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The Zif268 Cellular Transcription Factor Activates Expression of the Epstein-Barr Virus Immediate-Early BRLF1 Promoter

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The Epstein-Barr virus immediate-early protein BZLF1 mediates the switch from latent to lytic infection. BZLF1 transcription can be derived from either the BZLF1 promoter or the BRLF1 promoter (Rp). Productive viral infection of EBV-infected B cells can be induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment, as well as cross-linking of surface immunoglobulin with antiimmunoglobulin antibody. Both TPA and antiimmunoglobulin antibody are known to activate expression of the cellular transcription factor Zif268 in B cells. In this study, we have examined the regulation of BZLF1 transcription by Zif268. We show that Rp (but not the BZLF1 promoter) is activated by Zif268. Bacterially synthesized Zif268 binds strongly to an upstream sequence in the Rp promoter (located from −131 to −123 relative to the start site) and more weakly to a proximal sequence (−49 to −40). Zif268 activation of Rp requires these two Zif268 binding sites. TPA treatment of B cells induces the expression of Zif268 protein, which binds to Rp. Furthermore, TPA activation of Rp requires the upstream Zif268 site. These findings indicate that Zif268 can activate a critical Epstein-Barr virus immediate-early promoter and, therefore, may play a key role in the regulation of viral latency.

Epstein-Barr virus (EBV) is a human herpesvirus which can infect and replicate in oral epithelial cells (31, 38). In contrast, EBV infection of B cells is generally latent (31, 38). Reactivation of the lytic cycle in latently infected B cells can be achieved by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or cross-linking of surface immunoglobulin (51, 57). In addition, overexpression of the EBV immediate-early (IE) gene product, BZLF1 (Z), is sufficient to disrupt latency (8, 11, 30, 43, 44, 52).

The Z gene product can be derived from two different promoters (35). The more proximal promoter (Z promoter [Zp]) directs transcription of a 0.9-kb message which makes the Z gene product alone (35) and a spliced variant of Z missing the middle exon (32) (Fig. 1A). In addition, the Z gene product can also be derived from a second promoter, BRLF1 (R) promoter (Rp) (35). Rp directs transcription of two bicistronic messages (3.8 and 2.9 kb), which code for both the Z and the R IE proteins (35). The Z and R IE proteins have been shown to function synergistically in activating the expression of early EBV promoters (7, 8, 13, 28, 41). In addition, a 0.9-kb message which makes the RAZ protein (an inhibitor of Z activity [21]) is also derived from Rp (35).

Given the importance of the various EBV genes transcribed from Rp, understanding the transcriptional regulation of Rp may yield insight into the cellular mechanisms involved in the disruption of viral latency. Since high expression of the Z gene product is sufficient to disrupt viral latency, activation of Z expression through Rp could potentially lead to lytic infection. In addition, activation of Rp is required for the production of R and/or RAZ.

Currently, very little is known regarding the cellular and viral regulation of Rp. Both Zp and Rp are normally inactive in latently infected cells (2, 18, 33, 51). Agents which disrupt viral latency (such as TPA and antiimmunoglubulin antibody) also activate expression of Zp and Rp (2, 15, 18, 19, 33, 51), and it is presumed that disruption of viral latency proceeds through activation of these promoters (18, 19, 22, 49). TPA- and antiimmunoglobulin antibody-responsive elements have been mapped in Zp (15, 19, 48), but it is currently not understood how Rp is activated during disruption of viral latency. Although we have previously shown that the cellular transcription factor Sp1 is required for constitutive activity of Rp (55), this factor is unlikely to mediate activation of Rp during disruption of latency since Sp1 is ubiquitously expressed (12).

We have studied the regulation of Rp by the cellular transcription factor Zif268 (also known as EGR-1, Krox 24, and NGFI-A) (9, 34, 37, 50). Transcription of the Zif268 IE gene in B cells is induced by TPA and antiimmunoglobulin antibody (46, 47). The Zif268 gene product is a zinc finger protein which binds to GC-rich regions (10). Here, we show that Zif268 activates Rp but not Zp and that TPA activation of Rp requires a Zif268 binding site. Our data suggest that activation of Rp by Zif268 in latently infected cells may be a mechanism for inducing lytic EBV infection. The regulation of Zif268 expression in B cells may therefore play a critical role in the maintenance of viral latency.

MATERIALS AND METHODS

Cell lines. The D98/HE-R-1 cell line was formed by fusion of a HeLa cell subclone (D98) with the EBV-positive Burkitt's lymphoma cell line P3HR1 (39). The C-33 cell line is a human cervical carcinoma line (papillomavirus negative). B95-8 is an EBV-positive marmoset B-cell line. Schneider line 2 (SL2) is a *Drosophila* embryo cell line (45). NIH 3T3 is a murine fibroblast line. All lymphoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. All epithelial cell lines were maintained in Dulbecco modified Eagle medium H supplemented with 10% fetal calf serum. SL2 was maintained in Drosophila Schneider medium (obtained from GIBCO-BRL) supplemented with 10% fetal calf serum.

EBV plasmids. Plasmid RpBSCAT has been previously described (54) and contains the Rp sequences (from −962 to +5 relative to the mRNA start site) linked to the heterologous reporter gene, chloramphenicol acetyltransferase (CAT), in the pBS phagemid vector (Stratagene). A series of 5′ deletion mutants of Rp were also constructed as previously described (55) (the nomenclature of the above plasmids in regard to the Rp mRNA start site has been corrected slightly from our previous publication). Site-directed mutants of the RpBSCAT construct were made with the Bio-Rad MutaGene phagemid in vitro mutagenesis kit according to the manufacturer's instructions. Synthetic oligonucleotides com-

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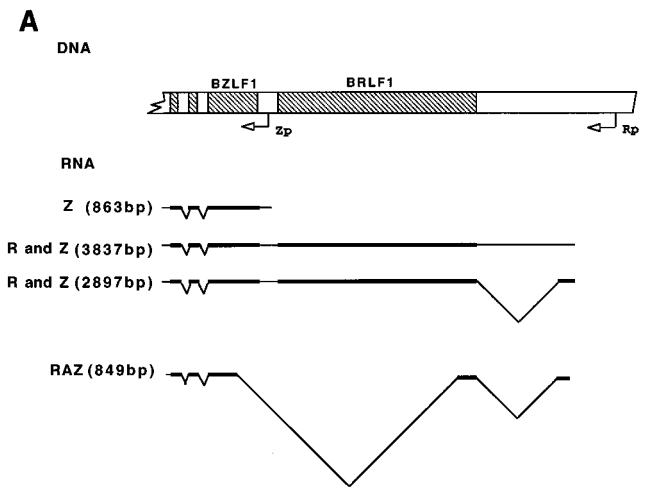


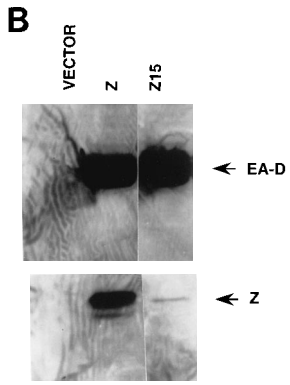


FIG. 1. (A) Sizes and structures of the messages originating from the Zp and Rp. Z and R exons in genomic DNA are hatched. The positions of the two promoters driving Z transcription (Zp and Rp) are indicated below the genomic map. The various cDNAs derived from Zp and Rp are based upon the mapping of Manet et al. (35). An additional cDNA (not shown) derived from Zp has the middle exon of Z deleted (32). (B) Viral latency can be disrupted by overexpression of the bicistronic message making the Z and R proteins. Latently infected D98/HE-R-1 cells were transfected with 5 μg of either vector DNA containing the cytomegalovirus promoter, a plasmid containing the 0.9-kb Z cDNA driven by the cytomegalovirus IE promoter, or a plasmid containing the 2.9-kb bicistronic Z15 cDNA. Whole-cell lysates of the transfected cells were prepared 48 h after transfection, loaded onto a sodium dodecyl sulfate-polyacrylamide gel, and transferred to nitrocellulose. The induction of the Z and proteins (a component of the early antigen diffuse complex) was then assessed by immunoblotting with monoclonal antibodies specific to either the Z (lower panel) or the BMRF1 (upper panel) protein. Although the transfected 0.9-kb Z cDNA produced significantly more Z protein than the 2.9-kb bicistronic Z15 cDNA, transfection of either cDNA resulted in efficient induction of BMRF1 expression in D98/HE-R-1 cells.

plementary to the region to be altered introduced a new restriction site to facilitate screening for mutants.

The Z-CAT plasmid has been previously described (30) and contains Zp (−552 to +12 relative to the mRNA start site) sequences linked to CAT. The EA-CAT plasmid (pEA-BS-CAT) has also been previously described and contains the early BMRF1 promoter sequences (−333 to +1) linked to CAT (28).

The CMV-Z plasmid has the Z cDNA under the control of the cytomegalovirus promoter in the pHD1013 vector as previously described (41). The pcDZ15 plasmid contains the bicistronic 2.9-kb cDNA (derived from Rp) driven by the simian virus 40 promoter in the pcDV vector as previously described (35). This construct was a gift from Alain Sergeant.

Zif268 plasmids. The pCMVEgr-1 plasmid contains the mouse Zif268 cDNA under the control of the cytomegalovirus promoter in the pCMV5 vector as previously described (26). The Egr-1(Δ331-374) control vector is identical to pCMVEgr-1 except that it is lacking some of the Zif268 zinc fingers. Both Zif268 expression vectors were gifts from Vikas Sukhatme. The pΔ56 EBS-1[3] vector (a gift from Vikas Sukhatme) contains three copies of the consensus Zif268 site upstream of a minimal *fos* promoter driving CAT. Bacterial Zif268 expression was achieved with a vector containing the murine Zif268 cDNA inserted under the control of the T7 promoter in plasmid Pet8C (a gift from Barbara Christy) (10).

Sp1 plasmids. Plasmids pPacSp1 (a gift from Robert Tjian) and pPacSp1FX have been described previously (12, 55).

DNA transfection. Plasmid DNA was purified through either Qiagen columns as described by the manufacturer (Qiagen, Inc.) or double-banded cesium chloride gradients. Ten micrograms of DNA was transfected into cells by electroporation with a Zapper electroporation unit (medical electronics shop, University of Wisconsin) at 1,500 V. Epithelial cells were harvested and suspended into RPMI 1640 medium prior to electroporation. NIH 3T3 and SL2 cells were transfected by the calcium phosphate precipitation method as described previously (55).

CAT assays. Cell extracts were prepared 48 h after transfection and incubated at 37°C with [^{14}C]chloramphenicol in the presence of acetyl coenzyme A as described previously (24). The percent acetylation of chloramphenicol was quantitated by thin-layer chromatography followed by Phosphorimage screening or by scintillation counting.

Production of bacterial Zif268 protein. The plasmid containing the Zif268 cDNA in the Pet8C vector was grown in *Escherichia coli* BL21(DE3) to an optical density of 0.6 before addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside. Solubilized Zif268 protein was obtained by the procedure of Christy et al. (10).

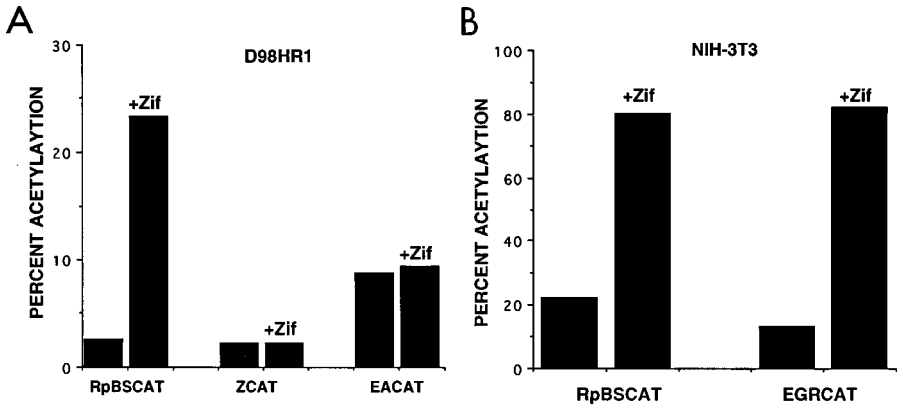
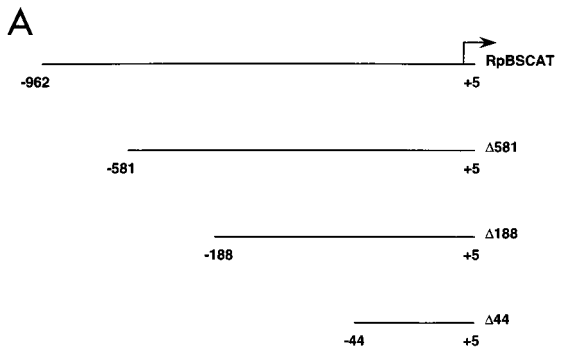


FIG. 2. The Zif268 transcription factor activates Rp. (A) Promoter constructs (5 μg) containing either Rp (RpBSCAT), Zp (ZCAT), or the early BMRF1 promoter (EACAT) linked to the CAT reporter gene were cotransfected into D98/HE-R-1 cells with either 5 μg of a Zif268 expression vector, CMVEgr-1 (+ZIF), or an equal amount of a control vector, CMVEgr-1 (Δ331-374), containing mutant Zif268. Two days after transfection, CAT activity was measured as previously described (24). Rp is activated by cotransfection with Zif268, whereas Zp and the BMRF1 promoter are not affected. (B) Five micrograms of either the Rp construct, RpBSCAT, or a positive control construct containing three consensus Zif268 binding sites (EGRCAT) was cotransfected with 5 μg of either a Zif268 expression vector (+ZIF) or the control vector into the NIH 3T3 fibroblast line. Rp, as well as the control promoter, is efficiently activated by Zif268.



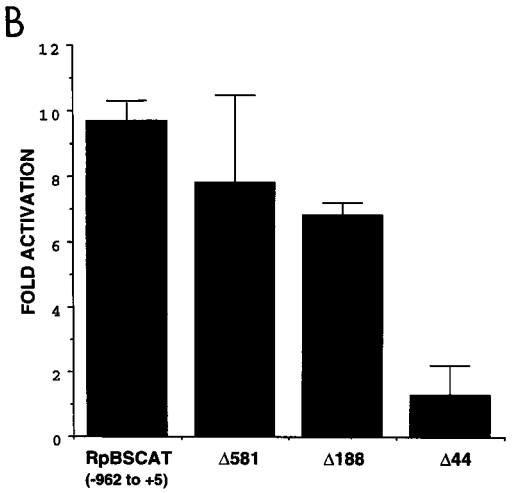


FIG. 3. Mapping the Zif268 responsive region in Rp. (A) A series of 5′ deletions in the RpBSCAT vector (containing Rp sequences from −962 to +5 linked to CAT in the pBS-CAT vector) was constructed as shown. (B) The ability of Zif268 to activate the RpBSCAT and the 5′ deletion plasmids was examined in D98/HE-R-1 cells. The average fold transactivation after cotransfection with the Zif268 expression vector versus the control vector is shown. Zif268-induced transactivation of Rp is significantly decreased when the promoter sequences between −188 and −44 are deleted.

Preparation of cellular extracts. EBV-positive B95-8 cells were induced with 30-ng/ml TPA and 5 mM sodium butyrate for 5 h. Whole-cell protein extracts were made by washing the cells twice with ice-cold phosphate-buffered saline and then resuspending the cell pellet in a volume of 2.5×10^7 cells per ml in a buffer consisting of 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.0), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by freeze-thawing twice, and the lysate was cleared by centrifugation for 10 min at 4°C. The resulting supernatant was used for electromobility shift assays.

Zif268 binding assays. Electromobility shift assays were performed as previously described (23). DNA probes spanning various regions of Rp were end labeled with ^{32}P. Binding reactions were carried out in a buffer consisting of 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA), 50 mM KCl, 7% glycerol, 2.5 mM phenylmethylsulfonyl fluoride, and 0.04 mM leupeptin. Poly(dI-dC) · poly(dI-dC) (4 μg) was added as a nonspecific competitor DNA. Cellular extracts or *E. coli* extracts containing the Zif268 protein were incubated in binding buffer with nonspecific competitor DNA (with or without specific competitor DNA) at room temperature for 15 min, and then the labelled probe (20,000 cpm) was added. The reaction mixture was further incubated for an additional 15 min at room temperature, loaded onto a 5% polyacrylamide gel, and run in 0.5× Tris borate buffer at room temperature. In reactions using specific antibodies, 2 μl of polyclonal antibody against either Zif268, Sp1, or NF-κB (Santa Cruz Co.) was added after the second 15-min incubation and the mixture was incubated for an additional 45 min at room temperature prior to electrophoresis.

DNase I footprinting. Binding reactions consisted of 50,000 cpm of the 5′-end-labelled probe incubated with 2 volumes of the reaction mixture used under the electrophoretic mobility shift assay conditions described above. The reaction mixture was incubated for 15 min at room temperature. The end-labelled probe containing Rp sequences from +5 to −188 was then added, and binding was allowed to proceed for an additional 15 min at room temperature. DNase I RQ1 (Promega) was added to the reaction mixture for 60 s. After further processing, the DNA was loaded on a 6% polyacrylamide–7 M urea gel and subjected to electrophoresis followed by autoradiography.

Oligonucleotide probes and competitors. Labelled oligonucleotide probes included an Rp probe (promoter sequences −143 to −114 relative to the RNA start site) (AGCTTGCCTCTCTGCTGCCCACTCATACTTAAGCT and CTAGAGCTTAAGTATGAGTGGGCAGCAGAGAGGCA) and a probe containing the CCAAT motif derived from EBV oriLyt (AGCTTGGGGGCTTCTTATTGGTTAATTCAGGTA and AGCTTACCTGAATTAACCAATAAGAAGCCCCCA). The Zif268 competitor oligonucleotide contains the Zif268 binding site (GGATCCAGCGGGGGCGAGCGGGGGCGA and CCTAGGTCGCCCCCGCTCGCCCCCGCT) (Santa Cruz Biotechnology). The mutant Zif268 oligonucleotide contains the sequence GGATCCAGCTAGGGCGAGCTAGGGCGA and CCTAGGTCGATCCCGCTCGATCCCGCT. The YY1 competitor oligonucleotide contains the YY1 binding site from the murine leukemia virus long terminal repeat (CTGCAGTAACGCCATTTTGCAAGGCAT and ATGCCTTGCAAAATGGCGTTACTGCAG) (17).

Immunoblot analysis. Detection of the EBV Z and BMRF1 proteins by immunoblot analysis was performed as previously described (56). The Z monoclonal antibody BZ.1 (diluted 1:50) (a gift from Alan Rickinson) was used to detect Z expression, and the monoclonal antibody 9240 (diluted 1:40) (NEN, Dupont) was used to detect induction of BMRF1 (EA-D). After immunoblotting, proteins were visualized by the chemiluminescence method (Amersham).

RESULTS

Overexpression of the bicistronic message making the Z and R IE proteins is sufficient to disrupt latency. It has been previously shown that the 2.9-kb Z15 cDNA derived from Rp can be translated into both Z and R after transfection into COS cells (35). Northern (RNA) blot analysis from transfection experiments suggested that Z protein is derived from the larger (bicistronic) message rather than from use of the Zp promoter contained within the Z15 cDNA (35). In order to assess whether overexpression of the bicistronic Z15 cDNA is sufficient to induce the viral lytic cycle, we transfected latently infected D98/HE-R-1 cells with either vector DNA, the CMV-Z plasmid (containing the 0.9-kb Z cDNA), or the pcDZ15 construct (containing the 2.9-kb Z15 cDNA). Cells were harvested 48 h after transfection, and the samples were processed for immunoblot analysis.

As shown in Fig. 1B, both the 0.9-kb cDNA making Z alone and the 2.9-kb cDNA making Z and R were capable of inducing expression of an early EBV protein (BMRF1) when transfected into latently infected D98/HE-R-1 cells. The 0.9- and 2.9-kb cDNAs both induced early protein expression efficiently, in spite of the fact that the amount of Z produced by the bicistronic Z15 cDNA was much less than that produced by the smaller, 0.9-kb Z cDNA. These results suggest that activation of Rp in latently infected cells could potentially lead to disruption of latency due to the production of Z protein from the 2.9-kb bicistronic message.

The Zif268 transcription factor activates Rp. We have previously shown that Rp is bound and regulated by the Sp1 transcription factor (55). Further analysis of the Rp sequence suggested the possibility of one (or more) Zif268 binding sites. To examine whether the cellular Zif268 transcription factor can activate Rp function, we performed transient transfection assays. As shown in Fig. 2A, the intact Rp reporter construct (RpBSCAT) is significantly activated by cotransfection with a Zif268 expression vector in the EBV-positive cell line D98/HE-R-1. In contrast, a construct containing the alternative promoter (Zp) for Z transcription (Z-CAT) was not activated by cotransfection with the Zif268 vector. The early BMRF1 promoter construct was also not activated significantly by Zif268.

In order to examine whether an EBV gene product is required for the activation of Rp by Zif268, we performed similar cotransfection experiments in EBV-negative NIH 3T3 murine fibroblasts. A control construct containing three Zif268 bind-

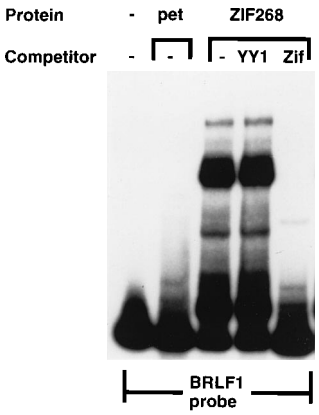


FIG. 4. Bacterially synthesized Zif268 binds to Rp. A ^{32}P-labelled probe containing Rp sequences (from −188 to +5) was incubated with bacterially synthesized Zif268 protein or bacterial extract containing the Pet8C vector alone. Binding was assessed by an electromobility shift assay. Bacterially expressed Zif268 binds efficiently to the Rp probe, and this binding is specifically inhibited by unlabelled oligonucleotide competitor DNA (Zif) containing a known Zif268 binding site but not by unlabelled oligonucleotide DNA containing a YY1 binding site (YY1).

ing sites upstream of the minimal *fos* promoter was activated by Zif268 as expected (Fig. 2B). The Rp reporter construct was also activated efficiently by cotransfection with the Zif268 expression vector in NIH 3T3 cells. These results indicate that no EBV gene product is required for activation of Rp by Zif268.

5′ deletion analysis of Rp. In order to define the region of Rp required for activation by Zif268, we cotransfected a series of 5′ Rp deletion constructs into D98/HE-R-1 cells with the Zif268 expression vector (Fig. 3). In this set of experiments, the intact Rp reporter construct was activated 10-fold by cotransfection with Zif268. When the Rp sequences between −962 and −188 were deleted, Zif268 activation was not significantly altered. However, Zif268 activation was significantly lost when the promoter sequences between −188 and −44 were deleted. These results suggest that the region between −188 and −44 in Rp contains potential sequences required for Zif268 activation.

The Zif268 protein binds directly to Rp. To determine if the Zif268-responsive region in the Rp contains Zif268 binding sites, electromobility shift assays were performed with an end-labelled probe containing the Rp sequences from −188 to +5. As shown in Fig. 4, bacterially synthesized Zif268 protein bound efficiently to the Rp probe. This binding was specifically inhibited by unlabelled oligonucleotide DNA containing the consensus Zif268 sequence but not by unlabelled oligonucleotide DNA containing a YY1 binding motif. Similar results were obtained with in vitro-translated Zif268 protein (data not shown).

To determine the precise sites of Zif268 binding in Rp, DNase I footprinting was performed with bacterially synthesized Zif268 protein and an end-labelled Rp probe containing the promoter sequences from −188 to +5. As shown in Fig. 5, two regions of protection were observed in the presence of Zif268 protein. A very strong region of protection was mapped to the promoter sequences between −114 and −143. The center of this strong footprint contains the sequence TGCCCA CTC, which is a seven-ninths match with the consensus Zif268 binding motif, CGCCCACGC. A weak area of protection was also observed over the Rp sequences from −36 to −53. This weaker footprint contains the sequence CGCCCATGC, which is an eight-ninths match with the consensus Zif268 sequence.

Zif268 is induced in TPA-treated B95-8 cells and binds to Rp. To determine if Zif268 protein in cellular extracts can bind to Rp, we performed electromobility shift assays using cellular extracts prepared from either untreated B95-8 cells (an EBV-positive B-cell line) or B95-8 cells which had been treated with TPA and sodium butyrate for 5 h. In the TPA-treated extracts, a complex was observed binding to a labelled probe containing Rp sequences between −113 and −141 which was specifically sensitive to competition with unlabelled oligonucleotide competitor DNA containing the Zif268 consensus binding site but not to competition with unlabelled DNA containing a mutated Zif268 site (Fig. 6A, lanes 5 to 7). A much smaller amount of this complex was also present in untreated B95-8 cell extracts (Fig. 6A, lanes 2 to 4). In contrast to the Rp probe, uninduced and TPA-induced extracts produced identical complexes binding to a labelled control probe containing the CCAAT motif (Fig. 6A, lanes 8 to 10).

To confirm the identity of the TPA-induced protein binding to the Rp probe, we used polyclonal antibodies specifically directed against Zif268, NF-κB, or Sp1. As shown in Fig. 6B, the TPA-induced protein binding to Rp was specifically supershifted by polyclonal antibody directed against Zif268 but not affected by polyclonal antibodies directed against NF-κB or

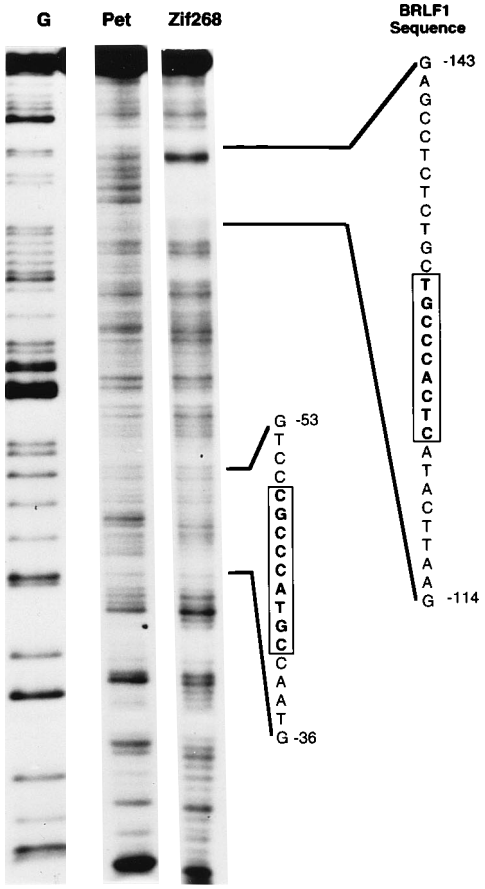


FIG. 5. Mapping of Zif268 binding sites in Rp. A ^{32}P-end-labelled probe containing the Rp sequences from −188 to +5 was incubated with bacterially synthesized Zif268 protein (Zif268) or bacterial extracts containing the Pet8C vector alone (Pet), and the incubation was followed by treatment with DNase I. After processing of the samples, reaction mixtures were loaded onto a 6% sequencing gel. The regions of protection induced by Zif268 binding and the sequence within each footprint with homology to the consensus Zif268 binding motif (CGCCCACGC) are indicated. Lane G, G sequence ladder.

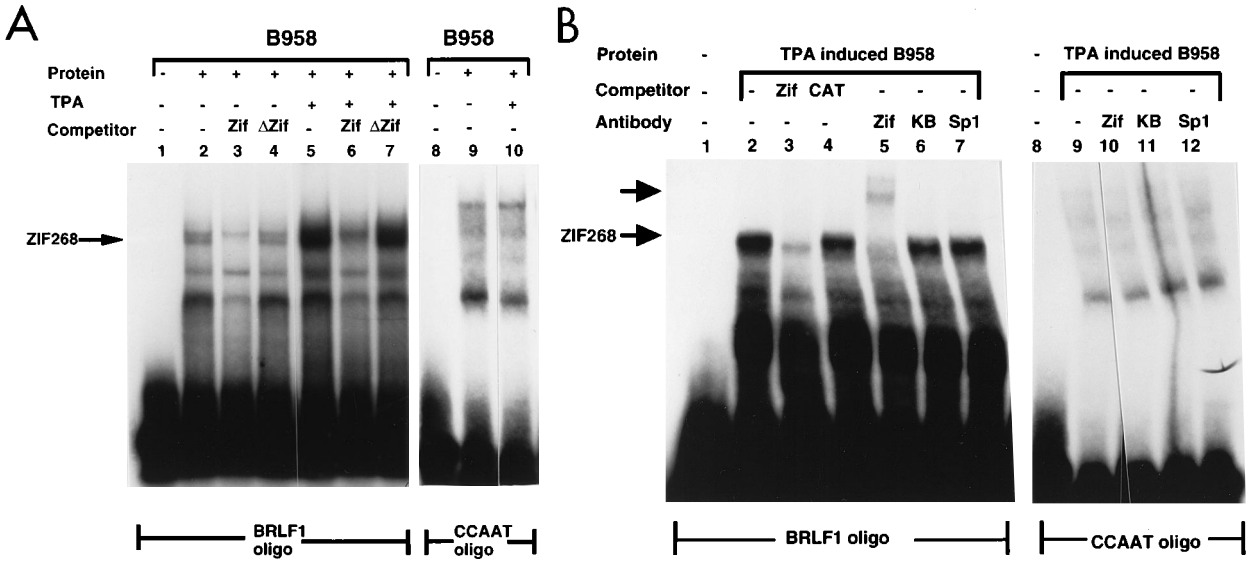


FIG. 6. Zif268 is induced in TPA-treated B95-8 cells and binds to Rp. (A) A ^{32}P-labelled oligonucleotide probe spanning the Rp sequences from −143 to −114 was incubated with whole-cell extracts derived from either untreated B95-8 cells or B95-8 cells treated with TPA-sodium butyrate for 5 h. Binding was analyzed by electromobility shift assay. TPA-treated extracts (lanes 5 to 7) contain increased amounts of a complex binding to the Rp probe which is specifically sensitive to competition with unlabelled oligonucleotide competitor DNA containing the Zif268 binding motif (Zif) but not to competition with a mutant Zif268 motif (ΔZif). In contrast, the untreated and TPA-treated extracts produced identical binding complexes with a labelled oligonucleotide probe containing the CCAAT motif (lanes 8 to 10). (B) Protein extract from TPA-treated B95-8 cells was incubated in binding buffer at room temperature for 15 min with various competitor oligonucleotides as indicated; then, a labelled oligonucleotide probe (20,000 cpm) containing Rp sequences from −143 to −114 was added. The reaction mixture was further incubated for an additional 15 min at room temperature. For lanes 5 to 7, 2 μl of polyclonal antibody against either Zif268, Sp1, or NF-κB was then added and the mixtures were incubated for an additional 45 min at room temperature prior to electrophoresis. The Zif268 specific antibody (but not the other antibodies) specifically supershifted the TPA-induced complex binding to the Rp probe. In contrast, none of the antibodies affected binding of complexes to a labelled oligonucleotide probe containing the CCAAT motif (lanes 8 to 12).

Sp1 (Fig. 6B, lanes 5 to 7). In contrast, none of the antibodies affected binding of proteins to another labelled probe containing the CCAAT motif (Fig. 6B, lanes 8 to 12).

Interestingly, we also observed a faster-migrating complex binding to the Rp probe which is partially inhibited by Zif268 competitor DNA (Fig. 6A). However, since this faster-migrating complex is constitutively present in B95-8 cells, is not induced by TPA, and is not recognized by Zif268 antibody, it is clearly not Zif268. Although we have not yet precisely determined the identity of this protein, other members of the zinc finger protein family (such as Wilms' tumor protein [WT1] and NGFI-C) have been previously shown to bind to Zif268 motifs (14, 42).

Site-directed mutations of the Zif268 binding sites in Rp reduce Zif268 activation. To examine the functional significance of the two potential Zif268 binding sites in Rp, each Zif268 site in the RpBSCAT vector was specifically altered by site-directed mutation as shown in Fig. 7A. In D98/HE-R-1 cells, neither of these site-directed mutations significantly affected the constitutive activity of the RpBSCAT plasmid (data not shown). In addition, neither mutation significantly decreased Sp1 responsiveness in comparison with the wild-type promoter (Fig. 7B). However, the ability of Zif268 to activate each of these mutant constructs was significantly decreased in comparison with the wild-type promoter. Mutation of the upstream Zif268 site (which produced the stronger footprint) reduced Zif268 responsiveness of Rp by 80% (Fig. 7C), and mutation of the weaker downstream Zif268 binding site reduced Zif268 responsiveness by 60%. Mutation of both sites together produced a complete inhibition of Zif268 activation. These data indicate that Zif268 activation of Rp is mediated through two specific Zif268 binding sites.

TPA activation of Rp requires the upstream Zif268 site. Finally, we examined whether TPA activation of Rp is mediated through Zif268 induction. C-33 cells were transfected with either the wild-type RpBSCAT vector or the site-directed mutants, and 24 h after transfection the cells were transferred to media containing 0.5% fetal calf serum in the presence or absence of TPA (20 ng/ml) (Fig. 7D). In this set of experiments, the wild-type Rp was activated an average of eightfold by TPA. In comparison, the mutant construct in which the upstream Zif268 site was altered was not significantly activated by TPA. Mutation of the downstream Zif268 site also inhibited TPA activation, although to a lesser degree. Thus, TPA activation of Rp appears to require Zif268 binding sites.

DISCUSSION

In this report, we have demonstrated that the cellular protein Zif268 can activate expression of one of the two promoter elements directing Z transcription. The regulation of Z is of critical importance in the biology of the EBV, because overexpression of the Z gene product leads to disruption of viral latency and consequent death of the host cell. Therefore, the cellular factors which activate (or repress) the transcription of Z must play a key role in the regulation of EBV latency.

The Z IE gene product is a member of the bZip (leucine zipper) family and functions as a transcriptional activator (5, 16). The Z protein has been shown to regulate early EBV promoters by binding directly to upstream elements and activating transcription (13, 28, 29, 30, 41). Although Z can also activate its own proximal promoter, Zp, through a direct binding mechanism (20, 53), it is not transcribed in latently infected cells. Therefore, disruption of viral latency must initially be mediated by a cellular factor(s) which activates Z transcription.

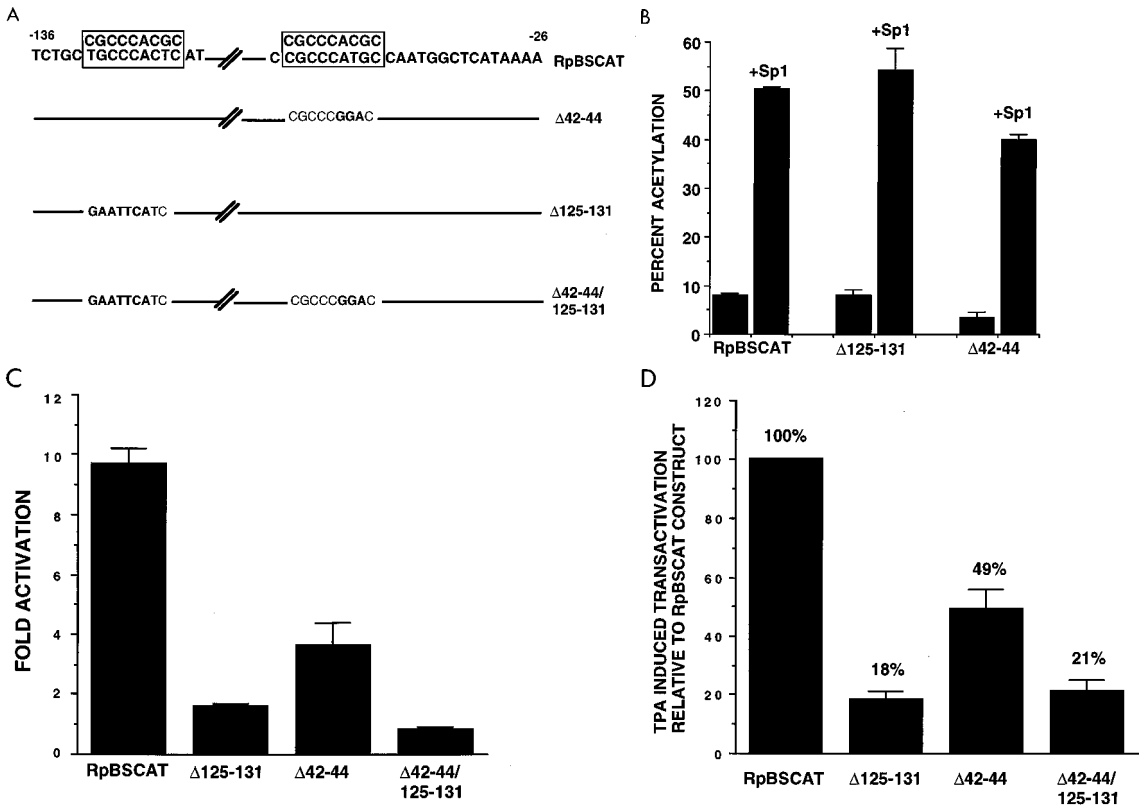


FIG. 7. The Zif268 sites in Rp are required for Zif268 activation. (A) Site-directed mutations of the RpBSCAT vector were constructed as shown. The sequences of the Zif268 binding sites in Rp (shown next to the consensus Zif268 binding motif) are indicated. The site-directed mutations disrupt each of the two Zif268 binding sites in Rp (either alone or in combination) as shown. Mutant designations reflect the Rp sequences (relative to the start site) affected. (B) Ten micrograms of the RpBSCAT plasmid with or without a site-directed mutation altering either the proximal or the distal Zif268 binding site was transfected into *Drosophila* SL2 cells with 1 μg of either an Sp1 expression vector (pPacSp1) or a control vector (pPacSp1FX). Two days after transfection, CAT activity was measured as previously described (24). Mutation of the Zif268 binding sites in the RpBSCAT plasmid does not significantly affect Sp1 activation. (C) Five micrograms of the RpBSCAT construct without or with a site-directed mutation altering either the upstream Zif268 binding site (Δ125-131), the proximal Zif268 binding site (Δ42-44), or both sites together (Δ42-44/125-131) was transfected into D98/HE-R-1 cells with 5 μg of either the Zif268 expression plasmid or a negative control vector. CAT activity was determined 2 days after transfection. The relative activity induced by Zif268 versus the negative control vector is shown. Mutation of either Zif268 site alone in Rp significantly reduced activation by Zif268; mutation of both sites simultaneously completely inhibited Zif268 activation. (D) Five micrograms of the RpBSCAT construct without or with a site-directed mutation altering either the upstream Zif268 binding site (Δ125-131), the proximal Zif268 binding site (Δ42-44), or both sites together (Δ42-44/125-131) was transfected into C-33 cells. Twenty-four hours after transfection, the cells were transferred to media containing 0.5% fetal calf serum (0.5%) in the presence (20 ng/ml) or absence of TPA. CAT activity was determined after an additional 24 h (after correction for protein concentration). The relative activation induced by TPA in comparison with the wild-type promoter is shown (the wild-type RpBSCAT plasmid was activated an average of eightfold by TPA treatment in this set of experiments). Mutation of the upstream Zif268 site in Rp significantly reduced activation by TPA.

Subsequently, autoregulation of Z transcription by the Z transactivator function presumably leads to irreversible activation of the lytic viral cascade.

Very little work has been previously published regarding the cellular regulation of Rp. We have previously shown that Rp (but not Zp) is responsive to the Sp1 transcription factor (55). Sp1 activation of Rp is mediated through a series of upstream Sp1 binding sites. The most proximal Sp1 site (located between −45 and −50) overlaps the proximal Zif268 site and appears to be critical for both Sp1 activation in *Drosophila* Schneider cells and constitutive activity of Rp in epithelial cells. However, since Sp1 is a ubiquitous housekeeping protein, the activation of Rp function during the disruption of viral latency is unlikely to be mediated through the Sp1 transcription factor.

In this report we have demonstrated that the cellular Zif268 protein can activate transcription of Rp. Zif268 is an IE gene which is induced by growth factors and a variety of cell surface stimuli (4). Both TPA and antiimmunoglobulin antibody treatment can activate Zif268 transcription in B cells (46, 47). Interestingly, these factors are also used to disrupt EBV latency, making this transcription factor an attractive candidate for playing a role in EBV regulation. Our results suggest that TPA-induced activation of Rp does indeed appear to be mediated through Zif268.

Zif268 is a member of a gene family that includes the IE genes NGFI-C and Krox 20, as well as the Wilms' tumor gene, WT-1 (6, 14, 42). Zif268 is a phosphorylated nuclear protein (54). The Zif268 DNA binding domain is composed of three zinc fingers of the C2H2 subtype and binds to a GC-rich motif, CGCCCACGC (9). Although the consensus Zif268 binding motif is not present in Rp, we have identified two closely related Zif268 binding sites in Rp and demonstrated that these binding sites are required for Zif268 activation. Other genes previously shown to be regulated by Zif268 include the Zif268 gene itself (10), adenosine deaminase (1), and α-myosin heavy chain (26).

It has sometimes been presumed that disruption of EBV latency is initially mediated through activation of Zp, followed by activation of Rp (19, 22, 49). However, to our knowledge, induction of the 0.9-kb message (derived from Zp) and that of the 2.9- and 3.8-kb messages (derived from Rp) have always been observed simultaneously (18, 36, 49, 51). Therefore, dis-

ruption of viral latency could potentially be mediated through activation of Rp, since the bicistronic messages derived from this promoter could potentially make a small amount of Z protein, which then leads to activation of Zp. We have demonstrated in this report that overexpression of the Rp-derived 2.9-kb message in latently infected cells does result in detectable production of Z protein and is sufficient to disrupt viral latency. The hypothesis that Rp activation may actually precede Zp activation is also supported by previous findings from our laboratory which suggest that Zp, but not Rp, is activated by Z (55). However, our findings are in contrast to those of another group (49), which found that Z induces low-level activation of Rp in cotransfection assays.

Alternatively, it is quite possible that disruption of latency requires the simultaneous activation of both Zp and Rp. In this case, activation of Rp could be required primarily for expression of R rather than the production of Z. The EBV IE gene product, R, is a transcriptional activator which activates expression of early EBV promoters containing upstream R binding sites (3, 13, 25, 27–29, 41). It has been reported by several groups that the combination of Z and R is required for maximal activation of a variety of early EBV promoters in transient transfection assays (7, 8, 13, 28, 41). Optimal disruption of latency may, likewise, require the simultaneous expression of both Z and R.

Rp also appears to be the major promoter regulating transcription of RAZ mRNA. Therefore, activation of Rp could potentially increase expression of RAZ (in addition to R and Z), although the relative abundance of the 0.9-kb RAZ message versus the 2.8-kb bicistronic R/Z message is likely to be controlled at the level of RNA splicing. RAZ is currently thought to function as a negative regulator of Z (21). However, the biological function of RAZ remains relatively unexplored, and it remains possible that RAZ expression actually plays a role in the disruption of viral latency. For example, RAZ could potentially mediate the transition from early to late gene expression.

There is still some controversy in regard to whether Z and R are true IE genes (i.e., their expression is independent of protein synthesis) (18, 33, 40). Since Zif268 is an IE gene, rather than an IE protein, the induction of Zif268 protein by TPA or antiimmunoglobulin antibody clearly does require protein synthesis. One study has found that induction of Z and R transcription by TPA requires protein synthesis (33), suggesting that Z and R are not true IE genes. Two other studies have found that, although the great majority of Z/R transcription (following antiimmunoglobulin induction) is inhibited in the absence of protein synthesis, a very small amount still occurs (18, 40). However, in the latter two studies, it is possible that protein synthesis inhibition was not complete.

Although the relative importance of Rp versus Zp as a source of Z protein remains unclear, the fact that Rp and Zp activation occurs essentially simultaneously during disruption of viral latency (18, 40) suggests that Rp plays an important role in this event. Our finding that Zif268 can activate Rp suggests that the cellular regulation of Zif268 may be one of the factors which influences the stringency of EBV latency. Whether Zif268 activation is the critical first step which initiates disruption of viral latency remains to be determined.

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