JOURNAL OF VIROLOGY, Nov. 1994, p. 7628–7633
0022-538X/94/$04.00+0
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Vol. 68, No. 11

Activation of the Epstein-Barr Virus BMRF1 and BZLF1 Promoters by ZEBRA in *Saccharomyces cerevisiae*

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Received 26 May 1994/Accepted 28 July 1994

ZEBRA has been shown to activate model reporter genes consisting of synthetic oligomerized ZEBRA response elements upstream of a minimal *CYC1* promoter fused to β-galactosidase in the yeast *Saccharomyces cerevisiae*. Here it is shown that in *S. cerevisiae* ZEBRA activates transcription of natural Epstein-Barr virus promoters. Two Epstein-Barr virus promoters were shown to be activated by ZEBRA in *S. cerevisiae*: Zp, the promoter that regulates expression of BZLF1, which encodes ZEBRA; and EAp, the promoter controlling expression of BMRF1, which encodes diffuse early antigen, EA-D. These observations indicate that neither mammalian-specific nor virally encoded coactivators are obligatory for ZEBRA to stimulate expression from these two promoters. Zp was also strongly activated by endogenous yeast factors. EAp was not activated by yeast factors. The results show that in *S. cerevisiae* and in B cells, ZEBRA dominates the response of EAp; ZEBRA plus endogenous cell factors activate Zp.

Epstein-Barr virus (EBV) immortalizes human B lymphocytes and thereafter remains in a latent state of limited gene expression (23, 31). Inducing agents, including phorbol esters, sodium butyrate, calcium ionophores, and anti-immunoglobulin, activate the lytic EBV life cycle in B lymphocytes in vitro (9, 21, 29, 49). Induction of the lytic phase of viral growth results in an orderly cascade of expression of immediate-early and early genes, followed by lytic viral DNA replication, expression of late genes, and viral maturation (23, 31). ZEBRA, the immediate-early lytic cycle gene product, is pivotal in this cascade (6, 32). Induction of the lytic cycle is accompanied by expression of the BZLF1 gene encoding ZEBRA (45). ZEBRA then activates its own transcription as well as transcription of several viral early genes (5, 7, 10, 12, 26, 27, 36, 39, 44). ZEBRA also plays a direct role in lytic viral DNA replication by binding to the lytic replication origin, *oriLyt* (11, 40, 41).

ZEBRA, a DNA-binding transcriptional activator, is a member of the bZIP family (4, 10, 25). ZEBRA binds to the same heptamer DNA site (TGAGTCA) that is recognized by cellular AP-1 bZIP transcription factors (4, 10, 25). ZEBRA also binds degenerate heptamer sequences called ZEBRA response elements (ZREs), whose consensus is T G/t A/T G C/T A/C/g A. Many ZREs are not bound by mammalian cellular AP-1 transcription factors, such as Fos (25, 27, 46). EBV genes that respond to ZEBRA in B cells contain ZREs and AP-1 sites in various numbers, configurations, and spacings in their 5′ upstream regions (7, 22, 26, 36, 39, 44). It is not yet understood what combination, orientation, spacing, or affinity of AP-1 sites and ZREs confers ZEBRA responsiveness to a gene. Nor is it known whether ZEBRA alone activates its responsive genes, in concert with the basal transcription machinery, or whether the combinatorial action of ZEBRA and other general or specific cellular transcriptional activators is required for activation of ZEBRA-responsive promoters and lytic cycle gene expression. Some viral genes are activated in synergy by ZEBRA and the EBV-encoded Rta protein (5, 7, 14, 17, 39).

Experiments with *Saccharomyces cerevisiae* have helped to define the domain structure of mammalian viral activators and to delineate activating regions that are conserved in all eukaryotic cells (24, 34, 48). ZEBRA has recently been found to activate transcription in *S. cerevisiae* from model reporter genes containing one or more oligomerized ZIIIB sites (TTAGCAA) from Zp, the promoter of the BZLF1 gene (33). Synthetic promoters do not reproduce the environment of complex naturally occurring regulatory regions. In order to exploit *S. cerevisiae* as an experimental system to study regulation of EBV lytic cycle genes, it was first essential to determine whether ZEBRA could activate natural promoters of EBV genes that were known to be ZEBRA responsive in mammalian cells. Two promoters were selected for initial study. Zp regulates the expression of ZEBRA and thereby is likely to be the pivotal control point for the maintenance of latency. The promoter of the BMRF1 gene, EAp, was of interest because it has been reported to be activated synergistically by ZEBRA together with viral activators, such as Rta, and cellular activators, such as c-*myb* (22).

The capacity of ZEBRA to activate transcription of EBV promoters in *S. cerevisiae* in two experimental systems was studied. In one system the ZEBRA expression cassette was integrated into the *S. cerevisiae* chromosome; in the other experiments ZEBRA was expressed from a low-copy-number plasmid. In both systems ZEBRA was expressed from the galactose-inducible *GAL1-GAL10* promoter (20). An inducible promoter and low-level expression were required because constitutive overexpression of ZEBRA is toxic to *S. cerevisiae* (33). Reporter genes in both experimental systems were derivatives of pCZΔ, a plasmid containing a minimal yeast *CYC1* promoter fused to *lacZ* (28). Portions of EBV DNA (Fig. 1A) were placed in a multiple cloning site located upstream of the *CYC1* TATA element. For reference, activation of natural EBV promoter regions was compared with activation of a

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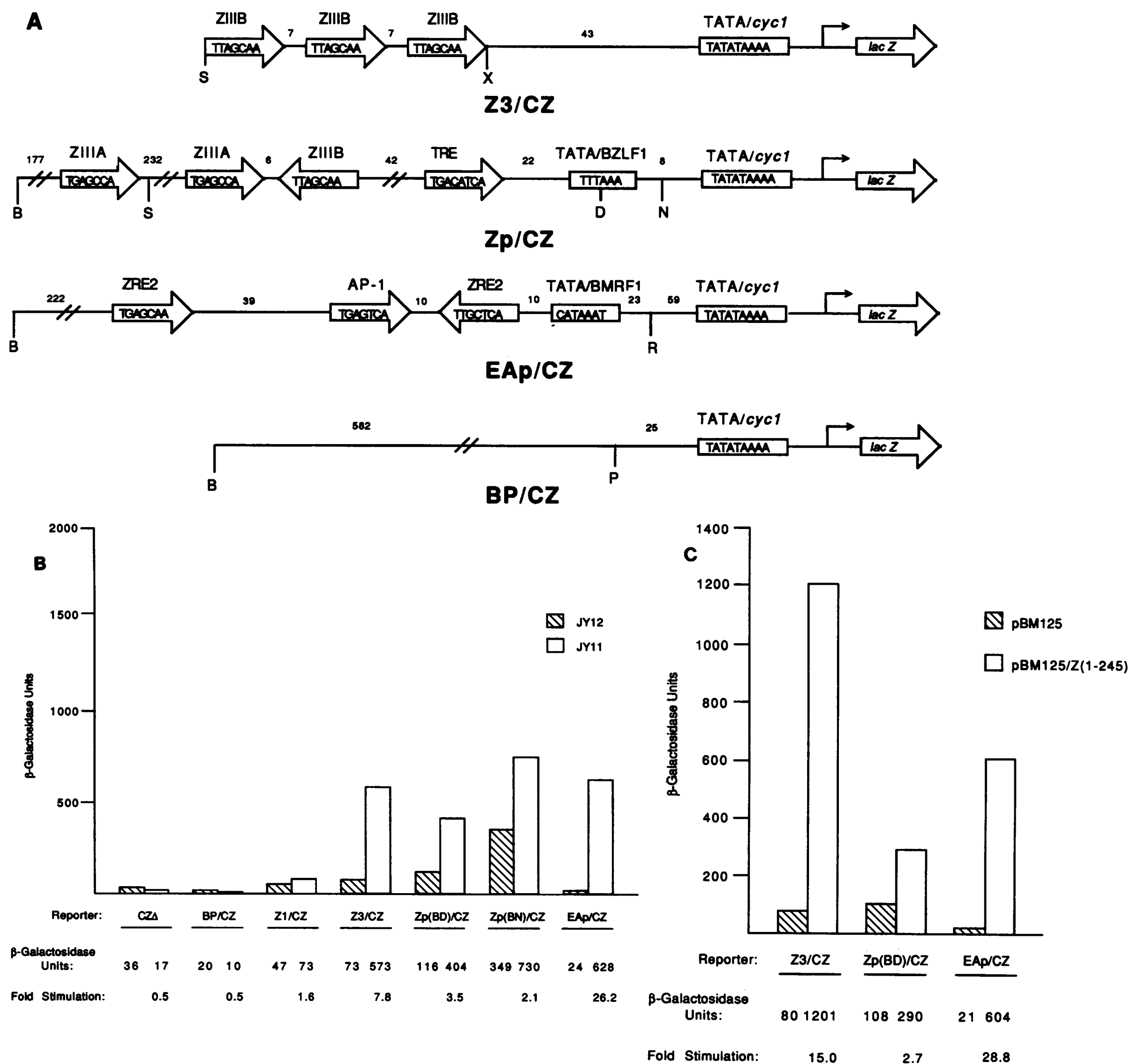


FIG. 1. Reporter plasmids and their activation in *S. cerevisiae*. (A) Reporter plasmids containing EBV DNA were constructed in the parental plasmid, pCZΔ (28). This plasmid contains a 144-bp minimal promoter from the yeast *CYC1* gene upstream of *lacZ*. It also contains the selectable *URA3* gene, a yeast chromosomal origin of replication (*ARS1*), and a centromere (*CEN4*). Reporters containing one or three oligomerized ZIIIB sites inserted upstream of the *CYC1-lacZ* fusion have been described previously (33). Zp/CZ reporters contained restriction fragments from the upstream region of the BZLF1 gene inserted into the *Xba*I-*Xho*I sites of pCZΔ. Thus, Zp(BN)/CZ contains a 563-bp *Bam*HI-to-*Nae*I fragment that includes the TATA element upstream of BZLF1 and Zp(BD)/CZ contains a 516-bp *Bam*HI-to-*Dra*I fragment that eliminates the TATA element. Zp(SN)/CZ contains a 234-bp *Sph*I-to-*Nae*I fragment representing positions −221 to +13 relative to the transcriptional start. EAp/CZ contains a 333-bp *Bam*HI-to-*Rsa*I fragment located upstream of the BMRF1 gene inserted into the blunted *Xba*I site of pCZΔ. BP/CZ contains a 582-bp *Bam*HI-to-*Pvu*II fragment, lacking ZREs, from the left end of the *Bam*HI Z fragment inserted into the *Xba*I-*Xho*I sites of pCZΔ. ZIIIA, ZIIIB, ZRE2, and AP-1 are sites to which ZEBRA binds. The TRE does not bind ZEBRA (12, 46). BP/CZ contains no known ZREs. Numbers above the lines indicate distance in base pairs. (B) Activation by Z(1-245). Reporter plasmids were transformed into JY11, a yeast strain that inducibly expresses Z(1-245), and JY12, a strain containing a nonfunctional integrated copy of Z(1-245). JY11 and JY12 were constructed by cotransformation of pJH180/Z(1-245) and Z3/CZ into yeast strain W3031A (*MAT***a** *can1-100 his3-11 his3-15 leu2-3 leu2-112 trp1-1 ura3-1 ade2-1*). pJH180 contains the *TRP1* gene but no yeast origin of replication or centromere; therefore, in the absence of exogenously added tryptophan (Trp), auxotrophic yeast cells will only form macroscopic colonies on synthetic complete medium without Trp following integration of pJH180 into yeast chromosomal DNA. To facilitate integration, JH180/Z(1-245) was linearized at a unique *Bst*XI site located within the *TRP1* gene. Colonies were cured of the Z3/CZ reporter plasmid and analyzed for the presence of BZLF1 DNA by Southern blot hybridization. Southern blot colonies were rescreened for their ability to activate expression of Z3/CZ as an indication that the ZEBRA protein was active. The stimulation index is the ratio of β-galactosidase units measured in JY11 to β-galactosidase units measured in JY12. The data are median values from four experiments. (C) ZEBRA expressed from a plasmid that was cotransformed with the reporters into yeast strain W3031A. Activation of the reporters was compared with that of the same reporters following cotransformation with the pBM125 vector. Data represent median values from eight experiments. Competent yeast cells were produced and transformations were performed according to published methods (19, 43). β-Galactosidase assays were done as previously described (33).

model reporter, Z3/CZ, containing three tandem copies of a 14-bp oligonucleotide encoding the ZIIIB site from the BZLF1 promoter (3, 12). pCZΔ lacking EBV DNA was used to measure basal transcription. β-Galactosidase activity served as a measure of transcription.

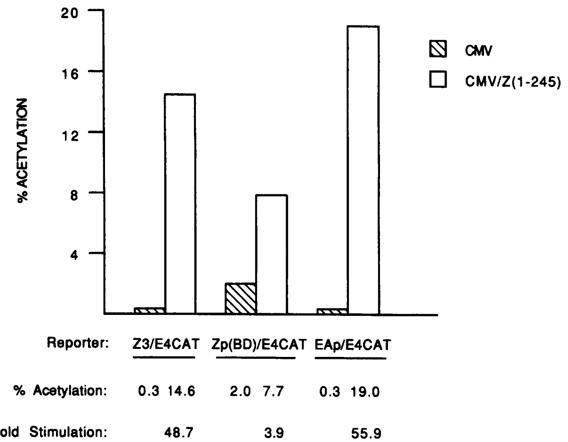
When ZEBRA was expressed from a chromosomal location in *S. cerevisiae*, the extent of transcriptional stimulation was measured by comparing activation of reporter genes placed in a yeast strain expressing ZEBRA with activation of the same reporters transformed in a syngeneic strain that did not express ZEBRA. In the experiments in which ZEBRA was expressed from a low-copy-number plasmid, transcriptional activation of a cotransformed reporter gene was compared with activation of the same reporter gene cotransformed with the vector plasmid lacking ZEBRA-coding sequences.

A yeast strain (designated JY11) that expressed Z(1-245) upon galactose induction was constructed (33). A syngeneic yeast strain (designated JY12) contained an integrated Z(1-245) that did not express ZEBRA. Figure 1B shows that ZEBRA expressed in yeast strain JY11 activated oligomerized sites; maximal stimulation (eightfold) was observed on a reporter with three tandem ZIIIB sites. ZEBRA in strain JY11 also activated natural EBV promoters, Zp about 3-fold and EAp more than 26-fold. The reporter, BP/CZ, containing a fragment of EBV DNA lacking ZREs was not activated. Thus, two well-characterized promoters of EBV lytic genes were activated by ZEBRA in *S. cerevisiae* in the absence of mammalian-specific, B-cell-specific, or EBV-specific factors.

ZEBRA's capacity to activate Zp and EAp in an *S. cerevisiae* strain expressing ZEBRA from an integrated copy and its capacity to activate them in a strain expressing ZEBRA from a cotransformed plasmid were compared (Fig. 1C and data not shown). Results were not dependent on the yeast strain or the nature of the ZEBRA expression plasmid. ZEBRA activated EAp 10-fold more than it activated Zp. These differences in transcriptional stimulation of the two EBV promoters were attributable to a higher level of activity in the presence of ZEBRA on EAp, the more responsive promoter, and to a higher level of background stimulation on Zp, the less responsive promoter. The background level of stimulation on the EAp/CZ reporter did not differ from basal transcriptional stimulation of pCZΔ, containing only the minimal *CYC1* promoter. In contrast, the background level of stimulation on Zp was four- to sixfold higher than that measured on a reporter without EBV DNA. These experiments indicated that in *S. cerevisiae*, ZEBRA alone drives EAp, while cellular factors and ZEBRA both activate Zp and oligomerized ZIIIB sites.

When ZEBRA was examined for its capacity to activate transcription of the same two EBV promoters in the EBV-negative human B lymphocyte cell line BJAB, the results were comparable to those found with *S. cerevisiae*. Figure 2 shows that EAp was stimulated most efficiently in B cells and that Zp was fourfold less responsive than EAp. The lower level of stimulation of Zp was the combined result of a twofold increase in the background level of activation in the absence of added ZEBRA and a twofold-lower level of stimulation in the presence of ZEBRA. In B cells ZEBRA activated EAp in the absence of any spontaneous activation by cell factors, a result similar to that observed with *S. cerevisiae*. Moreover, in B cells as in yeast cells, Zp was activated by the mixed action of cellular factors and ZEBRA.

To attempt to identify regions of Zp responsible for activation by endogenous yeast factors, we analyzed eight deletional and site-directed Zp mutants (12, 13). Several regions of Zp appeared to contribute to the background activation. One region was located between positions −221 and −159; deletion



Reporter:	Z3/E4CAT	Zp(BD)/E4CAT	EAp/E4CAT
% Acetylation:	0.3 14.6	2.0 7.7	0.3 19.0
Fold Stimulation:	48.7	3.9	55.9

FIG. 2. Activation of a model promoter and two natural EBV promoters in human B cells. Reporter plasmids contained three oligomerized ZIIIB sites or portions of the upstream regions of the BZLF1 (ZpBD) or BMFR1 (EAp) genes fused to a minimal promoter from the adenovirus E4 gene linked to chloramphenicol acetyltransferase. The reporter Z3/E4CAT has previously been described (3, 15). The reporter Zp(BD)/E4CAT was made by releasing Zp(BD) from CZΔ with *Hin*dIII and *Xho*I and ligating it into E4CAT digested with *Hin*dIII and *Sal*I. The reporter EAp/E4CAT was made by excising the EAp fragment from CZΔ with *Sph*I and *Xho*I and cloning it into E4CAT digested with *Sph*I and *Sal*I. The activators were CMV-Z(1-245) or, as a control, the vector pHD1013, containing the cytomegalovirus (CMV) immediate-early gene promoter/enhancer (8). EBV-negative BJAB cells were cotransfected by electroporation with 10 μg of one reporter and 10 μg of one activator. Chloramphenicol acetyltransferase activity was measured after 48 h by using 30 μl of cell extract. The stimulation index is the ratio of the percent acetylation on a given reporter transfected with CMV-Z(1-245) to the percent acetylation after cotransfection of the same reporter with pHD1013. The data represent median values from seven experiments.

of this region caused a 40% reduction in background activity. Another important site was the 12-*O*-tetradecanoylphorbol-13-acetate response element (TRE). Point mutation of this site significantly reduced the background in the context of the −221 fragment and in the −105 deletion mutant.

The same Zp mutants were examined for their capacity to be activated by ZEBRA. In the experiments illustrated in Fig. 3, ZEBRA was expressed from the plasmid Z(2-245), which contains a translation enhancer that presumably augments expression. This construct stimulates Zp about 1.5-fold better than Z(1-245) does (Fig. 4 and data not shown). Therefore, Z(2-245) was preferable to Z(1-245) for assessing small changes in activation due to Zp mutations. Two Zp constructs that permitted wild-type activation by ZEBRA were MTRE and −159, in which the ZIIIA or ZIIIB sites, to which ZEBRA is known to bind in vitro, were retained (12, 46). If the ZIIIA or ZIIIB sites were removed by point mutation or deletion, there was a 60 to 70% reduction in the ability of Z(2-245) to activate Zp in *S. cerevisiae*. By contrast, a Zp mutant lacking the TRE, a site to which ZEBRA does not bind efficiently in vitro, was unaffected in its ability to be activated by ZEBRA (46). This evidence indicated that the ZIIIA and ZIIIB sites were important components of ZEBRA's capacity to activate Zp in yeast cells, as previously found for B cells (12). Similar data were obtained when the same Zp mutants in strain JY11 were analyzed (data not shown).

We measured the ability of a set of deletion mutants of ZEBRA to activate Zp/CZ and EAp/CZ (Fig. 4). Deletion of

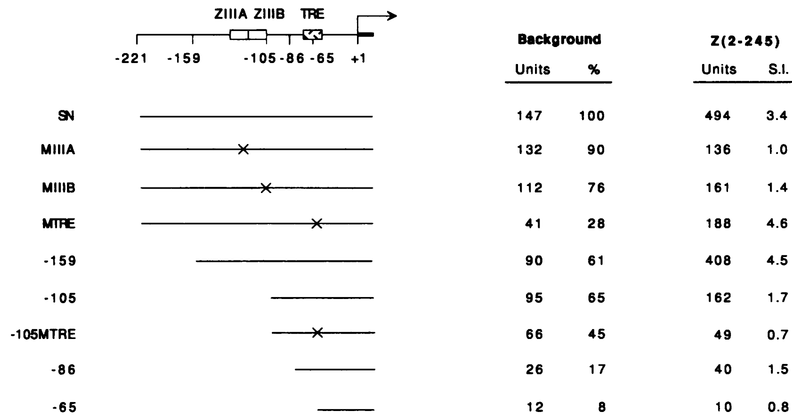


FIG. 3. Activation of the upstream region of the BZLF1 gene by endogenous factors and by ZEBRA in *S. cerevisiae*. Reporter plasmids contained an *Sph*I-to-*Nae*I fragment (positions −221 to +13) upstream of the BZLF1 gene fused to *CYC1-lacZ*. A set of Zp point and deletion mutants were kind gifts from Eric Flemington. Each mutant was excised from −41 β-globin CAT plasmid as a *Hin*dIII-*Sac*I fragment, after the *Sac*I end had been filled in with T4 DNA polymerase, and cloned into Bluescript KS^+ at the *Hin*dIII and *Eco*RV sites (13). Each mutant was excised from KS^+ as a *Xho*I-*Xba*I fragment and ligated into these sites in pCZΔ. Point mutations in this fragment, located in the ZIIIA, ZIIIB, and TRE sites, are indicated with an X (12, 13). Deletion mutants started at the indicated base pairs upstream of the transcriptional start (30). The reporter plasmids were cotransfected into strain W3031A with pBM125 or pBM125/Z(2-245). The data represent duplicate experiments. Basal transcription of pCZΔ, containing only the minimal *CYC1* promoter, was 38 U. S.I., stimulation index, the ratio of β-galactosidase units in the presence of Z(2-245) to units in the presence of pBM125 vector.

the N-terminal 130 amino acids destroyed the capacity of ZEBRA to activate either natural promoter. Internal deletion of amino acids 26 to 89 in *S. cerevisiae* was tolerated as long as the N-terminal 25 amino acids and C-terminal 18 amino acids were present. However, this deletion markedly impaired the capacity of ZEBRA to activate EAp or Zp in B cells (data not shown). Further internal deletion, such as that in the mutant Z(1-25,141-245), caused a decrease in activity.

Point mutations in the basic region that result in the loss of ZEBRA's capacity to bind DNA in vitro also eliminated the ability of ZEBRA to activate either Zp or EAp in *S. cerevisiae* (Fig. 4 and data not shown). The N-terminal and C-terminal

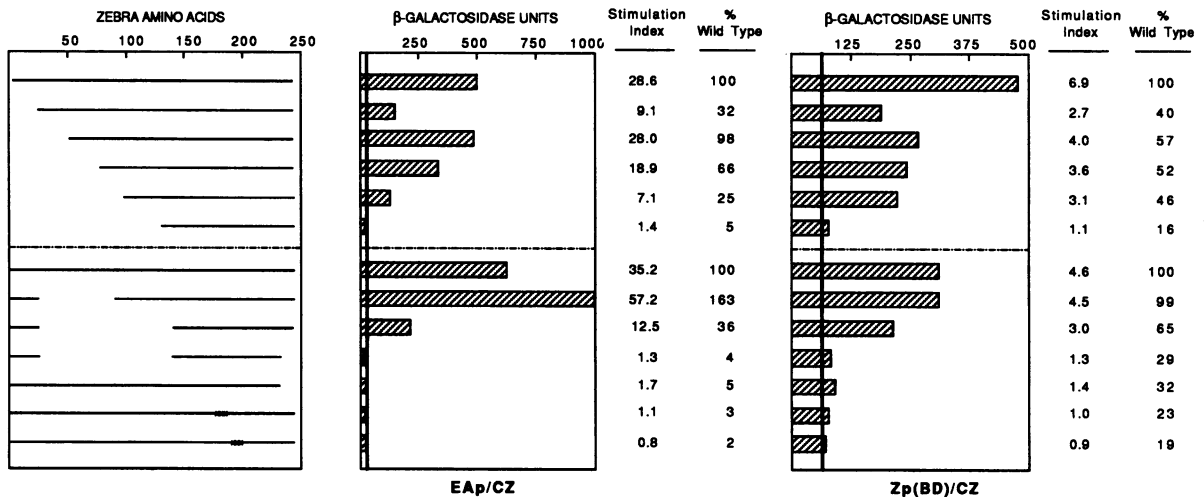


FIG. 4. Activation of the EBV BMRF1 and BZLF1 promoters by ZEBRA deletion mutants in *S. cerevisiae*. The left panel indicates the boundaries of N-terminal, C-terminal, internal deletion, and point mutants of ZEBRA. pBM125 plasmids carrying the intact BZLF1 cDNA; Z(2-245); various N-terminal, internal, and C-terminal deletion mutants of ZEBRA; and DNA-binding-deficient mutants of ZEBRA, Z(M178-180) and Z(M187-189), have been described previously (36). The dotted horizontal line separates mutants which contained different translation initiation sequences. An X denotes the location of a point mutation within the DNA-binding domain. For the middle panel ZEBRA mutants were cotransformed into strain JPY5 (*MATα ura3-52 his3Δ200 leu2Δ1 trp1Δ63 lys2Δ385*) with the reporter EAp/CZ, and for the right panel the ZEBRA mutants were cotransformed into JPY5 with the reporter Zp(BD)/CZ. The stimulation index is the ratio of the β-galactosidase units measured with the wild-type or mutant ZEBRA construct to the β-galactosidase units measured with pBM125. The basal level of activity, that measured with pBM125, is indicated by the heavy vertical lines. Data are derived from duplicate experiments with each reporter.

activating regions and the basic DNA recognition domain of ZEBRA have previously been found to be required for activation of reporter genes containing oligomerized ZIIIB sites (33). Thus, the interactions of ZEBRA with the transcription apparatus on natural promoters and on model genes are likely to be similar.

We find that ZEBRA activates naturally occurring EBV promoter regions in *S. cerevisiae*. Of the mammalian viral activators, only the human T-cell leukemia virus type I Tax protein has been reported to activate natural promoter regions in similar assays (48). The results imply that ZEBRA is a universal activator displaying no absolute requirement for mammalian or B-cell-specific coactivators even when acting on complex regulatory regions (38). Furthermore, the experiments show that ZEBRA stimulates EAp and Zp in the absence of any viral cofactors. It had previously been reported that ZEBRA required the R transactivator (Rta) to drive the BMRF1 promoter efficiently in lymphocytes (17). We found that ZEBRA alone strongly stimulated transcription of the BMRF1 promoter in *S. cerevisiae* and in B cells in the absence of Rta (Fig. 3 and 4).

The behavior of Zp and EAp in human B cells is similar to that in *S. cerevisiae*. EAp is stimulated strongly by ZEBRA, about 20- to 30-fold in yeast cells (Fig. 1B) and 50-fold in B cells (Fig. 2). Zp is stimulated weakly, about three- to fourfold in yeast cells and in B cells. In both B cells and in *S. cerevisiae* the relatively poor response of Zp, compared with the response of EAp, is the result of two variables: a higher level of stimulation of Zp by cellular factors and a lower level of activation by ZEBRA. Yeast factors stimulate Zp about 5- to 20-fold, depending on the yeast strain and Zp fragment used. Although this effect is less dramatic in B cells, endogenous factors stimulated Zp sixfold above the basal level of transcription.

These experiments suggest that EAp is likely to be driven solely by ZEBRA, while activation of Zp appears to involve the combined action of ZEBRA and cellular factors. These differences in behavior of Zp and EAp in yeast cells are consistent with EBV biology in human lymphocytes. Zp is likely to be regulated primarily by host cell factors and later, perhaps, to be autostimulated by ZEBRA. EAp is a downstream target gene regulated primarily by ZEBRA. The differences in the extents of stimulation of EAp and Zp in response to ZEBRA are likely to be the consequence of the number, disposition, and affinity of ZREs as well as the number, disposition, and affinity of binding sites for cellular factors on these two promoters.

EAp contains two ZREs flanking an AP-1 element, all three of which are known to bind ZEBRA in vitro (Fig. 1A) (39). Thus, the high level of stimulation of EAp could result from synergistic action of ZEBRA bound at all three sites, as has been described for oligomerized sites (3). Furthermore, all three potential ZEBRA binding sites in EAp are located within 160 bp of the *CYC1* TATA element; in *S. cerevisiae* the effects of weak activators, such as ZEBRA, are more apparent at shorter distances (37).

Zp also contains three ZREs, but deletion of the ZRE farthest upstream has no effect on the level of activation by ZEBRA in *S. cerevisiae* (data not shown). Thus, Zp may have only two functional ZEBRA binding sites. The relative affinities for the ZREs contained within EAp and Zp may also play a critical role in the levels of activation observed. EAp contains an AP-1 site and two ZRE2 sites, while Zp has a ZIIIA site and a ZIIIB site. Of these four types of sites, the ZIIIA site had the weakest ability to stimulate transcription in vitro in a HeLa cell extract (2). Thus, EAp has two high-affinity sites, AP-1 and ZRE2, while Zp only has one, ZIIIB.

An additional explanation for the difference in response between EAp and Zp may lie in the TRE, a site that is not bound by ZEBRA in vitro (46). However, yeast factors stimulate transcription from this site. Since the TRE is closer to the start of transcription than are the ZREs, the yeast factors that bind the TRE may inhibit the action of ZEBRA on Zp. The identities of the yeast factors that recognize the TRE in Zp are not known; they could include YAP1, GCN4, or CAD1, all of which are yeast bZ1P transcription factors (1, 16, 18, 35, 47).

We have studied the behavior of several other previously characterized ZEBRA-responsive promoters such as BSLF2/BMLF1 (MS-EAp), BRLF1 (Rp), BHRF1, and BHLF1 in *S. cerevisiae* (41, 42). These promoters of EBV lytic cycle genes vary in levels of activation by endogenous yeast factors as well as in extents of activation by ZEBRA. Some regions, such as those encompassing the bidirectional promoters of BHRF1 and BHLF1 and *oriLyt*, are similar to Zp in being activated to a high level by yeast factors. Other regions, e.g., Rp, are like EAp and are not activated by yeast factors. Some promoters (e.g., MS-EAp) are activated strongly by the addition of ZEBRA. Other promoters, such as those colinear with *oriLyt*, are not stimulated by the addition of ZEBRA in *S. cerevisiae*, a finding which may indicate the requirement for a viral or cell-specific coactivator. These analyses seem to classify ZEBRA-responsive promoters into two groups: those dominated by ZEBRA and those in which activation is shared by ZEBRA and a cellular coactivator. Experiments with *S. cerevisiae* could help to identify cellular proteins that participate in the control of ZEBRA-responsive promoters.

We thank M. Carey, E. Flemington, S. Speck, S. Kenney, and J. Wang for gifts of plasmids. We are particularly grateful to Mark Ptashne and Joe Pearlberg for the yeast strain JPY5. We thank M. Carey for critical reading of the manuscript and helpful discussions.

This work was supported by National Institutes of Health grants CA12055 and CA52228 and American Cancer Society grant VM-24N.

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