JOURNAL OF VIROLOGY, Aug. 1991, p. 4033–4041
0022-538X/91/084033-09$02.00/0

Vol. 65, No. 8

ZEBRA and a Fos-GCN4 Chimeric Protein Differ in Their DNA-Binding Specificities for Sites in the Epstein-Barr Virus BZLF1 Promoter

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Received 12 October 1990/Accepted 16 April 1991

Epstein-Barr virus (EBV) encodes a protein, ZEBRA, which enables the virus to switch from a latent to a lytic life cycle. The basic domain of ZEBRA is homologous to the Fos/Jun oncogene family, and both proteins bind the canonical AP-1 site (TGAGTCA). However, ZEBRA does not contain a leucine zipper dimerization domain which has been shown to be necessary for DNA binding of Fos/Jun proteins. Additionally, ZEBRA binds to sites which deviate from the AP-1 consensus sequence. Thus, it was of interest to define the domain of the ZEBRA protein required for DNA binding. We have determined by mutagenesis that ZEBRA residues 172 to 227, representing the basic domain and a putative dimerization domain, are required for specific binding to AP-1 and divergent sites. Mutagenesis of the basic amino acids 178 to 180 or 187 to 189 abrogates ZEBRA binding to all DNA target sequences. These residues are conserved in Fos and are also necessary for Fos DNA-binding activity. We have found that a Fos-GCN4 chimera and ZEBRA have different cognate binding specificities. The autoregulated BZLF1 promoter contains three divergent AP-1 sequences, ZIIIA (TGAGCCA), ZIIIB (TTAGCAA), and Z-AP-1-octamer (TGACATCA). ZEBRA binds with high specificity to ZIIIA and ZIIIB but weakly to the Z-AP-1 octamer. Conversely, the Fos-GCN4 chimera recognizes only the Z-AP-1 octamer. ZEBRA binds the ZIIIA and ZIIIB sites together in a noncooperative fashion, while Fos-GCN4 binds these sites as a higher-order complex. Additionally, we have found that flanking sequences influence binding of Fos-GCN4 to a degenerate AP-1 site (TGAGCAA). The characteristic binding specificities of ZEBRA and cellular AP-1 proteins suggest that they differentially affect viral and cellular transcription.

Epstein-Barr virus (EBV) encodes a protein, ZEBRA, which enables the virus to switch from a latent to a lytic life cycle (8, 9, 41). EBV is latent in lymphoid cells but can be activated in vitro by phorbol esters, butyrate, or expression of the ZEBRA gene (8, 9, 15, 29, 41, 46). The mechanism by which ZEBRA initiates the cascade of viral replication has been the focus of much study. Numerous groups have found that ZEBRA directly transactivates several promoters of lytic cycle genes, including a region integral to the EBV lytic origin of replication (7, 8, 13, 15–17, 20, 28, 41, 43).

Exon II of ZEBRA is homologous to the basic domain of the Fos/Jun proto-oncogene protein family (11). In Fos/Jun complexes, this domain has been shown to interact directly with a DNA heptamer known as the tetradecanoyl phorbol acetate (TPA) response element or AP-1 site (TGAGTCA) (3, 26, 34). It was determined that ZEBRA shares the ability of the Fos/Jun complex to bind an AP-1 consensus site (11). An AP-1 sequence (MS-AP-1) is located in the promoter of an EBV early gene, BSLF2-BMLF1 (MS-EA) (11). ZEBRA can transactivate transcription through this site, although it is not yet clear whether AP-1 sequences are required for transactivation of EBV genes in infected lymphocytes (18, 43).

ZEBRA also binds sites on the EBV genome which deviate from the AP-1 consensus sequence (11, 13, 27, 28, 37, 43). The autoregulated BZLF1 (ZEBRA) promoter contains three divergent AP-1 elements (13). Two sites, TGAGCCA (ZIIIA) and TTAGCAA (ZIIIB), 129 and 116 bp upstream of the transcriptional start site are arranged in a tail-to-tail fashion and are separated by six nucleotides. An additional AP-1 sequence, TGACATCA (Z-AP-1 octamer), is located further downstream at −67. This sequence, which was first identified in the *jun* promoter by Angel et al. (2), mediates activation by TPA and Jun/AP-1. Recent work has shown that although this site is TPA inducible in the ZEBRA promoter, it does not appear to be transactivated by ZEBRA (12, 13).

Since both ZEBRA and Fos/Jun are involved in transcriptional regulation of gene expression, it was important to compare the DNA-binding activities of these proteins. Therefore, we studied the relative DNA-binding affinities of ZEBRA and a Fos-GCN4 chimera for the divergent AP-1 sites found in the autoregulated BZLF1 promoter.

MATERIALS AND METHODS

Plasmids. The full-length BZLF1 cDNA (amino acids [aa] 1 to 245) (30) was cloned as a *Nae*I-*Bam*HI fragment into the *Sma*I-*Bam*HI sites of the *trpE* bacterial expression vector pATH 11. The Raji BZLF1 cDNA and the expression vector were the gifts of A. Sergeant and T. J. Koerner, respectively. BZLF1 deletion mutants were constructed as follows: $Z_{141-245}$ was cloned as a *Nhe*I-*Bam*HI fragment, $Z_{172-245}$ was cloned as a *Bsm*I-*Bam*HI fragment, Z_{1-198} was cloned as a *Nae*I-*Pst*I fragment, and Z_{1-227} was cloned as a *Bam*HI-

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*Hin*cII fragment. All fragments were cloned into the appropriate sites of pATH vectors. $Z_{\Delta 111-159}$ was constructed by bidirectional Bal 31 exonuclease digestion of Z_{1-245} at the unique *Nhe*I site (aa 141). Exon I of the BZLF1 open reading frame, WZ_{1-167}, was cloned into the pATH vector from WZhet (42), and exon II of BZLF1, $Z_{167-202}$, was cloned from genomic *Bam*HI Z as a *Pvu*II-*Hin*cII fragment.

Two additional mutant ZEBRA proteins were constructed by site-directed mutagenesis of the B95-8 BZLF1 cDNA cloned in SP64. This cDNA was the gift of P. Farrell (11). Amino acids 178 to 180 and 187 to 189 were mutated from KRY to EEL and RKC to EES, respectively. Site-directed mutagenesis was performed as described by the manufacturer (Bio-Rad).

A Fos-GCN4 chimera which contains the Fos basic DNA-binding domain, aa 126 to 162, fused in frame to the GCN4 leucine zipper domain, aa 251 to 281, was the kind gift of T. Kouzarides and E. Ziff (24). A *Sau*3AI-*Xba*I fragment containing the Fos-GCN4 chimera was cloned into the *Bam*HI-*Xba*I sites of pATH1.

Protein expression. Fusion proteins were expressed as described previously (22, 42). *Escherichia coli* AG1 containing the various TrpE-BZLF1 deletion mutants was grown in 5 ml of M9 medium containing 1% Casamino Acids (Difco) and induced for 4 h in the presence of indoleacrylic acid (20 μg/ml). Proteins from 0.5 ml of cells were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and stained with Coomassie brilliant blue. The level of induced protein in each preparation was estimated visually, and volumes of cells containing equivalent amounts of protein (3.5 to 5 ml) were pelleted and resuspended in 1 ml of 6 M urea (1). Cells were sonicated twice for 20 s each time. The urea-solubilized protein fraction was dialyzed three times for 45 min each time at 4°C against 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9)–75 mM KCl–0.2 mM EDTA–1 mM dithiothreitol–7.5% glycerol.

Constructs in pBluescript KS were transcribed in vitro (31) and then translated in a wheat germ extract as described by the manufacturer (Promega).

Synthetic oligonucleotides. The synthetic double-stranded oligonucleotides used in binding studies were as follows (sequence reflects top strand in a 5'-to-3' direction; core sequences are underlined and mutated bases are noted in bold type).

MS-AP-1: TCTTCATGAGTCAGTGCTTC
MS-AP-1: TCTTCATTAGTCAGTGCTTC
ZIIIA: CTAGCTATGCATGAGCCACAGATC
ZIIIA*: CTAGCTATGCATGAGCAACAGATC
ZIIIAm: CTAGCTATGCAGAATTCACAGATC
ZIIIB: CTAGCAGGCATTGCTAATGTACCGATC
ZIIIB*: CTAGCAGGCATTGCTCATGTACCGATC
ZIIIBm: CTAGCAGGATCCGCTAATGTACCGATC
Double: GACTATGCATGAGCCACAGGCATTGCTAATGTACCGA
(contains ZIIIA + ZIIIB)
Z-AP-1 octamer: CTAGAAACCATGACATCACAGAGGATC
Control oligonucleotide: TGGCATGCTGCTGACATCTGGC

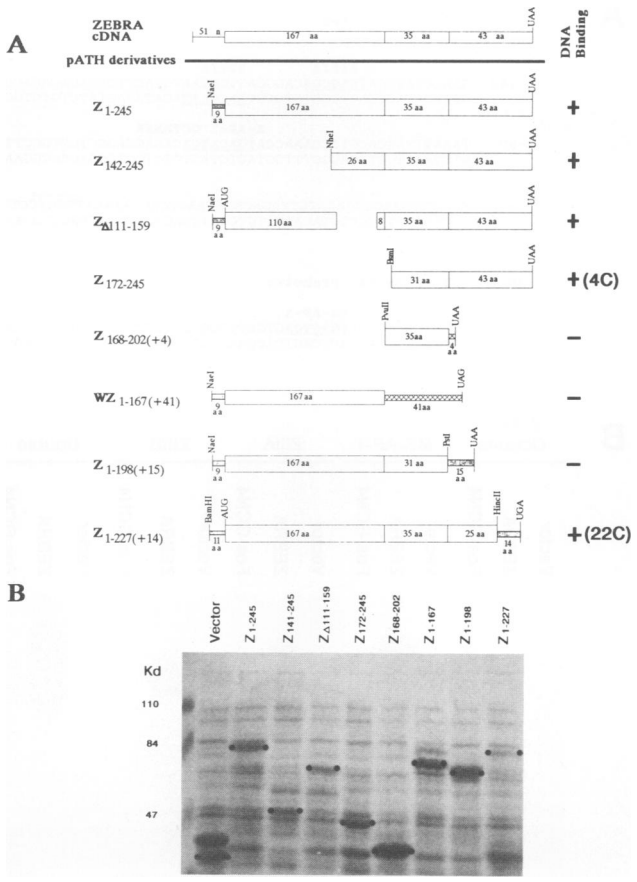
All oligonucleotides had *Xba*I sites at the 5' end and *Bam*HI sites at the 3' end, with the exception of the AP-1 and Double oligonucleotides, which had CT and AG overhangs at the 5' and 3' ends, respectively. Radiolabeled oligonucleotides were prepared by using the Klenow fragment of *E. coli* DNA polymerase and ^{32}P-labeled deoxynucleotides.

Gel retardation assays. DNA binding reactions were performed as described previously (11). Reaction mixtures were incubated at room temperature (22°C) or 4°C for 30 min. A 4×10^{-13} M concentration of the appropriate ^{32}P-end-labeled double-stranded oligonucleotide and 2 μl of protein (approximately 100 ng) were incubated in a 25-μl volume of 15 mM HEPES (pH 7.9)–75 mM KCl–0.2 mM EDTA–4 mM dithiothreitol–7.5% glycerol–1 μg of poly(dI-dC). Gel retardation competition assays were conducted with 5-, 20-, and 50-fold molar excesses of cold competitor over the ^{32}P-labeled MS-AP-1 probe. The Double oligonucleotide was used in 2.5-, 10-, and 25-fold molar excesses since it contains two potential binding sites (ZIIIA and ZIIIB). Samples were electrophoresed through 4% polyacrylamide–0.5× TBE gels at room temperature or 4°C. Gels were then dried and autoradiographed. Films from two oligonucleotide competition experiments were quantified by densitometer scanning on a Kodak BioImage densitometer with Visage 2000 software. Disruption of complex formation was determined by comparing the ratios of shifted complex in lanes with and without competitor.

RESULTS

Determination of the ZEBRA DNA-binding domain by deletional mutagenesis. A BZLF1 cDNA obtained from Raji cells was cloned in frame with the *trpE* coding sequences contained in the pATH 11 expression vector (Fig. 1B). The 75-kDa fusion protein bound a 20-bp oligonucleotide which encompasses an AP-1 consensus sequence (MS-AP-1) derived from the promoter of the EBV early gene, MS-EA (Fig. 1C and D, Z_{1-245}). Several deletion mutants defined the domain of the protein which bound the AP-1 site (Fig. 1C and D). All of the mutants exhibited some temperature-sensitive DNA-binding activity. Mutant $Z_{141-245}$ encoded the smallest protein, which behaved like the wild-type ZEBRA (Z_{1-245}) in binding DNA at room temperature and 4°C (Fig. 1A). However, even this mutant did not bind DNA as well at 4°C as did the wild-type Z_{1-245}. The region of ZEBRA encoded by exon II, which shares homology with the Fos basic domain (11), was insufficient to elicit DNA-binding activity (Fig. 1C and D, $Z_{168-202}$). Although $Z_{\Delta 111-159}$ bound DNA unstably (smear in Fig. 1C) at room temperature (22°C) and $Z_{172-245}$ did not bind DNA under these conditions (Fig. 1C), both mutant proteins bound DNA at 4°C (Fig. 1D). Deletion of the 18 carboxyl amino acids of ZEBRA resulted in a mutant (Z_{1-227}) which bound the AP-1 site at room temperature but not at 4°C (Fig. 1C and D). Furthermore, all of the ZEBRA deletion mutants showed the same pattern of binding to the divergent AP-1 sites, ZIIIA and ZIIIB, present in the BZLF1 promoter (data not shown). Thus, ZEBRA amino acids 172 to 227 were required for DNA binding, although neighboring sequences may have influenced protein folding or stability under some temperature conditions.

Site-directed mutagenesis of the ZEBRA basic domain. The region of ZEBRA encoded by exon II of BZLF1 (aa 168 to 202) is partially homologous to the basic region of Fos (Fig. 2A). It has recently been shown that aa 144 to 146 and 154 to 156 are required for the DNA-binding ability of Fos (35). These basic amino acids are conserved at positions 178 to 180 and 188 to 190 of ZEBRA. Therefore, we determined whether the same basic amino acids in this region contribute to the DNA binding of ZEBRA. These residues were mutated from Lys-Arg-Tyr to Glu-Glu-Leu and from Arg-Lys-Cys to Glu-Glu-Ser, respectively (Fig. 2A). These mutations abrogated DNA binding to the consensus (MS-AP-1) as well as divergent AP-1 sites (ZIIIA and ZIIIB) (Fig. 2B). Equivalent amounts of the in vitro-translated wild-type and mutant proteins were used in each reaction (data not shown). These results demonstrate that the same basic residues in ZEBRA



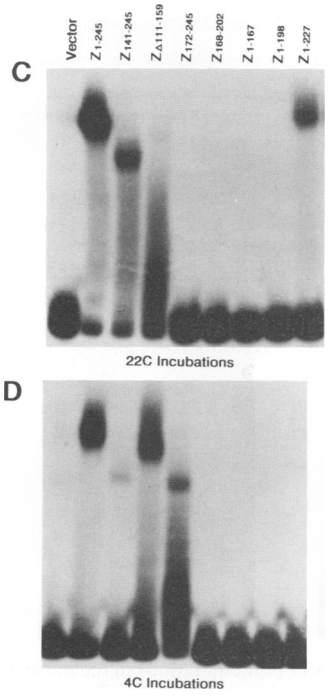


FIG. 1. DNA-binding activities of ZEBRA mutants. (A) Structures of ZEBRA deletion mutants. The ZEBRA cDNA is shown at the top (30), and clones are designated according to the ZEBRA amino acids present in the construct. $Z_{\Delta 111-159}$ is named according to the amino acids that have been deleted. Z_{1-167} was derived from genomic DNA (42). Translated sequences that are derived from ZEBRA intron sequences are indicated by a hatched bar, and sequences from the expression plasmid polylinker are indicated by a stippled bar. (B) Expression of TrpE-ZEBRA fusion proteins in bacteria. Total protein from bacteria that carried various TrpE-ZEBRA deletion proteins was resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The fusion protein is indicated with dots in each lane. (C) Gel retardation assay showing binding of TrpE-ZEBRA deletion mutants to a 20-bp ^{32}P-labeled oligonucleotide (4×10^{-13} M) containing the AP-1 consensus sequence (11). Extract from bacteria transformed with the *trpE* plasmid pATH 11 was used as a negative control (vector lane). The various proteins were each incubated with the oligonucleotide probe and 1 μg of poly(dI-dC) in binding buffer for 30 min prior to electrophoresis. Binding reactions and electrophoresis were performed at room temperature (22°C). Complexes were electrophoresed on a 4% acrylamide gel in 0.5× TBE. (D) Gel retardation assay of the same TrpE-ZEBRA deletion mutants performed as for panel C except that binding reactions and electrophoresis were carried out at 4°C.

and Fos are required for binding to AP-1 and related sequences. However, deletional mutagenesis showed that these basic residues are necessary but not sufficient for DNA binding (Fig. 1).

Binding of ZEBRA and Fos-GCN4 to oligonucleotides bearing single AP-1 and AP-1-like sequences. It was previously shown that ZEBRA footprinted sites ZIIIA at −129 (TGAGCCA) and ZIIIB at −116 (TTAGCAA) but not the Z-AP-1 octamer sequence (TGACATCA) 67 bp upstream of the BZLF1 transcriptional start site (13; Fig. 3A). A gel retardation assay was used to compare the relative affinities of ZEBRA and Fos-GCN4 for these sites in the BZLF1 promoter. The Fos basic domain has been shown to bind the AP-1 consensus site as a homodimer when fused to the GCN4 leucine zipper (18, 24, 40). A Fos-GCN4 chimera, expressed as a TrpE fusion protein, bound the MS-AP-1 consensus site but not ZIIIB or ZIIIA. ZIIIB differs from the consensus AP-1 site at 3 bp and ZIIIA contains a single base pair change from the consensus heptanucleotide AP-1 sequence (Fig. 3B). Fos-GCN4 bound the Z-AP-1 octamer with high affinity, whereas ZEBRA interacted only weakly with this sequence. Thus, Fos-GCN4 binds the AP-1 octamer and heptanucleotide sequences while ZEBRA binds the MS-AP-1, ZIIIA, and ZIIIB sites with relatively high affinity (Fig. 3B). The TrpE-ZEBRA and TrpE-Fos-GCN4 fusion proteins were derived from the urea-solubilized fraction of total *E. coli* protein extracts. ZEBRA and Fos-GCN4, expressed in this system, bind DNA as homodimers (23). A control extract of bacterial protein did not exhibit specific binding to any of these sites (data not shown).

Binding of ZEBRA and Fos-GCN4 to an oligonucleotide containing two ZEBRA response elements, ZIIIA and ZIIIB. Since the ZIIIA and ZIIIB sites are separated by only six nucleotides in the ZEBRA promoter, we studied the ability of ZEBRA and Fos-GCN4 to bind both sites simultaneously. An oligonucleotide termed Double, which includes both sites in the orientation found in the BZLF1 promoter, was used in gel band shift assays. ZEBRA was able to bind two sites on this oligonucleotide simultaneously, as demonstrated by the appearance of two band shift products in a mobility shift assay (Fig. 3B). Surprisingly, the Fos-GCN4 chimera, which did not bind a single ZIIIA or ZIIIB site, was able to bind the Double oligonucleotide (ZIIIA plus ZIIIB), generating a more slowly migrating complex (Fig. 3B).

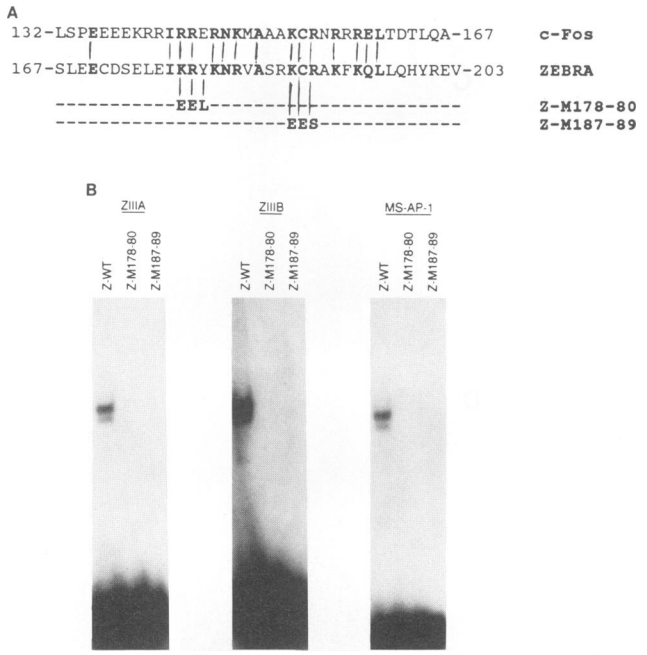


FIG. 2. Effects of point mutations in the basic domain of ZEBRA. (A) Amino acid sequences of human c-Fos exon III (aa 132 to 167) (44) and BZLF1 exon II (aa 168 to 202) (5). The conserved residues in this highly basic domain are indicated by lines (11). ZEBRA mutants were obtained by site-directed mutagenesis of the BZLF1 cDNA cloned in SP64. Amino acids 178 to 180 and 187 to 189 were changed as shown for Z-M178–80 and Z-M187–89, respectively. (B) Gel retardation assay demonstrating the inability of ZEBRA constructs mutated at aa 178 to 180 (Z-M178–80) or 187 to 189 (Z-M187–89) to bind MS-AP-1, ZIIIA, or ZIIIB. The wild-type (Z-WT) and mutated ZEBRA constructs (Z-M178–180 and Z-M187–189) were transcribed in vitro with SP6 polymerase and translated in a wheat germ extract. Equivalent amounts of the resulting translated proteins were each incubated with the following ^{32}P-labeled oligonucleotide probes: MS-AP-1, ZIIIA, and ZIIIB (see Materials and Methods for sequences). These complexes were then resolved by gel electrophoresis on a 4% acrylamide gel in 0.5× TBE.

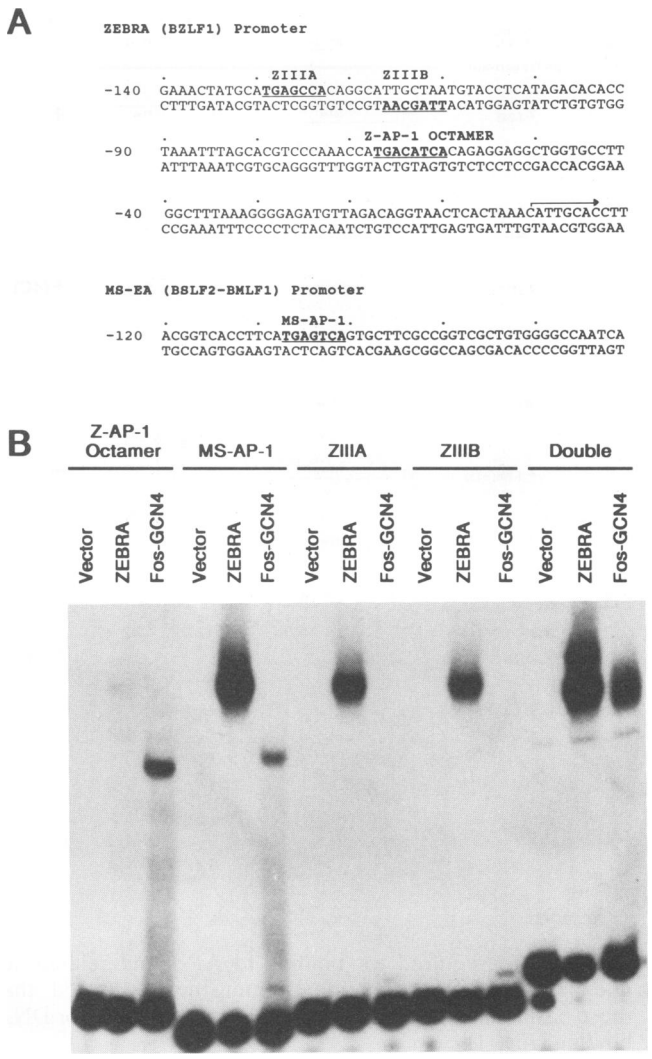


FIG. 3. Binding specificities of ZEBRA and Fos-GCN4 for AP-1 and BZLF1 promoter sequences. (A) Sequences of the ZEBRA (BZLF1) and MS (BSLF2-BMLF1) promoters. AP-1-like sequences are underlined (11, 13), and the transcriptional start site of the BZLF1 gene is marked. (B) Gel retardation assay showing the specificities of TrpE (vector), TrpE-ZEBRA (Z_{1-245}), and TrpE-Fos-GCN4 (Fos-GCN4) proteins for oligonucleotides containing the AP-1 and BZLF1 promoter sequences: Z-AP-1 octamer, ZIIIA, and ZIIIB (13). The Double oligonucleotide contains sites ZIIIA and ZIIIB in the orientation found in the BZLF1 promoter. Equivalent amounts of bacterially expressed fusion proteins were incubated with each oligonucleotide (4×10^{-13} M) for 30 min prior to analysis of the retardation complexes by gel electrophoresis.

We next studied the order of binding to sites on the Double oligonucleotide. Serial dilutions of ZEBRA and Fos-GCN4 were incubated with 4×10^{-13} M ^{32}P-labeled Double probe. At low protein concentrations (0.25 μl), ZEBRA bound only a single site on the oligonucleotide (one shifted complex), while at high concentrations (5 μl), it began to interact with both sequences on a single oligonucleotide molecule (two shifted complexes) (Fig. 4A). However, Fos-GCN4 generated a single complex of slower mobility with the Double probe, regardless of protein concentration (Fig. 4B).

Nucleotide specificity of ZEBRA and Fos-GCN4 binding sites. Point mutations were introduced into the MS-AP-1, ZIIIA, and ZIIIB oligonucleotides in an attempt to establish which base pairs mediate specific DNA binding by ZEBRA and Fos-GCN4. The oligonucleotides created were MS-AP-1* (TTAGTCA), ZIIIA* (TGAGCAA), and ZIIIB* (TGAGCAA). ZIIIA* and ZIIIB* contain an identical 7-bp core recognition site (TGAGCAA) but differ in their surrounding sequences (see Materials and Methods). Additionally, the ZIIIA and ZIIIB oligonucleotides were mutated at four positions to generate the ZIIIAm and ZIIIBm oligonucleotides.

ZEBRA bound with relatively high affinity to the MS-AP-1, ZIIIA, and ZIIIB sequences containing a single point mutation (MS-AP-1*, ZIIIA*, and ZIIIB*) (Fig. 5A). However, it was unable to bind either the ZIIIAm or ZIIIBm site, each of which contains 4-bp changes (Fig. 5A). The lack of binding to the ZIIIAm or ZIIIBm site confirms the importance of the identified core element. Fos-GCN4 was more fastidious in its binding site requirements; it recognized the ZIIIB* (TGAGCAA) sequence but did not interact with any of the other mutated oligonucleotides (Fig. 5B). ZIIIA* and ZIIIB* differ from the AP-1 consensus at two nucleotides, while ZIIIB differs at three positions and ZIIIA differs at

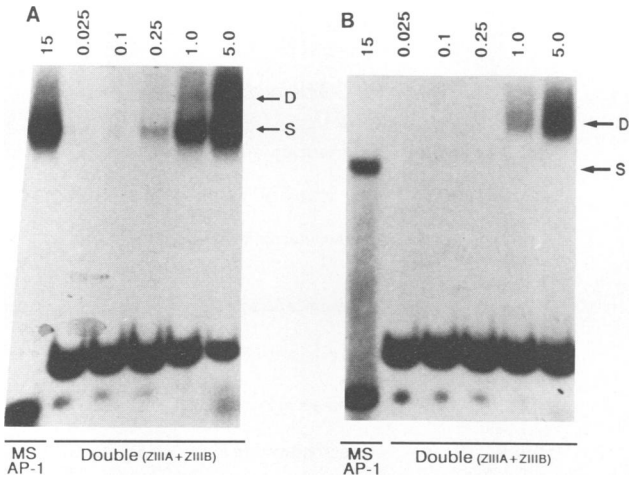


FIG. 4. Binding of ZEBRA (A) and Fos-GCN4 (B) to the Double oligonucleotide. ZEBRA and Fos-GCN4 proteins were titrated in a gel retardation assay with 4×10^{-13} M ^{32}P-labeled MS-AP-1 or Double (ZIIIA plus ZIIIB) probe. Various volumes of protein extract (indicated in microliters) were incubated with the MS-AP-1 or Double probe in the presence of poly(dI-dC). ZEBRA and Fos-GCN4 both bind to the MS-AP-1 site as a single complex, indicated as S. Mobility shift complexes with a slower mobility are indicated as D.

only one. ZIIIA* and ZIIIB* contain the same 7-bp recognition sequence, but their surrounding sequences vary. These results suggest that ZEBRA is considerably more tolerant of changes within the 7-bp recognition sequence than is Fos-GCN4.

Competition analysis to determine the affinities of ZEBRA and Fos-GCN4 for AP-1 and ZEBRA promoter sequences. The relative affinities of ZEBRA and Fos-GCN4 for AP-1-related sequences were assayed by competition analysis (Fig. 6). Complex formation between ZEBRA or Fos-GCN4 and the MS-AP-1 probe was assayed in the presence of three concentrations of cold competitor oligonucleotides. The relative reduction of AP-1 complex formation is proportional to the affinity of ZEBRA or Fos-GCN4 for the oligonucleotide competitor. Disruption was quantified by comparing the integrated intensity of each shifted complex as a function of the concentration of oligonucleotide competitor (Fig. 6C and D). The ZEBRA complex formed with the MS-AP-1 site was most efficiently competed for with MS-AP-1, ZIIIA*, and ZIIIB* (Fig. 6A and C). A 5-fold molar excess of these oligonucleotides eliminated ZEBRA binding to ^{32}P-labeled MS-AP-1, whereas a 50-fold molar excess of ZIIIA, ZIIIB, or Double oligonucleotide was required to achieve a similar level of inhibition (Fig. 6A and C). The ZEBRA/MS-AP-1 complex was minimally reduced by the addition of a 50-fold molar excess of Z-AP-1 octamer and was not affected by the addition of unlabeled ZIIIAm, ZIIIBm, or a control oligonucleotide with no AP-1-like sequences (Fig. 6A and C and data not shown). Thus, ZEBRA binds with highest affinity to TGAGTCA (MS-AP-1) and TGAGCAA (ZIIIA* and ZIIIB*) sites and with a lower affinity to TTAGCAA (ZIIIB), TGAGCCA (ZIIIA), TTAGTCA (MS-AP-1*), and the Double oligonucleotide (Fig. 6C and 7).

The Fos-GCN4/MS-AP-1 complex was efficiently competed for with a 5-fold molar excess of Z-AP-1 octamer, 20-fold molar excess of MS-AP-1, or 50-fold molar excess of ZIIIB* (Fig. 6B and D). Surprisingly, the Double oligonucleotide, which was bound by Fos-GCN4 in a gel retardation assay, was unable to disrupt the formation of a complex at the MS-AP-1 site (Fig. 4; Fig. 6B and D). None of the remaining divergent AP-1 sites competed with the formation of a Fos-GCN4/MS-AP-1 at a 50-fold molar excess (data not shown). A second competition experiment was also quanti-

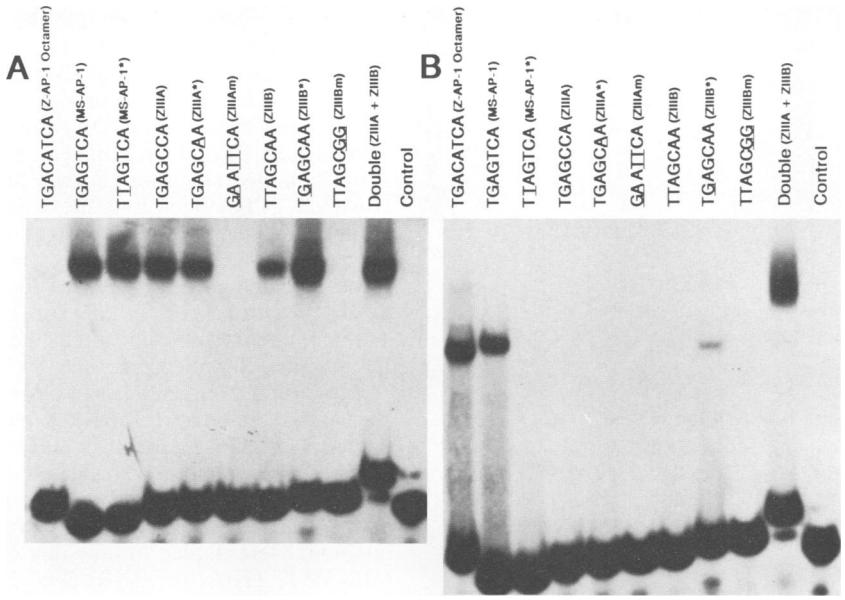


FIG. 5. Gel retardation assay illustrating the binding specificities of ZEBRA and Fos-GCN4 for mutant recognition sequences. MS-AP-1, ZIIIA, and ZIIIB oligonucleotides were mutated at a single position (noted by *); the base substitution is underlined. Two oligonucleotides mutated at multiple positions are designated ZIIIAm and ZIIIBm. The control oligonucleotide contains no AP-1-related sequences. Bacterially expressed TrpE-ZEBRA (A) or TrpE-Fos-GCN4 (B) was incubated with these ^{32}P-labeled oligonucleotides, and binding was analyzed by a mobility shift assay.

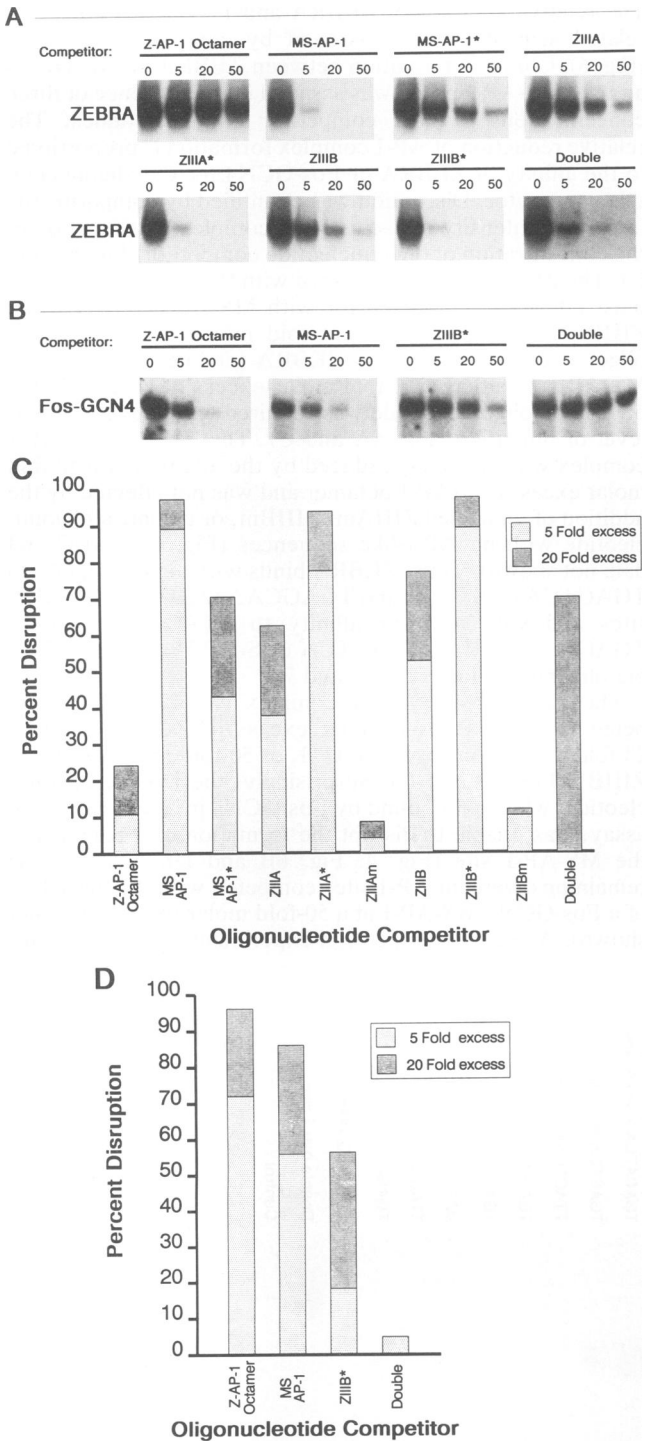


FIG. 6. Competition experiment to evaluate the affinities of ZEBRA and Fos-GCN4 for AP-1 and ZEBRA promoter sites. A ^{32}P-labeled MS-AP-1 oligonucleotide (4×10^{-13} M) was incubated with bacterially expressed TrpE-ZEBRA (A) or TrpE-Fos-GCN4 (B) in the presence of three concentrations of cold competitor DNA. Complex formation was resolved by gel electrophoresis. Only the shifted complexes are shown, and the nature of the competitor and its molar excess over the MS-AP-1 probe are indicated. (C) Results quantified by scanning of the relative intensity of each shifted complex. The percent disruption of the ZEBRA/MS-AP-1 complex is plotted for each oligonucleotide competitor. (D) Results quantified as for panel C for disruption of the Fos-GCN4/MS-AP-1 complex.

Name	Promoter	Oligonucleotide	ZEBRA	Fos/GCN4
MS-AP-1	BSLF2	TCTTCA**TGAGTCA**GTGCTTC	1	2
MS-AP-1*	—	TCTTCA**TTAGTCA**GTGCTTC	2	–
Z-AP-1 (Octamer)	BZLF1	CTAGAAACCA**TGACATCA**CAGAGGATC	3	1
ZIIIA	BZLF1	CTAGCTATGCA**TGAGCCA**CAGATC	2	–
ZIIIA*	—	CTAGCTATGCA**TGAGCAA**CAGATC	1	–
ZIIIB	BZLF1	CTAGCAGGCA**TTGCTAA**TGTACCGATC	2	–
ZIIIB*	—	CTAGCAGGCA**TTGCTCA**TGTACCGATC	1	3

FIG. 7. Evaluation of ZEBRA and Fos-GCN4 binding site affinities. Affinities were determined from the competition analysis in Fig. 6. Nucleotide substitutions in the recognition sequences are noted by bold type. A relative affinity of 1 represents the highest affinity; – indicates that the protein did not bind the sequence specifically.

fied; the relative affinities of ZEBRA and Fos for the various oligonucleotides were found to be the same in the two experiments (data not shown).

DISCUSSION

Comparison of ZEBRA and Fos DNA-binding domains. The construction and expression of ZEBRA deletion mutants in *E. coli* enabled us to determine the domains within the ZEBRA protein which are required for DNA binding. Unlike all other identified transcription factors which bind the AP-1 consensus sequence, ZEBRA does not contain a leucine zipper directly adjacent to the basic region (25, 45). However, these studies indicate that the region 3′ to the ZEBRA basic domain (aa 199 to 227) is required for DNA binding. The C-terminal 18 amino acids (228 to 245) may also contribute to dimer formation or protein stability, since mutants lacking this region bind DNA in a temperature-sensitive fashion (Fig. 1C and D). This region, exon III (aa 199 to 245), is not homologous to any known dimerization domain (45). However, it has been hypothesized that this domain forms an alpha helix and that dimerization involves a coiled-coil interaction (14, 23, 27, 36). These results are consistent with those of Packham et al., who found that mutants which deleted the same regions of exon I or III bound DNA in a salt-dependent manner, whereas the wild-type protein bound DNA under all conditions (37).

Site-directed mutagenesis of c-*fos* previously identified the amino acids necessary for DNA recognition of the Fos/Jun complex (35). Mutagenesis of Fos amino acids 144 to 146 or 154 to 156 completely abrogated DNA binding (35). The corresponding amino acids are conserved in the basic domain of ZEBRA exon II, and proteins mutated in these positions were unable to bind any of the DNA-binding sites (MS-AP-1, ZIIIA, or ZIIIB) (Fig. 2). These results suggest that while the DNA-binding specificities of Fos-GCN4 and ZEBRA differ, the same two clusters of basic amino acids are necessary for the DNA-binding abilities of both proteins. Further work is needed to define the amino acids in the basic region which confer the unique binding specificities of Fos-

GCN4 and ZEBRA. We have conducted dimerization domain swap experiments to show that DNA target specificity, in vitro, is contained exclusively within the basic region and is not influenced by the nature of the ZEBRA or GCN4 dimerization domain (23).

Binding site specificities of ZEBRA and Fos-GCN4. ZEBRA and Fos bind similar DNA sequences, and both ZEBRA expression and TPA treatment result in activation of EBV lytic viral DNA replication (8, 9, 15, 41, 46). An underlying biological question is whether Fos, or other cellular AP-1 proteins, mediates TPA induction of the EBV lytic cycle. While we have not addressed this question directly, we have begun by assessing the binding affinities of ZEBRA and Fos-GCN4 for several sites in the BZLF1 and MS-EA promoters. We chose to compare ZEBRA binding to that of Fos-GCN4, since the first indication that ZEBRA was a DNA-binding transactivator came from a recognition of the homology between the basic domains of ZEBRA and c-Fos (11). While Fos generally binds DNA as a Fos/Jun heterodimer, we used an artificial Fos-GCN4 construct, containing the Fos basic domain and the GCN4 leucine zipper, to allow Fos to bind DNA as a homodimer. Many groups have previously shown that the target site specificity of a protein is not affected by the nature of its leucine zipper (24, 32, 34, 40).

Several groups have recently examined ZEBRA binding activity in EBV promoters and the origin of EBV lytic replication (ori lyt) (11, 13, 17, 21, 27, 28, 37, 43), but there had been no studies directly comparing the ability of Fos and ZEBRA to bind the same sites. Since our work was completed, Chang et al. (6) reported on the binding specificities of ZEBRA and Fos/Jun in ori lyt. Our work on the binding specificities of ZEBRA and Fos for sites in the BZLF1 and MS-EA promoters provides both complementary and novel observations. Chang et al. demonstrated that ZEBRA binds an AP-1 consensus site with higher affinity than it binds divergent heptamer sites in ori lyt, whereas we have found that it binds equivalently to an AP-1 site and a divergent sequence (ZIIIA* or ZIIIB*) with high affinity (6). ZIIIA* and ZIIIB* contain the same core heptamer sequence found in ZRE2 in ori lyt (6). Their data suggest that ZEBRA binds ZRE2 with high affinity (6). Both studies show that Fos/Jun and Fos-GCN4 are restrictive in binding divergent AP-1 heptamer sites. However, we have found novel binding of a Fos-GCN4 homodimer to a divergent heptamer sequence flanked by dyad symmetry (ZIIIB*).

ZEBRA bound a much broader range of DNA sequences than did Fos-GCN4 (Fig. 4). ZEBRA bound the palindromic AP-1 site, but it also bound the ZIIIA and ZIIIB sites, which do not contain perfect dyads. Unexpectedly, oligonucleotides with base pair substitutions which disrupt the partial dyad nature of ZIIIA or ZIIIB (TGAGCCA to TGAGCAA or TTAGCAA to TGAGCAA) were bound by ZEBRA with increased affinity (Fig. 5 and 7). This departs from the proposed scissors-grip model of DNA binding by dimeric proteins, in which each protein monomer is hypothesized to bind an identical DNA half-site (45). Although it is known that ZEBRA (expressed in vitro or as a TrpE fusion protein) binds DNA as a homodimer (6, 14, 23, 27), it is unclear whether both monomers contact or recognize symmetrical units on DNA. There are two possibilities which could explain ZEBRA binding to asymmetric sites: first, only one subunit of the ZEBRA homodimer contacts DNA; or second, each subunit recognizes a wide variety of half-site sequences, thereby recognizing asymmetric sequences. Our preliminary data suggest that the second hypothesis accounts for ZEBRA's asymmetrical recognition of DNA (23). It has also been found that the homodimeric glutocorticoid receptor binds DNA asymmetrically and is more sensitive to mutations in the right half of its binding site (reviewed in reference 4).

Fos-GCN4 binds as a homodimer to the palindromic AP-1 site (19, 24, 40; Fig. 3). Both Fos/Jun heterodimers and artificial Fos homodimers have been shown to recognize the AP-1 consensus sequence asymmetrically (39). This suggests that the Fos basic domains in a homodimer interact with heptamer target sequences in a manner equivalent to a Fos/Jun heterodimer. However, a Fos-GCN4 homodimer did not bind any of the divergent AP-1 sites with high affinity. It was unable to recognize ZIIIA, ZIIIB, or ZIIIA*, all of which were recognized by ZEBRA homodimers (Fig. 4 and 7). Fos-GCN4 did bind ZIIIB* (TGAGCAA), although it did not bind ZIIIA*; both oligonucleotides contain the same 7-bp core recognition sequence. This result suggests that flanking nucleotides influence binding ability. ZIIIB*, but not ZIIIA*, contains the bases CA and TG adjacent to the recognition sequence increasing the dyad symmetry of the site, **CATGAGCAATG**. The importance of flanking sequences in the binding affinity of Fos was noted by Nakabeppu and Nathans (32). They found that substitutions of cytosine and guanosine for adenine and thymine bases at flanking positions 1 and 10 of the cyclic AMP response element (CRE) resulted in greater than a 90% decrease of homodimeric Fos or Fos/Jun binding activity (32). Our novel result indicates that the binding site specificity of Fos is broader than originally proposed; Fos-GCN4 can bind an asymmetric heptamer sequence with two mismatches from the AP-1 consensus in an appropriate flanking oligonucleotide environment.

While ZEBRA has a broader specificity than Fos-GCN4 in recognizing various heptamer sequences related to the AP-1 consensus, only Fos-GCN4 binds the Z-AP-1 octamer sequence (TGACATCA) located in the BZLF1 promoter with high affinity. This is interesting since this site was originally defined as the Jun/AP-1 response element in the *jun* promoter and is TPA responsive (2). Thus, ZEBRA is unable to bind a subset of the known AP-1/TPA-responsive elements.

Fos/Jun as well as artificial Fos homodimers are known to bind the octamer CRE (TGACGTCA), which differs from the Z-AP-1 octamer at position 5 (G to A) (6, 12, 32, 33, 38, 39). Although the sites are similar, Deutsch et al. have shown that they have different signal-responsive properties; that is, CREs are not responsive to TPA (10). Chang et al. have shown that ZEBRA does not bind a CRE (6). Although ZEBRA does not bind the TPA-responsive Z-AP-1 octamer site, binding to this site may be important in the induction of the EBV lytic cascade. For example, Fos or another cellular AP-1 protein may bind this site in vivo (2, 3, 10, 26), leading to low levels of transcription from the BZLF1 promoter. ZEBRA can then bind to the ZIIIA and ZIIIB sites in the BZLF1 promoter, resulting in autoactivation of this gene (13).

Binding of ZEBRA and Fos-GCN4 to an oligonucleotide with two binding sites. We studied ZEBRA and Fos-GCN4 binding to multiple sites as they are arranged in the BZLF1 promoter. The arrangements of ZEBRA binding sites in its own promoter and in the lytic-origin divergent promoter are very similar (28). ZRE1 (TGTGTAA) and ZRE2 (TGAG CAA) (identical to the heptamer core sequence in the ZIIIA* and ZIIIB* oligonucleotides) in the lytic origin are arranged in a tail-to-tail fashion separated by 10 bp (28). Sites ZIIIA (TGAGCCA) and ZIIIB (TTAGCAA) are arranged in the

same manner in the BZLF1 promoter, separated by 6 bp. This arrangement may be a common mode of targeting ZEBRA transcriptional control in the EBV genome.

We found that two ZEBRA homodimers can bind ZIIIA and ZIIIB simultaneously on the Double oligonucleotide (Fig. 3). We have not determined which site is filled initially, although footprinting data have demonstrated that ZEBRA binds ZIIIB with higher affinity than ZIIIA (13, 27). Gel shift experiments with limiting dilutions of ZEBRA protein show that both are bound only after a significant portion of single sites are filled. Furthermore, competition experiments indicate that ZEBRA does not have a greater affinity for the Double oligonucleotide than for either single site (Fig. 6A and C). Thus, ZEBRA binds noncooperatively to these sites. However, binding of ZEBRA to these two adjacent sites may allow for synergistic transactivation.

Fos-GCN4, which did not bind oligonucleotides bearing single ZIIIA or ZIIIB sites, bound the Double oligonucleotide (containing ZIIIA and ZIIIB) as a high-molecular-weight complex (Fig. 3 and 4). The association of this slow-mobility complex can be disrupted by addition of ZEBRA protein, suggesting that the two proteins compete for a similar sequence on the Double probe (unpublished data). However, the Double oligonucleotide did not interfere with Fos-GCN4/MS-AP-1 complex formation (Fig. 6B and D). One explanation for this phenomenon is that these associations are noncompetitive because the two probes bind different Fos-GCN4 complexes. The MS-AP-1 probe is bound by a Fos-GCN4 homodimer, whereas the Double probe may bind only a Fos-GCN4 complex with more than two monomers and perhaps with other proteins.

The interaction of ZEBRA with cellular AP-1 sequences may result in the displacement of Fos or other cellular AP-1 factors, thereby precluding their activity. Alternatively, by binding AP-1 sites, ZEBRA may transactivate (or possibly transrepress) cellular genes in a manner analogous to that with cellular AP-1 transcription factors. AP-1 proteins are probably not able to activate transcription of EBV genes through ZEBRA-responsive elements because at least one AP-1-binding protein, Fos-GCN4, is unable to bind those sites. The divergent nature of the ZEBRA-responsive sites makes it unlikely that cellular AP-1 proteins can have an ongoing role promoting viral transcription and activating the EBV lytic cycle. Additionally, the ability of Fos to bind the TPA-responsive Z-AP-1 octamer and CRE sites, which are not bound by ZEBRA, indicates that ZEBRA cannot simply substitute for Fos. The ability of these related proteins to bind similar as well as distinct sites adds yet another level of complexity to the relationship of these transactivators in regulating cellular and viral gene expression.

ACKNOWLEDGMENTS

We are grateful to E. Manet, A. Sergeant, and P. Farrell for the gifts of BZLF1 cDNAs, T. Kouzarides and E. Ziff for the Fos-GCN4 construct, and T. J. Koerner for the pATH expression plasmids. We also thank D. Crothers for his support and advice. N.T. would like to acknowledge the support of E. & E. Lichtenstein.

This work was supported by Public Health Service grants CA12055, CA16038, and AI22959 (to G.M.); RO1CA143 and RO1CA52004 (to S.H.S.); IF32CAO8482-01 (to E.F.); and CA09159-15 (to R.B.) from the National Institutes of Health and by grant NP 669K (to G.M.) from the American Cancer Society. N.T. is supported by the Medical Scientist Training Program of the National Institutes of Health.

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