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The Epstein-Barr Virus EBNA-LP Protein Preferentially Coactivates EBNA2-Mediated Stimulation of Latent Membrane Proteins Expressed from the Viral Divergent Promoter

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The mechanistic contribution of the Epstein-Barr virus (EBV) EBNA-LP protein to B-cell immortalization remains an enigma. However, previous studies have indicated that EBNA-LP may contribute to immortalization by enhancing EBNA2-mediated transcriptional activation of the LMP-1 gene. To gain further insight into the potential role EBNA-LP has in EBV-mediated B-cell immortalization, we asked whether it is a global or gene-specific coactivator of EBNA2 and whether coactivation requires interaction between these proteins. In type I Burkitt's lymphoma cells, we found that EBNA-LP strongly coactivated EBNA2 stimulation of LMP-1 and LMP2B RNAs, which are expressed from the viral divergent promoter. Surprisingly, the viral LMP2A gene and cellular CD21 and Hes-1 genes were induced by EBNA2 but showed no further induction after EBNA-LP coexpression. We also found that EBNA-LP did not stably interact with EBNA2 in coimmunoprecipitation assays, even though the conditions were adequate to observe specific interactions between EBNA2 and its cellular cofactor, CBF1. Colocalization between EBNA2 and EBNA-LP was not detectable in EBV-transformed cell lines or transfected type I Burkitt's cells. Finally, no significant interactions between EBNA2 and EBNA-LP were found with mammalian two-hybrid assays. From this data, we conclude that EBNA-LP is not a global coactivator of EBNA2 targets, but it preferentially coactivates EBNA2 stimulation of the viral divergent promoter. While this may require specific transient interactions between these proteins that only occur in the context of the divergent promoter, our data strongly suggest that EBNA-LP also cooperates with EBNA2 through mechanisms that do not require direct or indirect complex formation between these proteins.

Epstein-Barr virus (EBV) is a causative agent or cofactor in the etiology of several human malignancies, including endemic Burkitt's lymphoma, nasopharyngeal carcinoma, some forms of Hodgkin's disease, and lymphomas in the immunosuppressed (6, 56). The virus has an intrinsic ability to immortalize human B cells through expression of several gene products known as latent genes (4). One or more latent gene products are expressed in EBV-associated malignancies, so elucidation of latent protein activities may yield insight into EBV-mediated oncogenesis (4, 6, 56).

Two key latency proteins involved in EBV-induced immortalization of human B cells are EBNA2 and LMP-1 (36). EBNA2 stimulates the viral latency C promoter, the viral bidirectional promoter, and the LMP2A promoter, making it a pivotal transcriptional activator of latent viral gene expression (12, 30, 42, 67, 76, 78, 87, 88). EBNA2 also stimulates the expression of cellular genes that may be important for B-cell immortalization and include hes-1, runx3, and c-*myc* (26, 31, 60, 65). EBNA2 is not a direct DNA-binding protein but reaches its target promoters through an interaction with the cellular DNA-binding protein known as CBF1 (17, 23, 44, 74, 86). Promoter-bound complexes between CBF1 and EBNA2 activate transcription through recruitment of several components of the basal transcription machinery by EBNA2's carboxy-terminal acidic activation domain and chromatin remodeling machinery through a domain in the divergent region (40, 71-73, 77, 79, 80). The cellular membrane protein known as Notch induces cellular genes, which include hes-1 and CD21 (26, 28, 60, 66), in a ligand-dependent fashion through interactions with CBF1 (45). Thus, through the shared interaction with CBF1, EBNA2 possesses constitutively active Notch signaling activity. LMP-1 is a potent oncoprotein and is, like EBNA2, important for B-cell immortalization (4, 8, 11, 41). LMP-1 functions as a constitutively active tumor necrosis factor (TNF) receptor through its ability to interact with TNF receptor-associated factors via the cytoplasmic carboxy-terminal domain which resembles signaling mediated by the cellular CD40 receptor (4, 11, 41). Association with TNF receptor-associated factors results in induction of pathways that stimulate NF-κB, JNK, and STAT activities (4, 11, 41). In EBV-immortalized B cells, LMP-1 expression appears to be tightly linked to EBNA2 expression (35). Proper regulation of LMP-1 expression is important, since it is cytostatic for cellular proliferation when overexpressed (14). On the other hand, the initial expression of LMP-1 during the early stages of B-cell infection or the maintenance of expression during various stages of the cell cycle is also likely to be important (2, 3). Consequently, the virus has evolved several mechanisms to ensure proper regulation of LMP-1 expression. These mechanisms include (i) domains within the EBNA2 protein itself, such as the polyproline domain, which serves to temper EBNA2's stimulation of LMP-1 (16), (ii) down-regulation of EBNA2 activity by EBNA3 sequestration of CBF1 (43, 57, 75, 85), (iii) down-regulation through EBNA2 hyperphosphoryla-

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tion (84), and (iv) coactivation of EBNA2 by the viral EBNA-LP protein (22, 50, 52, 53). The mechanisms by which EBNA-LP coactivates EBNA2 have yet to be fully realized.

EBNA-LP is a highly enigmatic protein composed of 22- and 44-amino-acid repeats derived from the W1 and W2 exons found in the large internal repeated region in the virus (IR1) (61, 64). The number of repeats varies depending on the EBV isolate or EBV-immortalized cell line. The carboxy-terminal domain of the protein is encoded by two unique exons, Y1 and Y2, encoding 45-amino-acid residues. During initial infection, multiple isoforms of EBNA-LP can be detected due to the intrinsic number of repeats in the viral genome and alternative splicing (7, 13). However, most established lymphoblastoid cell lines (LCLs) ultimately express only one or a few isoforms (7, 13).

EBNA-LP function has been investigated by using genetic and cell-based assays. Genetic studies indicated that deletion of the carboxy-terminal 45-amino-acid residues resulted in viruses that immortalized cells with much lower efficiency and required feeder cells (19, 47). Once established, however, these cell lines were not markedly different from wild-type virus-immortalized cells in phenotype or growth properties (1). More recent studies have found that EBNA-LP can stimulate EBNA2-mediated activation of LMP-1 in EBV-positive type I Burkitt's lymphoma cells or LMP-1 and C promoter (Cp) reporter plasmids in transient-transfection assays (22, 50, 52, 53). Furthermore, EBNA-LP also cooperated with EBNA2 to induce cyclin D2 in transfected primary B cells (63). Several nonhuman primate lymphocryptoviruses have also been shown to encode EBNA-LP homologs, and the ability to coactivate EBNA2 appears to be conserved (52). More recent studies have suggested that EBNA-LP may induce and repress cellular genes independently of EBNA2, although these effects do not appear to be global in nature (9, 32).

In addition to deletion of the carboxy-terminal region encoded by the Y1 and Y2 exons, we and others have introduced multiple mutations into the W repeats of EBNA-LP, focusing on regions that are evolutionarily conserved among the lymphocryptovirus homologs (49, 53). These analyses revealed that EBNA-LP has a bipartite nuclear localization signal and that a conserved serine and a region known as conserved region 3 are critical for coactivation function. Additional studies have confirmed that EBNA-LP is serine phosphorylated, and phosphorylation on at least one serine residue (e.g., S35 and 101 in a 2-W repeat EBNA-LP isoform) is required for EBNA2 coactivation function (37, 54, 82). Surprisingly, the carboxy-terminal domain(s) encoded by Y1Y2 are not required for coactivation function (22, 50). In addition, EBNA-LP isoforms with at least 2 copies of the W1W2 repeated domain are required for coactivation function (50, 53), and viruses with 2 W repeats are immortalization competent (83). EBNA-LP isoforms with only a single W1W2 repeat localize to the cytoplasm, which may account for its inability to coactivate EBNA2 (53). A potential role for this smallest form of EBNA-LP has not yet been defined.

The mechanism for EBNA-LP coactivation function has yet to be fully characterized. Potential cofactors that mediate EBNA-LP activity include pRb, p53, hsp72/hsc73, hsp27, Hax-1, ERR1, p14ARF, DNA-Pkcs, α-tubulin, β-tubulin, prolyl-4-hydroxylase, and HA95 (10, 20, 21, 27, 29, 33, 34, 38, 48, 68, 70). So far, however, no correlation has been made between association with these factors and their ability to coactivate EBNA2 by using well-characterized coactivation-deficient EBNA-LP proteins, especially those containing mutations in the W repeats. Recent studies with reporter plasmids and in vitro binding studies with truncated portions of EBNA2 and EBNA-LP have suggested that EBNA-LP may mediate coactivation through interactions with EBNA2 (51). The implication of these studies suggests that EBNA-LP may also be a global coactivator of EBNA2. To address this question, we have examined EBNA-LP coactivation activity on EBNA2 target genes resident in the viral genome or cellular chromosomes in EBV-positive and EBV-negative Burkitt's lymphoma cell lines. EBNA-LP appeared to preferentially coactivate EBNA2 only on the viral divergent promoter and was not a global coactivator. These results prompted us to examine whether EBNA2 and EBNA-LP may associate with each other under conditions where functional coactivation occurred. Data derived from immunoprecipitation assays, high-resolution microscopy, and mammalian two-hybrid assays were largely negative for EBNA-LP–EBNA2 interactions. Consistent with our data, we suggest that EBNA-LP may stimulate EBNA2-mediated gene activation through mechanisms that do not require direct or indirect interactions with EBNA2.

MATERIALS AND METHODS

Cell culture and transfections. Eli-BL cells, an EBV type I Burkitt's lymphoma line, and DG75 cells, an EBV-negative Burkitt's cell line, were maintained in RPMI (Invitrogen) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Invitrogen). Cells were incubated at 37°C and 5% CO_2. EBV-immortalized IB4 and MHK cells were maintained similarly. MHKs were generated by infecting peripheral blood B cells from a human immunodeficiency virus-positive donor with the B95-8 strain of EBV. The cells are negative for human immunodeficiency virus. Approximately 10^7 cells were electroporated with a Bio-Rad GenePulser II for each transfection, as we have described previously (53). Transient transfections were harvested 24 to 48 h posttransfection. For transfections with reporter plasmids, luciferase activity was determined as previously described (53).

Plasmids. Expression plasmids included pSG5 vector (Stratagene), pSG5 expressing wild-type EBNA-LP with two repeated W domains (pJT125) (53), an isogenic mutant EBNA-LP (S35/101A, pRSP87) (53), or wild-type EBNA2 (pPDL176A). For some experiments, wild-type and CBF1-binding-deficient mutant EBNA2 expression plasmids pPDL151 (wild type) and pPDL152 (WW323SR) were used, which have been described previously (44). These EBNA2 expression plasmids contain approximately 30 bases of 5′ untranslated sequences preceding the initiation codon compared to pPDL176A. For some coimmunoprecipitation experiments, an SG5-driven wild-type EBNA2 with a carboxy-terminal epitope hemagglutinin (HA) tag (pAG155) was used and has been described previously (15). Gal4-CBF1 fusion plasmids have been described previously (25), and a plasmid expressing a Gal4–EBNA-LP fusion (pRSP364) was generated by PCR from an EBNA-LP isoform with two repeats and cloned into the identical Gal4 vector plasmid used for generating Gal4-CBF1. The Gal4-EBNA2 fusion was made by subcloning a BglII fragment from pPDL151 into the Gal4 vector pGH251 (a generous gift from Gary Hayward), generating pJT167. To generate a VP16-CBF1 fusion protein, we PCR amplified the VP16 activation domain from the pVP16 vector (Clontech) and subcloned it into pSG5 to create plasmid pJT163. The activation domain is also fused to the simian virus 40 (SV40) large-T nuclear localization signal. The CBF1 open reading frame (ORF) was PCR amplified with primers that also added an HA epitope tag at the carboxy terminus of CBF1 and then subcloned into pJT163 to generate pJT170. A Gal4-responsive luciferase reporter plasmid containing five Gal4 binding sites and an E1b TATA element was a generous gift from S. D. Hayward. The enhanced yellow fluorescent protein (YFP) gene was amplified by PCR and cloned into a BglII site that was introduced just before the termination codon in the EBNA2 expression plasmid (pPDL176A) to produce a carboxy-terminal EBNA2-YFP fusion protein (plasmid pRSP258). The cyan fluorescent protein (CFP) gene was amplified by PCR and cloned into the EcoRI site of the

TABLE 1. Oligonucleotide sequences used for RT-PCR, Southern blotting, and real-time PCR

Gene	Oligonucleotide (sequence [5′–3′])	Source
LMP1	Forward (CTTCAGAAGAGACCTTCTCT)	B95-8, bp 168785–168804
	Reverse (ACAATGCCTGTCCGTGCAAA)	B95-8, bp 168623–168642
	Probe (CCTACTGATGATCACCCTCCTG)	B95-8, bp 168749–168759, 168660–168670
LMP2A	Forward (CTCCCTACTCTCCACGGGAT)	B95-8, bp 166398–166417
	Reverse (GAGGTAGGGCGCAACAATTA)	B95-8, bp 81–100
	Probe (CAGAGGAAGTATGAATCCAG)	B95-8, bp 166450–166458, 58–68
LMP2B	Forward (GAGTGCGTAGTGTTGTGGGA)	B95-8, bp 169336–169355
	Reverse (GAGGTAGGGCGCAACAATTA)	B95-8, bp 81–100
	Probe (GGGGGTTCAGTATGAATCCAGTA)	B95-8, bp 169439–169448, 58–68
Cp	Forward (CCTCATCGCAGGGTTCTTAC)	B95-8, bp 11380–11399
	Reverse (ATGCTCACGTGCAGGAGGCT)	B95-8, bp 11638–11657
	Probe (CATCTAAACCGACTGAAGAA)	B95-8, bp 11470–11480, 11627–11635
CD21	Forward (ACCAACTTCTCCATGAACGG)	Exon2/3
	Reverse (CTTGTGTGATGTCCATTGTGG)	
	Probe (CTGTGTAAGTGTTTTCCCTCTC)	
HES1	Forward (TCTGAGCCAGCTGAAAACAC)	Exon 2/4
	Reverse (TACTTCCCCAGCACACTTGG)	
	Probe (GCAGATGACGGCTGCGCTGAGC)	
LMP1[a]	Forward (TGATCATCTTTATCTTCAGAAGAGACCTT)	B95-8, bp 168789–168817
	Reverse (CCTGTCCGTGCAAATTCCA)	B95-8, bp 168629–168647
	Probe (6FAMTGATGATCACCCTCCMGBNFQ)	B95-8, bp168749–168754, 168662–168670
LMP2A[a]	Forward (CTCCCTACTCTCCACGGGAT)	B95-8, bp166398–166417
	Reverse (AGGTAGGGCGCAACAATTACA)	B95-8, bp 79–99
	Probe (6FAMCAGAGGAAGTATGAATCCAMGBNFQ)	B95-8, bp167091–167098, 56–67

[a] Real-time PCR oligonucleotides.

EBNA-LP expression plasmid (pJT125A) to produce an amino-terminal CFP–EBNA-LP fusion protein (plasmid pSCM3.5).

RNA extraction. Transfected cells were pelleted, snap-frozen on dry ice, and stored at −80°C. Pellets were then resuspended in 1 ml of Trizol (Invitrogen) per 0.5×10^7 to 2×10^7 cells. Chloroform was added at 200 μl/1 ml of Trizol. Samples were then shaken vigorously and centrifuged for 15 min at 13,200 rpm in an Eppendorf 5417C centrifuge. The upper phase was removed to a new tube, and RNA was precipitated with isopropanol, followed by a 70% ethanol wash. The RNA pellet was resuspended in water and DNase treated. DNase was next inactivated by heating with the addition of EDTA followed by phenol-chloroform extraction or a second round of Trizol extraction. The final RNA pellet was ethanol precipitated, washed in 70% ethanol, and resuspended in water. The RNA was stored at −20°C in the presence of an RNase inhibitor (Invitrogen).

RT-PCR and Southern blotting. RNA (0.5 to 1 μg) extracted from transfected cells was reverse transcribed at 37°C with avian myeloblastosis virus reverse transcriptase (Invitrogen). PCRs typically included a 5-μl reverse transcription (RT) reaction mixture as a template in a 25-μl total volume. The primer and probe sequences used are listed in Table 1. Except for Hes-1, primers were designed to amplify short sequences on either side of an intron. Probes were designed to detect mRNA versus genomic DNA or unprocessed RNA by hybridizing to sequences spanning an exon-exon junction. PCR products were separated on 2% agarose gels and transferred by upward capillary transfer to a Nytran (Schleicher and Schuell) or Zeta probe (Bio-Rad) positively charged membrane. Buffer systems used for gel transfer consisted of denaturing and transfer in 0.4 N NaOH or denaturing in 0.5 M NaOH and 1.5 M NaCl, neutralization in 1 M Tris–1.5 M NaCl (pH 7.5), and transfer in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membranes were then UV cross-linked (Stratalinker-Stratagene).

Probes for hybridizations were prepared by T4-polynucleotide kinase (Invitrogen) end-labeling of oligonucleotides with [γ-^{32}P]ATP (Amersham). Labeled probes were purified over Sephadex. Membranes were prehybridized in buffer containing 5× Denhardt's, 0.2% sodium dodecyl sulfate (SDS), 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), and salmon sperm DNA (Invitrogen). Hybridization buffer consisted of 5× Denhardt's, 0.8% SDS, 6× SSC, and either herring or salmon sperm DNA. Membranes were prehybridized at 68°C and hybridized overnight at 37°C or incubated entirely at room temperature. Membranes were then washed in 2× SSC–0.1% SDS and in 0.2× SSC–0.1% SDS. Following the washes, the blots were exposed by using X-ray film (Kodak). Relative signal intensities from the Southern blots were quantitated with a PhosphorImager (Molecular Dynamics).

Real-time PCR. Following RT of RNA, 5 μl of each sample was amplified by real-time PCR in 25-μl reaction mixtures. Oligonucleotide sequences, as manufactured by Integrated DNA technologies or Applied Biosystems (AB) are described in Table 1. AB Primer Express software was used to aid in the selection of primers and probes. β-Actin was detected by using primers and probes supplied by Applied Biosystems reagent Hs99999903m1. AB Universal Master Mix was used at a 2× concentration, and the samples were run on an AB model 7700 sequence detection system. No-RT samples were included as negative controls. The results were analyzed according to the calibrator method for relative quantitation as detailed in AB User Bulletin 2. Briefly, cycle threshold values from duplicate reactions were entered into the standard curve equation for each primer-probe set to obtain a log input template as a unitless, relative quantity (log input). Input duplicates were averaged, and normalization to actin was calculated as the input transcript divided by the input actin. Induction relative to the EBNA2-only sample was expressed as a quotient of the actin-normalized value for the sample divided by the actin-normalized value for the EBNA2-only sample.

Western blots and immunoprecipitations. For Western blot analysis, transfected cells or cell lines were pelleted by centrifugation, resuspended in Laemmli sample buffer, and boiled for 5 min, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). A prestained protein molecular weight marker (Invitrogen) was included on each gel to determine relative protein sizes. Unless otherwise specified, 7.7% polyacrylamide gels were used to resolve EBNA2, LMP-1, and CBF1, while 12% gels were used to resolve EBNA-LP. Following transfer to nitrocellulose membranes, the blots were hybridized in phosphate-buffered saline containing 5% nonfat dried milk for at least 1 h. Primary antibodies used in these studies included anti-HA (Covance), anti-Flag (Sigma), anti-EBNA2 (R3) (39), anti-LMP-1 (S12) (46), and anti-EBNA-LP (JF186) (13). The dilutions used were according to the manufacturers' protocols, except for the EBNA2, LMP-1, and EBNA-LP antibodies, which were determined empirically. The blots were developed by using a chemiluminescent ECL detection kit (Pierce). For immunoprecipitations, we lysed transfected cells in either radioimmunoprecipitation assay buffer or a 1% NP-40 buffer (10 mM Tris-Cl [pH 7.4], 1 mM EDTA, 150 mM NaCl, 3% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5-mg/ml leupeptin, 10-mg/ml aprotinin). However, both buffers yielded similar results. Transfected cell lysates were incubated with a primary antibody at 4°C overnight, followed by incubation with protein G-Sepharose beads for 1 h at room temperature (Pierce). The beads were washed several times in the immunoprecipitation lysis buffer, and the bound proteins were then solubilized by the addition of 2× Laemmli sample buffer and boiling for 5 min. The proteins were then subjected to SDS-PAGE and Western blotting as described above.

Indirect immunofluorescence. Transfected Eli-BL or EBV-immortalized cells (50,000 to 100,000) were washed in phosphate-buffered saline and spun onto no.

1 thickness 12-mm-diameter round coverslips (Fisher Scientific) at 1,000 rpm for 1 min with a cytospin 3 centrifuge (Shandon). The cells were then fixed in 4% formaldehyde in PEM buffer [80 mM potassium–piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.8), 5 mM EGTA, and 2 mM $MgCl_2$] for 30 min on ice. Subsequently, the cells were permeabilized by incubation in PEM containing 0.5% Triton X-100 for 5 to 30 min at room temperature and blocked in TBST buffer (20 mM Tris-Hcl [pH 7.4], 137 mM NaCl, 0.1% Tween 20) with 5% powdered milk. The cells were then incubated with primary antibody at 37°C for 2 h at room temperature, followed by incubation with secondary antibody for 40 min at room temperature. The cells were fixed again in 4% formaldehyde in PEM buffer for 10 to 30 min. Autofluorescence was quenched by incubation with $HaBH_4$ in PEM at a concentration of 1 mg/ml. In some cases, the DNA was counterstained with 1× 4′,6′-diamidino-2-phenylindole (DAPI) diluted in TBST for 30 s to 1 min. The coverslips were mounted to slides by using a Slowfade antifade kit (Molecular Probes).

Microscopy and image processing. Deconvolution microscopy was performed on a Carl Zeiss AxioVert S100 TV microscope as described previously (24). The images were digitally processed by using Adobe Photoshop for presentation, and the images shown represent single Z sections. For standard fluorescence, a Zeiss AxioPlan2 microscope equipped with PC-controlled, high-resolution black and white and color charge-coupled device cameras was used. Images were digitally processed with Adobe Photoshop.

RESULTS

Effect of EBNA-LP coexpression on EBNA2-induced target genes. Studies by Nitsche et al. (50) originally demonstrated that EBNA-LP strongly stimulated or coactivated EBNA2 induction of the viral LMP-1 gene in type I Burkitt's lymphoma cells, which is an observation confirmed by several laboratories, including ours (53, 81, 82). Preliminary RT-PCR analysis also indicated that this effect seemed to occur predominantly at the RNA level (50). We wished to confirm and extend these results by examining the effect of EBNA-LP expression on other viral and cellular genes that are induced by EBNA2. As expected, transfection of Eli-BL cells with plasmids expressing EBNA-LP alone resulted in no induction of LMP-1 protein, while expression of EBNA2 resulted in a modest induction of LMP-1 (Fig. 1A). Coexpression of both EBNA2 and EBNA-LP resulted in a substantial increase in LMP-1 protein that depended on a wild-type version of EBNA-LP (Fig. 1A, lane 4). A coactivation mutant generated previously in our laboratory (53) was unable to stimulate LMP-1 to levels above EBNA2 expression alone (Fig. 1A). RNA extracted from the same transfected cells analyzed by Western blotting in Fig. 1A was then subjected to RT-PCR analysis. By RT-PCR Southern analysis with primers complementary to the 3′ end of LMP-1 exon 1 and the 5′ end of exon 2, we determined that LMP-1 RNA levels correlated with observed protein levels (Fig. 1B). LMP-1 RNA detected with primers that amplified between exons 2 and 3 also showed similar results (data not shown). RT-PCR amplification of the cellular β-actin gene was relatively unaffected by EBNA2 or EBNA-LP expression, and amplification of samples expressing the highest level of LMP-1 (Fig. 1B, lane 4) for additional PCR cycles indicated that the reactions had not yet plateaued (Fig. 1B, lane 7). LMP-1 is expressed from a viral promoter known as the bidirectional promoter that also controls expression of the LMP-2B gene. By RT-PCR analysis, we also found that EBNA-LP coactivated EBNA2 stimulation of LMP2B (Fig. 1B). When we examined the viral LMP2A gene or the cellular Hes-1 gene, we found that EBNA2 induced the expression as expected. Surprisingly, however, coexpression with EBNA-LP did not result in further induction of these RNAs, even though coactivation was observed from the same cell extracts for RNAs expressed from the viral bidirectional promoter (Fig. 1B). To control for the possibility that our PCRs had not plateaued (and masked possible differences between samples), samples from lane 4 (Fig. 1B) were amplified for additional PCR cycles (Fig. 1B, lane 7). In all cases, an increased signal was observed, indicating that our reactions had not yet reached this stage. Attempts to detect activity from the other viral latency promoter known as Cp were unsuccessful in these cells and may be due to high levels of DNA methylation at this promoter (58, 62) (data not shown). Coexpression of EBNA2 and EBNA-LP in DG75 cells, an EBV-negative Burkitt's cell line, also demonstrated that EBNA2 was able to induce both CD21 and Hes-1 but that EBNA-LP had no observable effect (Fig. 1C). Real-time PCR was also used to confirm the RT-PCR and Southern blotting results. Using this assay, EBNA-LP enhanced EBNA2 stimulation of the LMP-1 gene fivefold, while little to no cooperation was observed for LMP-2A (Table 2). We conclude from these results that EBNA-LP appears to preferentially coactivate EBNA2 stimulation of genes controlled by the viral bidirectional latent promoter.

EBNA2 and EBNA-LP do not form stable complexes in cells. One possible mechanism for the coactivating effects of EBNA-LP on EBNA2 may involve interactions between these proteins. To investigate this possibility, we immunoprecipitated either EBNA-LP or EBNA2 from transfected cells coexpressing both proteins followed by Western blot analysis. As a control, we also transfected cells with an HA epitope-tagged version of CBF1 and either wild-type EBNA2 or a mutant version of EBNA2 that is unable to interact with CBF1 (44). The expression levels of each protein were easily detected in transfected DG75 or Eli-BL cells (Fig. 2A). Immunoprecipitation of CBF1 resulted in coprecipitation with wild-type EBNA2 (Fig. 2B, lane 1) but not the CBF1-binding-deficient mutant (Fig. 2B, lane 3). Under similar conditions, we were unable to detect significant coprecipitation of EBNA2 following initial precipitation of EBNA-LP with an anti-Flag antibody followed by immunoblotting with an anti-EBNA2 antibody (Fig. 2B, lane 5). However, extremely long exposures revealed that low levels of the mutant EBNA2 protein associated with CBF1, as did wild-type EBNA2 with EBNA-LP (lanes 3 and 5, respectively). We also transfected cells with plasmids expressing an HA epitope-tagged version of EBNA2 and a Flag epitope-tagged EBNA-LP (Fig. 2A, lane 4). Precipitation of these extracts with an anti-HA antibody followed by immunoblotting with an anti-EBNA-LP antibody demonstrated that trace amounts of EBNA-LP associated with EBNA2 (Fig. 2C, lane 3). The levels detected are similar to those appearing in precipitations with no antibody (Fig. 2C, lane 4), suggesting that the levels of EBNA-LP detected were due to nonspecific binding to the protein A-Sepharose beads rather than specific associations with EBNA2. In these experiments, we used an EBNA-LP isoform that contained 4 W repeats. We also repeated similar coimmunoprecipitation experiments by using EBNA-LP isoforms with 2 W repeats as well as a coactivation-deficient EBNA-LP isoform that was coexpressed with an HA-tagged EBNA2 protein. Using these reagents, no detectable interaction between EBNA2 and EBNA-LP was observed (Fig. 2D). Taken together, the results indicate that trace levels of EBNA-LP may associate with

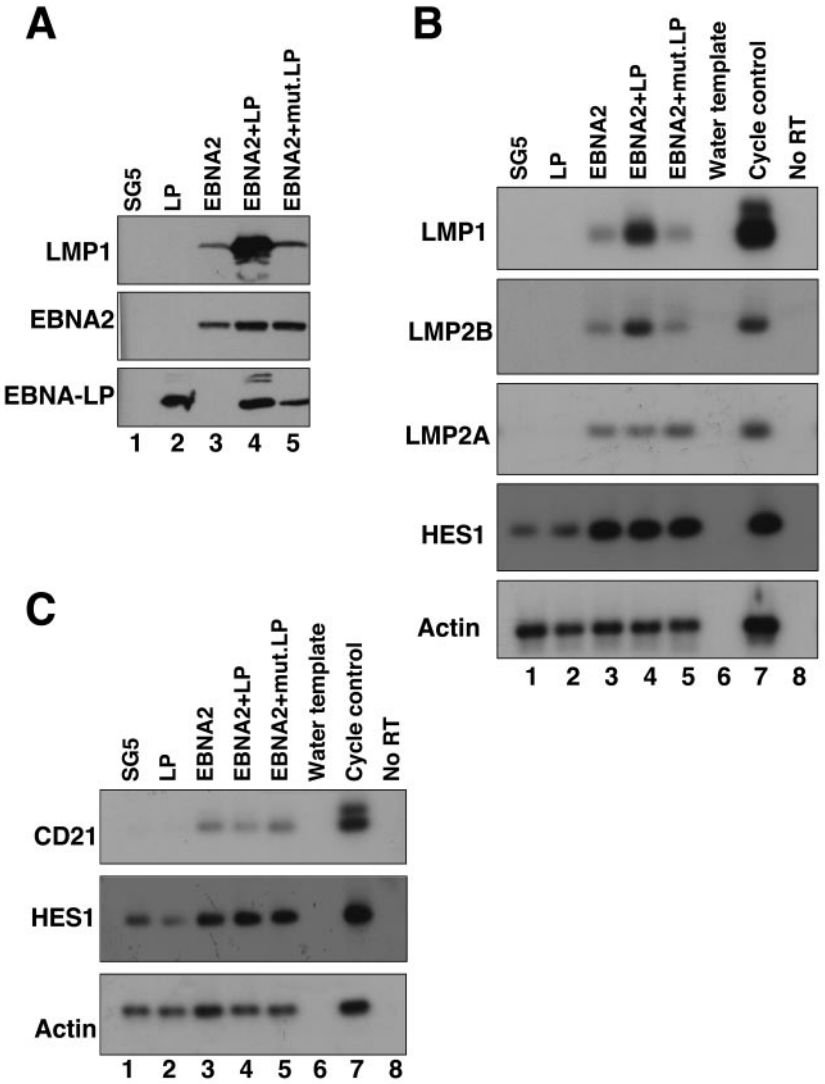

FIG. 1. EBNA-LP preferentially coactivates EBNA2 stimulation of the viral divergent promoter. (A) Eli-BL cells were transfected with pSG5 vector (lane 1) or plasmids expressing LP (lane 2), EBNA2 (lane 3), EBNA2 plus EBNA-LP (lane 4), or EBNA2 plus a mutant version of EBNA-LP (S35/101A) (lane 5). All versions of EBNA-LP had 2 W repeats. Approximately 20% of the transfected cells were processed for Western blotting as described in the Materials and Methods. Replicate blots were probed with anti-LMP-1 (S12), anti-EBNA2 (R3), and anti-EBNA-LP (JF186) monoclonal antibodies as indicated. (B) RT-PCR and Southern blotting of RNA derived from transfected samples in panel A. The specific genes amplified and probed for each blot are indicated to the left of each panel. Primers and probes are indicated in Table 1. In addition, RNA from EBNA2- and EBNA-LP-cotransfected cells (lane 4) was subjected to 5 to 10 additional PCR cycles to make sure that the reactions in lanes 1 to 5 had not yet plateaued (lane 7). PCRs were also performed with water as a template or RNA that had not been reverse transcribed (lanes 6 and 8, respectively). (C) DG75 cells, an EBV-negative cell line, were transfected as described for Eli-BL cells in the legend to panel A. However, the EBNA2 effector plasmid pEΔA6 was used instead of an SG5-EBNA2 expression plasmid, since EBNA-LP induces protein levels of the SG5-driven EBNA2 in these cells and pEΔA6 is refractory to this induction. RT-PCR and Southern blotting was performed as described for panel B.

EBNA2 in transient transfections that overexpress these proteins.

EBNA2 and EBNA-LP do not colocalize in transfected type I Burkitt's cells or in EBV-immortalized B cells. Although EBNA2 and EBNA-LP did not appear to form stable or robust interactions in a cell, we hypothesized that if EBNA-LP operated by forming transient or unstable contacts with EBNA2, then it may still colocalize with EBNA2 in cellular compartments. To investigate this possibility, we generated an amino-terminal CFP fusion with EBNA-LP and a carboxy-terminal YFP fusion with EBNA2. In transient-transfection experiments, the EBNA2-YFP fusion protein was able to induce LMP-1 in Eli-BL cells and coexpression with the CFP–EBNA-LP protein resulted in a substantial increase in LMP-1 protein (Fig. 3A, lanes 5 and 6). The total levels of LMP-1 induced were less than observed when wild-type proteins were expressed in these cells (Fig. 3B, lanes 2 and 3), but the overall effect of EBNA-LP was similar. These data imply that the fusion proteins function similarly to wild-type proteins and that visualization of their localization in transfected cells was likely to be representative of wild-type proteins. Using high-resolution deconvolution microscopy, EBNA2-YFP fusion proteins appear as 5 to 40 defined spots (green) that are relatively evenly distributed in the nucleus of transfected cells (Fig. 3B).

TABLE 2. Real-time RT-PCR detection of β-actin, LMP-1, and LMP2A

Sample[a]	Transcript	C_T[c]	Input	Avg input ± SD	Fold induction[b]
EBNA2	Actin	20.40	0.1892	0.2005 ± 0.0160	
EBNA2	Actin	20.22	0.2119		
EBNA2 + LP	Actin	20.31	0.2002	0.2074 ± 0.0101	
EBNA2 + LP	Actin	20.20	0.2146		
EBNA2	LMP-1	26.28	4.9671	5.1025 ± 0.1909	1.000 ± 0.0883
EBNA2	LMP-1	26.17	5.2372		
EBNA2 + LP	LMP-1	22.76	26.9153	27.111 ± 0.2781	5.138 ± 0.0501
EBNA2 + LP	LMP-1	22.73	27.3086		
EBNA2	LMP2A	31.81	6.7019	6.6091 ± 0.1312	1.000 ± 0.0824
EBNA2	LMP2A	31.85	6.5163		
EBNA2 + LP	LMP2A	31.67	7.3978	7.5569 ± 0.2250	1.105 ± 0.0574
EBNA2 + LP	LMP2A	31.61	7.7161		

[a] RNA from cells transfected with the indicated plasmids was reverse transcribed, and the cDNA was amplified by real-time PCR as described in Materials and Methods.

[b] Relative (*n*-fold) induction was calculated by using the AB User Bulletin 2 calibrator method for relative quantitation; relative transcript induction was normalized to that of actin and then expressed as a ratio to the EBNA2-only sample.

[c] C_T, number of PCR cycles at the threshold.

In contrast, CFP–EBNA-LP appears as 5 to 15 intense nuclear spots (blue) that are larger on average than the EBNA2 spots (Fig. 3B). In many cells, these spots appeared to be localized along the rim of the nucleus but also can be found randomly throughout the nucleus depending on the focal plane observed. Under no circumstances did we observe colocalization between the EBNA2 and EBNA-LP fusion proteins.

To determine whether this may be an artifact of transient expression or the fusion proteins, we also examined EBNA2 and EBNA-LP localization in EBV-immortalized cell lines. To do this, we utilized the mouse monoclonal antibody for EBNA-LP (JF186) and the rat monoclonal antibody for EBNA2 (R3). To make sure that the fluorescently tagged secondary antibodies directed against each of these antibodies did not cross-react, we stained LCLs (IB4) with various combinations of primary and secondary antibodies. Staining with the anti-rat EBNA2 followed by a secondary anti-rat antibody conjugated with Alexa 488 produced a characteristic staining pattern for EBNA2 which consisted of multiple punctate spots distributed evenly throughout the nucleus (Fig. 4A). Staining with the mouse anti-EBNA-LP antibody followed by a secondary anti-mouse antibody conjugated with Alexa dye 594 produced a staining pattern typical for EBNA-LP consisting of a few punctate nuclear spots that are likely to colocalize with promyelocytic leukemia (PML) bodies (Fig. 4B). None of the secondary antibodies cross-reacted with the primary antibodies, and they did not appear to cross-react with any cellular proteins either (Fig. 4B). Two independently generated LCLs were then tested for localization of EBNA2 and EBNA-LP in the same cells. IB4 cells have been described previously, and MHKs were derived from another donor and have been grown in our laboratory for several months (see Materials and Methods). Western blot analysis confirmed that both cell lines synthesize EBNA2, EBNA-LP, and LMP-1 as expected (Fig. 5B). High-resolution microscopy indicated that in both LCLs, EBNA2 localized in multiple punctate spots in the nucleus, like in transfected cells, but were generally in much greater numbers. EBNA-LP staining showed again that it localized in a few concentrated foci similar to the cells transfected with CFP–EBNA-LP. Like the cells transfected with the functional fusion proteins, no significant colocalization was observed between EBNA2 and EBNA-LP in LCLs (Fig. 5A).

EBNA2 and EBNA-LP do not functionally interact in a mammalian two-hybrid assay. Although the immunoprecipitation and colocalization results suggested that EBNA-LP appears to coactivate EBNA2 through a mechanism that does not involve a physical interaction, we decided to test whether these proteins may functionally interact, perhaps in a transient manner, by using a mammalian two-hybrid assay. A Gal4–EBNA-LP fusion protein was tested as a bait protein in transient-transfection experiments with DG75 cells. Cotransfection of this bait with EBNA2 did not result in detectable induction of a Gal4-responsive reporter plasmid (Fig. 6). Both proteins localize to the nucleus (data not shown) and are also detectable by Western blot analyses (Fig. 6). In contrast, cotransfection of a Gal4-CBF1 bait together with EBNA2 resulted in an average 14-fold stimulation of the Gal4-responsive reporter plasmid (Fig. 6). Cotransfection with a CBF1-binding-deficient EBNA2 mutant and the Gal4-CBF1 bait plasmid was unable to stimulate the Gal4-responsive plasmid as expected. It is interesting that in these assays the Gal4–EBNA-LP fusion protein appeared to have no intrinsic transcriptional activation function by itself. To rule out that the Gal4–EBNA-LP fusion protein exists in a conformation that is incompatible with forming an interaction with EBNA2, we also reversed the bait and prey fusion proteins. In these experiments, we fused the entire EBNA2 open reading frame with the Gal4 DNA-binding domain. As a control, we also generated a CBF1 protein fused with the VP16 activation domain. Cotransfection of Gal4-EBNA2 with VP16-CBF1 resulted in a consistent activation of a Gal4-luciferase reporter plasmid as expected (Fig. 7). No activation was observed when the VP16-CBF1 protein was coexpressed with the Gal4 DNA-binding domain alone. In contrast to the previous two-hybrid assay (Fig. 6), coexpression of EBNA-LP with Gal4-EBNA2 resulted in a 15-fold induction of the Gal4-responsive luciferase reporter, suggesting a functional interaction between EBNA2 and EBNA-LP (Fig. 7A). EBNA-LP also stimulated activity from the Gal4 DNA-binding domain alone. However, there were two anomalies in these experiments. First, EBNA-LP had a pronounced effect on the internal control vector expressing *Renilla* luciferase (CMVR-luc) by down-regulating its expression 4- to 5-fold and, in some cases, up to 10-fold (Table 3). Interestingly, this effect was much more pronounced in cells transfected with Gal4 fusion proteins than in cells expressing the Gal4 DNA-binding protein alone (Table 3). In addition, EBNA-LP had an opposite effect on expression of the Gal4-EBNA2 bait by increasing its expression over levels seen in the other cotransfected cells (Fig. 7B, compare lane 3 to lanes 1 and 2). To more reliably calculate the effect of EBNA-LP expression on the activity of the Gal4-EBNA2 bait, we tested several *Renilla* expression plasmids and found one, known as RL-null, which was more refractory to the effects of EBNA-LP than *Renilla* luciferase plasmids driven by SV40, CMV, or thymidine kinase promoters. An example of a typical transfection experiment with pRL-null is shown in Table 4, and the average of several experiments is shown in Fig. 8A. Coexpression of Gal4-EBNA2 and VP16-CBF1 resulted in stimulation of the Gal4-responsive reporter five- to sevenfold as observed in earlier experiments. In

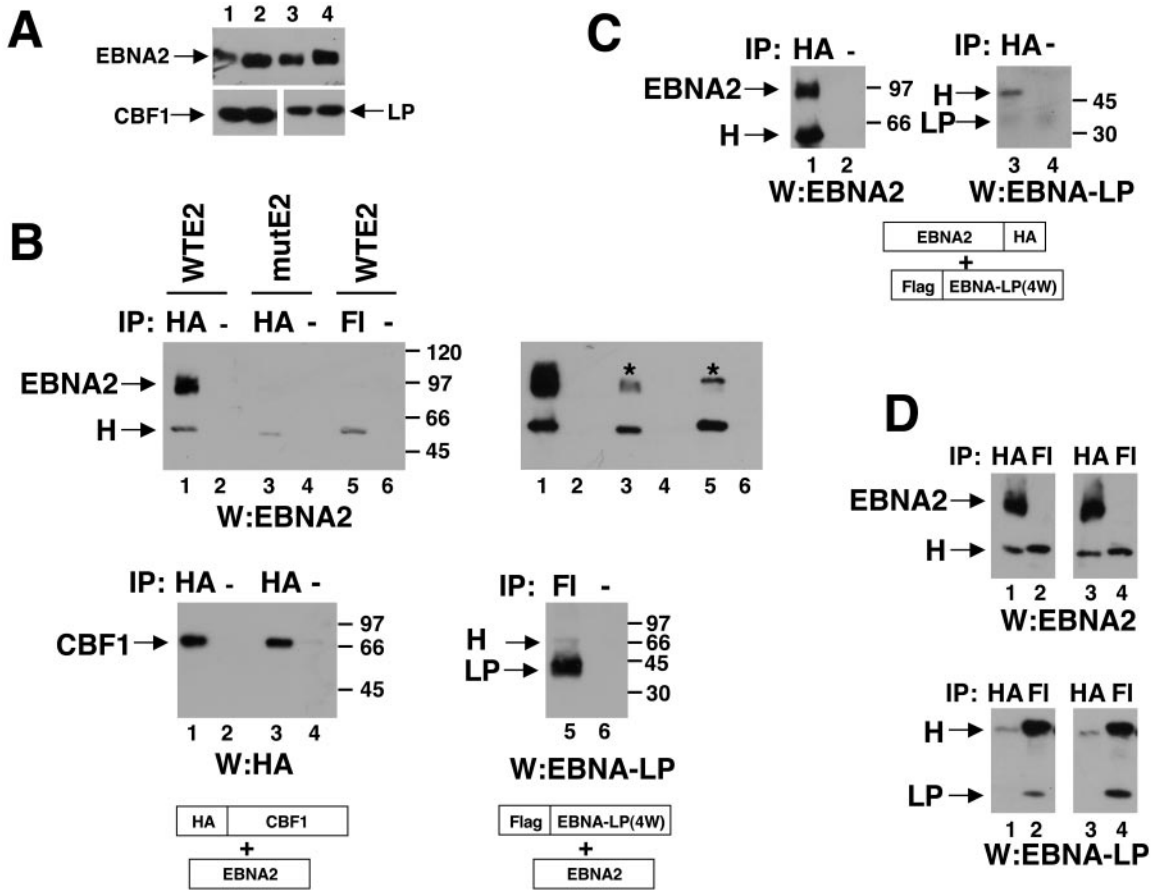

FIG. 2. Association of EBNA2 and EBNA-LP in cells is minimal. (A) DG75 cells were cotransfected with the following pairs of expression plasmids. Lane 1, wild-type EBNA2 (pPDL151) and HA-CBF1; lane 2, mutant EBNA2 (pPDL152) and HA-CBF1; lane 3, wild-type EBNA2 (pPDL151) and SG5LP; lane 4, wild-type EBNA2-HA (pAG155) and SG5LP. The transfected cells were solubilized in either radioimmunoprecipitation assay or NP-40 lysis buffer (see Materials and Methods), resolved by SDS-PAGE, and blotted, and replicate blots were probed with anti-EBNA2, anti-HA (for CBF1), or anti-EBNA-LP monoclonal antibody as indicated for each blot. (B) Extracts described for panel A containing wild-type (wt) EBNA2 and CBF1 (lanes 1 to 2), mutant (mut) EBNA2 and CBF1 (lanes 3 to 4), and wild-type EBNA2 and Flag–EBNA-LP (lanes 5 to 6) were analyzed by immunoprecipitation (IP) followed by Western blotting (W). The extracts were precipitated with an anti-HA antibody (lanes 1 and 3) or anti-Flag (lane 5) followed by incubation with protein G-Sepharose beads or beads only (lanes 2, 4, and 6). The precipitates were divided into two parts, and replicate blots were probed with anti-EBNA2, anti-HA (for CBF1), or anti-EBNA-LP antibody as indicated below each panel. A longer exposure of the blot probed with anti-EBNA2 is shown in the panel on the right, and the asterisks indicate trace amounts of EBNA2 that were detected after maximum exposures to the X-ray film. Cross-reactivity with the immunoglobulin heavy chain (H) by the secondary antibody is indicated for some of the immunoblots. Fl, Flag. (C) Extracts described for panel A (lane 4) containing wild-type EBNA2-HA and Flag–EBNA-LP were analyzed by immunoprecipitation followed by Western blotting. The panel on the left shows immunoprecipitation with anti-HA followed by immunoblotting with anti-HA (lane1) or precipitation with protein G beads alone (lane 2). The other half of the immunoprecipitation was immunoblotted with an anti-EBNA-LP antibody (IP anti-HA in lane 3 or beads alone lane 4). Cross-reactivity with the immunoglobulin heavy chain (H) by the secondary antibody is indicated for some of the immunoblots. (D) DG75 cells were transfected with a wild-type EBNA-LP (2W repeats) and EBNA2-HA (lanes 1 and 2) or a mutant EBNA-LP (S35/101A, 2 W repeats) and EBNA2-HA (lanes 3 and 4). The extracts were prepared as described in Materials and Methods. The panels on top show the results of extracts precipitated with anti-HA or anti-Flag antibody followed by Western blotting with an anti-EBNA2 antibody. The panels below are identical but instead were probed with an anti-EBNA-LP antibody. Cross-reactivity with the immunoglobulin heavy chain (H) by the secondary antibody is indicated for some of the immunoblots.

contrast, EBNA-LP now averaged only five- to sixfold induction when normalized over vector-transfected cells (Fig. 8A). Since the levels of Gal4-EBNA2 were still increased relative to the other samples, we suspected that the effects of EBNA-LP may be due to increasing levels of the bait protein rather than to some direct effect of EBNA-LP itself. To address this possibility, we performed dose-response experiments. In one experiment (Fig. 8B), several doses of the Gal4-EBNA2 expression plasmid with and with out coexpression of EBNA-LP were tested. Levels of luciferase activity from the Gal4-responsive promoter were measured and compared with the levels of bait protein synthesized. Cells transfected with 4 and 8 μg of the Gal4-EBNA2 bait plasmid appeared to synthesize levels of protein that were similar to cells transfected with 1 and 2 μg of Gal4-EBNA2 plus coexpression with EBNA-LP (Fig. 8B, compare lanes 4 and 5 to lanes 7 and 8, respectively). When cells expressing equivalent levels of the Gal4-EBNA2 protein were compared, the cells expressing EBNA-LP appeared to induce reporter gene activity approximately 30% above those cells that did not express EBNA-LP (Fig. 8B). These results suggest that a significant contribution of EBNA-LP toward activation of a Gal4-EBNA2 effector protein in transient-transfection

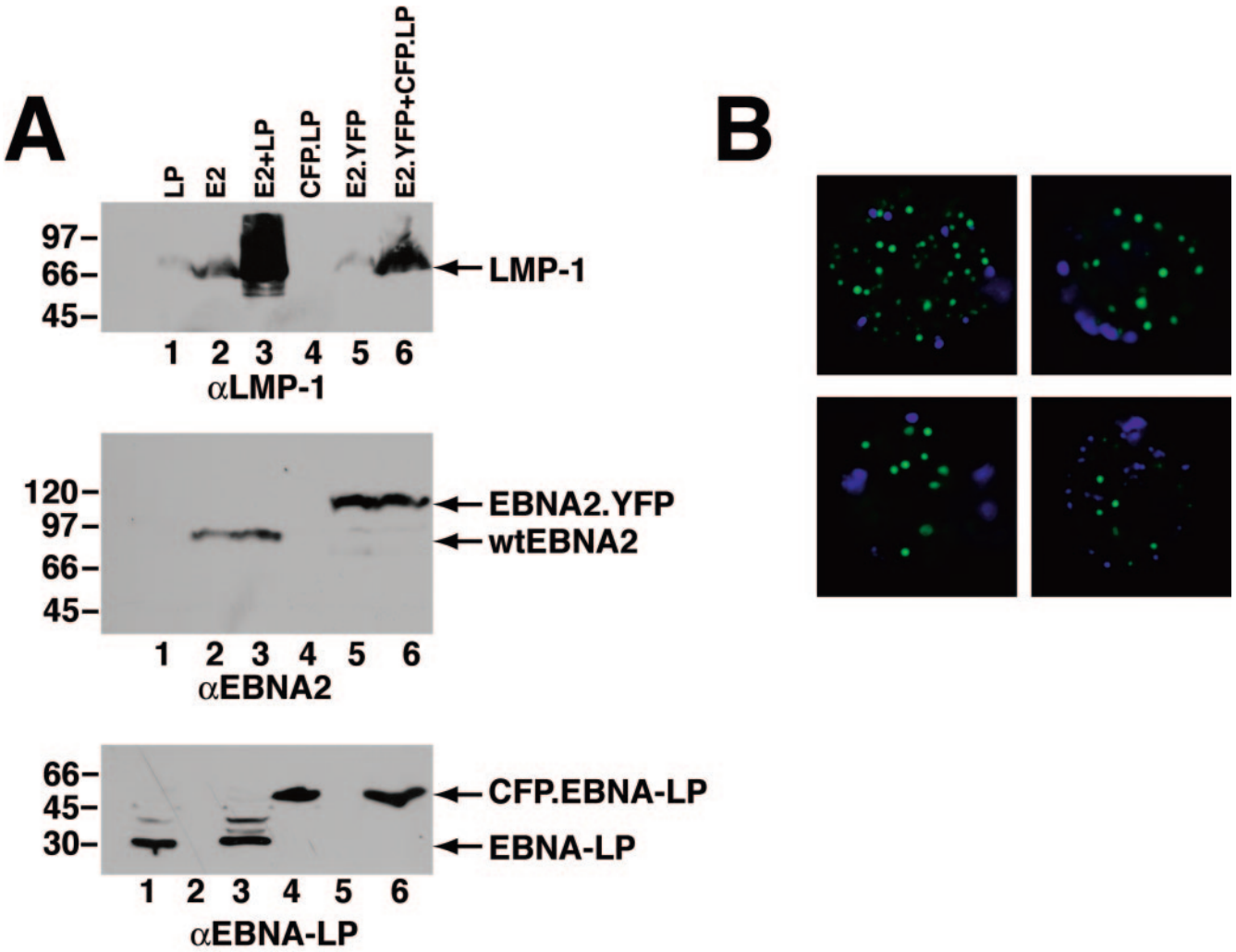

FIG. 3. CFP–EBNA-LP and EBNA2-YFP fusion proteins are transcriptionally competent but do not colocalize in cells. (A) Eli-BL cells were transfected with plasmids expressing EBNA-LP (lane 1), wild-type (wt) EBNA2 (lane 2), both effectors (lane 3), CFP–EBNA-LP (lane 4), EBNA2-YFP (lane 5), or both fusion proteins (lane 6). The cells were processed for Western blot analysis, and replicate blots were probed for LMP-1, EBNA2, and EBNA-LP with the appropriate antibodies. Bands corresponding to LMP-1 expression or each of the wild-type or fusion proteins are indicated on the right of each blot. The migration of molecular weight standards is shown on the left of each blot. α, anti. (B) Using high-resolution deconvolution microscopy, merged images of EBNA2 (green) and EBNA-LP (blue) cellular localization are shown. Images are single Z planes from four different cells.

assays is due to its ability to increase effector protein levels. In addition, several internal control expression plasmids are down-regulated by EBNA-LP which may further complicate interpretation of results.

DISCUSSION

In this study, we have demonstrated that EBNA-LP is a gene-specific coactivator of EBNA2, preferentially coactivating EBNA2 target genes expressed from the viral bidirectional promoter up to 10-fold with little effect on other viral or cellular genes (Fig. 1 and Table 2). Mechanistically, EBNA-LP may coactivate EBNA2 through mechanisms that do not involve direct or indirect interactions between these proteins. Evidence for this conclusion is based on an inability to detect significant interactions between EBNA-LP and EBNA2 in coimmunoprecipitation assays (Fig. 2), mammalian two-hybrid assays (Fig. 6 and 8; Tables 3 and 4), and a failure to observe significant colocalization between these proteins in immunofluorescence assays (Fig. 3 and 5).

The fact that EBNA-LP appears to be a gene-specific rather than a global coactivator is not unusual. Viral transactivators that share similar properties with EBNA-LP like the herpes simplex virus ICP0 protein or cytomegalovirus IE1 protein, which apparently lack specific DNA-binding activity but possess an ability to cooperate with other transactivators, do not globally affect the diversity of cellular and viral genes in an infected cell (5, 18). The preferential targeting of the viral bidirectional promoter is yet another example of a multitude of control mechanisms the virus has evolved to regulate LMP-1 expression. LMP-1 is essential for EBV-induced immortalization and B-cell proliferation, so the evolution of pathways that enable its efficient expression is not unexpected. Although LMP2A and B are not required for efficient B-cell immortal-

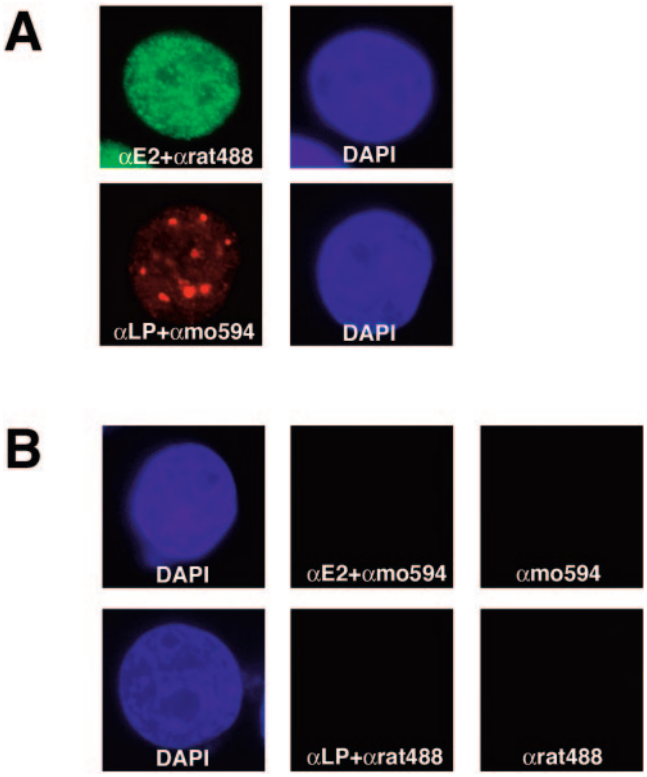

FIG. 4. Specific detection of EBNA2 and EBNA-LP in EBV-immortalized B cells. (A) The anti-EBNA2 (αE2) monoclonal antibody R3 and the secondary anti-rat Alexa 488 (αrat488)-conjugated antibody (Molecular Probes) were used to detect EBNA2 in IB4 cells. Indirect immunofluorescence with an EBNA-LP-specific antibody JF186 (αLP) and an anti-mouse Alexa 594 (αmo594)-conjugated antibody (Molecular Probes) was used to detect EBNA-LP in IB4 cells. DAPI staining for each cell is shown in the panel on the right. (B) In the top row of panels, IB4 cells were stained with DAPI (first panel) or the rat anti-EBNA2 antibody followed by the anti-mouse Alexa 594-conjugated antibody or the anti-mouse Alexa 594-conjugated antibody alone (second and third panels, respectively). In the lower set of panels, a DAPI-stained cell is shown (first panel). The second and third panels show the same cell stained with the mouse anti-EBNA-LP monoclonal and a secondary anti-rat Alexa 488-conjugated antibody or the anti-rat Alexa 488-conjugated antibody alone.

ization by EBV, it is interesting that LMP2B is also strongly induced by EBNA-LP. Temporally, EBNA-LP, along with EBNA2 is one of the first latent proteins expressed after B-cell infection. Our data suggest that expression of LMP2B may be important, particularly during early infection in vivo. The lack of significant interactions between EBNA2 and EBNA-LP found by coimmunoprecipitation assays in this study is similar to earlier findings (51) and supports the idea that EBNA-LP is not a global coactivator of EBNA2. In addition, we are unaware of extensive studies implying that EBNA2 and EBNA-LP may localize in similar compartments in the nucleus (50, 55). Thus, our data showing that these proteins do not colocalize are also consistent with those of previous studies and the interaction data. Finally, our data from mammalian two hybrid analyses also indicated a lack of significant functional interactions between EBNA2 and EBNA-LP, independently confirming results from the coimmunoprecipitation and immunofluorescence assays. Therefore, a picture emerges that is highly suggestive that the bulk of EBNA2 and EBNA-LP do not associate in cells and that the mechanisms of EBNA-LP coactivation that do not involve interactions with EBNA2 should be considered.

One previous study has suggested that perhaps EBNA-LP makes transient unstable interactions with EBNA2 that result in recruitment of factors that help induce transcription (51). However, three observations from our data are not consistent with this model. First, general interaction between EBNA2 and EBNA-LP would be expected to result in EBNA-LP coactivation of all EBNA2 target genes, but our observations suggest this does not occur. Second, if EBNA-LP worked by recruiting additional transcription factors to EBNA2 complexes, then it may be expected to contain some intrinsic transcriptional activation potential by itself when fused to the DNA-binding domain of Gal4. However, in mammalian two-hybrid assays from this study and yeast two-hybrid assays published previously (27, 33, 34, 81) and confirmed by our laboratory (data not shown), Gal4–EBNA-LP fusion proteins do not possess any transcriptional activating function. Finally, in our laboratory, we do not detect significant functional interactions between these proteins by two-hybrid analyses that may be consistent with the ability of EBNA-LP to coactivate EBNA2 on the order of at least one-half to one log.

It is clear that in some of our experiments we could detect small amounts of EBNA-LP interacting with EBNA2 (Fig. 2) and that even after normalizing to controls, a small effect of EBNA-LP was observed in the two-hybrid assays (Fig. 8). In LCLs, we could also occasionally see some partial overlap in colocalization (data not shown). However, we only observed interactions in the coimmunoprecipitations upon extremely long exposures and only when EBNA-LP was used to coprecipitate EBNA2 (Fig. 2B) but never when EBNA2 was used to coprecipitate EBNA-LP (Fig. 2C). In addition, we also never observed interactions in the coimmunoprecipitations when EBNA-LP isoforms containing 2 W repeats were tested (Fig. 2D). In the two-hybrid assays, we were also only able to detect possible interactions between EBNA-LP and EBNA2 when EBNA2 was fused to the DNA binding domain of Gal4 and EBNA-LP functioned as a prey protein (Fig. 8) but not the reverse (Fig. 7). Using YFP and CFP fusion proteins, we never observed any colocalization between EBNA2 and EBNA-LP. Thus, when interactions between these proteins were observed, they were significantly lower than those occurring between EBNA2 and CBF1 and they also occurred only under specific conditions in our assays. Our conclusions from these data are that relatively low-level interactions between EBNA2 and EBNA-LP can occur and may make minor contributions to the overall coactivation potential of EBNA-LP. However, given the specific and powerful coactivation effect of EBNA-LP on EBNA2 for the viral divergent promoter, we believe that alternative mechanisms that account for EBNA-LP coactivation may be relevant.

A major discrepancy exists between our results with the mammalian two-hybrid system and those obtained by Peng et al. (51). In the previous study, EBNA-LP was able to simulate the activity of a Gal4-EBNA2 fusion protein on Gal4-responsive reporters in transient-transfection assays. In our experiments, the bulk of this activation could be accounted for by a potent ability of EBNA-LP to induce levels of the bait protein

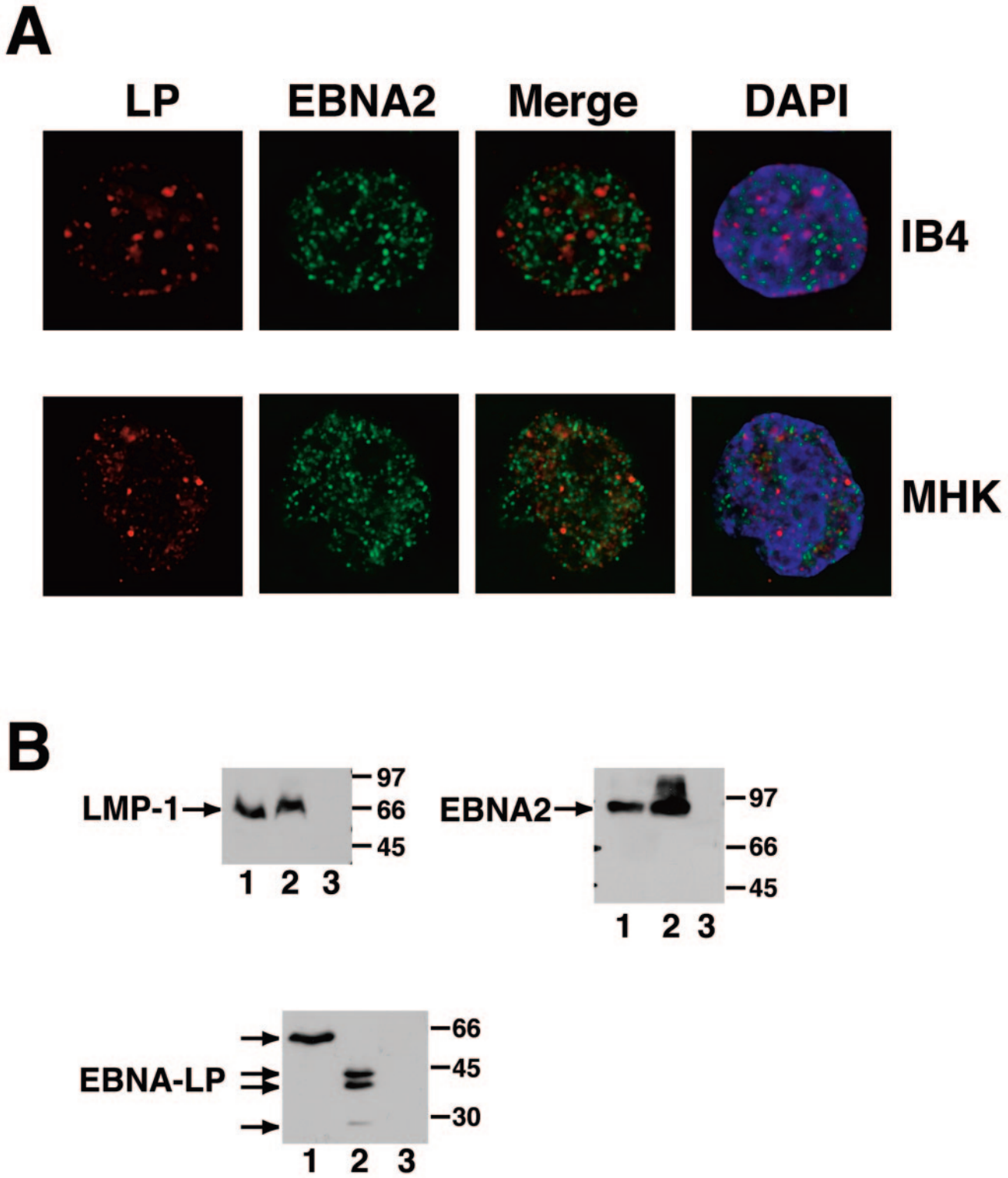

FIG. 5. EBNA protein expression and subcellular localization in LCLs. (A) High-resolution images from deconvolution microscopy are shown for MHK or IB4 cells stained with anti-EBNA2 or anti-EBNA-LP antibody followed by anti-rat Alexa 488 (green) and anti-mouse Alexa 594 (red) secondary antibodies, respectively. Images are representative of the population and are single Z planes. (B) Western blot analysis of MHK (lane 1 in all panels), IB4 (lane 2 in all panels), and DG75 (lane 3 in all panels) cells. Cell extracts from each cell line were resolved by SDS-PAGE, and replicate blots were produced. The blots were probed for LMP-1, EBNA2, or EBNA-LP with S12, R3, and JF186 monoclonal antibodies, respectively. The specific proteins detected on each blot are indicated by the arrow(s) on the left. IB4 cells expressed at least 3 EBNA-LP isoforms.

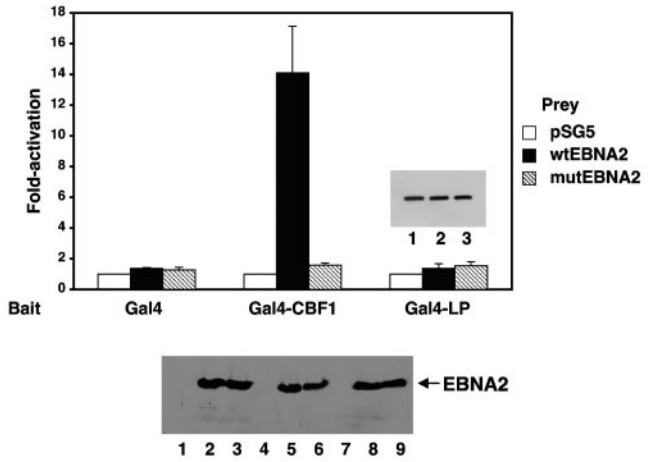

FIG. 6. EBNA2 does not functionally interact with a Gal4–EBNA-LP fusion protein in mammalian two-hybrid assays. Plasmids expressing Gal4, a Gal4-CBF1 fusion protein, or a Gal4–EBNA-LP fusion protein were cotransfected with pSG5 vector, wild-type (wt) EBNA2, or mutant (mut) EBNA2 and a Gal4-responsive luciferase reporter plasmid. An internal control plasmid driving the *Renilla* luciferase protein under the control of an SV40 promoter was also included. At 48 h posttransfection, luciferase activity was measured from transfected cell extracts and normalized to *Renilla* luciferase to control for transfection efficiencies. Results are reported as activation relative to the activity detected from the bait protein alone (e.g., cotransfected with pSG5 vector). Error bars indicate standard deviations from the means from at least three independent experiments. The levels of EBNA2 were detected by Western blot analysis from the transfected cells as shown in the panel below the graph with the R3 anti-EBNA2 monoclonal antibody. Cells were transfected with prey plasmids pSG5 (lanes 1, 4, 7), wild-type EBNA2 (lanes 2, 5, 8), and mutant EBNA2 (lanes 3, 5, 9) and bait plasmids Gal4 (lanes 1 to 3), Gal4-CBF1 (lanes 4 to 6), and Gal4-LP (lanes 7 to 9). Levels of the Gal4-LP fusion protein were also detected by immunoblotting with a monoclonal antibody to EBNA-LP and are shown in the panel above the bars corresponding to its activity in these assays.

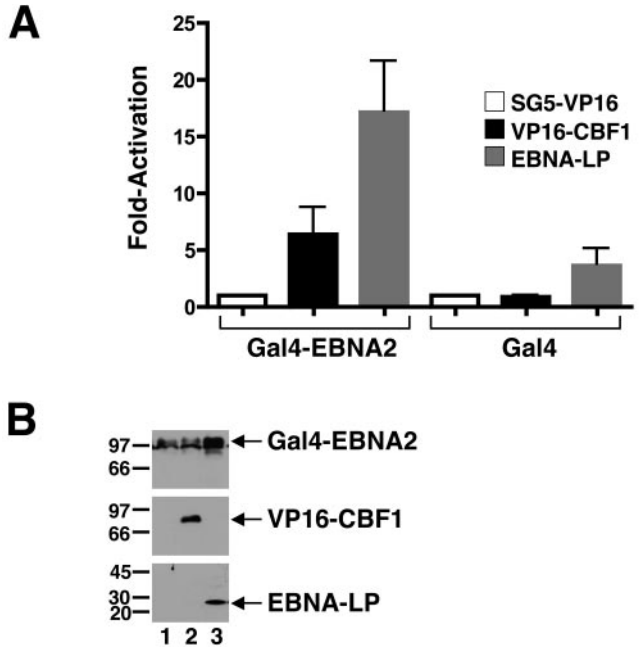

FIG. 7. EBNA-LP and VP16-CBF1 enhance Gal4-EBNA2 stimulation of a Gal4-responsive reporter. (A) Gal4-EBNA2 or Gal4 was cotransfected with an SG5-VP16 vector, VP16-CBF1, or EBNA-LP in DG75 cells together with a Gal4-responsive reporter plasmid (5xGal4E1bLuc). Results are shown as activation relative to that of vector-transfected cells. Error bars indicate standard deviations from the means from at least three independent experiments. (B) A portion of the lysates from panel A were analyzed by Western blotting. Three identical blots were probed with anti-EBNA2, anti-HA, or anti-EBNA-LP antibody. Lane 1, Gal4-EBNA2 and VP16-SG5; lane 2, Gal4-EBNA2 and VP16-CBF1; lane 3 Gal4-EBNA2 and EBNA-LP.

(Gal4-EBNA2) and also down-regulate the internal control plasmid. Differences in two-hybrid reagents may account for these observed discrepancies. Our two-hybrid system employed the entire EBNA2 ORF fused to the Gal4 DNA-binding domain while Peng et al. (51) used a Gal4 fusion with an EBNA2 ORF with a deletion of the first 19 amino acid residues. In addition, we used a *Renilla* luciferase internal control, while the previous study used a CMV–β-galactosidase internal control. The observed down-regulation of the *Renilla* reporter control is consistent with observations published by others (9). In addition, our two-hybrid assay conditions were further validated, since specific interactions between EBNA2 and CBF1 were readily and consistently observed, while significant interactions between EBNA2 and EBNA-LP were largely negative or very small when normalized to controls. A second discrepancy is our finding that the CFP–EBNA-LP fusion protein localized in discrete punctate spots in the nuclei of transfected Burkitt's cells rather than in a more diffuse nuclear pattern as reported earlier (50, 53, 55). One possibility is that the CFP domain of this fusion protein contributes to this phenotype. Alternatively, indirect immunofluorescence with the anti-EBNA-LP monoclonal antibody may be more sensitive than direct observation of fluorescence emission from the fusion protein. Protein overexpression in transiently transfected cells may also yield aberrant protein localization phenotypes that may be corrected when expressed at lower levels as, for example, in stably expressing cell lines. Nonetheless, even if we were unable to detect significant low-level diffuse staining from the expressed CFP–EBNA-LP protein, our results clearly showed that predominant amounts of this protein localized to distinct foci, and under these assay conditions, where it retained significant coactivation activity, no significant overlap with EBNA2 was apparent.

An important assumption we made for determination of the specificity of EBNA-LP coactivation was that the conditions in

TABLE 3. Down-regulation of an internal reference control by EBNA-LP

Effector plasmid	Fluc (RLU)[a]	Rluc (RLU)[b]	Fold activation
Gal4-EBNA2 + SG5/VP16	54	1,921	1
Gal4-EBNA2 + VP16/CBF1	280	2,151	4.6
Gal4-EBNA2 + EBNA-LP	184	392	16.7
Gal4 + SG5/VP16	103	1,100	1
Gal4 + VP16/CBF1	73	911	0.9
Gal4 + EBNA-LP	200	932	2.3

[a] Luminescence (in relative luciferase units [RLU]) from cells transfected with 5 μg of an E1b firefly luciferase reporter plasmid with five Gal4 binding sites.
[b] Luminescence (in relative luciferase units [RLU]) from cells transfected with pRL-SV40. Similar results were observed with pRL-CMV, pRL-TK, and phRL-TK (Promega).

TABLE 4. The RL-null internal reference plasmid is refractory to the down-regulating effects of EBNA-LP

Effector plasmid	Fluc (RLU)[a]	Rluc (RLU)[b]	Fold activation
Gal4-EBNA2 + SG5/VP16	21	23	1
Gal4-EBNA2 + VP16/CBF1	227	27	8.8
Gal4-EBNA2 + EBNA-LP	134	28	4.9

[a] Luminescence (in relative luciferase units [RLU]) from cells transfected with 5 μg of an E1b firefly luciferase reporter plasmid with five Gal4 binding sites.
[b] Luminescence (in relative luciferase units [RLU]) from cells transfected with pRL-null (Promega).

type I Burkitt's cell lines recapitulate the environment encountered by EBNA2 and EBNA-LP during natural infection more accurately than transient transfections with reporter genes. This is an issue open for debate; however, there is a vast literature describing potential pitfalls of using reporter gene plasmids to investigate gene regulation in certain circumstances, particularly herpesvirus promoters that are temporally regulated (59). One advantage of our assay was that we were able to measure the effect of EBNA-LP simultaneously on several viral and cellular genes where we observed a clear-cut preference for EBNA-LP to coactivate EBNA2 on the viral divergent promoter. Unfortunately, we were unable to evaluate the effects of EBNA-LP on the viral C promoter in this study due to an inability to consistently stimulate this promoter by EBNA2 with our cell lines. Examination of other cell lines more amenable to Cp induction may yield insight into whether EBNA-LP can regulate it. Although our data are highly suggestive that EBNA-LP works primarily on the viral bidirectional promoter, this conclusion is also limited to the number of genes analyzed in this study. While the viral targets of EBNA2 have been well characterized, surprisingly few direct cellular targets of EBNA2 have been identified and verified by multiple laboratories. A more global approach to identifying EBNA2 target genes in the presence and absence of EBNA-LP may identify additional specific targets coactivated by EBNA-LP.

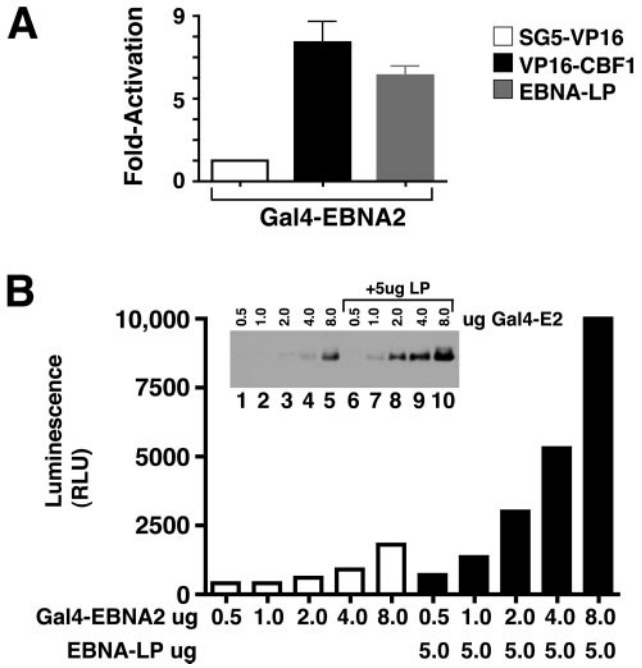

FIG. 8. The bulk of EBNA-LP effects on a Gal4-EBNA2 bait protein are due to down-regulation of most internal control plasmids and induction of bait protein levels. (A) Gal4-EBNA2 was cotransfected with the indicated plasmids expressing various prey proteins. Cotransfections were performed as described for Fig. 7A, but only the RL-null internal control was used to control for transfection efficiency. (B) The Gal4-EBNA2 bait plasmid was transfected with the amounts indicated below the graph or cotransfected with 5 μg of EBNA-LP. Luminescence (in relative luciferase units [RLU]) levels are indicated for each sample on the left. The inset shows a Western blot from the transfected cell lysates probed with an EBNA2 antibody to detect the Gal4-EBNA2 bait protein. Samples transfected with 0.5 μg (lane 1 and 6), 1.0 μg (lanes 2 and 7), 2.0 μg (lanes 3 and 8), 4.0 μg (lanes 4 and 9), and 8.0 μg (lanes 5 and 10) are shown. Five micrograms of EBNA-LP was cotransfected in samples shown in lanes 6 to 10.

Another limitation in our studies concerns the immunofluorescence assays. The sensitivity of our antibodies may not have been sufficient to detect low levels of EBNA2 or EBNA-LP that colocalized. In addition, transient interactions may still occur, and if they are dependent on particular stages of the cell cycle, they may be difficult to observe in an asynchronously growing population of cells. Despite this limitation, we believe that the bulk of the evidence from three different assays is highly suggestive that most of the EBNA2 and EBNA-LP, whether in transiently overexpressing cells or in EBV-immortalized cell lines, seems not to be associated with each other.

Based on the data from this study, we would like to suggest an alternative hypothesis, which, along with direct interactions, may account for the strong EBNA2 coactivation observed on the viral bidirectional promoter. One attractive idea is that EBNA-LP may sequester a repressor or stimulate modification of a repressor that regulates the divergent promoter. In such a model, EBNA-LP would not have to interact with EBNA2 or even the viral genome and would be consistent with the observations made in this study. Inactivation of a repressor activity may not be sufficient to observe any activation of the divergent promoter without expression of a positive activator like EBNA2 and would also be consistent with observations that EBNA-LP does not generally stimulate genes when expressed alone. Equally possible is the idea that EBNA-LP may stimulate pathways that result in activating a transcriptional activator that cooperates with EBNA2, although on the surface this seems less likely, since EBNA-LP, as mentioned previously, does not contain intrinsic LMP-1-activating function by itself. A mechanism by which EBNA-LP may operate distinct from interactions with EBNA2 would be consistent with the published observation that it colocalizes with PML or ND10 bodies (69). PML bodies contain multiple transcriptional regulators that could be targets for EBNA-LP, permitting it to function in transcriptional control from a promoter distal site. Precedents for manipulation of PML bodies, perhaps to create a favorable environment for viral gene expression and productive infection include the herpesvirus proteins ICP0 and IE1 (5, 18). An attractive idea is that EBV has also evolved mechanisms to manipulate ND10/PML, through EBNA-LP, that may be required for establishment of latent infection.

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