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Negative Regulation of the BZLF1 Promoter of Epstein-Barr Virus

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The Epstein-Barr virus BZLF1 gene product (ZEBRA) is a transcriptional activator whose expression in latently infected B cells is sufficient to induce the viral lytic cycle. Since there is no transcription of BZLF1 during latency, we carried out experiments to determine whether *cis*-acting negative elements in the BZLF1 promoter contribute to the lack of expression during this phase of the virus cycle. A series of deletion plasmids encompassing positions −551 to +14 of the BZLF1 promoter region were constructed and tested for the ability to drive chloramphenicol acetyltransferase (CAT) gene expression in the absence of inducing agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and anti-immunoglobulin. Expression from the intact 551-bp region was very weak in most of the cell lines tested, but deletion of 165 bp from the 5′ end caused a sevenfold increase in expression of CAT. Within these 165 bp, a minimal 48-bp region was sufficient to down regulate the expression of a simian virus 40/CAT fusion plasmid. The 48-bp negative element consists of 7-bp dyad symmetry elements separated by 27 bp. The rightmost half of the dyad symmetry element partially overlaps a region which has a 14-of-15-bp homology to the human cytoskeletal γ-actin promoter.

Epstein-Barr virus (EBV) is the etiological agent of infectious mononucleosis and is closely associated with Burkitt's lymphoma and nasopharyngeal carcinoma. The virus infects B-cell lymphocytes and certain epithelial cells, where it may either undergo productive replication or establish latency. Studies of the EBV life cycle have been carried out with lymphoblastoid cell lines which harbor multiple episomal copies of the EBV genome. In these cell lines, the virus is usually latent but can be activated to enter the replicative cycle by either chemical induction (e.g., by 12-*O*-tetradecanoylphorbol-13-acetate [TPA] or butyrate), superinfection with defective P3HR1 virus, or incubation with anti-immunoglobulin G or M (14, 19, 29, 30, 36, 42).

Activation of the EBV lytic cycle is characterized by the appearance of as many as 80 virus-specific RNA species (36). Depending on their time of appearance postinduction, these transcripts are designated immediate early, delayed early, or late. At least three immediate-early transcripts, which correspond to the BMLF1, BRLF1, and BZLF1 open reading frames, have been identified. Only two of these immediate-early genes, BRLF1 and BZLF1, are transcribed in the presence of protein synthesis inhibitors. In immunoglobulin-induced cultures, transcription of BZLF1 and BRLF1 appears to be transient and is detectable at high levels at 2 h posttreatment but returns to undetectable levels by 8 h. By contrast, the BMLF1 transcript appears at 2 h but levels remain high throughout the viral replicative cycle (36). All three of these genes are known to encode transactivating proteins, a feature common to many herpesvirus immediate-early genes (23, 25). However, in transfection experiments, only plasmids containing the BZLF1 gene under control of a strong heterologous promoter are capable of inducing the EBV lytic cycle (1).

Targets for BZLF1 transactivation, designated z-responsive elements, have been identified within the promoters of several EBV early genes, including the BZLF1 promoter itself and the c-*fos* promoter (9, 10, 16, 22). The BZLF1 gene product ZEBRA (also known as EB1 or Zta) was first shown to bind specifically to a consensus AP1 site within the BMLF1 promoter (7), but subsequent analyses of ZEBRA binding domains revealed that binding is merely confined to AP1-like sites (21). Because ZEBRA expression is sufficient to induce the EBV lytic cycle (1, 29, 30), regulation of the BZLF1 promoter, in essence, controls the EBV life cycle within an infected cell. Thus far, all studies regarding the regulation of BZLF1 expression have investigated regions of the BZLF1 promoter which are involved in positive regulation (8, 9, 39). Elements responsive to either TPA or ZEBRA have been mapped and shown to be distinct for these two inducers (8, 9).

Negative regulation of the BZLF1 promoter during latency has not been investigated. The dual regulation of a promoter by positive and negative factors has been shown for several cellular genes, including those (e.g., c-*myc*, c-*jun*, and c-*fos*) whose transient expression occurs during the cell cycle (2, 26, 27, 32). These effectors may function either by binding to *cis*-acting elements or by interacting with a protein(s) already bound to DNA (15, 33). In some cases, the same genetic element (e.g., a glucocorticoid-responsive element or a serum response element [SRE]) can confer both positive and negative regulation (4, 28).

In this report, we identify within the BZLF1 promoter a 48-bp *cis*-acting negative regulatory element which consists of a 7-bp dyad symmetry region separated by 27 bp. This 48-bp sequence confers negative regulation on a heterologous promoter and partially overlaps a region which is homologous to the SRE of the γ-actin promoter. However, the SRE is not essential for negative regulation.

MATERIALS AND METHODS

Cell lines and transfections. HeLa cells were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 100 U of penicillin, 100 μg of streptomycin per ml, and 10% fetal bovine serum. BALB/c 3T3 cells were maintained under the same conditions but in calf serum. B95-8, Raji, and IB4 cells were grown in RPMI medium supplemented with 10% fetal bovine serum. Transfections of B-lymphocyte cells were carried out as previ-

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ously described (8). Transfections of chloramphenicol acetyltransferase (CAT) expression vectors into BALB/c 3T3 and HeLa cells were carried out by the calcium phosphate precipitation technique (13) using the amounts of DNA designated in the figure legends. Dishes (100-mm diameter) were seeded with either 10^6 HeLa or 7×10^5 BALB/c 3T3 cells approximately 24 h prior to transfection. Cell monolayers were incubated with calcium phosphate precipitates overnight, washed with phosphate-buffered saline, and incubated at 37°C for an additional 36 h.

CAT assays. CAT assays were done as previously described by Gorman, with minor modifications (12). At 48 h posttransfection, cells were washed twice with phosphate buffered saline and dislodged from petri dishes with a rubber policeman in 10 ml of phosphate-buffered saline. The cells were centrifuged at 3,000 × *g* for 10 min, and the cell pellet was suspended in 75 μl of 0.25 M Tris (pH 8.0). Lysis was carried out by subjecting the cells to three freeze-thaw cycles. After removal of cell debris by centrifugation, extracts were incubated with 0.1 μCi of [^{14}C]chloramphenicol for 1 h at 37°C. Acetylated substrate was separated from unacetylated forms by ascending thin-layer chromatography, and thin-layer gels were then exposed to XAR5 film. Percent acetylation was calculated as previously described (41).

Plasmids. pzCAT was constructed by cloning the 551-bp *Bam*HI-*Nae*I fragment of the BZLF1 promoter into the *Bam*HI-*Eco*RV sites of pCAT (a generous gift from Mark Labow). pCAT contains the CAT gene and the polyadenylation and splice sites of simian virus 40 (SV40) (18). All pzCAT deletion constructs were made by double digestions at unique restriction endonuclease sites. The following combinations of enzymes were used to construct the designated plasmids: *Bam*HI-*Nde*I for p386, *Bam*HI-*Sph*I for p222, and *Bam*HI-*Nsi*I for p130. After digestion, the ends of DNAs were blunted with Klenow or T4 polymerase and religated with T4 DNA ligase. For promoter fusions between the SV40 promoter and sequences in the BZLF1 promoter, the 165-bp *Bam*HI-*Nde*I fragment of the BZLF1 promoter (−551 to −386) was isolated, gel purified, and digested with either *Ava*I or *Mst*II. Fragments of 58 and 107 bp were isolated from *Ava*I-digested DNA; 48- and 117-bp fragments were isolated from *Mst*II-digested DNA. Each of the four fragments were then made blunt with Klenow polymerase and cloned into the *Bam*HI site of pSVCAT, which contains the SV40 promoter and origin from the *Pvu*II site to the *Hin*dIII site cloned into pCAT. Each new plasmid was then designated on the basis of the size of the BZLF1 DNA inserted upstream of the SV40 enhancer.

Preparation of cell extracts and chromatography. Nuclear extracts were prepared by the method of Dignam et al. (5), with modifications (20). B lymphocytes were washed twice with phosphate-buffered saline and suspended in 1 ml of buffer A (0.5 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM KCl, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9]). Cells were then lysed by being expunged through a 23-gauge needle with a 1-ml syringe. Lysed cells were pelleted at 12,000 × *g* for 20 s, and the nuclear pellet was suspended in two-thirds of a volume of buffer C (400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% [vol/vol] glycerol, 209 mM HEPES [pH 7.9]). After incubation for 30 min, nuclear debris was removed by centrifugation at 12,000 × *g* for 5 min. The extract was then dialyzed against buffer D (100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 20 mM HEPES [pH 7.9]) for 2 h and frozen in liquid nitrogen. HeLa cell extracts for chromatography experiments were prepared in a similar fashion. The cell pellet was washed, suspended in an equal volume of buffer A, and subjected to 10 strokes in a Dounce homogenizer. Nuclei were recovered by centrifugation at 27,000 × *g* for 10 min and suspended in an equal volume of buffer C. After 45 min on ice, cell debris was removed by centrifugation at 27,000 × *g* for 30 min. The extract was then dialyzed for 4 h against buffer D. For partial purification of proteins that interact with the 48-bp *cis* negative regulatory element, nuclear extracts of HeLa cells were subjected to chromatography on a TSK phenyl-5PW matrix hydrophobic interaction column in an UltraPac high-performance liquid chromatography (HPLC) column (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.). Ammonium sulfate was added to the nuclear extract to a final concentration of 0.80 M, which was then used as input material for the hydrophobic interaction column. Proteins were eluted in a linear salt gradient of 0.80 to 0.00 M ammonium sulfate.

Gel retardation assays. The probe for gel retardation assays was made by labeling with either [^{32}P]ATP and polynucleotide kinase or [^{32}P]dCTP and Klenow. Free nucleotides were removed by centrifugation through a G50 Sephadex column. Nuclear extracts were mixed with radioactive probe (10 ng) in the presence or absence of cold DNA for 30 min at ambient temperature in a 10-μl volume containing 50 mM NaCl and 2 mM $MgCl_2$. Samples were then electrophoresed in 4% acrylamide gels in 0.25× TBE (22 mM Tris, 22 mM boric acid, 0.5 mM EDTA). After electrophoresis, gels were dried and exposed to XAR5 film.

DNase I footprinting. Footprinting was performed essentially as previously described (24). *Bam*HI-digested pzCAT was labeled with [^{32}P]ATP and Klenow polymerase. After a second digestion with *Sph*I, the 327-bp *Bam*HI-*Sph*I fragment was isolated and gel purified. Binding reactions were done in a 50-μl reaction volume containing 1 μg of poly(dI-dC), 1 mM $MgCl_2$, 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 10 mM HEPES (pH 7.9). Extracts (either 25 μg of crude extract or 10 μg of partially purified extract) were preincubated in the reaction mixture for 15 min on ice prior to addition of the probe. Binding reactions were carried out at 25°C for 10 min. After DNase I digestion for 1 min, the reaction was stopped by addition of 250 μl of stop buffer (300 mM NaCl, 20 mM EDTA, 50 μg of tRNA per ml, 1% [vol/vol] sodium dodecyl sulfate, 10 mM Tris [pH 7.4]). The samples were extracted three times with phenol-chloroform (1:1), precipitated with ethanol, and analyzed by electrophoresis on a 6% acrylamide denaturing gel.

Synthetic oligonucleotides. The synthetic oligonucleotides used in the experiments whose results are depicted in Fig. 5 and 6 were synthesized as single strands containing the following oligonucleotides: site I, CTAGTCAGCCATCT TCCCTTAG and CTAGCTAAGGGAGATGGCTGA; site II, ATCTGTCCACATATGGCTGCT and AGCAGCCATA TGTGGACAGAT; mutant site I, CTAAGGGAGATTGT TGA and TCAACAATCTCCCTTAG; SPI, GGCGTGTTA AATGGGGCGGGGCTTAAAGGGT and ACCCTTTAAGC CCCGCCCCATTTAACACGCC; wild-type (wt) SRE-wt AP1, CAGGATGTCCATATTAGGACATCTGCGTCAG CA and TGCTGACGCAGATGTCCTAATATGGGACATC CTG; mutant SRE-wt AP1, CAGGATGTGGATATTACCA CATCTGCGTCAGCA and TGCTGACGCAGATGTGGTA ATATCCGACATCCTG; wt SRE-mutant AP1, CAGGATG TCCATATTAGGACATCTGCCAGTCA and TGACTGGC AGATGTCCTAATATGGACATCCTG; and AP1, GGATG

TTATAAAGCATGAGTCAGACACCTCTGGCT and AGC CAGAGGTGTCTGACTCATGCTTTATAACATCC.

RESULTS

Identification of a *cis*-acting negative regulatory region. Since BZLF1 expression is sufficient to disrupt EBV latency (1, 29, 30), we were interested in identifying the mechanisms involved in BZLF1 regulation. Specifically, we wanted to determine whether *cis*-acting negative regulatory elements contribute to the absence of BZLF1 expression in latently infected cells. To this end, we first constructed a plasmid, designated pzCAT, in which the CAT gene was placed under the control of the BZLF1 promoter. For these studies, the BZLF1 promoter is defined as the region extending from the transcription start site to −551 bp. Because deletion of *cis*-acting negative control regions may allow increased expression from a promoter, deletions were then made in pzCAT extending from the 5′ end of the promoter to various downstream restriction endonuclease cleavage sites. These deletions resulted in removal of 165 (p386), 329 (p222), or 421 (p130) bp, respectively. These constructions (Fig. 1A) were subsequently used in transfection experiments and tested for CAT expression.

Expression of the parental plasmid pzCAT and the derivative deletion plasmids was assayed in BALB/c 3T3 and HeLa cells. Cell monolayers were transfected by the calcium phosphate precipitation method and harvested 48 h later. Lysates were then prepared, and the extracts were examined for CAT activity by thin-layer chromatography. An example of an assay with either BALB/c 3T3 or HeLa cells is shown in Fig. 1B. The corresponding quantitative measurement, determined from three independent experiments, is given below each sample tested. In BALB/c 3T3 cells, the intact BZLF1 promoter (pzCAT) was very weak relative to the control SV40 promoter (pSVCAT). However, deletion of 165 bp from the 5′ end of its promoter domain (p386) caused a sevenfold increase in CAT activity, suggesting the presence of a negative control element(s) within the deleted region. Removal of an additional 164 bp from the 5′ end (p222) caused the levels of CAT expression to return to those seen with the entire promoter. When only 130 bp were present upstream of the BZLF1 transcription start site, virtually undetectable levels of CAT activity were observed. Taken together, the results of BALB/c 3T3 transfections suggested that (i) a *cis*-acting negative control element was present within the leftmost 165 bp of the BZLF1 promoter, (ii) a positive control element(s) was present within a 164-bp region between the *Sph*I and *Nde*I sites, and (iii) an element(s) necessary for minimal expression was present between −130 and −222.

Similar experiments were then carried out with HeLa cells. HeLa cells, however, were less efficient in expression of the BZLF1 promoter plasmids. The relative differences between the activities of pzCAT and its derivatives compared with the control (pSVCAT) suggested that the lower levels of expression were not due to differences in transfection efficiency. A comparison of the CAT activities produced by pzCAT and p386 is shown in Fig. 1B (rightmost panel). p386 was sixfold more active than pzCAT. The relative levels of enzyme activity in p222-transfected cultures were similar to those of pzCAT-transfected cells, whereas virtually no activity was detected in p130-transfected cells. These results were consistent with those obtained from BALB/c 3T3 transfections and support the conclusion that a *cis*-acting negative control element was located within a 165-bp fragment flanked by the *Bam*HI and *Nde*I restriction sites. Furthermore, repression of the BZLF1 promoter was seen in both murine and human cell lines.

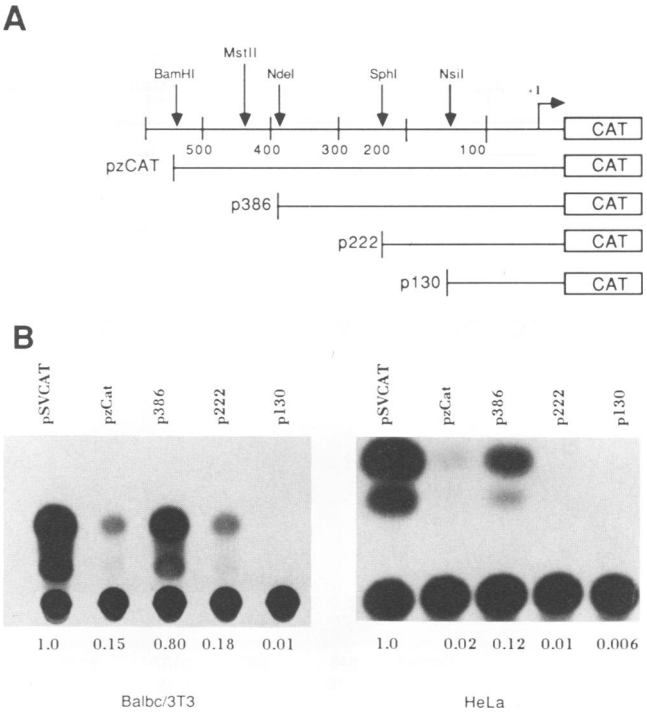

FIG. 1. Construction and analysis of BZLF1 promoter/CAT fusion plasmids. (A) The BZLF1 promoter (−551 to +12) was fused to a plasmid containing the coding region for the CAT gene and designated pzCAT. Deletions were then made from the 5′ end of the insert to various sites downstream. pzCAT was constructed by cloning the *Bam*HI-*Nae*I fragment of the *Bam*HI Z fragment into the pCAT vector, which contains the CAT coding sequence and the SV40 polyadenylation and splice sites. Deletions in pzCAT were made by excising from the 5′ end of the BZLF1 promoter to various sites downstream as shown here. We made p386 by deleting the *Bam*HI-*Nde*I fragment (165 bp), p222 by deleting a *Bam*HI-*Sph*I fragment (329 bp), and p130 by deleting the *Bam*HI-*Nsi*I fragment (421 bp). (B) A 20-μg sample of pzCAT or the corresponding deletion plasmid was transfected into either BALB/c 3T3 or HeLa cells by calcium phosphate precipitation. Cell monolayers were harvested 48 h posttransfection and assayed for CAT activity by the method of Gorman et al. (12). The level of CAT activity relative to that produced by pSVCAT is given below each sample tested.

Repression in a heterologous promoter. Given the apparent repression of pzCAT in BALB/c 3T3 and HeLa cells, we next asked whether a region(s) of the 165 bp deleted in p386 could exert a negative regulatory effect on a heterologous promoter. To answer this question, we isolated the 165-bp *Bam*HI-*Nde*I fragment from the BZLF1 promoter and dissected this region into four distinct but overlapping fragments. By taking advantage of two unique restriction endonuclease sites, we isolated fragments of 48, 58, 107, and 117 bp. Each of these fragments was then individually cloned upstream of the SV40 promoter in pSVCAT as depicted in Fig. 2A. The fragments were inserted into a polylinker site directly upstream of the 72-bp repeats of the SV40 enhancer, and the ensuing vectors were designated N48, N58, N107, and N117. These four plasmids were transfected into HeLa cells, which were then assayed for CAT activity.

An example of a HeLa cell transfection experiment is

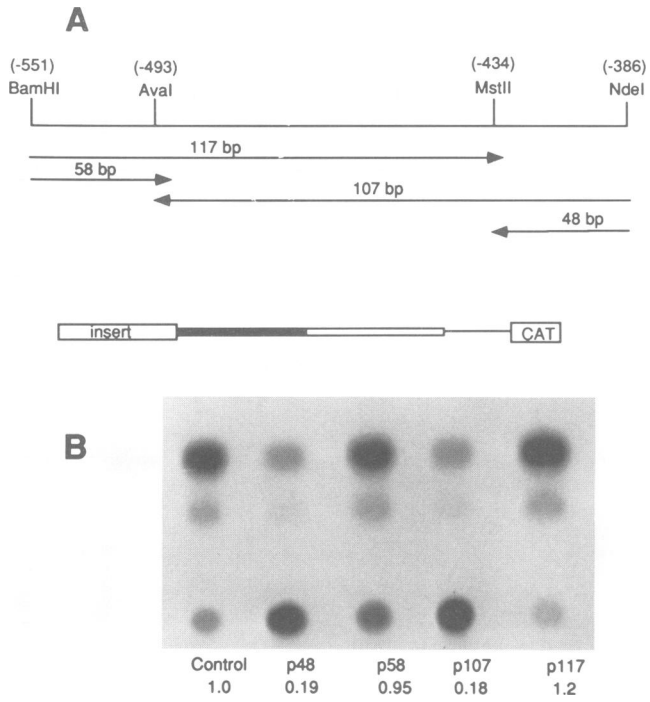

FIG. 2. Heterologous repression by a BZLF1 promoter fragment. (A) The 165-bp *Bam*HI-*Nde*I fragment was excised from the pzCAT vector and gel purified. The DNA was then digested with either *Ava*I or *Mst*II. Two fragments corresponding to 107 and 58 bp were isolated from *Ava*I-digested DNA, whereas *Mst*II digestions produced fragments of 117 and 48 bp. The fragments were individually cloned upstream of the SV40 enhancer in pSVCAT as shown. (B) Five micrograms of DNA from each of the plasmids shown was transfected into HeLa cells that were then assayed for CAT activity as described in the legend to Fig. 1. The level of CAT activity relative to that produced by pSVCAT is given below each sample.

shown in Fig. 2B. Plasmid vector N117, which contained all but the rightmost end of the 165-bp region of the BZLF1 promoter, failed to repress SV40 promoter activity in a promoter/CAT fusion vector. Rather than repressing promoter activity, insertion of the 117-bp fragment upstream of the SV40 promoter consistently caused slightly elevated levels of CAT activity. N58, which completely overlaps the 5′ end of N117 CAT, had no effect on the relative enzyme activity. In contrast, the levels of CAT expression in N48- and N107-transfected cultures were five- to sevenfold less than those obtained from the parent vector (on the basis of the results of three independent experiments). Similar results for all of these pSVCAT-derivative plasmid constructions were also obtained in BALB/c 3T3 transfection experiments (data not shown). Since the 48-bp insertion in N48 was identical to the 3′ end of the 107-bp insertion sequence, these results suggested that there was indeed a *cis*-acting negative control element within the BZLF1 promoter. Furthermore, this region conferring negative regulation was localized to the 48-bp fragment between the *Mst*II and *Nde*I restriction endonuclease sites.

To determine whether the 48-bp negative control domain was active in B cells, three different EBV-positive cell lines were transfected with either pSVCAT or N48. The results from these experiments are shown in Table 1. In B95-8 cells, the CAT activity in N48-transfected cultures was 7.5-fold less than in cells transfected with the parental plasmid

TABLE 1. Relative CAT activities in B cells[a]

Cell line	Mean relative CAT activity ± SEM	
	pSVCAT	N48
B95-8	1.0	0.13 ± 0.01
IB4	1.0	0.39 ± 0.12
Raji	1.0	1.20 ± 0.18

[a] B-lymphocyte lines were transfected by the DEAE-dextran method as previously described (8). The amount of CAT activity for pSVCAT was normalized to a value of 1.0 for each individual experiment. CAT expression in N48-transfected cultures is given as relative expression compared with that of pSVCAT. The values represent averages of three experiments.

pSVCAT. Insertion of the 48-bp *Mst*II-*Nde*I fragment upstream of the SV40 promoter caused a 2.5-fold reduction in CAT activity in IB4-transfected cultures. In contrast, the levels of CAT activity were essentially the same in pSVCAT- and N48-transfected Raji cells. Thus, the 48-bp domain can function as a negative regulator in lymphoblastoid cell lines B95-8 and IB4 but not in Raji cells. We do not know why repression was not seen in the Raji cell line.

Analysis of the 48-bp *Mst*II-*Nde*I region. Once it was determined that there was a *cis*-acting negative domain present within a 48-bp segment of the BZLF1 promoter, we decided to determine the specific binding domains within the fragment and then test each of the specific domains for the ability to repress a heterologous promoter. To search for proteins that could specifically bind the 48-bp region, we carried out a gel shift analysis with HeLa cell extracts fractionated by HPLC (5PW column) on the basis of their hydrophobic properties. The 48-bp DNA segment was gel purified from pzCAT and radiolabeled with ^{32}P with Klenow polymerase. Radioactive probe was then incubated with each of the fractions obtained by HPLC for 20 min, and the complexes were subsequently analyzed by electrophoresis in a low-ionic-strength polyacrylamide gel. An HPLC elution profile of DNA-protein complexes is shown in Fig. 3. The leftmost lane represents the formation of complexes in crude cell extracts, and the second lane contains complexes formed when the probe was incubated with the flowthrough fraction of the hydrophobic interaction column. Lanes 17 through 40 represent individual elution fractions tested for binding activity. Competition experiments indicated that the complexes seen in fractions 17 through 29 were due to nonspecific binding, while specific DNA-binding activities were present in fractions 31 through 40. As many as four distinct complexes were found to represent specific DNA-protein interactions (see fraction 34). The electrophoretic mobility of the slowest migrating complex decreased (fractions 36 to 40) as the salt concentration used for elution of proteins was decreased. Fraction 34, which contained at least three specific complexes, was used in the other experiments described in this report.

After confirmation of DNA-binding activity, footprint analysis of the 48-bp region was carried out. To perform this analysis, we digested pzCAT with *Bam*HI (Fig. 1) and radiolabeled it with T4 DNA kinase. After a second digestion with *Sph*I, radiolabeled probe was gel purified and incubated with either no protein, 20 μg of HeLa cell crude extract, or 10 μg of fraction 34 from the 5PW HPLC fractionation. After incubation for 10 min on ice, the samples were digested with DNase I and analyzed by electrophoresis through a polyacrylamide-urea sequencing gel. The results of our DNase I footprint analysis are shown in Fig. 4A. We were unable to

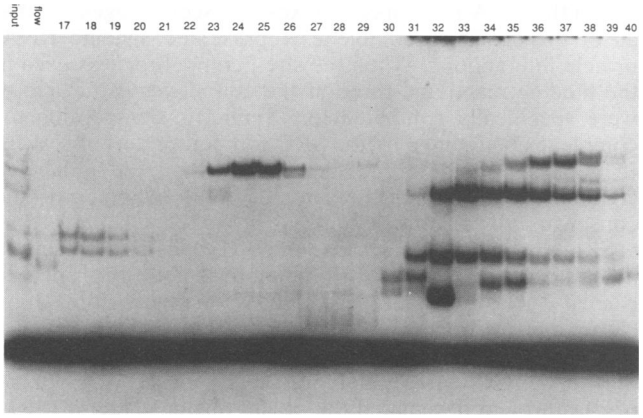

FIG. 3. Protein binding to the 48-bp *Mst*II-*Nde*I fragment. Proteins from HeLa cell nuclear extracts were partially purified by HPLC. Extracts were prepared as described in Materials and Methods and then separated by HPLC using a Pharmacia LKB phenyl-5PW column and an LKB 2152 HPLC system. Binding of partially purified protein to the 48-bp probe was tested by gel shift analysis. Final concentrations in binding reactions were 50 mM NaCl, 2 mM $MgCl_2$, 1 μg of poly(dI-dC), and 10 fmol of ^{32}P-labeled double-stranded probe. DNA-protein complexes were analyzed on a 4% acrylamide gel. The input and flowthrough lanes are labeled accordingly. The numerical designation above each lane represents the elution fraction from the phenyl-5PW column.

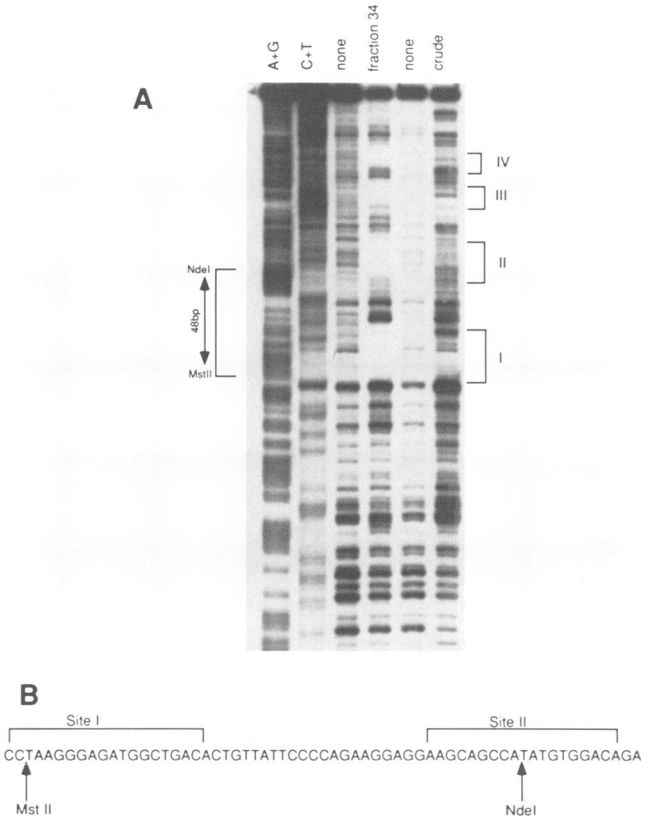

FIG. 4. DNase I footprinting of the 48-bp region. (A) Plasmid pzCAT was digested with *Bam*HI and radiolabeled with [^{32}P]ATP and Klenow. After a second digestion with *Sph*I, the 327-bp *Bam*HI-*Sph*I fragment was gel purified. The DNA was incubated with either crude nuclear extract or partially purified protein and digested with DNase I as described in Materials and Methods. Electrophoresis was carried out on a 6% polyacrylamide–urea sequencing gel. The sample analyzed in each reaction tube is designated above each lane. The Maxam-Gilbert sequencing reactions, A+G and C+T, are labeled. Binding reactions were carried out in the absence (lanes labeled none) or presence (either 10 μg of partially purified fraction 34 protein or 20 μg of crude extract) prior to DNase I digestion. Two different concentrations of DNase I were used to digest DNA incubations with crude extract. Four regions were protected from DNase I digestion with partially purified protein, designated sites I, II, III, and IV. (B) Schematic representation corresponding to sites I and II in the DNase I footprint analysis. The *Mst*II and *Nde*I sites are shown to indicate the region corresponding to the 48-bp negative regulatory domain.

obtain a footprint when crude HeLa extracts were used in binding reactions. However, four DNase I-protected regions were found when partially purified protein (fraction 34 from the column profile displayed in Fig. 3) was used for the DNA-protein incubations. Two of these regions, designated sites I and II, were contained within the 48-bp domain that was able to confer negative regulation on the BZLF1 promoter, as well as heterologous promoters. These sites are schematically shown in Fig. 4B. Upon inspection of the site I and II sequences, we discovered that these two sites were related, representing a 7-bp dyad symmetry region separated by 27 bp.

Two methods were then used to ascertain the relationship between sites I and II. First, an oligonucleotide which contained the site I sequence with 2 bp changes was synthesized. The 7-bp sequence ATGGCTG was changed to AT TGTTG. The mutant oligomer was then used to test the specificity of the binding reaction. In addition, four other oligonucleotides were tested in competitive binding experiments. These four competitors contained either wt site I, site II, a consensus SP1-binding site, or the −317 to −285 region of the c-*fos* promoter, which contains an SRE and an AP1-like binding domain (SRE-AP1). Nuclear extracts from B95-8 cells (an EBV-infected B-lymphocyte cell line) were mixed with radioactive probe in the presence or absence of competitor DNA, and complexes were then examined by gel shift analysis. The results from this experiment are shown in Fig. 5A. As predicted, the unlabeled site I oligodeoxynucleotide effectively competed with the radiolabeled site I probe for protein binding. Five complexes were observed in these reactions. Complex a represented both specific and nonspecific DNA-protein interactions which could be separated by HPLC fractionation (Fig. 3). By competition analysis, we determined that the other four complexes (b, c, d, and e) were results of specific DNA-protein interactions. The site II competitor also competed for protein binding, albeit at a lower efficiency than the homologous DNA. These results suggested that sites I and II could bind the same protein. To confirm that the specific competition by the heterologous DNA occurred via the 7-bp region, competition analysis was performed with the mutant site I oligomer. In this case, no competition for binding was observed. Similarly, no effect was seen when the consensus SP1 oligomer was present in the binding reactions. Unexpectedly, a slight amount of competition was observed with the SRE-AP1 competitor, particularly with the faster-migrating complexes.

Similar experiments were then carried out with the radioactive site II probe (Fig. 5B). In these experiments, five distinct complexes were observed. Since additional sequences are located outside the 48-bp region, we reasoned that some of these complexes represent additional binding

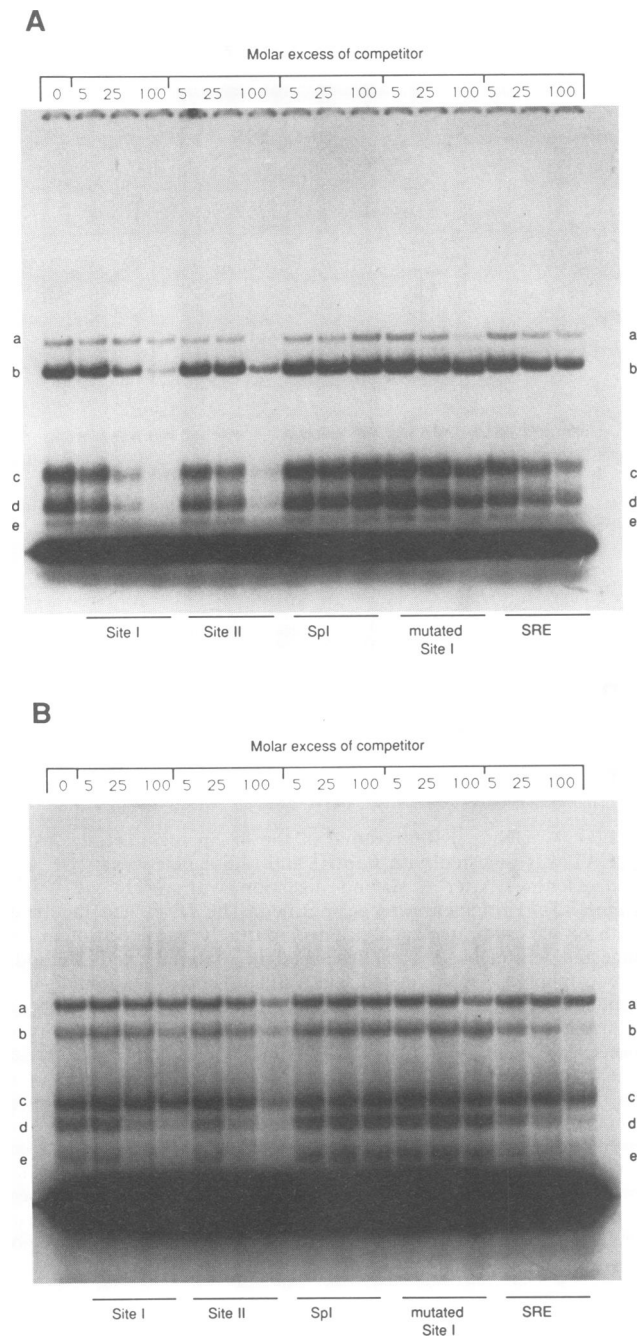

FIG. 5. Sites I and II compete with each other for protein binding. Radiolabeled site I (A) or site II (B) probe was incubated with B95-8 nuclear cell extracts in the presence or absence of cold competitor, and DNA-protein complexes were analyzed on a 4% nondenaturing polyacrylamide gel. Binding reactions were carried out in a 10-μl reaction containing 4 μg of protein, 10 fmol of probe, 1 μg of poly(dI-dC), 20 mM $MgCl_2$, and 50 mM NaCl. The specific competitors are designated below the lanes. The sequence of each competitor is described in Materials and Methods.

downstream of the *Nde*I site. In competition experiments, three of the unlabeled competitors (sites I and II and the SRE) affected the binding of a protein(s) to the radioactive site II oligomer. Homologous competition by site II for all five of the DNA-protein complexes was seen, suggesting that all of the observed complexes represented specific DNA-protein interactions. When the site I competitor was used in the binding reactions, three of the complexes (b, d, and e) were specifically competed for. Similarly, competition for complexes b, d, and e by the SRE competitor was observed. In contrast, no difference was seen when the unlabeled competitor was either the mutant site I oligomer or the consensus SPI oligonucleotide.

SRF binds to site II. Because the SRE oligonucleotide was able to compete linearly for binding to the site II probe, we carried out a more detailed analysis of this region. Upon closer examination, we discovered that site II contained a region which contained a 14-of-15-bp homology to the SRE of the human cytoskeletal γ-actin promoter (Fig. 6). A 67-kDa protein designated the serum response factor (SRF) has been shown to interact specifically with the SRE of the c-*fos* promoter, which is composed of a dyad symmetry element located at positions −320 to −299 (34, 38). The central core of the SRE contains the sequence $CC(A/T)_6GG$, known as the CArG box. Mutations at C and G residues in the CArG box of the dyad symmetry element (Fig. 7A) abolish binding of the SRF (11, 31). To determine whether mutations which affect SRF binding influenced the formation of site II DNA-protein complexes, competition experiments were done with either the wt SRE or the mutant SRE as the unlabeled competitor DNA. The results of these experiments are shown in Fig. 7B. Under the conditions used in these experiments (i.e., HeLa cells), we were unable to detect the upper and lower complexes previously detected with B95-8 extracts and the site II probe. Competitor DNA containing an SRE and an AP1-like sequence affected the formation of complexes a and c visualized in these experiments. In contrast, the mutant SRE oligomer failed to compete with site II for protein binding. Similarly, no effect on binding activity was seen when an oligonucleotide containing a consensus AP1 element was used as a labeled competitor. However, an oligonucleotide with a deleted AP1 site but an intact SRE competed effectively for protein binding. Two of the three complexes were also affected by the presence of an SRE in the reaction mixture. These results therefore suggested that the putative SRE binds the cellular transcription factor SRF. Furthermore, this putative SRE in the BZLF1 promoter partially overlaps one-half of a dyad symmetry element which apparently confers negative regulation on this EBV promoter.

DISCUSSION

The ZEBRA protein is known to be a viral transactivator whose expression in latently infected cells is sufficient to induce virus replication (1, 29, 30). Because of this integral role in the EBV life cycle, recent studies have begun to address the mechanisms which regulate the expression of this viral gene product. At least two distinct sets of *cis*-positive elements which are responsive to either TPA or the BZLF1-encoded protein itself have recently been described (8, 9). In this study, we identified a region of the BZLF1 promoter which negatively regulates expression. This region is a 48-bp sequence (Fig. 6), located at positions −433 to −386, which contains at least two distinct factor-binding sites.

By two different criteria, our data strongly suggest that the 48-bp region is a *cis*-acting negative regulatory domain: (i) Deletion of this region caused an increase in BZLF1 promoter activity (Fig. 1B), and (ii) insertion of the 48-bp region

FIG. 6. Schematic representation of the 48-bp negative regulatory region and the adjoining SRE. Regions of dyad symmetry are represented by arrows. The sequences of the human γ-actin and c-*fos* SREs are shown for comparison. The asterisks below the c-*fos* SRE indicate the contact points for the SRF.

upstream of the SV40 enhancer repressed the SV40 promoter (Fig. 2B). Footprint analysis of the 48-bp region revealed at least two protein-binding domains (Fig. 4). These domains are closely related in that they form a 7-bp dyad symmetry which is separated by 27 bp (Fig. 6). Competition experiments with heterologous competitor and mutant oligonucleotides (Fig. 5) imply that the 7-bp sequence is directly involved in the formation of protein complexes. However, insertion of one-half of the dyad symmetry (i.e., 7 bp) was not sufficient to down regulate the SV40 promoter (data not shown), implying that both binding sites are essential for efficient repression.

The right half of the dyad symmetry element partially overlaps a 15-bp region which is homologous to the SRE of the human γ-actin promoter (6). SREs have been identified in several mammalian promoters (3, 35, 37, 40), and they all contain a 10-bp sequence [CC(A/T)$_6$GG] known as the CArG box. In the c-*fos* SRE, the CArG box element is sufficient for both serum induction and repression by c-*fos* (28). The BZLF1 promoter contains an imperfect CArG box in that one of the (A/T)$_6$ bases is replaced by a guanine residue (i.e., ATATGT). However, competition experiments with either the wt or mutant c-*fos* SRE suggest that the SRF binds the putative SRE of the BZLF1 promoter (Fig. 7).

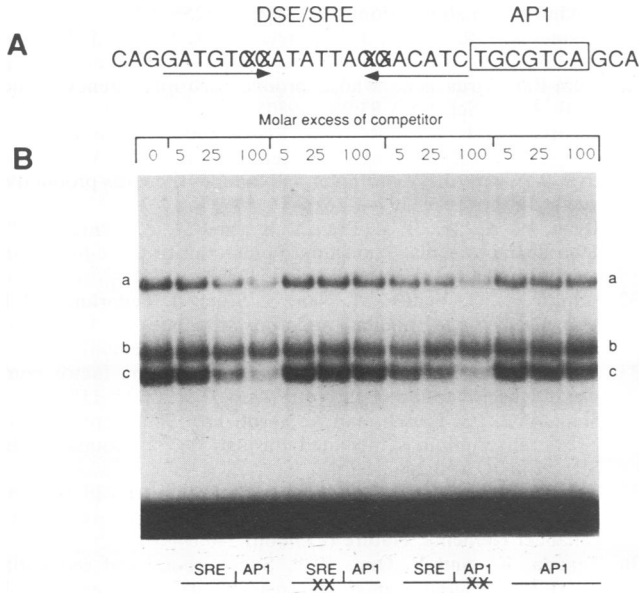

FIG. 7. The c-*fos* SRE competes with site II. (A) DNA sequence of the c-*fos* promoter representing positions −320 to −288. The dyad symmetry composing the c-*fos* SRE is shown by the arrows. The crossed-out C and G residues refer to the mutations which are known to affect binding of the SRF. Immediately 3′ of the dyad symmetry element (DSE) is an AP1-like site. Crossed-out mutations at this site abolish AP1 binding activity. (B) Competition of the c-*fos* SRE was analyzed by gel shift analysis. Binding reactions were carried out as described in the legend to Fig. 5.

We have identified and purified a 68-kDa protein which specifically binds sites I and II described in this report (unpublished data). We are currently investigating the possibility that the SRF and the 68-kDa protein can form a complex which represses BZLF1 promoter activity. Although the 48-bp BZLF1 region is sufficient to mediate repression of the SV40 promoter, we have not excluded the possibility that SRF binding can contribute additional down regulation. Preliminary results suggest that the BZLF1 SRE does not significantly contribute to negative regulation. However, lack of SRF binding may contribute to the enhanced expression seen when the 5′ 165 bp are deleted from the BZLF1 promoter. In our initial BZLF1 promoter deletions, a *Bam*HI-*Nde*I deletion caused an increase in promoter activity. Restriction endonuclease *Nde*I cleaves within the SRE, which would prevent binding of the SRF. In the c-*fos* promoter, mutations which abolish SRF binding also lead to higher levels of constitutive expression in some cell lines (34). Presumably, this is due to binding at an AP1-like site immediately downstream of the SRE (17). Interestingly, the BZLF1 promoter also contains a similar AP1-like site downstream of the putative SRE.

It is conceivable that the BZLF1 gene product, which is in some sense homologous to c-*fos*, is transcriptionally regulated by mechanisms which mediate c-*fos* expression. The relationship between these two proteins is further intertwined by a recent study which demonstrated transactivation of c-*fos* by BZLF1-encoded protein ZEBRA (10). Two of the sites identified as ZEBRA-binding domains are positioned at either side of the c-*fos* SRE. At least one of these sites is also present 3′ of the BZLF1 SRE. Further analysis of this region should determine whether similar sites in the BZLF1 promoter serve as transactivating targets for BZLF1 autoregulation. Regulation of BZLF1, in turn, may be provided by c-*fos*, which negatively autoregulates its own expression. The site for this c-*fos* autoregulation is the CArG box of the SRE (28). The BZLF1 promoter also contains a CArG box which could represent a site for turning off ZEBRA transcription. That ZEBRA transcription is, in fact, transient has been shown in anti-immunoglobulin-treated cultures in which RNAs were detected 2 h after treatment but returned to undetectable levels after 8 h. Whether c-*fos* and ZEBRA regulate each other's expression has not been delineated. However, simultaneous analysis of these two products in induced EBV-infected cells should answer these questions.

In sum, we propose that the 48-bp region of the BZLF1 promoter described in this report represents a negative regulatory domain which functions, together with positive

elements described by other investigators, in a complex regulatory network which determines whether the latent or lytic cycle will prevail in a virus-infected cell.

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