Cellular Transcription Factor Oct-1 Interacts with the Epstein-Barr Virus BRLF1 Protein To Promote Disruption of Viral Latency[▽]

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The Epstein-Barr virus (EBV) latent-to-lytic switch is an essential part of the viral life cycle, but the cellular factors that promote viral reactivation are not well defined. In this report, we demonstrate that the cellular transcription factor Oct-1 cooperates with the EBV immediate-early protein BRLF1 (R, Rta) to induce lytic viral reactivation. We show that cotransfected Oct-1 enhances the ability of BRLF1 to activate lytic gene expression in 293 cells stably infected with a BRLF1-defective EBV mutant (BRLF1-stop) and that Oct-1 increases BRLF1-mediated activation of lytic EBV promoters in reporter gene assays. We find that Oct-1 interacts directly with BRLF1 *in vitro* and that a mutant BRLF1 protein (the M140A mutant) attenuated for the ability to interact with Oct-1 *in vitro* is also resistant to Oct-1-mediated transcriptional enhancement in 293 BRLF1-stop cells. Furthermore, we show that cotransfected Oct-1 augments BRLF1 binding to a variety of lytic EBV promoters in chromatin immunoprecipitation (ChIP) assays (including the BZLF1, BMRF1, and SM promoters) and that BRLF1 tethers Oct-1 to lytic EBV promoters. In addition, we demonstrate that an Oct-1 mutant defective in DNA binding (the S335D mutant) still retains the ability to enhance BRLF1 transcriptional effects. Finally, we show that knockdown of endogenous Oct-1 expression reduces the level of constitutive lytic EBV gene expression in both EBV-positive B-cell and EBV-positive epithelial cell lines. These results suggest that Oct-1 acts as a positive regulator of EBV lytic gene expression and that this effect is at least partially mediated through its interaction with the viral protein BRLF1.

Epstein-Barr virus (EBV), or human herpesvirus 4 (HHV4), is a double-stranded DNA gammaherpesvirus which infects approximately 90% of the world's population (66). It causes a relatively mild primary disease if acquired early in life and infectious mononucleosis if acquired after adolescence. EBV is also associated with several B-cell and epithelial cell cancers, including Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma (NPC), and gastric carcinoma (66, 101).

EBV infection persists for the life of the host as a chronic asymptomatic infection by establishing latency in memory B cells (77). However, to be transmitted from host to host, and from cell to cell, the virus periodically reactivates from latency and converts to the lytic form of replication (66). Reactivation of lytic EBV infection in host cells is controlled at the level of the BZLF1 (Z, Zta, ZEBRA, EB1) and BRLF1 (R, Rta) viral promoters, as well as the activity of the BZLF1 and BRLF1 gene products (24, 42, 95). The BZLF1 and BRLF1 genes encode transcription factors that cooperatively turn on the expression of the early lytic viral genes involved in lytic viral replication (2, 9, 16, 17, 23, 24, 26, 29, 31, 41, 50, 62, 68), and the overexpression of either BZLF1 or BRLF1 is sufficient to induce lytic gene expression in many latently infected cell lines (13–15, 63, 83, 96). Since the combination of both BZLF1 and BRLF1 is commonly required to induce the expression of early lytic genes in the context of the virus, BZLF1 and BRLF1 must

first activate one another's promoters in order to induce full lytic gene expression (2, 24, 51, 96).

The BRLF1 protein is a homolog of the Kaposi's sarcoma-associated herpesvirus (KSHV) ORF50 immediate-early (IE) gene product (Rta), which mediates viral reactivation in KSHV-infected cells (54, 81, 94, 100). Both KSHV Rta and EBV BRLF1 contain a DNA-binding and homodimerization domain at the amino terminus and a transcriptional activation domain at the carboxy terminus (29, 53, 56, 73, 86). However, the mechanisms by which KSHV Rta and EBV BRLF1 activate transcription are quite distinct. BRLF1 activates some lytic EBV promoters (including the SM, BMRF1, and BHRF1 promoters) by binding directly to a GC-rich sequence (consensus GNCCN9GGNG) known as the BRLF1-responsive element (RRE) (12, 28–30, 62). Direct BRLF1 binding to promoters often results in very strong promoter activation, since BRLF1 can function as an enhancer factor when bound directly to certain RREs (16, 29, 40). The BRLF1 protein is also thought to activate the transcription of some genes (including the BZLF1 gene and the BRLF1 gene) without binding directly to their promoters (1, 17, 48, 51, 64). Although the mechanism(s) by which BRLF1 activates some promoters indirectly has not been as well studied, the ability of BRLF1 to activate its own promoter has been reported to be mediated through SP1 binding sites and to involve direct interactions between SP1, MCAF1, and BRLF1 (9, 10, 64, 97). In addition, BRLF1 has been shown to activate the BZLF1 promoter by enhancing signal transduction cascades (including the JNK, ERK, and p38 map kinases) that induce the phosphorylation of transcription factors such as c-Jun/ATF2 (1, 17, 48).

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KSHV Rta also activates some promoters through a direct binding mechanism, but its consensus DNA-binding motif (AACAATAATGTT) is quite different from that of the EBV homolog (6, 11, 19, 21, 52, 76, 79), and most Rta-responsive KSHV promoters do not contain this motif. Instead, Rta activation of most viral promoters is mediated through an interaction between Rta and various cellular transcription factors (49, 60, 87, 88, 98). The most well documented of these interactions is with the cellular factor RBP-Jkappa, which serves to tether Rta to RBP-Jkappa (Notch) binding sites in KSHV promoters (49, 60). In contrast, the EBV BRLF1 protein is not thought to interact directly with RBP-Jkappa (49) or activate promoters through Notch binding motifs, although a large number of Notch binding sites have been reported in the EBV genome (60).

The KSHV Rta protein also interacts directly with the cellular Pit-Oct-Unc (POU) domain transcription factor Oct-1, and this interaction is required for the ability of KSHV Rta to activate the early lytic K-bZIP (K8) promoter (7), as well as its own promoter (70), in certain cell lines. Interestingly, Oct-1 also interacts directly with herpes simplex virus (HSV)- and varicella-zoster virus (VZV)-encoded tegument proteins (VP16 and ORF10, respectively), and this interaction is required for the efficient activation of the HSV and VZV immediate-early promoters (34, 45, 46, 58, 93). While Oct-1 and the related POU domain transcription factor, Oct-2, have been previously suggested to play a role in regulating the type of EBV latency (3, 55, 90), neither protein has been shown to play a role in EBV lytic reactivation.

The POU family of transcription factors shares a highly conserved POU domain consisting of two characteristic regions: the specific domain and the homeodomain. The POU domain mediates binding to the octamer DNA motif (consensus site ATGCAAT) (39, 61, 69). Oct-1 is ubiquitously expressed and is involved in dynamic cellular processes such as chromatin structuring (39, 74). In B cells, Oct-1 has been shown to activate immunoglobulin gene transcription in association with the B-cell-specific coregulator, Bob-1 (8, 43). A role for Oct-1 as a stress sensor that responds to DNA damage has also been suggested, since Oct-1 protein expression is increased in response to stress and Oct-1 modulates genes important for the cellular stress response (72, 85, 99). Recently, Oct-1 has been reported to recruit the KDM3A histone lysine demethylase, as well as the NuRD chromatin-remodeling complex, to DNA (74).

Here, we report that Oct-1 enhances BRLF1-mediated disruption of EBV latency. We find that Oct-1 increases the amount of BRLF1 complexed to a variety of different lytic EBV promoters, including the BZLF1 promoter. Furthermore, using a variety of approaches, we show that the ability of Oct-1 to boost BRLF1-mediated lytic induction involves a direct protein-protein interaction between Oct-1 and BRLF1 but does not require Oct-1 itself binding to DNA. Finally, we show that short hairpin RNA (shRNA)- and small interfering RNA (siRNA)-mediated knockdown of endogenous Oct-1 expression decreases constitutive lytic gene expression in both EBV-infected B cells and epithelial cells. These results suggest that Oct-1 acts as an important positive regulator of EBV lytic reactivation through direct protein-protein interactions with BRLF1.

MATERIALS AND METHODS

Construction of the BRLF1-stop EBV mutant. BRLF1 stop codon mutations were inserted into the EBV B95.8 bacmid p2089 (a gift from W. Hammer-schmidt, Helmholtz Zentrum München), which has the complete genome of B95.8 strain EBV, plus inserted green fluorescent protein (GFP) and hygromycin resistance genes, as previously described (18). Stop codons were inserted in residues 4 and 5 in the BRLF1 open reading frame in the EBV bacterial artificial chromosome by site-directed mutagenesis as previously described (57, 75, 95). The wild-type (WT) EBV sequence (104575 to 105907) flanking the BRLF1 start site was PCR amplified (using the primers 5'-AAGAGCTCCTCGAGGACGG GATAGGTGAACACC-3' and 5'-GCTCTAGACTCGAGGTGAGGAGCT GGGAG-3') and cloned into pSP65 by using XbaI and SacI restriction enzymes to make pSP65-R1.28. Site-directed mutagenesis was performed on pSP65-R1.28 to convert the BRLF1 lysine residues at amino acids 4 and 5 to stop codons (using the primers 5'-CTAGCCATGAGGCCTTAATAGGATGGCTTGGAA GACTTTCTG-3' and 5'-CAGAAAGTCTTCCAAGCCATCCTATTAAGGCC TCATGGCTAG-3') according to the manufacturer's protocol (Stratagene). The DNA containing the mutated BRLF1 region was cut out of pSP65 by using XbaI and SacI and ligated into the shuttle vector pGS284 (a gift from W. Hammer-schmidt, Helmholtz Zentrum München), cut with NheI and SacI to yield pGS284-BRLF1-Stop. pGS284-BRLF1-Stop in S17 λ pir *Escherichia coli* was conjugated with the wild-type EBV bacmid p2089 in GS500 *E. coli*. Cointegrates were selected in LB broth containing carbenicillin and chloramphenicol. Cultures were then recovered in LB broth containing chloramphenicol only and plated on LB plates containing 5% sucrose and chloramphenicol. Colonies were screened by PCR using oligonucleotides to detect the mutated DNA sequence (5'-GAGCCATTTGAGGAACTGA-3'; 5'-GCGGATCCCTTTAGCCGCACA TCCAGATCTT-3'). Colonies containing the correct mutations were further screened by DNA sequencing, and restriction enzyme analysis was performed comparing the BRLF1-stop bacmid with the wild-type bacmid DNA (by using BamHI, HindIII, SalI, and EcoRI independent digestions) to make certain that no deletions or rearrangements of the viral genome had occurred.

Creation of the 293 BRLF1-stop cell line. 293 cells were transfected with the EBV BRLF1-stop mutant bacmid construct by using Lipofectamine 2000 transfection reagent (Invitrogen) as described in the manufacturer's protocol. Selection of stable 293 cell clones that carry the EBV BRLF1-stop plasmid was performed in Dulbecco's modified Eagle medium (DMEM) with 100 μ g/ml hygromycin. Clones were then examined for their ability to produce infectious virus by transfecting them with expression plasmids for BZLF1 and/or BRLF1 (plus gp110) to induce lytic replication, and the supernatant from cells was used to infect Raji cells. The 293 BRLF1-stop cell clone that produced the highest virus titer in the presence of both BZLF1 and BRLF1 was frozen at early passage and used for further study.

Cell lines and culture. EBV-negative HEK 293 and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. EBV-positive 293 BRLF1-stop cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 100 μ g/ml hygromycin. B. Mutu1 cells (a gift from Jeff Sample, Penn State) are an EBV-positive type I Burkitt lymphoma cell line and were maintained in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin. Hone-Akata cells (a gift from Lawrence Young, University of Birmingham) and CNE-2 Akata cells (a gift from K. W. Lo, The Chinese University of Hong Kong [received via Diane Hayward]) are NPC epithelial cell lines superinfected with the Akata strain of EBV and were maintained in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, and G418 (400 μ g/ml). Raji cells (ATCC), an EBV-positive type III Burkitt lymphoma cell line, were maintained in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin.

Plasmids, cloning, and site-directed mutagenesis. Plasmid DNA was purified on maxiprep columns according to the manufacturer's protocol (Qiagen). pSG5 and pcDNA3.1(+) were obtained from Stratagene and Invitrogen, respectively. The SG5-BRLF1 expression vector (a gift from S.D. Hayward, Johns Hopkins University) contains the B95.8 BRLF1 open reading frame under the control of the simian virus 40 (SV40) early promoter in the pSG5 vector (Stratagene) and was constructed as previously described (31). The cDNA-Oct-1 expression vector was cloned by PCR amplifying Oct-1 from pCGN-Oct-1 (a gift from Winship Herr, Cold Spring Harbor Laboratory) (84) and inserting it into pcDNA3.1(+) (Invitrogen) by using BamHI and XhoI restriction sites. Plasmids SG5-BRLF1(M140A), SG5-BRLF1(aa1-550), and cDNA-Oct-1(S335D) were constructed by using the Stratagene QuikChange II XL site-directed mutagenesis kit and the following primer sets: BRLF1(M140A) forward (5'-GGCACCGCTGC GTACAAGCTCCTAAACACAGTCGG-3') and reverse (5'-CCGACTGTG TTTTAGGAGCTTGTACGACGCGGTGCC-3'); BRLF1(aa1-550) forward

(5'-CCCCTCGTGGCCATTGTAGGAAGTACCACAACACTAGAGTCC-3') and reverse (5'-GGACTCTAGTGTGTGGTCAGTTCCTACAATGGC CACGAGGGG-3'); and Oct-1(S335D) forward (5'-GAAGCCTGAACCTCG ACTTAAAGAACATGTGCAAGTTGAAGCC-3') and reverse (5'-GGCTTCA ACTTGACATGTTCTTAAAGTCGAGGTTCAAGGCTTC-3'). pGEX-KG was a gift from Eric Turner (University of California-San Diego) (65). GST-Oct-1 was cloned by PCR amplifying the POU domain of Oct-1 from the cDNA-Oct-1 plasmid and inserting it into pGEX-KG by using HindIII and SalI restriction sites. The Smp and BMR1p-luciferase reporter gene constructs contain the B95.8 strain sequence from -595 to +15 and -553 to +16, respectively (relative to transcription start site) inserted upstream of the luciferase gene in pGL3-basic (Promega) using MluI and BglII restriction sites. The BHLF1p and BHRF1p-luciferase reporter gene constructs were constructed by PCR amplifying the divergent BHLF1 and BHRF1 promoter sequences (from 52781 and 53797) within the oriLyt region from the EBV B95.8 genomic DNA and inserted upstream of the luciferase gene in pGL3-basic (Promega) by using BamHI restriction sites. The BZLF1p-luciferase reporter gene construct contains the B95.8 strain BZLF1p sequence from -221 to +28 (relative to the BZLF1 transcription start site) inserted upstream of the luciferase gene in pGL3-basic (Promega) and was constructed as previously described (4).

Transient transfections. 293 BRLF1-stop cells were transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to manufacturer's instructions. HEK 293 cells were transfected using FuGENE6 Transfection Reagent (Roche) according to manufacturer's instructions. Each transfection experiment was performed at least three separate times with similar results. In general, cells were transfected in a 12-well dish with limiting amounts of wild-type or mutant BRLF1 (50 to 75 ng), wild-type or mutant Oct-1 (400 ng), or control expression vectors (500 ng of total DNA per well). Since Oct-1 regulates the SV40 promoter (27) (which drives BRLF1 in the SG5-BRLF1 vectors), immunoblot assays were performed to identify conditions with similar levels of BRLF1 expression, and only conditions with similar BRLF1 expression were used in the various assays.

Immunoblotting. Immunoblotting was performed as previously described (5, 82). Cell lysates were harvested in SUMO lysis buffer containing proteasome inhibitor cocktail (Roche) and quantitated by SUMO protein assay (Bio-Rad). Equivalent amounts of protein were separated in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and transferred to membranes. Membranes were blocked in phosphate-buffered saline (PBS) containing 5% milk and 0.1% Tween 20 solution and incubated with primary antibody. The following antibodies were used: anti- β -actin (Sigma; 1:5,000), BMRF1 (Vector; 1:250), Oct-1 (Santa Cruz, sc-71744; 1:250), BRLF1 (Argene; 1:250), and BZLF1 (Santa Cruz, sc-53904; 1:250). The polyclonal rabbit antibody against SM (1:1,000) was a gift from S. Swaminathan (University of Utah). The secondary antibodies used were horseradish peroxidase (HRP) goat-anti-mouse (Fisher Scientific; 1:5,000) and HRP goat-anti-rabbit (Fisher Scientific; 1:10,000). Quantification of immunoblots was performed using ImageJ software.

Virus lytic replication assays. Virus lytic replication titration assays were performed as previously described (35). 293 BRLF1-stop cells were transfected in a 6-well dish with 100 ng BRLF1, 400 ng Oct-1, or vector controls; an expression vector for the EBV gp110 protein (500 ng) was also cotransfected (total DNA of 1 μ g). Again, since Oct-1 regulates the SV40 promoter (27) (which drives BRLF1 in the SG5-BRLF1 vectors), immunoblot assays were performed to identify conditions with similar levels of BRLF1 expression, and only conditions with similar BRLF1 expression were used in the viral titration assay. After 72 h, supernatant from the transfected cells was collected and filtered through a 0.8- μ m-pore-size filter. The filtered virus was used to infect Raji cells (4×10^5 cells/infection). Phorbol-12-myristate-13-acetate (TPA; 20 ng/ml) and sodium butyrate (3 mM final concentration) were added at 24 h postinfection. Green fluorescent protein (GFP)-positive Raji cells were counted 48 h after infection to determine viral titer. Experiments were performed twice under each condition.

Reporter gene assays. For Smp, BMRF1p, BHRF1p, and BHLF1p-luciferase assays, cells were transfected in a 12-well dish with 50 ng of luciferase construct, 2 ng of BRLF1, 200 ng of Oct-1, or control expression vectors (500 ng total DNA per well). For BZLF1p-luciferase assays, cells were transfected in a 12-well dish with 50 ng of luciferase construct, 20 ng of BRLF1, 200 ng of Oct-1, or control expression vectors (500 ng total DNA per well). Cells were harvested 48 h posttransfection in reporter lysis 5 $\times$ buffer (Promega). Relative luciferase units were measured in a BD Monolight 3010 luminometer (BD Biosciences) by using Promega luciferase assay reagent. Each condition was performed in triplicate.

GST pulldown assays. Glutathione S-transferase (GST) and GST-Oct-1 expression vectors were propagated in DH5 α *E. coli* overnight. Cultures were diluted 1:10, grown 2 h, and then induced using 0.4 mM IPTG (isopropyl- β -D

thiogalactopyranoside) for an additional 2 h. GST proteins were collected by sonication followed by incubation with glutathione-agarose beads (Sigma-Aldrich), rocking for 1 h at room temperature. The beads were washed 3 times in NETN⁺ buffer (7) (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, protease inhibitors) and added to ³⁵S-labeled *in vitro*-translated BRLF1 protein. *In vitro*-translated proteins were generated using the TNT T7 quick coupled transcription/translation system (Promega) in accordance with the manufacturer's instructions. The reaction mixture was incubated for 1 h with rocking at 4°C. The beads were washed 3 times in NETN⁺ buffer. An equal volume of 2 $\times$ SDS sample buffer was added, and proteins were extracted by heating at 95°C for 10 min.

EMSA. T4 polynucleotide kinase (NEB) and [γ -³²P]ATP (PerkinElmer) were used to label double-stranded, annealed DNA oligonucleotides for use in DNA-protein binding experiments. The Smp RRE probe consisted of a 37-bp sequence containing the BRLF1-responsive element (underlined) in the SM promoter (SM positions -365 to -401; 5'-GGCCAGATGTCCTCTATCATG GCGCAGACATTCTC-3'). The Oct probe consisted of an 18-bp sequence containing the octamer consensus sequence (underlined) surrounded by random nucleotides (5'-CAGTGATGCAAATCTTGT-3'). The protein samples were used in electrophoretic mobility shift assays (EMSAs) were whole-cell extracts harvested from transfected HeLa cells, as previously described (12), or *in vitro*-translated protein (made using TNT T7 quick coupled transcription/translation system [Promega]).

ChIP assays. 293 BRLF1-stop cells were transfected in 10-cm dishes (1 μ g BRLF1, 5 μ g Oct-1, or control vector). Cells were first cross-linked in 1.5 mM EGS (ethylene glycolbis[succinimidyl succinate]) for 30 min at room temperature followed by cross-linking in fresh 1% paraformaldehyde for 10 min at room temperature. The cross-linking reaction was quenched using 125 mM glycine. Following cell lysis and DNA fragmentation by sonication, DNA-protein complexes were immunoprecipitated with anti-BRLF1 (Argene), a mix of anti-Oct-1 (Millipore and Santa Cruz), and control anti-IgG (Santa Cruz) antibodies. Immunoprecipitated DNA-protein complexes were washed using sequential low-salt, high-salt, lithium chloride and Tris-EDTA (TE) wash buffers. Protein-DNA cross-linking was reversed at 65°C overnight. DNA was purified using the Qiagen gel extraction kit. The presence of specific DNA fragments in each precipitate was detected using PCR. Primers used for amplifying the SM promoter were 5'-CGTGACATGGAGAACTGGGGG-3' and 5'-CCTCTTACATCACTCA CTGCACG-3'; those for the BMRF1 promoter were 5'-ATGCCAGAAACC TGAGCAAGTAGCC-3' and 5'-CCTGTGGTGGATGTGCGACCATAAAG-3'; those for the BZLF1 promoter were 5'-GGTGTAATTTTACCTCTTC-3' and 5'-GCTAATGTACCTCATAGACACACC-3'; those for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were 5'-TCACCACCATGGAGAAGG CT-3' and 5'-GCCATCCACAGTCTTCTGGG-3'; and those for Hs Gadd45a were 5'-CTCCTCTCAACCTGACTCCAGAG-3' and 5'-TCCGGGGTTATC CTGCCAAC-3'. Quantification of each chromatin immunoprecipitation (ChIP) experiment was performed using ImageJ software.

Infection with lentiviruses expressing shRNAs. Lentiviral vectors expressing 5 different Oct-1-directed short hairpin RNAs (shRNAs) (target set RHS4533-NM_002697) and the universal negative control pLKO.1 (RHS4080) were purchased from Open Biosystems (ThermoScientific) and propagated according to the manufacturer's instructions. 293T cells were cotransfected with lentiviral vector(s) plus DNA vectors encoding HIV Gag/Pol and vesicular stomatitis virus G (VSV-G) (for the packaging of lentiviruses) in 10-cm dishes. Medium (containing lentivirus) was harvested 72 h later and filtered through 0.8- μ m-pore-size filters. Mutu1 and Hone-Akata cells were infected by incubation with filtered medium containing the lentivirus. After 72 h, stable cell lines were selected for 7 days by treatment with 1 μ g/ml puromycin. Infection with lentivirus shRNAs was performed in duplicate for each cell type.

siRNA experiments. Small interfering RNAs (siRNAs) were transfected into cells by using Xtreme gene (Roche) according to the manufacturer's protocol. Control 2 siRNA targeting a nonspecific sequence was obtained from Ambion. Oct-1 siRNA (Santa Cruz Biotechnology; sc-36119) contains a mixture of Oct-1 siRNA sequences which target human Oct-1.

RT-PCR. RNA was harvested from control and shOct-1 Mutu1 cells by using the Qiagen RNeasy mini kit according to the manufacturer's instructions. Isolated RNA was quantitated and DNase treated. Reverse transcriptase (RT)-PCR analysis was performed to determine the transcript levels of the endogenous lytic viral BRLF1, BZLF1, and BMRF1 genes and the cellular β_2 -microglobulin gene. PCR primers used to detect BRLF1 transcript were 5'-CCGGAGTGTGGA GATTACTCTGA-3' and 5'-TCTTGGACGAGACCATAGTCTGGA-3'; those for BZLF1 transcript were 5'-CACGGTAGTGCTGCAGCAGTTGC-3' and 5'-CCCAGATCAACAGACTAACCAGCCG-3'; those for BMRF1 transcript were 5'-GGCGGCCCGCAGTACGGCC-3' and 5'-TTAATGAGGGGGTTA

FIG. 1. Oct-1 enhances BRLF1-mediated disruption of viral latency. (A) 293 cells stably infected with an EBV mutant that does not express BRLF1 (293 BRLF1-stop cells) were transfected with 50 ng BRLF1, 400 ng Oct-1, or control expression vectors as indicated. Immunoblot analysis was performed 2 days after transfection to compare the levels of transfected Oct-1 and BRLF1, as well as the lytic viral proteins BMRF1, SM, and BZLF1 derived from the endogenous viral genome. β -Actin expression was used as a loading control. (B) 293 BRLF1-stop cells were transfected with control vector, Oct-1, or decreasing amounts (250, 150, 100, 75, and 50 ng) of BRLF1 protein as indicated. Immunoblot analysis was performed 2 days after transfection to compare the levels of transfected Oct-1 and BRLF1, as well as the lytic viral proteins BMRF1 and BZLF1 derived from the endogenous viral genome. β -Actin served as a loading control. BMRF1 and BZLF1 protein bands were quantified using ImageJ software and are represented as numerical values in bar diagrams at the bottom of the gel; the amount of each protein obtained in cells transfected with 50 ng of BRLF1 alone was set as 1. (C) 293 BRLF1-stop cells were transfected with BRLF1, Oct-1, or a control vector as indicated (plus an EBV gp110 expression vector), and the amount of infectious virus produced by each condition was determined using the green Raji cell assay as described in Materials and Methods.

AAGGCCTG-3'; and those for β_2 -microglobulin transcript were 5'-TTCTGGC CTGAGGGCATCC-3' and 5'-ATCTTCAAACCTCCATGATG-3'.

RESULTS

Oct-1 enhances BRLF1-mediated disruption of viral latency. To determine if Oct-1 has an effect on BRLF1-mediated disruption of viral latency, we transfected limiting amounts of BRLF1 with or without cotransfected Oct-1 into 293 cells stably infected with a BRLF1-defective EBV mutant (BRLF1-stop). The BRLF1-stop mutant, which contains two stop codons inserted into the BRLF1 open reading frame, was constructed as described in Materials and Methods and cannot replicate unless it is rescued by a cotransfected BRLF1 expression vector (data not shown). In contrast to a previously described BRLF1 knockout mutant (24, 35), the BRLF1-stop mutant does not alter the promoter sequence or open reading frame of the BRRF1 gene (encoded on the opposite strand of the BRLF1 gene intron) and thus can be used to specifically examine BRLF1 function.

As shown in Fig. 1A, cotransfected Oct-1 improved the abil-

ity of BRLF1 to activate the expression of the other EBV immediate-early protein, BZLF1, in 293 BRLF1-stop cells. The combination of Oct-1 and BRLF1 also increased the expression of two different early lytic EBV proteins (BMRF1 and SM) in comparison to the effect of BRLF1 alone. We also examined the effect of Oct-1 in the presence of increasing amounts of transfected BRLF1 (Fig. 1B). The lytic induction effect of 50 ng BRLF1 plus cotransfected Oct-1 was similar to that of 150 to 250 ng of BRLF1 alone. Furthermore, cotransfected Oct-1 increased the ability of BRLF1 to activate BMRF1 and BZLF1 expression by at least 35-fold and 15-fold, respectively, when a limiting amount (50 ng per 12-well dish) of the BRLF1 expression vector was transfected. These results suggest that Oct-1 acts as a positive regulator of EBV lytic reactivation by enhancing BRLF1-mediated gene activation.

To determine if Oct-1 increases the ability of BRLF1 to induce lytic viral replication, 293 BRLF1-stop cells were transfected with limiting amounts of BRLF1 in the presence or absence of Oct-1 (along with a gp110 expression vector required for efficient replication in B95.8 bacmid-infected cells)

(24), and the amount of infectious virus released into the supernatant was titrated 3 days later using the green Raji cell assay. As shown in Fig. 1C, the combination of Oct-1 and BRLF1 resulted in a greater-than-4-fold increase in the amount of infectious virus produced compared to the effect of BRLF1 alone. The levels of BRLF1 protein expressed in the 293 BRLF1-stop cells were similar in the presence or absence of cotransfected Oct-1 (Fig. 1A and B; also data not shown). Together, these results indicate that Oct-1 enhances BRLF1-mediated lytic viral reactivation.

Physiologic levels of Oct-1 enhance BRLF1-mediated lytic viral reactivation. The experiments shown in Fig. 1, which used 400 ng of transfected Oct-1 expression vector (per 12-well dish), resulted in Oct-1 levels that were greatly above physiologic levels (data not shown). To determine if a physiologically relevant level of Oct-1 also enhances BRLF1-mediated lytic reactivation, we titrated the amount of Oct-1 expression vector (following transfection into 293 BRLF1-stop cells) required to match the level of Oct-1 constitutively expressed in the Burkitt lymphoma Mutu1 cell line. These experiments indicated that 50 ng of Oct-1 vector results in a level of Oct-1 expression in 293 BRLF1-stop cells similar to that constitutively expressed in Mutu1 cells (Fig. 2A). Importantly, this amount of transfected Oct-1 retained the ability to improve BRLF1-mediated lytic reactivation in 293 BRLF1-stop cells (Fig. 2B).

Given the observation that Oct-1 levels are variable between different cell types (for example, Mutu1 cells express more Oct-1 than 293 BRLF1-stop cells) (Fig. 2A), we next examined if the Oct-1 protein level is increased during lytic viral reactivation. Lytic viral reactivation was induced using different methods (TPA/sodium butyrate [SB], transforming growth factor β [TGF- β], or BZLF1 transfection) in a variety of different EBV-positive B-cell or epithelial cell lines (Fig. 2C). Although each treatment resulted in early lytic BMRF1 protein expression as expected, there was no consistent effect on Oct-1 expression. This result suggests that treatments which induce lytic reactivation do not increase the level of total Oct-1 protein, although the possibility that such treatments induce post-translational modifications of Oct-1 cannot be excluded.

Oct-1 increases BRLF1 activation of a variety of EBV lytic promoters in EBV-negative cells. Given that BRLF1 and BZLF1 cooperate to turn on the expression of most early lytic EBV genes in the context of the intact viral genome, the ability of Oct-1 to increase BRLF1-induced lytic gene transcription in the context of the intact virus could potentially involve Oct-1 effects on BRLF1 and/or BZLF1 function. To examine the effect of Oct-1 on BRLF1 transcriptional function in the absence of BZLF1, we performed reporter gene assays in EBV-negative cells. As shown in Fig. 3, cotransfected Oct-1 enhanced the ability of BRLF1 to activate each of the EBV early lytic promoters tested, including the BZLF1 promoter, in EBV-negative 293 cells. However, Oct-1 by itself did not significantly affect the constitutive activity of any lytic EBV promoter. While the SM, BMRF1, BHLF1, and BHRF1 promoters are known to contain RREs which are directly bound by BRLF1 *in vitro* (12, 28, 30), the BZLF1 promoter is not thought to be bound by BRLF1 directly (1). These results suggest that Oct-1 can increase BRLF1-mediated transcriptional effects in the presence or absence of direct BRLF1 binding to promoters and/or that Oct-1 enhances BRLF1 bind-

FIG. 2. Physiologic levels of Oct-1 enhance BRLF1-mediated lytic viral reactivation. (A) 293 BRLF1-stop cells were transfected with control vector or 50 ng of Oct-1 expression vector (with or without BRLF1) as indicated. Immunoblot analysis was performed 2 days after transfection to compare the levels of transfected and endogenous Oct-1 in 293 BRLF1-stop cells and untreated Mutu1 cells. β -Actin served as a loading control. (B) Immunoblot analysis was performed on the same 293 BRLF1-stop extracts shown in panel A to compare the levels of transfected BRLF1, as well as the lytic viral proteins BMRF1 and BZLF1 derived from the endogenous viral genome. β -Actin served as a loading control. (C) Various EBV-positive epithelial (CNE-2 Akata, Hone-Akata) and B-cell (Mutu1, Raji) lines were lytically induced either with chemical inducers (TPA/sodium butyrate) or with TGF- β (5 μ g/ml) or were transfected with BZLF1 as indicated. Immunoblot analysis was performed 2 days after induction/transfection to compare the levels of endogenous Oct-1, viral BMRF1, and β -actin (loading control).

ing to a weak RRE in the BZLF1 promoter. In addition, the finding that Oct-1 regulates BRLF1 in EBV-negative cells shows that Oct-1 enhances BRLF1 transcriptional function independent of any effect of Oct-1 on BZLF1 transcriptional function.

BRLF1 interacts directly with Oct-1 *in vitro*, and methionine residue 140 contributes to this interaction. Given that the KSHV homolog of BRLF1, KSHV Rta, directly interacts with Oct-1 (7), we next examined whether EBV BRLF1 is likewise able to associate with Oct-1 directly. Using GST pulldown assays performed with *in vitro*-translated 35 S-labeled BRLF1 protein and GST or GST-Oct-1 fusion proteins, we found that BRLF1 is indeed able to associate with GST-Oct-1 but not

FIG. 3. Oct-1 increases BRLF1 activation of a variety of EBV lytic promoters in EBV-negative cells. EBV-negative 293 cells were transfected with SMP-luciferase (A), BMRF1p-luciferase (B), BHLF1p-luciferase (C), BHRF1p-luciferase (D), or BZLF1p-luciferase (E) constructs in the presence or absence of cotransfected BRLF1, Oct-1, or control vectors as indicated. The fold luciferase activity for each condition is shown relative to that of the control vector; the value for the activity of the promoter construct plus the vector control is set at 1. Values are given as means $\pm$ standard deviations of results from three replicates. Immunoblot analysis was performed to determine the amounts of Oct-1, BRLF1, and β -actin (loading control) in the extracts used for the luciferase assays; BRLF1 levels were similar in the presence or absence of cotransfected Oct-1 (data not shown).

with the control GST protein (Fig. 4A). A BRLF1 mutant missing the C-terminal transactivator domain, BRLF1(aa1-550), retains the ability to interact with Oct-1 (Fig. 4B).

Since a specific region in the KSHV Rta DNA-binding domain (residues 136 to 144) has been shown to be important for its interaction with Oct-1 (7), we next determined if the same region of BRLF1 (based on EBV BRLF1 sequence homology with the Oct-1-interacting region of KSHV Rta) is also required (Fig. 4C). BRLF1 point mutations that alter amino acid residues predicted to mediate the interaction with Oct-1 (amino acids 136, 140, 143, and 144) were generated, and each mutant was compared with wild-type BRLF1 for the ability to interact with Oct-1 in a GST pull-down assay. As shown in Fig. 4D, the BRLF1(M140A) mutant has a reduced interaction with the GST-Oct-1 protein in comparison to the wild-type BRLF1 protein. Three other BRLF1 mutants (the L143A, L144A, and L143/144A mutants) were also found to be impaired for interaction with Oct-1 *in vitro* (data not shown), but since these mutants were found to be unstable when expressed in mammalian cells, they were not investigated further. These results indicate that a region of the EBV BRLF1 protein required for mediating the direct interaction with Oct-1 (residues 140 to 144) is similar to that mediating the Oct-1 interaction in the homologous KSHV Rta protein (Fig. 4C).

Since BRLF1 methionine residue 140 is within the DNA-binding and dimerization domain, we also investigated whether mutation of BRLF1 residue 140 disrupts its ability to bind DNA *in vitro*. As shown in Fig. 4E, whole-cell extracts obtained from HeLa cells transfected with wild-type or BRLF1(M140A) mutant expression vectors contained similar levels of BRLF1 binding activity in EMSAs. We cannot observe Oct-1 complexed to BRLF1 in EMSAs (presumably because the complex

does not survive the harsh EMSA conditions). Taken together, these results indicate that EBV BRLF1 interacts with Oct-1 *in vitro* and that BRLF1 methionine residue 140 is important for mediating this interaction, but this residue is not essential for BRLF1 binding to DNA *in vitro*.

BRLF1 residue M140 contributes to the Oct-1 enhancement of BRLF1 activity. To determine whether the protein-protein interaction between Oct-1 and BRLF1 is important for the ability of Oct-1 to enhance BRLF1 transcriptional function, we compared the ability of Oct-1 to increase wild-type BRLF1-versus BRLF1(M140A) mutant-mediated disruption of viral latency. 293 BRLF1-stop cells were transfected with a limiting amount of wild-type or BRLF1(M140A) mutant protein in the presence or absence of cotransfected Oct-1 and the level of induced BZLF1, SM, and BMRF1 lytic viral protein expression was examined by immunoblotting (Fig. 5A). As expected, the presence of cotransfected Oct-1 improved the ability of wild-type BRLF1 to disrupt viral latency. The BRLF1(M140A) mutant protein, which is attenuated for the ability to interact directly with Oct-1 (Fig. 4), was also defective in the ability to induce lytic gene expression in the presence of cotransfected Oct-1 (Fig. 5A). This result suggests that the ability of Oct-1 to enhance BRLF1-mediated disruption of viral latency relies on the protein-protein interaction between Oct-1 and BRLF1.

To examine the importance of the Oct-1/BRLF1 protein interaction in the absence of other EBV proteins, we compared the abilities of wild-type and BRLF1(M140A) mutant proteins, in the presence or absence of cotransfected Oct-1, to activate the SMP-luciferase and BZLF1p-luciferase constructs in EBV-negative cells. As expected, Oct-1 improved wild-type BRLF1 transcriptional activation of SMP and BZLF1p luciferase constructs (Fig. 5B and C). In comparison to its effect on

FIG. 4. BRLF1 interacts directly with Oct-1 *in vitro* and methionine residue 140 contributes to this interaction. (A) (Left) GST pull-down assays were performed using GST or GST-Oct-1 fusion proteins incubated with ³⁵S-labeled, *in vitro*-translated BRLF1 protein. Twenty percent of the direct load was used for autoradiography. (Right) A Coomassie stain demonstrating the levels of GST or GST-Oct-1 protein used in the GST pull-down assays. (B) GST pull-down assays were performed using GST and GST-Oct-1 fusion proteins incubated with ³⁵S-labeled, *in vitro*-translated wild-type BRLF1 or a BRLF1 truncation mutant containing amino acids 1 to 550 [BRLF1(aa1-550)]. Twenty percent of the direct load was used for autoradiography. (C) Alignment of octamer-interacting domains from HSV-1 VP16 and KSHV Rta with EBV BRLF1. Numbers represent amino acid positions. The amino acids in boldface type (aa 136, 140, 143, and 144) indicate positions of site-directed mutations. (D) GST pull-down assays were performed using GST and GST-Oct-1 fusion proteins incubated with increasing amounts of ³⁵S-labeled, *in vitro*-translated wild-type or BRLF1(M140A) mutant protein, as indicated. Twenty percent of the direct load was used for autoradiography. The amount of BRLF1 binding in each condition, quantified using ImageJ software, is shown on the bottom of the gel; the level of binding obtained with 30 μ l of the

the wild-type BRLF1 protein, Oct-1 had a reduced ability to increase BRLF1(M140A) mutant transcriptional activation, consistent with its reduced (but not totally absent) ability to interact with Oct-1 *in vitro* (Fig. 4). Again, these results support the hypothesis that protein-protein interactions between Oct-1 and BRLF1 enhance BRLF1 transcriptional function.

Oct-1 DNA-binding activity is not required to enhance BRLF1-mediated lytic reactivation. Since the capacity of Oct-1 to bind DNA is important for its ability to promote both HSV and KSHV lytic reactivation (7, 45, 70, 93), we next examined whether Oct-1 DNA-binding is required to enhance BRLF1-mediated lytic reactivation. For these studies, we constructed an Oct-1 mutant containing an aspartic acid in place of serine residue 335, since this change was previously reported to abolish Oct-1 DNA binding to octamer binding sites (38). We confirmed that the *in vitro*-translated Oct-1(S335D) mutant protein is impaired for DNA binding in an EMSA (Fig. 6A).

Next, we compared the abilities of the wild-type and Oct-1(S335D) mutant proteins to enhance the activity of cotransfected wild-type BRLF1 protein in 293 BRLF1-stop cells (Fig. 6B). As expected, wild-type Oct-1 augmented the ability of BRLF1 to disrupt viral latency. Somewhat surprisingly, the Oct-1(S335D) mutant protein increased the ability of BRLF1 to activate lytic gene expression to a similar degree as (if not more than) the wild-type Oct-1 protein (Fig. 6B). This result suggests that Oct-1 direct DNA-binding activity is not necessarily required for the ability of Oct-1 to enhance the BRLF1 activation of lytic EBV genes and is consistent with a model in which BRLF1 can tether Oct-1 to lytic viral promoters through a protein-protein interaction.

Oct-1 augments BRLF1 DNA-binding *in vivo*. To determine if Oct-1 enhances BRLF1 binding to lytic EBV gene promoters *in vivo*, we performed ChIP assays using extracts from 293 BRLF1-stop cells transfected with wild-type or BRLF1(M140A) mutant protein in the presence or absence of cotransfected Oct-1 (Fig. 7A). In the absence of cotransfected Oct-1, wild-type BRLF1 was associated with both the SM and BMRF1 promoters (shown in longer exposures) (which contain strong RRE binding sites) (12) but not the BZLF1 promoter or the control GAPDH gene. Consistent with its ability to enhance BRLF1-mediated transcription, Oct-1 increased the amount of wild-type BRLF1 associated with both the SM and BMRF1 promoters. Interestingly, BRLF1 was also tethered to the BZLF1 promoter in the presence of cotransfected Oct-1, even though this promoter is not known to be directly bound by BRLF1. Furthermore, the effect of Oct-1 on binding of the BRLF1(M140A) mutant protein (which has a reduced but not absent interaction with

wild-type BRLF1 protein is set at 100%. nd, Binding not detectable; Retic., reticulocyte lysate. (E) (Left) An EMSA was performed using whole-cell extracts from HeLa cells transfected with vector control, wild-type BRLF1, or BRLF1(M140A) mutant expression vectors and a ³²P-labeled oligonucleotide containing the RRE from the SM promoter, in the presence or absence of an anti-BRLF1 antibody as indicated. Protein-DNA complexes, including those supershifted by antibody, are indicated by arrows. (Right) Immunoblot analysis was performed to compare the levels of the wild type and the BRLF1(M140A) mutant (as well as β -actin) in the extracts used in the EMSA.

FIG. 5. BRLF1 residue M140 contributes to the Oct-1 enhancement of BRLF1 activity. (A) 293 BRLF1-stop cells were transfected with 50 ng wild-type BRLF1 and the BRLF1(M140A) mutant, 400 ng Oct-1, or a control vector as indicated. Immunoblot analysis was performed 2 days after transfection to compare the levels of transfected BRLF1 and Oct-1, as well as the levels of the BMRF1 and BZLF1 proteins induced from the endogenous viral genome. β -Actin served as a loading control. BMRF1 and BZLF1 protein bands were quantified using ImageJ software and are represented as numerical values in bar diagrams at the bottom of the gel (with the amount of protein induced by transfected wild-type BRLF1 alone set as 1). (B) EBV-negative 293 cells were transfected with a SMp-luciferase construct in the presence or absence of cotransfected wild-type BRLF1, the BRLF1(M140A) mutant, Oct-1, or control vectors as indicated. The relative luciferase

FIG. 6. Oct-1 DNA-binding activity is not required to enhance BRLF1-mediated lytic reactivation. (A) (Left) An EMSA was performed using *in vitro*-translated wild-type or Oct-1(S335D) mutant protein and a 32 P-labeled oligonucleotide probe containing the consensus Oct-1 binding site. Protein-DNA complexes are indicated by arrows. (Right) Immunoblot analysis was performed to compare the levels of the wild type and the Oct-1(S335D) mutant in the *in vitro*-translated samples. (B) 293 BRLF1-stop cells were transfected with 50 ng BRLF1, 400 ng wild-type Oct-1, 400 ng Oct-1(S335D) mutant, or a control vector as indicated. Immunoblot analysis was performed 2 days after transfection to compare levels of transfected BRLF1 and Oct-1, as well as the level of BMRF1 and BZLF1 proteins derived from the endogenous viral genome. β -Actin served as a loading control.

Oct-1 *in vitro*) was decreased in comparison to its effect on the wild-type BRLF1. These results suggest that a direct protein-protein interaction between BRLF1 and Oct-1 increases BRLF1 association with a variety of different lytic EBV gene promoters, including the BZLF1 promoter.

To determine if Oct-1 must bind directly to DNA in order to enhance BRLF1 binding, we compared BRLF1 binding to various lytic EBV gene promoters in the presence or absence

activity for each condition is shown; the value for the activity of each promoter construct plus the vector control is set at 1. Values are given as means $\pm$ standard deviations of results from three replicates. (C) EBV-negative 293 cells were transfected with a BZLF1p-luciferase construct in the presence or absence of cotransfected wild-type BRLF1, the BRLF1(M140A) mutant, Oct-1, or control vectors as indicated.

FIG. 7. Oct-1 augments BRLF1 DNA-binding *in vivo*. (A) A ChIP assay was performed using 293 BRLF1-stop cells transfected with Oct-1, wild-type BRLF1, the BRLF1(M140A) mutant, or a vector control as indicated. Cross-linked DNA-protein complexes were immunoprecipitated using antibodies against BRLF1 or an IgG control. Antibody-bound DNA sequences were then PCR amplified using primers spanning the EBV SM, BMRF1, or BZLF1 promoters or the GAPDH gene (negative control). Wild-type and BRLF1(M140A) mutant binding to both the SM and BMRF1 promoters was observed in the absence of cotransfected Oct-1 on longer exposures (data not shown). Binding bands were quantified using ImageJ software and are represented as numerical values in bar diagrams. The amount of wild-type BRLF1 binding to each promoter in the presence of cotransfected Oct-1 is set as 100. (B) A ChIP assay was performed with 293 BRLF1-

of cotransfected wild-type Oct-1 or the DNA-binding-defective Oct-1(S335D) mutant. Consistent with the abilities of both the wild-type and Oct-1(S335D) mutant proteins to enhance BRLF1-mediated transcription (Fig. 6B), both the wild type and the Oct-1(S335D) mutant increased BRLF1 binding to the SM, BMRF1, and BZLF1 promoters (Fig. 7B).

BRLF1 tethers Oct-1 to lytic EBV promoters *in vivo*. To determine if BRLF1 can tether wild-type Oct-1, or DNA-binding-defective Oct-1(S335D), to lytic EBV promoters, we performed ChIP assays to compare the levels of Oct-1 binding to the SM, BMRF1, and BZLF1 promoters in the presence or absence of cotransfected BRLF1. As expected, wild-type Oct-1, but not the Oct-1(S335D) DNA-binding mutant, bound to the known Oct-1-responsive promoter, Gadd45a (22) (Fig. 8). Both the wild-type and Oct-1(S335D) mutant proteins were complexed to the SM and BMRF1 promoters in the presence, but not absence, of cotransfected BRLF1. Similarly, although wild-type Oct-1 was bound to the BZLF1 promoter even in the absence of BRLF1, the DNA-binding-defective Oct-1(S335D) mutant protein was complexed to the BZLF1 promoter only in the presence of cotransfected BRLF1.

Loss of endogenous Oct-1 reduces constitutive lytic gene expression in EBV-infected B cells and epithelial cells. Finally, to examine the importance of physiologic levels of Oct-1 in promoting lytic viral gene expression, we utilized siRNA and shRNA technology to knock down endogenous Oct-1 protein levels in an EBV-positive B-cell line, Mutu1, and in EBV-positive epithelial cell lines Hone-Akata and CNE-2 Akata. These cell lines were chosen for study because each line has some degree of constitutive lytic viral protein expression (allowing us to determine if Oct-1 contributes to this lytic gene expression). As shown in Fig. 9A, siRNA-mediated loss of endogenous Oct-1 expression in the nasopharyngeal carcinoma cell line CNE-2 Akata resulted in decreased expression of the early lytic viral proteins BZLF1, BRLF1, and BMRF1. Similarly, shRNA-mediated knockdown of Oct-1 expression in the Burkitt lymphoma cell line, Mutu1, and the NPC cell line Hone-Akata also decreased the level of lytic viral protein expression (Fig. 9B and C). To confirm that the Oct-1 effect is mediated at the level of lytic gene transcription, we also examined the level of lytic RNA transcripts in the Mutu1 cells containing the control versus Oct-1 shRNAs (Fig. 9D). As expected, knockdown of Oct-1 resulted in decreased levels of three different lytic EBV gene transcripts (BZLF1, BRLF1, and BMRF1) but did not affect the level of the control cellular β_2 -microglobulin transcript. These results indicate that Oct-1 acts as a positive regulator of EBV lytic reactivation when expressed at normal levels in both B cells and epithelial cells.

In summary, our experiments indicate that Oct-1 interacts

stop cells transfected with BRLF1, wild-type Oct-1, the Oct-1(S335D) mutant, or a vector control as indicated. Cross-linked DNA-protein complexes were immunoprecipitated using antibodies against BRLF1 or an IgG control. Antibody-bound DNA sequences were then PCR amplified using primers spanning the EBV SM, BMRF1, or BZLF1 promoters or the GAPDH gene (negative control). Binding bands were quantified using ImageJ software and are represented as numerical values in bar diagrams. The amount of BRLF1 binding to each promoter in the presence of cotransfected wild-type Oct-1 is set as 100.

FIG. 8. BRLF1 tethers Oct-1 to lytic EBV promoters *in vivo*. A ChIP assay was performed with 293 BRLF1-stop cells transfected with BRLF1, Oct-1, or a vector control. Cross-linked DNA-protein complexes were immunoprecipitated using antibodies against Oct-1 or an IgG control. Antibody-bound DNA sequences were then PCR amplified using primers spanning the EBV SM, BMRF1, or BZLF1 promoters, the cellular Gadd45a promoter (positive Oct-1 DNA-binding control), or the GAPDH gene (negative control). Binding bands were quantified using ImageJ software and are represented as numerical values in bar diagrams. The amount of wild-type Oct-1 binding to each promoter in the presence of cotransfected BRLF1 is set as 100.

with the viral protein BRLF1 and through this interaction augments BRLF1 transcriptional activation of lytic EBV gene promoters. These results identify the cellular transcription factor Oct-1 as an important positive regulator of BRLF1 function and therefore EBV lytic reactivation.

DISCUSSION

EBV lytic viral gene expression can be reactivated by expression of either the BZLF1 or BRLF1 EBV immediate-early gene products (42). In contrast, only the KSHV BRLF1 homolog (Rta) can reactivate lytic gene expression in cells with the latent form of KSHV infection (54, 79, 94, 100). In this report, we have investigated whether the EBV BRLF1 protein, like the KSHV Rta protein, uses the cellular transcription factor Oct-1 as a cofactor during lytic viral reactivation. We find that EBV BRLF1 interacts directly with Oct-1 and demonstrate that this interaction is important for the BRLF1-mediated activation of several different early lytic EBV promoters. Furthermore, we show that knockdown of constitutive Oct-1 expression reduces lytic gene expression in both EBV-positive B-cell lines and EBV-positive epithelial cell lines. Thus, Oct-1 plays an important role in facilitating EBV viral reactivation.

Although the KSHV Rta and EBV BRLF1 proteins share amino acid homology and perform similar functions in regard to viral reactivation, the mechanism(s) by which the two proteins activate transcription are actually quite distinct. For example, EBV BRLF1 and KSHV Rta bind to different consensus DNA motifs and cannot activate one another's latent genomes (36). In addition, the critical interaction of the KSHV Rta protein with the cellular RBP-Jkappa transcription factor (which is required for the ability of Rta to disrupt viral latency) (49) is not shared by the EBV BRLF1 protein. Instead, EBV employs an interaction between viral latent proteins (EBNA2 and EBNA3A to -C) and RBP-Jkappa as a mechanism for activating essential viral latent promoters (32, 33, 37, 67, 80).

While the KSHV Rta protein primarily activates early lytic

FIG. 9. Loss of endogenous Oct-1 reduces constitutive lytic gene expression in EBV-infected B cells and epithelial cells. (A) CNE-2 Akata cells were transfected with a pool of siRNAs directed against Oct-1 or equal amounts of a control siRNA. Immunoblot analysis was performed 4 days later to compare the levels of endogenous Oct-1, endogenous viral proteins BZLF1, BRLF1, and BMRF1, and β-actin (loading control) in each condition. (B and C) Hone-Akata (B) and Mutu1 (C) cells were infected with a pool of lentiviral vectors expressing five different Oct-1-directed shRNAs, or control shRNAs, and cells were selected for 7 days using puromycin. Immunoblot analysis was performed to compare the levels of endogenous Oct-1, endogenous viral proteins BZLF1, BRLF1, and BMRF1, and β-actin (loading control) in each condition. (D) The expression level of the lytic viral BRLF1, BZLF1, and BMRF1 genes was examined by RT-PCR in Mutu1 cells stably expressing the control versus Oct-1 shRNAs. The β₂-microglobulin gene was used as a control.

KSHV promoters through Notch/RBP-Jkappa binding sites, its ability to activate its own promoter, as well as the K-bZIP (K8) promoter, has been shown to require a direct interaction with the cellular transcription factor Oct-1 in some cell types (7, 70). Oct-1 also promotes both HSV and VZV lytic gene expression (34, 58, 59, 93). Thus, we hypothesized that Oct-1 may likewise contribute to EBV lytic reactivation. To investigate the effect of Oct-1 on BRLF1 function, we constructed a new EBV mutant (BRLF1-stop) which has stop codons replacing the normal BRLF1 lysine residues at amino acids 4 and 5. In contrast to a previously described BRLF1 knockout virus (24, 35), the BRLF1-stop mutant does not affect the promoter or open reading frame of the overlapping BRRF1 (Na) gene. The BRLF1-stop mutant can thus be used to examine the functional role of specific BRLF1 amino acids in viral reactivation in the absence of cotransfected Na.

Our results here show that Oct-1 enhances the ability of wild-type BRLF1 to induce lytic EBV gene expression, as well as viral replication, from the endogenous viral genome in 293 BRLF1-stop cells (Fig. 1 and 2). Oct-1 also increases the ability of BRLF1 to activate a variety of different lytic EBV promoters in EBV-negative cells (Fig. 3), confirming that Oct-1 regulates BRLF1 transcriptional activity in the absence of any other EBV gene products. Interestingly, this Oct-1 effect was observed using promoters (SM, BHRF1, BHLF1, and BMRF1) with known BRLF1 response elements (RREs) as well as the BZLF1 promoter (which is not known to contain an RRE).

We find that, similar to KSHV Rta, EBV BRLF1 also interacts directly with Oct-1 *in vitro* (Fig. 4). Furthermore, we show that the BRLF1 domain homologous to a KSHV Rta domain required for interaction with Oct-1 (7) is likewise important for the EBV BRLF1/Oct-1 interaction (Fig. 4). Importantly, a BRLF1(M140A) mutant protein that is attenuated for interaction with Oct-1 *in vitro* was also found to be impaired for the ability to cooperate with Oct-1 in activating lytic transcription *in vivo* (Fig. 5). Thus, the ability of Oct-1 to increase BRLF1 transcriptional function likely requires a direct protein-protein interaction between BRLF1 and Oct-1. Nevertheless, we have not been able to coimmunoprecipitate the BRLF1 and Oct-1 proteins from cell extracts, suggesting that the interaction may be relatively weak and/or transient or that the protein-protein interaction obscures the antibody binding site for our antibodies. In contrast to what is seen for KSHV Rta, direct Oct-1 binding to BRLF1-responsive EBV promoters does not seem to be required for the ability of Oct-1 to enhance BRLF1 transcriptional effects, since a DNA-binding-defective Oct-1(S335D) mutant was found to enhance BRLF1 function as well as the wild-type Oct-1 protein (Fig. 6). This result further supports a model whereby Oct-1 augments BRLF1 function through a direct protein-protein interaction.

To further explore the mechanism(s) by which the BRLF1/Oct-1 interaction increases BRLF1 transcriptional function, we performed ChIP assays to examine the effect of Oct-1 on BRLF1 binding to lytic EBV promoters *in vivo*. Oct-1 was found to increase the binding of BRLF1 to both the SM and BMRF1 promoters *in vivo*, and this effect was stronger for the wild-type BRLF1 protein than for a BRLF1(M140A) mutant that is attenuated for interaction with Oct-1 (Fig. 7). ChIP assays also showed that Oct-1 is complexed to the lytic SM and BMRF1 EBV promoters in the presence but not the absence

of BRLF1 (Fig. 8). Thus, in the case of the SM and BMRF1 promoters, BRLF1 binding to strong RREs helps to tether Oct-1 to these promoters. Presumably, the BRLF1/Oct-1 complex binds more strongly and more efficiently activates these promoters than does BRLF1 alone.

Somewhat surprisingly, ChIP assays also showed that cotransfected Oct-1 results in BRLF1 association with the BZLF1 promoter (which does not contain a known RRE) (Fig. 7). This result suggests that the Oct-1/BRLF1 complex enhances BRLF1 binding *in vivo* to a weak RRE in the BZLF1 promoter that is poorly bound by BRLF1 *in vitro*. Alternatively, the BRLF1/Oct-1 complex may have an enhanced ability (relative to BRLF1 alone) to be tethered to the BZLF1 promoter through interactions with other cellular proteins bound to the BZLF1 promoter *in vivo*. In contrast to the SM and BMRF1 promoters, Oct-1 was found to be complexed to the BZLF1 promoter even in the absence of BRLF1, although the presence of BRLF1 increases the amount of Oct-1 complexed to the promoter (Fig. 8). Nevertheless, the fact that the DNA-binding-defective Oct-1(S335D) mutant also enhances BRLF1 activation of the BZLF1 promoter argues against the possibility that direct Oct-1 binding to the BZLF1 promoter is required for the ability of BRLF1 to bind to this promoter.

Importantly, knockdown experiments using either siRNA or shRNA directed against Oct-1 confirmed that a decrease in the level of constitutive Oct-1 expression results in reduced constitutive lytic EBV gene expression (Fig. 9). This result was observed in both an EBV-positive B-cell line (the Mutu1 Burkitt lymphoma line) and in two different EBV-positive epithelial cell lines (the Hone-Akata and CNE-2 Akata NPC lines). Thus, Oct-1 contributes to constitutive lytic EBV gene expression when expressed at a physiologic level and in both B-cell and epithelial cell environments.

Although Oct-1 acts as a positive regulator of lytic gene transcription in both alphaherpesvirus and gammaherpesvirus family members, the mechanism(s) by which Oct-1 promotes lytic gene expression appears to be somewhat distinct in each case. In the case of HSV and VZV, Oct-1 forms a complex with the viral tegument transcription factors (VP16 and ORF10, respectively) and the cellular coactivator HCF-1. This complex then binds to and activates the transcription of the immediate-early gene promoters (34, 45, 46, 58, 59, 93). In the case of the gammaherpesviruses, Oct-1 interacts directly with the EBV BRLF1 and KSHV Rta immediate-early proteins and enhances their ability to activate a variety of different lytic gene promoters. Whether Oct-1 likewise promotes lytic gene expression of betaherpesviruses is currently unknown.

While Oct-1 is expressed in many different cell types, a closely related family member, Oct-2, is expressed primarily in B cells and neuronal cells (44, 47, 78, 89, 92). Interestingly, since both EBV and KSHV establish viral latency in B cells, this leads to the question of whether Oct-2 has an effect similar to that of Oct-1 in regulating lytic viral reactivation. In the case of KSHV, a recent report showed that Oct-2 functions as a negative regulator of viral reactivation by competing with Oct-1 for viral promoter octamer binding sites and thereby helps to promote viral latency in B cells (20). We have likewise found that Oct-2 inhibits, rather than promotes, lytic EBV reactivation (A. R. Robinson, S. Kwek, and S. C. Kenney, unpublished data). Thus, the Oct family members appear to

play key roles in activating (Oct-1), as well as inhibiting (Oct-2), lytic EBV reactivation.

Reactivation of EBV, as well as of other herpesviruses, is often associated with conditions that induce cellular stress. For example, both chemotherapy and irradiation can induce EBV reactivation (25, 91). Given its constitutive expression in many cell types, Oct-1 may seem like an unlikely candidate for playing such an important role in mediating lytic reactivation. Interestingly, Oct-1 expression in cells is increased following DNA damage and other stressful stimuli through a posttranscriptional mechanism (99). In addition, Oct-1 is phosphorylated *in vivo* over a variety of Ser/Thr residues (including serine 335) following oxidative and genotoxic stress, and this phosphorylation (at least partially mediated by DNA-PK) can regulate the selectivity of Oct-1 for complex DNA sequences (38, 39, 71). Given our finding that the Oct-1(S335D) DNA-binding mutant retains the ability to promote BRLF1 transcriptional function, future studies should determine whether the phosphorylated form of Oct-1 residue S335 likewise retains this ability. If so, stress-induced phosphorylation may serve to redirect limiting amounts of cellular Oct-1 to lytic EBV promoters in conjunction with BRLF1.

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