Alteration of a Single Serine in the Basic Domain of the Epstein-Barr Virus ZEBRA Protein Separates Its Functions of Transcriptional Activation and Disruption of Latency

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The ZEBRA protein from Epstein-Barr virus (EBV) activates a switch from the latent to the lytic expression program of the virus. ZEBRA, a member of the bZIP family of DNA-binding proteins, is a transcriptional activator capable of inducing expression from viral lytic cycle promoters. It had previously been thought that ZEBRA's capacity to disrupt EBV latency resided primarily in its ability to activate transcription of genes that encode products required for lytic replication. We generated a point mutant of ZEBRA, Z(S186A), that was not impaired in its ability to activate transcription; however, this mutation abolished its ability to initiate the viral lytic cascade. The mutant, containing a serine-to-alanine substitution in the DNA-binding domain of the protein, bound to several known ZEBRA-binding sites and activated transcription from reporters bearing known ZEBRA-responsive promoters but did not disrupt latency in EBV-infected cell lines. Therefore, initiation of the EBV lytic cycle by the ZEBRA protein requires a function in addition to transcriptional activation; a change of serine 186 to alanine in the DNA-binding domain of ZEBRA abolished this additional function and uncovered a new role for the ZEBRA protein in disruption of EBV latency. The additional function that is required for initiation of the lytic viral life cycle is likely to require phosphorylation of serine 186 of the ZEBRA protein, which may influence either DNA recognition or transcriptional activation of lytic viral promoters in a chromatinized viral episome.

The two phases in the life cycle of a herpesvirus consist of latency, during which only a few viral genes are expressed, and lytic replication, during which many genes required for virus maturation are induced. Epstein-Barr virus (EBV), a gamma-herpesvirus, remains latent in B lymphocytes, but a number of B-cell lines can be triggered into the lytic cycle by treatment with inducing agents such as tetradecanoyl phorbol acetate (TPA) and sodium butyrate (33, 51). These agents promote expression of the viral BZLF1 gene product, ZEBRA. Transfer of ZEBRA-expressing plasmids into latently infected cell lines is sufficient to disrupt latency as measured by expression of lytic cycle genes and production of mature virus (12, 40, 45). The precise mechanism by which the ZEBRA protein initiates the cascade of events leading to virus production is not known. ZEBRA is a transcriptional activator; it is thought to disrupt EBV latency by activating the transcription of viral lytic cycle promoters (8, 22, 27, 41).

The ZEBRA protein is a member of the bZIP family of proteins and shares extensive homology with c-Fos and c-Jun in a highly basic region that serves as the DNA-binding domain for this class of proteins (20). Like c-Fos and c-Jun, ZEBRA binds to AP-1 heptamer sites as a dimer; however, ZEBRA also binds specifically to several other sites that diverge from the AP-1 consensus sequence (6, 22, 31, 37, 46). These sites are referred to as ZEBRA response elements (ZREs) and comprise a steadily growing list of different sequences with a consensus of T(G/T)(T/A)G(T/C)(C/A)A (4a). However, AP-1 activators, such as c-Fos and c-Jun, do not bind to ZREs (6).

ZEBRA has been shown to activate transcription from het-

erologous promoters containing oligomerized AP-1 sites or ZREs as well as from intact promoter sequences from EBV early lytic cycle genes (5, 8, 22, 27, 41). ZEBRA's transcriptional activation domain resides primarily in the N-terminal 100 amino acids of the protein and can function in a wide range of eukaryotic cells, from *Saccharomyces cerevisiae* to human B lymphocytes (13, 24, 36). Additionally, ZEBRA's role in transcriptional activation has been studied in vitro with purified basal transcription components, and ZEBRA has been shown to stabilize the TFIIA-TFIID complex bound to promoter DNA (9, 10, 28, 32).

A chimeric protein in which the basic region of ZEBRA was replaced with that of c-Fos did not retain the capacity to disrupt latency (30). This result suggested that transcriptional activation through ZREs that are uniquely recognized by ZEBRA and not by ZEBRA/Fos chimeras are important for entry into the lytic cycle. The X-ray crystal structure of Fos-Jun bound to an AP-1 site provided a starting point in identifying the amino acids in the basic region of ZEBRA that might contribute to its capacity to recognize ZREs specifically (25). Five amino acids in the basic domain of each partner in the Fos-Jun heterodimer made specific contacts with bases in the AP-1 DNA element. ZEBRA shares identity in four of these contacting amino acids at the level of primary structure (Fig. 1). Whereas both c-Fos and c-Jun have highly conserved alanines that contact a thymine at position ± 1 from the center of the DNA recognition element, tgAcTca (contacting bases are in uppercase letters), ZEBRA has a serine at the equivalent position, amino acid 186. This difference in a crucial amino acid in the DNA recognition domain of the protein may account for ZEBRA's diversity of binding compared with other AP-1 factors. Thus, it was hypothesized that a substitution of alanine for the serine at position 186 (S186A) would convert the DNA-binding specificity of ZEBRA to that of c-Jun and

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a.a. 139	K	R	R	I	R	R	E	R	N	K	M	A	A	A	K	C	R	N	R	R	R	E	L	T	D	Fos
263	K	A	E	R	K	R	M	R	N	R	I	A	A	S	K	C	R	K	R	K	L	E	R	I	A	Jun
227	P	A	A	L	K	R	A	R	N	T	E	A	A	R	R	S	R	A	R	K	L	Q	R	M	K	GCN4
174	E	L	E	I	K	R	Y	K	N	R	V	A	Ⓢ	R	K	C	R	A	K	F	K	Q	L	L	Q	ZEBRA
174	E	L	E	I	K	R	Y	K	N	R	V	A	A	R	K	C	R	A	K	F	K	Q	L	L	Q	Z(S186A)

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FIG. 1. Comparison of the amino acid sequences of the basic DNA-binding regions of members of the bZIP family of proteins. Amino acids in the GCN4 homodimer and the Fos-Jun heterodimer that were shown to contact DNA bases in crystal structures are boxed (19, 25). The serine at amino acid position 186 in the ZEBRA protein that differs from the highly conserved alanine found at the equivalent position in the majority of bZIP proteins is circled. Z(S186A) is a PCR-generated point mutant [Ser (TCC) → Ala (GCC)] of the ZEBRA protein that restores the conserved alanine at position 186.

c-Fos. Since the DNA-binding specificity of ZEBRA would be expected to affect its function in vivo, we assessed the phenotype of such a mutant, Z(S186A), with respect to DNA binding in vitro, transcriptional activation, and the ability to disrupt EBV latency in B cells.

Interestingly, the mutation of amino acid 186 of ZEBRA to alanine did not dramatically change the DNA-binding specificity or transcriptional activation properties of the protein; however, unexpectedly, this mutation abolished the protein's ability to promote entry of EBV into its lytic cycle. Thus, alteration of a single amino acid in the basic domain of ZEBRA can separate its transcriptional activation and disruption of latency functions. This suggests that a property in addition to transcriptional activation is required for initiation of the lytic cascade by ZEBRA.

MATERIALS AND METHODS

Constructs for overexpression of ZEBRA and its mutants in bacteria. Bacterial expression constructs of ZEBRA, Z(S186A), ZΔ131, and ZFZ [Z(1–171)/Fos(137–162)/Z(198–245)] were cloned into the *Nco*I and *Bam*HI sites of pET-11d, allowing for expression from T7 promoter sequences (9). Inserts were generated by PCR with the BZLF1 cDNA in pSP64 or ZFZ in pHD1013 as a template (30, 41). All PCR reactions were carried out with *Taq* DNA polymerase (Boehringer Mannheim) for 30 cycles with an annealing temperature of 50°C. The S186A mutation was introduced by a primary PCR step in which two separate PCR reactions were carried out with the BZLF1 cDNA as a template. A 568-bp fragment from the 5' end of the BZLF1 cDNA was generated with a primer complementary to the 5' end of the BZLF1 cDNA that introduced an *Nco*I site and a mutagenic primer of the sequence 5'TTTTCTGGCAGCCACC CGATTCTT3' which was complementary to the DNA-binding region of the BZLF1 gene between codons for amino acids 181 to 188 of the ZEBRA protein. The C in boldface type represents a mismatch to the template at the first nucleotide of codon 186 which introduced a change from TCC (serine) to GCC (alanine). A 201-bp fragment of the 3' end of the BZLF1 cDNA was generated with a primer complementary to the 3' end of the BZLF1 cDNA that introduced a *Bam*HI site and a mutagenic primer of the sequence 5'GTGGCTGCCAGAA AATGCCGG3' which was complementary to codons for amino acids 184 to 190 of the ZEBRA protein with a mismatch at the G in boldface to introduce the TCC (serine)-to-GCC (alanine) mutation in this fragment. After agarose gel electrophoresis and purification of 5' and 3' PCR fragments on glass beads, a secondary PCR step was performed. The purified 5' and 3' fragments of the BZLF1 cDNA were denatured and annealed by virtue of their 15-bp overlap (codons 184 to 188) and extended by *Taq* DNA polymerase to generate a 755-bp product [Z(S186A)] that represented the full-length BZLF1 cDNA with a TCC→GCC change at codon 186 and a 5'-end *Nco*I site and 3'-end *Bam*HI site. This product served as the template for PCR amplification with the 5'-end *Nco*I and 3'-end *Bam*HI primers and was cloned into pET-11d to generate Z(S186A). The Z(S186A) coding sequence that was cloned into pET-11d was sequenced in its entirety and found to be identical to the wild-type BZLF1 cDNA except at codon 186 (where there was a T-to-G change at the first position).

Eukaryotic expression constructs. The EBV genomic BZLF1 sequence contained on the *Nae*I-to-*Nco*I fragment (EBV 102115 to 103181) of EBV *Bam*HI Z (B95-8) was cloned into pHD1013, which utilizes the cytomegalovirus (CMV) immediate-early gene promoter-enhancer for constitutive expression of gene products in mammalian cells (1a, 16). Genomic ZVP expressed a fusion between amino acids 1 to 227 of the ZEBRA protein (encoded by EBV genomic sequence 102266 to 103181) and the C-terminal 80 amino acids of the herpes simplex virus type 1 activator VP16. Genomic clones of Z(S186A) and Z(S186T) were generated by a two-step PCR process with genomic BZLF1 in BXG1 as a template (5). Genomic Z(S186A) was constructed with the same mutagenic primers described above and nonmutagenic primers that introduced a 5'-end *Xba*I site at the *Nae*I site of Bam Z and a 3'-end *Bam*HI site at the *Nco*I site of Bam Z. Genomic

Z(S186T) was constructed with the same nonmutagenic 5'-end *Xba*I and 3'-end *Bam*HI primers and mutagenic primers that introduced a TCC (serine)-to-ACC (threonine) change at codon 186 of BZLF1 by the same strategy as described for Z(S186A). Genomic inserts bearing mutations at codon 186 were cloned into the *Xba*I and *Bam*HI sites in the polylinker of pHD1013. The genomic Z(S186A) insert was sequenced in its entirety and found to be identical to wild-type genomic BZLF1 except at codon 186 (where there was a T-to-G change at the first position).

Preparation of protein extracts. *Escherichia coli* BL21(DE3), featuring inducible T7 RNA polymerase expression, was transformed with constructs expressing ZEBRA, Z(S186A), or ZFZ from pET-11d or with pET-11d alone. BL21 (DE3)pLysS was used to overexpress ZΔ131. Ampicillin-resistant cultures were grown to an optical density at 600 nm of 0.6 to 0.7 and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM for 4 h. Induced cultures were harvested at 4°C, and pellets were resuspended in 1/5 volume of 6 M urea and sonicated on ice for eight rounds of 15 s each. Extracts were then dialyzed three times (45 min each) against DNA-binding buffer, containing 75 mM KCl 5 mM MgCl₂, 10 mM HEPES (pH 7.5), 0.5 mM EGTA, 0.5 mM dithiothreitol, and 15% glycerol. Aliquots of protein extracts were stored at –70°C until use in binding reactions. The relative amounts of ZEBRA, Z(S186A), and ZFZ present in equivalent volumes of extract were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with rabbit antiserum to ZEBRA and ¹²⁵I-protein A. Quantitation of immunoreactive bands on the Western blot was accomplished by phosphorimaging. Induced extracts contained roughly equivalent amounts of ZEBRA and Z(S186A); ZFZ extracts were roughly half as concentrated.

DNA-binding assays. Binding reaction mixtures contained 1 μl of each protein extract (except where indicated) and 5 × 10⁴ cpm of duplex oligonucleotide that had been labelled with [γ-³²P]dATP by T4 polynucleotide kinase (New England Biolabs). The binding reactions were carried out in DNA-binding buffer in the presence of 1 μg of poly(dI-dC) for 10 min at room temperature with the exception of the ZΔ131 competition reactions, which involved incubation for 30 min at room temperature. Reaction mixtures were subjected to electrophoresis on a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 200 V. The gels were dried, exposed to film overnight, and then subjected to phosphorimager analysis (Molecular Dynamics). Quantitated bands representing free and bound probe were corrected for background and used to calculate the percentage of probe shifted by binding to protein. Probes containing the heptamer ZEBRA recognition elements ZRE-R (TGAGCGA), ZIIIA (TGAGCCA), and ZIIIB (TTAGCAA) and the AP-1 octamer (TGACATCA) were synthesized to contain identical top-strand (5'GATCCAGGCA[XXXX(X)XXX]TGTACC3') and bottom-strand (5'GATCGGTACA[XXX(X)XXXX]TGCCTG3') flanking sequences (22, 37) (where each X represents a nucleotide of an inserted heptamer or octamer).

Cell culture and transfections. The EBV-positive human Burkitt's lymphoma line Raji, which is limited to expression of early viral genes, was grown in RPMI 1640 medium containing 8% fetal bovine serum. A total of 1.5 × 10⁷ cells were transfected with CsCl-purified DNA by electroporation according to published methods (30).

SDS-PAGE and immunoblotting. Protein extracts were prepared from transfected cells that had grown in culture for 3 days. Cells were harvested by centrifugation, resuspended in SDS sample buffer, and sonicated for 15 s. Samples containing 3 × 10⁶ cells were boiled and electrophoresed through a 10% polyacrylamide-SDS gel before the proteins were transferred to nitrocellulose. The Western blot was reacted with a 1:200 dilution of a monospecific polyclonal antiserum raised against ZEBRA, and this was followed by incubation with a 1:100 dilution of the R3.1 mouse monoclonal antibody directed against the EA proteins and a rabbit-antimouse bridge (11, 45). Immunoreactive bands were detected with ¹²⁵I-protein A and autoradiographed.

CAT assays. Cells transfected with reporter and activator DNAs were grown in suspension for 3 days prior to assaying for chloramphenicol acetyltransferase (CAT) activity. Cells were lysed with reporter lysis buffer, and extracts were prepared via the Luciferase Assay System (Promega). A 5-μl aliquot of lysed-cell supernatant was assayed for CAT activity for 1 h at 37°C. Stimulation was calculated by dividing the percent acetylation in the presence of each activator by

FIG. 2. Comparison of the DNA-binding activities of ZEBRA and the Z(S186A) mutant protein. Shown are the results of an EMSA of lysates containing bacterially expressed ZEBRA, Z(S186A), or ZFZ protein with duplex oligonucleotide probes harboring one of the known ZEBRA binding sites, ZRE-R, ZIIIA, or ZIIIB, or the AP-1 octamer sequence. Only the probe that was shifted by binding to protein is shown.

that seen with the CMV vector alone with BMRF1-CAT. CAT assays were performed in four separate transfection experiments.

RNA preparation and Northern blotting. Cytoplasmic RNA was prepared from 3×10^6 cells 20 h after transfection and subjected to Northern blotting according to published methods (30). Blots were probed with a 531-bp *EagI* restriction fragment of DNA containing sequences from the BMRF1 and BaRF1 genes (EBV genomic sequence 80141 to 80672) and a 370-bp *NcoI*-to-*PstI* restriction fragment from the cDNA for the H1 RNA component of human RNase P (3). The blot was reprobed with a 720-bp *TaqI*-to-*SalI* fragment of the BRLF1 gene (EBV genomic sequence 104577 to 105297). Probes were random-prime labelled with [α - 32 P]dCTP and Klenow enzyme (Boehringer Mannheim).

RESULTS

DNA binding by ZEBRA and basic-domain mutant proteins. The effect of substitution of alanine for serine at position 186 in the DNA-binding domain of ZEBRA was studied by electrophoretic mobility shift assays (EMSAs). BL21(DE3) bacterial extracts containing overexpressed ZEBRA, Z(S186A), or ZFZ (which contains a substitution of the basic domain of ZEBRA for that of c-Fos [30]) or pET11d vector alone were assessed for their ability to shift oligonucleotide probes containing three known ZEBRA binding sites and an AP-1 octamer site (TRE). Protein extracts were incubated with 32 P-labelled 27-mer duplex oligonucleotides harboring either a ZRE-R, ZIIIA, or ZIIIB site or a 28-mer containing an AP-1 octamer site. No bacterial proteins present in *E. coli* BL21 (DE3) were able to shift any of the oligonucleotide probes (Fig. 2, lane 1). As expected, ZEBRA shifted ZRE probes (ZRE-R, ZIIIA, and ZIIIB) very efficiently but bound poorly to an AP-1 octamer site (Fig. 2, lane 2). The Z(S186A) mutant recognized all ZRE probes and, in addition, produced a shifted band of greater intensity than wild-type ZEBRA on an AP-1 octamer probe (Fig. 2, lane 3). The ZEBRA/Fos chimeric protein ZFZ bound poorly to ZRE-R and ZIIIB probes, somewhat better to the ZIIIA site, which

more closely resembles an AP-1 site, and very efficiently to an AP-1 octamer site.

Comparison of affinities of ZEBRA and Z(S186A). A method of determining the relative affinities of ZEBRA and Z(S186A) for ZREs involved subjecting both ZEBRA and Z(S186A) binding complexes to competition with increasing amounts of the same competitor DNA-binding protein, Z Δ 131. This truncation mutant, lacking the N-terminal 131 amino acids of the protein, still bound to DNA efficiently but produced a shifted complex with much faster mobility than ZEBRA or Z(S186A) on 6% acrylamide native gels. Therefore, a reaction mixture containing Z Δ 131 and Z(S186A) as well as labeled ZRE probe produced two shifted bands after electrophoretic separation, with the upper complex containing Z(S186A)-shifted probe and the band of faster mobility containing Z Δ 131-shifted probe. A shifted product with intermediate mobility, representing Z(S186A)-Z Δ 131 heterodimers, was not apparent; past experiments suggest that dimerization partner exchange is unlikely to occur in the absence of denaturing agents such as urea. When binding reactions containing ZEBRA or Z(S186A) and labeled ZRE2 probe were challenged with increasing amounts of Z Δ 131 extract, the intensity of ZEBRA- or Z(S186A)-shifted probe decreased and the intensity of the band representing Z Δ 131-shifted probe increased (Fig. 3). Quantitation of the percentage of total probe present in each complex showed that Z Δ 131 competed with ZEBRA and with Z(S186A) in an identical manner. For example, after the addition of 0.5 μ l of Z Δ 131 extract to either

FIG. 3. EMSA showing competition of ZEBRA and Z(S186A) with Z Δ 131 on an oligonucleotide probe containing a ZRE2 site. The binding of ZEBRA and Z(S186A) is assessed alone (lanes 2 and 6) and in the presence of increasing amounts of Z Δ 131 extract (0.5, 1, or 2 μ l). The proportion of the labeled probe that was unbound or was bound by each protein was quantitated by phosphorimage analysis. The percentages of the total labeled probe that were bound by the various proteins are indicated below the gel, with the boldface boxes indicating the point at which Z Δ 131 shifts approximately the same amount of probe as ZEBRA or Z(S186A).

FIG. 4. Expression of transfected ZEBRA and Z(S186A) constructs. Shown is a Western blot exhibiting expression of the proteins ZEBRA and Z(S186A) in BJAB cells with respect to that of a cotransfected reference ZEBRA-VP16 fusion protein, gZ(1–227) + VP16 (ZVP). BJAB cells were cotransfected with 10 μ g of either genomic ZEBRA (gZ) (lane 7), gZ(S186A) (lane 9), or CMV vector (lane 5) and 10 μ g of ZVP. BJAB cells transfected with 20 μ g of CMV vector without ZVP were included in lane 3. P3J-HR-1 clone 16 cells induced with TPA and sodium butyrate (cl. 16 + T/B) served as a positive control for detection of the ZEBRA protein (lane 1). Whole-cell lysates from these cells were separated by SDS-PAGE, probed with monospecific BZLF1 antiserum, and reacted with 125 I-protein A.

the ZEBRA or Z(S186A) binding reactions (Fig. 3, lanes 3 and 7), approximately 30% of the total probe was present in each of the shifted complexes [ZEBRA, Z(S186A), and Z Δ 131]. Therefore, ZEBRA and Z(S186A) had the same

affinity for the ZRE2 oligonucleotide probe compared to Z Δ 131.

Expression of transfected ZEBRA and Z(S186A) constructs in human B lymphocytes. Protein expression from the BZLF1 cDNA and that from the genomic sequence which contains two introns were compared in preliminary experiments. Genomic constructs of ZEBRA (gZ) were expressed more abundantly than the cDNA in human B cell lines (1). For this reason, mutations at amino acid 186 were studied in the context of genomic ZEBRA for all B-cell experiments. To determine if the S186A substitution caused altered expression in mammalian cells compared with wild-type ZEBRA, CMV IE promoter-driven genomic constructs of ZEBRA or Z(S186A) were cotransfected with Z(1–227 + VP16) (ZVP) into the EBV-negative BJAB cell line. ZVP served as a tagged reference protein with slower mobility than ZEBRA and Z(S186A) to which expression of other constructs was standardized. Three days after transfection, protein extracts were immunoblotted with antiserum raised against ZEBRA that recognized all three proteins, ZEBRA, Z(S186A), and ZVP. The ZVP protein was expressed at similar levels in all transfections (Fig. 4). In addition, the ZEBRA and Z(S186A) proteins were expressed at identical levels when compared to the coexpressed ZVP (Fig. 4, compare lanes 7 and 9), a result that was confirmed by quantitation of bands by phosphorimage analysis (data not shown). This result indicated that the S186A mutation did not affect protein expression in EBV-negative B cells.

Abilities of ZEBRA and Z(S186A) to activate transcription of reporter constructs containing early lytic viral promoters. CMV IE promoter-driven genomic constructs of ZEBRA or Z(S186A) were transfected into EBV-negative BJAB cells together with a 333-bp region of the early viral lytic cycle pro-

FIG. 5. Comparison of transcriptional activation by ZEBRA and Z(S186A). (A) Location of AP-1 sites, ZREs, and Rta response elements (RREs) in the 333-bp fragment of the BMRF1 promoter from EBV in BMRF1p-CAT (39). (B) Representative data showing CAT activity in BJAB cells cotransfected with BMRF1p-CAT (5 μ g) and CMV vector (10 μ g) or increasing amounts (0.1, 1.0, or 10 μ g) of genomic ZEBRA (gZ) (lanes 2 to 4, respectively) or gZ(S186A) (lanes 5 to 7, respectively).

moter BMRF1 that was cloned upstream of the CAT gene. While transfection of the CMV vector alone led to little background chloramphenicol acetylation, transfection of 0.1 μ g of ZEBRA or Z(S186A) was able to activate transcription of this reporter at levels 28-fold greater than background in BJAB cells (Fig. 5). In fact, at inputs of 1 and 10 μ g of plasmid DNA, Z(S186A) expression led to two to three times higher stimulation of CAT activity than did the wild-type ZEBRA protein (approximately 100-fold compared to 40-fold [Fig. 5, compare lanes 3 and 6 and lanes 4 and 7]). Thus, substitution of alanine at amino acid 186 enhanced, rather than diminished, the ability of ZEBRA to promote transcription from the early viral lytic cycle promoter BMRF1 in the context of a reporter construct in BJAB cells.

Capacities of ZEBRA and Z(S186A) to disrupt latency of EBV. The ZEBRA protein has the capacity to induce lytic cycle expression of EBV in several latently infected cell lines. This disruption of latency can be monitored by assessing the expression of lytic products at the RNA or protein level. We therefore examined the abilities of transfected ZEBRA and Z(S186A) to induce the appearance of early lytic cycle transcripts in Raji cells, which contain a latent EBV genome, by Northern analysis (Fig. 6). Transfection of Raji cells with increasing amounts of a ZEBRA expression construct led to corresponding increases in the levels of two viral mRNAs known to be induced during the lytic phase (Fig. 6A): the 3.6-kb BaRF1 mRNA, whose product is a component of ribonucleotide reductase, and the 2.5-kb BMRF1 mRNA, which encodes a DNA polymerase accessory factor originally described as diffuse early antigen EA-D (7, 38). However, despite the ability of Z(S186A) to activate the BMRF1 promoter in a CAT assay (Fig. 5), transfection of Raji cells with Z(S186A) did not lead to detectable BMRF1 or BaRF1 transcription from the intact viral genome (Fig. 6A, lanes 6 to 8). Additionally, Z(S186A) expression did not result in transcription of the immediate-early viral BRLF1 mRNA, which is the first known message induced after entry into the lytic cycle (Fig. 6B). The abundance of the ubiquitously expressed H1 RNA component of RNase P indicated that the same amount of cellular RNA was loaded in each lane (Fig. 6C). Therefore, changing the serine at position 186 of the ZEBRA protein to an alanine completely destroyed its ability to promote the entry of EBV into the lytic cycle in Raji cells. Identical results were obtained in another cell line containing a latent EBV genome, P3J-HR-1 clone 16 (data not shown).

Simultaneous assessment of transcriptional activation and disruption of latency. The apparent paradox presented by Z(S186A)'s ability to activate a lytic cycle promoter in a reporter construct in EBV-negative cells and its failure to induce lytic cycle transcription from the virus in EBV-positive cells compelled us to assess these functions simultaneously in Raji cells. Cells were transfected with BMRF1p-CAT and increasing amounts of ZEBRA, Z(S186A), or a second mutant with a conservative serine-to-threonine substitution at position 186, Z(S186T). After 3 days, both CAT activity and lytic cycle protein expression were measured in the same cell extracts. In Raji cells, all three constructs, ZEBRA, Z(S186A), and Z(S186T), very efficiently promoted transcription from the BMRF1 promoter upstream of CAT, although Z(S186A) had the most potent effect on CAT activity (Fig. 7A). Although expression from the ZEBRA constructs was comparable, as assessed by immunoblot analysis of whole-cell extracts, only ZEBRA and Z(S186T), but not Z(S186A), induced BMRF1 protein (EA-D) expression from the virus (Fig. 7B). At an input of 0.1 μ g of the ZEBRA or Z(S186T) expression plasmid, a comparable induction of EA-D protein was observed;

FIG. 6. Comparison of the abilities of ZEBRA and Z(S186A) to disrupt latency of EBV. (A) Northern analysis of BMRF1 and BaRF1 transcripts from Raji cells transfected with genomic ZEBRA or Z(S186A). Raji cells were transfected with CMV vector (lane 3) or increasing amounts (0.2, 2.0, or 20 μ g) of genomic Z (gZ) (lanes 4 to 6, respectively) or gZ(S186A) (lanes 8 to 10, respectively). At 20 h posttransfection, cytoplasmic RNA was subjected to Northern blotting and probed with a 32 P-labelled *EagI* restriction fragment to detect the BMRF1 and BaRF1 mRNAs. P3J-HR-1 clone 16 cells induced into lytic cycle transcription with TPA and sodium butyrate (cl. 16 + T/B) (lane 1) served as a positive control for detection of lytic cycle transcripts. (B) The same blot shown in panel A was re-probed with a 32 P-labelled *TaqI*-to-*SalI* restriction fragment to detect the BRLF1 mRNA. (C) Abundance of constitutively expressed cellular H1 RNA of RNase P (0.35 kb), which served as a control for RNA loading.

however, at an input of 1.0 μ g, ZEBRA appeared to be better than Z(S186T) at inducing EA-D expression. This experiment showed that mutation of serine 186 to an alanine abolished the capacity of the ZEBRA protein to initiate entry into the lytic cycle of EBV without affecting protein expression or impairing transcriptional activation from reporter plasmids in the same cells at the same time. In contrast, a conservative serine-to-threonine mutation did not have the same debilitating affect on the ability to disrupt EBV latency.

DISCUSSION

The major biological function attributed to ZEBRA is activation of the EBV lytic cascade. It induces expression of early viral genes and plays a role in viral replication (8, 21, 22, 27, 41, 42). We created a ZEBRA mutant, Z(S186A), that was competent in assays of DNA binding and transcriptional activation but was not sufficient to drive expression of early lytic cycle genes from the latent virus. Several explanations could account for the phenotype of the Z(S186A) mutant. These include alterations in DNA-binding specificity, transcriptional activa-

FIG. 7. Comparison of the abilities of ZEBRA, Z(S186A), and Z(S186T) to activate the BMRF1 promoter from a reporter construct and in the context of the intact viral genome in Raji cells. Cells were transfected with BMRF1p-CAT (5 μ g) and increasing amounts (0.1, 1.0, or 10 μ g) of genomic ZEBRA, Z(S186A), or Z(S186T). After 3 days, both CAT activity and lytic protein expression were measured in the same cell extracts. (A) Representative data showing CAT activity in cell extracts from transfected Raji cells expressing the CMV vector alone (lane 1), ZEBRA (lanes 2 to 4), Z(S186A) (lanes 5 to 7), or Z(S186T) (lanes 8 and 9) in the presence of BMRF1p-CAT. (B) Western blotting of whole-cell extracts with antibodies that specifically recognize the ZEBRA and BMRF1 (EA-D) proteins. Raji cells chemically induced with TPA and sodium butyrate (T/B) (lane 1) served as a positive control for expression of lytic cycle polypeptides.

tion potency, phosphorylation state, protein-protein interactions, and capacity to access promoter DNA sequences. This system can be likened to the studies of MyoD, in which DNA binding, transcriptional activation, and initiation of the myogenic differentiation program are events that can be separated by introducing mutations in the basic domain of the protein (17, 47).

We were unable to demonstrate a difference between the mutant and wild-type ZEBRA proteins' abilities to recognize ZREs (Fig. 2 and 3). However, Z(S186A) may have an increased ability to bind to an AP-1 octamer site, which is recognized by c-Fos and c-Jun but not by wild-type ZEBRA. These results suggest that the serine-to-alanine mutant may represent an intermediate between ZEBRA and the components of AP-1 binding activity, c-Fos and c-Jun. Our DNA-binding studies focused on five different recognition sites, including those that are present in the promoters for the ZEBRA gene itself, as it is there that the critical early event(s) in disruption of latency is likely to occur. The ZEBRA protein can be expressed from the BRLF1 promoter, which contains a ZIIIA site and a ZRE-R site, or from the BZLF1 promoter, which contains two ZIIIA sites, one ZIIIB site, and an AP-1 octamer site (22, 23, 43). Whereas replacement of the entire basic domain of ZEBRA with that of c-Fos abolished binding to synthetic oligonucleotides containing ZIIIB and ZRE-R sites and reduced binding to ZIIIA sites *in vitro*, it destroyed the ability to disrupt EBV latency; binding to these three sites was unchanged by the mutation of serine 186 of ZEBRA to alanine (30). Although based on these studies of several important ZREs we believe that DNA-binding effects are unlikely to explain the failure of Z(S186A) to disrupt latency, it remains possible that this mutation affects binding to other, as

yet unidentified, ZREs that are important for the switch to the lytic phase of the viral life cycle. In addition, changes in DNA-binding affinity rather than site selection could affect the ability to disrupt latency. The DNA-binding affinities of ZEBRA and Z(S186A) for an oligonucleotide containing a ZRE2 site appear to be identical (Fig. 3); however, the mutant may have altered affinities on other sites that are crucial for the disruption of latency.

An unexpected finding was that the S186A mutant retained the ability to bind ZREs. The mutation altered the basic domain so that all five of the amino acids of c-Fos and c-Jun that contacted DNA in the X-ray crystal structure were now identical in ZEBRA. c-Fos and c-Jun are unable to bind ZREs; therefore, the capacity of Z(S186A) to recognize ZREs must involve residues or a tertiary structure other than that found to be important for binding of c-Fos and c-Jun to an AP-1 site. This hypothesis is also supported by the finding that phosphorylation or mutation of serine 173 in ZEBRA, a residue outside of the canonical basic region, abrogated DNA binding (29). This suggests that the ZEBRA protein recognizes its specific sites in DNA in a manner that is distinct from that used by two other members of the bZIP family of transcription factors, c-Fos and c-Jun.

A prevalent model of ZEBRA's action held that the capacity to disrupt latency was tightly linked to its transcriptional activation potency (4). However, Z(S186A) was not impaired in transcriptional activation of reporters containing promoters of several EBV early lytic cycle genes. In fact, Z(S186A) had an enhanced ability to activate BMRF1-CAT in comparison to the wild type but could not promote expression of viral BMRF1 transcripts or their protein product, EA-D, from the viral genome. The Z(S186A) mutant could also activate the promoters of the BRLF1 and BZLF1 genes and synergize with the EBV Rta transcription factor in activating the BHLF1-BHRF1 divergent promoter when linked to CAT as well as wild-type ZEBRA (data not shown). Our finding that Z(S186A) bound DNA and activated transcription makes it unlikely that folding

of the mutant protein is significantly altered from that of the wild type. A formal possibility that has not been excluded is that none of the promoters used in our reporter assays contains critical upstream or downstream information that discriminates the functions of wild-type ZEBRA and the Z(S186A) mutant.

ZEBRA has been shown to be phosphorylated on serine residues *in vivo*; however, the role of phosphorylation in ZEBRA's biological activity has not been established (14). It is possible that phosphorylation of serine is required for ZEBRA-mediated disruption of latency. Interestingly, the serine at position 186 of the ZEBRA protein fits the consensus for a potential site for phosphorylation by protein kinase C (PKC) (48). This observation is intriguing in light of the fact that the PKC signal transduction pathway has been implicated in induction of EBV lytic cycle transcription following cross-linking of surface immunoglobulins in Akata cells. PKC inhibitors block the anti-immunoglobulin G inducibility of the BZLF1 promoter in CAT assays and prevent the appearance of lytic cycle transcripts from the virus (15, 34). Preliminary results reveal that bacterially expressed ZEBRA protein can indeed serve as a substrate for PKC *in vitro* (24a). Notably, the mutant Z(S186T), in which serine 186 is replaced by threonine, retains a consensus PKC site and also retains the ability to disrupt latency (Fig. 7B). Thus, the presence of either a serine or a threonine at position 186 enables ZEBRA to disrupt latency, a feature which cannot be conferred by alanine at the same position. Phosphorylation of serine or threonine at position 186 could change the DNA-binding affinity or specificity of the ZEBRA protein, or it could be required for interaction with another protein. Although our data suggest that an intact PKC phosphorylation site at amino acid 186 of the ZEBRA protein correlates with the ability of the protein to disrupt EBV latency, it is not yet known if serine 186 is a target for phosphorylation *in vivo*.

There is much evidence that ZEBRA directly interacts with other proteins (26, 28, 35, 44, 49, 50). The mutation of serine 186 to alanine in the ZEBRA protein could destroy a contact that is required for disruption of latency, thereby resulting in the observed phenotype. There are several examples of protein-protein contacts that are made through the basic DNA-binding region of proteins. The human immunodeficiency virus transactivator Tax is capable of binding to the basic region of peptides containing the GCN4 bZIP region. This interaction stabilizes the GCN4 homodimer and increases the DNA-binding affinity of the protein (2). Similarly, the cellular high-mobility-group protein HMG I(Y) can bind to the bZIP region of activating transcription factor 2. This interaction stimulates the binding of activating transcription factor 2 to a site in the beta interferon promoter by promoting dimerization of this transcription factor (18). A similar mechanism involving protein-protein contacts in the basic domain of ZEBRA may affect its capacity to bind or activate transcription from the latent viral DNA.

Our results revealed a dramatic difference in the level of transcription scored from a viral promoter in the context of a transfected reporter construct and that observed from the same promoter as part of the viral genome in response to Z(S186A). Therefore, there may be a property of the promoter DNA itself that is responsible for the observed differences in the activities of ZEBRA and the S186A mutant. ZEBRA's initial role in disruption of latency may be to alter the state of the viral DNA in order to allow initiation of lytic cycle gene transcription. Transfected plasmid DNA may not require such a reorganization event. The phenotype of the Z(S186A) mutation could be explained if the amino acid change impaired

ZEBRA's ability to facilitate chromatin reorganization of the latent viral genome while leaving unchanged its ability to carry out the downstream events of DNA binding and transcriptional activation.

A ZEBRA-mediated nucleosomal rearrangement of viral promoter DNA could involve phosphorylation on serine residues and/or interactions with other proteins through its basic region. Further study is required to elucidate which of these properties may be involved in induction of the EBV lytic cycle by ZEBRA. Nonetheless, the serine-to-alanine mutation in the DNA-binding domain of the ZEBRA protein distinguishes its functions in transcriptional activation and disruption of EBV latency.

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