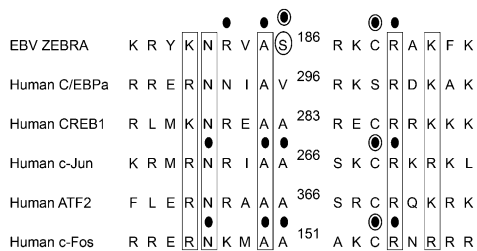
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**Fig. 1.** Comparison of amino acid sequences in the basic domains of EBV ZEBRA and five cellular bZIP proteins. Boxed amino acids are identical or similar in all of the bZIP proteins. Dots indicate amino acids that contact bases in the crystal structure of ZEBRA (5) and the c-Fos/c-Jun heterodimer (12). Circled dots indicate amino acids that were mutated in the crystal structures. ZEBRA (S186) was mutated to alanine. The cysteines at positions ZEBRA (C189), c-Jun (C269), and c-Fos (C154) were all changed to serine. The unique serine 186 of ZEBRA is circled.

Providing wt ZEBRA to BZKO cells led to synthesis of *BRLF1* mRNA detected on a Northern blot (Fig. 2A, lane 1). *BRLF1* mRNA was not detected when wt c-Fos or wt c-Jun were introduced individually or together (Fig. 2A, lanes 3, 5, and 7). However, Jun(A266S) promoted synthesis of *BRLF1* mRNA to levels comparable to those induced by wt ZEBRA (Fig. 2A, lane 6). The mutant Fos(A151S) did not by itself promote expression of *BRLF1* mRNA but was able to do so when cotransfected with wt Jun (Fig. 2A, lane 8). It is known that maximal DNA-binding activity of AP-1 requires heterodimerization of c-Fos with c-Jun (22).

Jun(A266S) alone, or in combination with Fos(A151S), yielded two- to fourfold higher levels of Rta protein than that obtained with ZEBRA (Fig. S24). To determine whether Jun (A266S) promoted a high level of Rta expression in a few cells or expression of Rta in many cells, the Jun(A266S) mutant was introduced into 293 cells containing EBV bacmids; the cells were examined simultaneously for expression of the Jun(A266S) and Rta proteins by immunofluorescence (Fig. 2B). Approximately

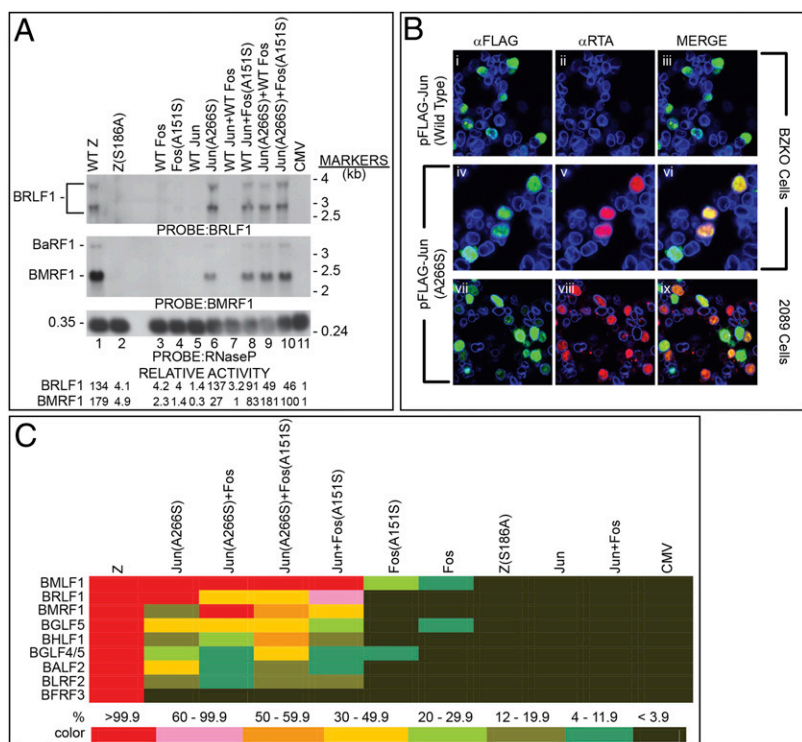
40% (88/219) of BZKO cells that expressed Jun(A266S) also expressed Rta. Rta was not seen in cells that expressed wt c-Jun. In a representative profile of 293 cells containing an intact EBV bacmid (2089 cells), more than 70% (88/120) of cells that expressed Jun(A266S) protein also expressed Rta protein.

The EBV gene *BMRFL1*, encoding DNA polymerase-processivity factor, is activated in synergy by ZEBRA and Rta (23). Because Jun(A266S), and Fos(A151S) in combination with wt Jun, could substitute for ZEBRA to stimulate synthesis of Rta protein, we asked whether the AP-1 mutants could act synergistically with Rta synthesized from the EBV genome in producing expression of *BMRFL1* mRNA. Jun(A266S) by itself, Fos(A151S) together with wt c-Jun, and the combination of both AP-1 protein mutants all activated expression of *BMRFL1* mRNA (Fig. 2A, lanes 6, 8, 10). The combination of the two AP-1 mutants was 56% as active as wt ZEBRA.

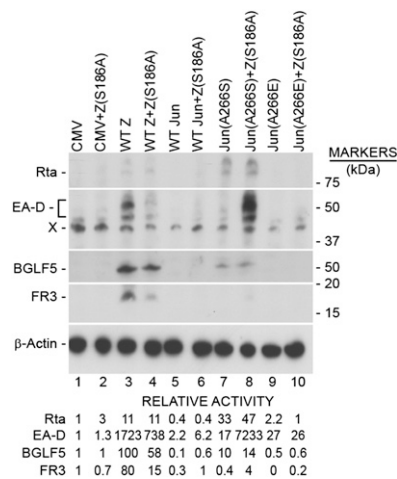
Jun(A266S) by itself, the Fos(A151S) plus wt c-Jun mixture, and the combination of the two AP-1 mutants activated expression of early antigen-diffuse (EA-D) protein, the product of the *BMRFL1* gene, considerably less efficiently than did ZEBRA. The level of EA-D protein produced by Jun(A266S) by itself was only 1% the level stimulated by ZEBRA (Fig. 3, lane 7); the combination of Jun(A266S) and Fos(A151S) activated expression of the EA-D protein to 9% the level produced by ZEBRA (Fig. S2B). Thus, the AP-1 mutants activated expression of EBV lytic cycle proteins in a gene-specific manner.

#### Jun and Fos Mutants Activate Early but Not Late EBV Gene Expression in the Absence of ZEBRA.

The experiments illustrated in Fig. 2A were expanded to examine the capacity of Jun(A266S) and Fos (A151S) to activate selected early and late EBV genes in BZKO cells. (These data are represented as a heat map in Fig. 2C; the Northern blots from which the heat map was derived are presented in Fig. 2A and Fig. S3; the functions of the genes analyzed are summarized in Table S1.) The AP-1 mutants substituted for ZEBRA in activating expression of mRNAs of at least six early viral lytic-cycle genes encoding proteins. These included the *BMLF1* gene, the product of which plays a role in the processing and transport of viral mRNAs, *BRLF1*, the Rta gene, *BGLF5*,



**Fig. 2.** Point mutants in the basic domain of c-Jun and c-Fos substitute for ZEBRA in initiation of the EBV lytic cascade. (A) Plasmids encoding the indicated proteins were transfected in BZKO cells. The transfected cells were assessed for expression of EBV *BRLF1*, *BMRFL1*, and *BaRF1* mRNAs by Northern blotting. Relative activity was determined by densitometry of autoradiographs. (B) Jun (A266S) but not wt c-Jun activates expression of EBV Rta in individual cells. FLAG-tagged wt c-Jun (i-iii) or FLAG-tagged Jun(A266S) (iv-ix) were transfected into BZKO or 2089 cells that were fixed 43 h after transfection and incubated with primary antibodies to FLAG, Rta, and lamin B and appropriate secondary antibodies conjugated with FITC, DyLight 549, or DyLight 645. Images were obtained by confocal microscopy. (C) Heat map illustrating the relative capacity of wt and mutant forms of ZEBRA and cellular AP-1 proteins to activate expression of eight representative EBV lytic genes. The primary data were generated from Northern blots presented in A and Fig. S2; the kinetic class and functions of these genes are described in Table S1.



**Fig. 3.** Defect in the capacity of c-Jun(A266S) to activate expression of EBV early protein EA-D can be complemented by ZEBRA mutant Z(S186A). BZKO cells were transfected with plasmids encoding wt or mutant c-Jun(A266S) in the presence or absence of Z(S186A), a ZEBRA mutant that by itself is unable to activate the lytic cycle. The transfected cells were examined by Western blot for Rta, EA-D, and *BGLF5* early proteins and *BFRF3* late protein.

encoding a protein with alkaline exonuclease and host shutoff activity (24), *BMRF1*, *BGLF4* encoding a kinase (25), and *BALF2*, an early gene encoding the single-stranded DNA-binding protein. The AP-1 mutants also promoted expression of *BHLF1*, a non-coding RNA that is important in lytic viral replication (26). *BMRF1*, *BALF2*, *BGLF5*, and *BHLF1* are synergistic targets of ZEBRA and Rta. The AP-1 mutants, however, were significantly impaired in activation of late gene expression. The Jun mutant was only 14% as active as ZEBRA in its capacity to promote synthesis of the transcript of *BLRF2*, a gene encoding a viral tegument protein. This gene can be weakly activated by Rta alone during early stages of the viral lytic cycle but is only strongly activated late in the viral life cycle following lytic viral DNA replication (27, 28). The AP-1<sup>(A/S)</sup> mutants did not activate either the transcript (Fig. S3B) or the protein (Fig. 3) of *BFRF3*, a true late gene (29). Because *BFRF3* was not expressed, a likely hypothesis is that in the absence of ZEBRA protein the Jun and Fos mutants were unable to activate lytic DNA replication that is a requirement for late gene expression. The experiments suggest that mutant AP-1 proteins can assume the functions of ZEBRA as a transcription factor but not its functions in viral DNA replication.

**ZEBRA Mutant Z(S186A) Complements Defective Expression of an Early Viral Protein by the Jun(A266S) Mutant.** Although the mixture of Jun and Fos (A-to-S) mutants induced expression of the *BMRF1* mRNA at levels averaging 54% of those induced by wt ZEBRA (Fig. 2A, lane 10), they were markedly deficient at promoting expression of EA-D protein, the product of the *BMRF1* mRNA (Fig. 3 and Fig. S3B). These results suggested that the AP-1 mutants lacked a function that is possessed by ZEBRA in promoting expression of the early protein encoded by *BMRF1*. To address this hypothesis, BZKO cells were cotransfected with wt or mutant Jun genes with or without the ZEBRA mutant Z(S186A) (Fig. 3). The Z(S186A) mutant by itself is unable to activate *BMRF1* expression but when Rta is provided *in trans*, Z(S186A) synergizes with Rta to activate *BMRF1* mRNA and EA-D protein (15, 16). Because Jun(A266S) is competent to activate expression of endogenous Rta protein, we reasoned that Jun(A266S) might synergize with Z(S186A) to activate EA-D. Coexpression of Z(S186A) with Jun(A266S) did not enhance the amount of *BMRF1* mRNA (Fig. S2C, lanes 7 and 8) but caused a 3.5-fold increase in *BMRF1* mRNA compared with Jun(A266S) alone (Fig. S2C, lanes 7 and 8). In noted contrast, the stimulatory effect of the combination of ZEBRA

mutant Z(S186A) and Jun mutant (A266S) on the EA-D protein was dramatic (>1,000-fold) (Fig. 3, lane 8). When mutant ZEBRA and c-Jun proteins were coexpressed, the amounts of EA-D protein were stimulated to levels greater than seen with wt ZEBRA protein (Fig. 3, compare lanes 3 and 8). In five replicate experiments, the combination of Z(S186A) and Jun(A266S) induced expression of EA-D protein to levels 0.35- to 4.2-fold the level activated by wt ZEBRA. The level of EA-D protein stimulated by Jun(A266S) alone was only 1% the level produced by ZEBRA (Fig. S2B); the level of EA-D after introduction of Z(S186A) was at background levels.

A surprising result was that whereas coexpression of Z(S186A) with the Jun mutant markedly enhanced expression of EA-D protein, this combination did not enhance the level of *BGLF5* protein (Fig. 3, lanes 7 and 8). Because the defect of Jun(A266S) in expression of some, but not all, early lytic proteins was enhanced by Z(S186A), ZEBRA selectively exerts a posttranscriptional role in promoting the translation of certain viral mRNAs or stabilizing certain viral proteins.

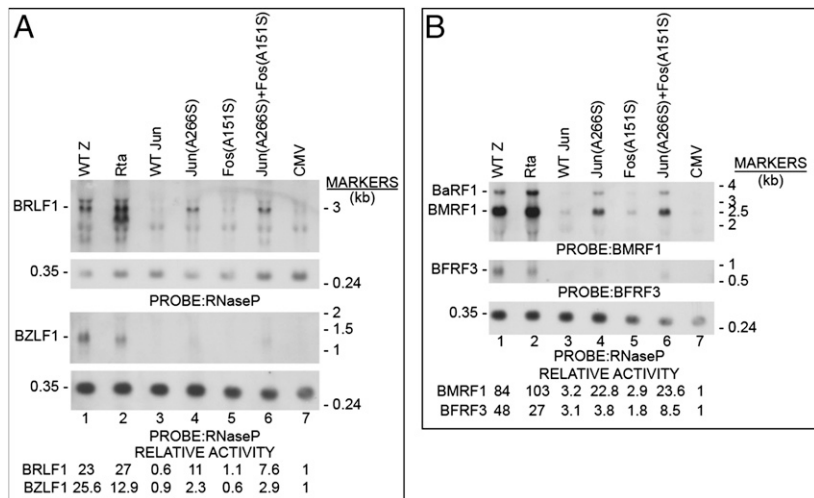
The amount of viral DNA measured after introduction of Jun(A266S) and Z(S186A) was not above the background (Fig. S2D). The failure of the Z(S186A) protein to complement the defect of the Jun mutant in activating viral DNA amplification (Fig. S2D) or *BFRF3* late gene expression may be linked to the observation that addition of Z(S186A) suppressed DNA amplification (Fig. S2D, lanes 3 and 4) and late gene expression (Fig. 3) by wt ZEBRA.

**Mutant AP-1 Proteins Activate the EBV Lytic Cycle in Cultured Cells from Burkitt Lymphoma.** The previously described experiments were conducted in the genetically tractable but biologically artificial system of 293 human embryonic kidney cells containing EBV bacmids. We also studied whether the mutants Jun(A266S) and Fos(A151S) could activate the EBV lytic cycle in natural host cells for EBV, a B-cell line from Burkitt lymphoma (BL). In HH514-16 BL cells, ZEBRA activates synthesis of *BRLF1* mRNA and Rta activates expression of *BZLF1* mRNA. Thus, each of the two viral activators is competent to initiate and to complete the EBV lytic cycle in this cell background (30). If introduction of the AP-1 mutants were to activate either *BZLF1* or *BRLF1* in HH514-16 cells, there should be evidence of late gene expression.

Fig. 4A shows that transfection of HH514-16 cells with Jun(A266S) activated *BRLF1* mRNA expression approximately one-third to one-half as efficiently as did transfection of ZEBRA. Although Jun(A266S) by itself did not detectably activate expression of Rta protein, the combination of Jun(A266S) and Fos(A151S) activated expression of Rta protein (Fig. S4A, lane 6). Jun(A266S) and the combination of AP-1<sup>(A/S)</sup> mutant proteins also activated low levels of *BZLF1* mRNA (Fig. 4A, lanes 4 and 6) and ZEBRA protein (Fig. S4A, lane 6). The mutant Jun(A266S) alone or in combination with Fos(A151S) activated expression of the mRNA of the early gene *BMRF1* but only weakly activated EA-D protein (Fig. S4A, lane 6), as had been observed in BZKO cells (Fig. 3). Transfection of the combination of Jun(A266S) and Fos(A151S) led to expression of *BFRF3* mRNA (Fig. 4B, lane 6) and *BFRF3* protein (Fig. S4A, lane 6). A Southern blot showed that Jun(A266S) by itself and in combination with Fos(A151S) caused a low but reproducible increase viral DNA above background levels (Fig. S4B).

In a subsequent experiment, cells transfected with Jun(A266S) were cotransfected with mGFP and sorted; the GFP-positive cells expressed *BFRF3* mRNA to a 124-fold higher level than GFP-negative cells (Fig. S4C). These experiments allowed two conclusions that were not possible in the 293 cell/EBV bacmid system: first, the AP-1 mutants were active at inducing lytic gene expression from the EBV genome in the setting of a natural host cell; and second, under conditions in which introduction of the AP-1 mutants stimulated expression of both Rta and ZEBRA protein, they could promote low levels of lytic viral DNA replication and late gene expression.





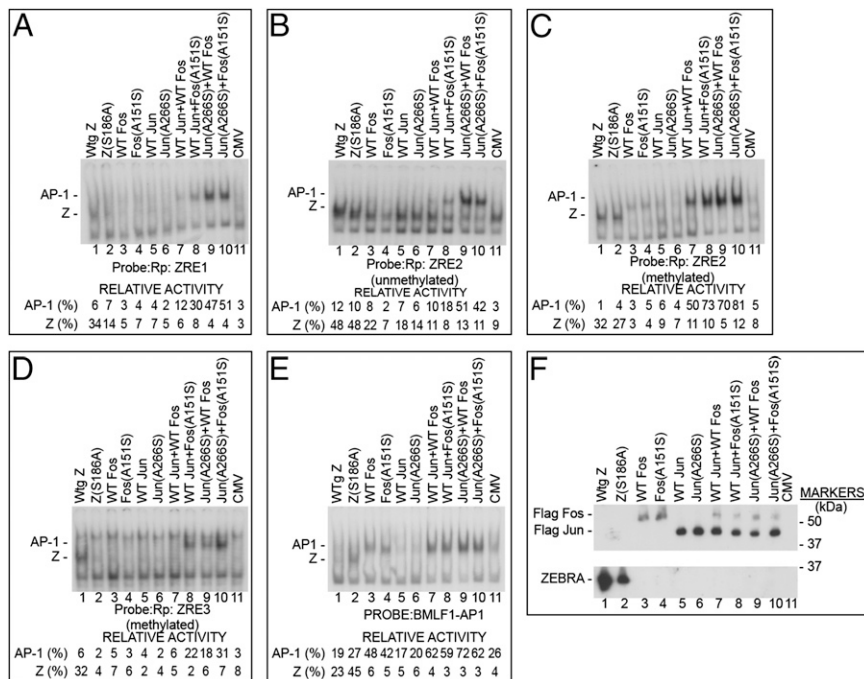
**Fig. 4.** c-Jun(A266S) activates the EBV lytic cycle in a cell line derived from BL. HH514-16 cells were nucleofected with plasmids encoding wt ZEBRA, Rta, wt Jun, or the basic domain mutants c-Jun(A266S) or c-Fos(A151S). The cells were assessed for expression of *BRLF1* and *BZLF1* mRNAs (A) and *BMRF1*, *BaRF1*, and *BFRF3* mRNAs (B).

**Alanine-to-Serine Mutations in AP-1 Proteins Alter Their DNA-Binding Affinity to ZREs in the Promoter of *BRLF1*.** ZEBRA binds to well-defined ZREs, designated ZRE-1, ZRE-2, and ZRE-3, in the promoter of *BRLF1* (Rp). ZRE-2 and ZRE-3 both contain CpG motifs that can be methylated. ZEBRA binds unmethylated and methylated ZREs in Rp (19). Binding by ZEBRA, Z(S186A), and wt and mutant AP-1 proteins to the three ZREs in Rp was compared using electrophoretic mobility-shift assays (EMSA) with duplex oligonucleotides 20 bp in length (Fig. 5 and Table S2). ZRE-2 and ZRE-3 were studied in an unmethylated and methylated state. wt ZEBRA bound more avidly to ZRE-1 (Fig. 5A) and methylated ZRE-3 (Fig. 5D and Fig. S5.4) than did the mutant Z(S186A), which is incapable of activating *BRLF1* expression. The Jun(A266S) mutant that by itself strongly activates *BRLF1* did not detectably bind to any of the ZREs. Therefore, the DNA-binding activity revealed by EMSA does not account for the activating phenotype of the Jun(A266S) mutant. However, the mixture of Jun(A266S) mutant with wt Fos or with mutant Fos(A151A) bound strongly to ZRE-1, unmethylated and methylated ZRE-2, and methylated ZRE-3 (Fig. 5A–D, lanes 9 and 10, and Fig. S5.4).

The Fos mutant (A151S) did not bind ZREs by EMSA; however, when accompanied by wt Jun, Fos(A151S) bound to ZRE-1, unmethylated and methylated ZRE-2, and methylated ZRE-3 (Fig. 5A–D, lane 8). A surprising result was that the mixture of wt Jun and wt Fos, which does not activate *BRLF1* expression, bound strongly to methylated ZRE-2 DNA (Fig. 5C, lane 7). Therefore, binding to methylated ZRE-2 does not explain the phenotypic difference between wt and mutant AP-1. Neither ZEBRA nor AP-1 proteins bound to unmethylated ZRE-3 (Fig. S5B).

The EMSA data demonstrated that DNA-binding affinity was altered as the result of mutations in c-Fos and c-Jun. Because the mixture of Jun(A266S) and Fos(A151S) bound to all of the promoter sequences tested in a methylated state, the results provide evidence that enhanced binding to methylated ZREs can be acquired as the result of specific alanine-to-serine mutations in cellular AP-1 proteins.

**Both wt and Mutant AP-1 Proteins Bind with Similar Affinity to a Classical AP-1 Site.** Additional experiments addressed the question of whether the same mutations that affected binding to ZREs



**Fig. 5.** Comparative binding of wt ZEBRA, c-Fos, and c-Jun and basic domain mutants Z(S186A), Fos(A151S), and Jun(A266S) to unmethylated and methylated ZEBRA response elements. Cell extracts of BZKO cells transfected with the indicated expression plasmids were prepared for EMSA. The probes were derived from sequences in the promoter of the *BRLF1* gene (Rp) or the promoter of the *BMLF1* gene (Table S2). These were Rp ZRE-1 (A), unmethylated Rp ZRE-2 (B), methylated Rp ZRE-2 (C), methylated ZRE-3 (D), and *BMLF1*p AP-1 (E). Relative activity was determined by densitometry of autoradiographs. (F) Immunoblot with antibody to FLAG in extracts prepared for the EMSA reactions.

in Rp also altered binding to a classical AP-1 site, TGACTCA, such as that found in the promoter of the EBV *BMLF1* gene (2). On a classical AP-1 site, the Z(S186A) mutant was enhanced in binding relative to wt ZEBRA (Fig. 5E). The binding of wt and mutant AP-1 proteins was similar on the classical AP-1 site. The results of Fig. 5, therefore, show that the alterations in DNA-binding affinity attributable to alanine-to-serine mutations of the AP-1 proteins are specific to some binding sites and not to others.

**Fos/Jun Mutants Bind Preferentially to Methylated DNA.** Because ZRE-1 is not methylated, and neither ZEBRA nor the Fos/Jun mutants bind to unmethylated ZRE-3, binding by ZEBRA and the AP-1 mutants to the same site in an unmethylated or methylated state can be compared only on ZRE-2. We used EMSA with cold competitors to compare binding of ZEBRA and the Fos/Jun mutants to unmethylated and methylated ZRE-2 (Fig. S5 C–E). In these experiments, the probe was unmethylated ZRE-2; the cold competitors were titrated from 5× to 80× molar excess relative to the probe. Competition with 20× methylated ZRE-2 inhibited binding by the AP-1<sup>(A/S)</sup> mutants by 83%, but competition with 20× unmethylated ZRE-2 did not inhibit binding (Fig. S5C). In a similar titration using cell extracts that expressed ZEBRA (Fig. S5D), 20× cold competitor strongly inhibited binding whether or not the competitor was methylated. These findings suggested that ZEBRA bound equally to both to methylated and unmethylated DNA, whereas the AP-1<sup>(A/S)</sup> mutants bound preferentially to methylated ZRE-2. By mixing cell extracts expressing either ZEBRA or the Fos/Jun mutants, the effects of cold unmethylated or methylated competitor could be compared on the same gel (Fig. S5E). A 20× excess of unmethylated ZRE-2 and a 10× excess of methylated ZRE-2 inhibited binding by ZEBRA by more than 50%. A 40× excess of unmethylated ZRE-2 and a 20× excess of methylated ZRE-2 inhibited binding by the AP-1 mutants to a comparable extent. From these experiments, we conclude that the AP-1 mutants and ZEBRA each preferentially bind to methylated ZRE-2 but they also bind, with lower avidity, to unmethylated ZRE-2. The binding affinity of ZEBRA appears to be somewhat higher than the AP-1 mutants.

## Discussion

Mutants of two cellular AP-1 proteins, c-Jun and c-Fos, containing single alanine-to-serine missense mutations in the DNA-recognition domain assume critical functions of ZEBRA that are required for activation of the EBV viral lytic cascade. The AP-1 mutants strongly activate the EBV *BRLF1* gene, the product of which, Rta, stimulates transcription of many viral genes and participates in viral DNA replication during the lytic cycle (28, 31). A plausible mechanism for the ability of the AP-1 proteins to activate expression of Rta is the acquisition the capacity to bind methylated ZEBRA response elements embedded in the promoter of the *BRLF1* gene encoding Rta. The mutant AP-1 proteins also activate expression of several viral early genes that are involved in lytic-cycle processes such as viral DNA replication (*BMRF1*, *BHLF1*), mRNA processing (*BMLF1*), host cell shutoff (*BGLF5*), and phosphorylation of viral and cellular proteins (*BGLF4*) (Table S1). Many of these genes are activated in synergy between the mutant AP-1 proteins and Rta.

The mutant AP-1 proteins, however, do not substitute for all functions of ZEBRA. The AP-1 mutants do not efficiently stimulate lytic viral DNA replication and late gene expression. They are profoundly defective in expression of an early viral lytic protein, EA-D. Because this defect can be complemented by a ZEBRA mutant that by itself does not stimulate EA-D, the experiments with the AP-1 mutants have revealed previously unrecognized roles of ZEBRA in facilitating protein expression from certain viral lytic transcripts.

**Mutant AP-1 Proteins Are Defective in Promoting DNA Amplification and Late Gene Expression.** A number of hypotheses might account for the defect of the mutant AP-1 proteins in stimulating lytic DNA replication and late gene expression. The defect of the AP-1

mutants in lytic DNA synthesis may be linked to the defect in promoting expression of early proteins, such as EA-D, which is involved in DNA replication. If this were the case, it should be possible to rescue lytic DNA replication by providing one or more of the group of viral proteins that are essential for lytic replication *in trans*. A second possibility is that although the AP-1 mutants can substitute for the transcriptional activity of ZEBRA, they may not function as origin binding proteins and promote assembly of the complex of replication proteins on oriLyt (origin of lytic replication). Future experiments must examine whether Jun(A266S) can directly interact with oriLyt and replication proteins. A third possibility is that the AP-1 mutants are unable to stimulate expression of transcripts, such as *BHLF1* and *BHRF1*, that are adjacent to oriLyt and have been found to facilitate the function of oriLyt (26). However, the AP-1 mutants did induce the *BHLF1* mRNA (Fig. S3C). A fourth possibility is that the AP-1 mutants, although having gained function as activators of transcription of viral lytic genes, behave as repressors of lytic DNA replication.

**Experimental Observations Linking the Capacity of the AP-1 Mutants to Bind to Methylated ZREs with Their Capacity to Initiate the EBV Lytic Cycle.** Alterations in DNA-binding affinity occurred as the result of introduction of alanine-to-serine mutations into c-Fos and c-Jun at positions corresponding to ZEBRA S186. When expressed under conditions that favor the formation of Fos/Jun heterodimers, the AP-1 mutants bound more strongly to unmethylated ZRE-1 and to unmethylated ZRE-2 than did wt AP-1 proteins (Fig. 5 A and B). When the mutants Jun(A266S) and Fos(A151S) were coexpressed, they also bound to oligonucleotides consisting of methylated ZRE-2 and methylated ZRE-3 from Rp, the promoter of the *BRLF1* gene, more strongly than wt AP-1 proteins (Fig. 5 C and D and Fig. S5A). Particularly striking were the results showing that the mutant, but not wt AP-1 proteins, bound to methylated ZRE-3 (Fig. S5A). These observations show that a single missense mutation in a cellular protein can promote the capacity to recognize methylated DNA. They clearly demonstrate that the serine in the DNA-binding domain of the mutated bZIP proteins is crucial in recognizing methylated DNA.

In cell lines from BL, the EBV genome is extensively methylated (32). Interaction of both viral and cellular transcription factors with methylated ZRE-3 in Rp may be one of the crucial events for *BRLF1* activation. Notably, the Jun/Fos mutants are not different from wt AP-1 proteins in their capacity to bind a classical AP-1 site (Fig. 5E). Earlier experiments with ZEBRA/Fos chimeras demonstrated that binding to and transcriptional activation through an AP-1 site were not linked to disruption of EBV latency (11). Therefore, there is a strong correlation between binding to methylated ZREs, but not to AP-1 sites, and capacity to disrupt latency.

Although the mutant Jun/Fos proteins bind five- to sevenfold more strongly to methylated ZRE-3 than do wt AP-1 proteins (Fig. 5D and Fig. S5A), the AP-1 mutants also bind more strongly to the unmethylated ZRE-1 probe than do wt Jun/Fos (Fig. 5A). Therefore, a significant change in DNA-binding affinity of the AP-1 mutants is observed on both unmethylated and methylated ZREs. The activity required to disrupt EBV latency is likely to require interaction with both methylated and unmethylated DNA. For example, the promoters of the *BHLF1* and *BHRF1* genes that overlap oriLyt and are required for replication (26) contain ZREs without CpGs.

Our EMSA experiments did not evaluate interactions with more than one ZRE-binding site on the same probe or DNA containing ZREs and binding sites for other transcription factors. For example, EGR1 binds near ZRE-1, YY1 near ZRE-2, and Sp1/Sp3 near ZRE-3 (33–36). Detailed studies of other “enhancesomes” emphasize the importance of many transcription factors bound together on adjacent regions of DNA (37).

**Broader Implications and Further Questions.** The experiments presented here suggest the hypothesis that mutations in cellular genes in some individuals may account for high levels of viral replication that are known to precede most cancers associated with EBV. Although EBV infection is nearly universal in the human population, EBV-associated cancers are relatively rare. When followed prospectively, the sera of patients who develop EBV-associated cancers, such as nasopharyngeal cancer, BL, and Hodgkin disease, contain higher antibody titers to lytic EBV gene products than those of control subjects (38–41). These observations from seroepidemiology imply that more active lytic viral replication and a higher viral load precede the development of the cancer in susceptible individuals. The conventional explanation for this observation is that a defect in immunosurveillance or an exposure to an environmental agent promotes lytic viral replication in patients who develop cancer. An alternative hypothesis is that patients who develop virally induced cancer might have activating mutations in genes encoding cellular proteins that promote lytic viral reactivation or inactivating mutations of cellular proteins that repress viral reactivation. Such mutations could also occur in genes that modify the levels of the proteins or their posttranslational modifications.

A second hypothesis of general interest is that because the mutations introduced into the AP-1 proteins changed their

capacity to interact with binding sites containing methylated CpGs, some cellular genes might be activated rather than repressed by promoter DNA methylation. The mutant AP-1 proteins might aid in the discovery of a class of cellular genes that could be positively regulated by AP-1 mutants competent to bind methylated DNA.

## Materials and Methods

Human embryonic kidney 293 cells containing EBV bacmids and the HH514-16 BL cell line were transfected with plasmids expressing wt or mutant ZEBRA, c-Fos, or c-Jun under control of the CMV immediate-early promoter. Activation of the EBV lytic cycle was assessed by Northern, Western, and Southern blotting and by indirect immunofluorescence. DNA-binding affinity was evaluated by EMSAs with radiolabeled duplex oligonucleotides that were synthesized in an unmethylated or methylated state (*SI Materials and Methods*).

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