The Epstein-Barr Virus Immediate-Early Promoter BRLF1 Can Be Activated by the Cellular Sp1 Transcription Factor

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Disruption of viral latency in Epstein-Barr virus-infected cells is mediated through the activation of the BZLF1 (Z) immediate-early gene product. The Z protein can be derived from either of two promoters: the BZLF1 promoter, which directs transcription of a 1.0-kb mRNA encoding the Z gene product alone, or the upstream BRLF1 promoter, which directs transcription of a 2.8-kb bicistronic mRNA encoding the BRLF1 and BZLF1 immediate-early proteins. In this study we have examined the regulation of the BRLF1 promoter by viral and cellular factors. We found that the BRLF1 promoter is autoregulated by the BRLF1 transactivator through a nonbinding mechanism. We show that the BRLF1 (but not the BZLF1) promoter is highly responsive to the Sp1 transcription factor. Sp1 activation of the BRLF1 promoter is mediated through a consensus Sp1-binding site located from -39 to -44 (relative to the mRNA start site). We demonstrate that the BRLF1 promoter has high constitutive activity in C-33 cells (an epithelial cell line) and that the proximal Sp1-binding site is required for this activity. Despite the ubiquitous presence of Sp1 in many cell types, we found that the BRLF1 promoter has essentially no activity in lymphoid cell lines, suggesting that factors other than Sp1 may negatively regulate the BRLF1 promoter in these cells. Our findings demonstrate that the two potential promoters directing BZLF1 transcription are differentially regulated and that Sp1 can activate the BRLF1 promoter but not the BZLF1 promoter.

Epstein-Barr virus (EBV) is a human herpesvirus which infects both epithelial and lymphoid cells (38). Infection of epithelial cells can lead to nasopharyngeal carcinoma (56, 72), whereas infection of B cells is associated with Burkitt's lymphoma and B-cell lymphoma (52, 72). EBV infection of epithelial cells is often associated with productive viral expression (43, 64). In contrast, EBV infection of B cells is generally latent, although overexpression of the EBV immediate-early (IE) gene product, BZLF1 (Z), is sufficient to disrupt latency (8-10, 57, 58, 66). The cellular control of BZLF1 transcription therefore plays a crucial role in the disruption of viral latency.

The BZLF1 gene product can be derived from either of two promoters (50). The more proximal (3') promoter, BZLF1, drives transcription of a 1.0-kb mRNA which encodes only the BZLF1 gene product, whereas an upstream promoter, BRLF1, drives transcription of a 2.8-kb bicistronic mRNA which encodes both the BZLF1 and BRLF1 (R) gene products (2, 18, 50, 51, 65). The BZLF1 IE gene product is a leucine zipper protein (71) which activates a number of different early EBV promoters (8, 19, 37, 46, 47, 57, 68, 70) through a direct binding mechanism (5, 17, 19, 21, 25, 40, 45, 46, 57, 68, 70). The R IE protein is also a transcriptional activator (6-8, 31, 36) and sequence-specific DNA-binding protein (27, 28, 49). The combination of BZLF1 and BRLF1 has been shown to induce synergistic transactivation of several early EBV promoters (8, 13, 25, 32, 55).

It is not yet clear whether disruption of viral latency is mediated primarily by activation of the BZLF1 promoter or activation of the BRLF1 promoter. Sinclair et al. have reported that the BZLF1 gene product can activate BRLF1

promoter activity and have suggested that disruption of latency may be mediated primarily at the BZLF1 promoter, with the Z protein subsequently activating the BRLF1 promoter (63). However, analyses of the time course of the induction of the 1.0- and 2.8-kb mRNAs have shown that they are both already present even at the earliest time points assayed (51, 63). The Z gene product has been previously shown to activate BZLF1 promoter expression (19). Therefore, it is plausible that activation of the BRLF1 promoter sometimes precedes activation of BZLF1 and that the resultant translation of the Z gene product from the bicistronic 2.8-kb mRNA subsequently activates BZLF1 promoter function. Nevertheless, the ability of Z protein derived from the bicistronic 2.8-kb mRNA to disrupt viral latency has not yet been firmly documented. Regardless of which promoter is activated first in the disruption of viral latency, the BRLF1 promoter plays a key role because it is the only promoter known to drive transcription of the R IE gene product.

To date, we are aware of only one report which has been published regarding the regulation of the BRLF1 promoter (63). In this report the BRLF1 promoter was found to contain two Z-binding sites and to be activated by both the BZLF1 and BRLF1 viral IE transactivators (63). However, nothing is known about the cellular regulation of BRLF1. In the work described in this paper, we have studied the regulation of the BRLF1 promoter by viral and cellular factors. We show here that the cellular transcription factor, Sp1, strongly activates the BRLF1 promoter. Despite the ubiquitous presence of Sp1 in all cell types, we found that the BRLF1 promoter is inactive in B cells (in which EBV infection tends to be latent) but quite active in C-33 cells (an epithelial cell line). Therefore, the BRLF1 promoter is likely to be negatively regulated by additional cellular factors.

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MATERIALS AND METHODS

Cell lines. The cell lines used include EBV-negative Burkitt's lymphoma B-cell lines (Louckes and Ramos), the Jurkat T-cell line, a latently infected EBV-positive B-cell line (Raji), the cervical epithelial cell lines HeLa (papillomavirus positive) and C-33 (papillomavirus negative), the EBV-positive epithelial cell line NPC-KT (67), and the *Drosophila* embryo cell line Schneider line 2 (SL2) (62). 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. The Schneider line 2 (provided by C. Benyajati) was maintained in *Drosophila* Schneider media obtained from GIBCO-BRL with 10% fetal calf serum.

Plasmids. We initiated our studies by constructing a vector (RpBSCAT) containing the presumed BRLF1 promoter sequences (from -959 to +11 relative to the mRNA start site) linked to the heterologous reporter gene, chloramphenicol acetyltransferase (CAT) in the pBS phagemid vector (Stratagene). Our promoter sequence was based on previously published cDNA mapping which showed that the mRNA start site of the bicistronic mRNA encoding the Z and R transactivators was located at EBV sequence 106,175 (50). A series of 5' deletions of this promoter was constructed by cutting at convenient restriction sites and religating, as shown in Fig. 1A.

Site-directed mutants of the BRLF1 promoter construct (Fig. 1B) were made by using a Bio-Rad Muta Gene phagemid in vitro mutagenesis kit, which is based on the method of Kunkel (41). Synthetic oligonucleotides complementary to the region to be altered, except for a limited internal mismatch, were designed. We introduced a new restriction site in the oligonucleotide to screen for mutants.

The construction of the R and Z transactivating plasmids has been previously described (32). The Z plasmid (pEBV-ZIE) contains the *Bam*HI Z gene fragment in the pGEM2-based vector pHD1013 such that the BZLF1 gene is under the control of the strong cytomegalovirus IE promoter. The R plasmid (pEBV-RIE) contains the BRLF1 gene linked to cytomegalovirus IE promoter in a pUC-based plasmid (32).

The plasmid pPacSp1 (a gift from Robert Tjian) contains a 2.1-kb fragment encoding the C-terminal 696 amino acids of Sp1 driven by the *Drosophila* actin promoter (12). As a vector control for Sp1 transfection studies, we made a frameshift mutation within the *Bam*HI site of the Sp1 open reading frame and named this plasmid pPacSp1FX.

DNA transfections. Plasmid DNA was purified through two sequential cesium chloride gradients. DNA was transfected in both lymphoid and epithelial cells by electroporation (69), with 10 μ g of DNA and 10^7 cells per condition. Cells were shocked at 1,500 V with a Zapper electroporation unit (Medical Electronics Shop, University of Wisconsin). Epithelial cells were harvested and suspended into RPMI 1640 medium prior to electroporation.

Schneider line 2 (SL2) cells were transfected by the calcium phosphate precipitation method (15). Cells were replated onto petri dishes at a density of 10^7 cells per plate 20 to 24 h before transfection. For each condition, 10 μ g of reporter CAT plasmid and 1 μ g of Sp1 expression plasmid were used. The cells were harvested 48 h after addition of DNA.

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

(26). The percent acetylation of chloramphenicol was quantitated by thin-layer chromatography followed by scintillation counting or AMBIS scanning (AMBIS, Inc.).

Gel mobility shift assays. Gel retardation studies were performed as previously described (22, 24). Cellular nuclear extracts were prepared by a modification of the method of Dignam et al. (14). The binding reactions consisted of 20,000 cpm of 32 P-end-labeled probes spanning the entire BRLF1 promoter incubated with 4 μ g of nuclear extract in a buffer consisting of 50 mM Tris (pH 7.9), 250 mM NaCl, 2.5 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl₂, and 50% glycerol. Poly(dI-dC) · poly(dI-dC) (4 μ g) was added as a nonspecific competitor DNA, and the final volume was adjusted to 20 μ l. The reaction mixture was loaded on a 4% polyacrylamide-1% Tris glycine gel and run in 1× Tris glycine buffer (0.025 M Tris, 0.19 M glycine) at 20 mA at room temperature. The gel was dried and subjected to autoradiography.

For studies of R protein binding, we used a bacterial fusion protein (pGR356) containing the first 356 amino acids of R ligated into the PGEX-3X vector as previously described (29). The negative control vector, PGR-, contains the R reading frame in an antisense orientation. These plasmids were induced for 2 h in *Escherichia coli* DH5 α F' at 30°C with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The bacteria were lysed by sonication in buffer containing 50 mM KCl, 50 mM phenylmethylsulfonyl fluoride, and 8 μ M leupeptin. The samples were centrifuged, and the supernatants were assayed for protein concentration (3) prior to use in gel retardation studies. The R-binding reaction consisted of 20,000 cpm of probe and 10 μ g of GR356 or PGR protein in a buffer consisting of 50 mM KCl, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.3), 5% Ficoll, 5 mM MgCl₂, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride and 1 μ g of poly(dI-dC) · poly(dI-dC) in a final volume of 30 μ l. The binding reaction was incubated at 4°C for 15 min, and the reaction mixture was loaded onto a 5% polyacrylamide gel and run in 0.5× Tris borate buffer.

Methylation protection assays. Methylation protection was performed by a modification of the methylation interference procedure previously described (1). For these experiments, the binding reaction consisted of 100,000 cpm of probe incubated with 2 volumes of the reaction mixture used in gel retardation assays described above. Just prior to loading on a 4% polyacrylamide-1% Tris glycine gel, 1 μ l of dimethyl sulfate was added to the binding reaction. The bands representing the Sp1 bound (determined by competition assays) and the free probe were cut separately from the gel and transferred to DEAE membrane paper. The membrane was incubated with 1 M piperidine at 90°C for 15 min and then rinsed twice with 1 ml of sterile water. The probe fragments were eluted from the membrane with 250 μ l of elution buffer consisting of 1 M NaCl, 0.1 mM EDTA, and 20 mM Tris (pH 8.0) at 67°C. Equal amounts of radioactive bound and free probes were then loaded on a 6% polyacrylamide-7 M urea gel and subjected to autoradiography.

RESULTS

The BRLF1 promoter is constitutively active in epithelial cells but not in lymphoid cells. Our initial analysis of the BRLF1 promoter sequence (Fig. 1) suggested that there is a TATA box (CATAAAA) located 26 bp upstream of the RNA start site. In addition, we noted that there is a consensus Sp1-binding site (CCGCCC) (4, 12, 16, 34) positioned at -39 bp relative to the start site and two copies of another

FIG. 1. Construction of RpBS plasmids and 5' deletions. (A) The parental vector, RpBSCAT, contains the BRLF1 promoter sequences (from -959 to +11 relative to the mRNA start site) linked to CAT in the pBS vector. The locations of three potential Sp1-binding sites in the BRLF1 promoter are indicated. A series of 5' deletions was constructed as shown. (B) A series of site-directed mutants of the RpBSCAT vector was constructed as shown. The locations of the TATA box, the CCAAT box, and the proximal Sp1 motif are as indicated. The previously mapped (63) proximal Z-binding site is underlined. The site-directed mutants are named to reflect the BRLF1 promoter sequences (relative to the start site) affected.

sequence recently shown to bind Sp1 (CCACCC) (44) positioned at -272 and -490 bp. There is also a consensus CCAAT box sequence (61) located between the TATA box and the consensus Sp1 motif.

To study BRLF1 promoter function, we constructed the vector RpBSCAT, as shown in Fig. 1, which contains the BRLF1 promoter sequences from -959 to +11 (relative to the mRNA start site) linked to the CAT reporter gene. Initially we examined the level of constitutive BRLF1 promoter activity in a variety of cell types. As a positive control, we used the RSV-CAT construct (a gift from Bruce Howard), which contains the Rous sarcoma virus long terminal repeat linked to CAT and has strong promoter activity in a variety of cell types (26). As a negative control, we used the pBS-CAT construct, which is identical to the RpBSCAT construct except that it contains no eukaryotic promoter sequences.

The activity of the BRLF1 promoter construct in a variety of cell types is shown in Fig. 2. The highest level of constitutive BRLF1 promoter activity was observed in C-33 cells (a papillomavirus-negative cervical carcinoma cell line). In this cell type, the BRLF1 promoter construct (RpBSCAT) produced an average of 17% acetylation in CAT assays incubated for 1 h, a value which was approximately sevenfold greater than the activity produced by the promot-

FIG. 2. Constitutive activity of the BRLF1 promoter in different cell types. The activity of the CAT vector containing the BRLF1 promoter (RpBSCAT) (■) was compared with that of the positive control vector containing the Rous sarcoma virus promoter (RSV-CAT) (▨) and that of the promoterless negative control vector, pBS-CAT (□) in different cell types. Cells were transfected with 5 μg of each plasmid and analyzed for CAT activity (in a 1-h incubation) 48 h after transfection as previously described (26). C-33, HeLa, and NPC-KT are epithelial cell lines. Jurkat is a T-cell line. Louckes, Raji, and P3HR1 are B-cell lines. The numbers are presented as average percent acetylation of at least two experiments for each cell type.

erless CAT vector, pBSCAT. In the two other epithelial lines studied, the EBV-positive NPC-KT line and the EBV-negative HeLa cell line, the level of RpBSCAT activity was also greater than that of the negative control vector. However, in several different lymphoid cell types tested (the T-cell line Jurkat, the EBV-negative B-cell line Louckes, and the EBV-positive B-cell lines Raji and P3HR1), the level of CAT activity produced by the RpBSCAT construct was lower than that produced by the negative control construct. Thus, it appears that the BRLF1 promoter may be subject to negative regulation in lymphoid cells.

Effect of the Z transactivator on BRLF1 promoter activity. We next examined the effect of the Z transactivator on BRLF1 promoter activity in different cell types. Sinclair et al. have previously reported that the BRLF1 promoter contains two Z-binding sites (centered at -28 and -189) and can be activated by cotransfection with the Z gene product (63). However, the Z transactivation of the BRLF1 promoter reported by these investigators was quite low (only a three-fold effect in C-33 cells) compared with that of other Z-responsive promoters (8, 19, 37, 46, 47, 57, 68, 70). The results of our own experiments comparing the effect of the Z transactivator on the RpBSCAT construct with that on the promoterless control vector, pBSCAT, in a variety of cell types are shown in Fig. 3A. We were unable to document significant activation of the BRLF1 promoter (with respect to the negative control vector) in any cell type. In control experiments (data not shown), as previously reported (32, 47), we could easily detect strong transactivation of the BMRF1 and BHRF1 CAT constructs by the Z vector. It thus appears that Z-induced transactivation of the BRLF1 promoter is minimal in comparison with the effect of Z on the BMRF1 and BHRF1 promoters or that such transactivation may require a particular cellular environment or dosage of Z.

The R transactivator activates BRLF1 promoter activity through a nonbinding mechanism. We also studied the effect of the R transactivator on BRLF1 promoter activity. Sinclair et al. have previously reported that the R transactivator produces low-level (approximately fourfold) activation of the BRLF1 promoter (63). This group did not investigate whether the R protein can bind directly to the BRLF1 promoter. As shown in Fig. 3B, we likewise observed increased BRLF1 promoter activity (in comparison with that of the negative control vector, pBSCAT) in the presence of R in a variety of cell types. However, this R-induced transactivation of the BRLF1 promoter is much lower than the effect that we previously noted when using the EBV early promoter, BMLF1 (36).

Because the R IE protein has been recently shown to be a sequence-specific DNA-binding protein which binds directly to the R-responsive BMLF1 and DR enhancer elements (27, 28, 49), we investigated the possibility that the low-level R transactivation of the BRLF1 promoter is also mediated by a direct binding mechanism. We used a bacterial fusion protein containing the first 356 amino acids of the R protein as a source of protein and performed gel retardation assays with various probes spanning the entire BRLF1 promoter (-1 to -182, -48 to -390, and -390 to -959 bp). As a positive control for R binding, we made a probe containing the R-responsive region of the BMLF1 promoter (27). As shown in Fig. 4, we observed R binding to the BMLF1 probe, which was specifically inhibited (data not shown) by an oligonucleotide sequence containing another known R-binding site from the DR enhancer element. However, using probes of the BRLF1 promoter sequence region, we could not see any direct R binding to the BRLF1 promoter. It thus appears

FIG. 3. Effect of the Z and R transactivators on BRLF1 promoter function. (A) The RpBSCAT plasmid (5 μ g) ($\square$) or the promoterless negative control plasmid, pBSCAT ($\blacksquare$), was cotransfected with 5 μ g of either the pHD1013 vector DNA or the Z transactivator plasmid (pEBV-ZIE). The average fold transactivation induced by the pEBV-ZIE plasmid (compared with that induced by the pHD1013 vector DNA) is shown for different cell types. (B) The average fold transactivation induced by the R transactivator plasmid (pEBV-RIE) compared with vector DNA (pHD1013) in cotransfections with pBSCAT ($\blacksquare$) and RpBSCAT ($\square$) is shown for different cell types.

likely that the low-level R-induced transactivation of the BRLF1 promoter is mediated through an indirect mechanism.

The Sp1 transcription factor activates the BRLF1 promoter but not the BZLF1 promoter. Because the BRLF1 promoter contains several potential Sp1-binding sites, as shown in Fig. 1, we next examined whether the cellular Sp1 transcription factor can activate BRLF1 promoter function. Sp1 transfection studies were performed with SL2 cells as previously reported (15), because these cells contain no endogenous Sp1 activity. As shown in Fig. 5, the BRLF1 promoter was highly responsive to the Sp1 transactivator. The simian virus 40 (SV40) early promoter construct, pA10CAT (42), which has six Sp1-binding sites (16), served as a positive control and, as expected, was also activated by the Sp1 expression plasmid. In contrast, two other EBV promoters, the IE BZLF1 promoter and the early BMRF1 promoter, were not found to be significantly activated by Sp1. These experiments confirm that the Sp1 transcription factor specifically activates the BRLF1, but not the BZLF1, IE EBV promoter.

Defining the Sp1-responsive region(s) in the BRLF1 pro-

FIG. 4. The R protein does not bind directly to the BRLF1 promoter. A bacterial fusion protein was constructed containing the amino-terminal 356 amino acids of BRLF1 (which contains the DNA-binding domain) in the pGEX-3X vector. The BRLF1 fusion protein binds to a known R-binding site within the BMLF1 (27) promoter as shown. This binding is specifically inhibited by an oligonucleotide containing a known R-binding site in the DR enhancer (28) but not by the identical oligonucleotide in which the R-binding sequence has been mutated (data not shown). The R fusion protein does not bind to a probe containing the BRLF1 promoter sequences from -1 to -182 or additional BRLF1 probes spanning the promoter sequences between -179 and -959 (data not shown).

motor. We next examined which regions of the BRLF1 promoter are required for Sp1 responsiveness. We initially cotransfected the series of 5' BRLF1 promoter deletion mutants (Fig. 1A) into SL2 cells with the Sp1 expression plasmid and measured the resultant increase in the BRLF1 promoter activity. In this series of experiments (Fig. 6), the parent plasmid, RpBSCAT, was activated an average of 114-fold by cotransfection with Sp1. When the promoter sequences between -959 and -575 were deleted, Sp1 responsiveness was not decreased (in fact, Sp1 responsiveness

FIG. 5. Effect of the Sp1 transcription factor on different EBV promoters. Promoter constructs (10 μ g) containing either the BRLF1 IE promoter (RpBSCAT), the SV40 early promoter (pA10CAT) (42), the EBV BZLF1 IE promoter (BZLF1-CAT) (37), the EBV BMRF1 early promoter (BMRF1-CAT) (37), or the promoterless CAT vector (pBSCAT) were cotransfected into *Drosophila* SL2 cells with either 1 μ g of the Sp1 expression vector, pPacSp1 (12), or an equal amount of the pPacSp1FX vector containing a frameshift Sp1 mutation. The SV40 and BRLF1 promoters were activated by the Sp1 transcription factor, whereas the BZLF1 and BMRF1 promoters were not affected. Repeat experiments gave similar results.

increased to 139% of that of the parental construct). In contrast, 80% of the Sp1-induced transactivation was lost when sequences between -575 and -379 were deleted. This deleted region contains one potential Sp1 site. The Sp1 responsiveness of the promoter was totally lost when the promoter sequences between -182 and -33 (which contain the consensus Sp1-binding site) were deleted. These results suggest that two regions of the BRLF1 promoter, one located between -33 and -182 and the other located between -379 and -575, contribute to total Sp1 responsiveness.

A site-directed mutation of the proximal Sp1-binding site decreases Sp1 responsiveness. To examine more directly the functional significance of the proximal Sp1 site, we introduced a site-directed mutation in the proximal Sp1 site at -39, leaving the rest of the promoter intact, as shown in Fig. 1B. In addition to the mutation specifically abolishing the proximal Sp1 motif, we constructed several other site-directed mutations in the BRLF1 promoter, as shown in Fig. 1B, and tested the ability of Sp1 to activate each of these mutant constructs. As shown in Fig. 6, removal of the proximal Sp1 site (positions -39 to -44) reduced the Sp1 responsiveness of the BRLF1 promoter by 89%. In contrast, mutation of the potential Sp1-binding site (CCACCC) located at -272 (positions -272 to -277), which was not found to be important in the 5' deletion analysis, had no significant effect on the Sp1 responsiveness of the BRLF1 promoter. Other than the mutation specifically abolishing the proximal Sp1 motif, none of the site-directed mutations significantly reduced Sp1 responsiveness in SL2 cells.

From the above results it is clear that the consensus Sp1-binding site located at -39 is required for the Sp1 response of the BRLF1 promoter. Although we have not yet determined the exact sequence (mapped by the 5' deletion analysis) contributing to the Sp1 response in the -379 to -575 region of BRLF1, it appears that both the proximal and distal Sp1-responsive regions are required for maximal Sp1 transactivation of BRLF1.

Sp1 responsiveness can be transferred to a heterologous promoter. Once we had determined that the region between -30 and -182 in the BRLF1 promoter contains an Sp1 response element, we also asked whether this region of the promoter was sufficient to transfer Sp1 responsiveness to a heterologous promoter. We inserted one copy of this sequence upstream of the adenovirus E1B TATA box in the E1BCAT construct (48) (a gift from Katherine Martin). As shown in Fig. 7, the E1B promoter alone is minimally responsive to the Sp1 transcription factor. However, when the -30 to -182 sequence of BRLF1 was inserted upstream of the E1B TATA element, the promoter was activated 13-fold by cotransfection with Sp1. The -30 to -182 region of the BRLF1 promoter is therefore sufficient to transfer Sp1 responsiveness to a heterologous promoter.

The Sp1 protein binds directly to the BRLF1 promoter. As outlined above, our functional studies suggested that there are two different Sp1-responsive regions in the BRLF1 promoter: a proximal site from -39 to -44 containing the consensus Sp1 motif (CCGCCC), and the -379 to -575 region. To confirm that the Sp1 protein, as expected, binds directly to the BRLF1 promoter, we performed gel retardation assays. Binding studies were initially performed by using commercially available purified Sp1 protein (Promega). Purified Sp1 protein bound to a BRLF1 probe containing the promoter sequences from -1 to -182, as well as to a BRLF1 probe containing the sequences from -179 to -575 (Fig. 8). Similar data were obtained with HeLa cell

FIG. 6. Mapping the Sp1-responsive regions in the BRLF1 promoter. The ability of Sp1 to activate a series of RpbSCAT deletion plasmids in *Drosophila* SL2 cells was analyzed. The 5' deletions were constructed as shown in Fig. 1A, and the site-directed mutations were constructed as shown in Fig. 1B. The results are presented as the average fold transactivation induced by Sp1 (versus the frameshift mutant vector) relative to the Sp1 transactivation of the intact promoter construct (RpbSCAT). In this set of experiments, the average transactivation induced by Sp1 on the parental RpbSCAT construct was 114-fold.

extracts as a source of protein; in these extracts both probes were bound to proteins specifically inhibited by the Sp1 sequence (data not shown; see Fig. 11). It thus appears that there are at least two Sp1-binding sites within the BRLF1 promoter.

We subsequently used methylation protection (1) to map the proximal region of the BRLF1 promoter contacted by the Sp1-inhibited bands in HeLa cell extracts. Using a probe containing the BRLF1 sequences from -1 to -182, we were able to observe partial protection of the proximal Sp1 motif in the bound probe, as shown in Fig. 9. We have not yet mapped the precise sequence bound by Sp1 in the -182 to -575 probe.

The proximal Sp1-binding site is required for constitutive activity in C-33 cells. The above results suggested that the proximal Sp1-binding site is required for efficient Sp1 activation in *Drosophila* cells. To determine whether this Sp1

motif is also required for efficient BRLF1 promoter activity in C-33 cells, we examined the constitutive activity of the series of site-directed BRLF1 mutants (Fig. 1B) in C-33 cells. In this set of experiments (Fig. 10), the C-33 cell extracts were incubated overnight with [¹⁴C]chloramphenicol (in contrast to the experiment in Fig. 2, in which incubations were performed for 1 h), resulting in a constitutive level of RpbSCAT activity of 61%. The mutation abolishing the proximal Sp1 motif (positions -39 to -44) completely inhibited the constitutive activity of the RpbSCAT plasmid (relative to the negative control plasmid, pBSCAT) in C-33 cells. Therefore it appears that the proximal Sp1 site is required for maintaining the high constitutive

FIG. 7. Sp1 responsiveness can be transferred to a heterologous promoter. The BRLF1 promoter sequences from -30 to -182 were inserted upstream of the E1B TATA box in the E1BCAT construct (48) to create the E1BCAT-Sp1 construct. The constructs were transfected into SL2 cells in the presence and absence of the Sp1 expression vector. The E1B promoter by itself responds minimally to the Sp1 factor. However, the -30 to -182 BRLF1 promoter sequence confers Sp1 responsiveness to the E1B promoter.

FIG. 8. Purified Sp1 protein binds to the BRLF1 promoter. Purified Sp1 protein (Promega) was incubated with ³²P-labeled probes containing the BRLF1 sequence from -1 to -182 or from -179 to -575. Sp1 protein produced a retarded complex with both probes, which was specifically inhibited by cold oligonucleotide DNA containing the consensus Sp1 site but not by cold oligonucleotide containing the Ap1-binding site.

FIG. 9. Mapping of the proximal Sp1-binding site in the BRLF1 promoter by methylation protection. A ^{32}P -labeled probe containing the BRLF1 promoter sequences from -1 to -182 was incubated with HeLa cell nuclear extract and then treated with dimethyl sulfate immediately prior to gel electrophoresis. The retarded bands specifically inhibited by the Sp1 motif were removed separately (B) from the free probe (F), cleaved with piperidine, and analyzed on a sequencing gel. The area of partial protection is indicated next to the sequencing gel (○).

activity of the BRLF1 promoter in C-33 cells. One other mutation, which affected the -36 to -38 region of the BRLF1 promoter, also abolished BRLF1 constitutive activity. Although this mutation (being adjacent to the Sp1 motif) could potentially decrease Sp1 binding, our finding that Sp1-induced activation in *Drosophila* SL2 cells was not significantly affected by this mutation suggests that another

cellular factor required for C-33 activity may bind to this site. The four other mutations each moderately decreased promoter activity in C-33 cells.

The level of Sp1 binding to the BRLF1 promoter is similar in several cell types. Our initial analysis of BRLF1 promoter activity in different cell types suggested that the BRLF1 promoter had constitutive activity in C-33 cells but had no activity in Raji cells (Fig. 2). Given that the Sp1-binding motif was subsequently shown to be required for constitutive BRLF1 promoter activity in C-33 cells, we examined whether differences in the level of Sp1 binding to the BRLF1 promoter in C-33 and Raji cells could account for the difference in constitutive promoter activity. We performed gel retardation studies comparing the proteins binding to the BRLF1 promoter in C-33, HeLa, and Raji cell extracts.

When we incubated protein extracts from each of these three cell types with the -1 to -182 ^{32}P -labeled BRLF1 probe, we observed several retarded bands which were specifically inhibited by cold oligonucleotide DNA containing the consensus Sp1-binding site but not by cold DNA containing the consensus Ap1 site (Fig. 11). Since the level of Sp1-inhibited binding to the BRLF1 promoter in each of the three cell extracts was very similar, the low level of BRLF1 activity in Raji cells may not be due to insufficient Sp1 binding. A probe containing the -179 to -575 region of the BRLF1 promoter was also shown to bind to Sp1-inhibited complexes which were present in equal amounts in all three cell types (data not shown). Interestingly, the Raji extracts were found to contain an increased level of an additional gel-retarded band (complex A) binding to the -1 to -182 probe; this band was present in only minimal amounts in the HeLa and C-33 extracts, suggesting that this protein might serve to negatively regulate BRLF1 activity in Raji cells. We have not yet identified the exact binding site of this potential negative regulator of BRLF1.

DISCUSSION

The Epstein-Barr virus BZLF1 gene product is unique because overexpression of BZLF1 is sufficient to convert

FIG. 10. The proximal Sp1 motif is required for constitutive BRLF1 promoter activity in C-33 cells. A series of site-directed deletions of the RpBSCAT construct was constructed as shown in Fig. 1, and the activities of these deletion constructs in the C-33 cells were compared. The number above each bar represents the average percent activity relative to that of the parental RpBSCAT plasmid. The promoterless negative control plasmid, pBSCAT, has 16% of the activity of RpBSCAT in this series of experiments. The BRLF1 promoter mutation abolishing the proximal Sp1 motif (-39 to -44) reduced the level of BRLF1 activity to that of the promoterless construct. The mutant Δ36-38 also reduced the level of promoter activity to that of the negative control construct.

FIG. 11. Comparison of the level of Sp1 binding to the BRLF1 promoter in different cell extracts. HeLa, C-33, and Raji cell extracts were prepared by using a modification of the method of Dignam et al. (14). A 32 P-labeled probe containing the BRLF1 promoter sequences from -1 to -182 was incubated with an equal amount of protein from each extract. The amount of the retarded complexes which were specifically inhibited by the Sp1 binding sequence was similar in all three cell types. However, another retarded band (complex A) was present at much higher levels in Raji cells than in HeLa or C-33 cells.

latent infection to productive infection (8-10, 30, 57, 58, 66). Latent viral infection in B cells can also be disrupted by a variety of other compounds, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA), butyrate, and cross-linking surface immunoglobulin (36, 52). Although it has been generally assumed that these alternate methods of disrupting viral latency are mediated by activation of the BZLF1 promoter (23), it is equally plausible that disruption of viral latency in certain situations is mediated by activation of the BRLF1 promoter. Overexpression of the bicistronic mRNA has not yet been shown to disrupt viral latency. In principle, however, expression of the 2.8-kb RNA driven from the BRLF1 promoter should be sufficient to express both the Z and R proteins, thus activating the lytic cycle. In addition, the regulation of the BRLF1 promoter plays a key role in EBV reactivation because this is the only known promoter to direct transcription of the BRLF1 IE gene product (50).

Although the regulation of the BZLF1 promoter has been relatively well studied (20, 21, 53), little has been published regarding the regulation of the BRLF1 promoter. The BZLF1 promoter has been shown to be activated by TPA through TPA-responsive elements (20, 63) and to be auto-regulated by the Z gene product through two Z-binding sites (19, 70). In addition, a 48-bp negatively regulating *cis* element has been located (between -433 and -386) in the BZLF1 promoter; this element binds to the serum response factor and another unidentified protein (53). Although the BRLF1 promoter has been specifically shown not to be activated by TPA treatment (63), essentially no information has been previously published regarding the cellular factors that do regulate BRLF1 activity. The possibility exists that certain types of inducing agents preferentially activate the BRLF1 promoter, whereas other inducing agents (such as TPA) preferentially activate the BZLF1 promoter. Our studies examining the constitutive activity of the BRLF1 promoter in different cell types demonstrated that the BRLF1

promoter is constitutively active in several different epithelial cell lines but has no activity in a variety of different B-cell lines. The finding of low constitutive BRLF1 promoter activity in lymphoid cells correlates with the tendency of EBV to remain latent in B cells and suggests that disruption of viral latency in this cell type may require removal of a negative regulator of BRLF1 promoter function.

This report provides the first identification of the cellular Sp1 transcription factor as a positive regulator of BRLF1 function. Although a number of other viruses such as SV40, herpes simplex virus type 1, and human immunodeficiency virus have been previously shown to be regulated by the Sp1 factor (16, 33), this is the first EBV promoter (to our knowledge) shown to be regulated by Sp1. In addition to showing that the BRLF1 promoter is activated by the Sp1 factor, we have specifically shown that the BZLF1 IE promoter is not responsive to Sp1. Although the Sp1 transcription factor is expressed in a variety of different cell lines, it has recently been shown that the level of Sp1 activity is quite variable in different tissues (59) and that in certain cell types the level of Sp1 activity can be limiting (60). Sp1 activity has been recently shown to be positively regulated by several factors, including the retinoblastoma gene product (35, 39, 60). The ability of Sp1 to activate the BRLF1 but not the BZLF1 promoter suggests that a cellular environment favoring high Sp1 activity might preferentially disrupt viral latency through the BRLF1 (versus the BZLF1) promoter.

Our analysis of the BRLF1 promoter sequences required for Sp1 activation suggest that there are at least two Sp1-responsive elements in the promoter and that both of these elements are required for efficient Sp1 activation. We have specifically identified one of these elements as the consensus Sp1 motif located at positions -39 to -44 in the promoter. The other element has not yet been precisely identified, but by 5' analysis it has been shown to reside between positions -379 and -575. This region contains one copy of an alternate Sp1-binding site (CCACCC) recently identified in the papillomavirus (44). Our demonstration that two different Sp1-binding sites are required for maximal BRLF1 activation by Sp1 is in keeping with recent results showing that Sp1 bound to distal binding sites can interact synergistically with Sp1 bound at proximal sites through direct protein-protein contacts between Sp1 molecules (11, 54).

Although we have found that the BRLF1 promoter is virtually silent in B cells, our gel retardation assays indicate that the amount of Sp1 binding to the BRLF1 promoter is essentially the same in Raji, HeLa, and C-33 extracts. Therefore, it does not appear to be a relative lack of Sp1 binding which accounts for the low constitutive activity of the BRLF1 promoter in B cells. However, since a variety of factors (such as the retinoblastoma gene product and the human immunodeficiency virus TAT protein [35, 39]) have recently been shown to modulate Sp1 transactivation function, it is possible that the availability of such Sp1 regulatory factors accounts for the observed variability of BRLF1 activity. Interestingly, we have observed the presence of a BRLF1 promoter-binding complex which is present in largest amounts in Raji cells (in which there is no BRLF1 constitutive activity) and in smaller amounts in HeLa cells (in which there is some constitutive activity) and is essentially absent in C-33 cells (in which there is high constitutive activity). This protein could potentially serve as a negative regulator of BRLF1 function, and its presence in larger amounts in Raji extracts may account for the total loss of

BRLF1 constitutive activity. The binding site and nature of this protein will be the focus of subsequent studies.

Unlike the findings of another group (63), we have been unable to demonstrate that the Z gene product significantly activates the BRLF1 promoter in any cell type. As reported previously (63), we also found that the Z protein binds directly to the BRLF1 promoter (unpublished data). The reasons for the discrepancy between our own results and those previously reported are not clear. One possibility is that our Z expression vectors make significantly different amounts of protein. The effect of Z on BRLF1 promoter function may be dose dependent, with activation occurring at lower doses of Z and repression occurring at high doses. The proximal Z-binding site overlaps the CCAAT box, which, from our mutational analysis, has some effect on constitutive BRLF1 promoter activity in C-33 cells. The sequences between -36 and -38 (adjacent to the proximal Z-binding site) were also shown by our mutational analysis to be essential for constitutive BRLF1 promoter activity, and Z binding could potentially interfere with binding by an essential cellular factor to this site. In addition, the proximal Z-binding site is near the proximal Sp1-binding site and could potentially interfere with Sp1 binding. Further studies are necessary to determine whether both the Sp1 and Z factors can simultaneously bind to the BRLF1 promoter and, if so, whether such binding results in synergistic (versus antagonistic) activation of BRLF1.

As previously reported (63), we have also shown that the R gene product can activate its own promoter. In this report, we have extended the analysis of R transactivation of the BRLF1 promoter by showing that the R protein does not bind directly to the BRLF1 promoter. For two other EBV promoters known to be activated by the R transactivator, BMLF1 (27, 36) and DR (28), it has been shown that the R protein binds directly to these promoters and that this binding is required for R transactivation. Our finding that the R protein can activate the BRLF1 promoter through a nonbinding mechanism is the first suggestion that the R protein may activate EBV promoters by more than one mechanism, one involving direct binding and the other being mediated indirectly through modulation of cellular transcription factors. We have recently found that the R transactivator also activates the *c-myc* promoter through an apparently indirect mechanism (29). Although we have not yet mapped the region of the BRLF1 promoter required for R response, it is possible that R activates the BRLF1 promoter through modulation of Sp1 activity. The SV40 T antigen has been recently shown to activate certain promoters through induction of cellular Sp1 activity (60).

In conclusion, we have shown that the cellular factor Sp1 activates the BRLF1 promoter but not the BZLF1 promoter. We have identified a binding site for Sp1 in this promoter and shown that mutation of this site leads to loss of constitutive promoter activity and response to Sp1. The activation of the BRLF1 promoter by Sp1 could be a potential mechanism for disruption of viral latency.

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