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Activation Domain Requirements for Disruption of Epstein-Barr Virus Latency by ZEBRA

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Latent infection of B lymphocytes by Epstein-Barr virus (EBV) can be disrupted by expression of the EBV ZEBRA protein. ZEBRA, a transcriptional activator, initiates the EBV lytic cascade by activating viral gene expression. ZEBRA is also indispensable for viral replication and binds directly to the EBV lytic origin of replication. The studies described herein demonstrate that the activation domain of ZEBRA is not unique and can be replaced by a heterologous acidic, proline-rich, or glutamine-rich activation domain. ZEBRA activation domain swap constructs retain ZEBRA's native abilities to activate specific EBV promoters, to disrupt EBV latency, and to stimulate replication at the EBV lytic origin. Additional work, employing sequential and internal deletions of ZEBRA's N-terminal activation domain, indicates that its separate activities are not attributable to specific subdomains but are spread throughout its N terminus and therefore cannot be inactivated by deleting localized regions.

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, is the causative agent of infectious mononucleosis. It has been implicated as a cofactor in the genesis of several neoplasms and is strongly associated with lymphoproliferative disorders in immunocompromised individuals (33). EBV infects and establishes lifelong latency in B lymphocytes. During latency the viral genome is maintained as a multiple-copy episome, and only a small fraction of EBV's genes are expressed. Replication of latent viral DNA occurs at a low level and is initiated at the origin of plasmid maintenance (oriP) (44). In contrast, when EBV enters the lytic cycle, most EBV genes are expressed; DNA replication occurs at a much higher level and is initiated at a unique origin of replication designated oriLyt (9–11, 14, 20, 22, 23, 29, 30, 40).

Initiation of the EBV lytic cycle in latently infected B lymphocytes is a rare event. However, diverse agents can activate latent EBV, including phorbol esters, *n*-butyrate, anti-immunoglobulin, superinfection with another virus, or the expression of the EBV ZEBRA protein (*Bam*HI Z Epstein-Barr replication activator; also known as EB1 and Zta) (13). ZEBRA is unique among herpesvirus activators in its capacity to disrupt viral latency. ZEBRA, a bZIP-like transcriptional activator, binds as a homodimer to ZEBRA-responsive elements (ZREs) which are located upstream of specific EBV genes and also within the oriLyt region (30). ZEBRA performs a dual role in the EBV life cycle, functioning both as a transcriptional activator of viral gene expression and as an origin binding protein essential for EBV DNA replication (16, 17). Furthermore, ZEBRA has been shown to interact with a variety of cellular proteins, including TATA-binding protein (TBP), TFIIA, the retinoic acid receptor, NF-κB/p65, and p53 (21, 31, 42, 47). ZEBRA has also been shown to alter the regulation of cell gene expression and to arrest the cell cycle in epithelial cells (6–8).

The ZEBRA protein consists of three exons which roughly correspond to its three functional domains. The DNA recognition and dimerization domains are located in exons 2 and 3, respectively, while exon 1 contains the activation domain (Fig. 1). Deletion studies have shown that the activation domain is encompassed within amino acids 25 to 152 (18), with the most potent region localized to amino acids 25 to 42 (19). However, the localization of the domain(s) important for DNA replication is not as clear. In different cell types there are distinct portions of ZEBRA required for DNA replication. In the D98/HR1 cell line (a HeLa cell line derivative) amino acids 28 to 103 are essential for DNA replication (41), while in Vero cells (an African green monkey cell line) deletion of amino acids 13 to 19 of ZEBRA abolishes its ability to support replication of DNA (39).

In these studies we address the domain requirements of ZEBRA in human B lymphocytes latently infected with EBV. Previous work from this laboratory has shown that ZEBRA's activation domain can be replaced by potent acidic activation regions (5). Here those studies are extended to nonacidic activation domains. Heterologous glutamine-rich and proline-rich activation domains, when appended to a ZEBRA mutant lacking the wild-type activation region, restored its capacity to induce viral early antigen and to stimulate replication of a plasmid containing the EBV origin of replication. In addition, deletion analysis of ZEBRA's large activation region indicated that no subdomains specific for early-antigen induction or the stimulation of lytic replication could be identified. These data suggest that ZEBRA's activation domain is not unique and can be replaced by heterologous activation domains of several classes. In addition, ZEBRA's ability to function as a transcriptional activator is intimately linked to both its role as an inducer of viral gene expression and its role as an essential component in viral lytic replication.

MATERIALS AND METHODS

Cells. The B-cell lymphoma lines Clone 16 (CL16), Raji, BL41/CL16, and BJAB were maintained in RPMI medium supplemented with 8% fetal calf serum and antibiotics at 37°C under a 5% CO_2 atmosphere (4). CL16 cells are human B lymphocytes which harbor the nontransforming P3HR-1 strain of EBV in the latent state (36). Raji cells are human B lymphocytes which contain a deleted EBV episome that can be induced to express early antigen, but which cannot replicate (24). The BL41/CL16 cell line is a derivative of the EBV-negative Burkitt's lymphoma BL41 cell line, which has been stably infected with the EBV CL16 virus. After prolonged culture in vitro, the EBV in BL41/CL16 cells has become progressively more tightly latent and can no longer be induced by

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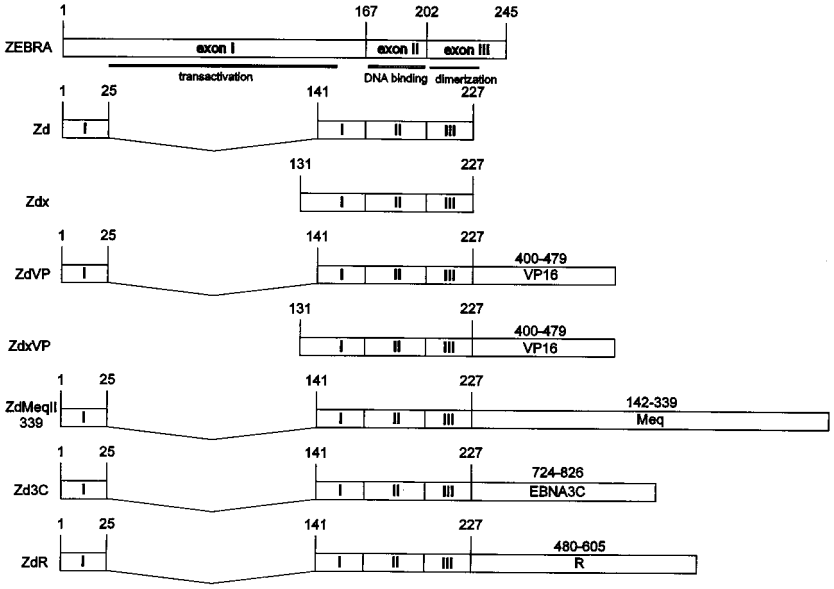


FIG. 1. Schematic diagram depicting the ZEBRA chimeras. ZEBRA's exon structure is shown in cDNA form at the top of the diagram. Clone designations are given at the left. Numbers above each diagram represent amino acids. All constructs were expressed from the CMV promoter enhancer element in a derivative of vector pHD1013 (3, 15).

chemical or other means (2, 37). BJAB is an EBV-negative human B-cell line (36).

Electroporation. B-cell lines were transfected by using a Bio-Rad Gene Pulser apparatus with a capacitance extender, at 960 μF and 0.25 kV in 0.4 ml of complete medium. After electroporation, cells were maintained in RPMI medium with 8% fetal calf serum for 2 to 4 days at 37°C under a 5% CO_2 atmosphere (5).

Plasmid constructs. The Zd clone lacks the activation domain of ZEBRA. It contains amino acids 1 to 25 and 142 to 227 of the wild-type ZEBRA protein (Fig. 1) (3, 5). The ZdVP and ZdR clones have been described before (5); briefly, ZdVP contains amino acids 400 to 479 of herpes simplex virus's VP16 protein fused to the carboxyl terminus of Zd, and ZdR contains amino acids 480 to 605 of EBV's R protein fused to the carboxyl terminus of Zd. The Zd chimeras, ZdMeqII339 and Zd3C, contain the proline-rich region of the Marek's disease virus Meq protein (amino acids 142 to 339) (35) and the glutamine-rich region of EBV's EBNA3C protein (amino acids 724 to 826) (32), respectively. The Zdx clone contains amino acids 131 to 227 of wild-type ZEBRA, while ZdxVP contains amino acids 400 to 479 of VP16 fused to the carboxyl terminus of Zdx. The ZdMeq deletions were generated with *Bal* 31 exonuclease (3). Standard cloning techniques were used to generate all the constructs noted here (38). Where necessary, constructs were confirmed by double-stranded DNA sequencing (Sequenase 2; Amersham). All constructs were expressed downstream of the cytomegalovirus (CMV) promoter enhancer in a derivative of the expression vector pHD1013 (3, 15). The ZEBRA sequential deletions were a kind gift from the laboratory of M. Carey (12). The ZEBRA internal deletions were a kind gift from the laboratory of E. Flemington (18).

CAT assays. Chloramphenicol acetyltransferase (CAT) assays were performed by using a liquid phase assay as described previously (3, 5). Briefly, separate plasmids containing the effector (30 μg) and the target (5 μg) were electroporated into 5×10^6 to 10×10^6 cells. Two days posttransfection, cell extracts were incubated in a reaction mixture containing 0.25 μCi of [^{3}H]acetyl-coenzyme A (Amersham) at 37°C for 2 h. After incubation ^{3}H-labelled acetylated chloramphenicol was extracted with toluene and quantitated in a scintillation counter. The targets used were EABSCAT and DPCAT. EABSCAT contains the promoter of the EBV BMRF1 early antigen gene (EA-D), which is a native target of ZEBRA (26). DPCAT contains a native target region for ZEBRA which includes seven ZREs and EBV oriLyt (5, 29).

Western blots. Equal numbers of cells (5×10^6 to 10×10^6) were electroporated with 30 μg of each plasmid. Three days after electroporation, cells were harvested, washed, and resuspended in protein sample buffer, and the proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and subjected to immunoblot analysis. Immunoblots to monitor ZEBRA expression utilized a rabbit anti-trpE ZEBRA exon 1 polyclonal serum (1:7,500 in 15% dry milk) as the primary antiserum (43). Immunoblots to monitor early antigen utilized a mouse anti-EA-D monoclonal antibody (1:10,000 in 10% dry milk; Advanced Biotechnologies Inc.) as the primary antiserum. Amersham's ECL kit, which contains the secondary antisera, was used for immunoblot detection according to the manufacturer's specifications.

Replication assays. Lymphocyte origin replication (LOR) assays were performed as described previously (5). Briefly, effector plasmid (30 μg), encoding ZEBRA or a ZEBRA derivative, and plasmid p526 (10 μg), which contains the EBV lytic origin of replication (22), were electroporated into CL16 cells. Four days posttransfection, total DNA was isolated and digested with *Bam*HI to linearize the vector DNA and with *Dpn*I to detect newly replicated plasmid DNA. *Dpn*I cleavage requires that DNA be methylated at adenine residues. Adenine methylation occurs in Dam$^+$ bacteria but not in eukaryotes. Therefore, all newly replicated p526 DNA will be resistant to *Dpn*I digestion, while input p526 DNA (which was grown in bacteria) will be sensitive. Control plasmid was added to all samples prior to restriction enzyme digestion to ensure that each digest was complete. Samples were resolved on a 0.8% agarose gel and transferred to nylon membranes (Hybond N$^+$; Amersham). The p526 plasmid was labelled with an ECL Southern blot kit (Amersham) and used as a probe for hybridization. Following hybridization, filters were washed and treated with chemiluminescent reagents according to the manufacturer's specifications. Bands were visualized by autoradiography (exposure, 1 to 30 min).

Figure preparation. All autoradiograms were scanned on a Hewlett-Packard Scanjet 4C scanner with Deskscan II software at a resolution of 900 dots/in. Images were saved as TIFF files and imported into CorelDraw 5.0 for annotation. Negatives were made directly from CorelDraw files by using a Management Graphics 8XP film recorder.

RESULTS

Small internal deletions of the ZEBRA activation domain do not impair its ability to activate latent EBV. The protein sequences responsible for ZEBRA activation have been mapped to amino acids 25 to 152 by reporter gene assays. Deletion analysis suggests that within this large region, amino acids 27 to 42 make the greatest contribution to ZEBRA's activation potency. However, previous studies have not addressed the capacity of various ZEBRA mutants to activate gene expression directly from a latent viral genome and to disrupt viral latency. Here, experiments were performed to determine if subdomains exist within the diffuse activation region of ZEBRA which are specific in determining ZEBRA's ability to disrupt latency or to stimulate EBV lytic replication.

A series of ZEBRA constructs bearing sequential 25-amino-acid internal deletions in the ZEBRA activation domain were

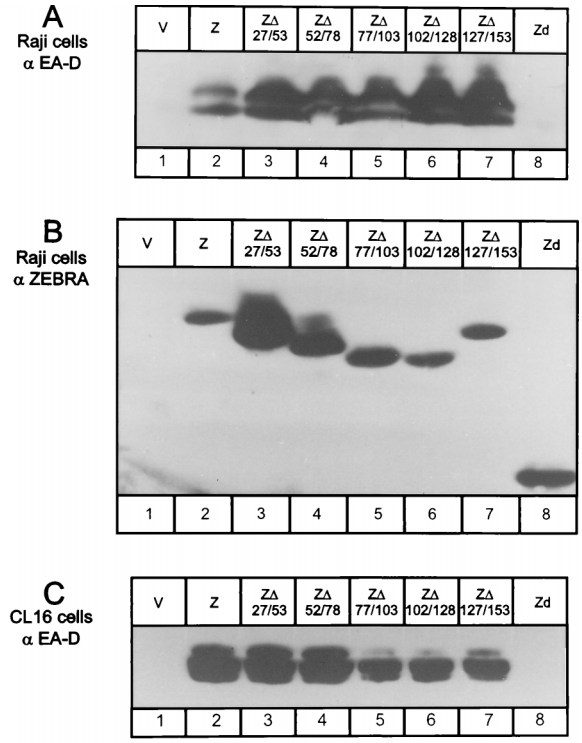
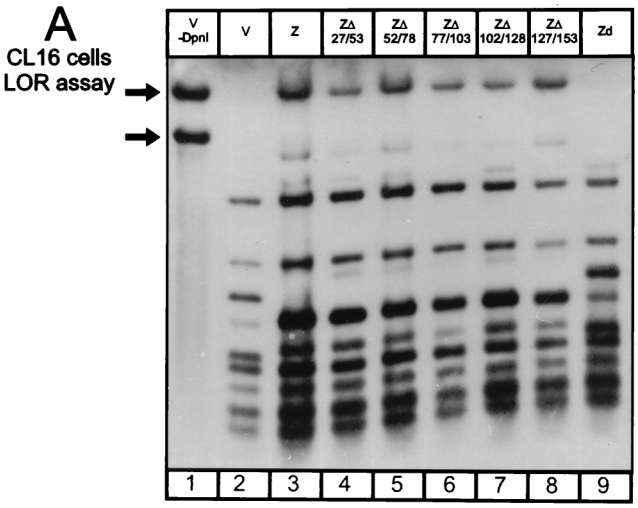


FIG. 2. Effects of the small internal deletions in the ZEBRA activation domain on ZEBRA function. (A) Western blot analysis of EA-D induction in Raji cells by ZEBRA internal-deletion mutants. Cells were electroporated with 30 μg of each construct and harvested in protein sample buffer 3 days after transfection. Protein samples (equivalents of 2×10^6 cells) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose filter. Primary immunoblot analysis on the filter was performed with a mouse monoclonal antibody directed against EA-D (α EA-D; Advanced Biotechnologies). The bands were visualized with a goat antimouse monoclonal antibody as the secondary antibody and with an ECL kit (Amersham). The ZEBRA internal-deletion mutants are designated by the amino acids that have been deleted from wild-type ZEBRA (e.g., ZΔ 27/53 lacks amino acids 27 to 53). Lane V, vector; lane Z, ZEBRA. (B) Expression of ZEBRA internal deletions in Raji cells. Samples were processed as described in the legend to panel A and subjected to immunoblot analysis with a rabbit anti-ZEBRA exon 1 polyclonal antiserum (α ZEBRA) as the primary antibody (43). (C) Western blot analysis of EA-D induction by ZEBRA internal deletion mutants in CL16 cells. The procedures employed were the same as those described for panel A.

tested for the ability to initiate the disruption of EBV latency as judged by the capacity to induce early antigen-diffuse (EA-D) expression. EA-D expression is a marker of the disruption of EBV latency and the initiation of the lytic cycle, since early-antigen expression does not occur during latent EBV infection (33). Furthermore, the EA-D promoter has been shown to be responsive to ZEBRA (26). Each clone was electroporated in 30-μg amounts into EBV-positive Raji cells, and EA-D expression was assessed by immunoblot analysis 3 days later. All five constructs with internal deletions of ZEBRA induced early-antigen expression (Fig. 2A, lanes 3 to 7). Both the vector without an insert and Zd (ZEBRA without an activation domain) failed to induce EA-D expression (Fig. 2A, lanes 1 and 8). In addition, as shown in Fig. 2B, all constructs were abundantly expressed in Raji cells. EA-D induction was also tested in the CL16 B-cell line and shown to be identical (Fig. 2C), indicating that this pattern of early-antigen induction is reproducible and is not unique to Raji cells.

The early-antigen assay tests the ability of each ZEBRA deletion mutant to induce expression of a single protein from the EBV genome. A more stringent functional assay of these mutants is to test their ability to stimulate lytic replication, because this requires the induction of the expression of at least seven EBV proteins which are essential for EBV origin replication (16, 17). Hence, the LOR assay was used to test the five ZEBRA internal-deletion mutants. Thirty micrograms of each construct and 10 μg of the p526 plasmid, which contains an EBV origin of replication (22), were electroporated into EBV-positive CL16 cells. Four days posttransfection, samples were tested for p526 replication by Southern blot analysis as described in Materials and Methods. In agreement with EA-D analysis, all five ZEBRA internal-deletion constructs were active in the LOR assay (Fig. 3A, lanes 4 to 8).

The ZEBRA internal deletions were also tested in reporter gene assays to determine if their capacity to transcriptionally activate specific targets mirrored their ability to function in the EA-D induction and the LOR bioassays. EABSCAT, which contains the promoter for the EA-D gene, was assayed in Raji cells, and DPCAT, which contains the promoter for the BHRF1 gene, included within the oriLyt region, was assayed in CL16 cells. These two targets were selected for reporter gene



B
CAT assay

	V	Z	ZΔ 27/53	ZΔ 52/78	ZΔ 77/103	ZΔ 102/128	ZΔ 127/153	Zd
EABSCAT (Raji cells)	1	216.5	88.0	49.4	114.8	183.6	184.4	1.5
DPCAT (CL16 cells)	1	34.5	76.2	68.6	38.5	73.7	54.0	1.7

FIG. 3. Effects of small internal deletions in the ZEBRA activation domain on its function. (A) LOR analysis of the ZEBRA internal-deletion clones in CL16 cells. Cells were electroporated and processed as described in Materials and Methods. DNA was isolated from each transfected sample, and the equivalent of 0.8×10^6 cells was digested with *Dpn*I and *Bam*HI (with *Bam*HI only in the first lane) and subjected to Southern blot analysis with a chemiluminescence detection kit (ECL; Amersham). Plasmid p526, which contains the EBV lytic origin of replication, was used as a probe. For demonstration purposes the complete blot is shown. The upper arrow indicates the position of linearized p526. The lower arrow indicates the position of control plasmid incorporated into all samples to monitor for complete digestion. Lanes are as explained in the Fig. 2 legend. The faster-migrating bands present in lanes 2 to 9 are *Dpn*I digestion products of unreplicated input plasmid DNA, which are expected from this type of analysis. For simplicity, subsequent figures depicting LOR assay results will display only the pertinent upper replicated band and the control band. (B) Reporter gene assays of the internal-deletion mutants. The target EABSCAT was used in Raji cells. The target DPCAT was used in CL16 cells. Numbers indicate fold activation relative to the CMV vector (V), which was taken to be 1.0. Each number represents the average of at least two independent experiments.

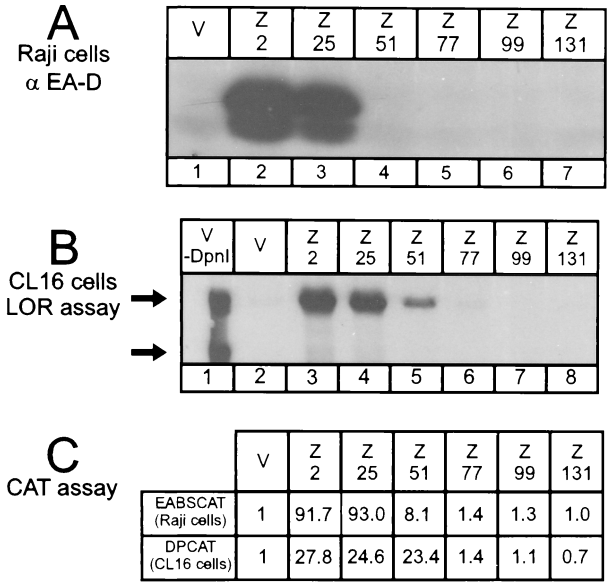


FIG. 4. Evaluation of progressive deletions of the ZEBRA activation domain. (A) Western blot analysis of EA-D induction by ZEBRA deletion mutants in Raji cells. Bands were visualized by using the Western blot ECL kit (Amersham) according to the manufacturer's specifications and as described in the legend to Fig. 2A. (B) Induction of replication of the p526 plasmid in CL16 cells (LOR assay) by the ZEBRA deletion mutants. Southern blot analysis was performed as described in the legend to Fig. 3A. V, vector. In lane 1 no *Dpn*I enzyme was added. Clones are designated by initial amino acid, with all clones ending with amino acid 245; for example, Z2 denotes ZEBRA amino acids 2 to 245. The upper arrow indicates the position of linearized p526. The lower arrow indicates the position of control plasmid incorporated into all samples to monitor for complete digestion. (C) Reporter gene analysis of the ZEBRA progressive deletion mutants. Results are presented as explained in the legend to Fig. 3B.

assays because they match the promoter regions tested in the bioassays employed in these studies and might therefore provide a more relevant standard for comparison. Again, all five clones were active in the CAT assay independent of the target or the cell line employed (Fig. 3B). Taken together, these results indicate that small deletions in the ZEBRA activation region do not destroy its ability to disrupt latency. Furthermore, no distinct subdomain that was required specifically for transcriptional activation, the induction of early antigen, or the stimulation of lytic replication could be identified.

The amino-terminal 50 amino acids of ZEBRA are required for latency disruption. To identify the region required for the disruption of EBV latency, ZEBRA mutants with progressive deletions from the amino-terminal end were tested for activity. The clones are designated by the initial amino acid retained from wild-type ZEBRA [e.g., Z(2) contains amino acids 2 to 245 of ZEBRA] (12). Clones were first assayed to determine their capacity to induce EBV early antigen in Raji cells. The ability to induce EA-D was lost in construct Z(51), which lacks the first 50 amino acids of ZEBRA (Fig. 4A, lane 4), but EA-D induction was retained by clones Z(2) and Z(25) (Fig. 4A, lanes 2 and 3). When tested by immunoblot analysis, all clones were found to be expressed in abundance (2). Furthermore, this pattern of EA-D induction was identical in CL16 cells (2).

The deletion mutants were next tested in the LOR assay. Interestingly, in addition to Z(2) and Z(25), Z(51) was also able to induce replication of the p526 plasmid containing the EBV oriLyt in the LOR assay, albeit at a reduced level (Fig. 4B, lanes 3 through 5). CAT assays using DPCAT (oriLyt promoter region) and EABSCAT (EA promoter) as targets supported results obtained with the LOR and EA-D bioassays (Fig. 4C), respectively. Notably, activation was apparent with the Z(51) construct on DPCAT, in agreement with results obtained with this construct in the LOR assay. We believe that the activity of Z(51) in the oriLyt assay reflects the increased sensitivity of this assay over EA-D analysis. Similarly, DPCAT contains an excellent promoter target for ZEBRA, the oriLyt region, which harbors seven ZREs and is highly sensitive to ZEBRA activation. Deletion of the first 25 amino acids did not alter the ability of ZEBRA to function in human B lymphocytes in any of the assays used here. These results were of interest because a recent report has shown that deletion of amino acids 13 to 19 of ZEBRA abolishes its ability to induce viral DNA replication in Vero cells (39).

Nonacidic activation domains can substitute for ZEBRA's activation domain. If, as the above data suggests, the ZEBRA activation domain functions as a unit without subdomains, then other discrete activation domains may be able to completely substitute for its activity. In agreement with this, this laboratory has previously demonstrated that potent acidic activation domains can replace the ZEBRA activation region (5). In the current study, two nonacidic activation domains, the glutamine-rich domain of the EBV EBNA 3C protein and the proline-rich domain of the Marek's disease virus Meq protein, were fused to a ZEBRA mutant which lacks an activation domain (Zd) (Fig. 1) (3). The resulting Zd3C and ZdMeqII339 chimeras (Fig. 1) were first tested for the capacity to initiate the disruption of EBV latency as judged by the induction of EA-D expression in Raji cells. When compared to the potent acidic Zd chimeras, ZdVP and ZdR (Fig. 5A, lanes 4 and 7), the nonacidic chimeras were found to be equally effective at

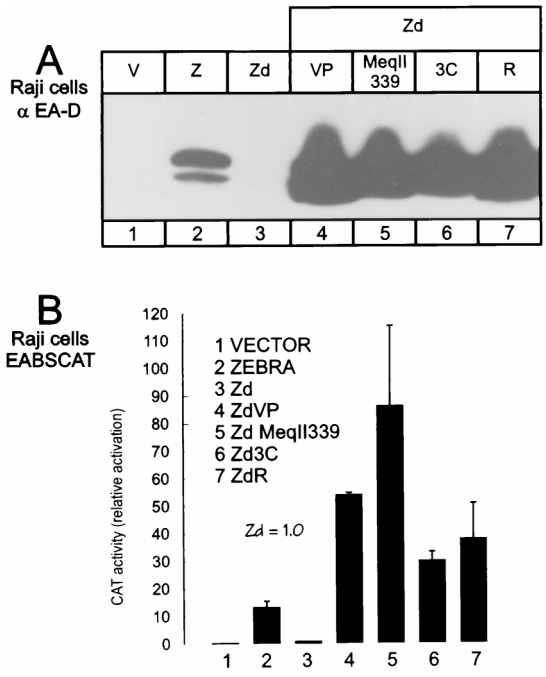


FIG. 5. Analysis of ZEBRA activation domain substitution constructs. (A) EA-D induction by ZEBRA chimeric clones in Raji cells. Electroporation and Western blot analysis were performed as described in the legend to Fig. 2A. V, vector; Z, ZEBRA. (B) Reporter gene analysis of ZEBRA chimeric constructs in Raji cells. The target employed was EABSCAT. Fold activation relative to Zd activity, which was arbitrarily taken to be 1.0-fold, is given on the vertical axis. Clone designations are shown within the figure. Results are averages of two independent experiments, and error bars indicate 1 standard deviation.

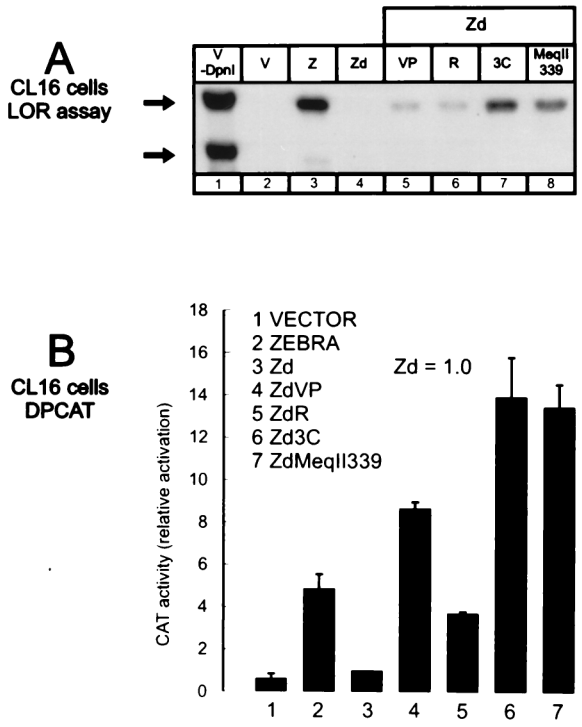


FIG. 6. Analysis of the ZEBRA chimeras. (A) oriLyt analysis of the ZEBRA chimeras in CL16 cells. oriLyt analysis was conducted as described in the legend to Fig. 3A. The upper arrow indicates the position of linearized p526. The lower arrow indicates the position of control plasmid incorporated into all samples to monitor for complete digestion. V, vector; Z, ZEBRA. (B) Activation of the DPCAT target by the ZEBRA chimeras. DPCAT contains the EBV oriLyt region. Results are presented as explained in the legend to Fig. 5B.

inducing EA-D expression (Fig. 5A, lanes 5 and 6). Identical results were obtained with CL16 cells (2). Next, the nonacidic Zd chimeras were assayed for the ability to activate the EA-D promoter from the reporter gene construct EABSCAT (Fig. 5B, columns 5 and 6). Both constructs display activation ability in Raji cells which is similar in magnitude to that seen for the potent acidic activation constructs ZdVP and ZdR. Furthermore, immunoblot analysis indicated that all constructs were abundantly expressed (2). These studies demonstrate that nonacidic activation domains can restore Zd's ability to activate the EA-D promoter both in the context of the intact viral genome and in the context of a plasmid construct independent of the viral genome. In addition, since expression of EA-D is a marker for the EBV lytic cycle, these experiments also suggest that the ZEBRA chimeras can initiate the disruption of EBV latency.

Zd3C and ZdMeqII339 were also subjected to the more stringent LOR assay, which tests for the ability to induce the full repertoire of gene products necessary for lytic viral replication. Constructs were electroporated into EBV-positive CL16 cells along with the p526 plasmid, which contains an EBV oriLyt. Southern blot analysis of *Dpn*I/*Bam*HI-digested DNA samples indicated that these Zd fusions with nonacidic activation domains could induce EBV replication in the LOR assay. Both Zd3C and ZdMeqII339 were able to drive replication of the oriLyt-containing plasmid (p526), while vector alone and Zd were inactive, as shown in Fig. 6A (lanes 2, 4, 7, and 8). ZdVP and ZdR, which have previously been shown to be active in this assay (5), were used as positive controls (lanes 5 and 6). Finally, the Zd chimeras were tested in CAT assays against the target DPCAT, which contains an oriLyt sequence upstream of the CAT gene (5). All the Zd chimeric clones activated this target more than fourfold compared to Zd alone (Fig. 6B, columns 4 through 7).

It is important that all the ZEBRA domain substitution constructs (Fig. 1) contain the first 25 amino acids of ZEBRA. As has been previously discussed, this region has been found to be important for replication of an EBV origin-containing plasmid in African green monkey cells (39). Therefore, two additional clones were constructed, Zdx and ZdxVP, which lack this 25-amino-acid region. Zdx contains amino acids 131 to 227 of ZEBRA, and ZdxVP contains amino acids 400 to 479 of VP16 fused to the carboxy-terminal end of Zdx. ZdxVP was capable of inducing oriLyt replication to the same levels as ZdVP (Fig. 7, lanes 5 and 7), which argues that the N-terminal 25 amino acids do not contribute to the ability of ZdVP to induce DNA replication at the oriLyt in B lymphocytes. Control assays containing vector, Zd, or Zdx could not induce replication in the LOR assay.

Deletion analysis of the Meq activation region. As discussed in a previous section, sequential deletion of the ZEBRA activation region failed to reveal any subdomains that were required for reporter gene assays, EA-D induction, or oriLyt replication. Sequential deletions of the Meq activation domain in ZdMeqII339 were also generated to determine if this pattern would hold true for a fully functional Zd chimera. Therefore, deleted derivatives of ZdMeqII339 were used to determine if the regions of the Meq activation domain which are required for transactivation are also essential for the induction of viral replication. The ZdMeq clones were first tested for the ability to activate the ZEBRA targets DPCAT and EABSCAT in CL16 and Raji cells, respectively. As shown in Fig. 8A, with progressive deletion of the Meq domain, activity is gradually reduced on the DPCAT target. Then, after more than 100 amino acids have been removed, activation capacity is abolished (ZdMeqII238 construct). On the EABSCAT target, activity is substantially reduced for the ZdMeqII283 construct but is completely lost for construct ZdMeqII262. As discussed below, this overall pattern is reproduced in the bioassays (Fig. 8B and C).

In the preceding sections experimentation has focused on two cell lines, Raji and CL16, both of which are EBV positive and both of which contain viral genomes that can be reactivated from latency. The ZdMeq clones were also tested by CAT assay in two additional cell lines: the BL41/CL16 cell line, which harbors latent EBV which cannot be induced from latency (2, 37) and the BJAB cell line, which is EBV negative. As shown in Fig. 8A, the overall patterns of activation with EABSCAT are similar in these two cell lines. These data indicate

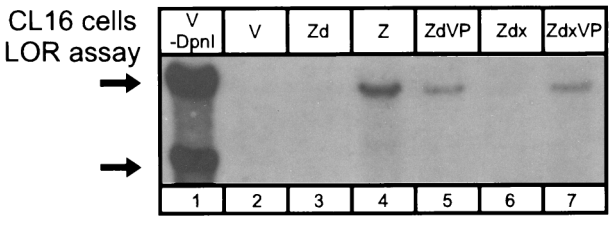
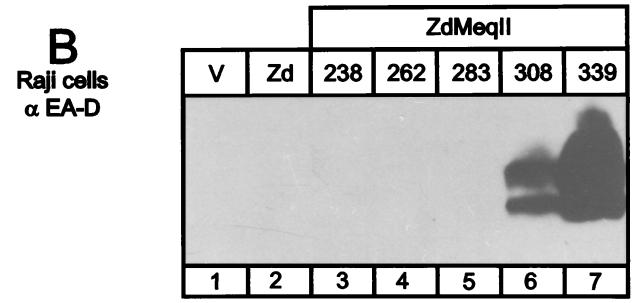


FIG. 7. oriLyt analysis of a ZEBRA chimera lacking the first 25 amino acids of the ZEBRA activation domain. The LOR assay was performed in CL16 cells as described in the legend to Fig. 3A. The upper arrow indicates the position of linearized p526. The lower arrow indicates the position of control plasmid incorporated into all samples to monitor for complete digestion. V, vector; Z, ZEBRA.

A
CAT assay

	V	Zd	Z	ZdMeqII 238	262	283	308	339
CL16 DPCAT	0.4	1	4.1	0.8	3.3	6.3	12.2	12.3
Raji EABSCAT	0.8	1	6.5	1.0	1.4	5.2	14.6	22.8
BL41/CL16 EABSCAT	N/D	1	10.6	0.7	1.1	1.0	14.6	10.2
BJAB EABSCAT	1.5	1	N/D	1.2	2.0	4.6	13.3	9.5



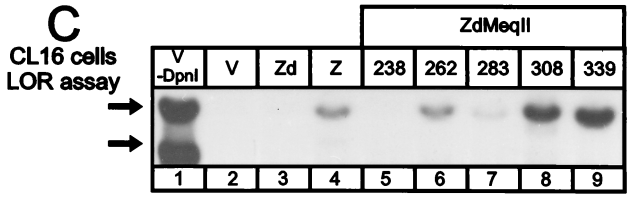


FIG. 8. Analysis of the deletion mutants of the ZEBRA chimera ZdMeqII339, designated by the last amino acid retained. (A) CAT assay analysis of the ZdMeqII subclones. The target DPCAT was employed in CL16 cells. EABSCAT was used as a target in Raji, BL41/CL16, and BJAB cells. Results are given as fold activation relative to Zd activity, which was arbitrarily set to a value of one. Each value represents the average of at least two independent experiments. V, vector; Z, ZEBRA; N/D, not determined. (B) Induction of EA-D in Raji cells by ZdMeqII339 deletion mutants. Whole-cell extracts were prepared 3 days post-transfection. Equivalents of 2×10^6 cells were loaded into each lane and subjected to Western blot analysis. The primary antibody was mouse anti-EA-D (Advanced Biotechnologies), and the secondary antibody was goat antimouse antibody (Amersham). Bands were visualized by using an ECL kit according to the manufacturer's specifications (Amersham). (C) Induction of replication of the p526 plasmid by the ZdMeqII clones in CL16 cells. DNA from 0.8×10^6 cells was digested with *Dpn*I and *Bam*HI and subjected to Southern blot analysis as described in the legend to Fig. 3A. The upper and the lower arrows represent the positions of p526 and of the digestion control plasmid, respectively.

that the results are not dependent on the individual cell line used or on the presence of latent EBV.

The ZdMeq constructs were next tested for the ability to induce EA-D (Fig. 8B). The results from the EA-D induction assay are in general agreement with those seen for activation of the EA-D promoter in CAT assays, although the loss of activity in the EA-D assay seems to be more precipitous after deletion beyond amino acid 283 of Meq. In addition, these experiments suggest that amino acids 309 to 339 of Meq are dispensable for its ability to activate EA-D from the latent viral genome. These results cannot be explained by a difference in the expression of the ZdMeq clones, since immunoblot analysis with polyclonal antisera directed against ZEBRA demonstrated that all constructs are expressed in abundance (2).

Finally, the ZdMeq chimeras were tested in the LOR assay. Interestingly, in this assay clone ZdMeqII262, which was clearly inactive at EA-D induction and in CAT assays against the EABSCAT target (Fig. 8A and B), was able to induce EBV oriLyt replication (Fig. 8C). In support of these results, ZdMeqII262 was also active when tested against the DPCAT (oriLyt) target (Fig. 8A). As discussed above for the N-terminal ZEBRA mutants, we believe that these differences are attributable to a higher sensitivity of the oriLyt assay and to a greater responsiveness of the DPCAT target. In summary, the various targets of ZEBRA seem to show individually consistent specificities and sensitivities that are independent of the type of assay employed (reporter gene assay or bioassay). In general, the results seen with reporter gene assays accurately reflected gene activation from the context of the viral genome as tested by the bioassays employed here (Fig. 9). Lastly, the results of deletion analysis with the ZdMeq chimera were consistent with the pattern seen for the deletion of the wild-type ZEBRA domain. Specifically, the capacity to function as a transcriptional activator could not be separated from, and was a prerequisite for, the ability to disrupt virus latency and to stimulate replication at the EBV oriLyt.

DISCUSSION

Activation domains share very little direct amino acid sequence homology and are often grouped on the basis of their

	BIO ASSAYS		CAT ASSAYS	
	LOR	EA-D	DPCAT	EABS CAT
	CL16	Raji	CL16	Raji
VECTOR	-	-	-	-
ZEBRA	+	+	+	+
ZΔ 27/53	+	+	+	+
ZΔ 52/78	+	+	+	+
ZΔ 77/103	+	+	+	+
ZΔ 102/128	+	+	+	+
ZΔ 127/153	+	+	+	+
Z 2-245	+	+	+	+
Z 25-245	+	+	+	+
Z 51-245	+	-	+	+
Z 77-245	-	-	-	-
Z 99-245	-	-	-	-
Z 131-245	-	-	-	-
Zd	-	-	-	-
ZdVP	+	+	+	+
Zd3C	+	+	+	+
ZdR	+	+	+	+
ZdMeqII 339	+	+	+	+
ZdMeqII 308	+	+	+	+
ZdMeqII 283	+	-	+	+
ZdMeqII 262	+	-	+	-
ZdMeqII 238	-	-	-	

FIG. 9. Schematic diagram summarizing the results of assays performed with CL16 and Raji cells. The various constructs employed are given in the left column.

amino acid content, into acidic, proline-rich, and glutamine-rich classes (34). The activation domain of ZEBRA cannot be classified into any of these groups. Not surprisingly, an amino acid homology search of the activation domain of ZEBRA revealed no significant homology to any protein in the GenBank database (1). Studies on basal transcription have suggested that acidic and glutamine-rich activation regions bind to specific TBP-associated factors within the TFIID complex and thus mediate the activation of transcription (25). ZEBRA itself has been shown to bind TBP and TFIIA through interaction with protein sequences within its first exon that overlap its activation domain (31).

This report, together with previously published papers (3, 5), clearly demonstrates that the activation domain of ZEBRA can be replaced by heterologous nonacidic activation domains, so that the resulting ZEBRA chimeric proteins have been restored to wild-type activity. Both the glutamine-rich domain of the EBNA 3C protein and the proline-rich domain of the Meq protein were capable of substituting for the wild-type ZEBRA activation region. The ZEBRA chimeras were capable of disrupting EBV latency, as demonstrated by their ability to induce virus early antigen and to stimulate replication of a plasmid containing an EBV lytic origin. These experiments also address the broad biological question of whether activation regions confer specificity or participate in the uniqueness of a protein's function. In the system employed here, activation domains are readily exchangeable and do not confer obvious functional specificity.

The results described here contrast with the study of Schepers et al. (40a) in which the ZEBRA activation domain was found to be required for replication of a modified EBV oriLyt region. In that study all ZEBRA binding sites in the oriLyt region were mutated to GAL4 binding sites; and GAL4 chimeras containing heterologous activation domains (other than ZEBRA's) fused to the GAL4 DNA binding domain were shown to be incapable of driving oriLyt plasmid replication in D98/HR1 cells. The different result obtained in our study may reflect fundamental differences in the GAL4 and ZEBRA proteins. Alternately, the introduction of mutations into the EBV lytic origin may have altered its response to direct stimulation. Lastly, these variant experimental outcomes may reflect cell type-specific differences in the requirements for EBV lytic replication.

The ease with which ZEBRA's activation region can be exchanged in the B-lymphocyte system may be related to the unique properties of the ZEBRA bZIP-like region. In addition to mediating dimerization and DNA recognition, the bZIP-like region in ZEBRA has been found to be essential for binding to the cellular proteins p53 and NF-κB/p65 and to the viral BMRF1 (EA-D) protein (21, 46, 47). The bZIP region has also been recently demonstrated to mediate growth arrest at the G0/G1 phase in epithelial cells (6, 8). Perhaps ZEBRA's specificity for altering cellular and viral gene expression pathways resides in its multifunctional bZIP-like carboxyl-terminal region.

Our analysis of small internal deletions within exon 1 of ZEBRA indicated that the activities associated with this exon are not contributed by specific subdomains but are spread throughout exon 1 and therefore cannot be inactivated by deleting localized regions. Studies with Vero cells (39) and D98/HR1 cells (41) have suggested that the ZEBRA activation domain is unique and contains essential sequences which cannot be substituted for. In contrast, these studies, conducted with EBV-positive human lymphocyte lines, suggest that both acidic and nonacidic heterologous activation regions can fully substitute for ZEBRA's activation domain. Furthermore, deletion of the N-terminal 25 amino acids of ZEBRA did not obviously impair its ability to support DNA replication at the EBV lytic origin in B lymphocytes. In addition, our conclusions are independent of the particular B-cell line employed, as the four distinct cell lines employed here yielded similar results. These cell lines included EBV-negative and -positive lines, as well as a tightly latent lymphocyte line (BL41/CL16). Three distinct assays were used to test each of the various clones employed in these studies: reporter gene assays, EA-D induction, and a stringent oriLyt replication assay. Although minor differences were noted for some of the sets of clones, we attribute these to the observation that the CAT assays and the origin assays appeared slightly more sensitive than Western blot analysis of EA-D induction. Also, the two targets employed in the CAT assays represent unique native EBV promoters that differ in their responsiveness to ZEBRA transactivation in different cell types, an observation which may explain some of the minor differences observed in our studies. However, as summarized in Fig. 9, overall the results are in excellent agreement among the various assays performed.

Our results may differ from those of other investigators because of the inherent differences in the cell lines utilized. Human B cells, which are the natural host cells for latent EBV, may provide a unique environment for the disruption of EBV latency and the induction of lytic replication which is not duplicated by other cell lines. One obvious possible complication of this system, which utilizes EBV-infected cells, is the potential for the induction of endogenous ZEBRA expression by ZEBRA derivatives. We feel that it is highly unlikely that the induction of endogenous ZEBRA expression can account for our results for two reasons: (i) Western blot analysis fails to detect any induction of endogenous ZEBRA expression in the assays performed here (2), and (ii) previously published studies from several different laboratories have demonstrated that exogenously introduced ZEBRA cannot induce endogenous ZEBRA expression in human B-cell lines (27, 28, 45). Finally, it must be stated that lytic replication of the endogenous viral genome was not directly tested for in the studies described herein. However, it should be reiterated that the oriLyt plasmid assay employed here is dependent on the expression of seven lytic-cycle viral-gene products that must be provided in *trans* by the endogenous genome, an event unlikely to occur in the absence of true lytic replication in EBV-positive cells.

In summary, these studies indicate that ZEBRA's capacity to activate transcription cannot be readily separated from its role in the induction of lytic replication and that ZEBRA's function as a transactivator may be intimately involved in all its activities. Overall, the results from reporter gene assays, early antigen induction, and the LOR assays were in strong agreement with each other. Finally, the ability of ZEBRA to function when fused to many different types of activation regions suggests that ZEBRA may be useful for mapping putative activation regions (3).

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. Myers, and D. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Askovic, S., and R. Baumann.** Unpublished data.
3. **Askovic, S., and R. Baumann.** 1997. An alternative system for detecting and mapping activation domains. BioTechniques **22:**948–951.
4. **Baumann, R., E. Grogan, M. Ptashne, and G. Miller.** 1993. Changing Ep-

stein-Barr viral ZEBRA protein into a more powerful activator enhances its capacity to disrupt latency. Proc. Natl. Acad. Sci. USA **90:**4436–4440.
5. **Baumann, R., G. Warren, and S. Askovic.** 1995. Restoration of the Epstein-Barr virus ZEBRA protein's capacity to disrupt latency by the addition of heterologous activation regions. Virology **211:**64–72.
6. **Cayrol, C., and E. K. Flemington.** 1996. The Epstein-Barr virus bZIP transcription factor Zta causes G0/G1 cell cycle arrest through induction of cyclin-dependent kinase inhibitors. EMBO J. **15:**2748–2759.
7. **Cayrol, C., and E. K. Flemington.** 1995. Identification of cellular target genes of the Epstein-Barr virus transactivator Zta: activation of transforming growth factor βigh3 (TGF-βigh3) and TGF-β1. J. Virol. **69:**4206–4212.
8. **Cayrol, C., and E. K. Flemington.** 1996. G0/G1 growth arrest mediated by a region encompassing the basic leucine zipper (bZIP) domain of the Epstein-Barr virus transactivator Zta. J. Biol. Chem. **271:**31799–31802.
9. **Chavrier, P., H. Gruffat, A. Chevallier-Greco, M. Buisson, and A. Sergeant.** 1989. The Epstein-Barr virus early promoter DR contains a *cis*-acting element responsive to the EBV transactivator EB1 and an enhancer with constitutive and inducible activities. J. Virol. **63:**607–614.
10. **Chevallier-Greco, A., E. Manet, P. Chavrier, C. Mosnier, J. Daillie, and A. Sergeant.** 1986. Both Epstein-Barr virus (EBV)-encoded trans-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. EMBO J. **5:**3243–3249.
11. **Chevallier-Greco, A., H. Gruffat, E. Manet, A. Calender, and A. Sergeant.** 1989. The Epstein-Barr virus (EBV) DR enhancer contains two functionally different domains: domain A is constitutive and cell specific, domain B is transactivated by the EBV early protein R. J. Virol. **63:**615–623.
12. **Chi, T., and M. Carey.** 1993. The ZEBRA activation domain: modular organization and mechanism of action. Mol. Cell. Biol. **13:**7045–7055.
13. **Countryman, J. K., and G. Miller.** 1985. Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogenous viral DNA. Proc. Natl. Acad. Sci. USA **82:**4085–4089.
14. **Cox, M. A., J. Leahy, and J. M. Hardwick.** 1990. An enhancer within the divergent promoter of Epstein-Barr virus responds synergistically to the R and Z transactivators. J. Virol. **64:**313–321.
15. **Davis, M. G., and E. Huang.** 1988. Transfer and expression of plasmids containing human cytomegalovirus immediate-early gene 1 promoter enhancer sequences in eukaryotic and prokaryotic cells. Biotechnol. Appl. Biochem. **10:**6–12.
16. **Fixman, E. D., G. S. Hayward, and S. D. Hayward.** 1992. *trans*-acting requrements for replication of Epstein-Barr virus ori-Lyt. J. Virol. **66:**5030–5039.
17. **Fixman, E. D., G. S. Hayward, and S. D. Hayward.** 1995. Replication of Epstein-Barr virus oriLyt: lack of a dedicated virally encoded origin-binding protein and dependence on Zta in cotransfection assays. J. Virol. **69:**2998–3006.
18. **Flemington, E. K., A. M. Borras, J. P. Lytle, and S. H. Speck.** 1992. Characterization of the Epstein-Barr virus BZLF1 protein transactivation domain. J. Virol. **66:**922–929.
19. **Giot, J., I. Mikaelian, M. Buisson, E. Mante, I. Joab, J. Nicholas, and A. Sergeant.** 1991. Transcriptional interference between the EBV transcription factors EB1 and R: both DNA-binding and activation domains of EB1 are required. Nucleic Acids Res. **19:**1251–1258.
20. **Gruffat, H., N. Moreno, and A. Sergeant.** 1990. The Epstein-Barr virus (EBV) ORI_{lyt} enhancer is not B-cell specific and does not respond synergistically to the EBV transcription factors R and Z. J. Virol. **64:**2810–2818.
21. **Gutsch, D. E., E. A. Holley-Guthrie, Q. Zhang, B. Stein, A. A. Blanar, A. S. Baldwin, and S. C. Kenney.** 1994. The bZIP transactivator of Epstein-Barr virus, BZLF1, functionally and physically interacts with the p65 subunit of NF-κB. Mol. Cell. Biol. **14:**1939–1948.
22. **Hammerschmidt, W., and B. Sugden.** 1988. Identification and characterization of oriLyt, a lytic origin of DNA replication of Epstein-Barr virus. Cell **55:**427–433.
23. **Hardwick, J. M., P. M. Lieberman, and S. D. Hayward.** 1988. A new Epstein-Barr virus transactivator, R, induces expression of a cytoplasmic early antigen. J. Virol. **62:**2274–2284.
24. **Hatfull, G., A. T. Bankier, B. G. Barrell, and P. J. Farell.** 1988. Sequence analysis of RAJI Epstein-Barr virus DNA. Virology **164:**334–340.
25. **Hoey, T., R. O. J. Weinzieri, G. Gill, J. L. Chen, B. D. Dynlacht, and R. Tjian.** 1993. Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell **72:**247–260.
26. **Kenney, S., J. Kamine, G. E. Holley, J. C. Lin, E. C. Mar, and J. Pagano.** 1989. The Epstein-Barr virus (EBV) BZLF1 immediate-early gene product differentially affects latent versus productive EBV promoters. J. Virol. **63:** 1729–1736.
27. **Kolman, J. L., N. Taylor, L. Gradoville, J. Countryman, and G. Miller.** 1996. Comparing transcriptional activation and autostimulation by ZEBRA and ZEBRA/c-Fos chimeras. J. Virol. **70:**1493–1504.
28. **Le Roux, F., A. Sergeant, and L. Corbo.** 1996. Epstein-Barr virus (EBV) EB1/Zta protein provided in trans and competent for the activation of productive cycle genes does not activate the BZLF1 gene in the EBV genome. J. Gen. Virol. **77:**501–509.
29. **Lieberman, P. M., J. M. Hardwick, and S. D. Hayward.** 1989. Responsiveness of the Epstein-Barr virus Not1 repeat promoter to the Z transactivator is mediated in a cell-type-specific manner by two independent signal regions. J. Virol. **63:**3040–3050.
30. **Lieberman, P. M., J. M. Hardwick, J. Sample, G. S. Hayward, and S. D. Hayward.** 1990. The Zta transactivator involved in induction of lytic cycle gene expression in Epstein-Barr virus-infected lymphocytes binds to both AP-1 and ZRE sites in target promoter and enhancer regions. J. Virol. **64:**1143–1155.
31. **Lieberman, P. M., and A. J. Berk.** 1991. The Zta transactivator protein stabilizes TFIID association with promoter DNA by direct protein-protein interaction. Genes Dev. **5:**2441–2454.
32. **Marshal, D., and C. Sample.** 1995. Epstein-Barr virus nuclear antigen 3C is a transcriptional regulator. J. Virol. **69:**3624–3630.
33. **Miller, G.** 1990. Epstein-Barr virus: biology, pathogenesis and medical aspects, p. 1921–1958. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology. Raven Press, Ltd., New York, N.Y.
34. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245:**371–378.
35. **Qian, Z., P. Brunovskis, F. Rauscher III, L. Lee, and H.-J. Kung.** 1995. Transactivation activity of Meq, a Marek's disease herpesvirus bZIP protein persistently expressed in latently infected transformed T cells. J. Virol. **69:** 4037–4044.
36. **Rabson, M., L. Heston, and G. Miller.** 1983. Identification of a rare Epstein-Barr virus variant that enhances early antigen expression in Raji cells. Proc. Natl. Acad. Sci. USA **80:**2762–2766.
37. **Rooney, C. M., D. T. Rowe, T. Ragot, and P. J. Farrell.** 1989. The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J. Virol. **63:**3109–3116.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Sarisky, R., Z. Gao, P. M. Lieberman, E. D. Fixman, G. S. Hayward, and D. Hayward.** 1996. A replication function associated with the activation domain of the Epstein-Barr virus Zta transactivator. J. Virol. **70:**8340–8347.
40. **Schepers, A., D. Pich, J. Mankertz, and W. Hammerschmidt.** 1993. *cis*-acting elements in the lytic origin of DNA replication of Epstein-Barr virus. J. Virol. **67:**4237–4245.

40a. **Schepers, A., D. Pich, and W. Hammerschmidt.** 1993. A transcription factor with homology to the AP-1 family links RNA transcription and DNA replication in the lytic cycle of Epstein-Barr virus. EMBO J. **12:**3921–3929.

41. **Schepers, A., D. Pich, and W. Hammerschmidt.** 1996. Activation of oriLyt, the lytic origin of DNA replication of Epstein-Barr virus, by BZLF1. Virology **220:**367–376.
42. **Sista, N. D., J. S. Pagano, W. Liao, and S. Kenney.** 1993. Retinoic acid is a negative regulator of the Epstein-Barr virus protein (BZLF1) that mediates disruption of latent infection. Proc. Natl. Acad. Sci. USA **90:**3894–3898.
43. **Taylor, N., J. Countryman, C. Rooney, D. Katz, and G. Miller.** 1989. Expression of the BZLF1 latency-disrupting gene differs in standard and defective Epstein-Barr viruses. J. Virol. **63:**1721–1728.
44. **Yates, L., N. Warren, D. Reismann, and B. Sugden.** 1984. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81:**3806–3810.
45. **Zalani, S., E. Holley-Guthrie, and S. Kenney.** 1996. Epstein-Barr viral latency is disrupted by the immediate-early BRLF protein through a cell-specific mechanism. Proc. Natl. Acad. Sci. USA **93:**9194–9199.
46. **Zhang, Q., Y. Hong, D. Dorsky, E. Holley-Guthrie, S. Zalani, N. A. Elshiekh, A. Kiehl, T. Le, and S. Kenney.** 1996. Functional and physical interactions between the Epstein-Barr virus (EBV) proteins BZLF1 and BMRF1: effects on EBV transcription and lytic replication. J. Virol. **70:**5131–5142.
47. **Zhang, Q., D. Gutsch, and S. Kenney.** 1994. Functional and physical interactions between p53 and BZLF1: implications for Epstein-Barr latency. Mol. Cell. Biol. **14:**1929–1938.