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Comparing Transcriptional Activation and Autostimulation by ZEBRA and ZEBRA/c-Fos Chimeras

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The lytic cycle of Epstein-Barr virus (EBV) can be activated by transfection of the gene for ZEBRA, a viral basic-zipper (bZip) transcriptional activator. ZEBRA and cellular AP-1 bZip activators, such as c-Fos, have homologous DNA-binding domains, and their DNA-binding specificities overlap. Moreover, EBV latency can also be disrupted by phorbol esters, which act, in part, through AP-1 activators. It is not known whether ZEBRA and AP-1 factors play equivalent roles in the initial stages of reactivation. Here, the contribution of ZEBRA's basic DNA recognition domain to disruption of latency was analyzed by comparing ZEBRA with chimeric mutants in which the DNA recognition domain of ZEBRA was replaced with the analogous domain of c-Fos. Chimeric ZEBRA/c-Fos proteins overexpressed in *Escherichia coli* bound DNA with the specificity of c-Fos; they bound a heptamer AP-1 site and an octamer TPA response element (TRE). ZEBRA bound the AP-1 site and an array of ZEBRA response elements (ZREs). In assays with reporter genes, both ZEBRA and ZEBRA/c-Fos chimeric mutants activated transcription from Zp, a promoter of the ZEBRA gene (BZLF1) that contains the TRE and multiple ZREs. However, despite their capacity to activate reporters bearing Zp, neither ZEBRA nor the c-Fos chimeras activated transcription from Zp in the context of the intact latent viral genome. In contrast, ZEBRA but not ZEBRA/c-Fos chimeras activated Rp, a second viral promoter that controls ZEBRA expression. Hence, transcriptional autostimulation by transfected ZEBRA occurred preferentially at Rp. Both ZEBRA and the ZEBRA/c-Fos chimeras activated transcription from reporters with multimerized AP-1 sites. However, in the context of the virus, only ZEBRA activated the promoters of two early lytic cycle genes, BMRF1 and BMLF1, that contain an AP-1 site. Thus, overexpression of an activator that recognized AP-1 and TRE sites was not sufficient to activate EBV early lytic cycle genes.

The switch between Epstein-Barr virus (EBV) latency and lytic replication, as studied in cultured human B-lymphoid cells, is hypothesized to consist of three events. The first step, occurring within 1 to 2 h after addition of an inducing stimulus, is activation of viral immediate-early gene expression through the action of cellular transcription factors binding to Zp, a promoter immediately upstream of BZLF1, the gene encoding ZEBRA, the immediate-early transactivator (14, 21, 43, 49, 58). The second stage is thought to be autostimulation of BZLF1 transcription at two promoters, Zp and Rp (7, 19, 39, 43, 58). The third stage results in activation by ZEBRA of early viral lytic cycle genes whose promoters contain ZEBRA binding sites (13, 17, 30, 32, 39–41, 50, 53, 55, 58, 63). ZEBRA also binds to the lytic origin of EBV DNA replication and acts as an essential replication factor (18, 56).

The first phase of the activation cascade may be triggered by addition of inducing agents, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), *n*-butyrate, and calcium ionophores, or by cross-linking of the surface of the B cell with anti-immunoglobulin (anti-Ig) (14, 16, 42, 59, 62, 65). The second and third stages of activation may be reproduced by infection with stocks of EBV that contain het DNA, from which ZEBRA expression is constitutive, or by transfection of plasmids that overexpress ZEBRA (11, 12, 29, 46, 54, 60).

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ZEBRA is a modular protein similar to other transcriptional activators of the bZip family, such as the cellular AP-1 activators c-Fos and c-Jun (1, 9, 31, 37, 48, 50, 52, 57, 64). The activating domain of ZEBRA consists of 93 amino acids, located in the amino terminus of the protein, that are indispensable for transcriptional activation and an accessory activation domain found in the carboxy-terminal 18 amino acids (aa) (10, 22, 45). ZEBRA's DNA-binding domain consists of three elements: aa 178 to 196 are basic amino acids, homologous to those of c-Fos and c-Jun, that determine the specificity of DNA recognition (9, 17, 50); aa 197 to 227, postulated to form a coiled-coil region, are responsible for homodimerization (20, 36); and a putative regulatory region, aa 167 to 177, contains a serine, S-173, which, when phosphorylated by casein kinase II, abrogates DNA binding in vitro (35). The nuclear targeting signal overlaps the basic domain (26, 44). The general organization of the DNA-binding domain of cellular bZip activators is similar to that of ZEBRA (17, 35).

In the present report, the technique of chimeric mutagenesis was employed to explore the specific requirement of the ZEBRA DNA recognition domain for disruption of latency. We asked whether substitution of the DNA recognition domain of ZEBRA with that of c-Fos would alter ZEBRA's capacity to promote entry into the EBV lytic cascade. Several observations provoked this inquiry. ZEBRA and c-Fos both bind a canonical AP-1 heptamer site, TGAGTCA, and activate transcription of reporter genes containing AP-1 sites in their upstream regions (9, 17, 41, 61). A number of ZEBRA-responsive EBV promoters contain AP-1 sites in addition to ZEBRA-specific response elements (ZREs) with the consensus **T** · G or T · A or T · **G** · T or C · C or A · **A** (invariant nucleotides are

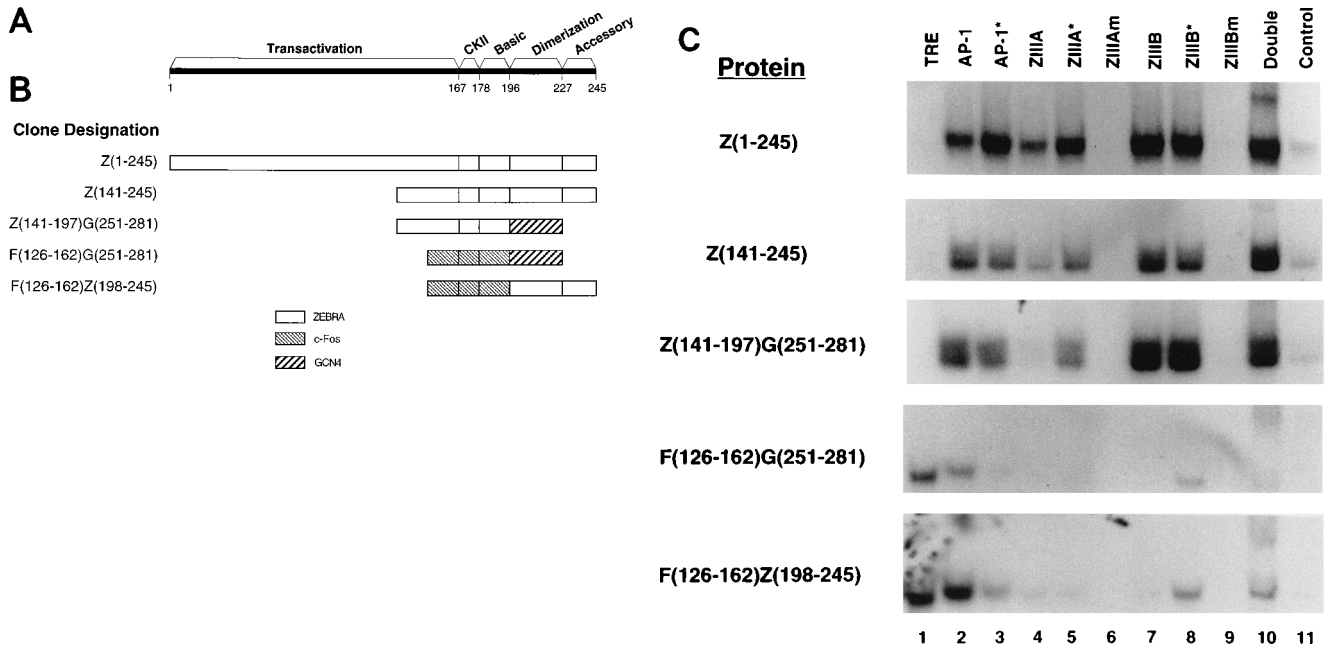


FIG. 1. DNA binding by ZEBRA, ZEBRA/GCN4, ZEBRA/c-Fos, and c-Fos/GCN4 chimeric proteins. (A) Schematic diagram of the functional domains of the ZEBRA protein. CKII, casein kinase II phosphorylation site. (B) Diagrams of proteins expressed in *E. coli* as TrpE fusions. Vertical lines demarcate the domains of ZEBRA. (C) DNA-binding assays. Equal amounts of each protein were tested for the capacity to cause electrophoretic mobility shifts of a panel of duplex oligonucleotides (see Table 1). Only the shifted portion of the gel is shown.

boldfaced) (13). Furthermore, in certain EBV-containing lymphoid cell lines, latency can be disrupted by TPA, whose effects are mediated by AP-1 transcription factors such as c-Fos and c-Jun (2, 3). Zp itself can be activated by TPA (21). The effect is partially mediated by an octamer sequence, TGACGTCA, located at −55 relative to the transcriptional start site (21). This octamer sequence, variably called a TPA response element (TRE), a ZII site, or a Z-AP-1 octamer, is identical to the TPA-responsive element in the c-Jun promoter and will be referred to here as a TRE (2). When present in a double-stranded oligonucleotide, this site is bound by proteins containing the c-Fos or c-Jun DNA recognition domain but not by ZEBRA (21, 61). These data suggested that recognition of the AP-1 and TRE sites might be sufficient to trigger entry of EBV into the lytic cycle.

In the present experiments, the early events in the EBV lytic cycle were analyzed following introduction of chimeric activators containing the DNA recognition domain of either ZEBRA or c-Fos. The chimeras were evaluated for DNA-binding specificity, transcriptional activation capacity, ability to disrupt EBV latency, and autostimulation of Zp and Rp. Chimeric mutants containing the c-Fos DNA-binding domain were similar to ZEBRA in their capacity to bind AP-1 sites and to activate transcription from Zp fused to reporter genes. Surprisingly, neither transfected ZEBRA nor ZEBRA/c-Fos chimeras stimulated Zp from the latent virus. Thus, the activity of Zp in reporter assays in response to ZEBRA or ZEBRA/c-Fos chimeras did not reflect its activity in the context of the viral genome. Moreover, only those activators with the ZEBRA DNA-binding domain drove EBV into the lytic cycle.

MATERIALS AND METHODS

Plasmids for expression in *Escherichia coli*. All proteins were expressed as TrpE fusions in pATH vectors (Fig. 1B) (34). ZEBRA-encoding sequences were derived from the BZLF1 cDNA (43, 55). The full-length ZEBRA cDNA, Z(1–245), and the deletion mutant Z(141–245) have been described elsewhere (61). A Fos-GCN4 chimera, F(126–162)G(251–281), contains the c-Fos basic DNA-binding domain fused to the GCN4 leucine zipper at an introduced *Xho*I site (37). A chimeric clone, Z(141–197)G(251–281), expressing the ZEBRA basic domain fused to the GCN4 leucine zipper was constructed by ligating a blunted *Xho*I site at the 5′ end of *GCN4* sequences to a blunted *Pst*I site at codon 197/198 in Z(141–245). A chimera expressing the c-Fos basic domain fused to the ZEBRA dimerization domain, designated F(126–162)Z(198–245), was made by ligating a blunted *Xho*I site at the 3′ end of c-*fos* to the blunted *Pst*I site of Z(141–245). The spacing between the basic region and the dimerization domain was conserved in the three chimeras F(126–162)G(251–281), F(126–162)Z(198–245), and Z(141–197)G(251–281).

Plasmids for eukaryotic expression. All genes were expressed following fusion to the cytomegalovirus (CMV) immediate-early gene promoter-enhancer in the vector pHD1013 (Fig. 2A) (15). The construction of Z(1–227)VP(411–490) has been described elsewhere (6). Z(1–171)F(137–162)Z(198–245) was constructed by PCR mutagenesis of the template F(126–162)Z(198–245). The 5′ oligonucleotide primer 5′TCTCCTGAATGCGAAGAG generated a *Bsm*I site in frame at codon 171 of ZEBRA. The carboxy termini of Z(1–245) and F(126–162)Z(198–245) were then swapped at the *Bsm*I site. Z(1–197)G(251–281) was constructed by substituting sequences beyond the *Bsm*I site of Z(1–245) with those of Z(141–197)G(251–281). Z(1–197)G(251–281)Z(229–245) was constructed by generating a PCR product encompassing all of Z(1–197)G(251–281) by using an oligonucleotide 5′ to ZEBRA coding sequences and the oligonucleotide 5′GCGGTT CGCCAACTAATTTC at the 3′ end of *GCN4* sequences. This DNA fragment was ligated to a second PCR product generated with 5′TCCATTATCCCCCGG ACAC at its 5′ end and another oligonucleotide consisting of ZEBRA 3′ coding sequences. In this way, ZEBRA's accessory activation domain, aa 228 to 245, was added. Z(1–177)F(143–162)Z(198–245) was constructed by ligating two PCR products. One product was generated with an oligonucleotide complementary to ZEBRA's N-terminal nucleotide sequence and 5′TATTTCTAGTTCAGAATC as the distal primer, with Z(1–245) as the template. The other PCR product was made from a template of F(126–162)Z(198–245) with 5′CGAAGAGAACGGA ATAAG and an oligonucleotide representing the C terminus of ZEBRA. The clone CMV Z(1–171)F(137–162)Z(198–227)VP(411–490) was made by ligating two fragments: (i) CMV Z(1–171)F(137–162)Z(198–245) cut with *Xho*I, blunted with Klenow, and then cut with *Bam*HI and (ii) CMV Z(1–227)VP(411–490) cut with *Pst*I, blunted with T4 DNA polymerase, and then cut with *Bam*HI. All inserts were sequenced by the dideoxynucleotide chain termination protocol. c-Junε, a c-Jun derivative that lacks the ε inhibitory domain, was expressed on a plasmid by using the Rous sarcoma virus long terminal repeat (5).

Reporter plasmids. Reporters for transcriptional activation in human B cells were derivatives of pE4CAT, which contains a minimal adenovirus E4 promoter fused to the chloramphenicol acetyltransferase (CAT) gene (Fig. 3A) (28). The reporter Zp/E4CAT contains a *Bam*HI-to-*Dra*I subfragment of EBV *Bam*HI Z.

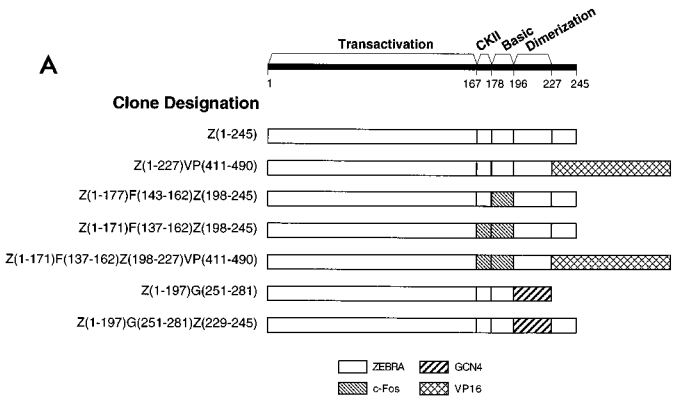
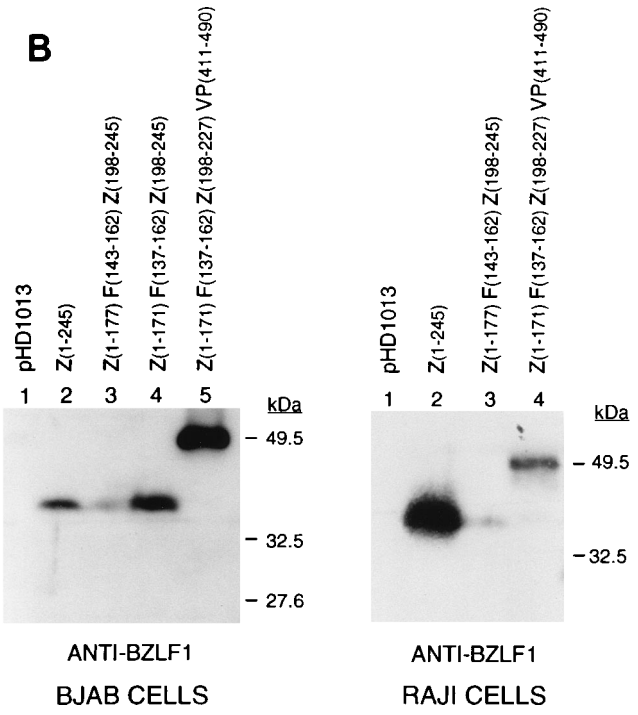


FIG. 2. Expression of ZEBRA/c-Fos chimeric proteins in B lymphocytes. (A) Diagram of chimeric ZEBRA proteins. Constructs were derivatives of the BZLF1 cDNA; they were driven by the CMV immediate-early promoter. (B) Expression of ZEBRA and ZEBRA/c-Fos chimeras. Approximately 10 μg of DNA was transfected by electroporation into BJAB or Raji cells. Cell extracts were prepared 20 h after transfection and analyzed by immunoblotting. ZEBRA was detected with a rabbit polyclonal antibody (60).



EAp/E4CAT contains a *Bam*HI-to-*Rsa*I subfragment of the EBV *Bam*HI M fragment. Reporter plasmids containing variable numbers of AP-1 sites were constructed by annealing two single-stranded oligonucleotides, 5′TCATGAGTC AGTGT and 5′GAACACTGACTCAT, and then self-ligating them to make oligomers of different lengths. The oligomers were blunted with T4 DNA polymerase and cloned into the *Eco*RV site of pBluescript (pBS KSII). The number of AP-1 repeats cloned into each pBS recombinant was determined by nucleotide sequencing. Inserts containing a desired number of repeats were excised with *Xho*I and *Pst*I and moved to pE4CAT cut with the same enzymes.

Protein expression. TrpE fusion proteins were expressed in *E. coli* AG1 as described before (61). Protein concentrations were measured by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels and equalized for use in DNA-binding assays. The amount of activator protein generated from eukaryotic expression plasmids in human BJAB and Raji cells was assessed by immunoblotting 20 h after electroporation (6). Cell extracts were electrophoresed through a 10% polyacrylamide–SDS gel, transferred to nitrocellulose, and reacted with a 1:200 dilution of rabbit antibody raised to TrpE/ZEBRA exon 1, followed by 1 μCi of ^{125}I-protein A (60).

EMSAs. For electrophoretic mobility shift assays (EMSAs), *E. coli* extract containing 300 ng of fusion protein was incubated with 4×10^{-13} duplex oligonucleotides (Table 1) that had been radiolabeled with ^{32}P at their 3′ recessed ends. The buffers and electrophoresis conditions have been described before (60).

Assays for transcriptional activation. Raji or BJAB cells (1.5×10^7) were suspended in 0.4 ml of growth medium (RPMI 1640 plus 8% fetal calf serum) in an electroporation cuvette with a 0.4-cm gap. Ten micrograms of reporter and of activator plasmid were added, and the cells were exposed to 0.25 kV and 960 μF with a Bio-Rad Gene Pulser. Extracts of cells harvested 48 h after electroporation were assayed for CAT activity. The stimulation index is the ratio of percent acetylation obtained with an activator to percent acetylation measured on the same reporter cotransfected with the vector pHD1013.

Assays for disruption of latency. Raji or B95-8 cells were electroporated with 10 μg of activator. Disruption of latency was assessed by expression of lytic cycle mRNAs or proteins. Cytoplasmic RNA samples were prepared 20 h after transfection by a modification of a previously described protocol (6). The lysis buffer contained 150 mM NaCl, 10 mM Tris (pH 8.0), and no $MgCl_2$. The SDS and proteinase K steps were substituted with a purification in 7 M urea–350 mM NaCl–10 mM Tris (pH 7.5)–20 mM EDTA. The RNA was electrophoresed in a 1% agarose–6% formaldehyde gel in 20 mM MOPS (morpholinepropanesulfonic acid; pH 7). The gel was transferred to Nytran (Schleicher and Schuell) and hybridized with a ^{32}P-labeled probe. Probes were derived from BMRF1, a 531-bp *Eag*I fragment (nucleotides 80141 to 80672 in the B95-8 sequence [4]), BMLF1 (82917 to 84233), and a *Taq*I-to-*Sal*I subfragment of BRLF1 (104577 to 105297). RNA blots were reprobed with a 1.8-kbp portion of the β-actin cDNA.

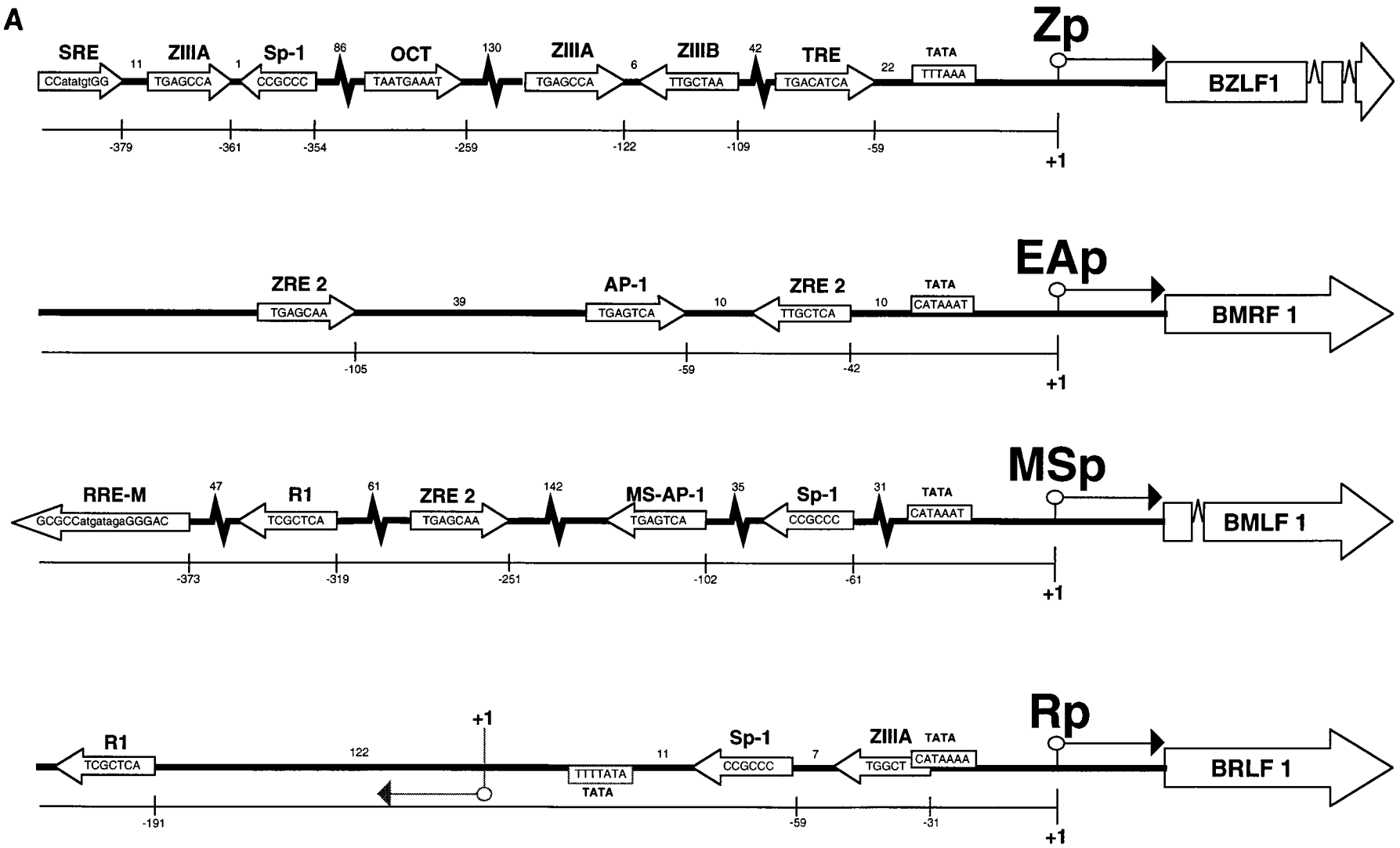
Protein extracts prepared 72 h after electroporation were assayed for EA-D (BMRF1) expression by immunoblotting. Extracts were electrophoresed in a 10% polyacrylamide–SDS gel and transferred to nitrocellulose. The R3.1 monoclonal antibody (51) was used at a 1:800 dilution, followed by a 1:100 dilution of rabbit anti-mouse Ig and then 1 μCi of ^{125}I-radiolabeled staphylococcal protein A.

Assays for autostimulation. Raji cells were transfected as described above. RNA was prepared 20 h later with the Trizol reagent (GIBCO). Northern (RNA) blots were hybridized with a 623-bp *Bam*HI-to-*Pst*I subfragment of the BZLF1 cDNA, with the BRLF1 probe, or with a 2.2-kb *Pst*I subfragment of the vector containing the CMV immediate-early gene promoter-enhancer.

RESULTS

DNA-binding specificity of ZEBRA and ZEBRA/c-Fos chimeras. The creation of ZEBRA/c-Fos chimeric mutants for use in latency disruption experiments was predicated on the assumption that the basic and dimerization domains of bZip proteins are modular. When the basic regions of two bZip proteins are exchanged, the resulting chimeric protein binds DNA with the specificity of the basic region (1, 9, 48). ZEBRA was tested for modular domain structure by swapping its basic domain with that of c-Fos. Chimeric proteins containing either the ZEBRA or the c-Fos DNA recognition domain linked to the dimerization domains of ZEBRA or the yeast bZip activator GCN4 were overexpressed in *E. coli*, normalized for protein content, and tested for DNA-binding specificity in EMSAs. These assays used a panel of duplex oligonucleotides (Table 1) that were derived from two viral promoters, Zp and MSp, the promoter of the BMLF1 gene (Fig. 3A).

Figure 1C shows that chimeric proteins containing the ZEBRA DNA-binding domain recognized a different subset of oligonucleotides than were recognized by chimeras containing the c-Fos DNA recognition domain. Full-length ZEBRA protein [Z(1–245)] bound to oligonucleotides containing a heptamer AP-1 site (lane 2) and naturally occurring heptamer ZREs, such as ZIIIA and ZIIIB (lanes 4 and 7). Chimeras containing the c-Fos basic domain also bound the heptamer AP-1 site but failed to bind ZIIIA and ZIIIB. Binding by ZEBRA was not markedly affected by single-point mutations in the oligonucleotides designated AP-1*, ZIIIA*, and ZIIIB* (lanes 3, 5, and 8). However, c-Fos-mediated DNA binding was decreased on AP-1* (lane 3) and slightly increased on ZIIIB* (lane 8). Only chimeras containing the c-Fos basic domain bound to the oligonucleotide containing the TRE site found in Zp (lane 1). When the dimerization regions of two bZip pro-


A
Zp
SRE
CCatatgtGG
11
ZIIIA
TGAGCCA
1
Sp-1
CCGCCC
86
OCT
TAATGAAAT
130
ZIIIA
TGAGCCA
6
ZIIIB
TTGCTAA
42
TRE
TGACATCA
22
TATA
TTTAAA
BZLF1
-379
-361
-354
-259
-122
-109
-59
+1
EAp
ZRE 2
TGAGCAA
39
AP-1
TGAGTCA
10
ZRE 2
TTGCTCA
10
TATA
CATAAAT
BMRF 1
-105
-59
-42
+1
MSp
RRE-M
GCGCCatgatagaGGGAC
47
R1
TCGCTCA
61
ZRE 2
TGAGCAA
142
MS-AP-1
TGAGTCA
35
Sp-1
CCGCCC
31
TATA
CATAAAT
BMLF 1
-373
-319
-251
-102
-61
+1
Rp
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R1
TCGCTCA
122
TTTTATA
TATA
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Sp-1
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CATAAAA
BRLF 1
-191
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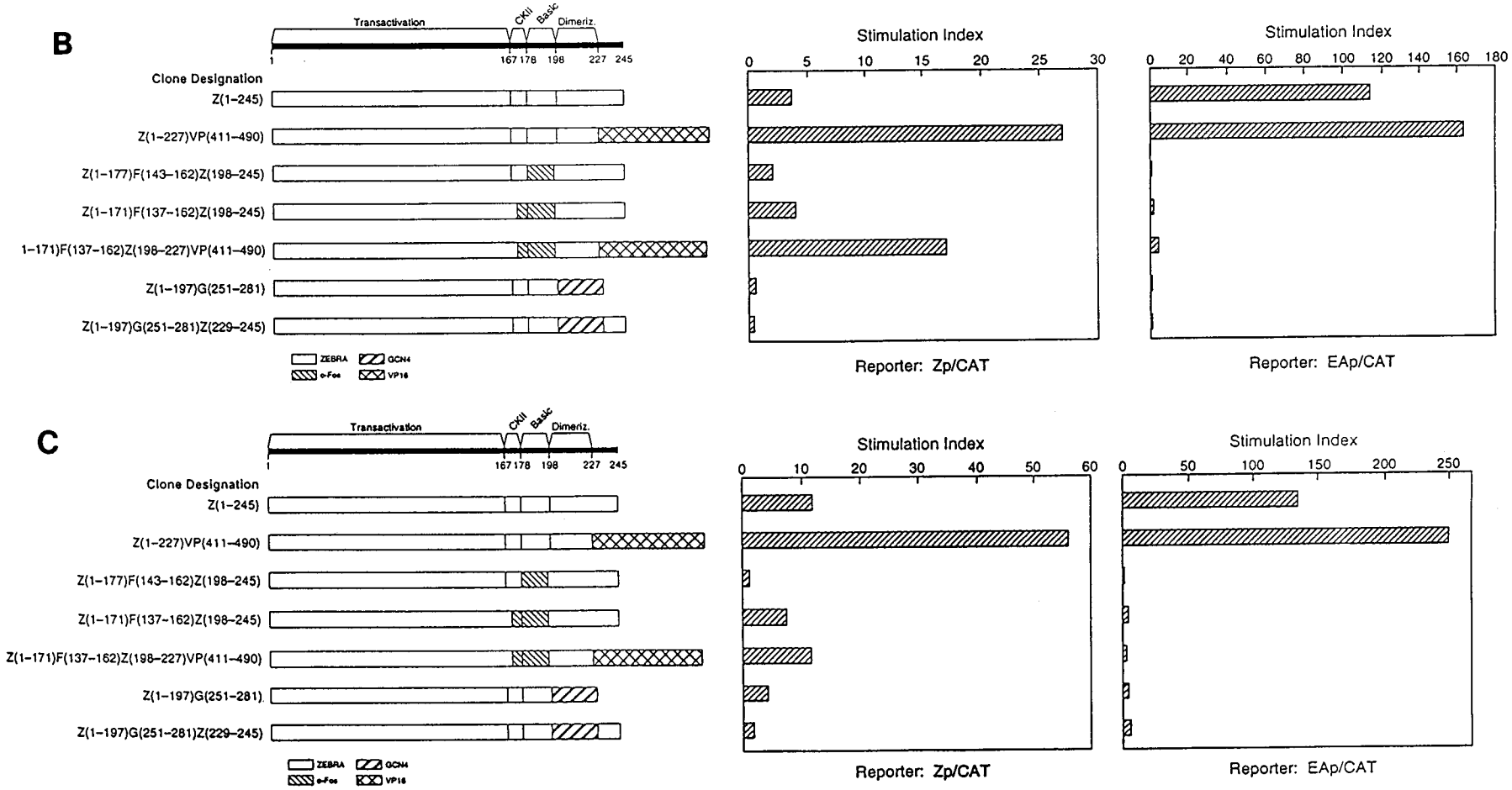


FIG. 3. Transcriptional activation by ZEBRA, ZEBRA/c-Fos, and ZEBRA/GCN4 chimeras. (A) Diagram of the Zp and EAp promoters that were fused to a CAT reporter. The promoters of the BMLF1 and BRLF1 genes, MSp and Rp, respectively, that were scored in disruption-of-latency experiments (Fig. 4 and 5) are also diagrammed. (B and C) Transcriptional activation assays. Cells were transfected by electroporation with 10 μg of each activator and Zp/E4CAT or EAp/E4CAT. Data are the means for three experiments. (B) In BJAB cells, the background with vector alone was 2.0% acetylation with Zp/E4CAT and 0.33% acetylation with EAp/E4CAT. (C) In Raji cells, the background activity was 1.95% acetylation on Zp/E4CAT and 0.44% acetylation on EAp/E4CAT.

TABLE 1. Duplex oligonucleotides used in EMSAs[a]

Designation	Oligonucleotide	Bound by:	
		ZEBRA	c-Fos
Zp TRE	CTAG**AAACCA**<u>**TGACATCA**</u>**CAGAGGA**TC	−	++
MSp AP-1	T**CTTCA**<u>**TGAGTCA**</u>**GTGCTTC**	++	++
MSp AP-1*	T**CTTCA***T*<u>**AGTCA**</u>**GTGCTTC**	++	+
Zp ZIIIA	CTAG**CTATGCA**<u>**TGAGCCA**</u>**CAG**ATC	++	−
Zp ZIIIA*	CTAG**CTATGCA**<u>**TGAGC***A*</u>**ACAG**ATC	++	−
Zp ZIIIAm	CTAG**CTATGCA***GA*<u>**A***TT*</u>**CACAG**ATC	−	−
Zp ZIIIB	CTAG**CAGGCA**<u>**TTGCTAA**</u>**TGTACC**GATC	++	−
Zp ZIIIB*	CTAG**CAGGCA**<u>**TTGCT***C*</u>**ATGTACC**GATC	++	+
Zp ZIIIBm	CTAG**CAGG***ATCC*<u>**GCTAA**</u>**TGTACC**GATC	−	−
Zp Double	G**ACTATGCA**<u>**TGAGCCA**</u>**CAGGCA**<u>**TTGCTAA**</u>**TGTACC**GA	++	+
Control	TGGCATGCTGCTGACATCTGGC	+	−

[a] Bold sequences are derived from EBV DNA. Underlined sequences are ZREs. Italicized sequences are mutant. *, mutations which allow ZEBRA to bind DNA (56). m, mutations which eliminate ZEBRA binding. Zp, promoter of the BZLF1 gene. MSp, promoter of the BMLF1 gene. ZIIIA and ZIIIB are sites in Zp previously found to confer a response to ZEBRA (19). Data for binding by ZEBRA and c-Fos/ZEBRA chimeras are shown in Fig. 1C. ++, strong binding; +, binding; −, no binding.

teins are swapped, the resulting chimera is affected in its selection of a dimerization partner but not in its DNA-binding specificity (37, 38, 57). Figure 1C shows that substitution of the ZEBRA dimerization region with that of the yeast activator GCN4, as in the chimera Z(141–197)G(251–281), had little effect on ZEBRA's DNA-binding specificity in vitro. The single exception was that Z(141–197)G(251–281) consistently bound a ZIIIA site (lane 4) with less affinity than did Z(141–245), a construct that contained ZEBRA's own dimerization domain. The DNA-binding specificities of the two chimeras with the c-Fos basic domain, F(126–162)G(251–281), which had the GCN4 dimerization domain, and F(126–162)Z(198–245), which had the ZEBRA dimerization domain, were identical in vitro.

Creation of ZEBRA/c-Fos chimeras and expression in human B cells. A set of constructs were designed to assess the capacity of chimeric proteins containing either ZEBRA's own DNA recognition domain or that of c-Fos to activate transcription and to disrupt EBV latency in human B-cell lines (Fig. 2A). All chimeras retained ZEBRA's amino-terminal activation domain. In one ZEBRA/c-Fos chimera, Z(1–171)F(137–162)Z(198–227)VP(411–490), the carboxy-terminal 18 aa of ZEBRA were replaced with VP16 sequence. This construct was compared with Z(1–227)VP(411–490), a construct previously shown to possess enhanced capacity to drive early-gene expression as the result of substitution of the strong activation domain of VP16 for the carboxy-terminal activation module of ZEBRA (6). We tested two other ZEBRA/c-Fos chimeras. In Z(1–177)F(143–162)Z(198–245), the basic region of ZEBRA was replaced with that of c-Fos. In the second chimera, Z(1–171)F(137–162)Z(198–245), both the basic and regulatory regions of ZEBRA were replaced with the comparable regions of c-Fos.

The ZEBRA/c-Fos chimeric proteins diagrammed in Fig. 2A were expressed to a level comparable to that of ZEBRA in EBV-negative human B cells (Fig. 2B and data not shown). Furthermore, addition of the VP16 activation domain to ZEBRA or to the ZEBRA/c-Fos chimeras did not affect the level of expression (data not shown). Higher ZEBRA protein levels were measured following expression of Z(1–245) in EBV-positive cells than in EBV-negative cells, possibly as a result of autostimulation of endogenous ZEBRA expression from the latent virus (see Discussion). No protein corresponding to the molecular weight of the endogenous ZEBRA was detected in Raji cells following transfection of Z(1–171)F(137–162)Z(198–227)VP(411–490), a ZEBRA/c-Fos chimera that also contained a VP16 activation domain (Fig. 2B, lane 4). Thus, unlike native ZEBRA, constructs with the c-Fos basic domain could not stimulate ZEBRA synthesis from the endogenous virus.

ZEBRA/c-Fos chimeras activate transcription from Zp but not EAp reporters. ZEBRA and the ZEBRA chimeras were assayed for their abilities to activate a synthetic reporter composed of five copies of an AP-1 heptamer fused to a minimal promoter and the CAT gene. The assays were conducted by cotransfection of activator and reporter into EBV-negative human B cells (BJAB). The chimera Z(1–171)F(137–162)Z(198–245) reproducibly activated the model reporter plasmid to two- to threefold the level of wild-type ZEBRA over a range of input doses of activator plasmid (data not shown). These experiments demonstrated that c-Fos could efficiently substitute for ZEBRA's DNA recognition domain in activation of transcription from multimerized AP-1 sites.

The activators were then examined for their capacity to stimulate transcription of reporter genes bearing the upstream sequences of two known ZEBRA-responsive EBV promoters. Zp (19) and EAp (30), the promoter of the BMRF1 gene, contain various numbers and orientations of ZREs, octamer TREs, and AP-1 sites (Fig. 3A). These assays were conducted in EBV-negative BJAB cells (Fig. 3B) and EBV-positive Raji cells (Fig. 3C).

ZEBRA itself stimulated transcription from Zp and EAp, while ZEBRA/c-Fos chimeras could activate transcription from Zp but not EAp (Fig. 3B and C). In response to ZEBRA, EAp was stimulated 10-fold more strongly than Zp. The lower level of activation of Zp was partially due to a four- to sixfold-higher level of background activation by cell factors on Zp than on EAp, as recently described (13). Substitution of the carboxy-terminal 18 aa of ZEBRA with VP16 sequence led to enhanced transactivation of Zp and EAp in both EBV-positive and EBV-negative cells. However, augmentation of the activation potency of the ZEBRA/c-Fos chimera with a comparable substitution of VP16 did not restore its capacity to activate EAp.

Nonetheless, all the ZEBRA/c-Fos chimeras were able to activate Zp. The chimera containing the c-Fos basic domain as well as VP16 was equivalent to ZEBRA in activating Zp in Raji cells (Fig. 3C) and about threefold more potent than ZEBRA in activating Zp in BJAB cells (Fig. 3B). These experiments indicated that both Zp and EAp could be activated through ZEBRA's DNA recognition domain; only Zp was

stimulated by chimeric activators containing the DNA recognition domain of c-Fos.

Disruption of latency. Transcription of two EBV early lytic cycle genes, BMRF1 and BMLF1, from a latent EBV genome following transfection of a clone expressing ZEBRA or a ZEBRA/c-Fos chimera was used as a measure of the ability to initiate the EBV lytic genetic program. ZEBRA is known to transactivate EAp, the BMRF1 promoter, directly, whereas activation of MSp, the BMLF1 promoter, requires other viral early lytic cycle gene products (32). Introduction of Z(1–245) or Z(1–227)VP(411–490) into Raji (Fig. 4A and B), B95-8 (Fig. 4C), or Daudi (not shown) cells resulted in transcription of BMRF1 (Fig. 4A and C) and BMLF1 (Fig. 4B). The abundance of these mRNAs was greater following transfection of ZEBRA activators containing VP16 than following introduction of ZEBRA alone (compare Fig. 4A and C, lane 2, with Fig. 4A and B, lane 7, and Fig. 4C, lane 3). Transfection of chimeric constructs containing substitutions of ZEBRA's basic domain with c-Fos failed to induce expression of lytic cycle mRNAs. These effects were tested over a wide range of input DNA concentrations. In addition, Z(1–245) and Z(1–227) VP16(411–490) each caused an increase in the early lytic cycle protein EA-D, but the chimeric ZEBRA/c-Fos/VP16 protein did not (Fig. 4D, lanes 3 to 5). Thus, a ZEBRA chimera containing the c-Fos basic domain was unable to drive EBV lytic gene expression even when its transcriptional activation potency was enhanced by the addition of VP16.

Similarly, a nonchimeric transcriptional activator which specifically binds TREs, c-Junε, was unable to activate lytic EBV gene expression from the endogenous virus (Fig. 4C, lane 6); however, this construct was able to activate transcription from reporter genes containing multimerized AP-1 sites (5) (data not shown).

Comparing autostimulation by ZEBRA and ZEBRA/c-Fos chimeras. The next series of experiments were prompted by an apparent paradox. ZEBRA/c-Fos/VP16 chimeras strongly activated Zp/E4CAT reporters (Fig. 3B and C); yet these same chimeras lacked the ability to disrupt latency, as assessed by transcription or translation of early lytic cycle genes (Fig. 4A to D). If ZEBRA/c-Fos chimeras could activate Zp, why were they unable to initiate the EBV lytic cycle through activation of expression of the BZLF1 gene encoding the endogenous ZEBRA protein? Therefore, we sought to determine whether ZEBRA with a native or c-Fos-substituted DNA recognition domain could stimulate expression of mRNAs containing BZLF1 from the latent viral genome.

By cloning cDNAs, Manet and her colleagues found that several BZLF1-specific mRNAs were transcribed following chemical induction of the lytic cycle in Raji cells (Fig. 5A) (43). A 4.0-kb and a 3.0-kb mRNA, differing in the presence or absence of an untranslated leader, encompass both the upstream BRLF1 open reading frame and the BZLF1 gene, while a 1.0-kb mRNA encodes only BZLF1. Transcription of the bicistronic mRNAs is controlled by Rp, a promoter which is upstream of the BRLF1 gene, while the smaller RNA is controlled by Zp. In addition, a 1.3-kb mRNA contains the BRRF1 lytic cycle gene. Deleted, bicistronic BZLF1/BRLF1 mRNAs, called RAZ, are of low abundance and are not regularly detected by Northern analysis (25). The presence of these BZLF1-specific mRNAs was assessed after transfection of Raji cells with ZEBRA or ZEBRA/c-Fos chimeras (Fig. 5B and C).

The previously described BZLF1 mRNAs were detected on Northern blots of RNA prepared from chemically induced cells (Fig. 5B, lane 1, and Fig. 5C, lanes 1 and 3). The 4.0- and 3.0-kb bicistronic mRNAs were seen following transfection of ZEBRA and were induced three- to fivefold more strongly by ZEBRA/VP16 than by ZEBRA (Fig. 5B, lanes 4 and 5 and 8 and 9, and Fig. 5C, lanes 6 and 7). However, the 1.0-kb BZLF1 mRNA was not detected following transfection of Z(1–245) or Z(1–227)VP(411–490) (Fig. 5B, lanes 4 and 5 and 8 and 9). Instead, 1.3- and 1.4-kb mRNAs were seen; these mRNAs represent the transcripts from the transfected expression plasmid, since they were also detected by a CMV probe (data not shown). Thus, in the context of the virus, ZEBRA autostimulates BZLF1 expression from Rp but not from Zp. In contrast, transfection of a plasmid expressing the ZEBRA/c-Fos/VP16 chimeric protein failed to induce the bicistronic transcripts from Rp or monocistronic transcript from Zp (Fig. 5B, lanes 6 and 10, and Fig. 5C, lane 8).

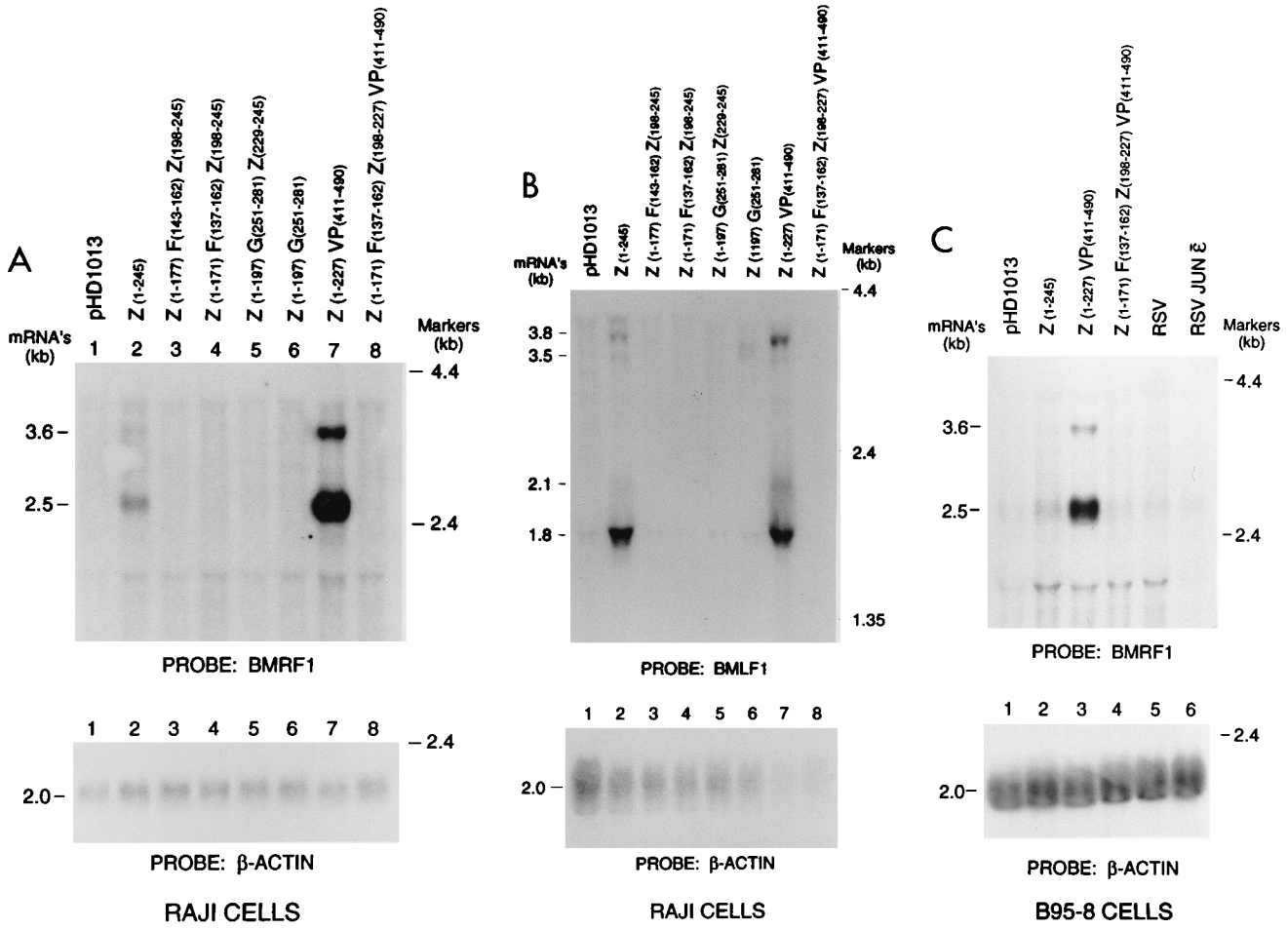
In summary, these experiments showed that activators with ZEBRA's own DNA recognition domain autostimulated Rp. They did not autostimulate Zp, even though they were able to activate Zp/E4CAT reporters. Chimeric mutants with the c-Fos DNA recognition domain did not activate Zp or Rp from the latent virus, even though these chimeric activators activated Zp in reporter constructs. Moreover, the ZEBRA/c-Fos chimeras could not bypass the requirement for endogenous ZEBRA. They could not directly stimulate promoters of EBV lytic genes even though the promoters contained AP-1 sites.

DISCUSSION

ZEBRA/c-Fos chimeras fail to disrupt latency. ZEBRA and cellular AP-1 activators bind AP-1 sites in vitro and activate transcription from reporter genes that contain oligomerized AP-1 sites (17, 41, 61). Furthermore, several ZEBRA-responsive promoters contain AP-1 sites or TREs (8). Therefore, our experiments sought to determine whether the EBV lytic cycle could be activated exclusively through AP-1 sites or TREs. Transfection of chimeric activators that recognize these sites in in vitro DNA-binding assays (Fig. 1) and activate reporter genes containing such sites (Fig. 3) nevertheless did not stimulate EBV to enter the lytic cascade (Fig. 4). The results of DNA-binding assays and assays for transcriptional activation with reporters with synthetic or natural promoters were discordant from those of assays for activation of natural promoters in the context of the latent viral genome.

The failure of the ZEBRA/c-Fos chimeras to disrupt latency presented several paradoxes. One paradox was the failure of ZEBRA/c-Fos chimeras to activate EAp or MSp, promoters containing AP-1 sites, in reporter constructs (Fig. 3B and C) or in the context of the intact EBV genome (Fig. 4A, C, and D); yet chimeric proteins with the c-Fos DNA recognition domain and the ZEBRA dimerization domain efficiently bound an AP-1 site derived from MSp in vitro (Fig. 1C). Furthermore, ZEBRA/c-Fos chimeric proteins strongly activated reporters with multimerized AP-1 sites in human B cells (data not shown). Binding of the single AP-1 sites in EAp or MSp may not be sufficient to activate these promoters (30, 32). Binding of the AP-1 site may need to be accompanied by contact with another cellular protein, such as c-Myb or the EBV activator Rta (32, 33, 53). Both the ZREs and the AP-1 site may need to be bound to activate transcription. The occupation of two or more sites might reflect transcriptional synergy by ZEBRA (8).

A second paradox was the capacity of ZEBRA/c-Fos chimeras to activate Zp when it was linked to a CAT reporter (Fig. 3) but their inability to activate Zp in the latent virus (Fig. 5B). Even augmentation of the activation potency of the ZEBRA/c-Fos chimera with VP16 did not enable it to activate Zp from the viral genome. There was a discrepancy between assays with plasmid reporters containing Zp and assays for transcriptional



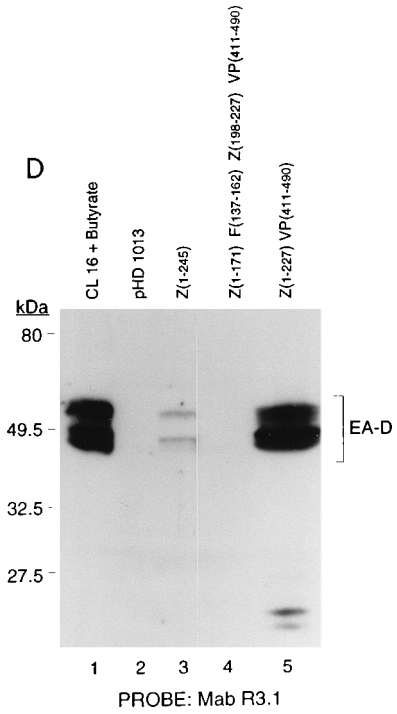


FIG. 4. Activation of expression of EBV early lytic cycle genes by ZEBRA, ZEBRA/c-Fos, and ZEBRA/GCN4 chimeras. (A to C) Activation of lytic cycle mRNAs. (A) BMRF1 transcripts in Raji cells. (B) BMLF1 transcripts in Raji cells. (C) BMRF1 in B95-8 cells. (D) Activation of EA-D (BMRF1) protein in Raji cells. The probe was monoclonal antibody R3.1.

activation of Zp from the viral genome. In the latent EBV genome, the TRE or other sites in Zp that are recognized directly or indirectly by c-Fos may be occluded by a specific cellular repressor or by chromatin. Alternatively, the TRE may be accessible, but binding of the TRE alone may not be sufficient to drive BZLF1 transcription from the viral genome. An unlikely explanation is that Zp is not functional in the viral genome. Zp activity, as evidenced by the presence of the monocistronic BZLF1 transcript, was readily detected following chemical induction (Fig. 5B).

Taken together, the experiments with the ZEBRA/c-Fos chimeras show that disruption of latency requires the ZEBRA basic domain and cannot be mediated by a potent activator which binds only to AP-1 and TRE sites. This conclusion is supported by experiments showing that introduction of c-Junε did not activate the lytic cascade (Fig. 4C). A clue to the importance of the ZEBRA basic domain in activating the EBV lytic cycle may be gleaned from a comparison of its amino acid sequence with that of c-Fos/c-Jun. The crystal structure of the c-Fos/c-Jun heterodimer bound to DNA (27) shows that four of the five amino acids of c-Fos/c-Jun that contact DNA are conserved in ZEBRA. These correspond to N-182, A-185,

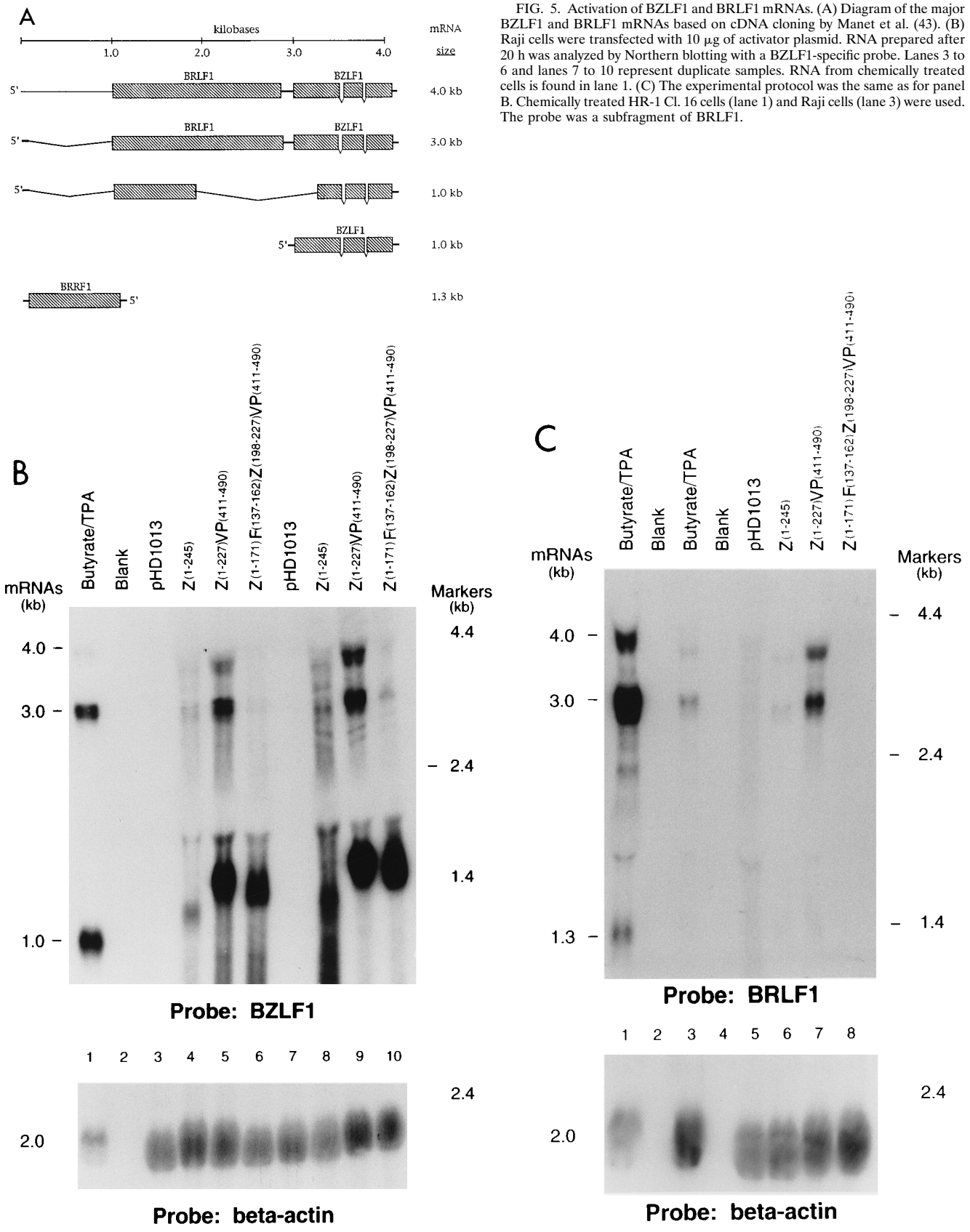


FIG. 5. Activation of BZLF1 and BRLF1 mRNAs. (A) Diagram of the major BZLF1 and BRLF1 mRNAs based on cDNA cloning by Manet et al. (43). (B) Raji cells were transfected with 10 μg of activator plasmid. RNA prepared after 20 h was analyzed by Northern blotting with a BZLF1-specific probe. Lanes 3 to 6 and lanes 7 to 10 represent duplicate samples. RNA from chemically treated cells is found in lane 1. (C) The experimental protocol was the same as for panel B. Chemically treated HR-1 Cl. 16 cells (lane 1) and Raji cells (lane 3) were used. The probe was a subfragment of BRLF1.

C-189, and R-190 of ZEBRA. The fifth amino acid, alanine in c-Fos/c-Jun, corresponds to S-186 in ZEBRA. This serine may account for the ability of ZEBRA to bind the ZREs and thus to drive EBV early lytic cycle gene expression.

ZEBRA autostimulation. The concept that ZEBRA autostimulates its own expression is derived from several previous experiments. In cells bearing het DNA, two polymorphic ZEBRA variants can be detected, one with the electrophoretic mobility of het ZEBRA and the other with the mobility of standard (HR-1) ZEBRA (60). This observation was interpreted as showing that ZEBRA expressed constitutively from het DNA stimulates expression of the standard gene. In transient-transfection assays, transfection of ZEBRA expression vectors stimulates expression of reporters containing Zp (13, 19) (Fig. 4). The ZREs in Zp (19) and Rp (58) are thought to mediate this autostimulation. ZEBRA has also been proposed to stimulate its own expression in a manner independent of binding DNA. For example, non-DNA-binding mutants of ZEBRA stimulate Zp reporters, presumably through interactions with cellular proteins bound to sites in Zp other than the ZREs (24). Also, inhibitors of protein synthesis block high-level expression of BZLF1 mRNAs in cells triggered into the lytic cycle by cross-linking cell surface Igs (23). High-level BZLF1 transcription is thought to be the result of autostimulation.

The experiments reported here are the first to analyze autostimulation by comparing activation of Zp reporters with activation of transcription of the BZLF1 gene in the latent viral genome. They also address whether autostimulation in the context of the viral genome takes place at Zp or Rp. Both ZEBRA and ZEBRA/VP16 chimeras strongly activated Zp/E4CAT, consistent with previous postulates about autostimulation at Zp (13, 19). However, when examined for their effects on the latent virus, neither of these activators induced expression from Zp at a time when mRNAs derived from Rp were abundant (Fig. 5B). This is in contrast to BZLF1 transcription in cells induced by surface Ig cross-linking (14, 23, 59) or chemical inducing agents (Fig. 5B). In these cells, Zp and Rp messages are equally abundant. Furthermore, they appear with the same kinetics following induction (14, 49, 59a) and have the same half life, about 3.5 h (data not shown).

One hypothesis for the absence of the 1.0-kb BZLF1 mRNA following transfection is that overexpression of ZEBRA suppresses transcription of BZLF1 from Zp. Such an effect is suggested by in vitro footprinting experiments, in which low-level binding of ZEBRA is seen at the start of transcription in Zp (39). This suppression may not occur when Zp/E4CAT reporters are used because the large amount of target DNA present in transiently transfected cells titrates the ZEBRA protein. However, suppression of Zp by ZEBRA could still occur in cells with latent EBV containing far less template. Another hypothesis is that Zp responds exclusively to cellular activators. These factors may only be transcribed or made active by posttranslational modification following stimulation of the cell by chemicals that mimic physiologic inducing stimuli.

We favor the hypothesis that the ZREs in Zp of the latent viral genome are blocked and hence unable to mediate stimulation by transfected plasmids that express ZEBRA. ZEBRA may activate the endogenous Zp only after the ZREs become accessible as the result of physiologic events that are mimicked by TPA and *n*-butyrate. By contrast, the ZRE in Rp is available at all times; therefore, Rp, and not Zp, is the major site of autostimulation following introduction of ZEBRA expression vectors.

Models for entry into the EBV lytic cascade. The simplest model (19, 39, 58, 63) proposed for regulation of the EBV lytic cycle suggests that the two promoters that control ZEBRA expression, Zp and Rp, are inactive during latency as the result of the absence of a positive activator. Upon induction, a signal is transmitted from the cell surface, and an activator of the AP-1 family binds Zp, causing ZEBRA to be expressed. ZEBRA then autostimulates its own synthesis through its action on Zp and Rp.

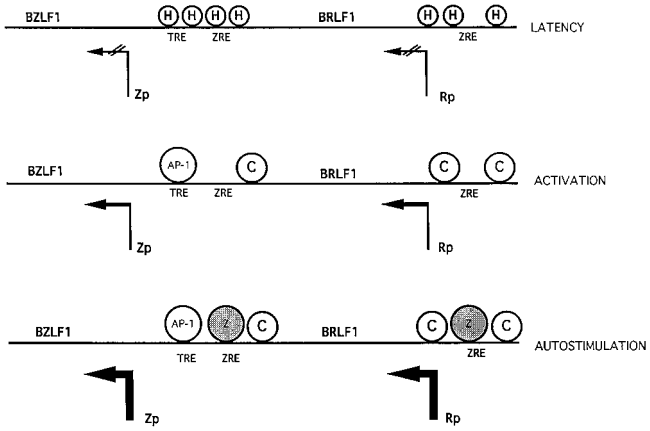


FIG. 6. Model for the entry pathway into the EBV lytic cascade. Zp, promoter of BZLF1; Rp, promoter of BRLF1; TRE, octamer TPA response element or ZII site (20); ZRE, ZEBRA response element; AP-1, an AP-1 family b-Zip activator such as c-Fos/c-Jun; Z, ZEBRA; H, host cell protein, possibly chromatin; C, host cell activator other than AP-1.

Two predictions of this simplified but heuristically useful model are inconsistent with the data presented here. One prediction is that the first stage of activation should be reproduced by introduction of an AP-1-type activator. We showed that introduction of a powerful composite activator, such as Z(1–171)F(137–162)Z(198–227)VP(411–490), is not sufficient to activate the endogenous viral locus or disrupt latency. The second prediction is that ZEBRA should autostimulate Zp in the latent genome by acting through the ZREs. However, following introduction of ZEBRA or ZEBRA/VP16 chimeras, Zp-specific mRNAs transcribed from the latent genome were not detected. Therefore, neither introduction of ZEBRA nor an AP-1 activator alone is a sufficient stimulus to activate Zp from the virus.

These experiments suggest an alternative model for entry of EBV into the lytic cascade (Fig. 6). During latency, Zp and possibly Rp are inactive as the result of repression mediated by chromatin or silencers. Candidate suppressors of BZLF1 expression include the cellular transcription factor YY1 (47) and the cellular factor which binds to ZI elements in both Zp and Rp (21). Whereas the ZREs and TRE in Zp are blocked, the ZRE in Rp is available, since Rp can be activated by transfection of ZEBRA (58) (Fig. 5). Thus, Rp may be silent simply because it lacks its specific activator, ZEBRA. The initial phase of the activation cascade is accompanied by changes in the structure of Zp and its associated proteins. Proteins normally bound in the latent state may be lost, and other cellular proteins may be added (19, 21, 50). Thereafter, BZLF1 is transcribed. Since kinetics experiments show that Zp and Rp are activated simultaneously after application of inducing stimuli (14, 19, 23, 49, 58), the two promoters are likely to be coordinately regulated, possibly through similar changes in promoter structure. Although autostimulation of BZLF1 expression may occur at Rp without changes in promoter structure, autostimulation is likely to occur at Zp only after chromatin changes or elimination of a specific repressor makes the ZREs and TRE

in Zp available for activation by ZEBRA, AP-1, and other cellular proteins. Finally, ZEBRA stimulates transcription from its other targets in the EBV genome and plays a role in lytic viral DNA replication. Thus, entry into the EBV lytic cascade is a complex process involving changes in Zp and Rp promoter structure, autostimulation, and, ultimately, activation of lytic gene expression.

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