JOURNAL OF VIROLOGY, Sept. 1997, p. 6619–6628 Vol. 71, No. 9
0022-538X/97/$04.00+0

Epstein-Barr Virus Leader Protein Enhances EBNA-2-Mediated Transactivation of Latent Membrane Protein 1 Expression: a Role for the W_1W_2 Repeat Domain

FIONA NITSCHE, ANDREW BELL, AND ALAN RICKINSON*

CRC Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TA, United Kingdom

Received 3 March 1997/Accepted 23 May 1997

The Epstein-Barr virus (EBV)-encoded leader protein EBNA-LP is made up of several 66-amino-acid repeats (the W_1W_2 domains) linked to a unique 45-amino-acid C-terminal sequence (the Y_1Y_2 domain). This protein is highly expressed along with a second nuclear antigen, EBNA-2, during the initial stages of virus-induced B-cell transformation. While EBNA-2's essential role in transformation as a transcriptional activator is well documented, very little is known about EBNA-LP function except that recombinant viruses lacking the EBNA-LP Y_1Y_2 exons show reduced, but still detectable, transforming ability. This was taken as evidence that EBNA-LP plays an auxiliary role but is not essential for transformation. A recent study showed that EBNA-LP could cooperate with EBNA-2 in activating cyclin D_2 transcription in resting B cells (A. J. Sinclair, I. Palmero, G. Peters, and P. J. Farrell, EMBO J. 13:3321–3328, 1994). Here we report that EBNA-LP can also cooperate with EBNA-2 in up-regulating expression of the major EBV effector protein of B-cell transformation, latent membrane protein 1 (LMP1). In transient-transfection assays, EBNA-LP enhanced the level of EBNA-2-induced LMP1 expression by 5- to 10-fold in one Latency I Burkitt's lymphoma cell line, Eli-BL, and was absolutely required, along with EBNA-2, to induce LMP1 in a second line, Akata-BL. These changes in LMP1 protein expression appeared to be reflected at the transcriptional level. A study of EBNA-LP mutants showed that this cooperative function mapped to the W_1W_2 repeat domain rather than to Y_1Y_2. Because a Y_1Y_2-deleted form of EBNA-LP may therefore retain some aspects of wild-type function, the original data from virus recombinants leave open the possibility that EBNA-LP is actually an essential transforming gene.

Epstein-Barr virus (EBV), a B-lymphotropic gammaherpesvirus of humans, can transform resting B cells to permanent growth in culture. The resultant lymphoblastoid cell lines (LCLs) carry the viral genome in an episomal form and constitutively express six viral nuclear antigens, EBNA-1, -2, -3A, -3B, -3C and -leader protein (LP), and three latent membrane proteins, LMP1, LMP2A, and LMP2B (22). The aim of much of the current work in the EBV field is to understand the individual roles which these proteins play in the transformation process. In this context, EBNA-1 binds to the origin of plasmid replication (*ori-p*) on the viral genome and is essential for maintenance of the viral episome in proliferating cells (53). Genetic analysis with virus recombinants has subsequently identified at least four other latent proteins, EBNA-2, EBNA-3A, EBNA-3C, and LMP1, as being essential for transformation (6, 13, 20, 47, 48). Of these, EBNA-2 is a transactivator of viral gene transcription, in particular the EBNA transcripts expressed from the *Bam*HI C promoter (Cp) plus the various LMP transcripts (19, 26, 41, 57), and also of certain cellular genes such as CD21, CD23, and c-*fgr* (7, 25). EBNA-2 is therefore an important effector in the process of B-cell transformation; indeed, the stronger in vitro transforming capacity of type 1 compared to type 2 EBV strains reflects type-specific differences at the EBNA-2 locus (6). Many, but not all, of the transactivating effects of EBNA-2 appear to be mediated through an interaction with the sequence-specific DNA binding protein RBPJκ (12, 16, 56). The capacity of EBNA-3A, -3B, and -3C also to bind RBPJκ (29, 33, 34, 55) suggests that these proteins are also transcriptional regulators, part of whose function in transformation may be to modify EBNA-2 activity. LMP1, whose expression in B cells is dependent upon EBNA-2 (1, 51), is the major protein effector of growth transformation, with the capacity to induce a plethora of cell phenotype changes (15, 37, 49); at least some of these effects are mediated through constitutive activation of pathways that normally transduce ligand-dependent signalling from the tumor necrosis factor receptor/CD40 family of cell surface molecules (30).

The present work concerns the latent protein EBNA-LP (also known as EBNA-5), which, although apparently not essential for transformation, greatly enhances the efficiency of the process (13, 27). EBNA-LP contains multiple copies of a 66-amino-acid repeat domain, encoded by the W_1 and W_2 exons within each of the *Bam*HI W repeats, and a 45-amino-acid unique domain encoded by the Y_1 and Y_2 exons within the downstream *Bam*HI Y fragment (39). In freshly infected cells, EBNA-LP and EBNA-2 appear as the first detectable virus-encoded proteins (2); at this stage, EBNA-LP is highly expressed relative to its level in the subsequently established LCL and is detectable in immunoblots as a ladder of related proteins, whose 6- to 8-kDa spacing suggests polypeptides carrying different numbers of W_1W_2 repeats (10). As the initially high levels of expression subside, most of the EBNA-LP detectable by immunofluorescence staining becomes concentrated in a small number of nuclear foci (50), now recognized as ND10 bodies, which also stain for an antigenically distinct form of the retinoblastoma RB protein (18), for 70-kDa heat shock proteins (hsp 70) (42), and for the promyelocytic leukemia-associated protein PML (43). A physical interaction between EBNA-LP and hsp 70 has been demonstrated by coimmunoprecipitation from LCL cell extracts (24, 28), and these same proteins redistribute to the nucleolus in stressed cells (42).

* Corresponding author. Mailing address: CRC Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TA, United Kingdom. Phone: 121-414-4492. Fax: 121-414-4486. E-mail: Williamsd@cancer.bham.ac.uk.

Another report has also described physical interactions between recombinant EBNA-LP and both RB and p53 (45), but this is less well substantiated, and in short-term transfection assays expression of EBNA-LP has failed to show direct effects upon RB- and p53-dependent transcription from reporter constructs (17).

The important auxiliary role played by EBNA-LP in transformation was first demonstrated in virus recombination experiments involving the laboratory virus strain P3HR1; this is a nontransforming deletion mutant which lacks the Y_1Y_2 exons of EBNA-LP and the entire EBNA-2 coding sequence. Reconstitution of both the EBNA-LP and EBNA-2 genes restores full transforming activity to P3HR1; by comparison, a virus reconstituted with the EBNA-2 gene alone initiates cell transformation at least 10-fold less efficiently and generates transformed colonies which grow poorly and remain feeder layer dependent (13, 27). The basis of this growth-deficient phenotype has not been identified, and, because long-term culture will itself select for faster-growing clonal variants, it has even been difficult to demonstrate specific complementation of the growth deficiency by EBNA-LP transfection (3, 13, 27). Furthermore, stable expression of EBNA-LP in EBV genome-negative B-lymphoma cell lines failed to induce detectable cell changes (49). As a consequence, there was no direct evidence for any effect of EBNA-LP upon cell phenotype until the observation in transient-transfection assays with resting human B cells that EBNA-LP and EBNA-2 could cooperate to activate cyclin D_2 expression, as measured by reverse transcription-PCR (RT-PCR) amplification of cyclin D_2 mRNA (40). This prompted us to look for evidence of EBNA-LP-mediated enhancement of another, more easily quantitated, effect of EBNA-2, namely, the activation of LMP1 expression (1, 51).

MATERIALS AND METHODS

Plasmids. The majority of EBNA-LP and EBNA-2 expression constructs were based on the pSG5 vector (Stratagene), which contains a simian virus 40 early promoter and a β-globin intron upstream of the multiple cloning site (11). The pSG5 expression constructs for type 1 EBNA-2 and for type 2 EBNA-2 contained the EBNA-2 open reading frame cloned from the B95.8 (type 1) and AG876 (type 2) EBV strains, respectively (51), and were kindly provided by F. Wang (Harvard University).

The standard EBNA-LP expression construct pSG5-EBNA-LP, used in all experiments except where otherwise stated, contained a complete EBNA-LP cDNA with four W_1W_2 repeats and the unique domain encoded by Y_1Y_2. This cDNA was obtained as an *Eco*RI/*Fnu*DII subfragment of an EBNA-LP cDNA clone, T65, originally derived from the B95.8 virus-transformed IB4 LCL (50); this subfragment was transferred from a pUC18 vector as a 1-kb *Eco*RI/*Bam*HI fragment into the pSG5 expression vector. The control plasmid, pSG5-EBNA-rLP, was generated by inserting the EBNA-LP cDNA into the same vector but in the reverse orientation. The EBNA-LP construct, pSNOC-LP7R (3), encoding a protein with seven W_1W_2 repeats plus Y_1Y_2, was kindly provided by P. Farrell (Ludwig Institute, London, United Kingdom). EBNA-LP constructs encoding proteins with either one or two W_1W_2 repeats linked to the Y_1Y_2 domain were generated by PCR amplification from pSG5-EBNA-LP as a template. As primers we used the oligonucleotides 5′-AAAAGAATTCATGGGAGACCGAAGT GAAGG-3′, which is complementary to sequences in W_1 and contains an *Eco*RI restriction site and an in-frame ATG initiation codon (underlined), and 5′-GCTGCAATAAACAAGTTCTGCTTA-3′, which is complementary to sequences immediately downstream of the pSG5 multiple cloning site. Four PCR products, ranging in size from 0.4 to 1.0 kb and corresponding to fragments with one to four W_1W_2 repeats, were obtained. The 0.4-kb fragment, comprising $W_1W_2Y_1Y_2$, and the 0.6-kb fragment, comprising $(W_1W_2)_2Y_1Y_2$, were each digested with *Eco*RI and *Bam*HI, gel purified, and cloned into pSG5.

The EBNA-LP stop codon mutants encoding Y_1Y_2-deleted and Y_2-deleted forms of the protein, were generated by using the Sculptor in vitro mutagenesis system (Amersham) according to the manufacturer's instructions. The standard EBNA-LP cDNA with four W_1W_2 repeats plus Y_1Y_2 was cloned as an *Eco*RI/*Bam*HI fragment into M13mp18. The mutagenic primer 5′-GTGGCC GTAGTTACCCTGAAGG-3′, which is complementary to sequences at the W_2/Y_1 junction but with an in-frame stop codon (underlined), was used to generate the Y_1Y_2-deleted EBNA-LP mutant $(W_1W_2)_4$. Similarly, the mutagenic primer 5′-CCACTCTCTAAGTTACCGAGCGGG-3′, complementary to sequences at the Y_1/Y_2 junction but with an in-frame stop codon (underlined), was used to generate the Y_2-deleted mutant $(W_1W_2)Y_1$. After mutagenesis, the entire mutant EBNA-LP cDNAs were sequenced to confirm the presence of the desired mutation prior to subcloning into pSG5 as *Eco*RI/*Bam*HI fragments.

Cell lines. Akata-BL (46) is derived from a sporadic Burkitt's lymphoma (BL) and carries a type 1 EBV strain; Eli-BL (35) is derived from an endemic BL and carries a type 2 strain. Both lines display a classical Latency I pattern of infection in which EBNA-1 is the only detectable viral protein (38). Additional experiments were conducted with a third line, P3HR1/BL30, which was generated by stable infection of the EBV-negative sporadic BL line BL30 with the transformation-defective P3HR1 virus strain (7). This line expresses EBNA-1, -3A, -3B, and -3C and trace levels of a 28-kDa EBNA-LP species (lacking Y_1Y_2) from the resident P3HR1 virus genome but shows no detectable expression of LMP1 (1, 31). The type 1 virus-transformed LCL X50-7 and the type 2 virus-transformed LCL JC5 were used as reference lines. All cell lines were routinely maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 8 μg of gentamicin per ml, and 10% (vol/vol) fetal calf serum (culture medium).

Transfections. A total of 10^7 cells in log-phase growth were resuspended in 0.5 ml of HEPES-buffered Dulbecco modified Eagle medium (supplemented as described above) at room temperature and placed in a 1-ml Gene Pulser cuvette with 0.4-cm spacing (Bio-Rad Laboratories, Richmond, Calif.). All transfections were carried out with a total of 25 μg of cesium chloride gradient-purified DNA; where necessary, the amount of DNA within the transfection mixture was made up to 25 μg by addition of the empty pSG5 vector. Cells were electroporated at 0.27 kV (or 0.2 kV in the case of P3HR1/BL30) and 960 μF and then suspended in a total of 9 ml of warm culture medium and cultured at 37°C in humidified 5% CO_2. Cells were harvested after 48 h, pelleted by centrifugation, and washed in phosphate-buffered saline (PBS).

Immunofluorescence. An aliquot of cells from each transfection was resuspended in PBS, smeared on microscope slides, air dried for 1 h, and fixed in 1:1 methanol-acetone for 10 min at −20°C. Slides were stained with murine monoclonal antibodies (MAbs) (hybridoma culture supernatants diluted up to 1:10 in PBS–10% normal rabbit serum) and incubated for 2 h at 37°C. EBNA-LP was detected with MAb JF186 (10), EBNA-2 was detected with MAb PE2 (54), and LMP1 was detected with MAbs CS1 to CS4 (36). The slides were then washed in PBS and, in a second step, incubated for 1 h at 37°C with the goat anti-mouse immunoglobulin G (IgG)–fluorescein isothiocyanate conjugate F-0257 (Sigma) diluted 1:50 in PBS–10% rabbit serum–10% normal human serum (EBV seronegative). Slides were washed as before and, in a third step, incubated for 1 h at 37°C with the rabbit anti-goat IgG–fluorescein isothiocyanate conjugate F-2016 (Sigma) diluted 1:50 as described above. Slides were counterstained with propidium iodide at 1 μg/ml for 15 min and observed by confocal microscopy on a Zeiss fluorescence microscope with a low-light level video camera and the Biovision software package (Improvision, Coventry, United Kingdom). The two channels were recorded independently, and pseudocolor images were generated.

Immunoblotting. Pellets of transfected cells were resuspended in urea buffer (9 M urea, 50 mM Tris, pH 7.5), solubilized by sonication, and mixed with urea gel sample buffer. Samples were boiled at 100°C for 2 min prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); 100 μg of protein was loaded on a 7.5% gel for EBNA-2 and LMP1 detection, on a 10% gel for routine EBNA-LP detection, and on a 15% gel for detection of EBNA-LP species with different numbers of repeats. Immunoblotting was carried out with the MAbs described above followed by incubation with rabbit anti-mouse IgG (Dako; 1:2,000 dilution) as a second step and with ^{125}I-labelled protein A (Amersham) as a third step (10, 36, 54). Levels of expression were quantitated with a Molecular Dynamics PhosphorImager.

RT-PCR assay of LMP1 transcription. Total RNA was extracted from pellets of transfected cells by using RNAzol B (Biogenesis), and aliquots of 2, 0.2, and 0.02 μg of RNA (with the latter two samples being made up to a total of 2 μg with yeast tRNA) were analyzed by RT-PCR amplification for LMP1 transcripts. The LMP1 5′ and 3′ primers, representing nucleotides 169262 to 169243 and 169081 to 169100 of the B95.8 genome sequence, amplify transcripts across the first intron of LMP1 mRNA to give a 104-bp product detected by a probe representing nucleotides 169118 to 169127/169207 to 169216 straddling the splice junction. Conditions for RT-PCR amplification and probing were as described previously (4).

RESULTS

Effect of EBNA-LP on EBNA-2-induced expression of LMP1. In preliminary experiments we carried out transient transfections with the standard pSG5-EBNA-LP vector in a number of EBV-positive BL cell lines, all with a Latency I pattern of infection in which EBNA-1 is the only latent protein detectably expressed from the resident virus genome. As judged by immunofluorescence staining, the two lines giving the best transfection efficiencies (10 to 15% of cells EBNA-LP positive) in these assays, Eli-BL and Akata-BL, were selected for the main body of experiments examining the effects of EBNA-2 and/or EBNA-LP on resident levels of LMP1 expres-

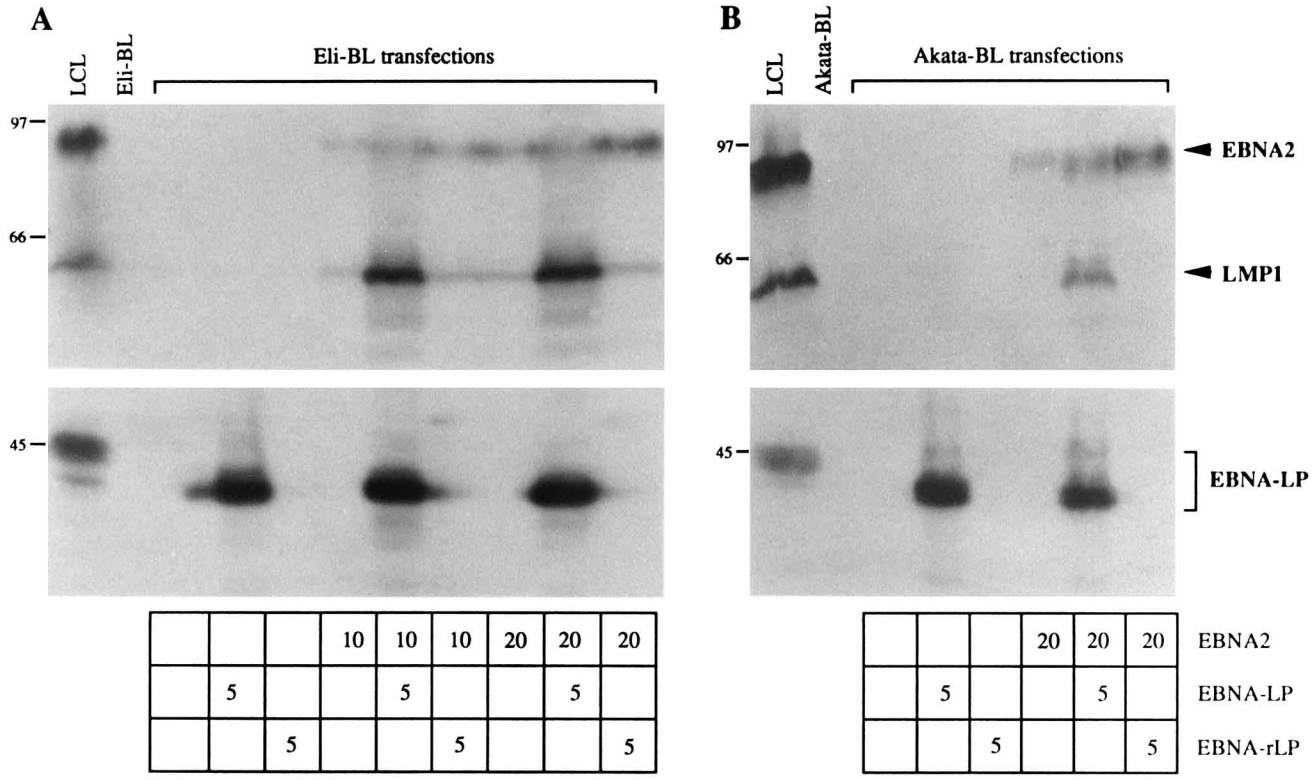

FIG. 1. Effect of EBNA-LP on EBNA-2-mediated induction of LMP1 in Eli-BL cells (A) and Akata-BL cells (B). Target cells were transfected with one or more of the following constructs: pSG5-EBNA-2 (expressing the type 1 EBNA-2 protein), pSG5-EBNA-LP, and the reverse-orientation control pSG5-EBNA-rLP. The amounts of each vector used are indicated below each lane as micrograms of DNA per transfection; where necessary, the total amount of DNA used per transfection was made up to 25 μg by addition of the empty pSG5 vector. Protein extracts prepared from cells 48 h posttransfection were separated by SDS-PAGE and immunoblotted with mixture of the EBNA-2-specific MAb PE2 and the LMP1-specific MAbs CS1 to CS4 on a 7.5% gel (upper gels) or the EBNA-LP-specific MAb JF186 on a 10% gel (lower gels). Control protein samples were prepared from the untransfected Eli-BL or Akata-BL line and from the reference type 1 EBV-transformed LCL X50-7. All lanes were loaded with 100 μg of protein; molecular weight markers (in thousands) are as shown.

sion. Cells were transfected with 10 or 20 μg of the type 1 EBNA-2 expression vector pSG5-EBNA-2 either alone or in combination with 5 μg of pSG5-EBNA-LP or an equivalent amount of the reverse-orientation construct pSG5-EBNA-rLP. Protein extracts were made after 48 h, subjected to SDS-PAGE, and screened for EBNA-2, EBNA-LP, and LMP1 levels by immunoblotting.

Figure 1A shows typical results obtained with the Eli-BL cell line. Transfection of the EBNA-2 vector alone produced detectable amounts of EBNA-2 protein and led to a clear up-regulation of LMP1 expression, although to levels lower than that seen in the X50-7 reference LCL. Such an effect was never observed following transient transfection with the EBNA-LP vector alone, despite strong expression of the 40-kDa EBNA-LP product detectable by immunoblotting. However, cotransfection of both EBNA-2 and EBNA-LP vectors produced levels of LMP1 which, by PhosphorImager analysis, were regularly 5- to 10-fold higher than those induced by EBNA-2 alone. As a control, the EBNA-rLP construct, from which no EBNA-LP expression was detectable, gave no augmentation of the EBNA-2-mediated effect. Figure 1B illustrates the pattern of results observed in similar experiments with Akata-BL cells. Here, expression either of EBNA-2 alone or of EBNA-LP alone had no effect, whereas the combination of both vectors clearly induced detectable levels of LMP1. Again, the reverse EBNA-rLP construct had no such activity. We also examined a third target cell line, P3HR1/BL30, in which the resident P3HR1 EBV genome expresses EBNA-1, the EBNA-3 proteins, and trace levels of a truncated EBNA-LP species (lacking the Y_1Y_2 domain) but no detectable LMP1 (1, 31). Transient transfection of the EBNA-2 vector induced low level up-regulation of LMP1 expression in this line, and the effect was again markedly enhanced by cotransfection with pSG5-EBNA-LP (data not shown).

Since all of the above-described assays were conducted with a vector expressing type 1 EBNA-2, we repeated the experiments to include a type 2 EBNA-2 vector. Figure 2 shows the results of such an experiment conducted with the Akata-BL cell line. Following transient transfection, we reproducibly observed higher levels of expression of the 75-kDa type 2 EBNA-2 protein than of the 85-kDa type 1 protein in transfected cells, but neither protein alone mediated a detectable induction of LMP1. However, when coexpressed with EBNA-LP, the two allelic forms of EBNA-2 showed equally strong LMP1 up-regulation. Similar experiments with Eli-BL cells confirmed that EBNA-LP could enhance LMP1 induction by either type 1 or type 2 EBNA-2 (data not shown).

Analysis of EBNA-LP mutants. Having established an assay of EBNA-LP function, we next aimed to determine which domains of the protein were required for the observed effect. Using as a template the original type 1 EBNA-LP cDNA (containing four copies of the W_1W_2 repeat), we generated PCR-amplified sequences containing one or two copies of W_1W_2 linked to the Y_1Y_2 unique region. These were inserted into the same pSG5 vector and tested alongside the original construct and also against an independently derived (3)

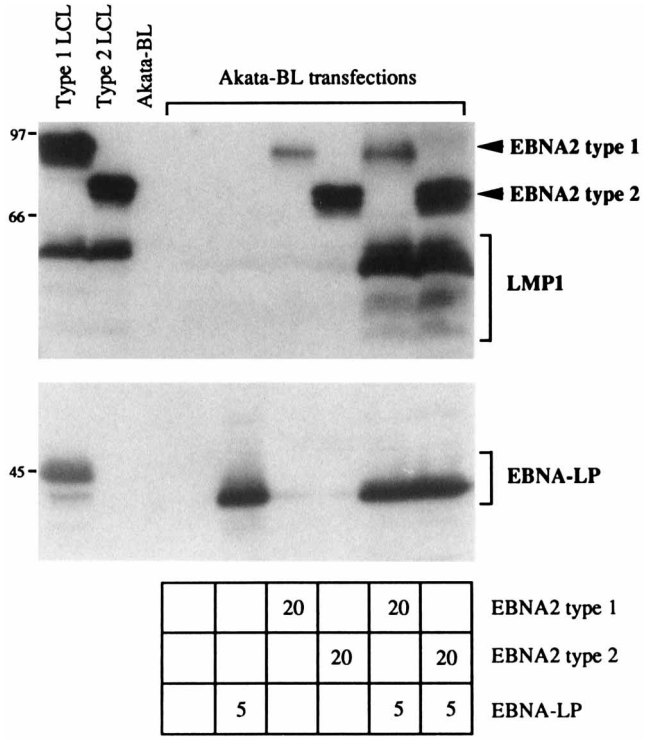

FIG. 2. EBNA-LP cooperates with both type 1 EBNA-2 and type 2 EBNA-2. Akata-BL cells were transfected with the pSG5-EBNA-LP vector and/or vectors expressing type 1 or type 2 EBNA-2. The amounts (in micrograms) of each vector used per transfection are indicated below each track. Expression of EBNA-2 and LMP1 (upper gel) and EBNA-LP (lower gel) was determined by SDS-PAGE and immunoblotting described for Fig. 1. Control protein samples were prepared from the untransfected Akata-BL line, from the type 1 EBV-transformed LCL X50-7, and from the type 2 EBV-transformed LCL JC5. Note that the type 2 virus carried by the JC5 LCL encodes an EBNA-LP which lacks the JF186 epitope. All lanes were loaded with 100 μg of protein; molecular weight markers (in thousands) are as shown.

EBNA-LP construct containing seven copies of the repeat. Figure 3 presents the results of such a transient-transfection assay carried out with Eli-BL cells. Expression of the different EBNA-LP species was confirmed as before by immunoblotting with MAb JF186, an antibody recognizing an epitope within the W_1W_2 repeat domain. The apparent sizes of the expressed proteins were consistent with the various EBNA-LP constructions used (Fig. 3, lower gel). None of these EBNA-LP species mediated any detectable induction of LMP1 when expressed alone, whereas EBNA-2 alone did up-regulate LMP1 expression, as previously observed in Eli-BL cells. Figure 3 (upper gel) shows that in cotransfection assays the EBNA-LP-mediated enhancement of LMP1 up-regulation was equally strong for LP species with seven, four, or two copies of the W_1W_2 repeat. No enhancement was observed with the minimal EBNA-LP containing a single repeat; however, this polypeptide appeared to be expressed at a lower level than the other EBNA-LP species. This pattern of results, indicating optimal activity of an EBNA-LP with only two W_1W_2 repeats, was confirmed on several occasions of testing.

Two further EBNA-LP mutants were generated from the original $(W_1W_2)_4Y_1Y_2$ construct by inserting stop codons so as to express proteins lacking either the entire 45-amino-acid Y_1Y_2 unique domain or the 34-amino-acid Y_2 domain alone. These mutants were then tested alongside the original EBNA-LP construct in transient-transfection assays. The results of one representative experiment, conducted with Eli-BL cells, are illustrated in Fig. 4. Expression of the mutant EBNA-LPs was again confirmed by immunoblotting with MAb JF186; note that both C-terminally deleted proteins run slightly above their predicted sizes of 29 and 30 kDa, respectively (Fig. 4, bottom gel). We reproducibly observed in such experiments that the Y_1Y_2-deleted EBNA-LP was as efficient as the full-length protein at enhancing the EBNA-2-mediated up-regulation of LMP1 (Fig. 4, middle gel). Interestingly, the Y_2-deleted species also had a significant enhancing effect, but this was never as strong as that of wild-type EBNA-LP or of the Y_1Y_2-deleted proteins. In the particular experiment shown in Fig. 4, there was relatively low expression of the Y_2-deleted species, but we observed the same intermediate enhancement of LMP1 levels by this mutant in other experiments where all three EBNA-LP species were equally strongly expressed (data not shown).

Analysis of LMP1 mRNA levels. To assess whether the cooperative effects of EBNA-LP and EBNA-2 on LMP1 protein expression were reflected at the level of transcription, we carried out RT-PCR amplification of serially diluted RNA samples from cells transiently transfected with EBNA-LP and/or EBNA-2 expression vectors. Figure 5 shows the results of one such experiment with Eli-BL cells and an RT-PCR primer combination which amplifies a 104-bp product across the first intron of the LMP1 gene. Transfection with the EBNA-LP vector alone did not induce detectable LMP1 transcription; in contrast, cells transfected with the EBNA-2 vector showed modest levels of the LMP1-specific product, whereas cells cotransfected with both vectors gave a much increased signal, at least as strong as that produced by the X50-7 reference LCL. Figure 5 also shows that the Y_1Y_2-deleted form of EBNA-LP was as efficient as the full-length protein at enhancing the EBNA-2-mediated up-regulation of LMP1 transcripts. These RT-PCR data directly mirror the effects previously observed at the level of LMP1 protein in Eli-BL cell transfections (Fig. 1 and 4). Extension of the analysis to Akata BL transfections likewise showed a clear correlation between the level of RT-PCR-amplifiable LMP1 transcripts and protein expression (data not shown).

Analysis of transient transfections at the single-cell level. In all of the experiments described above, small aliquots of transfected cells were used to prepare slides for immunofluorescence staining. This allowed us to monitor EBNA-2, EBNA-LP, and LMP1 expression at the single-cell level by MAb staining. Figure 6 shows representative results from such an analysis with Eli-BL target cells.

Transfection of the standard pSG5-EBNA-LP vector regularly gave EBNA-LP expression in 10 to 15% of the cells. At the single-cell level, EBNA-LP staining was significantly stronger than that seen in the cells of established LCLs and was distributed throughout the nucleus rather than concentrated in discrete foci. Transfection of the standard pSG5-EBNA-2 vector gave detectable EBNA-2 expression in 2 to 5% of the cells, with positive cells showing nuclear staining similar in intensity to that seen in LCL cells. Up-regulation of LMP1 expression from the resident EBV genome was never seen following EBNA-LP transfection but was detectable in 2 to 5% of cells following EBNA-2 transfection; these LMP1-positive cells showed membrane and cytoplasmic fluorescence, often aggregated into small dots or patches, that closely resembled the pattern and intensity of LMP1 staining shown by most cells in LCLs. In cultures cotransfected with both pSG5-EBNA-LP and pSG5-EBNA-2, levels of EBNA-LP and of EBNA-2 expression were not significantly different from those obtained when either vector was transfected alone. However, LMP1

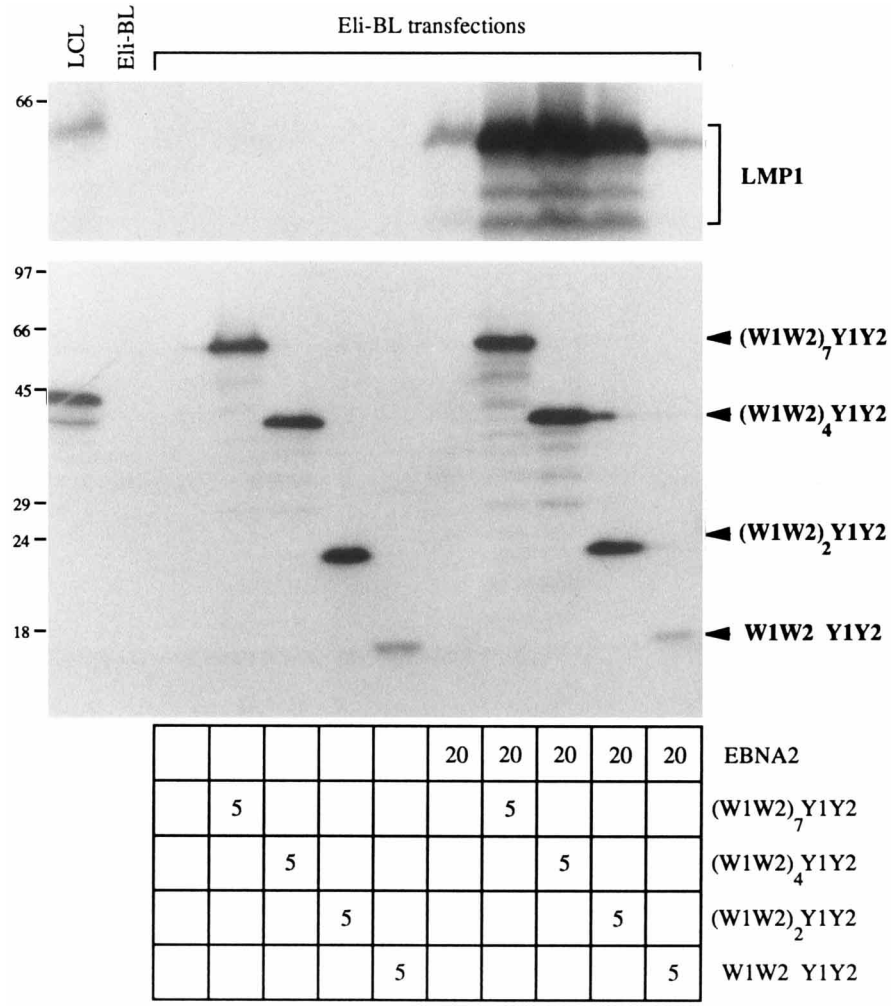

FIG. 3. Influence of the number of W_1W_2 repeats on the cooperative effect of EBNA-LP. Eli-BL cells were transfected with the pSG5-EBNA-2 vector and/or EBNA-LP expression vectors encoding proteins with 7, 4, 2, or 1 copy of the W_1W_2 repeat domain (in each case linked to the Y_1Y_2 unique domain). The amounts (in micrograms) of each vector used for transfection are indicated below each lane. Expression of LMP1 (upper gel) and of the various EBNA-LP species (lower gel) was determined by SDS-PAGE and immunoblotting as described for Fig. 1 except that EBNA-LP species were separated on a 15% gel. Control protein samples were prepared from the untransfected Eli-BL line and from the type 1 EBV-transformed LCL X50-7. All lanes were loaded with 100 μg of protein; molecular weight markers (in thousands) are as shown.

staining in these dually transfected cultures, although still restricted to 2 to 5% of the cells, was now much more intense. Figure 6 also shows typical results obtained from a cotransfection of pSG5-EBNA-2 with the vector expressing the Y_1Y_2-deleted form of EBNA-LP. This mutant protein was strongly expressed in the nuclei of transfected cells and, like the full-length EBNA-LP, was able to cooperate with EBNA-2, leading to much more intense LMP1 staining at the single-cell level.

DISCUSSION

The initiation of EBV infection in resting B cells is characterized by transcription from the *Bam*HI W promoter (Wp) (52) and by the preferential expression of two viral proteins, EBNA-LP and EBNA-2 (2, 10, 40). Only later do the other EBNA proteins, EBNA-1, -3A, -3B, and -3C, appear in detectable amounts, probably coincident with the switch from Wp to Cp as the major promoter of EBNA gene transcription (52). This sequence of events suggests that EBNA-LP and EBNA-2 play important complementary and/or cooperative roles very early in the infectious cycle. Vectored expression of EBNA-2 into BL or other B-lymphoma cell lines in vitro first indicated the ability of this protein to transactivate both viral and cellular genes (1, 7, 19, 25, 26, 51, 57), and the molecular mechanisms of EBNA-2-mediated transactivation are now becoming clear (12, 16, 19, 56). In contrast, expression of EBNA-LP on its own in a variety of cell systems has failed to reveal specific phenotypic changes (3, 17, 27, 49), and the function of the protein remains poorly understood. An important clue, however, came with the observation by Sinclair and colleagues (40) that cotransfection of EBNA-LP and EBNA-2 genes into resting B cells induced cyclin D_2 transcription, detectable by a sensitive RT-PCR assay, whereas transfection of either gene alone had no detectable effect. Here we show evidence of EBNA-LP/EBNA-2 cooperation in another physiologically relevant system, namely, the activation of LMP1 expression. This effect is more amenable to experimental assay and provides an opportunity for the genetic analysis of EBNA-LP's cooperative function.

As illustrated in Fig. 1, 2, and 6, EBNA-LP enhances the EBNA-2-mediated induction of LMP1 expression in Eli-BL

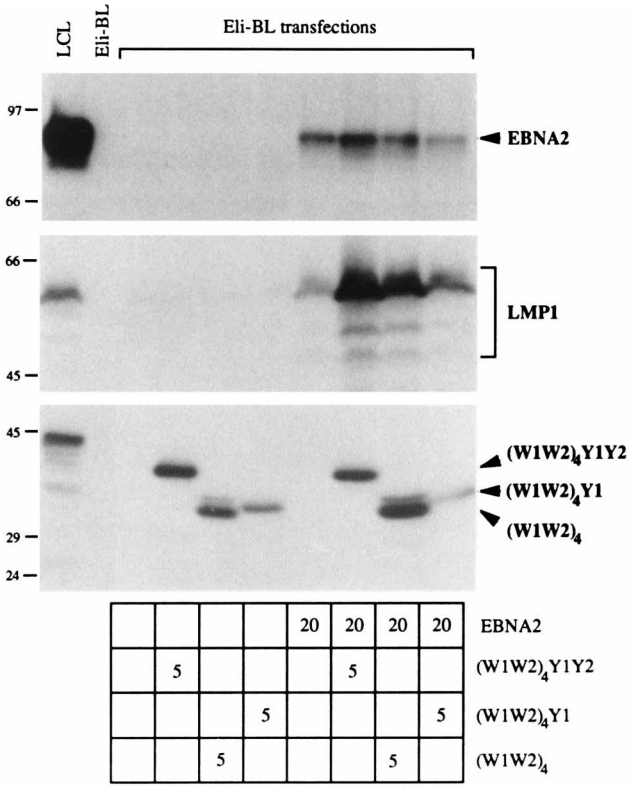

FIG. 4. Influence of Y_2 or Y_1Y_2 deletion on the cooperative effect of EBNA-LP. Eli-BL cells were transfected with the pSG5-EBNA2 vector and/or EBNA-LP expression vectors encoding the wild-type protein [$(W_1W_2)_4Y_1Y_2$], a Y_2-deleted mutant [$(W_1W_2)_4Y_1$], and a Y_1Y_2-deleted mutant [$(W_1W_2)_4$]. The amounts (in micrograms) of each vector used per transfection are indicated below each lane. Expression of EBNA-2 (top gel), LMP1 (middle gel), and EBNA-LP (bottom gel) was determined by SDS-PAGE and immunoblotting as described for Fig. 1. Control protein samples were prepared as described for Fig. 3. All lanes were loaded with 100 μg of protein; molecular weight markers (in thousands) are as shown.

cells and is absolutely required for LMP1 induction in a second cell line, Akata-BL. Note that in both of these lines, baseline EBV latent protein expression is restricted to EBNA-1, and so any possible regulatory effects of the EBNA-3 family of latent proteins, in particular EBNA-3C, upon EBNA-2 activity (29, 33, 55) would not come into play. Clearly, however, endogenous expression of the EBNA-3 proteins does not abrogate the activity of EBNA-LP in this type of assay, because EBNA-LP/EBNA-2 cooperation was also seen in a cell line positive for EBNA-3A, -3B, and -3C, P3HR1/BL30. The cooperative effect of EBNA-LP was observed with both type 1 and type 2 EBNA-2 proteins and was not due to any EBNA-LP-mediated enhancement of EBNA-2 expression in the transfected cells (cf. EBNA-2 levels in EBNA-2-transfected, EBNA-2/EBNA-LP-cotransfected, and EBNA-2/EBNA-rLP-cotransfected lanes in Fig. 1 and 2). It is worth noting that this activity of EBNA-LP was not recognized in either of the two original reports (1, 51) describing EBNA-2-mediated induction of LMP1 in BL cells. In the first study (1), stable EBNA-2-positive and stable EBNA-LP-positive transfectants were established on the P3HR1/BL30 cell background, and only the EBNA-2 transfectants gave evidence of LMP1 induction; however, dual transfectants were not included in the analysis. In the second study (51), transient transfections with EBNA-2 and/or EBNA-LP vectors were carried out, again in P3HR1/BL30 cells, but in contrast to the present work, this did not reveal any effect of EBNA-LP on the EBNA-2-mediated induction of LMP1 expression. Interestingly, the one discernible difference between the present work and the original study of Wang and colleagues (51) is the higher (greater than LCL) levels of EBNA-LP achieved by transient transfection in the present experiments. These levels are in fact much closer to those observed in EBV-infected cells during the early stages of the transformation process (10, 40, 44). We would therefore argue that our results may well reflect a cooperative action of EBNA-LP and EBNA-2 that is important for the initial induction of LMP1 expression in resting B cells.

The mechanism of EBNA-LP's cooperation with EBNA-2 remains to be determined, but the results of RT-PCR analysis (Fig. 5) indicate that the enhanced expression of LMP1 protein in dually transfected cells is reflected by an increased abundance of spliced LMP1 transcripts. An effect mediated at the RNA level is consistent with the earlier finding that EBNA-LP can cooperate with EBNA-2 to activate cyclin D_2 transcription (40). However, the results obtained to date do not allow one to discriminate between an effect of EBNA-LP on transcription per se or on the stability or processing of RNA transcripts. In this context, it is interesting that most EBNA-LP, at least in

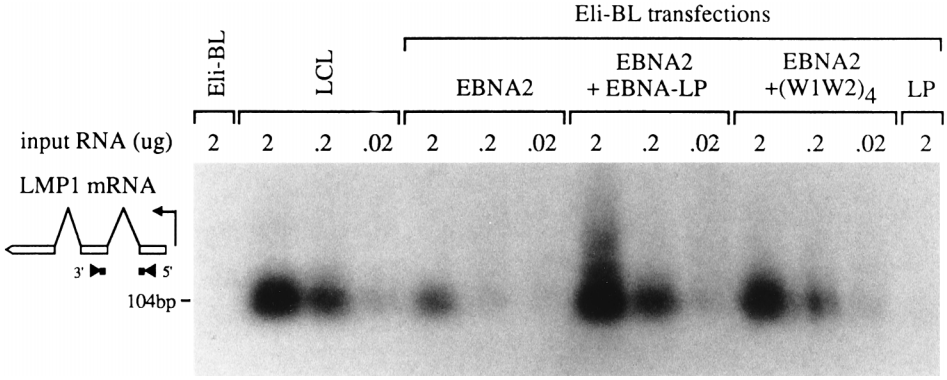

FIG. 5. RT-PCR analysis of LMP1 up-regulation at the RNA level. Eli-BL cells were transfected with the pSG5-EBNA2 vector and/or the EBNA-LP expression vectors encoding the wild-type protein $(W_1W_2)_4$ Y_1Y_2 and the Y_1Y_2-deleted mutant $(W_1W_2)_4$. RNA was prepared from cells 48 h posttransfection, and RT-PCR amplification for spliced LMP1 transcripts was carried out with 2, 0.2, and 0.02 μg of each RNA sample (with yeast tRNA to bring the total RNA content to 2 μg in each case). Amplification was carried out with 5′ and 3′ primers from exons 1 and 2 of the LMP1 mRNA as indicated, and then the amplified products were separated on a 2% agarose gel and Southern blotted with an LMP1 mRNA-specific oligonucleotide probe straddling the exon 1-exon 2 splice junction (4). Control RNA samples were prepared from untransfected Eli-BL cells and from the type 1 EBV-transformed LCL X50-7.

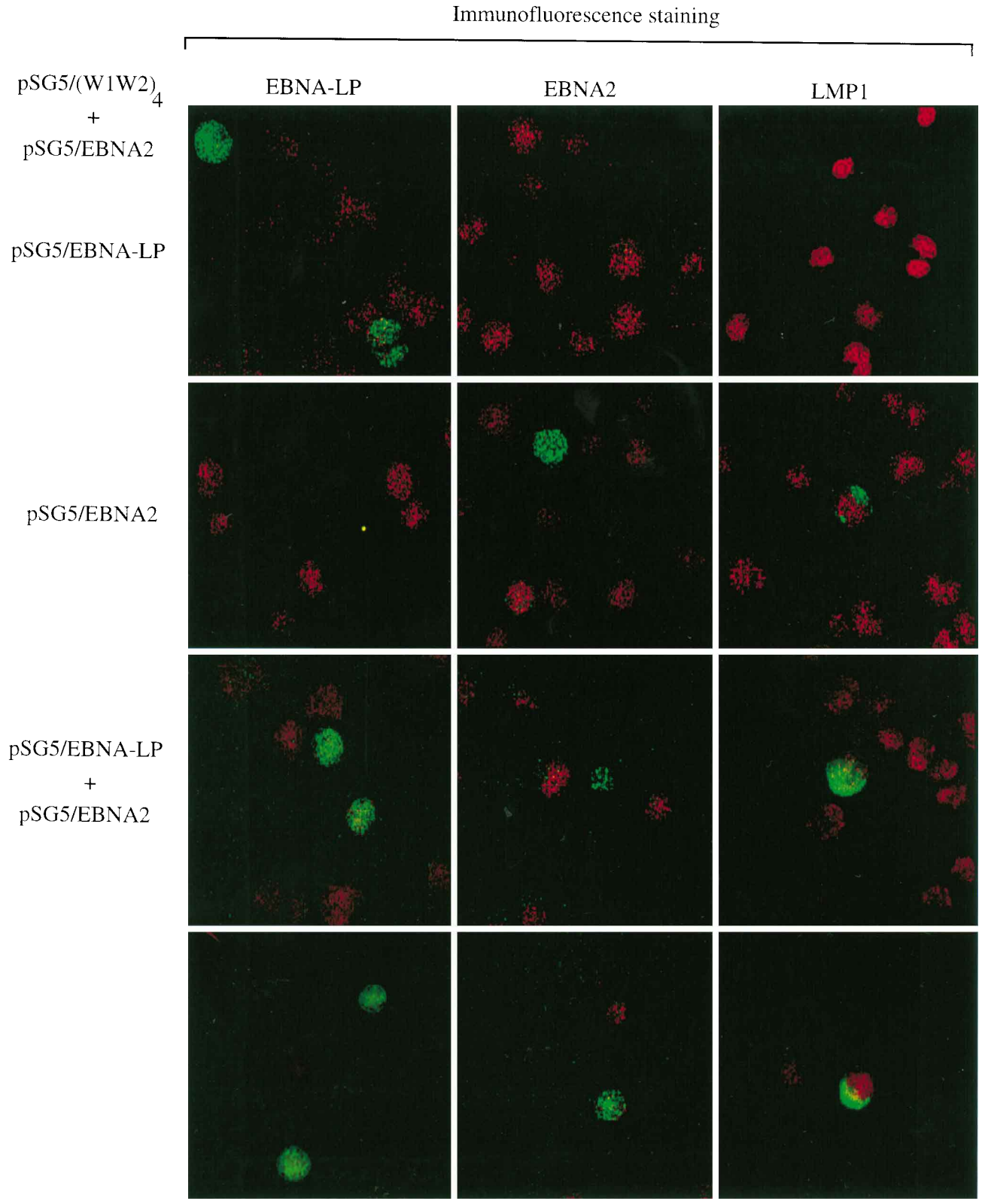

FIG. 6. Analysis of LMP1 induction by immunofluorescence staining at the single-cell level. Eli-BL cells were transfected with pSG5-EBNA-LP alone, pSG5-EBNA-2 alone, pSG5-EBNA-LP and pSG5-EBNA-2, or pSG5-$(W_1W_2)_4$ and pSG5-EBNA-2. Aliquots of cells were taken 48 h posttransfection, and separate slide preparations were stained for EBNA-LP, EBNA-2, or LMP1 with the relevant MAbs. Specific staining is shown in green, and nuclear counterstaining is shown in red. The images shown are from confocal microscopy.

LCL cells, is concentrated within the PML-containing nuclear ND10 bodies (43) and, like the PML protein, appears to be tightly bound to the nuclear matrix (32). The function of these ND10 bodies is still unknown, but it has been suggested that they may be involved in intracellular transport processes, either in the relocalization of transcription factors or in the processing of pre-mRNA molecules (8). In this regard, a number of other viral transactivators, such as simian virus 40 large T antigen, adenovirus E1A (5), and herpes simplex virus ICP0 (8), have been shown to associate with ND10 bodies either transiently during the early stages of the virus replicative cycle or when expressed by transfection in uninfected cells. The possibility remains that the targeting of EBNA-LP to ND10 bodies, perhaps mediated by the protein's interaction with hsp 70 (24, 28, 42), may be important for some aspects of EBNA-LP function.

In studying further EBNA-LP's cooperation with EBNA-2, it will be important to know whether the effect is general or restricted to a subset of EBNA-2-responsive genes. The present experiments do not bear upon this issue except to show that expression from another EBNA-2-responsive promoter within the EBV genome, Cp (16, 41), is not detectably induced by the same procedures that activate LMP1 expression. Thus, we observed no up-regulation of the endogenous type 2 (75-kDa) EBNA-2 protein in Eli-BL cells cotransfected with EBNA-LP and type 1 EBNA-2 vectors (Fig. 1A) or of the endogenous type 1 (85-kDa) EBNA-2 protein in Akata-BL cells cotransfected with EBNA-LP and type 2 EBNA-2 vectors (Fig. 2). As an alternative approach, we have more recently studied the effects of EBNA-2 and/or EBNA-LP in transient-transfection assays with various reporter gene constructs, including an LMP1 reporter, but have noticed that in such experiments EBNA-LP appears generally to enhance expression in an EBNA-2-independent manner (31a). The significance of these findings remains to be determined, but we note that such broad-ranging activity in transient-transfection assays is reminiscent of that shown by another ND10-localizing viral protein, herpes simplex virus ICP0 (9).

The present work, using as an assay the induction of LMP1 expression from a resident viral genome in Latency I BL cells, provides a tractable system in which to begin a genetic analysis of EBNA-LP's cooperative function. The experiments illustrated in Fig. 3 show that an EBNA-LP species containing two W_1W_2 repeats is as active in this assay as proteins with up to seven repeats. It is interesting in this regard that the mini-EBV episome constructed by Kempkes and colleagues (21) and shown to exhibit full transforming function also contains only two copies of the *Bam*HI W repeat. In our assays the single-repeat construct $W_1W_2Y_1Y_2$ appeared to be inactive, but its level of expression, at least as detected by the JF186 MAb, was consistently low in the transiently transfected cells (Fig. 3) and so no final conclusion can be made regarding this particular construct. Analysis of the mutants with C-terminal deletions clearly showed that an EBNA-LP with four copies of the W_1W_2 repeat but lacking the unique 45-amino-acid Y_1Y_2 domain retained wild-type cooperative activity (Fig. 4, 5, and 6). Although initially surprised by this result, we noted earlier reports showing that the endogenous Y_1Y_2-deleted form of EBNA-LP expressed in P3HR1 virus-positive cells is still capable of accessing the nucleus (see also Fig. 6), of localizing to discrete nuclear bodies (50), and (although contrary to the implications of a recent study with EBNA-LP fusion proteins [24]) of binding hsp 70 (28). The W_1W_2 domain also contains serine residues which appear to be targets for cell cycle-dependent phosphorylation by cellular kinases such as p34 cdc2 (23). Interestingly, the level of such W_1W_2 phosphorylation is exaggerated in EBNA-LP fusion proteins lacking the Y_1Y_2 domain (23), and this could possibly explain why the $(W_1W_2)_4$ protein expressed in our transient-transfection assays always included a minor species with a higher apparent molecular mass (Fig. 4). The concept that alterations in the Y_1Y_2 domain might somehow influence the activity of the W_1W_2 repeats was further emphasized by our finding that the $(W_1W_2)_4Y_1$ mutant showed levels of cooperativity with EBNA-2 that were always lower than those shown by the $(W_1W_2)_4$ mutant or by wild-type EBNA-LP.

We conclude that EBNA-LP can cooperate with EBNA-2 in enhancing expression of the viral LMP1 gene, a key event in virus-induced B-cell transformation. This activity of EBNA-LP is mediated primarily by the W_1W_2 repeat domain, and two copies of this domain appear to be sufficient for the effect. It will be interesting to see whether this is also true of the EBNA-LP/EBNA-2 cooperation leading to activation of the cellular cyclin D_2 gene (40). In any case, we anticipate that EBNA-LP will be found to have other functions which, unlike that described here, do depend upon the presence of Y_1Y_2 domain. Thus, recombinant viruses either with Y_1Y_2 deleted or containing a stop codon at the start of the Y_1 exon have much-reduced transforming ability (13, 27). Nevertheless the fact that such viruses do yield some transformed foci, albeit slow growing, has been taken to indicate that EBNA-LP is not essential for the transformation process. We would argue that such a conclusion should be reevaluated in the light of the present results. Thus, the Y_1Y_2-deleted form of EBNA-LP, which all such virus recombinants can still encode, may retain some aspects of wild-type function. Such a protein may be sufficient to cooperate with EBNA2 in certain key functions, thereby masking the fact that EBNA-LP may indeed be an essential transforming gene.

ACKNOWLEDGMENTS

This work is supported by the Cancer Research Campaign (CRC), London, United Kingdom, and F.N. holds a CRC-funded Ph.D. studentship.

We are grateful to F. Wang (Department of Microbiology and Molecular Genetics, Harvard Medical School) and to P. Farrell (Ludwig Institute, St. Mary's Hospital, London, United Kingdom) for cDNAs and/or expression constructs, to S. Roberts and G. Johnson for invaluable help with confocal microscopy, to Martin Rowe (Department of Medicine, Cardiff, United Kingdom) for regular discussions, and to Deborah Williams for excellent secretarial help.

ADDENDUM

An accompanying paper from Harada and Kieff (14) describes experiments in which EBNA-LP enhances EBNA-2-mediated activation of a reporter construct carrying regulatory sequences derived from the LMP1 promoter.

REFERENCES

1. **Abbot, S. D., M. Rowe, K. Cadwallader, J. Gordon, A. Ricksten, L. Rymo, and A. B. Rickinson.** 1990. Epstein-Barr virus nuclear antigen 2 induces expression of the virus-coded latent membrane protein. J. Virol. **64:**2126–2134.
2. **Alfieri, C., M. Birkenbach, and E. Kieff.** 1991. Early events in Epstein-Barr virus infection of human B lymphocytes. Virology **181:**595–608.
3. **Allan, G. J., G. J. Inman, B. D. Parker, D. T. Rowe, and P. J. Farrell.** 1992. Cell growth effects of Epstein-Barr virus leader protein. J. Gen. Virol. **73:**1547–1551.
4. **Brooks, L., Q. Y. Yao, A. B. Rickinson, and L. S. Young.** 1992. Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. J. Virol. **66:**2689–2697.
5. **Carvalho, T., J.-S. Seeler, K. Ohman, P. Jordan, U. Pettersson, G. Akusjarvi, M. Carmo-Fonseca, and A. Dejean.** 1995. Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. J. Cell Biol. **131:**45–56.

6. **Cohen, J. I., F. Wang, J. Mannick, and E. Kieff.** 1989. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc. Natl. Acad. Sci. USA **86:**9558–9562.
7. **Cordier, M. A., A. Calender, M. Billaud, U. Zimber, G. Rousselet, O. Pavlish, J. Banchereau, T. Tursz, G. Bornkamm, and G. M. Lenoir.** 1990. Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. J. Virol. **64:**1002–1013.
8. **Everett, R. D., and G. G. Maul.** 1994. HSV-1 IE protein Vmw 110 causes redistribution of PML. EMBO J. **13:**5062–5069.
9. **Everett, R. D., C. M. Preston, and N. D. Stow.** 1991. Functional and genetic analysis of the role of Vmw 110 in herpes simplex virus replication, p. 49–76. *In* E. K. Wagner (ed.), Herpes virus transcription and its regulation. CRC Press Inc., Boca Raton, Fla.
10. **Finke, J., M. Rowe, B. Kallin, I. Ernberg, A. Rosén, 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.
11. **Green, S., I. Issemann, and E. Sheer.** 1988. A versatile *in vivo* and *in vitro* eukaryotic expression vector for protein engineering. Nucleic Acids Res. **16:**369.
12. **Grossman, S. R., E. Johannsen, X. Tong, R. Yalamanchili, and E. Kieff.** 1994. The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the J_K recombination signal binding protein. Proc. Natl. Acad. Sci. USA **91:**7568–7572.
13. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalising 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, S., M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff, and A. B. Rickinson.** 1991. Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell **65:**1107–1115.
16. **Henkel, T., P. D. Ling, S. D. Hayward, and M. G. Petersen.** 1994. Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J_K. Science **265:**92–95.
17. **Inman, G. J., and P. J. Farrell.** 1995. Epstein-Barr virus EBNA-LP and transcriptional regulation properties of pRB, p107 and p53 in transfection assays. J. Gen. Virol. **76:**2141–2149.
18. **Jiang, W.-Q., L. Szekely, V. Wendel-Hansen, N. Ringertz, G. Klein, and A. Rosen.** 1991. Co-localisation of the retinoblastoma protein and the Epstein-Barr virus-encoded nuclear antigen EBNA5. Exp. Cell Res. **197:**314–318.
19. **Johansen, E., E. Koh, G. Mosialos, X. Tong, E. Kieff, and S. R. Grossman.** 1995. Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by Jκ and PU.1. J. Virol. **69:**253–262.
20. **Kaye, K. M., K. M. Izumi, and E. Kieff.** 1993. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc. Natl. Acad. Sci. USA **90:**9150–9154.
21. **Kempkes, B., D. Pich, R. Zeidler, B. Sugden, and W. Hammerschmidt.** 1995. Immortalization of human B lymphocytes by a plasmid containing 71 kilobase pairs of Epstein-Barr virus DNA. J. Virol. **69:**231–238.
22. **Kieff, E.** 1996. Epstein-Barr virus and its replication, p. 2343–2396. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology. Lippincott-Raven, Philadelphia, Pa.
23. **Kitay, M. K., and D. T. Rowe.** 1996. Cell cycle stage-specific phosphorylation of the Epstein-Barr virus immortalization protein EBNA-LP. J. Virol. **70:** 7885–7893.
24. **Kitay, M. K., and D. T. Rowe.** 1996. Protein-protein interactions between Epstein-Barr virus nuclear antigen-LP and cellular gene products: binding of 70-kilodalton heat shock proteins. Virology **220:**91–99.
25. **Knutson, J. C.** 1990. The level of c-fgr RNA is increased by EBNA2, an Epstein-Barr virus gene required for B-cell immortalization. J. Virol. **64:** 2350–2356.
26. **Laux, G., F. Dugrillon, C. Eckert, B. Adam, U. Zimber-Strobl, and G. W. Bornkamm.** 1994. Identification and characterization of an Epstein-Barr virus nuclear antigen 2-responsive *cis* element in the bidirectional promoter region of latent membrane protein and terminal protein 2 genes. J. Virol. **68:**6947–6958.
27. **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.
28. **Mannick, J. B., X. Tong, A. Hennes, and E. Kieff.** 1995. The Epstein-Barr virus nuclear antigen leader protein associates with hsp72/hsp73. J. Virol. **69:**8169–8172.
29. **Marshall, D., and C. Sample.** 1995. Epstein-Barr virus nuclear antigen 3C is a transcriptional regulator. J. Virol. **69:**3624–3630.
30. **Mosialos, G., M. Birkenbach, R. Yalamanchili, T. Van Arsdale, C. Ware, and E. Kieff.** 1995. The Epstein-Barr virus transforming protein LMP1 engages signalling proteins from the tumor necrosis factor receptor family. Cell **80:**389–399.
31. **Murray, R. J., L. S. Young, A. Calender, C. D. Gregory, M. Rowe, G. M. Lenoir, and A. B. Rickinson.** 1988. Different patterns of Epstein-Barr virus gene expression and of cytotoxic T-cell recognition in B-cell lines infected with transforming (B95.8) or nontransforming (P3HR1) virus strains. J. Virol. **62:**894–901.

31a. **Nitsche, F.** Unpublished observations.

32. **Petti, L., C. Sample, and E. Kieff.** 1990. Subnuclear localization and phosphorylation of Epstein-Barr virus latent infection nuclear proteins. Virology **176:**563–574.
33. **Robertson, E. S., S. Grossman, E. Johannsen, C. Miller, J. Lin, B. Tomkinson, and E. Kieff.** 1995. Epstein-Barr virus nuclear protein 3C modulates transcription through interaction with the sequence-specific DNA-binding protein Jκ. J. Virol. **69:**3108–3116.
34. **Robertson, E. S., J. Lin, and E. Kieff.** 1996. The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJκ. J. Virol. **70:**3068–3074.
35. **Rooney, C. M., C. D. Gregory, M. Rowe, S. Finerty, C. F. Edwards, H. Rupani, and A. B. Rickinson.** 1986. Endemic Burkitt's lymphoma: phenotypic analysis of Burkitt's lymphoma biopsy cells and of the derived tumour cell lines. J. Natl. Cancer Inst. **77:**681–687.
36. **Rowe, M., H. S. Evans, L. S. Young, K. Hennessy, E. Kieff, and A. B. Rickinson.** 1987. Monoclonal antibodies to the latent membrane protein of Epstein-Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells. J. Gen. Virol. **68:**1575–1586.
37. **Rowe, M., R. Khanna, C. A. Jacob, V. Argaet, A. Kelly, S. Powis, M. Belich, D. Croom-Carter, S. Lee, S. R. Burrows, J. Trowsdale, D. J. Moss, and A. B. Rickinson.** 1995. Restoration of endogenous antigen processing in Burkitt's lymphoma cells by Epstein-Barr virus latent membrane protein-1: co-ordinate up-regulation of peptide transporters and HLA class I antigen expression. Eur. J. Immunol. **25:**1374–1384.
38. **Rowe, M., D. T. Rowe, C. D. Gregory, L. S. Young, P. J. Farrell, H. Rupani, and A. B. Rickinson.** 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J. **6:**2743–2751.
39. **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.
40. **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.
41. **Sung, N. S., S. Kenney, D. Gutsch, and J. S. Pagano.** 1991. EBNA2 transactivates a lymphoid-specific enhancer in the *Bam*HI C promoter of Epstein-Barr virus. J. Virol. **65:**2164–2169.
42. **Szekely, L., W.-Q. Jiang, K. Pokrovskaja, K. G. Wiman, G. Klein, and N. Ringertz.** 1995. Reversible nucleolar translocation of Epstein-Barr virus-encoded EBNA5 and hsp 70 proteins after exposure to heat shock or cell density congestion. J. Gen. Virol. **76:**2423–2432.
43. **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.
44. **Szekely, L., K. Pokrovskaja, W.-Q. Jiang, G. Selivanova, M. Lowbeer, N. Ringertz, K. G. Wiman, and G. Klein.** 1995. Resting B-cells, EBV-infected B-blasts and established lymphoblastoid cell lines differ in their Rb, p53 and EBNA-5 expression patterns. Oncogene **10:**1869–1874.
45. **Szekely, L., G. Selivanova, K. P. Magnusson, G. Klein, and K. G. Wiman.** 1993. EBNA-5, an Epstein-Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins. Proc. Natl. Acad. Sci. USA **90:**5455–5459.
46. **Takada, K., and Y. Ono.** 1989. Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. J. Virol. **63:**445–449.
47. **Tomkinson, B., and E. Kieff.** 1992. Use of second-site homologous recombination to demonstrate that Epstein-Barr virus nuclear protein EBNA3B is not essential for B-lymphocyte infection or growth transformation in vitro. J. Virol. **66:**2893–2903.
48. **Tomkinson, B., E. Robertson, and E. Kieff.** 1993. Epstein-Barr virus nuclear proteins (EBNA) 3A and 3C are essential for B-lymphocyte growth transformation. J. Virol. **67:**2014–2025.
49. **Wang, F., C. Gregory, J. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. J. Virol. **64:**2309–2318.
50. **Wang, F., L. Petti, D. Braun, S. Seung, and E. Kieff.** 1987. A bicistronic Epstein-Barr virus mRNA encodes two nuclear proteins in latently infected, growth-transformed lymphocytes. J. Virol. **61:**945–954.
51. **Wang, F., S.-F. Tsang, M. G. Kurilla, J. J. Cohen, and E. Kieff.** 1990. Epstein-Barr virus nuclear antigen 2 transactivates latent membrane protein LMP1. J. Virol. **64:**3407–3416.
52. **Woisetschlaeger, M., C. N. Yandava, L. A. Furmanski, J. L. Strominger, and S. H. Speck.** 1990. Promoter switching in Epstein-Barr virus during the initial stages of infection of B lymphocytes. Proc. Natl. Acad. Sci. USA **87:**1725–1729.

53. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A *cis*-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently-infected cells. Proc. Natl. Acad. Sci. USA **81:**3806–3810.
54. **Young, L., C. Alfieri, K. Hennessy, H. Evans, C. O'Hara, K. C. Anderson, J. Ritz, R. S. Shapiro, A. Rickinson, E. Kieff, and J. I. Cohen.** 1989. Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. N. Engl. J. Med. **321:**1080–1085.
55. **Zhao, B., D. R. Marshall, and C. E. Sample.** 1996. A conserved domain of the Epstein-Barr virus nuclear antigens 3A and 3C binds to a discrete domain of Jκ. J. Virol. **70:**4228–4236.
56. **Zimber-Strobl, U., L. J. Strobl, C. Meitinger, R. Hinrichs, T. Sakai, T. Furukawa, T. Hong, and G. W. Bornkamm.** 1994. Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-Jk, the homologue of *Drosophila suppressor of hairless*. EMBO J. **13:**4973–4982.
57. **Zimber-Strobl, U., K.-O. Suentzenich, G. Laux, E. Eick, M. Cordier, A. Calender, M. Billaud, G. M. Lenoir, and G. W. Bornkamm.** 1991. Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene. J. Virol. **65:**415–423.