Viral Genome Methylation Differentially Affects the Ability of BZLF1 versus BRLF1 To Activate Epstein-Barr Virus Lytic Gene Expression and Viral Replication

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The Epstein-Barr virus (EBV) immediate-early proteins BZLF1 and BRLF1 can both induce lytic EBV reactivation when overexpressed in latently infected cells. Although EBV genome methylation is required for BZLF1-mediated activation of lytic gene expression, the effect of viral genome methylation on BRLF1-mediated viral reactivation has not been well studied. Here, we have compared the effect of viral DNA methylation on BZLF1- versus BRLF1-mediated activation of lytic EBV gene transcription and viral genome replication. We show that most early lytic viral promoters are preferentially activated by BZLF1 in the methylated form, while methylation decreases the ability of BRLF1 to activate most early lytic promoters, as well as the BRLF2 late viral promoter. Moreover, methylation of bacmid constructs containing the EBV genome enhances BZLF1-mediated, but decreases BRLF1-mediated, early lytic gene expression. Methylation of viral promoter DNA does not affect BRLF1 binding to a variety of different CpG-containing BRLF1 binding motifs (RREs) *in vitro* or *in vivo*. However, BRLF1 preferentially induces H3K9 histone acetylation of unmethylated promoters *in vivo*. The methylated and unmethylated forms of an oriLyt-containing plasmid replicate with similar efficiency when transfected into EBV-positive cells that express the essential viral replication proteins *in trans*. Most importantly, we demonstrate that lytic viral gene expression and replication can be induced by BRLF1, but not BZLF1, expression in an EBV-positive telomerase-immortalized epithelial cell line (NOKs-Akata) in which lytic viral gene promoters remain largely unmethylated. These results suggest that the unmethylated form of the EBV genome can undergo viral reactivation and replication in a BRLF1-dependent manner.

Epstein-Barr virus (EBV) is a double-stranded DNA gamma-herpesvirus that infects over 90% of the world population and causes infectious mononucleosis and oral hairy leukoplakia (1–3). Additionally, EBV is associated with several types of cancer, including nasopharyngeal carcinoma (NPC), gastric cancer, and B-cell lymphomas (2–4). The EBV genome is heavily methylated in many EBV-infected cancer cells (5). EBV-positive cancers are composed primarily of cells with the latent forms of viral infection (2, 3), in which the virus is replicated once per cell cycle by the host cell DNA polymerase and only a subset of the virally carried genes are expressed (2, 3, 6). In contrast, oral hairy leukoplakia lesions (which result from EBV infection of epithelial cells along the side of the tongue) contain the lytic form of EBV infection, in which the virus is replicated by the virally encoded DNA polymerase and infectious viral particles are produced (1–3, 7, 8).

EBV genomes produced by the lytic form of viral infection are not methylated, since the EBV-encoded DNA polymerase does not have the capacity to methylate the replicated viral genome (5, 9). Following infection of B cells, the EBV genome is initially unmethylated, but it becomes progressively methylated in cells that support the latent form of infection by host cell-encoded DNA methyltransferases (5, 10, 11). DNMT3A, a *de novo* methyltransferase which is upregulated by viral infection of germinal center B cells, may mediate methylation of the incoming EBV genome (12). EBV genome methylation begins to be detectable by methylated DNA immunoprecipitation (MeDip) approximately 2 weeks after primary infection of B cells (11).

The switch from latent to lytic infection is mediated by the EBV immediate-early (IE) proteins BZLF1 (also called Z, Zta, ZEBRA, or EB1) and BRLF1 (also called R or Rta) (2, 3). The BZLF1 and

BRLF1 proteins are transcription factors that cooperatively activate expression of the EBV lytic genes, many of which are involved in lytic replication (13–20). BZLF1 is a bZip transcription factor, homologous to c-jun and c-fos, that binds as a homodimer to the consensus AP-1 site as well as AP-1-like motifs known as BZLF1-responsive elements (ZREs) (13, 21–26). Interestingly, BZLF1 was the first transcription factor shown to preferentially activate the methylated forms of certain target promoters (27). Many early lytic EBV promoters have CpG-containing ZREs that must be methylated for efficient BZLF1 binding (10, 11, 24, 27–29). A BZLF1 mutant (S186A) that is defective for binding to methylated CpG-containing ZREs (but not the consensus AP-1 motif) cannot induce lytic reactivation (28, 30, 31), suggesting that the ability of BZLF1 to bind to methylated CpG-containing ZREs is essential for induction of lytic gene expression in cells latently infected with a methylated viral genome. However, recent evidence suggests that methylation is not uniformly required for efficient BZLF1 transactivation of all early lytic promoters (10, 24), and some highly BZLF1-responsive promoters (such as BHLF1 and BHRF1) are not thought to encode CpG-containing ZREs (10, 24, 25).

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Expression of the BRLF1 immediate-early protein (the homolog of the ORF50 gene product in Kaposi's sarcoma-associated herpesvirus [KSHV]) can also induce lytic reactivation in a subset of latently infected cell lines (20, 32). Although BRLF1 binds to and activates many of the same early lytic EBV promoters as BZLF1 (33–39), the effect of DNA methylation on BRLF1-mediated activation has not yet been examined. BRLF1 activates certain lytic promoters (for example, the BRLF1 promoter itself) through indirect mechanisms (40–43). BRLF1 also directly binds as a homodimer to BRLF1-responsive elements (RREs) contained within many early lytic viral promoters, with a consensus sequence of GNCCN₉GGNG (in which N₉ is a spacer sequence that can be any nucleotide) (33–39). Interestingly, a number of previously confirmed RREs contain CpG motifs, suggesting that DNA methylation may affect the ability of BRLF1 to bind to and/or activate lytic viral promoters.

During lytic replication, the virally encoded DNA polymerase (BALF5) replicates the viral genome via the oriLyt origin (2, 44). oriLyt contains two divergent early lytic promoters (BHLF1 and BHRF1) and has at least three RREs and seven ZREs (15, 25, 33, 36, 37, 44–46). BZLF1 binding to four ZREs located proximal to the BHLF1 promoter is essential for oriLyt replication independent of BZLF1 transcriptional function (45–47), and there is evidence that BZLF1 recruits core viral replication machinery to oriLyt (48, 49). Interestingly, a recent study showed that the highly transcribed BHLF1 transcript (which is not thought to encode a functional protein) is required in *cis* for effective lytic replication (50). However, the effect of viral genome methylation in *cis* on oriLyt replication remains uncertain. Recent studies found that infectious virions are not produced following infection of B cells until 13 days postinfection (coincident with the onset of viral genome methylation) (11) and suggested that completion of the viral lytic life cycle in B cells requires viral genome methylation (9). However, these results could be due to the inability of BZLF1 to activate expression of essential viral replication proteins (such as the virally encoded DNA polymerase) from an unmethylated viral genome in B cells, rather than an effect of methylation in *cis* on oriLyt-mediated replication.

In addition, the potential effect of viral genome methylation on late viral gene expression has not been examined. Since late genes are expressed after lytic viral DNA replication (2) and thus from an unmethylated template, DNA methylation could potentially be used by the virus as an inhibitory mechanism for restraining late gene expression prior to lytic viral DNA replication. Interestingly, BRLF1 activates a subset of late viral promoters in reporter gene assays performed with nonreplicating vectors and binds directly to at least two late gene promoters, BLRF2 and BFRF3 (31, 33, 37); however, the effect of promoter methylation on the ability of BRLF1 to activate late promoters is not known.

Here, we have compared the effect of viral genome methylation on the ability of BZLF1 versus BRLF1 to activate expression of a series of different early and late genes and have studied the mechanism(s) for the methylation effects. Consistent with previous reports, we show that most early lytic viral promoters are preferentially activated by BZLF1 in the methylated form (with some functionally important exceptions) and that methylation of the viral genome enhances BZLF1 binding to CpG-containing ZREs in early lytic promoters. In contrast, we show that DNA methylation decreases the ability of BRLF1 to activate many early lytic EBV promoters (as well as a late viral promoter), although methylation of the viral genome does not affect BRLF1 binding to CpG-containing RREs. Furthermore, we find that BRLF1 induces acetylation of histone H3K9 in the chromatin of unmethylated, but not methylated, viral promoters *in vivo*. Furthermore, we demonstrate that DNA methylation of an oriLyt-containing plasmid does not have a *cis*-acting effect on its replication efficiency when all of the essential viral replication proteins are supplied in *trans*. Most importantly, we have identified a cell line (the telomerase-immortalized normal oral keratinocyte cell line NOKs) that supports long-term viral infection with a largely unmethylated form of the EBV genome, and we demonstrate that BRLF1, but not BZLF1, expression is sufficient to induce the lytic form of viral replication in this cell line.

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MATERIALS AND METHODS

Cell lines and culture. HEK 293T, HeLa, and D98/HR-1 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (pen-strep). HONE-1 (a gift from Ron Glaser, Ohio State University) is an early-passage EBV-negative NPC cell line and was maintained in RPMI 1640 supplemented with 10% FBS and 1% pen-strep. The NOKs cell line (a gift from Karl Munger, Harvard University) is a telomerase-immortalized normal oral epithelial keratinocyte cell line that was derived as previously described (51) and was maintained in keratinocyte-SFM (Life Technologies, Inc.) supplemented with epidermal growth factor, bovine pituitary extract, and 1% pen-strep (K-SFM). Akata-GFP Burkitt lymphoma (BL) cells (a gift from Kenzo Takada [received from Bill Sugden]) were maintained in RPMI 1640 supplemented with 10% FBS, 1% pen-strep, and 500 µg/ml G418. Akata-GFP BL cells are derived from Akata BL, a type I latency Burkitt lymphoma line, that lost the endogenous EBV genome and then was superinfected with an Akata EBV containing inserted green fluorescent protein (GFP) and G418 resistance genes as previously described (52). HONE-Akata cells are derived from HONE cells stably infected with EBV produced from the Akata-GFP BL line and were maintained in RPMI 1640 supplemented with 10% FBS, 1% pen-strep, and G418 (400 µg/ml). NOKs-Akata cells were derived from the EBV-negative NOKs cell line. Briefly, 10⁵ NOKs cells were plated in a 6-well dish and cocultured with 2 × 10⁶ Akata-GFP BL cells in 2 ml of K-SFM for 24 h. Akata-GFP BL cells were removed by washing with phosphate-buffered saline (PBS), and EBV-positive NOKs cells were selected by adding 50 µg/ml of G418 to the medium starting 1 week after infection. The NOKs-Akata cell line used in these studies had been infected with EBV approximately 6 months prior to experimentation and maintained in G418 since the time of infection.

Plasmids and cloning. Plasmid DNA was prepared using the Qiagen Midi/Maxiprep kit according to the manufacturer's instructions. pSG5 was obtained from Stratagene. The SG5-BRLF1 expression vector (a gift from S. D. Hayward, Johns Hopkins University) was constructed as previously described (17) and carries the BRLF1 open reading frame under the control of the simian virus 40 (SV40) early promoter. SG5-BRLF1 aa1-550 (R550) was constructed using the Stratagene QuikChange II XL site-directed mutagenesis kit and the following primer set: BRLF1(aa1-550) forward (5'-CCCCTCGTGGCCATTGTAGGAAGTACCACAA CACTAGAGTCC-3') and reverse (5'-GGACTCTAGTGTGTGGTTCAG TTCCTACAAATGGCCACGAGGGG-3'). SG5-BZLF1 was a gift from Diane Hayward, Johns Hopkins University, and contains the BZLF1 genomic sequence under the control of the SV40 promoter (47). Flag-tagged-BZLF1 contains BZLF1 cDNA inserted into a p3XFLAG-myc-CMV24 vector (Sigma) for mammalian cell expression (a gift from Paul Lieberman, Wistar Institute). The promoterless luciferase reporter gene construct pCpGL-basic (a gift from Micheal Rehli, Universitätsklinikum Regensburg) was constructed as previously described (53) and contains no CpG motifs in the entire vector. Various EBV promoters (Table 1) were PCR amplified from the EBV B95.8 genome and cloned upstream of

TABLE 1 Function and expression kinetics of selected EBV lytic genes

Gene	Classification	Function
BALF2	Early	EBV single-stranded DNA binding protein
BARF1	Early/NPC latency	Macrophage colony-stimulating factor decoy receptor
BFLF2	Early	Envelope protein
BGLF4	Early	Protein kinase
BGLF5	Early	Alkaline exonuclease
BHLF1	Early	Most highly transcribed RNA that may have a role in replication, not known to produce a functional protein
BHRF1	Early	Bcl-2 homolog
BMLF1	Early	SM, RNA binding and export protein
BMRF1	Early	EAD, double-stranded DNA binding protein
BLRF2	Late	Virion protein
BRLF1	Immediate early	BRLF1 early gene transactivator
BRRF1	Early	Na, enhancer of lytic reactivation
BZLF1	Immediate early	BZLF1 early gene transactivator

the luciferase gene in pCpGL-basic using the SpeI and BglII restriction sites. The following promoters were cloned into pCpGL-basic (the position in the EBV genome is in parentheses): BALF2p (164776 to 165375), BARF1p (164825 to 165503), BFLF2p (56948 to 57548), BGLF4p (123619 to 124322), BGLF5p (122355 to 122966), BLRF2p (88203 to 88895), BMLF1p (84311 to 84922), BMRF1p (79317 to 79886), BRLF1p (106144 to 107250), and BRRF1p (104447 to 105161). The BHLF1p and BHRF1p-luciferase reporter gene constructs were constructed by PCR amplifying the divergent BHLF1 and BHRF1 promoter sequences (52781 to 53797) with the primer set 5'-CCCCAGATCTCGACGCTGGCGAGCCGGGC C-3' and 5'-CCCCAGATCTGTGATGAAACAGGCAACTCC-3' within the oriLyt region of EBV B95.8 genomic DNA and were inserted upstream of the luciferase gene in pCpGL-basic using the BglII restriction site.

EBV bacmid preparation. The B95.8 bacmid (p2089) contains the EBV B95.8 genome as well as the hygromycin resistance and GFP genes on an *Escherichia coli* F-factor-based plasmid as previously described (a gift from Henri-Jacques Delecluse) (54). The Akata bacmid (AK-BAC) contains the EBV Akata genome in addition to the GFP gene and chloramphenicol resistance gene, as previously described (52) (a gift from Kenzo Takada, Hokkaido University, via Clare Sample at Pennsylvania State University College of Medicine). EBV bacmid DNA was isolated from 2.5-liter bacterial cultures by alkaline lysis and purified with CsCl₂-ethidium bromide gradients (55).

In vitro methylation of reporter gene constructs and bacmid DNA. Reporter gene constructs and EBV bacmids were methylated *in vitro* using CpG methyltransferase M.SssI (NEB) according to the manufacturer's instructions. EBV bacmid DNA was methylated using the large-scale methylation protocol. After completion of the methylation reaction, the DNA was cleaned by phenol chloroform extraction and salt precipitation. Successful methylation was confirmed by enzymatic digestion with two restriction enzymes (NEB) that recognize the same cut site: HpaII (digestion is blocked by methylation) and MspI (cuts regardless of methylation status).

Transient transfection. HONE-1, HEK 293T, NOKs-Akata, and D98/HR-1 cells were transfected with Lipofectamine 2000 transfection reagent

(Invitrogen) according to the manufacturer's instructions. HeLa cells were transfected using FuGENE6 transfection reagent (Roche) according to the manufacturer's instructions.

Reporter gene assays. HONE-1 cells were transfected in 12-well dishes with 50 ng of pCpGL-basic promoter constructs, 10 ng of BZLF1 alone, 10 ng of BRLF1 alone, 5 ng of both BZLF1 and BRLF1 (synergy studies), and up to 500 ng of SG5 control expression vectors. The cells were washed with PBS and harvested in 1× Reporter lysis buffer (Promega) at 48 h posttransfection. Lysates were subjected to one freeze-thaw cycle, and relative luciferase units were quantified with a BD Monolight 3010 luminometer (BD Biosciences) using Promega luciferase assay reagent. For each condition, at least 3 independent experiments were performed in duplicate.

Immunoblotting. Immunoblotting was performed as previously described (27). Cells lysates were harvested in Sumo lysis buffer including proteasome inhibitor cocktail (Roche), and the protein concentration was determined using the Sumo protein assay (Bio-Rad). Equal amounts of protein were resolved with 10% or 4 to 20% gradient (Bio-Rad) sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were first blocked in a phosphate-buffered saline solution containing 5% milk and 0.1% Tween 20 and then incubated with primary antibody. The following antibodies were used: anti-β-actin (Sigma; 1:5,000), anti-BMRF1 (Vector; 1:250), anti-BRLF1 (Argene; 1:250), anti-BZLF1 (Santa Cruz, sc-53904; 1:250), and anti-tubulin (Sigma; 1:2000). The murine monoclonal antibody against BALF2 (1:250) was a gift from Jaap Middeldorp (VU University medical center). The secondary antibody used was horseradish peroxidase (HRP)—goat anti-mouse (Fisher Scientific; 1:5,000).

Reverse transcription-PCR (RT-PCR). HEK 293T cells were transfected in 6-well dishes with 550 ng of methylated or mock-treated EBV bacmid DNA and cotransfected with either 225 ng of BZLF1, 100 ng of BRLF1, or SG5 control vector. NOKs-Akata cells were transfected in 6-well dishes with SG5 control vector, 100 ng BZLF1, 100 ng BRLF1, or 20 ng of BZLF1 plus 20 ng BRLF1 (synergy studies). RNA was isolated at 2 days posttransfection using the RNeasy Minikit (Qiagen). The RNA concentration was determined, equivalent amounts of RNA were DNase treated, and cDNA was made using the Improm-II reverse transcription system (Promega) according to the manufacturer's instructions. PCR using the cDNA was performed to quantify relative transcript levels of multiple EBV lytic genes with the following primers: BALF2, 5'-TCAATGTC AAGGCTCTGCACAGGA-3' and 5'-ACCATATGGGCATTGTGGAAC ACG-3'; BLRF2, 5'-TGTCAGCTCCACGCAAGTCAGAT-3' and 5'-AG GACCTGTTGCTTCAGAGCCTTA-3'; BHLF1, 5'-ATGAGCTCCAGG ACCAGGCAA-3' and 5'-TAGGGTTCGAATGGGCGTGGT-3'; BMLF1, 5'-TCTCCCGAAGTAGCAGCATTTCT-3' and 5'-ATCGCAGTCTGT GTTGGTGTCTGA-3'; BMRF1, 5'-GCCGCCGTGTCATTTAGAAAC CTT-3' and 5'-TGTTGGTGGCTCTGGACACCTTAT-3'; BRLF1, 5'-TG GCTTGAAGACTTTCTGAGGCT-3' and 5'-AATCTCCACACTCCCG GCTGTAAA-3'; BZLF1, 5'-AATGCCGGGCCAAGTTAAAGCAAC-3' and 5'-TTGGGCACATCTGCTTCAACAGGA-3'; and beta-2 microglobulin (β2 M) cellular gene, 5'-TTCTGGCCTGGAGGGCATCC-3' and 5'-ATCTTCAAACCTCCATGATG-3'.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (33). Methylated and unmethylated probes were prepared as previously described (29) or commercially obtained from IDT. Whole-cell extracts containing BRLF1 protein (missing the inhibitory carboxy-terminal domain) were created by transfection of HeLa cells with the R550 deletion construct. Cells were harvested at 48 h posttransfection in lysis buffer (0.42 M NaCl, 20 mM HEPES [pH 7.5], 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1× proteasome inhibitor cocktail [Roche]) and spun down for 15 min at 10,000 rpm at 4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad), and supernatants were stored at −80°C. The whole-cell extracts were used to perform EMSAs with known and novel BRLF1-responsive elements

(RREs). The BMLF1 promoter probe spanned positions 84691 to 84728 (5'-GGCCAGATGTCCTCTATCATGGCGCAGACATTCTC-3'). BALF2 probe 1 spanned positions 165056 to 165093 (5'-GCACAGCACACCC TGAGCCGCGACAGTAGTCGTAG-3'), and BALF2 probe 2 spanned positions 165266 to 165303 (5'-CCGGGTGAACACCGCGTACATGGC CCTGAACATGAGG-3'). The BLRF2 promoter probe spanned positions 88638 to 88675 (5'-GCGCTTCCAGTCCCACAAACGCGGCGGCT TCCCT-3'). The underlined portion of the probes contains the site of the RRE, and the bold nucleotides are CpGs that were methylated or not. Double-stranded, annealed DNA oligonucleotides were labeled with [γ - 32 P]ATP (Perkin-Elmer) using T4 polynucleotide kinase (NEB) and desalted with G-25 Sephadex columns (GE Healthcare). Whole-cell extracts (1 μ g for BMLF1 reactions and 15 μ g for BALF2 and BLRF2 reactions) were incubated for 5 min in binding buffer containing 10 mM HEPES, (pH 7.5), 50 mM NaCl, 2 mM MgCl₂, 2.5 μ M ZnSO₄, 0.5 M EDTA, 1 mM DTT, 15% glycerol, and 0.5 μ g poly(dI-dC), and 30,000 cpm of labeled oligonucleotide was added to the reaction mixture and allowed to incubate for 20 min at room temperature. For supershift reactions, 1 μ l of anti-BRLF1 (Argene) was added, and reactions were allowed to incubate for an additional 20 min at room temperature. The reaction mixtures were loaded onto a 4% polyacrylamide gel in 0.5 $\times$ Tris-borate-EDTA buffer and electrophoresed at 35 mA. Gels were dried on Whatman paper under a vacuum and exposed to autoradiography film for 12 to 40 h at -80°C .

Chromatin immunoprecipitation (ChIP) assays. HEK 293T cells were transfected in 10-cm dishes with 200 ng methylated or mock-treated EBV bacmid DNA, 1 μ g BZLF1, 1 μ g BRLF1, or SG5 control vector (up to 6 μ g). Cells were cross-linked for 10 min at room temperature with fresh 1% paraformaldehyde at 48 h posttransfection. The cross-linking reaction was quenched using 125 mM glycine, and the cells were lysed. The lysate was sonicated to yield approximately 500-bp DNA fragments. DNA-protein complexes were immunoprecipitated with the following antibodies: anti-BRLF1 (Argene), anti-BZLF1 (Argene), anti-FLAG (Sigma; F1804), anti-acetyl H3K9 (Abcam), mouse isotype anti-IgG control (Santa Cruz), and rabbit isotype anti-IgG control (Santa Cruz). Immunoprecipitated DNA-protein complexes were washed with low-salt, high-salt, lithium chloride, and Tris-EDTA (TE) wash buffers. The protein-DNA cross-links were reversed at 65°C overnight, and the DNA was purified using the Qiagen gel extraction kit. PCR was used to determine the presence and relative amount of specific DNA fragments that were immunoprecipitated. Primers used for amplifying the for the BALF2 promoter were 5'-AAACACCACTGTGTAGCACAGCAC-3' and 5'-TGAGTCCAGCTAC CTCATGTTTCAG-3', those for the BHLF1 promoter were 5'-CTCTTTT TGGGGTCTCTGTG-3' and 5'-CCTCTCTCTCTCGTTATCC-3' (56), those for BLRF2 were 5'-ACTGAAGCCAGGACCAGTTCTA-3' and 5'-TAAGACAAGCGTCAGAAAGTGCCCA-3', those for BMLF1 were 5'-CG TGACATGGAGAACTGGGGG-3' and 5'-CCTCTTACATCACTCAC TGCACG-3', those for the BMRF1 promoter were 5'-ATGCCAGAAA CCTGAGCAAGTAGCC-3' and 5'-CCTTGGTGGATGTGCGAGCCAT AAAG-3', those for BRLF1 were 5'-CTCTTACCTGCGTCTGTTTGT G-3' and 5'-CTCTCTGTGCTGCCACTCATCT-3', those for BZLF1 were 5'-GGTGTAATTTTACATCTTC-3' and 5'-GCTAATGTACCTC ATAGACACACC-3', and those for β -globin were 5'-AGGGCTGGGCA TAAAGTCA-3' and 5'-GCCTCACCACCAACTTCATC-3'.

qPCR. Quantification of ChIP samples was performed by quantitative PCR (qPCR) analysis using SYBR green (Bio-Rad) according to the manufacturer's protocol. Samples were measured with an ABI Prism 7900 real-time PCR system (Applied Biosystems). BMRF1 was amplified with primers 5'-CACTGCGGTGGAGGTAGAG-3' and 5'-GGTGGTGTGCC ATACAAGG-3' (56). Input samples were diluted to 5%, 1%, and 0.2% into H₂O with 100 μ g/ml sonicated salmon sperm DNA (Agilent). A standard curve was calculated from the threshold cycle (C_T) of the input sample dilution series and used to calculate percent input bound in the tested samples. Each condition and input dilution was loaded in triplicate.

OriLyt plasmid-based replication assays. OriLyt plasmid replication assays were performed as previously described (44). Latently infected,

EBV-positive D98/HR-1 cells were transfected with 500 ng of the oriLyt-containing plasmid p588 (57) (a gift from Bill Sugden, University of Wisconsin-Madison), 1 μ g of BZLF1, or SG5 control vector (up to 6 μ g) in 10-cm dishes. Cells were harvested at various time points posttransfection, and nuclei were isolated using a modified REAP method (58). Briefly, cells were resuspended twice in a solution of cold PBS plus 0.1% NP-40. DNA was isolated from the nuclear pellets using the DNeasy blood and tissue kit (Qiagen), concentrated with salt precipitation, and quantified using spectrophotometry. Equivalent amounts (4 to 6 μ g) of DNA were digested overnight with 2 μ l of restriction enzymes (BamHI and DpnI) and spiked with an additional 1 μ l of restriction enzymes. The DNA was separated on 0.8% agarose gels at 25 V for 16 h. The gel was prepared for transfer by incubation with 0.25 N HCl for 30 min, denaturing buffer (0.5 M NaOH, 1.5 M NaCl) for 30 min, neutralizing buffer (0.5 M Tris-HCl, 1.5 M NaCl, [pH 7.0]) for 30 min, and 20 $\times$ SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]) for 30 min. The DNA was transferred to nylon membranes overnight using the Turboblotter rapid downward-transfer system (Whatman) and cross-linked with UV irradiation. Membranes were prehybridized in Church hybridization buffer (0.5 M Na₂HPO₄ [pH 7.2], 1% bovine serum albumin, 7% sodium dodecyl sulfate [SDS], 5 mM EDTA [pH 8.0]) for 1 h at 65°C . Membranes were then hybridized at 65°C overnight with a DNA probe directed against the hygromycin resistance gene labeled with [γ - 32 P]ATP using the random primer labeling system (GE Healthcare). After hybridization, membranes were washed with Church wash buffer (1% SDS, 20 mM Na₂HPO₄ [pH 7.2], 1 mM EDTA) one time at 65°C (15 min) and three times at 45°C (10 min for each wash). The membrane was exposed to film at -80°C overnight, and films were developed.

Methylation status of selected EBV promoters. The methylation status of various EBV promoters in HONE-Akata cells and NOKs-Akata cells was determined. Cells were treated for 3 days with 100 μ g/ml of acyclovir (Sigma) prior to DNA extraction. Genomic DNA was prepared using the Qiagen DNeasy blood and tissue kit. Two hundred nanograms of genomic DNA and 20 ng of methylated or mock-treated bacterial artificial chromosome (BAC) DNA (control) were digested with HpaII and then assayed by PCR amplification using the following primers: BALF2, 5'-GCG ACTAGTTGTTTGTGAGGACCCCGGTGCGAGGCGT-3' and 5'-CTGA GATCTCCAAGGTATCGCCCCGGCTCCCACT-3'; BHLF1, 5'-GAG ACTAGTGAGACCTGCATCTGCACACC-3' and 5'-CTGTGTAATAC TTTAAGGTTTGCTCAGGAG-3'; BLRF2, 5'-GCAACTAGTCGCTGAT TCTGGAGGATTAGCC-3' and 5'-GACAGATCTCAAACAGCCGAGA TTGCTGCC-3'; BMLF1, 5'-GCGACTAGTTGCGCCTCTTTGTCTGTC ATCCGAA-3' and 5'-CAGAGATCTTAGCTGGGATGTAGTGCTGT CTTGACTGGC-3'; BRLF1, 5'-AATAGATCTTGAGGTGTTGTGCTCT GTATGGTATTC-3' and 5'-CTGACTAGTCCCAACACCATGGGTGAT AACGTC-3'; and BZLF1, 5'-GCGACTAGTAGGTGTGTGTCAGCCAAAG AGGATCA-3' and 5'-GCGAGATCTCCGCAAGGTGCAATGTTTAG TGA-3'. The BMRF1 promoter does not contain an HpaII site and hence could not be assessed by this assay.

Virus titration assay. Virus titration assays were performed in NOKs-Akata cells as previously described (59). NOKs-Akata cells were plated onto a 12-well dish and then transfected with control SG5 vector, 50 ng of BZLF1, 50 ng of BRLF1, or 10 ng of BZLF1 plus 10 ng of BRLF1 expression vectors (for synergy studies). Supernatant was harvested at 48 h posttransfection and filtered through a 0.8- μ m-pore-size filter. Raji cells (2×10^5 cells/infection) were infected with 100 μ l of supernatant and incubated at 37°C . Phorbol-12-myristate-13-acetate (TPA) (20 ng/ml) and sodium butyrate (3 mM final concentration) were added 24 h after infection. GFP-positive Raji cells were counted at 48 h postinfection to determine the viral titer.

RESULTS

Methylation enhances BZLF1-mediated activation of many, but not all, early lytic promoters. To examine the effect of promoter methylation on the ability of BZLF1 to activate various different

FIG 1 DNA methylation enhances BZLF1 transactivation of most early lytic EBV promoters. (A and B) EBV-negative HONE-1 cells were transfected with BALF2p, BARF1p, BFLF2p, BGLF4p, BGLF5p, BMLF1p, BMRF1p, BRLF1p, and BRRF1p (A) and BHLF1p and BHRF1p (B) pCpGL luciferase constructs that were either methylated (dark bars) or mock treated (light bars). The reporter gene constructs were transfected in the presence or absence of BZLF1 and SG5 control vector as indicated. Luciferase assays were performed 2 days after transfection. The fold luciferase activity under each condition is shown relative to the activity of the unmethylated promoter in the presence of the SG5 control vector (set to 1). The error bars indicate +1 standard deviation calculated from 3 experiments performed in triplicate. (C) A representative immunoblot shows similar levels of cotransfected BZLF1 protein in the extracts used in the methylated (M) versus unmethylated (U) BHLF1 promoter luciferase assays; similar results were observed in other luciferase assays (data not shown).

early lytic EBV promoters, we cloned multiple different lytic promoters (Table 1) upstream of the luciferase gene in a CpG-free vector (53) and then methylated or mock treated the various promoter constructs *in vitro* as previously described using the CpG methyltransferase M.SssI (29). The CpG-free luciferase vector prevents nonspecific inhibitory effects of total plasmid DNA methylation on luciferase gene activity by ensuring that only the inserted EBV promoter sequences can be methylated. EBV-negative HONE-1 NPC cells were transfected with the methylated or the mock-treated promoter constructs in the presence or absence

of a limiting amount of cotransfected BZLF1 expression vector (10 ng/12-well dish), and the amount of luciferase activity for each condition was quantitated 2 days later.

As shown in Fig. 1A, methylation of promoter DNA increased the ability of BZLF1 to activate 9 out of 11 early lytic promoters tested (BALF2, BARF1, BFLF2, BGLF4, BGLF5, BMLF1, BMRF1, BRLF1, and BRRF1). We documented that similar levels of transfected BZLF1 were expressed under each condition (Fig. 1C and data not shown). Of note, while we previously reported that the BRLF1 promoter is more efficiently activated by BZLF1 in the

methylated form (28), the positive effect of methylation on BZLF1 activation of this promoter is even more apparent in the current study, likely reflecting the use of the CpG-free luciferase vector, as well as the limiting amount of BZLF1 used in the current (but not the former) study. Interestingly, using this lower level of transfected BZLF1 (which we found to be similar to that expressed in transforming growth factor β [TGF- β]-treated Mutu 1 Burkitt cells [data not shown]), we did not observe autoactivation of the BZLF1 promoter in either the methylated or unmethylated form (data not shown).

The oriLyt early lytic promoters BHLF1 and BHRF1 are more efficiently activated by BZLF1 in the unmethylated form. In contrast to the case for the majority of early lytic promoters, we also identified two early lytic EBV promoters that are activated by BZLF1 more efficiently in the unmethylated form (Fig. 1B). Interestingly, both of these promoters, BHRF1 and BHLF1, are located within the EBV lytic origin of replication (oriLyt), and in contrast to many early lytic promoters, the previously identified ZREs located upstream of the BHRF1 and BHLF1 promoters do not contain CpG motifs (10, 25, 45). These results suggest that while promoter methylation generally enhances BZLF1-mediated activation of early lytic promoters, the two oriLyt promoters are potentially important exceptions to this rule.

BRLF1 activation of early lytic promoters is inhibited by DNA methylation. Although BRLF1 can bind directly to, and activate, many of the same early lytic EBV promoters that are activated by BZLF1, the effect of DNA methylation on BRLF1-mediated activation has not yet been explored. We therefore examined the ability of limiting levels of BRLF1 (10 ng/12-well dish, which produced a level of BRLF1 similar to that in TGF- β -treated Mutu 1 cells [data not shown]) to activate a series of methylated and mock-methylated early and late lytic EBV promoters. As shown in Fig. 2A, we found that BRLF1 activated five different early lytic promoters (BALF2, BARF1, BFLF2, BMRF1, and BRRF1) much more efficiently in the unmethylated form than in the methylated form. Four other promoters (BGLF4, BHLF1, BHRF1, and BMLF1) were also activated more efficiently in the unmethylated forms, although the inhibitory effect of methylation was not as dramatic. We documented that similar levels of transfected BRLF1 were expressed under each condition (Fig. 2C and data not shown). At the low level of transfected BRLF1 used in these studies, we did not observe BRLF1 activation of the other early lytic promoters listed in Table 1 (including the BZLF1 and BRLF1 IE promoters) in either the methylated or unmethylated form (data not shown). Thus, all BRLF1-responsive early lytic promoters tested were more efficiently activated by the BRLF1 protein in the unmethylated form than in the methylated form, although the effect of methylation was more dramatic for some early lytic promoters (such as the BALF2 promoter) than for others (such as the BMLF1 promoter).

BRLF1 activation of the BLRF2 late lytic viral promoter is also inhibited by promoter DNA methylation. BRLF1 has also been reported to activate certain late gene viral promoters in a replication-independent manner in reporter gene assays, and it binds directly to at least two of these promoters (BLRF2 and BFRF3) (33, 37). Interestingly, the previously identified RRE in the BLRF2 promoter (GTCCCACAAACGCGGCG) contains several CpG motifs (33). Therefore, we examined how promoter DNA methylation affects the ability of BRLF1 to activate the BLRF2 promoter in the methylated versus the unmethylated

form. We found that promoter DNA methylation greatly inhibits the ability of BRLF1 to turn on the BLRF2 promoter (Fig. 2B). However, we did not observe BRLF1 activation of several other late lytic viral promoters tested (including the BcLF1, BDLF3, and BLLF1 promoters) in either the unmethylated or methylated form under the conditions used in our studies (data not shown). Thus, the ability of BRLF1 to activate at least one late lytic EBV promoter, BLRF2, requires that the viral promoter be in the unmethylated form.

Viral genome methylation differentially affects the ability of BZLF1 versus BRLF1 to induce early lytic gene expression in the context of the intact viral genome. To examine how methylation affects lytic gene expression in the context of the intact viral genome, purified EBV bacmid DNA was methylated or mock treated *in vitro* and then transfected into HEK 293T cells in the presence or absence of cotransfected BZLF1 or BRLF1. Immunoblotting was performed 3 days later to compare the ability of cotransfected BZLF1 versus BRLF1 to activate expression of the BMRF1 and BALF2 early lytic genes from the EBV bacmid genome in the methylated versus unmethylated state. As indicated in Fig. 3A, methylation of the EBV bacmid genome enhances BZLF1-induced BMRF1 and BALF2 protein expression, in agreement with the results of the reporter gene assays (Fig. 1). In contrast, methylation of the EBV genome decreases BRLF1-induced BMRF1 and BALF2 protein expression (Fig. 3A), as also predicted by the reporter gene assays (Fig. 2). Similar results were obtained using either the B95.8 or Akata bacmid DNA (data not shown). These results confirm that methylation differentially affects the ability of BZLF1 versus BRLF1 to induce early lytic viral protein expression in the context of the intact viral genome.

To determine if the differential effect of EBV bacmid methylation on the ability of BZLF1 versus BRLF1 to activate lytic protein expression is associated with differences in lytic viral gene transcription, we harvested RNA from 293T cells transfected with methylated or mock-methylated EBV bacmids (in the presence or absence of cotransfected BZLF1 or BRLF1) and performed RT-PCR to detect various EBV transcripts. As shown in Fig. 3B, BRLF1 preferentially activates expression of the BMRF1, BALF2, and BLRF2 transcripts from the unmethylated EBV bacmid, whereas BZLF1 preferentially activates expression of the BMRF1 and BALF2 early lytic EBV transcripts from the methylated EBV bacmid. Activation of the BLRF2 late transcript occurs only in response to BRLF1 expression, and this activation requires an unmethylated viral genome. Of note, the ability of transfected BZLF1 to induce BRLF1 gene transcription from the cotransfected EBV bacmid construct and, vice versa, the ability of BRLF1 to induce BZLF1 transcription from the cotransfected bacmid construct were very limited, for unclear reasons.

The combination of BZLF1 and BRLF1 synergistically activates early lytic BMRF1 protein expression from either the methylated or unmethylated EBV bacmid genome. Deletion of either the BZLF1 or BRLF1 protein severely inhibits expression of the early lytic BMRF1 protein in stably EBV-infected 293 cells (16), which contain a highly methylated viral genome (29). However, the mechanism(s) by which BZLF1 and BRLF1 cooperate to synergistically activate expression of early lytic viral proteins, and in particular whether this effect is dependent upon the DNA methylation state of the lytic viral promoters, is not well understood. To examine whether the combination of BZLF1 and BRLF1 can synergistically induce early lytic BMRF1 protein expression

FIG 2 BRLF1-mediated activation of lytic promoters is inhibited by CpG methylation. (A and B) HONE-1 cells were transfected with methylated or mock-treated BALF2p, BARF1p, BFLF2p, BMRF1p, BRRF1p, BGLF4p, BHLF1p, BHRF1p, and BMLF1p (A) and BLRF2p (B) pCpGL luciferase constructs in the presence or absence of BRLF1 and SG5 control vector, and luciferase assays were performed 2 days after transfection. The fold luciferase activity under each condition is shown relative to the activity of the unmethylated promoter in the presence of the control vector (set to 1). The error bars indicate ± 1 standard deviation calculated from 3 replicate experiments. (C) A representative immunoblot shows similar levels of cotransfected BRLF1 protein in the extracts used in the methylated (M) versus unmethylated (U) BHLF1 promoter luciferase assays; similar results were observed in other luciferase assays (data not shown).

from either the methylated or unmethylated forms of EBV bacmids, we transfected methylated or mock-treated EBV bacmid DNA into 293T cells in the presence of BZLF1 alone, BRLF1 alone, or the combination of both BZLF1 and BRLF1 and compared the amounts of BMRF1 protein expression derived from the transfected EBV bacmid DNA 3 days later. As shown in Fig. 4A, the combination of BZLF1 and BRLF1 synergistically activated expression of the BMRF1 protein from either the unmethylated (left) or methylated (right) form of the EBV genome; similar results were obtained using either B95.8 or Akata bacmid DNA.

To determine if the synergistic effect of the BZLF1/BRLF1

combination on BMRF1 protein expression is associated with an increase in BMRF1 transcription, we harvested RNA from 293T cells transfected with methylated or mock-methylated EBV bacmids (in the presence or absence of cotransfected BZLF1, BRLF1, or BZLF1 and BRLF1 together) and performed RT-PCR to examine the level of BMRF1 transcript (Fig. 4B). Somewhat surprisingly, for both the methylated and unmethylated forms of the EBV bacmid, the combination of BZLF1 and BRLF1 together resulted in only a relatively modest increase in the level of BMRF1 transcript relative to the effect of BZLF1 or BRLF1 alone, in contrast to the large effect observed at the BMRF1 protein level. These results

FIG 3 EBV genome methylation enhances BZLF1-mediated expression of lytic genes yet decreases BRLF1-induced lytic gene expression. 293T cells were transfected with methylated or mock-treated EBV bacmid DNA (with or without cotransfected SG5 control vector, BZLF1, or BRLF1 expression vectors) as indicated. (A) Immunoblot analysis was performed at 3 days posttransfection to compare the levels of BZLF1- and BRLF1-induced BMRF1 and BALF2, as well as the levels of transfected BZLF1 and BRLF1. β-Actin served as a loading control. (B) RNA was isolated from cells at 2 days posttransfection and DNase treated. RT-PCR was performed using primers to detect BZLF1 (transfected and EBV bacmid derived), BRLF1 (transfected and EBV bacmid derived), BMRF1, BALF2, BLRF2, or beta-2 microglobulin (β2 M) transcripts as indicated.

suggest that BZLF1 and BRLF1 may cooperate to enhance BMRF1 protein expression through at least a partially (as-yet-unknown) posttranscriptional mechanism(s).

DNA methylation does not affect BRLF1 binding to RREs *in vitro*. Given our finding that promoter DNA methylation decreases the ability of BRLF1 alone to activate lytic gene expression, we next asked if methylation of CpG-containing RREs inhibits BRLF1 binding. To examine the effect of RRE methylation on BRLF1 binding *in vitro*, we prepared extracts from HeLa cells transfected with a BRLF1 expression vector containing amino acids 1 to 550 (since it is difficult to detect BRLF1 binding activity by EMSA in cells transfected with the intact BRLF1 protein [33]) and performed EMSAs using unmethylated versus methylated RRE probes. As shown in Fig. 5A, BRLF1 binds similarly to the methylated and unmethylated forms of a CpG-containing RRE in the BMLF1 promoter. Likewise, the methylated versus unmethylated forms of two different CpG-containing RREs within the BALF2 promoter were bound similarly *in vitro* (Fig. 5B), even though

BRLF1 activation of this promoter *in vivo* is much more efficient for the unmethylated form of the promoter (Fig. 2 and 3). BRLF1 binding to the methylated and unmethylated forms of a CpG-containing RRE in the late BLRF2 promoter was also similar (by EMSA) (Fig. 5C), even though BRLF1 activates this promoter much more efficiently in the unmethylated form (Fig. 2 and 3).

DNA methylation does not affect BRLF1 binding to RREs *in vivo* but enhances BZLF1 binding to most ZRE-containing promoters. We next performed ChIP assays to examine the effect of viral genome methylation on BRLF1 (full length) versus BZLF1 DNA binding *in vivo* in 293T cells transfected with the methylated or unmethylated forms of the EBV bacmid DNA (Fig. 6). BRLF1 bound similarly to the methylated and unmethylated forms of the BALF2, BMLF1, and BMRF1 promoters *in vivo* (Fig. 6A), similar to the results of the EMSA studies (Fig. 5); quantitative PCR analysis of the BMRF1 promoter ChIP results (Fig. 6B) confirmed that BRLF1 binding to the methylated and unmethylated forms of this promoter is similar. Although BRLF1 clearly activates the un-

FIG 4 BZLF1 plus BRLF1 induce synergistic expression of the BMRF1 protein from both the methylated and unmethylated viral genomes. (A) Mock-methylated (left) or methylated (right) EBV bacmid DNA was transfected into 293T cells with or without SG5 control vector, BZLF1, BRLF1, or BZLF1 plus BRLF1 expression vectors, as indicated. Immunoblot analysis was performed at 3 days posttransfection to compare the levels of BZLF1- or BRLF1-induced BMRF1, as well as the transfected BZLF1 and BRLF1 proteins. β-Actin served as a loading control. (B) Unmethylated (U) or methylated (M) EBV bacmid DNA was transfected into 293T cells with or without SG5 control vector, BZLF1, BRLF1, or BZLF1 plus BRLF1 expression vectors, as indicated. Two days later, RNA was isolated from the cells and DNase treated, and RT-PCR was performed using primers to detect BRLF1, BZLF1, and EBV bacmid-derived BMRF1 transcripts as indicated. The cellular beta-2 microglobulin (β2 M) transcript was also measured as a control.

FIG 5 BRLF1 binds to unmethylated and methylated DNA similarly *in vitro*. BRLF1 binding to the unmethylated versus methylated forms of an RRE from the BMLF1 promoter (33) (A), two predicted RREs from the BALF2 promoter (B), and an RRE from the late BLRF2 promoter (33) (C) was measured by EMSAs performed with whole-cell extracts. Extracts were derived from HeLa cells transfected with a truncated mutant of BRLF1 (R550) or SG5 control vector. Anti-BRLF1 antibody was added to the indicated reaction mixtures to ensure that retarded probe was indeed bound by BRLF1. BRLF1-DNA complexes, as well as supershifted complexes, are designated by arrows. The underlined cytosines indicate methylated sites in each RRE sequence (shown below the respective EMSA image). Boxed nucleotides encompass the core binding site where BRLF1 directly contacts DNA. This sequence is separated by a 9-nucleotide spacer.

methylated forms of the BMRF1 and BALF2 promoters more efficiently than the methylated forms in reporter gene assays (Fig. 2A) and in the bacmid studies (Fig. 3), these results suggest that the inhibitory effect of DNA methylation on BRLF1-mediated activation of the BALF2 and BMRF1 promoters is not due to a decreased ability of BRLF1 to bind to methylated BALF2 or BMRF1 promoter DNA.

As shown in Fig. 6C, methylation of EBV bacmid DNA promotes BZLF1 binding to multiple CpG-containing early lytic viral

promoters (BMRF1, BRLF1, and BMLF1), consistent with the enhanced BZLF1-mediated activation of the methylated forms of these promoters in reporter gene assays (Fig. 1A) and bacmid studies (Fig. 3). Increased BZLF1 binding to the methylated versus unmethylated form of the BMRF1 promoter was confirmed by quantitative PCR analysis (Fig. 6D). Interestingly, although BZLF1 activates the unmethylated form of the BHLF1 promoter (which has CpG-free ZREs) more efficiently than the methylated form in reporter gene assays (Fig. 1B), it bound at least as well to

FIG 6 Viral genome methylation does not alter BRLF1 DNA binding *in vivo* but enhances BZLF1 binding. 293T cells were transfected with unmethylated (U) or methylated (M) EBV bacmid DNA in the presence or absence of SG5 control vector, BZLF1, or BRLF1 as indicated. ChIP assays were performed at 2 days posttransfection. (A and C) Cross-linked protein-DNA complexes were immunoprecipitated with anti-IgG isotype control and anti-BRLF1 antibodies (A) or anti-IgG isotype control and anti-BZLF1 antibodies (C) as specified. The relative presence of bound promoters was assayed by PCR amplification using primers spanning BALF2p, BMLF1p, BMRF1p, BRLF1p, BHLF1p, and β -globin (negative control) as indicated. (B) Quantitative PCR was performed on immunoprecipitated DNA to examine the amount of BRLF1 binding to the unmethylated versus methylated BMRF1 promoter. (D) Quantitative PCR was performed on the immunoprecipitated DNA to examine the amount of BZLF1 binding to the unmethylated versus methylated BMRF1 promoter. (E) 293T cells were transfected with methylated EBV bacmid DNA in the presence or absence of SG5 control vector, FLAG-BZLF1, BRLF1, or FLAG-BZLF1 plus BRLF1 as indicated. A ChIP assay was performed at 2 days posttransfection with anti-IgG isotype control, anti-BRLF1, and anti-FLAG (denoted BZLF1) antibodies as specified. The relative presence of bound promoters was assayed by PCR amplification using primers spanning BALF2p, BHLF1p, BLRF2p, BMLF1p, BMRF1p, BRLF1p, and β -globin (negative control) as indicated. Similar results were obtained with the unmethylated EBV bacmid (data not shown). (F) Quantitative PCR was performed on the immunoprecipitated DNA to examine the amount of BZLF1 and BRLF1 binding to the methylated BMRF1 promoter in the presence or absence of the other IE protein as indicated.

the methylated, versus unmethylated, form of this promoter *in vivo*. Thus, the inhibitory effect of DNA methylation on BZLF1 activation of the BHLF1 promoter is not associated with reduced BZLF1 DNA binding to this promoter.

We also performed ChIP assays to examine whether BRLF1 and BZLF1 increase one another's ability to bind to several different lytic EBV promoters. As shown in Fig. 6E and F, BZLF1 and BRLF1 did not significantly increase each other's ability to bind to

FIG 7 BRLF1 preferentially enhances acetylation of H3K9 on unmethylated viral promoters. 293T cells were transfected with unmethylated (U) or methylated (M) EBV bacmid DNA in the presence or absence of SG5 control vector, BZLF1, or BRLF1 as indicated. A ChIP assay was performed at 2 days post-transfection with anti-IgG isotype control and anti-H3K9Ac antibodies as specified. The relative presence of bound promoters was assayed by PCR amplification using primers spanning BALF2p, BHLF1p, BLRF2p, BMLF1p, BMRF1p, BZLF1p, and β -globin (negative control) as indicated.

any of the six different lytic promoters examined in the methylated EBV bacmid. Similar results were obtained in studies examining BZLF1 and BRLF1 binding to lytic promoters in the unmethylated EBV bacmid (data not shown).

BRLF1 induces an activating histone modification (H3K9 acetylation) more efficiently on unmethylated viral promoters. Since BRLF1 interacts directly with the histone acetyltransferase CBP (60), we hypothesized that BRLF1 binding to promoter DNA may induce the activating histone modification H3K9 acetylation. To determine if promoter DNA methylation affects the ability of BRLF1 to induce H3K9 acetylation, we performed ChIP assays (using an antibody that recognizes acetylated H3K9) in 293T cells transfected with the methylated or unmethylated forms of EBV bacmid DNA in the presence or absence of cotransfected BRLF1 or BZLF1. The results of these experiments confirmed that BRLF1 can induce H3K9 acetylation on numerous different early lytic EBV promoters (Fig. 7). Importantly, however, BRLF1 induced much more H3K9 acetylation on the unmethylated form of the EBV bacmid DNA than on the methylated form. These results indicate that while BRLF1 binds similarly to both the unmethylated and methylated forms of viral promoters, it preferentially confers H3K9 acetylation to the unmethylated forms of the promoters. Of note, BRLF1 also conferred H3K9 acetylation to the unmethylated (but not methylated) form of the BZLF1 promoter, even though it is not known to bind directly to this promoter. Interestingly, in comparison to BRLF1, binding by the BZLF1 protein to the unmethylated and methylated forms of EBV bacmid DNA induced relatively little H3K9 acetylation, even though we and others have shown that BZLF1 interacts directly with CBP and p300 (61, 62).

Methylation does not affect lytic replication of an oriLyt-containing vector in *cis*. Since the BHLF1 transcript has been shown to be required in *cis* for efficient lytic replication and BZLF1 binding to oriLyt is essential for efficient lytic replication independent of the transcriptional function of BZLF1 (45, 47), we also studied the effect of methylation in *cis* on oriLyt replication. A

vector containing the EBV BamHI fragment (which contains the entire EBV oriLyt), in addition to a hygromycin resistance gene, was methylated or mock treated *in vitro* and transfected into EBV-positive D98/HR-1 cells in the presence or absence of a BZLF1 expression vector. Nuclear DNA was harvested at various time points after transfection and digested with DpnI, and a Southern blot assay was performed using a probe directed against the hygromycin resistance gene (to avoid detection of the replicated endogenous D98/HR-1 viral genome). As previously described (44), the unreplicated oriLyt plasmid is sensitive to DpnI-mediated cutting (since plasmid DNA replicated in bacteria is dam methylated at the adenine in the GATC motif), whereas oriLyt plasmid DNA replicated by the viral DNA polymerase in human cells is not methylated at this site and is thus resistant to DpnI cutting.

As shown in Fig. 8A, the methylated and unmethylated oriLyt-containing vectors replicated similarly at all time points, in a BZLF1-dependent manner. Note that transfection of BZLF1 into D98/HR-1 cells results in strong expression of the BRLF1 protein (derived from the endogenous viral genome) (Fig. 8B), and hence both BZLF1 and BRLF1 are available in this replication assay. Since the *trans*-acting BZLF1-induced viral replication proteins in this experiment were all derived from the endogenous viral genome of D98/HR-1 cells, this oriLyt plasmid replication assay result indicates that DNA methylation of oriLyt does not alter the efficiency of lytic replication *in cis*.

BRLF1, but not BZLF1, expression results in lytic viral reactivation and release of infectious viral particles in a cell line infected with a highly unmethylated form of the EBV genome. We have recently identified a telomerase-immortalized oral keratinocyte cell line (NOKs) that can be stably infected with EBV in a latent form and maintains the lytic viral promoters on the EBV genome in a highly unmethylated state. To examine the methylation status of the various lytic viral promoters in NOKs-Akata cells, DNA was purified from the cells and cut or mock cut with the HpaII restriction enzyme (which can cut the unmethylated, but not methylated, form of the CCGG recognition sequence), and lytic EBV promoter sequences were then PCR amplified using primers located on either side of the HpaII restriction site(s). As shown in Fig. 9A, the methylated EBV bacmid DNA was resistant to HpaII cutting (and hence could be PCR amplified when exposed to the restriction enzyme), while the unmethylated form of the bacmid was sensitive to cutting (and hence could not be PCR amplified), as expected. The EBV DNA purified from NOKs-Akata cells could not be PCR amplified following HpaII cutting at any of a variety of different lytic EBV promoters tested (including the BZLF1, BRLF1, BALF2, BHLF1, BLRF2, and BMLF1 promoters), indicating that the CpG-containing HpaII sites present in each of these promoters are not methylated. In contrast, with the exception of the BZLF1 and BHLF1 promoters, each of the lytic viral promoters in EBV DNA purified from the HONE-Akata line was partially protected from HpaII digestion, suggesting that this cell line contains a mixture of methylated and unmethylated viral genomes. Interestingly the BZLF1 promoter was recently shown to be generally unmethylated in various EBV-positive tumors, even when other lytic viral promoters were methylated (5).

We next compared the ability of transfected BRLF1 versus BZLF1 expression vectors to induce early lytic protein expression in NOKs-Akata versus HONE-Akata cells. As shown in Fig. 9B, although the NOKs-Akata cells expressed at least as much transfected BZLF1 as the HONE-Akata cells, BZLF1 activated BRLF1

FIG 8 DNA methylation in *cis* does not alter the efficiency of lytic replication. Lytic replication of an oriLyt-containing plasmid, p588, was assayed as previously described (57). Methylated or mock-treated p588 was transfected into EBV-positive D98/HR-1 cells with and without SG5 control vector and BZLF1 as indicated. (A) DNA was isolated from nuclear extracts at the specified time points after transfection and digested with BamHI (to linearize the p588 plasmid) and DpnI (to differentiate replicated versus unreplicated p588 plasmid). Southern blotting employing a [γ - 32 P]ATP-labeled probe directed against the hygromycin resistance gene was performed. The positions of the replicated and unreplicated plasmids are indicated at the right. (B) Cell lysates were harvested in SUMO buffer, and the level of BRLF1 expression induced by BZLF1 transfection into D98/HR-1 cells was assayed by immunoblot analysis.

and BMRF1 expression from the endogenous EBV genome in HONE-Akata cells but had no effect whatsoever in the NOKs-Akata cells. In contrast, BRLF1 activated BZLF1 and BMRF1 expression from the endogenous viral genomes in both NOKs-Akata and HONE-Akata cells (Fig. 9B and data not shown). Similar results were obtained when lytic viral gene expression was examined using RT-PCR analysis (Fig. 9C).

To determine if the combination of BRLF1 and BZLF1 induces synergistic early lytic BMRF1 protein expression in NOKs-Akata cells (as was observed using the unmethylated as well as methylated EBV bacmids), cells were transfected with control vector, BZLF1 alone, BRLF1 alone, or the combination of BZLF1 and BRLF1. As shown in Fig. 9D, the combination of BZLF1 and BRLF1 together induced much more BMRF1 protein expression than either BZLF1 or BRLF1 alone. Interestingly, NOKs-Akata cells did not show a significant increase in lytic gene transcript levels (including the BMRF1 transcript) in cells transfected with BRLF1 alone versus the combination of BRLF1 and BZLF1 (Fig. 9C). This result is similar to that obtained using EBV bacmids (Fig. 4) and again suggests that the BRLF1/BZLF1 combination synergistically enhances BMRF1 protein expression (and perhaps other lytic viral proteins as well) through an at least partially post-transcriptional mechanism.

Finally, we also examined the amount of infectious viral particles released (using the Green Raji cell assay) from NOKs-Akata cells transfected with control vector, BZLF1 alone, BRLF1 alone, or the combination of BZLF1 and BRLF1. As shown in Fig. 9E, BZLF1 alone did not result in release of infectious viral particles (in comparison to cells transfected with a control vector), while BRLF1 alone induced release of infectious viral particles. However, the combination of BZLF1 and BRLF1 together resulted in the greatest number of infectious viral particles, consistent with the ability of this combination to increase expression of the essential viral replication protein BMRF1 (the viral DNA polymerase processivity factor). These results confirm that BRLF1 plays a critical

and primary role in initiating lytic gene expression in cells containing the unmethylated form of the EBV genome and show that cells infected with a highly unmethylated form of the EBV genome are capable of undergoing the lytic form of viral replication in response to BRLF1 but not BZLF1 expression.

DISCUSSION

DNA methylation enhances the ability of the EBV immediate-early BZLF1 protein to bind to, and activate, certain early lytic viral promoters, and viral genome methylation has previously been shown to promote virion production following infection of human B cells (9–11, 24, 27–29). However, while the EBV BRLF1 immediate-early protein can also induce lytic reactivation in many latently infected cell lines (20, 32), the effect of promoter DNA methylation on the ability of BRLF1 to activate various EBV lytic gene promoters has not been explored. In this study, we have investigated how viral genome methylation affects the ability of BZLF1 versus BRLF1 to activate transcription using a series of different lytic EBV promoters in reporter gene assays and using methylated versus unmethylated EBV bacmid DNA. We show that DNA methylation enhances BZLF1-mediated activation, but inhibits BRLF1-mediated activation, of most early lytic EBV promoters. We also demonstrate that methylation of oriLyt plasmid DNA does not have a *cis*-acting effect on its ability to replicate when essential viral replication proteins are provided in *trans*. Most importantly, we have identified an EBV-positive cell line (NOKs-Akata) stably infected with a highly unmethylated viral genome and have shown that BRLF1, but not BZLF1, expression in this line results in lytic viral gene expression and release of infectious viral particles. Together, these results suggest that in cellular environments that promote efficient expression of both the BRLF1 and BZLF1 proteins, lytic viral replication may occur in both the presence and absence of viral genome methylation.

In agreement with previous results reported by our own lab and others (9–11, 24, 27–29, 63), we found that methylation en-

FIG 9 The NOKs-Akata cell line contains a highly unmethylated form of the EBV genome and undergoes lytic reactivation in response to BRLF1, but not BZLF1, expression. (A) DNA isolated from NOKs-Akata cells (N/A) or HONE-Akata cells (H/A) was digested or mock digested with HpaII and then PCR amplified using primers located on either side of HpaII restriction sites in various different lytic EBV promoters as indicated. Methylated (M) or mock-methylated (U) EBV bacmid DNA was similarly treated and PCR amplified to serve as controls representing completely unmethylated and completely methylated viral DNA. Similar results were obtained in a second experiment (data not shown). (B) NOKs-Akata (N/A) and HONE-Akata (H/A) cells were transfected with SG5 control vector, BZLF1, or BRLF1 (50 ng of each vector in NOKs-Akata cells and 10 ng of each vector in HONE-Akata cells) as indicated. Immunoblotting was performed at 2 days posttransfection to compare the levels of BZLF1- or BRLF1-induced BMRF1 and the transfected BZLF1 and BRLF1 proteins. Tubulin served as a loading control. (C) NOKs-Akata cells were transfected with SG5 control vector, BZLF1, BRLF1, or the combination of both BZLF1 and BRLF1 as indicated. Two days later, RNA was isolated from the cells and DNase treated, and RT-PCR was performed using primers to detect BZLF1 (transfected and EBV genome-derived), BRLF1 (transfected and EBV genome-derived), BMRF1, BALF2, BHLF1, BLRF2, BMLF1, or beta-2 microglobulin (β 2 M) transcripts as indicated. (D) NOKs-Akata cells were transfected with SG5 control vector, BZLF1, BRLF1, or the combination of both BZLF1 and BRLF1 as indicated. Immunoblotting was performed at 2 days after transfection to compare the levels of BZLF1- or BRLF1-induced BMRF1 and the transfected BZLF1 and BRLF1 proteins. Tubulin served as a loading control. (E) NOKs-Akata cells were transfected with SG5 control vector, BZLF1, BRLF1, or the combination of both BZLF1 and BRLF1 as indicated. The number of infectious virions released into the supernatant under each condition was quantitated 3 days later using the Green Raji cell assay.

hances BZLF1 activation of the majority of early lytic promoters tested, with some functionally important exceptions. In particular, we found that the two early lytic promoters within oriLyt, BHLF1 and BHRF1, are preferentially activated by BZLF1 in the

unmethylated form. This result (also reported by another group [10]) likely reflects the fact that the ZREs in oriLyt do not contain CpG motifs, and thus viral genome DNA methylation does not increase BZLF1 binding to these sites. In contrast, as shown here

and previously (10, 27–29), methylation of CpG-containing ZREs is associated with greatly increased BZLF1 binding *in vitro* and *in vivo* (Fig. 6).

The BRLF1 protein binds to the consensus element, GNCCN₃GGNG, known as the R-responsive element (RRE), and RREs often contain CpGs motifs in either the nine-nucleotide spacer sequence or the 4-bp core sequences directly contacted by the R protein at either end of the motif. However, in EMSAs we did not find that methylation of CpG motifs located either in the RRE spacer region or within the core binding sites at either end of the motif affected BRLF1 DNA binding. *In vivo* ChIP assays confirmed that BRLF1 binding to promoters with RREs is similar in the presence or absence of viral genome methylation.

Although direct BRLF1 binding to DNA does not appear to be affected by methylation of RREs, we nevertheless found that BRLF1 activation of at least a subset of early lytic promoters is rather dramatically inhibited by DNA methylation (Fig. 2 and 3). Consistent with this result, we found that BRLF1 binding to unmethylated, but not methylated, promoters *in vivo* is associated with H3K9 acetylation (Fig. 7). This result suggests that repressive chromatin modifications associated with viral genome methylation may inhibit the ability of BRLF1 to recruit histone acetylases such as CBP and p300 to promoters. Interestingly, although BZLF1 has been reported by our own group and others to interact directly with the histone acetylases CBP and p300 (61, 62), we found that BZLF1 binding to lytic EBV promoters did not result in robust H3K9 acetylation. Likewise, another recent study found that BZLF1 promoter binding did not result in uniform acetylation of H3K9 and showed that BZLF1 is able to bind to and activate target promoters despite high levels of repressive chromatin modifications (56).

Similar to the results reported by another group (11), we found that DNA methylation inhibits BZLF1 activation of the BHRF1 and BHLF1 oriLyt promoters, which are not thought to have CpG-containing ZREs. We also found that BZLF1 binding to the DNA of these promoters *in vivo* is not affected by the viral genome methylation state (Fig. 6), even though transcriptional activation of these promoters is reduced by methylation (Fig. 1B). These results suggest the possibility that BZLF1 assumes different conformations when bound to different types of ZREs and that the conformation bound to methylated CpG-containing ZREs is particularly efficient in activating transcription in the context of a repressive chromatin environment.

Interestingly, while we found that EBV bacmid DNA methylation has the opposite effect on the ability of BZLF1 alone versus BRLF1 alone to activate BMRF1 expression, the combination of BZLF1 and BRLF1 synergistically activates BMRF1 protein expression regardless of the viral genome methylation state. Nonetheless, the mechanism(s) by which BZLF1 plus BRLF1 synergistically activates BMRF1 protein expression from both the methylated and unmethylated viral genomes appears to be at least partially posttranscriptional, since the effect on the protein level is much larger than the effect on the transcript level. We did not find that binding of BZLF1 and BRLF1 to either the unmethylated or methylated forms of the viral promoters was significantly enhanced when the other IE protein was also present (Fig. 6C and data not shown). The ability of the BZLF1/BRLF1 combination to enhance BMRF1 protein expression to a greater degree than the BMRF1 transcript level from both the unmethylated and methylated viral genomes could potentially reflect the known ability of

the virally encoded SM protein to enhance the level of protein expression from viral genes (such as BMRF1) that contain no introns (64–66). Since our lab has previously reported that the BZLF1/BRLF1 combination can synergistically activate the (unmethylated) BMRF1 promoter in some cell lines but not others (38), it remains possible that the BZLF1/BRLF1 combination synergistically activates BMRF1 at the transcriptional level in B cells.

The EBV oriLyt encompasses two divergent early lytic promoters, BHRF1 and BHLF1, and has both CpG-free ZREs and CpG-containing RREs. Although we initially hypothesized that methylation of oriLyt would inhibit its replication, since methylation of the BHLF1 promoter inhibits BZLF1-mediated transcriptional activation (Fig. 1) and the BHLF1 RNA transcript has been reported to form an RNA-DNA hybrid that promotes oriLyt replication *in cis* by inducing DNA strand separation (50), we did not find that oriLyt methylation affects the replication efficiency of an oriLyt-containing plasmid when the essential core viral replication proteins (and the BZLF1 and BRLF1 proteins) are supplied *in trans* (Fig. 8). Furthermore, we found that NOKs-Akata cells, which are stably infected with a highly unmethylated form of the viral genome, release infectious viral particles following transfection with a BRLF1 (but not BZLF1) expression vector (Fig. 9). Together, these results show that both the unmethylated and methylated forms of oriLyt can serve as templates for lytic viral replication, provided that the essential *trans*-acting viral replication proteins are expressed in the cell.

Although we are unaware of studies examining the viral genome methylation status in oral hairy leukoplakia (OHL) lesions, the EBV genome in these lesions is likely to be mostly (if not totally) unmethylated, given the high level of lytic protein expression and viral replication and the fact that there is no known reservoir of persistent latent infection in this lesion (1, 8, 67). Our findings here indicate that the combination of BZLF1 and BRLF1 can induce lytic gene expression from the unmethylated form of the EBV genome, and this is consistent with the high level of lytic EBV replication observed in OHL lesions. In contrast, the inability of EBV to replicate lytically in B cells prior to viral genome methylation may reflect insufficient activation of BRLF1 expression by cellular factors in this cell type, such that BRLF1 expression is instead activated primarily by BZLF1, which can occur only from a methylated viral genome. The results of our NOKs-Akata cell studies suggest that EBV can indeed replicate from a highly unmethylated viral genome when BRLF1 is not limiting, and in future studies it will be interesting to determine if this phenomenon is cell type dependent. We are in the process of more completely defining the precise methylation status of various latent and lytic EBV promoters in NOKs-Akata cells using the bisulfite conversion technique. In the meantime, the finding that lytic viral promoters remain highly unmethylated in this cell type suggests that it may be useful for identifying viral and/or cellular proteins that regulate EBV genome methylation.

Finally, our finding that promoter DNA methylation inhibits the ability of BRLF1 to activate at least one late viral gene promoter, if confirmed for other late viral promoters, suggests a mechanism by which late gene promoters can be expressed in a replication-dependent manner. Since late gene promoters in EBV generally do not contain ZREs, while at least a subset of late viral promoters contain RREs and can be activated by BRLF1 (33, 37), EBV late gene transcription may be primarily BRLF1 rather than BZLF1 dependent. If so, lytic viral replication, by converting the

late promoters to an unmethylated form, would then be predicted to enhance the ability of late viral promoters to be activated by BRLF1 in cells latently infected with highly methylated viral genomes.

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