NOTES

Epstein-Barr Virus BZLF1 *trans* Activator Induces the Promoter of a Cellular Cognate Gene, *c-fos*

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The Epstein-Barr virus BZLF1 gene product ZEBRA is a DNA-binding protein that is partially homologous to c-Fos, binds specifically to AP-1 sites, and can induce the lytic cycle in latently infected B lymphocytes. Induction of the viral lytic cycle can also be achieved by treatment with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, a reagent which activates gene expression in part through AP-1 (Jun/Fos). In this article the interrelationship between ZEBRA and AP-1 is extended by the demonstration that ZEBRA can induce c-Fos expression through AP-1 and "AP-1-like" sites present in the *c-fos* promoter. Induction of c-Fos may be necessary for the expression of other viral lytic genes and perhaps cellular genes whose products are required for viral replication.

Disruption of viral latency in Epstein-Barr virus (EBV)-infected B lymphocytes is determined by an intricate cascade of events initiating at the plasma membrane. Triggering of the viral lytic cycle can be signaled by a variety of reagents, including anti-immunoglobulin, calcium ionophore, butyrate, and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (2, 11, 15, 21). Of the viral genes known to be expressed during the onset of the lytic cycle, the BZLF1 gene product ZEBRA (also referred to as EB1) is unique in its ability to trigger the entire lytic cascade (3-5). Interestingly, ZEBRA contains two regions with striking homology to c-Fos, one of which is homologous to the DNA-binding domain of c-Fos (7). Moreover, it has been shown that ZEBRA specifically binds to a consensus AP-1 site located in the promoter of the early gene BMLF1 (7), and we have previously identified an "AP-1-like" site within the BZLF1 promoter to which ZEBRA can bind and auto-activate (9). The structural and functional similarities between ZEBRA and AP-1 suggest that EBV may be usurping a cellular transcription regulatory pathway. To investigate this relationship further, we addressed the question of whether ZEBRA affects the expression of other members of the AP-1 family which are known to be autoregulatory. In this article we show that ZEBRA can *trans* activate the *c-fos* promoter via multiple AP-1 and AP-1-like sequences.

ZEBRA *trans* activates the *c-fos* promoter in several different cell lines. Cotransfection of several cell lines with a *c-fos* promoter-*cat* gene construct (-404 to +42 base pairs [bp]; fospCAT) and either a ZEBRA expression vector (pSV40-BZLF1) or a control plasmid (pSV40) revealed *trans* activation of the *c-fos* promoter by ZEBRA (Fig. 1). Activation of fospCAT by ZEBRA ranged from 7- to 9.5-fold in the two B-cell lines tested, Ramos (EBV-negative Burkitt's lymphoma) and JC5 (EBV-infected lymphoblastoid cell line), and a similar level of induction was observed in the HeLa cell line. *trans* activation was also apparent in the

T-cell line Jurkat, although the effect was considerably smaller (twofold). In contrast, the human *c-jun* promoter (-132 to +170 bp) was not *trans* activated by ZEBRA in any of these cell lines (data not shown).

ZEBRA binds to three distinct sites in the *c-fos* promoter and enhancer. To localize ZEBRA-responsive elements in the *c-fos* promoter and enhancer, a TrpE-ZEBRA bacterial fusion protein (9) was used to identify ZEBRA-binding domains. DNase I protection analysis revealed three distinct footprints (Fig. 2), one within the promoter region which extended from -46 to -61 bp (FZ3) and two in the enhancer region which flanked the A+T-rich sequence located at the center of the dyad symmetry element (DSE) (20) (FZ1 [-333

FIG. 1. *trans* activation of the *c-fos* promoter-enhancer by ZEBRA in several cell lines. Cells were transfected with 2 μ g of fospCAT and 2 μ g of either a control plasmid, pSV40, or the ZEBRA expression plasmid pSV40-BZLF1. HeLa cells were transfected by liposome-mediated DNA transfer as per the vendor's protocol (Bethesda Research Laboratories). Ramos (an EBV-negative B-cell line), JC-5 (an EBV-positive B-cell line), and the T-cell line Jurkat were transfected by the DEAE-dextran method as described previously (8). fospCAT was generated by transferring the entire human *c-fos* promoter-enhancer and *cat* gene from FC4 (6) into the *Hind*III and *Bam*HI sites of the Bluescript vector (Stratagene). pSV40 and pSV40-BZLF1 have been described previously (9).

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FIG. 2. Identification of ZEBRA-binding domains within the human *c-fos* promoter-enhancer. TrpE-ZEBRA fusion protein was used in DNase I protection analysis of two different fragments of the *c-fos* promoter-enhancer, one which extends from -404 to -227 bp and another which extends from +42 to -222 bp. Footprinted regions are bracketed and are labeled FZ1, FZ2, and FZ3. Labeled DNA fragments were incubated either with partially purified TrpE protein (control) or the TrpE-ZEBRA fusion protein (ZEBRA). DSE refers to the dyad symmetry element in the *c-fos* enhancer. WT, Wild-type DNA; FM1, FM2, and FM3 refer to DNA fragments which bear mutations in the respective binding domains. The following mutations were introduced: FM1, from -327 TTACACA -321 to -327 TGAATC -321; FM2, from -299 TGGCTCA -293 to -299 TGGATCC -293; FM3, from -55 AACTCA -49 to -55 AGGATCC -49; FMP67 from -312 ATATTA -307 to -312 CATGGA -307. It should be noted that the altered DNase I cleavage pattern exhibited by the FM3 mutant compared with that of the unmutated promoter is due to the modified sequence and not ZEBRA binding, since an identical pattern was observed in the absence of added TrpE-ZEBRA fusion protein (data not shown). Mutant plasmids were generated by site-directed mutagenesis (10). Generation of fusion protein, preparation of extracts, and DNase I footprinting were performed as previously described (9).

to -314 bp] and FZ2 [-304 to -287 bp]) (Fig. 3A). Alignment of the core sequences for each of these footprints revealed significant sequence similarity to the AP-1 consensus sequence (Fig. 3B). Moreover, others have demonstrated binding of purified AP-1 to both FZ1 and FZ2 (19), and Piette and Yaniv (16) have reported binding of the mouse AP-1 homolog PEA-1 to FZ2.

With regard to the FZ3 domain, another study (18) demonstrated binding of purified CREB and AP-1 to a domain which overlaps FZ3. They identified the octanucleotide

FIG. 3. (A) Location of ZEBRA-binding domains with respect to *c-fos* promoter sequence. Overbrackets delineate regions protected from DNase I digestion (FZ1, FZ2, and FZ3; see Fig. 2). Previously described AP-1- and CREB-binding domains are indicated by underbrackets. (B) Alignment of ZEBRA-binding domains. ZEBRA-binding domains are aligned with respect to the heptanucleotide AP-1 consensus (cons.) sequence. AP-1 does not bind to the ZIIIB ZEBRA-binding site in the BZLF1 promoter.

sequence TGACGTTT, which bears similarity to the consensus CREB-binding domain, as the likely element in this domain to which both CREB and AP-1 bind. However, it is unclear whether binding is limited to this sequence or whether it also includes the heptanucleotide AP-1-like element in the FZ3 domain. It is clear, however, that ZEBRA does not bind to the CREB/AP-1 octanucleotide element, since the first three bases of the core sequence were not protected from DNase I digestion (Fig. 2). This is consistent with our previous results obtained with the BZLF1 promoter; it was observed that although ZEBRA could bind and *trans* activate through a heptanucleotide AP-1-like site, binding to an octanucleotide AP-1 site within the promoter could not be detected (8, 9). Furthermore, ZEBRA did not bind to the octanucleotide AP-1 site present in the *c-jun* promoter (data not shown) and, as indicated above, failed to *trans* activate this promoter. Thus, since AP-1 has been shown previously to bind with high affinity to octanucleotide AP-1 sequences (1), these observations suggest a fundamental difference in the activities of AP-1 and ZEBRA.

ZEBRA *trans* activation of the *c-fos* promoter correlates with ZEBRA binding. To determine whether ZEBRA *trans* activation of the *c-fos* promoter maps to the identified ZEBRA-binding domains, site-directed mutations were introduced into the relevant domains. Both single- and multiple-ZEBRA-binding domain fospCAT mutants were constructed. Before the effect of these mutations on ZEBRA *trans* activation was tested, they were assayed for their capacity to bind ZEBRA. As shown in Fig. 2, the mutations introduced into each ZEBRA-binding domain abrogated binding of ZEBRA to the respective site.

The effect of ZEBRA-binding domain mutations on ZEBRA *trans* activation was assayed in HeLa cells. The results of three separate experiments are shown in Table 1, and the values given for each experiment represent the average of duplicate transfections. We found that there was significant variation in the level of ZEBRA-*trans* activation of the *c-fos* promoter constructs between experiments, although duplicates within a given experiment were very

TABLE 1. *trans* activation of fospCAT and ZEBRA-binding domain mutants^a

Reporter plasmid	Cotransfection	Expt 1		Expt 2		Expt 3	
		Relative activity	Induction (fold)	Relative activity	Induction (fold)	Relative activity	Induction (fold)
fospCAT	C	1.0		1.0		1.0	
	Z	7.5	7.5	13.9	13.9	4.1	4.1
FM1	C	1.0		0.9		0.8	
	Z	6.3	6.3	11.3	12.6	2.2	2.8
FM2	C	1.5		1.4		1.8	
	Z	5.9	3.9	6.5	4.6	2.2	1.2
FM3	C	1.7		1.2		1.4	
	Z	5.8	3.4	15.4	12.8	3.7	2.6
FM1 + FM2	C	1.6		2.1		1.9	
	Z	4.6	2.9	8.2	3.9	2.4	1.2
FM1 + FM3	C	1.4		1.7		2.2	
	Z	3.2	2.3	6.7	3.9	2.3	1.1
FM2 + FM3	C	3.3		3.0		3.0	
	Z	5.5	1.7	6.2	2.1	3.2	1.1
FM1+ FM2 + FM3	C	2.9		2.8		3.4	
	Z	3.9	1.3	6.8	2.4	2.0	0.6
FMp67	C					3.6	
	Z					13.5	3.7

^a Results for each experiment represent the average of duplicate transfections. The indicated reporter plasmid was cotransfected with either pSV40 (C) or pSV40-BZLF1 (Z). Relative activity represents the CAT activity observed with the various ZEBRA-binding domain mutants cotransfected with either pSV40 or pSV40-BZLF1 versus that with fospCAT and pSV40. Fold induction indicates the increase in activity with pSV40-BZLF1 cotransfection.

consistent. The reasons for this variation in ZEBRA *trans* activation are unclear; however, they may be partly the result of a variable cytotoxic effect of ZEBRA. It is also possible that variations in the extent of ZEBRA induction of endogenous *c-fos* may influence the observed induction of the reporter construct, since c-Fos would be expected to *trans* repress the reporter construct through the DSE. Notwithstanding the complications that these effects introduce, analysis of ZEBRA-binding domain mutants mapped ZEBRA *trans* activation to these sites.

Of the single-domain mutations, mutation of the FZ2 domain (FM2, Table 1) caused the largest diminution in ZEBRA *trans* activation (two- to threefold), while the effect of the FM1 and FM3 mutations was smaller and more variable. However, mutation of any combination of two ZEBRA-binding domains had a greater effect on ZEBRA *trans* activation, and mutation of all three domains appeared to largely abrogate ZEBRA *trans* activation. Therefore, it appears that each of the ZEBRA-binding domains is involved in the *trans* activation of the *c-fos* promoter-enhancer by ZEBRA. Notably, the basal activity of the *c-fos* promoter increased with the FM2 and FM3 mutations. This effect has been observed previously with mutation of the AP-1 site in the FZ2 domain in both the mouse and human *c-fos* genes (12, 14). The increase in the basal activity observed with these mutations suggests that the FZ2 and FZ3 domains are involved in repressing the basal activity of the *c-fos* promoter.

Previous reports have demonstrated autoregulation of *c-fos* via the DSE (12, 17). The homology between c-Fos and ZEBRA led us to address the question of whether mutation of the DSE, through which Fos *trans* represses its own expression, affected ZEBRA *trans* activation. A fospCAT construct containing a mutation in the p67-binding domain (FMp67) was assayed for ZEBRA *trans* activation (Table 1). Consistent with previous reports (19), the basal activity of the promoter was significantly increased by this mutation. However, ZEBRA *trans* activation was not significantly affected.

Conclusions. Although the ramifications of *c-fos* induction by ZEBRA are unclear at this time, it is likely to be an

important component in the temporal sequence of gene expression during induction of the viral lytic cycle. Several AP-1 recognition sequences have been identified within the promoter regions of early genes (7), and we have identified two AP-1-binding sites within the promoter of the BZLF1 gene (9). As discussed above, the two AP-1 sites in the BZLF1 promoter (ZII and ZIIIA domains) differ in that one is an octanucleotide AP-1 site while the other is a heptanucleotide AP-1 site. Therefore, although the implications of ZEBRA and AP-1 binding to the same site (heptanucleotide AP-1 site) are unclear at this time, Fos and Jun can also bind to octanucleotide AP-1 sites, which ZEBRA cannot. Conversely, we have also shown that ZEBRA can bind to a second element within the BZLF1 promoter (ZIIIB domain; Fig. 3B) to which Fos and Jun do not appear to bind (9).

Previously we demonstrated autoregulation of the BZLF1 promoter by a mechanism involving the direct interaction of ZEBRA with the BZLF1 promoter (9). The data presented here suggest a second, albeit more circuitous, autoregulatory loop involving ZEBRA activation of the intermediary, *c-fos*. Since there are many cellular genes which contain AP-1 sites within their promoter regions, it seems likely that ZEBRA also affects the expression of other cellular genes. This may be important for initiating viral replication, either through direct interactions or through induction of *c-fos*.

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