

## Alternative System for Detection and Mapping of Activation Domains

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### ABSTRACT

The ZEBRA protein is a transcriptional activator that induces expression of viral lytic genes in cells harboring latent Epstein-Barr virus (EBV). In this report it is shown that a derivative of ZEBRA that cannot activate transcription (Zd) can be used to detect and characterize activation domains. Three expression vectors that allow the fusion of putative activation regions in any reading frame were constructed using Zd. These vectors were used to demonstrate the activity of different classes of activation domains using a chloramphenicol acetyltransferase (cat) reporter gene construct containing seven ZEBRA response elements (Z7). The Zd/Z7 system effectively detected proline-rich, glutamine-rich and acidic activation domains in a variety of cell lines and cell types. Using a bioassay unique to the EBV Zd/Z7 system, fusion constructs can also be tested for the ability to activate gene expression directly from a chromatin structure, the EBV genome. These studies indicate that the Zd/Z7 system is an alternative to GAL4 and can be a useful tool for identifying heterologous activation domains.

### INTRODUCTION

The GAL4 system from yeast (*Saccharomyces cerevisiae*) has been widely used to characterize activation domains (13). Here, an alternative system based on the Epstein-Barr virus (EBV) ZEBRA protein is described. ZEBRA consists of three domains: a transcriptional activation domain, a basic DNA recognition domain and a leucine zipper-like coiled-coil dimerization domain (bZIP). ZEBRA disrupts EBV latency by binding as a homodimer to 7-bp motifs, which are found in the promoter regions of numerous EBV lytic genes (References 2, 3 and 6 and references therein).

A ZEBRA mutant from which the activation domain has been deleted (Zd) can still dimerize and bind DNA, but cannot activate transcription or disrupt EBV latency (2,3,6). The activity of different domains fused to Zd can be tested in reporter gene assays with a target plasmid containing seven ZEBRA binding sites (Z7E4CAT). Furthermore, the biological activity of the constructs can be directly assessed by measuring the capacity of expressed chimeras to induce lytic viral protein expression in cells latently infected with EBV (2,3). The Zd/Z7 system was used to demonstrate the activity of different classes of activation domains (acidic, glutamine-rich and proline-rich) and to fine-map the activation domain of the Meq protein. Meq is a bZIP-type transcriptional activator protein encoded by an oncogenic herpesvirus of chickens known as Marek disease virus (MDV) (9,12). The results

indicate that the Zd/Z7 system can be used to map activation domains and that Zd is functional when fused to different classes of activation domains.

### MATERIALS AND METHODS

#### Cells

EBV-positive B-cell lymphoma lines BL41/CL16, CL16 (3) and Raji (3) were maintained in RPMI medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 8% fetal calf serum (FCS) and antibiotics under 5% CO<sub>2</sub>. LTK- (mouse fibroblast) cells were maintained in minimum essential medium (MEM) supplemented with 5% FCS.

#### Electroporations

Plasmids were introduced into lymphocytes using a Bio-Rad Gene Pulser<sup>®</sup> apparatus with a capacitance extender (Hercules, CA, USA). Cells were electroporated at 960  $\mu$ F and 0.25 kV in 0.4 mL of complete medium. After electroporation, the cells were incubated in RPMI/8% FCS for 2–3 days at 37°C under 5% CO<sub>2</sub>. LTK- cells were transfected by the calcium phosphate technique as described previously (3).

#### Plasmid Constructs

Plasmid construction was performed using standard techniques (2,3). Details concerning the generation of various clones are available upon request. Vectors can be obtained by request from Ray Baumann (Internet: [yar@fiona.umsmed.edu](mailto:yar@fiona.umsmed.edu)).

## Chloramphenicol Acetyltransferase (CAT) Assays

Plasmids containing the effector and the target (10–30  $\mu\text{g}$  and 5  $\mu\text{g}$ , respectively) were electroporated into  $0.5\text{--}1.0 \times 10^7$  cells. Two days posttransfection, the cells were resuspended in 100  $\mu\text{L}$  of Buffer I (250 mM Tris-HCl, pH 7.8, 5 mM EDTA) and lysed by three freeze-thaw cycles. Cell debris was pelleted, and supernatants were transferred to fresh tubes. Fifty microliters of cell lysate were mixed with 10  $\mu\text{L}$  of Buffer

I, and the following reagents were added: 20  $\mu\text{L}$  of 5 mM chloramphenicol in Buffer I, 20  $\mu\text{L}$  of a reaction mixture (16.25  $\mu\text{L}$  of  $\text{dH}_2\text{O}$ , 0.75  $\mu\text{L}$  of 4 mM acetyl CoA, 2  $\mu\text{L}$  of 750  $\mu\text{M}$  HCl and 1  $\mu\text{L}$  of [ $^3\text{H}$ ]acetyl CoA [Amersham, Arlington Heights, IL, USA]). The tubes were incubated for 2 h at  $37^\circ\text{C}$ , the samples were extracted with 0.5 mL toluene and the upper phase was counted in a scintillation counter (7,11,14). The target used for assaying Z*d* clones was Z7E4CAT [a synthetic target that has 7 ZEBRA binding sites

in front of the E4 promoter and *cat* gene (4)]. Assays were designed so that effector was expressed in excess, and activation of target was maximal. Furthermore, the abundance of effector expression was routinely monitored by Western blot.

## Western Blots

The cells ( $1.0 \times 10^7$ ) were electroporated with 30  $\mu\text{g}$  of each plasmid. Three days after electroporation, the cells were harvested, washed and resuspended in protein sample buffer, and the proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane and subjected to immunoblot analysis. The primary antibodies used were rabbit anti-trpE-ZEBRA exon 1 polyclonal [1 to 1000 in 15% dry milk (3)] and mouse anti-EA-D monoclonal (Advanced Biotechnologies, Columbia, MD, USA) (1 to 10000 in 10% dry milk). The ECL<sup>TM</sup> Kit (Amersham) was used according to the manufacturer's specifications.

## RESULTS

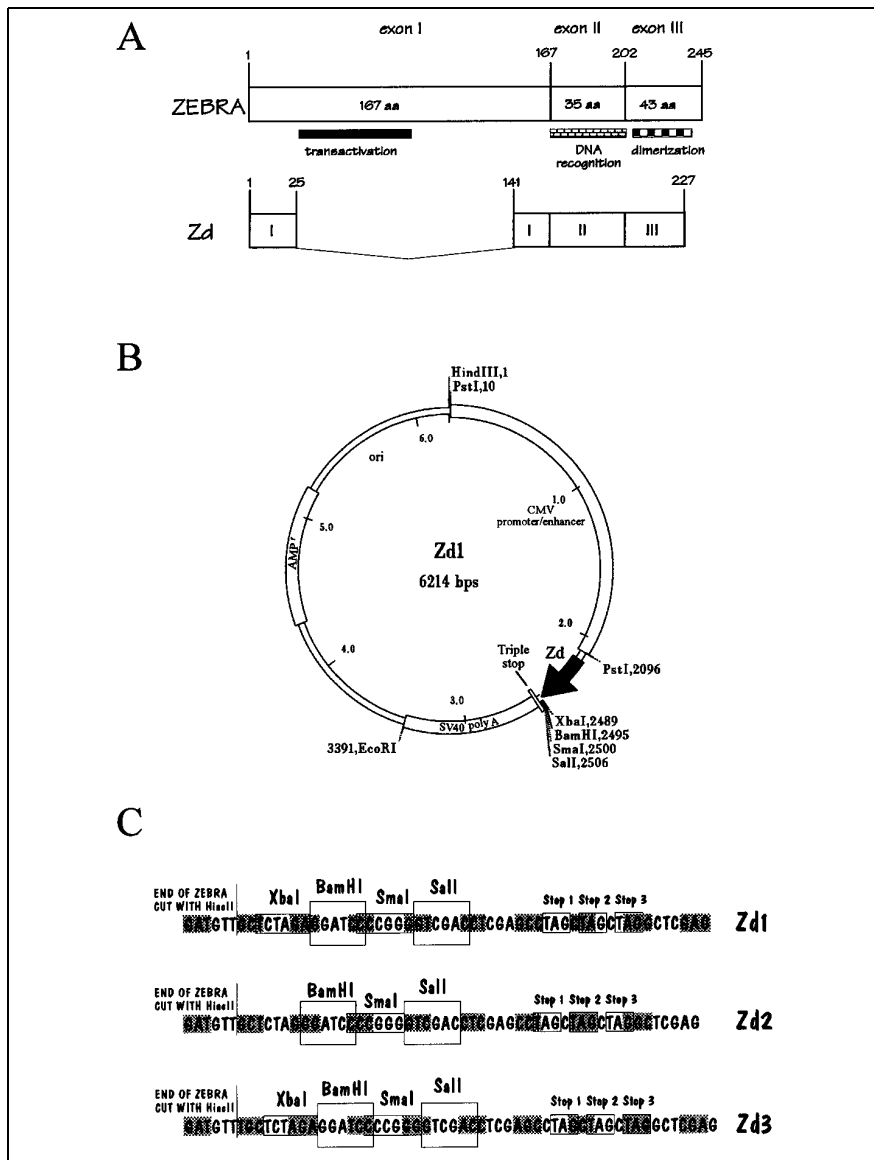
### Construction of the ZEBRA Expression Vectors

Three vectors were constructed that lack the activation domain of ZEBRA but retain the dimerization and DNA binding regions (Z*d*). The vector design permits any coding region to be fused in-frame with the appropriate vector. Expression is driven by the cytomegalovirus (CMV) promoter/enhancer (5). A triple stop linker and the simian virus 40 (SV40) poly(A) signal sequence are located downstream from the multiple cloning site. These constructs were designated as Z*d*1 and Z*d*3 (Figure 1).

All three deleted proteins are readily expressed after electroporation, but fail to activate ZEBRA-specific targets (data not shown). For clarity, the Z*d*1-3 constructs shown in Figure 1 will hereafter be collectively referred to as Z*d*.

### Z*d* Fusions are Functional

The activities of four different activation domains representing different classes of activators were compared



**Figure 1. Z*d* vectors.** (A) Schematic diagram of the Z*d* vectors. Full-size ZEBRA and ZEBRA deleted are shown at the top. (B) All three vectors contain the Z*d* sequence followed by a multiple cloning site, a triple stop sequence and an SV40 poly(A) signal sequence. Expression is driven by the CMV promoter/enhancer. (C) The reading frame in each vector starts after the first (Z*d*1), second (Z*d*2) or third (Z*d*3) base in the *Bam*HI site.

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using the Z<sub>d</sub>/Z<sub>7</sub> system. These domains included the acidic activation domain of the herpes simplex virus (HSV) VP16 protein, the proline-rich domain of MDV's Meq protein (12), the glutamine-rich domain of EBV's EBNA3C protein (10) and ZEBRA's activation domain. Z<sub>d</sub> clones were tested against the Z7E4CAT target, which contains seven ZEBRA binding sites upstream of the *cat* gene (4). Relative fold activity of these constructs is shown in Table 1. All of the activation domains tested were functional when fused to Z<sub>d</sub>. These results were not dependent on the cell line used (Table 1 and data not shown).

## Sequential Mapping with the Z<sub>d</sub> System

The Z<sub>d</sub>/Z<sub>7</sub> system was used to map the proline-rich domain of the Meq protein, which had previously been identified using the GAL4 system (12). The amino terminal protein region from Meq was fused to Z<sub>d</sub>. Two sets of constructs (Z<sub>d</sub>MeqI and Z<sub>d</sub>MeqII) were generated using Z<sub>d</sub> (Table 2). Both sets have progressive deletions from the C-terminal end of the Meq protein. The amino terminal end of the fused Meq region begins at amino acid 105 for the Z<sub>d</sub>MeqI constructs or amino acid 142 for the Z<sub>d</sub>MeqII constructs. The number at the end of each clone designation represents the final C-terminal amino acid derived from the Meq protein. The activity of each clone was determined in CL16 cells against the Z7E4CAT target. Both series of Z<sub>d</sub> deletions displayed a similar pattern of activation. The Z<sub>d</sub>Meq constructs lost activity when the 101 C-terminal amino acids were deleted (clones Z<sub>d</sub>MeqI-238 and Z<sub>d</sub>MeqII-238, Table 2) The results of these experiments were in general agreement with the GAL4 studies that localized the Meq activation domain to the 100 C-terminal amino acids (12).

## A Biological Assay for Activation Domains

Z<sub>d</sub>MeqII fusions were tested in a biological assay to determine their capacity to disrupt latency and to confirm results obtained in CAT assays. Thirty micrograms of each construct were electroporated into EBV-positive Raji cells, and cell extracts were subjected

**Table 1. Activity of Different Activation Domains in the Z<sub>d</sub>/Z<sub>7</sub> System**

Activation Domain <sup>a</sup>	Fold Activation <sup>b</sup>			
	BL41/CL16 Cells		LTK-	
Meq	876.6	(736.7–1021.2) <sup>d</sup>	44.6	(19.2–70.1)
3C	6.2	(5.2–7.9)	10.8	(3.3–18.4)
VP	981.9	(919.1–1044.8)	160.3	(148.1–172.6)
Z <sup>c</sup>	8.2	(4.7–10.1)	25.3	(5.2–45.5)

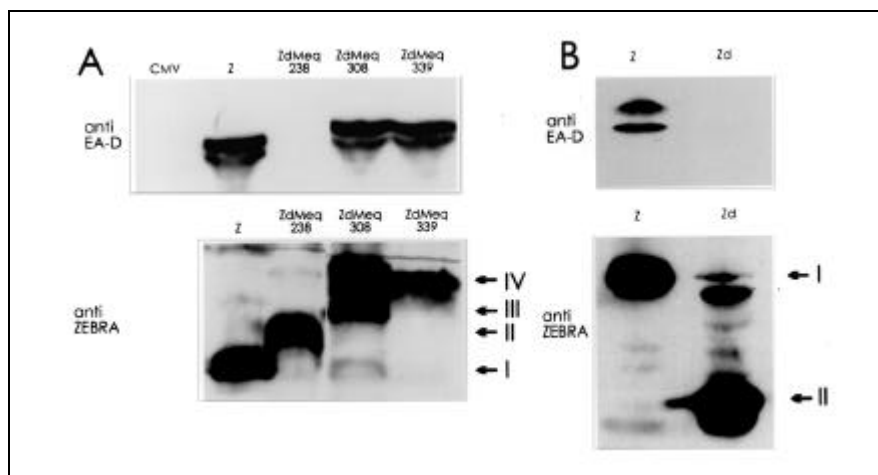
<sup>a</sup>Amino acids 142–339 of Meq, 724–826 of EBNA3C and 410–490 of VP16.  
<sup>b</sup>Fold activation is represented as the mean of at least two experiments where the value of Z<sub>d</sub> is set as one.  
<sup>c</sup>Wild-type, full-size ZEBRA (amino acids 1–245).  
<sup>d</sup>Numbers in parentheses represent minimum and maximum values obtained for the particular clone.

to Western blot analysis three days posttransfection to detect the expression of EBV early antigen [EA-D (3)]. As seen in Figure 2, A and B (top panels), Z<sub>d</sub>MeqII-308, Z<sub>d</sub>MeqI-339 and wild-type ZEBRA readily induce EBV early antigen, but Z<sub>d</sub> does not (Figure 2B) (2,3). The chimera Z<sub>d</sub>MeqII-238, which is negative for activation, is also unable to induce early antigen expression. All constructs are abundantly expressed in these cells (Figure 2, A and B [bottom panels]). Additional bands in the Z<sub>d</sub> lane represent dimerization products of Z<sub>d</sub> that remain associated despite boiling (5 min) and the presence of denaturing conditions (2,3).

## DISCUSSION

These results suggest that the Z<sub>d</sub>/Z<sub>7</sub> system may be an alternative to the GAL4 system for the mapping of activation regions. In this study, the Z<sub>d</sub>/Z<sub>7</sub> system was used to compare the activity of different heterologous classes of activation domains. Z<sub>d</sub> fusions with known proline-rich, glutamine-rich and acidic activation regions were all functional in reporter gene assays.

When the Meq proline-rich activation domain was mapped using sequential deletions, a progressive decrease in activity was seen similar to the pattern observed previously for GAL4 fusions



**Figure 2. Western blot analysis of early antigen induction in Raji cells.** Panels A and B (top): cells were electroporated with each construct and harvested three days later. Immunoblots were conducted with an anti EA-D monoclonal at a dilution of 1:10 000 (Advanced Biotechnologies) using the ECL detection system. Panels A and B (bottom): Western blot analysis of fusion protein expression with anti-ZEBRA exon I polyclonal antisera (2,3). Lane designations: CMV, vector alone; Z, wild-type ZEBRA; Z<sub>d</sub>MeqII-238; Z<sub>d</sub>MeqII-308; Z<sub>d</sub>MeqII-339; and Z<sub>d</sub>, ZEBRA deleted. Arrows in Panel A (bottom) indicate from top to bottom: Z<sub>d</sub>MeqII-339 (IV); Z<sub>d</sub>MeqII-308 (III); Z<sub>d</sub>MeqII-238 (II); and ZEBRA (I). Arrows in Panel B (bottom) indicate from top to bottom: ZEBRA wild-type (I); Z<sub>d</sub> (II).

**Table 2. Activity of Deletion Mutants of the Meq Protein in the Zd/Z7 System**

C-Terminal Amino Acid of Meq	Fold Activation <sup>a</sup>			
	ZdMeqI <sup>b</sup>		ZdMeqII <sup>c</sup>	
339	70.1	(68.2–84.4) <sup>d</sup>	195.5	(185.8–208.6)
308	28.9	(15.7–41.8)	89.0	(86.8–92.3)
283	20.8	(14.9–26.8)	31.4	(25.1–35.7)
262	4.0	(3.2–5.5)	13.1	(10.2–18.5)
254	5.1	(3.8–7.2)	N/D <sup>e</sup>	
238	1.4	(1.2–1.7)	1.2	(0.9–1.5)
226	0.8	(0.6–1.0)	N/D	
224	0.9	(0.8–1.4)	N/D	
208	0.7	(0.6–0.9)	N/D	
197	1.1	(1.1–1.1)	N/D	

<sup>a</sup>Fold activation is represented as the mean of at least two experiments where the value of Zd is set as one.  
<sup>b</sup>Meq sequences starting from amino acid 105 fused to Zd vector.  
<sup>c</sup>Meq sequences starting from amino acid 142 fused to Zd vector.  
<sup>d</sup>Numbers in parentheses represent minimum and maximum values obtained for the particular clone.  
<sup>e</sup>Not determined.

(12). These results suggest that the Zd/Z7 system is sufficiently sensitive to conduct fine-mapping of activation domains (Table 2).

A unique feature of the Zd/Z7 system is the capacity to test constructs in a bioassay that more faithfully replicates the conditions seen *in vivo*. The EBV genome exists as an episomal chromatin structure attached to the nuclear matrix (8). Numerous studies have suggested that histones and chromatin structure can participate in the regulation of gene transcription and expression (1). Western blot analysis verified that the ZdMeqII 308 and 339 fusions were capable of inducing expression directly from the EBV genome, while the ZdMeq 238 fusion was not.

The Zd/Z7 system provides a second choice for investigators interested in identifying potential activation regions. The ZEBRA protein on which this system is based has been extensively characterized by numerous researchers. In certain situations this system might prove to be a better choice for the detection of activation domains

than GAL4. Potential improvements to the Zd system may include: the addition of a larger multiple cloning site, the use of the luciferase gene as a reporter and the incorporation of an epitope tag or the construction of stable cell lines that harbor an integrated Zd target. The complete sequences and restriction enzyme maps of the Zd clones are available through Internet access at <http://fiona.umsmed.edu/~askovic/vectors/vectors.html>.

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