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The Epstein–Barr Virus (EBV) DNA Polymerase Accessory Protein, BMRF1, Activates the Essential Downstream Component of the EBV oriLyt

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The EBV DNA polymerase accessory protein, BMRF1, is an essential component of the viral DNA polymerase and is required for lytic EBV replication. In addition to its polymerase accessory protein function, we have recently reported that BMRF1 is a transcriptional activator, inducing expression of the essential oriLyt promoter, BHLF1. Here we have precisely mapped the BMRF1-response element in the BHLF1 promoter. We demonstrate that a region of oriLyt (the "downstream component"), previously shown to be one of two domains absolutely essential for oriLyt replication, is required for BMRF1-induced activation of the BHLF1 promoter. Furthermore, the downstream component of oriLyt is sufficient to confer BMRF1-responsiveness to a heterologous promoter. The downstream component contains Sp1 binding sites, and confers Sp1-responsiveness to a heterologous promoter. A series of plasmids containing various portions of the oriLyt downstream component were constructed and analyzed for their ability to respond to the BMRF1 versus Sp1 transactivators. Although the BMRF1-responsive region of the downstream component overlaps the Sp1-responsive element, certain oriLyt sequences required for maximal BMRF1-responsiveness were not required for maximal Sp1-responsiveness. In particular, a site-directed mutation altering the downstream component sequence GATGG (located from −588 to −592 relative to the BHLF1 transcription initiation site) did not affect Sp1-responsiveness, but reduced BMRF1-responsiveness by 75% and abolished oriLyt replication. Although BMRF1 possesses nonspecific DNA binding activity, we were unable to demonstrate specific BMRF1 binding to the downstream component of oriLyt. Our results suggest that BMRF1-induced activation of the essential downstream component of oriLyt may play an important role in oriLyt replication.

INTRODUCTION

Epstein–Barr virus (EBV), a human gammaherpesvirus, causes infectious mononucleosis and is closely associated with several types of human malignancy, including B-cell lymphomas and nasopharyngeal carcinoma (Miller 1990; Zur Hausen *et al.,* 1970). EB virus infects and immortalizes human primary B lymphocytes *in vitro* (Miller, 1990). In B cells, the virus is generally maintained in a latent state, and replicated as an episome using oriP and the cellular DNA replication machinery (Kieff and Leibowitz, 1990; Miller, 1990; Reissman *et al.,* 1985; Yates *et al.,* 1985). Chemical reagents, including TPA and sodium butyrate, or cross-linking of surface immunoglobulin, can induce lytic viral replication in at least a portion of EBV-infected B cells (Luka *et al.,* 1979; Takada and Ono, 1989; Zur Hausen *et al.,* 1979). In contrast to B cells, EBV infection of epithelial cells commonly results in lytic infection (Li *et al.,* 1992; Miller, 1990; Sixby *et al.,* 1984). During lytic infection, the virus is replicated by a virally encoded polymerase and uses a separate origin of replication, oriLyt (Gruffat *et al.,* 1995; Hammerschmidt and Sugden, 1988; Schepers *et al.,* 1993a and b). Lytic EBV infection can be induced by expression of the BZLF1 immediate-early protein in B cells (Chevallier-Greco *et al.,* 1986; Countryman and Miller, 1985; Kenney *et al.,* 1989; Rooney *et al.,* 1988; Takada and Ono, 1986), or expression of either BZLF1 or the BRLF1 immediate-early viral protein in epithelial cells (Zalani *et al.,* 1996). BZLF1 and BRLF1 transcriptionally activate the early viral proteins required for replication of oriLyt (Buisson *et al.,* 1989; Chevallier-Greco *et al.,* 1989; Cox *et al.,* 1990; Farrell *et al.,* 1989; Fixman *et al.,* 1992, 1995; Hardwick *et al.,* 1988, 1992; Holley-Guthrie *et al.,* 1990; Lieberman *et al.,* 1989; Quinlivan *et al.,* 1993).

Although most strains of EBV contain two copies of oriLyt, one copy (as found in the prototype B95-8 strain) is sufficient for replication (Hammerschmidt and Sugden, 1988). OriLyt overlaps the divergent promoters of the BHRF1 and BHLF1 genes. The BHRF1 gene encodes a bcl-2 homolog which inhibits apoptosis (Henderson *et al.,* 1993). The BHLF1 gene is the most actively transcribed gene during lytic EBV infection, although the precise function of the BHLF1 gene product remains unknown (Laux *et al.,* 1985; Lieberman *et al.,* 1989).

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Hammerschmidt *et al.* have published extensive mutational analyses of oriLyt, showing that only two regions of oriLyt, the "upstream component" and the "downstream component," are absolutely essential for replication (Gruffat *et al.,* 1995; Schepers *et al.,* 1993). The upstream component overlaps the BHLF1 proximal promoter and contains two BZLF1 binding sites, the BHLF1 TATA box and a CCAAT box motif (Schepers *et al.,* 1993). The downstream component is located from −554 to −632 relative to the BHLF1 mRNA start site and contains Sp1 binding motifs (Gruffat *et al.,* 1995). However, detailed mutational analysis of the downstream component has shown that sequences required for oriLyt replication do not correlate precisely with the Sp1 binding sites and cannot be functionally replaced with the canonical Sp1 sites from the SV40 promoter (Gruffat *et al.,* 1995). Therefore, it has been suggested that a cellular protein other than Sp1 (as yet undefined) may bind directly to the downstream component and be essential for oriLyt replication (Gruffat *et al.,* 1995).

OriLyt replication requires the gene products of six viral genes: BALF5 (the catalytic component of the viral DNA polymerase), BMRF1 (the polymerase accessory protein), BALF2 (single-stranded DNA-binding protein), BSLF1 (primase), BBLF4 (helicase), and BBLF2/3 (helicase-primase-associated protein) (Fixman *et al.,* 1992, 1995). In addition, the immediate-early gene product, BZLF1, plays an essential role in lytic EBV replication. BZLF1 binds directly to oriLyt and is also required for activation of the viral replicative genes (Guo and DePamphilis, 1992; Schepers *et al.,* 1993; Takada and Ono, 1989).

The BMRF1 gene product is a dsDNA binding protein which is essential for processive DNA synthesis by the BALF5 gene product and is the major early phosphoprotein induced during EBV lytic replication (Chen *et al.,* 1995; Chiou *et al.,* 1985; Cho *et al.,* 1985; Kallin *et al.,* 1985; Kiehl and Dorsky, 1991, 1995; Li *et al.,* 1987; Pearson *et al.,* 1983; Tsurumi, 1993a and b). We have recently reported that in addition to its role as the DNA polymerase accessory protein, BMRF1 transactivates the early BHLF1 promoter, while not affecting the BHRF1 promoter (Zhang *et al.,* 1996). Although the BMRF1 gene product alone induces significant BHLF1 activation, the combination of the BZLF1 and BMRF1 gene products is required for maximal activation (Zhang *et al.,* 1996). Given the overlap between the BHLF1 promoter elements and the essential domains of oriLyt, control of BHLF1 transcription could potentially play a pivotal role in oriLyt replication.

In this study, we have mapped the domain in the BHLF1 promoter required for BMRF1-responsiveness. We demonstrate that BMRF1-induced activation of BHLF1 is mediated through the essential downstream component of oriLyt. The downstream component is sufficient for transferring BMRF1- responsiveness to a heterologous promoter. Although the downstream component also confers Sp1-responsiveness, a mutation which significantly reduces BMRF1-responsiveness, without affecting Sp1-responsiveness, has been identified. This mutation (located between −588 and −592 relative to the BHLF1 initiation site) also abolishes oriLyt replication. Our results suggest that BMRF1-induced transcriptional activation of the downstream component of oriLyt may be required for lytic EBV replication.

MATERIALS AND METHODS

Cell lines

HeLa is a cervical epithelial cell line. The D98/HE-R-1 cell line was formed by fusion of a HeLa subclone (D98) with the EBV-positive Burkitt's lymphoma, P3HR-1 (Glaser and O'Neill, 1972). HeLa and D98/HE-R-1 cells were grown in Dulbecco modified Eagle medium H with 10% fetal calf serum. DG75, an EBV-negative Burkitt's lymphoma B-cell line, was maintained in RPMI 1640 medium with 10% fetal calf serum. Schneider line 2 (SL2) is a Drosophila embryo cell line (Schneider, 1972) and was grown in Drosophila Schneider media obtained from GIBCO-BRL with 10% fetal calf serum.

Plasmids

The oriLyt∆Kpn plasmid (used in plasmid replication assays) was made by ligating the EBV *Sst*II–*Hin*cII fragment (EBV sequences 52,623 to 53,819) (Baer *et al.,* 1984) into the *Hin*cII site of the pBS-CAT plasmid (a gift from Frank Funari). The control oriLyt plasmid contains the same *Sst*II–*Hin*cII fragment inserted into the pBS vector (Stratagene). The nonessential *Kpn*I–*Kpn*I fragment of oriLyt (EBV sequences 52,944 to 53,207) was subsequently deleted from both oriLyt plasmids. The BHLF1-CAT plasmid contains the *Nsi*I–*Sac*II DNA fragment of EBV (sequences 52,623 to 53,581; −789 to +165 relative to the RNA start site of the BHLF1 promoter) linked to the chloramphenicol acetyltransferase (CAT) gene in the pBS vector (Stratagene). The *Kpn*I–*Kpn*I fragment from 52,944 to 53,207 has been deleted from BHLF1-CAT. A series of deletions in the BHLF1-CAT plasmid were constructed by cutting at convenient restriction enzyme sites and religating (Fig. 3A). These constructs are named to reflect the nucleotides present in each plasmid (relative to the BHLF1 initiation site). A series of additional BHLF1 promoter constructs were made using the polymerase chain reaction (PCR) method to clone portions of the BHLF1 promoter into the pBS-CAT vector as shown in Fig. 3B. This series of constructs, which each contain the same 3′ end and progressively longer 5′ ends, is named to reflect the BHLF1 promoter sequences present in each construct. A series of BHLF1 promoter fragments amplified by PCR were also cloned into the *Pst*I and *Xba*I sites upstream of the E1b promoter in the E1b-CAT

construct (a gift from Michael Green) as shown in Fig. 4. The E1b-CAT construct contains the minimal adenovirus E1b promoter linked to the CAT gene (Lillie and Green, 1989). Site-directed mutants of BHLF1 and oriLyt constructs were made using a Bio-Rad Muta Gene phagemid *in vitro* mutagenesis kit as described by the manufacturer. The BHRF1-CAT construct contains the early EBV BHRF1 promoter sequences (from −972 to +46 relative to the RNA start site) linked to the CAT gene in the pBS-CAT vector. The BMRF1 expression plasmid has been previously described (Zhang *et al.,* 1996) and contains the 1341 base pair *Bcl*I–*Bgl*II fragment of the EBV *Bam*HI M fragment subcloned in the *Bam*HI and *Bgl*II sites of the pSG5 vector (Stratagene), under the control of the simian virus 40 (SV40) early promoter. The BMRF1 *in vitro* translation plasmid contains the identical *Bcl*I–*Bgl*II fragment inserted into the pBS vector (Stratagene). The construction of the glutathione *S*-transferase (GST)–BMRF1 fusion protein has been previously described (Zhang *et al.,* 1996). The GST–BMRF1 protein contains the first 303 residues of BMRF1 cloned in-frame in the pGEX-3X vector, downstream of the GST protein. The pPacSp1 plasmid contains a 2.1-kb fragment encoding the C-terminal 686 amino acids of Sp1 protein driven by the Drosophila actin promoter (Courey and Tjian, 1988). The pA10CAT construct contains the simian virus 40 early promoter (but not enhancer) driving the CAT gene (Laimins *et al.,* 1984).

DNA transfections

Plasmid DNA was purified using a QIAGEN Maxi kit as specified by the manufacturer. DNA was transfected by electroporation, using 10 μg of DNA and 10^7 cells per condition. Cells were shocked at 1500 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 as described previously (DiNocera and Dawid, 1983). Before transfection the cells were replated onto 10-mm petri dishes and allowed to grow for 20 to 24 hr.

CAT assays

Cell extracts were prepared 48 hr after transfection and incubated at 37° with [^{14}C] chloramphenicol in the presence of acetyl coenzyme A as described previously (Gorman *et al.,* 1982). The percentage of acetylation of chloramphenicol was quantitated by thin-layer chromatography followed by Phosphorimager scanning (Molecular Dynamics).

OriLyt plasmid replication assays

OriLyt plasmid replication assays were performed as previously described (Fixman *et al.,* 1992; Hammerschmidt and Sugden, 1988). The wild-type or mutant oriLytΔKpn plasmids (containing the wild-type or mutated oriLyt sequence in pBS-CAT) were transfected into D98/HE-R-1 cells with either pHD1013 vector DNA or the BZLF1 expression plasmid and a control oriLyt plasmid (containing the minimal oriLyt in pBS). Total cellular DNA was harvested 3 days after transfection, cut with the *Bam*HI and *Dpn*I restriction enzymes, separated on an agarose gel, and analyzed by the Southern blot technique using a ^{32}P-labeled single-stranded RNA probe spanning oriLyt. The efficiency of replication was determined by quantitating the amount of the replicated (*Dpn*-resistant) band in each condition, with the control oriLyt plasmid serving as a control for transfection and replication efficiency.

Electromobility shift assays

Electromobility shift assays (EMSA) were performed as previously described (Garner and Revzin, 1981). DG75 cell nuclear extracts were prepared by washing the cells in phosphate-buffered saline, resuspending the cell pellet in 5 vol of CE buffer (10 m*M* Hepes (pH 7.6), 60 m*M* KCl, 1 m*M* EDTA, 0.075% NP-40, 1 m*M* dithiothreitol, and 1 m*M* phenylmethylsulfonyl fluoride), incubating on ice 3 min, spinning at 1200 rpm for 4 min, removing the supernatant, washing the pellet with CE buffer lacking NP-40, and then lysing the nuclear pellet with NE buffer (20 m*M* Tris (pH 8.0), 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* phenylmethylsulfonyl fluoride, and 25% glycerol). Equal amounts of protein supernatant were incubated with 10,000 cpm of a ^{32}P end-labeled double-stranded oligonucleotide probe spanning a large portion of the downstream component of oriLyt (5′-AAGCTTGGAACCCTATAGTGTAATCCCTCCCCCCCCTACCCCCCCCTCCCT-3′) or a mutant oligonucleotide probe (5′-AAGCTTGGAACCCTATAGTGTAATCCCTCCCCCCCGAATTCCCCCCTCCCT-3′) which contains the same sequence as above except for a mutation of CTACC into GAATT. Binding reactions were incubated at room temperature for 15 min in a buffer consisting of 50 m*M* Tris (pH 7.9), 250 m*M* NaCl, 2.5 m*M* EDTA, 5 m*M* dithiothreitol, 5 m*M* MgCl2, and 50% glycerol. Salmon sperm DNA was added as a nonspecific competitor DNA. After adding the probe, the reaction was incubated 15 min at room temperature and then incubated for an additional 45 min at room temperature with 1 μl of antibody. The reaction was loaded onto a 4% polyacrylamide gel and run in 0.5× Tris glycine buffer (0.025 *M* Tris and 0.19 *M* glycine). The gel was dried and subjected to autoradiography. In some experiments, 1.5 μg of purified bacterial Glutathione *S*-transferase (GST) protein, or 1.5 μg of purified GST–BMRF1 fusion protein, were added to the DG75 cell extracts. GST and GST–BMRF1 fusion proteins were constructed and purified by affinity chromatography as previously described (Zhang *et al.,* 1996).

BMRF1 protein used in EMSA assays was produced using a pAc-BMRF1 baculovirus, containing the intact BMRF1 open reading frame under the control of the baculovirus PXIV promoter (Wang *et al.,* 1991). Three days after infection, Sf9 cells were washed in PBS, resuspended in lysis buffer containing 50 m*M* Tris (pH 8.0), 40% glycerol, 1 m*M* EDTA, 1 m*M* DTT, and 1 m*M* PMSF, and sonicated briefly. The supernatant was then purified by a two-step column procedure (a P-11 phosphocellulose column, followed by a dsDNA cellulose column). The BMRF1 peak fractions (eluted in 200–350 m*M* NaCl) were identified by Western blot analysis and pooled.

DNase I foot printing assays

Binding reactions consisted of 50,000 cpm of ^{32}P-end-labeled probes spanning the oriLyt sequence and 1 μl (1 footprint unit) of purified Sp1 protein (Promega) incubated in a reaction buffer containing 50 m*M* Tris (pH 7.9), 250 m*M* NaCl, 2.5 m*M* EDTA, 5 m*M* $MgCl_2$, and 50% glycerol, incubated for 15 min at room temperature. DNase I (0.1 unit) (Promega) was added to the reaction mixture for 60 sec, and then the reaction was terminated by adding the stop buffer (100 m*M* NaCl, 100 m*M* Tris (pH 8.0), 0.2 m*M* EDTA, 1% SDS, 50 μg/ml glycogen, and 50 μg/ml proteinase). The probe was precipitated by ethanol, dried, and equal amounts of counts were loaded on a 6% polyacrylamide gel containing 7 *M* urea and subjected to electrophoresis, followed by autoradiography.

RESULTS

Response of the oriLyt promoters, BHLF1 and BHRF1, to the BMRF1 and Sp1 transactivators

We recently reported that the BMRF1 gene product activates the BHLF1 promoter in oriLyt (Zhang *et al.,* 1996). Oguro *et al.* previously reported that BMRF1 activates the SV40 early promoter (Oguro *et al.,* 1987), although two other groups did not observe BMRF1 transactivator function (Chen *et al.,* 1995; Wong and Levine, 1986). The SV40 early promoter contains a series of Sp1 binding sites (Briggs *et al.,* 1986; Dynan and Tjian, 1983; Kadonaga *et al.,* 1987). The essential downstream component of oriLyt, located several hundred basepairs upstream of both the BHLF1 and BHRF1 mRNA start sites, also contains Sp1 binding sites (Gruffat *et al.,* 1995), although the functional significance of these Sp1 binding sites for BHLF1 versus BHRF1 transcription has not been reported. The presence of Sp1 binding sites in both of the known BMRF1-responsive promoters suggests that BMRF1-induced activation may be mediated through proteins which bind to the Sp1 motif, such as Sp1 or Sp3.

The effect of the BMRF1 and Sp1 transcription factors on BHLF1 versus BHRF1 promoter activity was compared in transient cotransfection assays (Fig. 1). In the epithelial HeLa cell line, the BMRF1 gene product activates the BHLF1 promoter, but not the BHRF1 promoter, as previously described (Zhang *et al.,* 1996) (Fig. 1A). As previously reported (Oguro *et al.,* 1987), the pA10CAT construct, containing the SV40 early promoter, is also activated by the BMRF1 gene product. In contrast, cotransfection studies done in Drosophila SL2 cells (which have no endogenous Sp1 activity (Courey and Tjian, 1988)) demonstrated that Sp1 activates the BHRF1 promoter, but not the BHLF1 promoter (Fig. 1B). We have previously shown that the pA10CAT construct, as expected, is also activated by Sp1 (Zalani *et al.,* 1992). The differential response of the BHRF1 and BHLF1 promoters to the Sp1 versus BMRF1 transactivators indicates that the BMRF1 effect on oriLyt transcription can be separated from Sp1-responsiveness.

Mapping Sp1 binding sites in oriLyt

To further determine if BMRF1-induced transactivation of the BHLF1 promoter is mediated through Sp1, we mapped the Sp1 binding sites in oriLyt. DNase I footprint analysis was performed with purified Sp1 protein (Promega), using a series of probes spanning the minimal oriLyt. As shown in Fig. 2, a probe spanning the BHLF1 promoter sequences from −152 to +201 (relative to the mRNA start site) identified three Sp1 sites located downstream of the BHLF1 mRNA initiation site (+20 to +44, +65 to +78, and +130 to +148). In addition, two large Sp1-protected regions covering nearly all of the essential downstream component of oriLyt (−565 to −605 and −608 to −630 relative to the BHLF1 mRNA start site) were mapped (Fig. 2). The downstream component of oriLyt, although extremely GC rich, does not contain the consensus Sp1 binding motif, GGGCGG.

Mapping the BHLF1 promoter domain(s) required for BMRF1 transactivation

To map the region(s) in the BHLF1 promoter required for BMRF1-induced transactivation, a series of BHLF1-CAT deletions were constructed as shown in Figs. 3A and 3B. The various BHLF1-CAT constructs were cotransfected into HeLa cells, or DG75 cells (an EBV-negative Burkitt lymphoma line), with the SG5 control vector or the BMRF1 expression vector. The parent BHLF1-CAT construct contains the promoter sequences from −789 to +165 (with the internal *Kpn*I–*Kpn*I fragment, from −157 to −420, deleted). Preliminary mapping results (Fig. 3A) indicated that a deletion construct (−644/+23) which removes BHLF1 promoter sequences upstream of −644 and downstream of +23 is still efficiently activated by BMRF1. However, a BHLF1 promoter construct containing sequences from −142 to +165 is completely unresponsive to the BMRF1 transactivator. These results suggested that sequences between −420 and −644, or

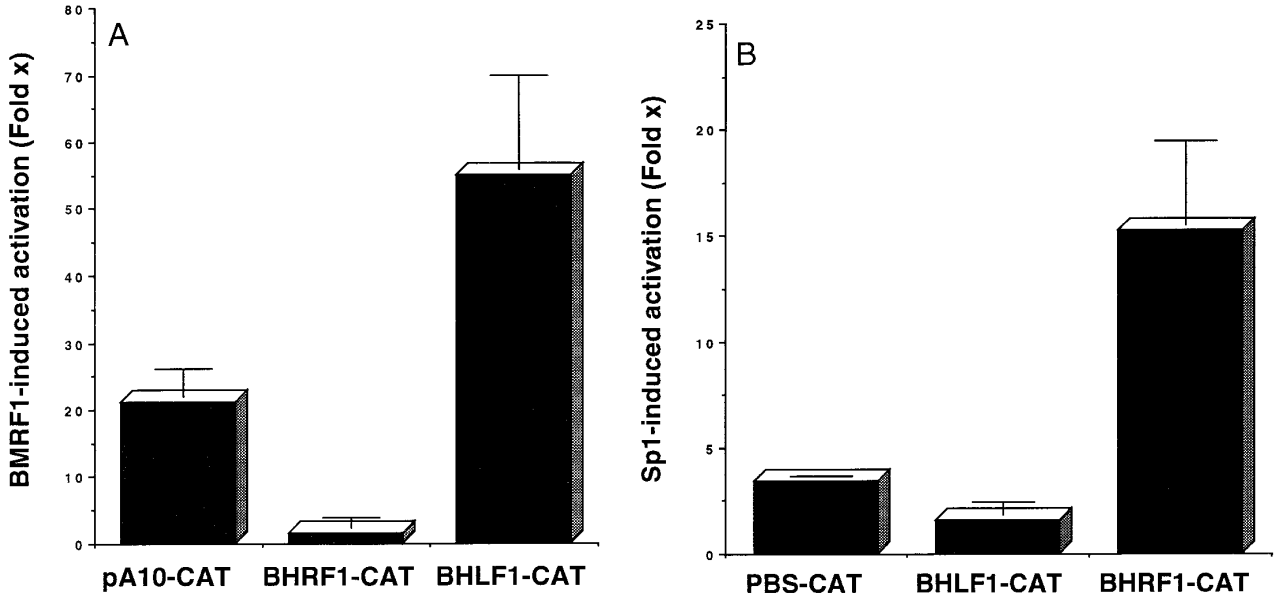

FIG. 1. Transactivation of the BHRF1 and BHLF1 promoters by BMRF1 and Sp1. (A) 5 μg of the BHLF1-CAT reporter plasmid (containing the early EBV BHLF1 promoter), the BHRF1-CAT reporter plasmid (containing the early EBV BHRF1 promoter), or the pA10CAT plasmid (driven by the SV40 early promoter) were transfected into HeLa cells with either 5 μg of pSG5 vector DNA or 5 μg of the BMRF1 expression vector. Two days after transfection, the percentage of acetylation of ^{14}C-labeled chloramphenicol in each condition was measured as previously described (Gorman *et al.,* 1982). (B) 3 μg of the negative control plasmid, pBS-CAT (containing the CAT gene with no promoter), the BHLF1-CAT reporter plasmid, or the BHRF1-CAT reporter plasmid were transfected into Drosophila SL2 cells with either 1 μg of the pPac-vector or 1 μg of the Sp1 expression vector. Two days after transfection, the percentage of acetylation of ^{14}C-labeled chloramphenicol in each condition was quantitated.

from −142 to −157, are required for BMRF1-responsiveness.

To more precisely map the BMRF1-response element, a series of BHLF1 promoter plasmids were constructed as shown in Fig. 3B. These plasmids each have the same 3′ end (+45 relative to the BHLF1 mRNA start site) but contain progressively shorter 5′ ends. The parent construct in this series, BHLF1-CAT −655/+45, is efficiently activated by BMRF1 in either HeLa or DG75 cells. The promoterless vector plasmid, pBS-CAT, is not activated by BMRF1 in either HeLa or DG75 cells (data not shown). Deletion of the sequences between −605 and −655 essentially abolished BMRF1-response in HeLa cells, while having a lesser effect in DG75 cells. Further removal of the sequences between −576 and −605 abolished BMRF1 response in DG75 cells (Fig. 3b). A weaker BMRF1-response element was also observed between −463 and −518 in both cell types. However, this second BMRF1-response element did not appear to be functional when sequences between −518 and −576 were included in the construct, suggesting that a negatively regulating element resides between −518 and −576.

These results suggest that in both HeLa and DG75 cells, BHLF1 promoter sequences between −576 and −655 confer BMRF1-responsiveness. Interestingly, this region of the BHLF1 promoter overlaps the essential downstream component of oriLyt and contains Sp1 binding sites (Fig. 2). To determine if the downstream component of oriLyt is sufficient to transfer BMRF1-responsiveness to a heterologous promoter, a series of plasmids were constructed as shown in Fig. 4. In this series of plasmids, BHLF1 promoter sequences (containing various portions of the essential downstream component of oriLyt) were inserted upstream of the minimal adenovirus E1b promoter in the E1b-CAT vector. The parent construct, E1b-CAT (−655/−558) contains the BHLF1 promoter sequences from −588 to −655 inserted upstream of the E1b promoter in the same orientation as the sequences would normally be relative to the BHLF1 promoter. Additional plasmids were constructed which remove progressively larger 3′ ends of the BHLF1 insert, as shown. In addition, the construct E1b-CAT (−558/−655) contains the BHLF1 promoter sequences from −558 to −655 placed upstream of the E1b promoter in the opposite (BHRF1) orientation. Finally, a site-directed mutation in the E1b-CAT (−655/−558) construct, altering sequences between −588 and −592, was constructed. This region of the BHLF1 promoter has been previously shown by Hammerschmidt's group to be essential for oriLyt replication (Gruffat *et al.,* 1995; Schepers *et al.,* 1993).

The ability of each of the above constructs to respond to the Sp1 versus BMRF1 transactivators was tested in cotransfection assays in SL2 and HeLa cells. As shown in Fig. 4, the BHLF1 promoter sequences from −558 to −655 transferred both Sp1- and BMRF1-responsiveness to the E1b promoter, regardless of orientation. Thus, sequences outside of −558 to −655 are required to inhibit

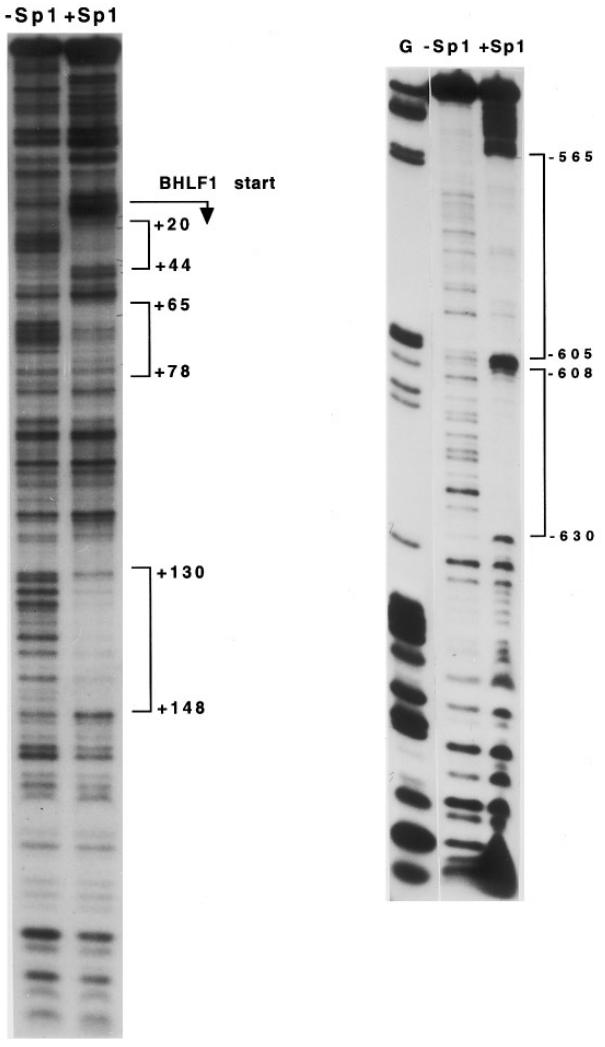

FIG. 2. Mapping Sp1 binding sites in oriLyt. A series of radioactively end-labeled probes spanning oriLyt were digested with DNase I in the presence (+Sp1) or absence (−Sp1) of purified Sp1. A G ladder ("G") was used to define the sequences protected for each probe. The Sp1-protected sequences are indicated in brackets, labeled relative to the position of the BHLF1 initiation site.

Sp1-responsiveness in the intact BHLF1 promoter and BMRF1-responsiveness in the intact BHRF1 promoter.

Removal of the sequences from −558 to −606 reduced BMRF1-responsiveness in HeLa cells about 7-fold. A site-directed mutation between sequences −588 and −592 (in the context of the intact −558 to −655 insert) reduced BMRF1-responsiveness 4-fold. However, this same mutation did not affect Sp1-responsiveness. These results suggest that the sequences between −588 and −592 are required for efficient BMRF1-response, but dispensable for Sp1 response. In addition, the region from −606 to −655 (which contains one or more Sp1 binding sites) is also clearly required for maximal BMRF1-responsiveness in HeLa cells, as shown in Fig. 3B. The sequences from −606 to −655 are sufficient to confer low-level (5-fold) BMRF1-responsiveness in HeLa cells, and a higher level (21-fold) of Sp1-responsiveness. Thus, the BMRF1-response element in oriLyt clearly overlaps, but is distinct from, the Sp1-response element.

BMRF1 does not bind specifically to the downstream component of oriLyt

BMRF1-induced transactivation of BHLF1 may be mediated through specific binding of BMRF1 to the downstream component, since BMRF1 possesses strong nonspecific DNA binding activity (Chen *et al.,* 1995; Kiehl and Dorsky, 1995; Tsurumi, 1993), although BMRF1 is not known to recognize a specific DNA binding sequence. To determine if BMRF1 can specifically bind to the downstream component of oriLyt, electromobility gel shift assays were performed using BMRF1 protein derived from a variety of sources. *In vitro*-translated full-length BMRF1 protein, or a GST–BMRF1 fusion protein containing the amino-terminal 303 amino acids of BMRF1, bound to all probes tested with equal affinity, including a probe encompassing the downstream component of oriLyt (data not shown). DNase I footprinting experiments performed with the GST versus GST–BMRF1 proteins and a probe containing the essential downstream component of oriLyt likewise did not show specific sites of BMRF1 binding (data not shown). Partially purified, baculovirus-derived full-length BMRF1 protein (Fig. 5A) did not bind more efficiently to a probe containing the wild-type downstream component sequences than to a probe containing a mutant downstream component sequence (altered from −588 to −592) which has much reduced BMRF1-responsiveness (Fig. 4B). Thus, BMRF1-induced activation of the BHLF1 promoter was not associated with specific BMRF1 binding to the BMRF1-response element in these assays.

Binding of cellular transcription factors to the oriLyt essential downstream component

To examine the binding of cellular transcription factors to the essential downstream component of oriLyt, nuclear extracts were prepared from DG75 cells and EMSA s were performed using wild-type and mutant (altered from −588 to −592) probes spanning the downstream component. As shown in Figs. 5B and 5C, the wild-type and mutant probes were both bound by two complexes (designated "Sp3") which were specifically competed by cold DNA containing the Sp1 binding motif and blocked by Sp3 antibody. These Sp3-like complexes likely represent binding by different versions of the Sp3 protein (Kennett *et al.,* submitted). The wild-type and mutant probes were also both bound by another complex (designated "A"), whose identity remains unknown. Interestingly, the wild-type, but not the mutant, probe bound to a third complex (designated "wt"). The "wt" complex was specifically competed by cold competitor DNA containing the wild-type downstream component oriLyt sequence but not com-

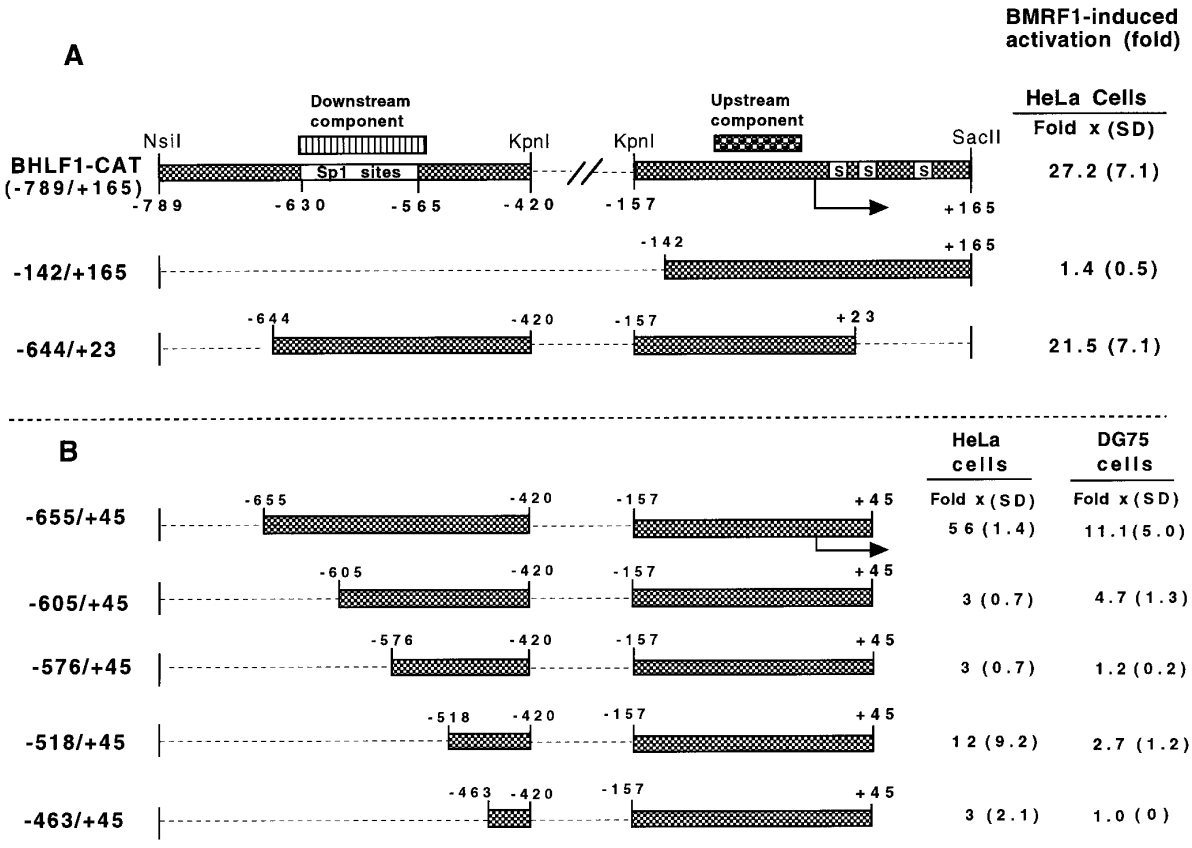

FIG. 3. Mapping the region of the BHLF1 promoter required for BMRF1-induced activation. (A) Deletion constructs of BHLF1-CAT were constructed as shown by cutting at convenient restriction enzyme sites and religating. In the map at the top of the figure, the position of the two essential domains of oriLyt (the upstream and downstream components) is shown, as well as the Sp1 sites (indicated by "S"). The constructs were cotransfected into HeLa cells with the SG5 or BMRF1 expression vectors, and the amount of CAT enzyme activity was calculated. The fold activation (and standard deviation) induced by BMRF1 for each CAT construct (relative to the SG5 vector) is shown. (B) A series of 5′ BHLF1-CAT deletion constructs were constructed by PCR amplification, using the same 3′ primer for each construct, and a series of different 5′ primers. The amplified fragments were inserted into the pBS-CAT vector upstream of the CAT gene. The various constructs were cotransfected with the SG5 and BMRF1 expression vectors into HeLa and DG75 cells (an EBV-negative Burkitt lymphoma line). 48 hr after transfection, CAT activity was determined and the fold activation (and standard deviation) induced by BMRF1 in each cell type is shown.

peted by competitor DNA containing the identical sequences except for the mutation between −588 and −592 (data not shown). Although the exact identity of the protein(s) forming the "wt" complex remains unknown, the fact that this complex binds BHLF1 promoter sequences required for efficient BMRF1-responsiveness suggests that it possibly plays a role in mediating BMRF1 transactivation.

Gruffat *et al.* (1995) have previously reported that binding of cellular proteins to the downstream oriLyt component is similar using nuclear extracts derived from latently infected cells (which would lack BMRF1 protein) and lytically EBV-infected cells (which would contain BMRF1 protein). We likewise observed similar levels of cellular protein binding to the downstream oriLyt component using extracts derived from latently versus lytically EBV-infected cells, or DG75 cells transfected with the pHD1013 expression vector versus the BMRF1 expression vector (data not shown). Thus, we did not find that the presence or absence of BMRF1 *in vivo* alters the binding of particular cellular proteins to oriLyt. Interestingly, in the presence of exogenously added GST–BMRF1 protein (but not GST protein alone), Sp3 binding (derived from DG75 cell extracts) to the downstream oriLyt component was decreased, whereas the binding of the "wt" and "A" complexes was not significantly affected (Fig. 5d). However, since a similar decrease in Sp3 binding was not observed in the lytically infected cell extracts, it remains unclear whether the GST–BMRF1 effect on Sp3 binding observed *in vitro* is actually relevant to the *in vivo* BMRF1 transactivator effect.

Mutation of the BHLF1 sequences from −592 to −588 abolishes oriLyt plasmid replication

The previous mapping studies clearly suggest that the BHLF1 promoter sequences from −588 to −592 are important for maximal BMRF1-responsiveness, with transactivation being reduced fourfold when these sequences are altered (Fig. 4). To determine if the mutation altering

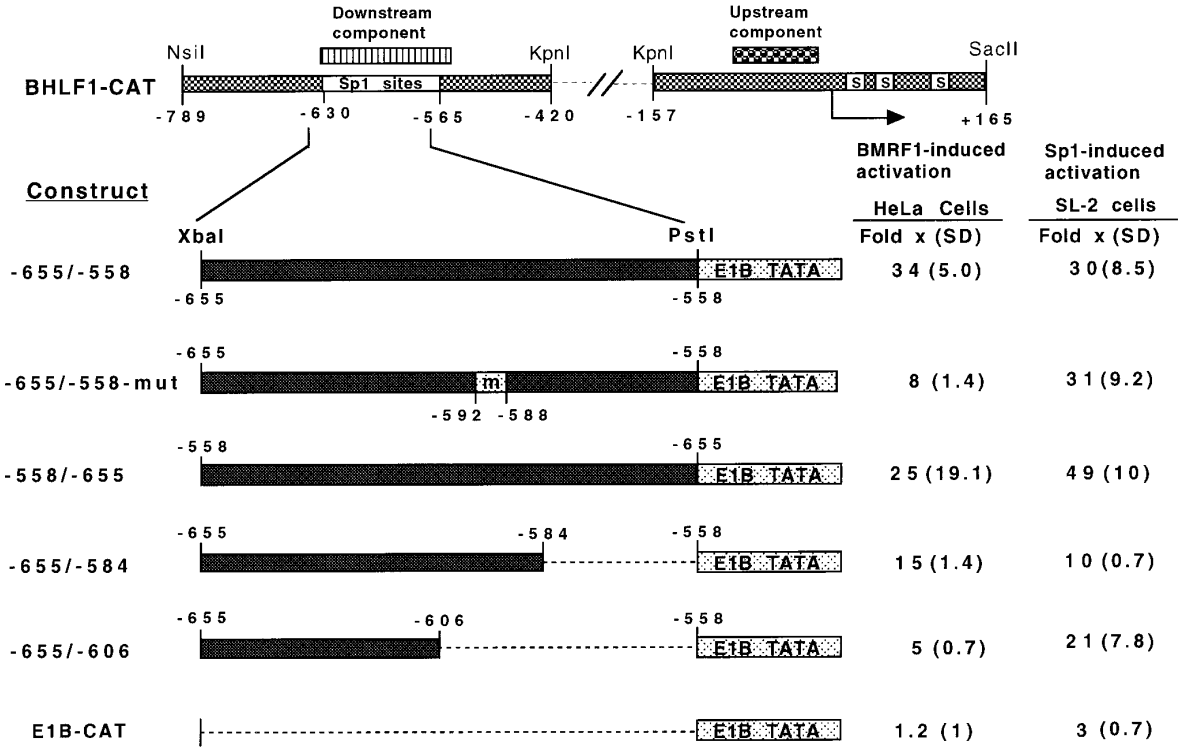

FIG. 4. Transferring BMRF1- and Sp1-response to a heterologous promoter. Various BHLF1 promoter sequences encompassing different portions of the essential downstream component of oriLyt were amplified by PCR as shown and inserted upstream of the adenovirus minimal E1b promoter in the E1b-CAT vector. The −655/−558-mut construct is identical to the −655/−558 construct, except for a site-directed mutation altering sequences −588 to −592 (as shown in Fig. 6). The various constructs were cotransfected into HeLa cells with the SG5 or BMRF1 expression vectors and into SL2 cells with the pPac- and Sp1 expression vectors. The fold-activation (relative to the control vector) induced by BMRF1 and Sp1 is shown.

the BHLF1 sequences from −588 to −592 also affects oriLyt replication, we inserted this mutation into the oriLytΔKpn plasmid as shown in Fig. 6 and examined the effect in plasmid replication assays. Two additional oriLytΔKpn plasmid mutants were also constructed, one which alters the BHLF1 downstream Sp1 sites from +21 to +44, and another which alters the downstream component sequences from −570 to −574.

The wild-type and mutant oriLytΔKpn plasmids were transfected into D98/HE-R-1 cells with or without a BZLF1 expression vector and plasmid replication was quantitated as previously described (Zhang *et al.,* 1996). DNA was harvested 3 days after transfection, cut with the *Dpn*I and Bam HI restriction enzymes, run on an agarose gel, transferred to a nitrocellulose blot, and probed with a radioactive probe specific for oriLyt sequences. The replicated plasmid is resistant to Dpn 1 cutting. A control construct, containing oriLyt inserted into the pBS vector, was also included in each transfection to serve as a control for transfection and replication efficiency.

As shown in Fig. 6, mutation of sequences from −574 to −570 (within the downstream component), or from +21 to +44, did not affect oriLyt replication. However, mutation of BHLF1 promoter sequences from −588 to −592 abolished oriLyt replication in two separate experiments. The replication of the control plasmid was similar in each condition. Hammerschmidt's group has observed similar results constructing mutations which overlap the −588 to −592 and −570 to −574 sequences (Gruffat *et al.,* 1995). These results indicate that BHLF1 promoter sequences from −588 to −592 are not only important for BMRF1-induced transactivation, but are also essential for oriLyt replication.

DISCUSSION

The EBV BMRF1 gene product is the viral DNA polymerase accessory protein and in this capacity plays an essential role in lytic replication (Chen *et al.,* 1995; Fixman *et al.,* 1992; Kiehl and Dorsky, 1991, 1995; Li *et al.,* 1987; Tsurumi, 1993). We have recently reported that the BMRF1 gene product transcriptionally activates the oriLyt promoter, BHLF1 (Zhang *et al.,* 1996). In this report, we have further explored the mechanism of BMRF1 transactivation by precisely mapping the BMRF1-response element in oriLyt. We show that one of the two essential domains of oriLyt, the downstream component, is required and sufficient for BMRF1 transactivation. A small mutation in the downstream component which inhibits BMRF1-induced transactivation also abolishes oriLyt replication. Our results suggest that transcriptional activation of the downstream component of oriLyt by BMRF1 may play an important role in lytic EBV replication.

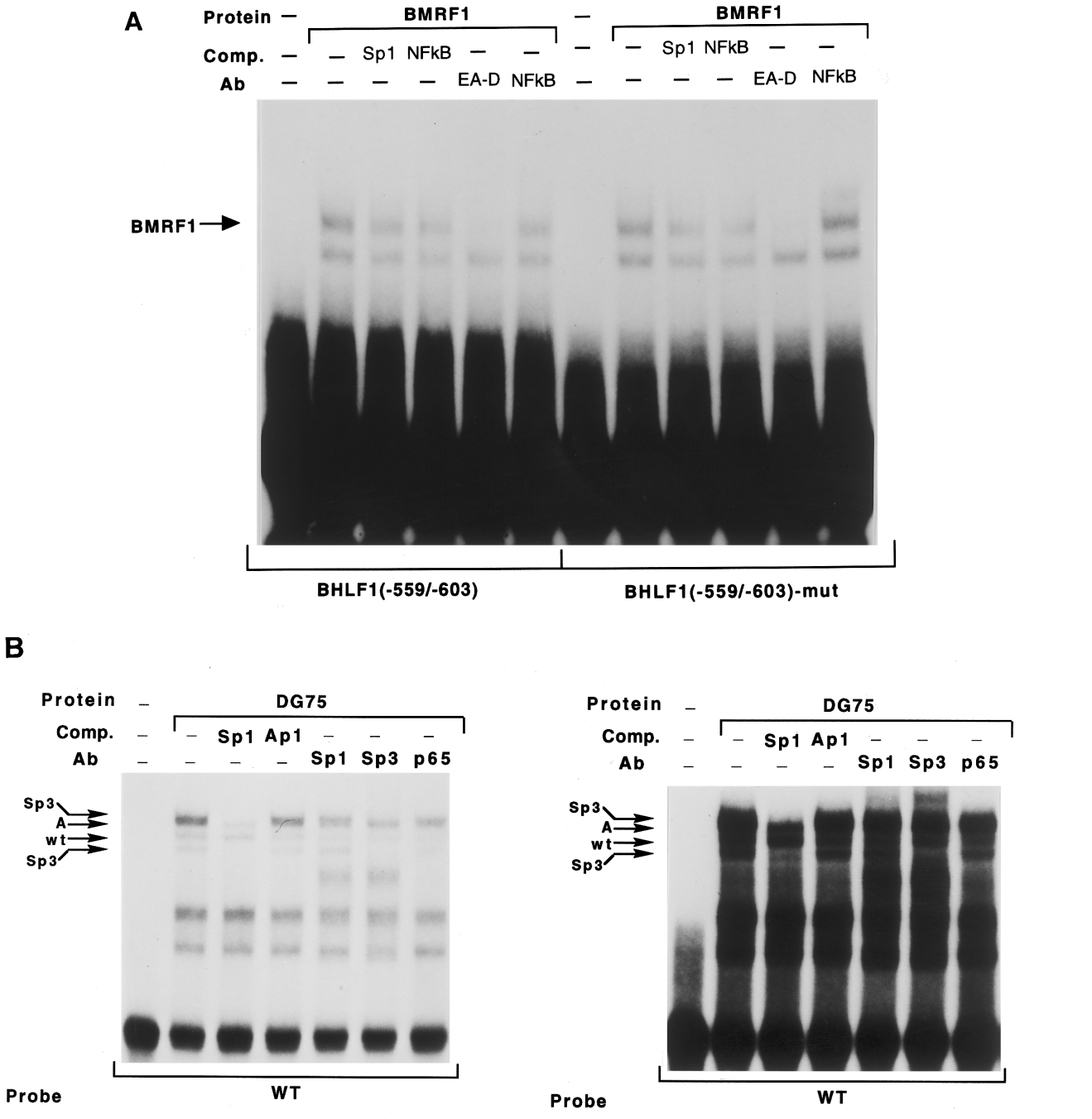

FIG. 5. Binding of BMRF1 and cellular proteins to the essential downstream component of oriLyt. (A) EMSA was performed using full-length BMRF1 protein expressed from a baculovirus vector in Sf9 cells and partially purified as described under Materials and Methods. The BMRF1 protein was incubated with either a probe spanning the wild-type oriLyt downstream component sequence ("BHLF1(−559/−603)" or a probe in which the downstream component sequences (from −582 to −592 relative to the BHLF1 start site) had been mutated as shown in Fig. 6 ("BHLF1 (−559/−603)-mut"). Various competitor DNA's ("Comp") containing the Sp1 or NF-KB consensus binding motifs, or various antibodies ("Ab"), directed against the NF-KB or BMRF1 proteins, were added to the binding reactions as indicated. Both probes bound BMRF1 with similar efficiency. The BMRF1 complex was specifically blocked by a monoclonal antibody ("E-AD," Capricorn) recognizing the BMRF1 protein, but not by an antibody directed against the NF-KB transcription factor. (B and C) DG75 cells nuclear extracts were prepared and tested for their ability to bind to the ^{32}P-end-labeled wild-type (WT) and mutant (MT) downstream component probes. Complexes were competed with cold competitor DNA containing the Sp1 or Ap1 binding motifs or incubated with rabbit serum containing antibodies directed against the Sp1 protein, the Sp3 protein, or the p65 (NF-KB) protein. The various binding complexes are indicated by arrows. The Sp3 and A complexes bind equally well to both the wild-type and mutant probes, whereas the "wt" complex binds preferentially to the WT probe. Shorter (left panel) and longer (right panel) exposures of the same gel are shown in Fig. 5B. (D) As in Figs. 5B and 5C, except that equal amounts (1.5 μg) of purified GST or BMRF1-GST proteins were added to the DG75 cell extract. The position of the GST-BMRF1 binding complex is indicated on the right of the figure.

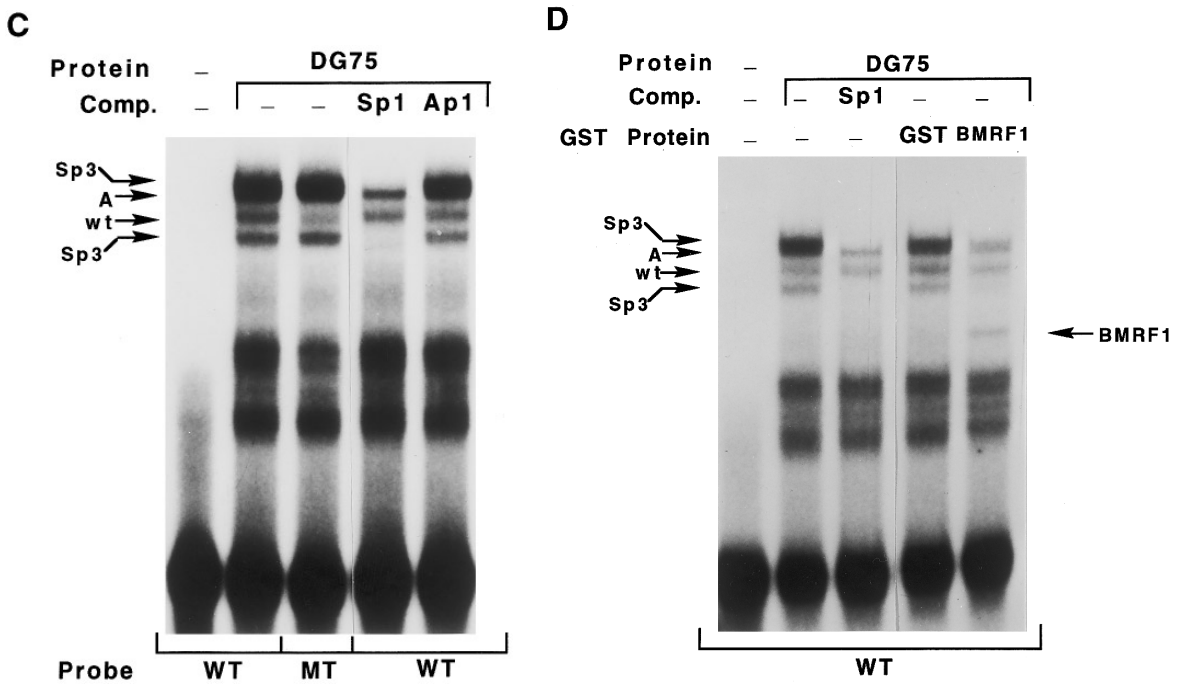

FIG. 5—*Continued*

The precise mechanism by which BMRF1 transcriptionally activates oriLyt remains unknown. Although our data clearly indicate that the BMRF1 versus Sp1 effects on oriLyt transcription can be separated, several lines of evidence suggest that BMRF1 may nevertheless modulate Sp1 function, or the function of other closely related members in the Sp1 transcription family. First, both the SV40 early promoter and the BHLF1 promoter contain Sp1 sites, and both are responsive to the BMRF1 transactivator. Second, the BHLF1 promoter region from −605 to −655 is bound by the Sp1 transcription factor (Fig. 2) and is also clearly required for BMRF1 transactivation (Fig. 3B). Third, the BHLF1 promoter region from −606 to −655 is sufficient to confer Sp1-responsiveness to a heterologous promoter, and likewise confers at least a low level (fivefold) of BMRF1-responsiveness to a heterologous promoter (Fig. 4). Sp1 transcriptional activity is regulated by several other virally encoded proteins, including the papilloma virus E2 protein (Li *et al.,* 1987) and the HIV TAT protein (Kamine *et al.,* 1991), and Sp1 augments replication of the SV40 and adenoviruses (Guo and DePamphilis, 1992; Hatfield and Hearing, 1993).

However, our data suggest that even if BMRF1 does act by modulating Sp1 (or Sp3) function, Sp1 sites alone are not sufficient for BMRF1-induced transactivation. Transfer of maximal BMRF1-responsiveness to a heterologous promoter requires additional sequences in the downstream component of oriLyt which are not required for Sp1 response. The BHLF1 promoter sequences from −584 to −606 are not important for Sp1 response and may in fact inhibit Sp1-induced activation (Fig. 4). In contrast, deletion of the −584 to −606 region reduces BMRF1 transactivation threefold. Site-directed mutation of the −588 to −592 sequence does not affect Sp1 response, yet reduces BMRF1 response by more than fourfold (Fig. 4). Finally, although the downstream component of oriLyt is located between the divergent BHLF1 and BHRF1 promoters, in the context of the intact oriLyt, only the BHLF1 promoter responds to the BMRF1 transactivator, and only the BHRF1 promoter responds to the Sp1 transactivator.

Given that our transfer experiments have not yet identified a sequence which has lost Sp1-responsiveness but which remains BMRF1-responsive (Fig. 4), our data are consistent with the hypothesis that efficient BMRF1-induced transactivation requires both an Sp1 site and an additional motif (possibly sequences spanning −588 to −592 in the BHLF1 promoter). Our binding assays suggest that in DG75 cells, at least, the downstream component of oriLyt is primarily bound by Sp3, rather than Sp1. Sp1 and Sp3 share extensive homology and bind to the same DNA sequences. Sp3 can function as either a positive or negative regulator of transcription, depending upon the site of Sp3 translational initiation (Kennett *et al.,* submitted). Purified GST–BMRF1 protein reduced Sp3 binding to the downstream oriLyt component *in vitro* (Fig. 5d). If Sp3 binding functions as a negative regulator of BHLF1 transcription *in vivo,* then BMRF1 could potentially activate BHLF1 transcription by reducing Sp3 binding. However, since we were unable to demonstrate reduced Sp3 binding to the downstream component in lytically versus latently infected cells, the observed *in vitro* effect

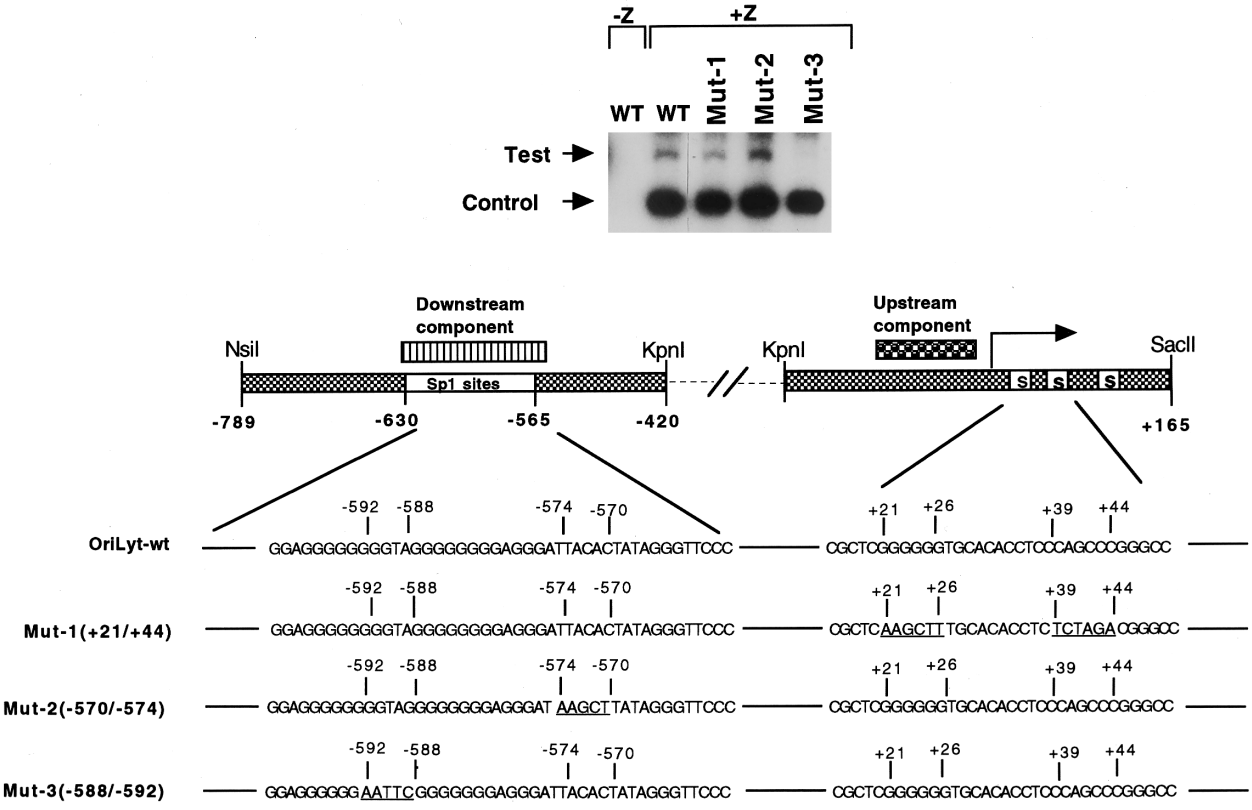

FIG. 6. Mutation of the BHLF1 sequence from −588 to −592 abolishes oriLyt replication. Three site-directed mutations in the oriLytΔKpn plasmid were constructed as shown. Mut-1 plasmid contains two mutations within Sp1 binding sites located downstream of the BHLF1 initiation site (+12 to +26 and +39 to +44). Mut-2 and Mut-3 alter sequences within the downstream component of oriLyt. Plasmids were cotransfected into D98-HE-R-1 cells with ("+Z") or without ("−Z") the BZLF1 expression vector. A control oriLyt plasmid ("control"), containing wild-type oriLyt inserted into the pBS vector, was also included in each transfection to serve as a control for transfection and replication efficiency. Three days after transfection, the DNA was harvested, cut with the *Bam*HI and *Dpn*I restriction enzymes, separated on an agarose gel, transferred to a nitrocellulose blot, and probed with a radioactive probe spanning oriLyt. The position of the Dpn1-resistant (replicated) control and test plasmids is indicated by arrows. The wild-type "WT" mut-1 and mut-2 plasmids replicate with similar efficiency. The mut-3 plasmid (in which BHLF1 promoter sequences from −588 to −592 have been altered) had severely reduced replication capacity in two independent replication experiments.

of the GST–BMRF1 protein on Sp3 binding is not necessarily physiologically relevant. Sp1 and Sp3 activity are regulated by multiple other mechanisms, including phosphorylation and interactions with other transcription factors (Kim *et al.,* 1992), and BMRF1 could potentially affect any of these other regulatory mechanisms.

BMRF1-induced activation of BHLF1 could also be mediated through cellular transcription factors other than Sp1 or Sp3 which bind to the GC-rich downstream component. Additional cellular factors (complexes "A" and "wt" in Fig. 5C) also clearly bind to this region of oriLyt and their contribution, if any, to BMRF1-induced transactivation remains unknown. Efficient binding by the "wt" complex was found to require BHLF1 sequences from −588 to −592, which are also required for efficient BMRF1-responsiveness. Thus the "wt" complex remains an excellent candidate for mediating BMRF1-responsiveness. The −588 to −592 motif could serve as a binding site for the "wt" cellular transcription factor, and binding by both Sp1 (and/or Sp3) and this putative transcription factor might be required for BMRF1 effect. Although we cannot completely exclude the possibility that BMRF1 itself specifically binds to the downstream component, we were unable to observe this in our electromobility shift assays. More likely, BMRF1 is indirectly tethered to the downstream component by transcription factors which do bind directly to oriLyt, and this interaction is labile under our EMSA conditions.

Regardless of the precise mechanism, we did find a significant correlation between BMRF1-responsiveness and oriLyt replication. The downstream component of oriLyt, which unquestionably plays an essential role in oriLyt replication, is required, and sufficient, for BMRF1-responsiveness. A site-directed mutation in the downstream component which inhibits BMRF1 response (altering BHLF1 promoter sequences from −588 to −592) also abolishes oriLyt replication. Therefore, BMRF1-induced transcriptional activation of the oriLyt downstream component may be important during lytic EBV infection.

Interestingly, the two essential domains of oriLyt are

both transcriptionally activated by lytic EBV proteins: the upstream component by BZLF1 and the downstream component by BMRF1. The BMRF1 and BZLF1 gene products colocalize within the intranuclear replication compartments during lytic infection (Takagi *et al.,* 1991) and can physically and functionally interact (Zhang *et al.,* 1996). Assuming that BMRF1 is tethered to the downstream component by interaction with a cellular transcription factor (or direct binding), direct interaction between BMRF1 and BZLF1 (bound to the upstream component) could potentially result in looping of oriLyt. Alternatively, BMRF1-induced transcriptional activation of the oriLyt downstream component could be required for opening the chromatin during the onset of replication.

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