JOURNAL OF VIROLOGY, July 2009, p. 7109–7116 Vol. 83, No. 14
0022-538X/09/$08.00+0 doi:10.1128/JVI.00654-09


# Nuclear-Cytoplasmic Shuttling Is Not Required for the Epstein-Barr Virus EBNA-LP Transcriptional Coactivation Function[▽]

Paul D. Ling,* Jie Tan, and RongSheng Peng

*Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas 77030*

Received 30 March 2009/Accepted 20 April 2009

**Epstein-Barr virus (EBV) EBNA-LP is a transcriptional coactivator of EBNA2 that works though interaction with the promyelocytic leukemia nuclear-body-associated protein Sp100A. EBNA-LP localizes predominantly in the nucleus through the action of nuclear localization signals in the repeated regions of the protein. EBNA-LP has also been detected in the cytoplasm, and a previous study suggested that some of the EBNA-LP coactivation function is mediated by relocalizing histone deacetylase 4 (HDAC4) from the nucleus to the cytoplasm. Although EBNA-LP can be found in the cytoplasm, it has no obvious nuclear export signal, and there is no direct evidence for active shuttling between these cellular compartments. Whether active shuttling between the nucleus and cytoplasm is required for coactivation remains to be clarified. To address these issues, we tested a variety of EBNA-LP isoforms and mutants for nuclear-cytoplasmic shuttling activity in an interspecies heterokaryon assay and for the ability to associate with HDAC4. EBNA-LP isoforms smaller than 42 kDa shuttle efficiently in the heterokaryon assay via a crm-1-independent mechanism. In addition, no specific EBNA-LP domain that mediates nuclear export could be identified. In contrast, an EBNA-LP 62-kDa isoform does not demonstrate detectable shuttling in the heterokaryon assay yet still coactivates EBNA2 similarly to the smaller EBNA-LP isoforms. All of the EBNA-LP mutants tested, including the coactivation-deficient ΔCR3 mutant and the nonshuttling 62-kDa isoform, were capable of associating with HDAC4. Taken together, our results suggest that simple diffusion may account for the nuclear export observed with smaller isoforms of EBNA-LP, that nuclear-cytoplasmic shuttling is not required for efficient EBNA-LP coactivation function, and that competence for HDAC4 association is not sufficient to mediate nuclear-cytoplasmic shuttling or EBNA-LP coactivation in the absence of a functional interaction with Sp100A.**

Epstein-Barr virus (EBV) is associated with several human malignancies, including endemic Burkitt lymphoma and lymphomas in the immunosuppressed (38, 42). However, the mechanisms of EBV-mediated lymphomagenesis remain to be clarified. EBV possesses an intrinsic ability to immortalize human B cells with high efficiency through the expression of several virus-encoded proteins (41). By unraveling the mechanisms by which EBV immortalizes B cells, we hope to understand how the virus induces cancer in humans.

Several viral gene products are made in EBV-immortalized B cells, including Epstein-Barr nuclear antigens (EBNA-LP, EBNA1, EBNA2, EBNA3a, EBNA3b, and EBNA3C), latent membrane proteins (LMP1, LMP2A, and LMP2B), EBV-encoded RNAs (Ebers 1 and 2 and BamA rightward transcripts), and EBV microRNAs (2, 3, 41). More recently, presumed lytic cycle genes, such as BHRF1, have been implicated in the immortalization process (1). Our laboratory is interested in understanding the role of EBNA-LP, a coactivator of EBNA2, for EBV-induced immortalization.

Genetic and biochemical evidence has shown that EBNA2 is essential for initiating and maintaining EBV-mediated B-cell immortalization while EBNA-LP is important but not essential for immortalization (11, 13, 19, 24). EBNA2 is a transcription-activating protein that regulates viral latency genes through mimicry of Notch signaling pathways (12, 16, 17), and EBNA-LP coactivates EBNA2, preferentially enhancing the expression of the major viral oncoprotein LMP1 (8, 14, 29, 30). EBNA-LP is an unusual protein comprised of a variable number of 66-amino-acid repeats derived from two exons (W1 and W2) in the viral internal repeated region 1 and a unique 45-residue carboxyl terminus derived from the Y1 and Y2 exons (Fig. 1; also see Fig. 5A) (33, 35). In newly infected B cells, a range of EBNA-LP isoforms are expressed (Fig. 1), but over time in culture, the number of expressed isoforms decreases (5, 9). In addition, EBNA-LP localizes diffusely throughout the nucleus within the first 24 to 48 h postinfection, but with time, it associates with promyelocytic leukemia nuclear bodies (PML NBs) (21, 37). EBNA-LP nuclear localization is mediated primarily thorough a bipartite nuclear localization signal located in evolutionarily conserved region 1c (CR1c) and CR2 (see Fig. 5A) (31). EBNA-LP mutants that fail to localize in the nucleus are coactivation deficient (31). EBNA-LP plays an important role in the establishment of B-cell immortalization, as mutant viruses lacking the Y1-Y2 exons, which code for the unique carboxy-terminal domain, are much less efficient at immortalizing B cells than wild-type viruses (13, 24). Interestingly, the EBNA-LP coactivation function does not require the carboxy-terminal unique domain, so previous genetic analyses did not evaluate the role of this function for EBV-mediated B-cell immortalization (14, 29). The EBNA2 coactivation function of EBNA-LP requires a minimum of two W repeats, and deletion of CR3 in EBNA-LP greatly diminishes its coactivation function (21, 27, 31). Recent evidence suggests that the PML NB-associated protein Sp100A is an important mediator

* Corresponding author. Mailing address: Department of Molecular Virology and Microbiology, Baylor College of Medicine, Mail Stop BCM-385, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-8474. Fax: (713) 798-3586. E-mail: pling@bcm.tmc.edu.

▽ Published ahead of print on 29 April 2009.

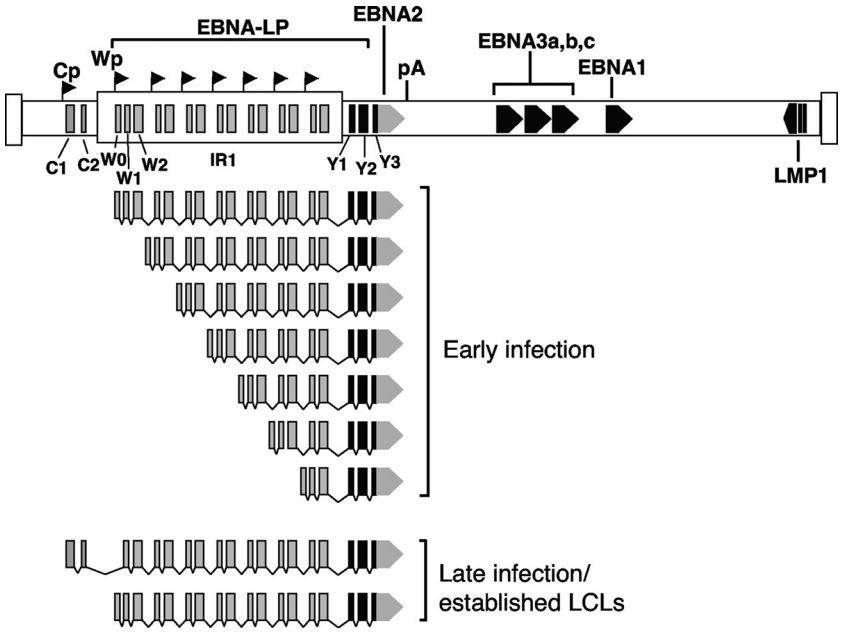


FIG. 1. Exon organization of EBNA-LP gene transcripts. Transcription of the EBNA-LP gene initiates from either the W promoter (Wp) or the C promoter (Cp). The different noncoding (C1, C2, and W0) and coding (W1, W2, Y1, and Y2) exons are indicated. During early stages of infection, transcription initiates from available Wps residing in each internal repeated region 1 (IR1) repeat, which results in the production of multiple EBNA-LP protein isoforms. During later stages of infection or in established lymphoblastoid cell lines (LCLs), transcription from Cp is stimulated and there is a bias toward the origin of replication (OriP)-proximal Wp. The level of Cp- versus Wp-initiated transcription varies depending on several circumstances. The viral latent OriP, the polyadenylation site (pA) for EBNA-LP/EBNA2 transcripts, and the locations of other EBNA genes and LMP are shown.

of the EBNA-LP coactivation function (7, 21). EBNA-LP specifically relocalizes Sp100A from PML NBs via interaction with CR3, and this activity correlates with EBNA2 coactivation (21). In addition, the heterochromatin protein 1 (HP1) binding domain of Sp100A is required for coactivating EBNA2, suggesting that modulation of chromatin is important for its mechanism of action (21). Consistent with this theme, a recent report suggested that EBNA-LP sequesters histone deacetylase 4 (HDAC4) in the cytoplasm, and this may also contribute to its coactivation function (32). However, it is unclear (i) if EBNA-LP actively moves HDAC4 from the nucleus to the cytoplasm, (ii) if cytoplasmic pools of EBNA-LP sequester HDAC4, or (iii) if HDAC4, which has been reported to shuttle between the nucleus and cytoplasm (40, 43), mediates EBNA-LP nuclear-cytoplasmic shuttling. EBNA-LP does not have a recognizable nuclear export signal, so the mechanisms governing nuclear export remain uncharacterized. To gain further insight into these issues, we investigated whether EBNA-LP actively shuttles between the nucleus and cytoplasm by using an interspecies heterokaryon assay. The results indicate that isoforms of EBNA-LP smaller than 42 kDa shuttle between the nucleus and cytoplasm by a crm-1 independent pathway. However, isoforms larger than 62 kDa have no detectable nuclear-cytoplasmic shuttling activity but still retain significant coactivation function. Moreover, the ability to interact with HDAC4 and shuttle between the nuclear and cytoplasmic compartments is not on its own sufficient to mediate the coactivation function. The data suggest that nuclear-cytoplasmic shuttling is not required for transcriptional coactivation function and that the nuclear export observed with smaller isoforms of EBNA-LP may occur by diffusion.

## MATERIALS AND METHODS

**Cell culture and plasmids.** NIH 3T3, 293, and HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and were grown in 5% $CO_2$ tissue culture incubators at 37°C. X50-7, DG75, and Eli-BL cells were maintained similarly, except RPMI was used instead of Dulbecco's modified Eagle medium. Expression plasmids for EBNA2 (pRSP363), EBNA-LP with two W repeats (pJT125), EBNA-LP with four W repeats (pSG5LP), and the EBNA-LP mutants ΔCR3, GD2/67AAA, PGP13/79AAA, GIG17/83AAA, GPE19/85AAA, EGP21/87AAA, S5/71A, and S35/101A have been described previously (21, 27, 31). The EBNA-LP mutant $S_{34, 36, 63}$-A, which contains alanine substitutions for three conserved serine residues in W1-W2, was generously provided by Andrew Bell and has been described previously (27). EBNA-LP with seven W repeats (pRSP693) was PCR amplified from a cDNA clone described previously (35) and cloned into the pSG5 expression vector. The expression plasmid for myc-tagged HDAC4 was generously provided by T. Kouzarides and has been described previously (28).

**Interspecies heterokaryon assay and indirect immunofluorescence.** To form heterokaryons, HeLa cells were transfected with 2.0 μg of expression plasmid DNA using Superfect (Qiagen) according to the manufacturer's protocol. Twenty-four hours posttransfection, the cells were trypsinized, and $1 \times 10^5$ transfected HeLa cells were mixed with an equal number of NIH 3T3 cells in 1 ml medium and plated onto glass coverslips in a 24-well tissue culture plate. The cells were allowed to settle onto the coverslips for 3 to 4 h in the presence of 100-mg/ml cycloheximide. In some experiments, leptomycin B (LMB) was added at a concentration of 25 ng/ml (Sigma) and was maintained in the medium throughout the rest of the experiment. Cell fusion was performed by adding 50% polyethylene glycol 8000 (Sigma) for 2 min, after which the cells were washed with complete medium and incubated in complete medium containing cycloheximide or cycloheximide plus LMB for an additional hour. The cells were then washed with phosphate-buffered saline and fixed at 4°C with 4% paraformaldehyde. The cells were then permeabilized using 1% Triton, washed, and blocked for 1 h at

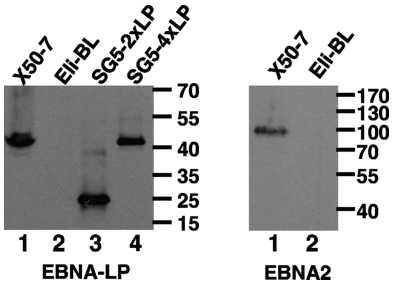


FIG. 2. EBNA2 and EBNA-LP expression in X50-7 cells. On the left is a Western blot of extracts from transfected and untransfected cells. Lane 1, X50-7 cells; lane 2, Eli-BL cells; lanes 3 and 4, DG75 cells transfected with an expression plasmid for EBNA-LP with two W repeats or four W repeats, respectively. The blot was probed with an EBNA-LP monoclonal antibody (JF186). On the right is a Western blot of X50-7 and Eli-BL cells probed with an EBNA2 monoclonal antibody (R3). Eli-BL cells are EBV-positive Burkitt lymphoma cells that do not express EBNA2 or EBNA-LP. DG75 cells are EBV-negative Burkitt lymphoma cells. Numbers on the right of each blot are molecular masses (in kilodaltons).

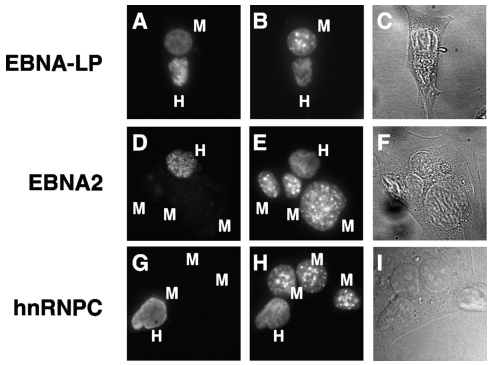


FIG. 3. Nuclear-cytoplasmic shuttling of EBNA-LP in heterokaryons between human B cells and mouse 3T3 cells. (A, D, and G) X50-7 cells were fused with murine 3T3 cells and stained with EBNA-LP, EBNA2, or hnRNPC antibodies as indicated. (B, E, and H) Human and mouse cell nuclei (H and M, respectively) were distinguished by staining them with Hoechst stain, which gives a diffuse staining pattern for human nuclei and diffuse/punctate staining for mouse nuclei. (C, F, and I) Heterokaryon formation was confirmed by differential interference contrast.

room temperature or at 4°C overnight with 5% dried milk in Tris-buffered saline. Following incubation with primary and secondary antibodies in blocking buffer, the cells were stained with Hoechst stain and Alexa Fluor 594-phalloidin and visualized on a Zeiss Axioplan II upright microscope. Primary antibodies against EBNA-LP (mouse monoclonal JF186), EBNA2 (rat monoclonal R3), hnRNPC (mouse monoclonal from Santa Cruz), and hemagglutinin (mouse monoclonal from Covance) were used for detection of the respective proteins. Alexa 488- or 594-conjugated anti-mouse or anti-rat antibodies (Molecular Probes) were used as secondary antibodies. Captured images were imported into and processed by Adobe Photoshop. For heterokaryons between X50-7 cells and 3T3 cells, $1 \times 10^5$ of each cell type were plated onto glass coverslips as described for the HeLa-3T3 heterokaryons and then processed for generation of heterokaryons as described above.

To visualize Sp100 localization following expression of EBNA-LP, DG75 or Eli-BL cells were electroporated with expression plasmids for EBNA-LP as described previously (7, 21). Twenty-four hours posttransfection, the cells were spun onto coverslips, and Sp100 localization was detected by indirect immunofluorescence (7, 21). Similar experiments were done in HeLa cells, except that they were transfected using Superfect (Qiagen).

**EBNA-LP coactivation.** EBNA-LP coactivation was tested by looking for enhancement of LMP1 protein induction by EBNA2 in Eli-BL cells. Eli-BL cells were transfected with expression plasmids for EBNA2 or EBNA2 and EBNA-LP (21, 31). Forty-eight hours posttransfection, the cells were harvested and analyzed for expression of LMP1, EBNA2, and EBNA-LP as described previously (21, 31).

**Immunoprecipitations.** 293 or DG75 cells were cotransfected with an HDAC4-myc expression plasmid and either the wild type or the coactivation-defective EBNA-LP mutant ΔCR3. Twenty-four to 48 h posttransfection, the cells were harvested and proteins were precipitated, followed by Western blot analysis as described previously (21).

## RESULTS

**EBNA-LP shuttles between the nucleus and the cytoplasm.** To determine whether EBNA-LP shuttles between the nucleus and cytoplasm, we used an interspecies heterokaryon assay. Heterokaryon formation between human B cells and murine 3T3 cells is difficult because most human B-cell lines are nonadherent. However, a significant proportion of EBV-immortalized X50-7 cells are adherent. X50-7 cells constitutively express all of the EBV latent proteins, including EBNA2 and EBNA-LP (Fig. 2). The EBNA-LP protein expressed in X50-7 cells migrates at approximately 42 kDa, similar to transiently expressed EBNA-LP with four W repeats, suggesting that it also has four W repeats (Fig. 2). As shown in Fig. 3A, fusion of X50-7 cells with mouse NIH 3T3 cells resulted in detectable shuttling of EBNA-LP between human and mouse nuclei in nearly 100% of human-mouse cell heterokaryons. EBNA2 was never found in any mouse nuclei (Fig. 3D). In addition, the heteronuclear protein hnRNPC, which is a nonshuttling 37-kDa protein, was found only in human cells (Fig. 3G). Interaction with crm-1 regulates the nuclear export of some nuclear-cytoplasmic shuttling proteins, which can be blocked with LMB (15, 20, 22, 39). Concentrations of LMB up to 25 ng/ml, which is sufficient to block crm-1-dependent shuttling of other proteins (15, 20, 22, 39), were unable to inhibit EBNA-LP nuclear-cytoplasmic shuttling and were sufficient to inhibit shuttling of an LMB-sensitive p53-green fluorescent protein fusion protein, as expected (references 4 and 36 and data not shown).

**No single EBNA-LP domain appears to mediate nuclear export.** To define the functional domains required for EBNA-LP nuclear-cytoplasmic shuttling, we generated heterokaryons between HeLa cells transfected with various mutant EBNA-LP expression plasmids and NIH 3T3 cells. Because the panel of mutant EBNA-LP proteins available for these analyses is in the context of proteins that have only two W repeats, we first compared the shuttling activity between wild-type EBNA-LP with two and four W repeats. As expected, EBNA-LP with two and four W repeats localized in the nucleus with a diffuse staining pattern (Fig. 4). In addition, nearly 100% of the human-mouse heterokaryons had detectable EBNA-LP in both human and mouse nuclei. Transiently expressed EBNA2 and endogenous hnRNPC were found only in human nuclei (Fig. 4). Coexpression of EBNA2 with EBNA-LP had no effect on EBNA-LP shuttling, and LMB was also unable to inhibit shuttling (data not shown).

To define EBNA-LP domains required for nuclear-cytoplasmic shuttling, we investigated the phenotypes of several well-characterized EBNA-LP mutants (31). Since we previously defined the domains required for nuclear import (31), our focus was to identify domains required for nuclear export.

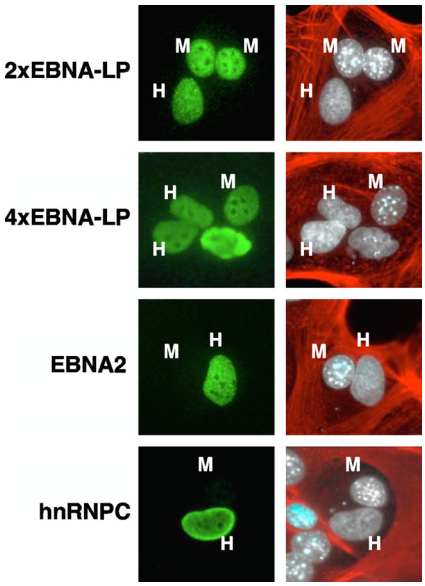


FIG. 4. Transiently expressed EBNA-LP with two or four W repeats shuttles between the nucleus and cytoplasm. HeLa cells were transfected with expression plasmids for EBNA-LP with two W repeats (2×) or four W repeats (4×) or EBNA2 and fused with murine 3T3 cells to form heterokaryons. The heterokaryons were stained with EBNA-LP, EBNA2, or hnRNPC antibodies as indicated (green). Heterokaryons and human versus mouse nuclei were stained with phalloidin 594 (red) and Hoechst (white) stains, respectively, and are shown on the right. Human and mouse nuclei are indicated (H and M, respectively).

Thus, we confined our analysis to mutants that are competent for nuclear localization. The mutants tested are shown in Fig. 5A, along with their coactivation status. In addition, a previous study suggested that HDAC4 interaction with EBNA-LP might mediate EBNA-LP export (32). An EBNA-LP $S_{34,\ 36,\ 63}$-A mutant, lacking three conserved serine residues, failed to interact with HDAC4 (32), so we attempted to test this mutant in the heterokaryon assay as well. However, it should be noted that the $S_{34,\ 36,\ 63}$-A mutant induces the degradation of EBNA2 by a mechanism that remains unknown, so the ability to evaluate its coactivation function is also compromised (27). Surprisingly, all of the mutants tested, including one lacking residues encoded by Y1 and Y2, shuttled between the nucleus and cytoplasm with frequencies that could not be differentiated from those of the wild-type proteins. A representative example of these proteins and their phenotypes in the heterokaryon assay is shown in Fig. 5B. Unfortunately, the $S_{34,\ 36,\ 63}$-A mutant localized in the cytoplasm of many transfected cells, and we were unable to evaluate it in the interspecies heterokaryon assay.

Since molecules up to 50 to 60 kDa can in principle enter or leave the nucleus by diffusion (23, 26), we hypothesized that this might account for nuclear export of the EBNA-LP isoforms we tested, especially since they are smaller than 42 kDa and no specific domain was found to mediate nuclear export (Fig. 5). We tested this idea with a 62-kDa EBNA-LP protein comprised of seven W1-W2 repeats in the heterokaryon assay. As predicted, the larger EBNA-LP protein did not exhibit detectable shuttling activity (Fig. 6A). Since most studies have used EBNA-LP isoforms with four or fewer W1-W2 repeats for functional assays, we tested the larger isoform for its ability to coactivate EBNA2 and relocalize Sp100A from PML NBs. As expected, the EBNA-LP isoform with seven W1-W2 repeats relocalized Sp100A from PML NBs (Fig. 6B) and coactivated EBNA2 with efficiency similar to that of the smaller EBNA-LP isoforms (Fig. 6C).

**Interaction of EBNA-LP and HDAC4.** To determine the significance of HDAC4 interaction for the EBNA-LP coactivation function, we investigated whether the EBNA-LP coactivation mutant ΔCR3 associated with HDAC4. Coimmunoprecipitation assays with 293 cells transfected with EBNA-LP and HDAC expression plasmids indicated that ΔCR3 EBNA-LP associated with HDAC4 similarly to wild-type EBNA-LP (Fig. 7). Similar results were obtained with cotransfected EBV-negative DG75 B cells (data not shown). The EBNA-LP 62-kDa isoform, which does not exhibit a detectable nuclear-cytoplasmic shuttling phenotype, was also able to associate with HDAC4. Since the ΔCR3 mutant efficiently shuttles between the nucleus and cytoplasm in the heterokaryon assay (Fig. 5B), the results suggest that nuclear-cytoplasmic shuttling and the ability to associate with HDAC4 are not sufficient to mediate EBNA-LP coactivation in the absence of interaction with Sp100A.

## DISCUSSION

Our results indicate that EBNA-LP isoforms smaller than 42 kDa efficiently shuttle between the nucleus and cytoplasm in a crm-1-independent fashion (Fig. 3 and 4), and no single domain that mediates shuttling activity could be identified (Fig. 5B). In contrast, we were unable to detect nuclear-cytoplasmic shuttling with EBNA-LP isoforms larger than 62 kDa (Fig. 6A). The data support the notion that EBNA-LP isoforms smaller than 42 kDa may exit the nucleus by diffusion rather than by any specific mechanism. In addition, because the 62-kDa nonshuttling EBNA-LP isoform coactivates EBNA2 with efficiency similar to that of the smaller forms (Fig. 6C), nuclear-cytoplasmic shuttling does not appear to be required for the EBNA-LP coactivation function. Finally, an Sp100A-binding-deficient EBNA-LP coactivation mutant shuttles and associates with HDAC4 with efficiency similar to that of wild-type EBNA-LP (Fig. 7). These results suggest that nuclear-cytoplasmic shuttling and competence for HDAC4 association are not sufficient to mediate EBNA2 coactivation in the absence of a functional interaction with Sp100A.

Our results lend credence to the idea that EBNA-LP isoforms smaller than 42 kDa may exit the nucleus by simple diffusion for the following reasons: (i) the EBNA-LP 62-kDa isoform did not have detectable nuclear-cytoplasmic shuttling activity, (ii) we failed to identify a specific nuclear export signal, and (iii) molecules smaller than 50 to 60 kDa can enter or leave the nucleus by diffusion (23, 26). Consistent with our results, Garibal et al. (10) showed that wild-type EBNA-LP isoforms larger than 60 kDa are detectable only in the nuclei of fractionated cells. Since EBNA-LP isoforms smaller than 42 kDa are made during early infection of B cells (5, 9), our results are consistent with the idea that cytoplasmic EBNA-LP can be found during natural infections. Regardless of the mechanism by which EBNA-LP enters the cytoplasm from the

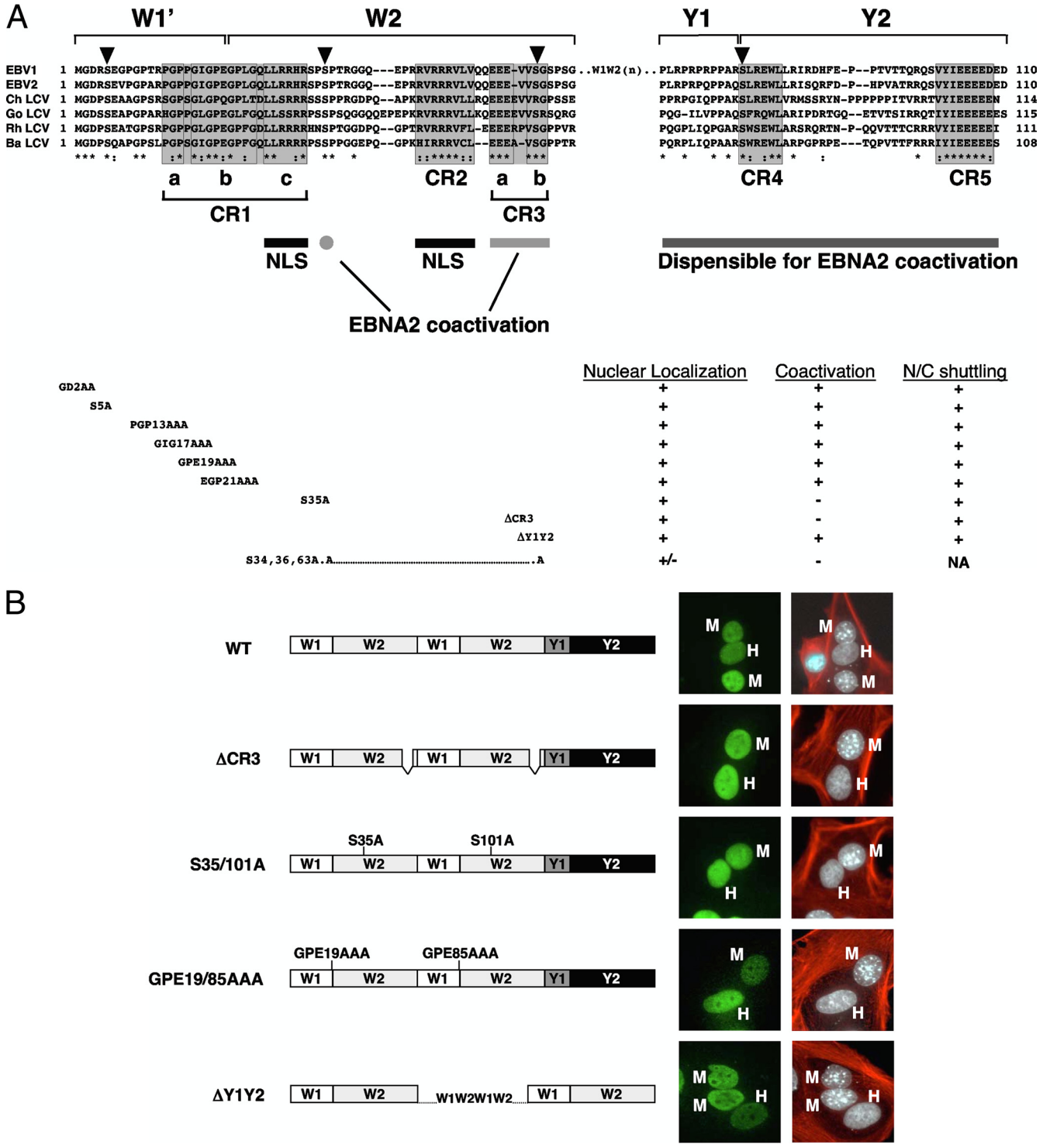


FIG. 5. Coactivation-functional and -nonfunctional EBNA-LP mutants shuttle in a heterokaryon assay. (A) Comparison of EBNA-LP primary amino acid sequences from EBV types 1 and 2 (EBV1 and EBV2) and several nonhuman primate lymphocryptoviruses (LCV). Ch, chimpanzee; Go, gorilla; Rh, rhesus macaque; Ba, baboon. Amino acid residues encoded by the W1, W2, Y1, and Y2 exons are indicated above. The sequence is shown with a single W repeat, which is comprised of W1 and W2. The arrowheads above the sequence indicate conserved serine residues, and below the sequences the various CRs are indicated. The black and gray bars below show regions of the protein required for coactivation function and nuclear localization (NLS). At the bottom of the diagram, the various mutants tested are shown and have been described previously (21, 31). The coactivation-competent ΔY1Y2 EBNA-LP contains four repeats because a smaller version with two repeats failed to localize efficiently in the nucleus. Nuclear localization, coactivation, and nuclear-cytoplasmic (N/C) shuttling statuses are indicated for each mutant. +, present; −, absent. (B) Heterokaryons between HeLa cells transfected with the indicated EBNA-LP proteins and murine 3T3 cells. Heterokaryons were stained with anti-EBNA-LP (green) in the images on the left. The images on the right show Hoechst (white) and phalloidin (red) staining to visualize the heterokaryons. WT, wild type. Human and mouse nuclei are indicated (H and M, respectively).

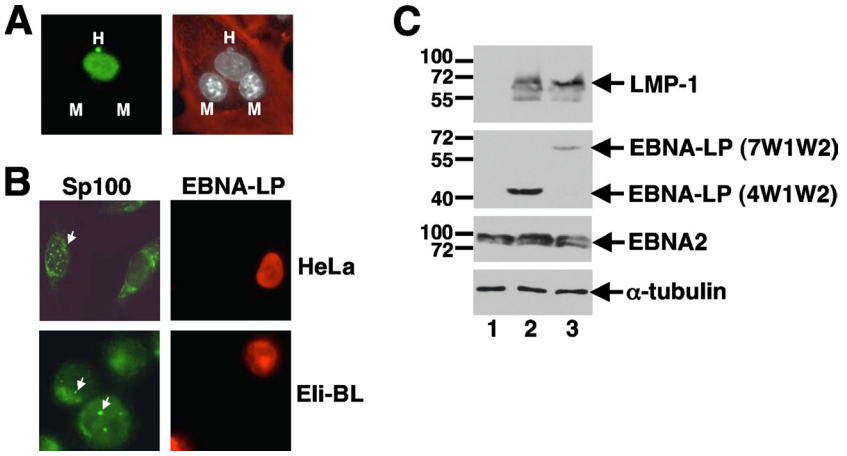


FIG. 6. Analysis of a 62-kDa EBNA-LP isoform with seven W repeats. (A) Heterokaryon between HeLa cells transfected with the seven-W1-W2 EBNA-LP protein and murine 3T3 cells. The heterokaryons were stained with anti-EBNA-LP (green) in the image on the left. The image on the right shows Hoechst (white) and phalloidin (red) staining to visualize the heterokaryon. Human and mouse nuclei are indicated (H and M, respectively). (B) HeLa or Eli-BL cells were transfected with a plasmid expressing the seven-W1-W2-repeat EBNA-LP and then fixed and stained with rabbit anti-Sp100 (green) and mouse anti-EBNA-LP (red). The arrows show the continued integrity of Sp100 nuclear dots in untransfected cells. (C) Western blots of transfected Eli-BL cells expressing EBNA-LP and/or EBNA2. The blots from top to bottom were probed with anti-LMP1, anti-EBNA2, Flag, and α-tubulin. Lane 1, EBNA2 only; lane 2, EBNA2 plus four-W EBNA-LP; lane 3, EBNA2 plus seven-W EBNA-LP. LMP1 expression was detected only when EBNA2 and EBNA-LP were coexpressed. Molecular weight markers (in kilodaltons) are shown on the left.

nucleus (or stays there after translation), how might this facilitate EBNA-LP function(s)? Cytoplasmic EBNA-LP may be involved in coactivation-independent functions through potential interactions with HAX-1 that modulate apoptosis (6, 18, 25). The role of cytoplasmic forms of EBNA-LP for coactivation, however, remains to be clarified. A previous study suggested that EBNA-LP might facilitate EBNA2 coactivation by either retaining the transcriptional corepressor HDAC4 in the cytoplasm or shuttling it out of the nucleus (32). Our data indicate that a 62-kDa EBNA-LP isoform, which has wild-type coactivation function, does not shuttle between the nucleus and cytoplasm (Fig. 6). Moreover, the coactivation mutant ΔCR3, which has only two W repeats and has a robust nuclear-cytoplasmic shuttling function, was able to interact with HDAC4 similarly to wild-type EBNA-LP. These results suggest that nuclear-cytoplasmic shuttling or the ability to interact with HDAC4 is not sufficient to mediate EBNA2 coactivation in the absence of a functional interaction with Sp100A. One possibility is that even though the ΔCR3 mutant protein can interact with HDAC4 and shuttle between the nucleus and cytoplasm, it fails to sequester HDAC4 in the cytoplasm. We have attempted to analyze this in previously generated DG75 cell lines that constitutively express wild-type and ΔCR3 proteins (21). While we could detect a small but perceptible increase in HDAC4 in the cytoplasm of both wild-type- and ΔCR3-expressing cell lines (data not shown), we have not been able to detect HDAC4 in the nuclei of these cell lines to see if levels are decreased relative to the non-EBNA-LP-expressing control cells (data not shown). Identification of specific EBNA-LP mutants that fail to interact with HDAC4 but still retain the ability to associate with Sp100A would help to clarify the role of HDAC4 for EBNA2 coactivation function.

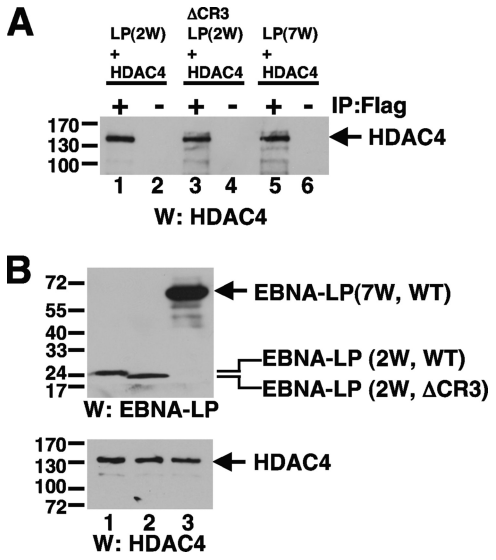


FIG. 7. Interaction of an EBNA-LP coactivation mutant with HDAC4. (A) Immunoprecipitation (IP) assay with 293 cells cotransfected with HDAC4 and wild-type (WT) or coactivation mutant EBNA-LP or an EBNA-LP with seven W repeats. Cell extracts were precipitated with an anti-Flag monoclonal antibody that reacts with Flag epitope-tagged EBNA-LP (+) or no antibody (−), followed by Western blot analysis with an HDAC4 monoclonal antibody. Molecular weights are shown on the left. (B) Western blot analysis of cell extracts used for the immunoprecipitations in panel A. The expression levels of EBNA-LP (top) and HDAC4 (bottom) in each of the three cotransfections are shown. Because the anti-EBNA-LP antibody reacts with the W repeats, the expression level of the 62-kDa EBNA-LP relative to the smaller isoforms is exaggerated. Molecular weights are shown on the left.

One limitation of our results is the sensitivity of the heterokaryon assay. We were unable to detect appreciable levels of seven-W1-W2 EBNA-LP in mouse cells of human-mouse heterokaryons, but this does not rule out the possibility that low levels of the protein shuttle and are below the detection limit

of our assay. However, combined with the observation that the ΔCR3 coactivation-defective mutant is competent for shuttling and interaction with HDAC4, we conclude that the nuclear-cytoplasmic shuttling function is not itself sufficient to mediate EBNA-LP coactivation. A second limitation is that nuclear-cytoplasmic shuttling was assessed largely in nonlymphoid cells. However, the heterokaryons between X50-7 cells and mouse 3T3 cells showed essentially that a 42-kDa EBNA-LP isoform could shuttle in a human B-cell–mouse cell heterokaryon. As we observed similar results between HeLa cell-mouse cell heterokaryons, it seems unlikely that our results are due to the presence or absence of some tissue-specific factor(s).

To our knowledge, most studies focusing on the EBNA-LP coactivation function have relied on 42-kDa or smaller EBNA-LP isoforms (i.e., four or two W repeats). Two previous studies have characterized an EBNA-LP isoform with seven W1-W2 repeats (6, 34), but none have looked at its coactivation function in the context of stimulation of LMP1 expression. Our results are the first to examine the effects of an EBNA-LP protein with seven W1-W2 repeats on its ability to displace Sp100A from PML NBs and EBNA2 coactivation in the context of LMP1 induction. In addition, our study is the first that we are aware of to describe heterokaryons formed between human B cells and mouse cells. The EBV-immortalized cell line X50-7 or other EBV-immortalized cell lines with an adherent phenotype might be useful for investigating lymphoid-specific nuclear-cytoplasmic shuttling factors.

In summary, our results show that EBNA-LP can shuttle between the nucleus and the cytoplasm, but this applies only to isoforms smaller than 42 kDa. Moreover, the mechanism for nuclear export may occur through simple diffusion. Finally, nuclear-cytoplasmic shuttling does not appear to be required for the EBNA-LP coactivation function, and the ability to associate with HDAC4 is not sufficient to mediate EBNA-LP coactivation in the absence of a functional interaction with Sp100A. Future studies aimed at the identification of specific EBNA-LP mutants that retain the ability to associate with Sp100A, but not HDAC4, will help to elucidate the relative contributions of these cofactors to the EBNA-LP coactivation function.

## ACKNOWLEDGMENTS

We thank Andrew Bell, Tony Kouzarides, and Sam Speck for their generous gifts of mutant EBNA-LP, HDAC4, and seven-W EBNA-LP cDNA plasmids, respectively.

This study was supported by NIH RO1 grant CA124309 (P.D.L.).

## REFERENCES

1. **Altmann, M., and W. Hammerschmidt.** 2005. Epstein-Barr virus provides a new paradigm: a requirement for the immediate inhibition of apoptosis. PLoS Biol. **3:**e404.
2. **Bornkamm, G. W., and W. Hammerschmidt.** 2001. Molecular virology of Epstein-Barr virus. Phil. Trans. R. Soc. Lond. B **356:**437–459.
3. **Cullen, B. R.** 2009. Viral and cellular messenger RNA targets of viral microRNAs. Nature **457:**421–425.
4. **DeLong, L. J., G. M. Bonamy, E. N. Fink, and L. A. Allison.** 2004. Nuclear export of the oncoprotein v-ErbA is mediated by acquisition of a viral nuclear export sequence. J. Biol. Chem. **279:**15356–15367.
5. **Dillner, J., B. Kallin, H. Alexander, I. Ernberg, M. Uno, Y. Ono, G. Klein, and R. A. Lerner.** 1986. An Epstein-Barr virus (EBV)-determined nuclear antigen (EBNA5) partly encoded by the transformation-associated Bam WYH region of EBV DNA: preferential expression in lymphoblastoid cell lines. Proc. Natl. Acad. Sci. USA **83:**6641–6645.
6. **Dufva, M., M. Olsson, and L. Rymo.** 2001. Epstein-Barr virus nuclear antigen 5 interacts with HAX-1, a possible component of the B-cell receptor signalling pathway. J. Gen. Virol. **82:**1581–1587.
7. **Echendu, C. W., and P. D. Ling.** 2008. Regulation of Sp100A subnuclear localization and transcriptional function by EBNA-LP and interferon. J. Interferon Cytokine Res. **28:**667–678.
8. **Fahraeus, R., A. Jansson, A. Ricksten, A. Sjoblom, and L. Rymo.** 1990. Epstein-Barr virus-encoded nuclear antigen 2 activates the viral latent membrane protein promoter by modulating the activity of a negative regulatory element. Proc. Natl. Acad. Sci. USA **87:**7390–7394.
9. **Finke, J., M. Rowe, B. Kallin, I. Ernberg, A. Rosen, J. Dillner, and G. Klein.** 1987. Monoclonal and polyclonal antibodies against Epstein-Barr virus nuclear antigen 5 (EBNA-5) detect multiple protein species in Burkitt's lymphoma and lymphoblastoid cell lines. J. Virol. **61:**3870–3878.
10. **Garibal, J., E. Hollville, A. I. Bell, G. L. Kelly, B. Renouf, Y. Kawaguchi, A. B. Rickinson, and J. Wiels.** 2007. Truncated form of the Epstein-Barr virus protein EBNA-LP protects against caspase-dependent apoptosis by inhibiting protein phosphatase 2A. J. Virol. **81:**7598–7607.
11. **Gordadze, A. V., C. W. Onunwor, R. Peng, D. Poston, E. Kremmer, and P. D. Ling.** 2004. EBNA2 amino acids 3 to 30 are required for induction of LMP-1 and immortalization maintenance. J. Virol. **78:**3919–3929.
12. **Gordadze, A. V., R. Peng, J. Tan, G. Liu, R. Sutton, B. Kempkes, G. W. Bornkamm, and P. D. Ling.** 2001. Notch1IC partially replaces EBNA2 function in B cells immortalized by Epstein-Barr virus. J. Virol. **75:**5899–5912.
13. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. Nature **340:**393–397.
14. **Harada, S., and E. Kieff.** 1997. Epstein-Barr virus nuclear protein LP stimulates EBNA-2 acidic domain-mediated transcriptional activation. J. Virol. **71:**6611–6618.
15. **Henderson, B. R., and A. Eleftheriou.** 2000. A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. Exp. Cell Res. **256:**213–224.
16. **Hofelmayr, H., L. J. Strobl, G. Marschall, G. W. Bornkamm, and U. Zimber-Strobl.** 2001. Activated Notch1 can transiently substitute for EBNA2 in the maintenance of proliferation of LMP1-expressing immortalized B cells. J. Virol. **75:**2033–2040.
17. **Hsieh, J. J., and S. D. Hayward.** 1995. Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. Science **268:**560–563.
18. **Kawaguchi, Y., K. Nakajima, M. Igarashi, T. Morita, M. Tanaka, M. Suzuki, A. Yokoyama, G. Matsuda, K. Kato, M. Kanamori, and K. Hirai.** 2000. Interaction of Epstein-Barr virus nuclear antigen leader protein (EBNA-LP) with HS1-associated protein X-1: implication of cytoplasmic function of EBNA-LP. J. Virol. **74:**10104–10111.
19. **Kempkes, B., D. Spitkovsky, P. Jansen-Durr, J. W. Ellwart, E. Kremmer, H. J. Delecluse, C. Rottenberger, G. W. Bornkamm, and W. Hammerschmidt.** 1995. B-cell proliferation and induction of early $G_1$-regulating proteins by Epstein-Barr virus mutants conditional for EBNA2. EMBO J. **14:**88–96.
20. **Kurooka, H., and Y. Yokota.** 2005. Nucleo-cytoplasmic shuttling of Id2, a negative regulator of basic helix-loop-helix transcription factors. J. Biol. Chem. **280:**4313–4320.
21. **Ling, P. D., R. S. Peng, A. Nakajima, J. H. Yu, J. Tan, S. M. Moses, W. H. Yang, B. Zhao, E. Kieff, K. D. Bloch, and D. B. Bloch.** 2005. Mediation of Epstein-Barr virus EBNA-LP transcriptional coactivation by Sp100. EMBO J. **24:**3565–3575.
22. **Lischka, P., C. Rauh, R. Mueller, and T. Stamminger.** 2006. Human cytomegalovirus UL84 protein contains two nuclear export signals and shuttles between the nucleus and the cytoplasm. J. Virol. **80:**10274–10280.
23. **Macara, I. G.** 2001. Transport into and out of the nucleus. Microbiol. Mol. Biol. Rev. **65:**570–594.
24. **Mannick, J. B., J. I. Cohen, M. Birkenbach, A. Marchini, and E. Kieff.** 1991. The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation. J. Virol. **65:**6826–6837.
25. **Matsuda, G., K. Nakajima, Y. Kawaguchi, Y. Yamanashi, and K. Hirai.** 2003. Epstein-Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) forms complexes with a cellular anti-apoptosis protein Bcl-2 or its EBV counterpart BHRF1 through HS1-associated protein X-1. Microbiol. Immunol. **47:**91–99.
26. **Mattaj, I. W., and L. Englmeier.** 1998. Nucleocytoplasmic transport: the soluble phase. Annu. Rev. Biochem. **67:**265–306.
27. **McCann, E. M., G. L. Kelly, A. B. Rickinson, and A. I. Bell.** 2001. Genetic analysis of the Epstein-Barr virus-coded leader protein EBNA-LP as a coactivator of EBNA2 function. J. Gen. Virol. **82:**3067–3079.
28. **Miska, E. A., C. Karlsson, E. Langley, S. J. Nielsen, J. Pines, and T. Kouzarides.** 1999. HDAC4 deacetylase associates with and represses the MEF2 transcription factor. EMBO J. **18:**5099–5107.
29. **Nitsche, F., A. Bell, and A. Rickinson.** 1997. Epstein-Barr virus leader protein enhances EBNA-2-mediated transactivation of latent membrane protein 1 expression: a role for the W1W2 repeat domain. J. Virol. **71:**6619–6628.
30. **Peng, R., S. C. Moses, J. Tan, E. Kremmer, and P. D. Ling.** 2005. The

Epstein-Barr virus EBNA-LP protein preferentially coactivates EBNA2-mediated stimulation of latent membrane proteins expressed from the viral divergent promoter. J. Virol. **79:**4492–4505.
31. **Peng, R., J. Tan, and P. D. Ling.** 2000. Conserved regions in the Epstein-Barr virus leader protein define distinct domains required for nuclear localization and transcriptional cooperation with EBNA2. J. Virol. **74:**9953–9963.
32. **Portal, D., A. Rosendorff, and E. Kieff.** 2006. Epstein-Barr nuclear antigen leader protein coactivates transcription through interaction with histone deacetylase 4. Proc. Natl. Acad. Sci. USA **103:**19278–19283.
33. **Sample, J., M. Hummel, D. Braun, M. Birkenbach, and E. Kieff.** 1986. Nucleotide sequences of mRNAs encoding Epstein-Barr virus nuclear proteins: a probable transcriptional initiation site. Proc. Natl. Acad. Sci. USA **83:**5096–5100.
34. **Sinclair, A. J., I. Palmero, G. Peters, and P. J. Farrell.** 1994. EBNA-2 and EBNA-LP cooperate to cause $G_0$ to $G_1$ transition during immortalization of resting human B lymphocytes by Epstein-Barr virus. EMBO J. **13:**3321–3328.
35. **Speck, S. H., A. Pfitzner, and J. L. Strominger.** 1986. An Epstein-Barr virus transcript from a latently infected, growth-transformed B-cell line encodes a highly repetitive polypeptide. Proc. Natl. Acad. Sci. USA **83:**9298–9302.
36. **Stommel, J. M., N. D. Marchenko, G. S. Jimenez, U. M. Moll, T. J. Hope, and G. M. Wahl.** 1999. A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. EMBO J. **18:**1660–1672.
37. **Szekely, L., K. Pokrovskaja, W. Q. Jiang, H. de The, N. Ringertz, and G. Klein.** 1996. The Epstein-Barr virus-encoded nuclear antigen EBNA-5 accumulates in PML-containing bodies. J. Virol. **70:**2562–2568.
38. **Thorley-Lawson, D. A.** 2005. EBV the prototypical human tumor virus—just how bad is it? J. Allergy Clin. Immunol. **116:**251–261.
39. **Verhagen, J., M. Donnelly, and G. Elliott.** 2006. Characterization of a novel transferable CRM-1-independent nuclear export signal in a herpesvirus tegument protein that shuttles between the nucleus and cytoplasm. J. Virol. **80:**10021–10035.
40. **Wang, A. H., and X. J. Yang.** 2001. Histone deacetylase 4 possesses intrinsic nuclear import and export signals. Mol. Cell. Biol. **21:**5992–6005.
41. **Young, L. S., and P. G. Murray.** 2003. Epstein-Barr virus and oncogenesis: from latent genes to tumours. Oncogene **22:**5108–5121.
42. **Young, L. S., and A. B. Rickinson.** 2004. Epstein-Barr virus: 40 years on. Nat. Rev. Cancer. **4:**757–768.
43. **Zhao, X., A. Ito, C. D. Kane, T. S. Liao, T. A. Bolger, S. M. Lemrow, A. R. Means, and T. P. Yao.** 2001. The modular nature of histone deacetylase HDAC4 confers phosphorylation-dependent intracellular trafficking. J. Biol. Chem. **276:**35042–35048.