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Epstein-Barr Virus Nuclear Antigen 2 Transactivates Latent Membrane Protein LMP1

FRED WANG,* SO-FAI TSANG, MICHAEL G. KURILLA, JEFFREY I. COHEN, AND ELLIOTT KIEFF

Departments of Medicine and Microbiology and Molecular Genetics, Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115

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Several lines of evidence are compatible with the hypothesis that Epstein-Barr virus (EBV) nuclear antigen 2 (EBNA-2) or leader protein (EBNA-LP) affects expression of the EBV latent infection membrane protein LMP1. We now demonstrate the following. (i) Acute transfection and expression of EBNA-2 under control of simian virus 40 or Moloney murine leukemia virus promoters resulted in increased LMP1 expression in P3HR-1-infected Burkitt's lymphoma cells and the P3HR-1 or Daudi cell line. (ii) Transfection and expression of EBNA-LP alone had no effect on LMP1 expression and did not act synergistically with EBNA-2 to affect LMP1 expression. (iii) LMP1 expression in Daudi and P3HR-1-infected cells was controlled at the mRNA level, and EBNA-2 expression in Daudi cells increased LMP1 mRNA. (iv) No other EBV genes were required for EBNA-2 transactivation of LMP1 since cotransfection of recombinant EBNA-2 expression vectors and genomic LMP1 DNA fragments enhanced LMP1 expression in the EBV-negative B-lymphoma cell lines BJAB, Loucks, and BL30. (v) An EBNA-2-responsive element was found within the −512 to +40 LMP1 DNA since this DNA linked to a chloramphenicol acetyltransferase reporter gene was transactivated by cotransfection with an EBNA-2 expression vector. (vi) The EBV type 2 EBNA-2 transactivated LMP1 as well as the EBV type 1 EBNA-2. (vii) Two deletions within the EBNA-2 gene which rendered EBV transformation incompetent did not transactivate LMP1, whereas a transformation-competent EBNA-2 deletion mutant did transactivate LMP1. LMP1 is a potent effector of B-lymphocyte activation and can act synergistically with EBNA-2 to induce cellular CD23 gene expression. Thus, EBNA-2 transactivation of LMP1 amplifies the biological impact of EBNA-2 and underscores its central role in EBV-induced growth transformation.

Epstein-Barr virus (EBV) can cause B-lymphoproliferative disorders in immunocompromised or genetically susceptible humans (6, 33) and in marmosets (42). In vitro, EBV infection efficiently induces immunoglobulin secretion and perpetual B-lymphocyte proliferation (17, 32).

The functions of the six nuclear proteins (EBV nuclear antigen [EBNA]-1, -2, -3A, -3B, -3C, and leader protein [EBNA-LP]) and three latent membrane proteins (LMP1, LMP2A, and LMP2B) expressed from the viral genome during latent, growth-transforming infection are being actively investigated (see reference 20 for a review). LMP1 appears to be important for growth transformation since transfection and expression in rodent fibroblasts results in reduced serum dependence, anchorage independence, and tumorigenicity in nude mice (2, 44, 45). In various EBV-negative Burkitt's lymphoma cells, LMP1 induces phenotypic changes characteristic of B-cell activation including induction of cellular adhesion molecules, transferrin receptor, CD21, CD23, CD39, CD44, and vimentin (3, 46, 49).

EBNA-2 or EBNA-LP is necessary for B-lymphocyte growth transformation. The Daudi and P3HR-1 EBV strains are deleted for EBNA-2 and part of the EBNA-LP genes (4, 15, 16, 19, 22, 34) and are unable to immortalize B lymphocytes (19, 28). P3HR-1 recombination with other EBV strains or cloned EBV DNA fragments which results in reconstitution of the P3HR-1 deletion restores the transforming phenotype (7, 13, 43). EBNA-2 is essential for growth transformation since truncation (13) or deletions within the EBNA-2 open reading frame (7) result in transformation-incompetent EBV. In rat-1 rodent fibroblasts, EBNA-2 reduces serum dependency but does not affect contact inhibition, anchorage independence, or tumorigenicity (9). In the EBV-negative B-lymphoma cell lines Loucks and BJAB, EBNA-2 alone induces CD21 and CD23 expression without concomitantly inducing other activation antigens or cellular adhesion molecules (47, 48).

Several experiments suggest that EBNA-2 or EBNA-LP also affects LMP1 expression. First, EBNA-2 and LMP1 are often difficult to detect in early passage Burkitt's tumor cell lines and coordinately increase in abundance with cell passage (38). Second, EBV-negative Burkitt's tumor cells stably infected with the P3HR-1 EBV strain do not express EBNA-2, a normal EBNA-LP, or LMP1, while cells stably infected with EBV strain B95-8 express EBNA-2, a complete EBNA-LP, and LMP1 (31). Third, B cells immortalized by recombinant P3HR-1 with a restored EBNA-2 and EBNA-LP express normal LMP1 levels (7). Fourth, *cat* reporter gene expression under control of the LMP1 promoter (−512 to +40) was higher in cells stably infected with B95-8 EBV, which expressed EBNA-2 and EBNA-LP, compared with cells stably infected with P3HR-1 EBV, which lacks EBNA-2 and a complete EBNA-LP (11). The objective of the experiments reported here is to investigate directly whether EBNA-2 and EBNA-LP, either individually or together, transactivate LMP1.

MATERIALS AND METHODS

Cell lines. IB4 is a lymphoblastoid cell line latently infected with EBV. BJAB is an EBV-negative B-lymphoma cell line (27). Loucks, BL30, and BL41 are EBV-negative Burkitt's lymphoma cell lines. Daudi (19) and P3HR-1 (HH514-16 kindly provided by G. Miller [35]) are EBV-infected Burkitt's lymphoma cell lines. BL30/P3HR-1, BL30/B95-8, BL41/P3HR-1, and BL41/B95-8 are EBV-neg-

* Corresponding author.

ative Burkitt's lymphoma cell lines which have been stably infected in vitro with the respective EBV (kindly provided by G. Lenoir [5]). M12 and A20 (kindly provided by A. Abbas) are spontaneous mouse B-lymphoma cell lines (21). MT-2 is a human T-cell-lymphotropic virus type I-transformed human T-cell line (kindly provided by G. Tosato and R. Yarchoan [29]). The human cervical carcinoma cell line HeLa was obtained from the American Type Culture Collection (Rockville, Md.).

Plasmids. The pSG5 expression vector (Stratagene, La Jolla, Calif.) contains the simian virus 40 (SV40) early promoter, a beta-globin intron, and an SV40 polyadenylation signal. EBV DNA fragments encoding the EBV type 1 EBNA-2 (nucleotides 48475 to 50306 of B95-8 EBV) or EBV type 2 EBNA-2 (an *Rsa*I partial digest of the Ag876 EBV *Bam*HI D1 fragment) were cloned into the pSG5 vector and are referred to as SV-EB1NA2 and SV-EB2NA2, respectively. The pZipNeoSV(X)-1 expression vectors containing the EBV type 1 and type 2 EBNA-2 open reading frames only have been previously described (9, 48) and are referred to here as pZip-EB1NA2 and pZip-EB2NA2. The *Hin*dIII fragments of each vector were also cloned into pUC18 to generate a smaller plasmid containing the Moloney murine leukemia virus (MuLV) long terminal repeat (LTR) and EBNA-2 gene (LTR-EB1NA2 and LTR-EB2NA2). The pZip-ELP expression vector contains the EBNA-LP open reading frame derived from an *Eco*RI-to-*Fnu*dII fragment of the T65 cDNA (39). These vectors express the appropriate EBV protein after stable transfection into human EBV-negative B-lymphoma cells (47, 48). The LTR-ELP vector is the *Hin*dIII fragment of pZip-T65 and contains the MuLV LTR and the bicistronic T65 cDNA. LTR-ELP expresses only EBNA-LP after transfection into EBV-negative B-lymphoma cells (47, 48).

EBNA-2 mutations were all derived from the B95-8 EBV EBNA-2 (nucleotides 48475 to 50306) and cloned into the pZipNeoSV(X)-1 expression vector. Deletions were constructed by restriction enzyme digestion and are named according to the deleted EBNA-2 amino acids (pZip-E2d19-115, *Hin*cII to *Bam*HI; pZip-E2d148-324, *Bst*EII to *Bal*I; pZip-E2d200-234, *Stu*I to *Stu*I).

The N_{Het} plasmid contains the *Bam*HI N_{Het} fragment of B95-8 EBV DNA from nucleotide 166,614 across the terminal repeats to nucleotide 3,955 in pUC18. The NdEco plasmid (nucleotides 166,614 to 1) was derived by digestion of N_{Het} with *Eco*RI and cloning into pUC18. Both constructs appear to have only one copy of the terminal repeat by restriction enzyme digests.

The −512/+40 LMPCAT construct was derived by cloning the −512 to +40 latent membrane protein (LMP1) upstream sequences relative to the BNLF1 start site into the *Bgl*II site of pCAT (11). pCAT and pSV2CAT contain the chloramphenicol acetyltransferase (CAT) gene (*cat*) without and with the SV40 early promoter, respectively.

Transfections. A total of 5×10^6 cells in log-phase growth were suspended in 0.3 ml of RPMI 1640 medium with 10% fetal bovine serum at room temperature and placed in a Gene Pulser cuvette (Bio-Rad Laboratories, Richmond, Calif.). A 15-μg sample of each uncut plasmid DNA which had been purified twice on cesium chloride gradients was added to the cuvette. Equal amounts of total DNA were added to each sample within any single experiment. Cells were electroporated with 0.2 V at 960 μF (Gene Pulser) and then suspended in 10 ml of RPMI 1640–10% fetal bovine serum and cultured at 37°C. After 4 days, cells were harvested, counted, and then assayed. Transfection efficiencies for EBNA-2 were determined in all experiments by immunofluorescence with an EBNA-2-specific monoclonal antibody. Each type of acute transfection experiment was repeated at least three times.

Immunoprecipitations. Cells were pelleted by centrifugation, suspended in 100 μl of buffer A (10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 0.5% Triton X-100), mixed with 0.09 g of urea and 4.2 μl of 2-mercaptoethanol, and vortexed to extract the detergent-soluble and -insoluble LMP1 fractions (23). Buffer B (1 ml) (10 mM Tris hydrochloride [pH 7.4], 250 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 0.1% sodium dodecyl sulfate) was added, and insoluble material was removed by centrifugation. Equal portions of the LMP1-specific murine monoclonal antibody S12 (26) were added to each sample and incubated at 4°C for 1 h. Protein A-Sepharose CL4B (Sigma Chemical Co., St. Louis, Mo.) was added, and samples were mixed for 30 min at 4°C. Immunoprecipitates were removed by gentle centrifugation and washed twice with buffer B. Immunoprecipitates were then suspended in gel loading buffer, boiled, and analyzed by immunoblotting.

After LMP immunoprecipitation, EBNA-2 was immunoprecipitated by diluting the sample with 5 ml of buffer A and adding the EBNA-2-specific murine monoclonal antibody PE2 (51). Subsequent steps were as described above except that protein G-Sepharose 4 (Pharmacia, Inc., Piscataway, N.J.) was used to immunoprecipitate PE2 and washes were with buffer A. All buffers contained protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.7 μg of pepstatin per ml, 0.5 μg of leupeptin per ml, 1.0 μg of aprotinin per ml).

LMP1 and EBNA-2 were quantitatively immunoprecipitated by these procedures with up to 5×10^6 EBV-infected cells, indicating that antibodies were in excess in immunoprecipitations from acute transfections. The LMP1 or EBNA-2 expression level in acute transfections was in all instances substantially less than that of 5×10^6 EBV-infected cells owing to the fact that only a minority of cells were successfully transfected.

Immunoblotting. Cell lysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretic transfer to nitrocellulose, and immunoblotting were performed as previously described (9). Pooled EBV-immune human sera followed by ^{125}I-protein A were used for detection of EBNAs. LMP1 was detected with the monoclonal antibody S12 (26) followed by a rabbit anti-mouse immunoglobulin antiserum and ^{125}I-protein A. All X-ray film was preflashed before autoradiography.

Immunofluorescence. Cells for immunofluorescence were pelleted by centrifugation, smeared onto microscope slides, air dried, and then fixed for 7 min in ice-cold 1:1 methanol-acetone. Slides were blocked with 10% goat serum in phosphate-buffered saline for 30 min and stained with either an EBNA-2-specific monoclonal antibody, PE2 (51), or LMP1-specific monoclonal antibodies CS1 to CS4 (kindly provided by M. Rowe and A. B. Rickinson [37]) for 1 h. Slides were washed for 30 min in phosphate-buffered saline and then stained with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin antiserum. EBNA-LP immunofluorescence was performed similarly with an affinity-purified monospecific human antiserum against EBNA-LP (49) followed by fluorescein isothiocyanate-conjugated anti-human immunoglobulin antiserum. Slides were washed extensively in phosphate-buffered saline and viewed under fluorescence microscopy.

CAT assays. CAT assays were as described previously (12) except that cells were harvested 4 days after transfection,

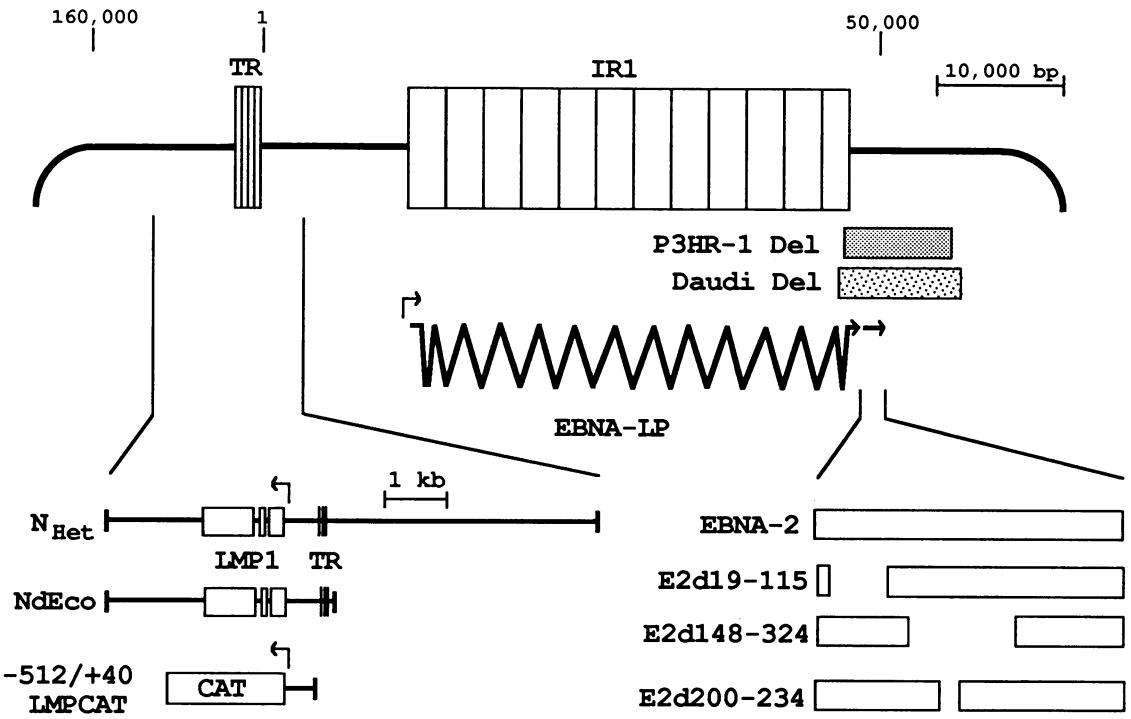

FIG. 1. Schematic diagram of the EBV episome from nucleotides 160,000 to 60,000 showing the map locations of EBNA-2, EBNA-LP, and LMP1. A portion of the EBV episome from nucleotide 160,000 across the terminal repeats (TR) and the major internal repeat region (IR1) to approximately nucleotide 60,000 is shown at the top. The extent of the deletions in P3HR-1 and Daudi EBV is represented by the stippled boxes. The EBNA-2 and the spliced EBNA-LP open reading frames are represented by the heavy lines with arrows. The deleted regions for the EBNA-2 deletion mutants E2d19-115, E2d148-324, and E2d200-234 are illustrated in the lower right. The N_{Het} and NdEco plasmids containing the LMP1 gene and either 5.4 or 1.3 kb of upstream sequences, respectively, are shown in the lower left. The −512/+40 LMPCAT plasmid contains the +40 to −512 LMP1 upstream sequences linked to the *cat* gene.

extracts were made by three cycles of freeze-thawing, and equal portions of cell lysate were assayed in 30-min enzyme reactions. Simultaneous immunofluorescence assays for EBNA-2 expression were performed in all appropriate CAT transfections to ensure equal and adequate transfection efficiencies. Percent acetylation was calculated by removing the nonacetylated and acetylated forms from each sample and determining the counts per minute for each form by liquid scintillation counting.

Northern (RNA) blotting. Cells were lysed in 0.5% Nonidet P-40–10 mM NaCl–10 mM Tris (pH 7.4)–3 mM $MgCl_2$. Nuclei were separated by centrifugation. The cytoplasmic fraction was adjusted to 1% sodium dodecyl sulfate, 100 mM Tris, and 0.2 M NaCl, extracted multiple times with phenol and chloroform, and ethanol precipitated. Cytoplasmic RNA (15 μg) was separated on a 1% agarose–formaldehyde gel, transferred to Genescreen Plus (Amersham Corp., Arlington Heights, Ill.), and hybridized with randomly primed ^{32}P-labeled probes (Amersham).

RESULTS

EBNA-2 induces LMP1 in Daudi and P3HR-1-infected cells, while EBNA-LP has no effect. P3HR-1 and Daudi EBV are notable for their deletion of a DNA fragment which encodes EBNA-2 and the two 3′ unique exons of EBNA-LP (Fig. 1) (4, 15, 16, 19, 22, 34). Although the LMP1 regulatory and coding regions are more than 50 kilobases (kb) 5′ to this deletion, LMP1 expression in the P3HR-1 cell line (Fig. 2, lane 3) was markedly reduced compared with that of an EBV-immortalized lymphoblastoid cell line, IB4 (Fig. 2, lane 1), and was undetectable in the Daudi cell line (Fig. 2, lane 4) (14) by immunoblotting. EBV-negative BL30 and BL41 cells stably infected with P3HR-1 EBV also expressed little, if any, LMP1 (Fig. 2, lanes 7 and 11), whereas the identical cells infected with the prototype B95-8 EBV expressed high levels of LMP1 (Fig. 2, lanes 6 and 10) (29). Thus, EBNA-2

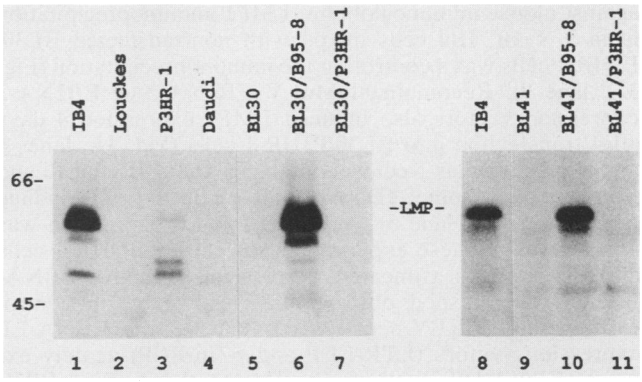

FIG. 2. LMP1 immunoblot of P3HR-1 cells, Daudi cells, and P3HR-1- or B95-8 EBV-infected BL30 or BL41 Burkitt's lymphoma cells. Protein extracts from 2.5×10^5 cells were loaded into each lane, and immunoblots were probed with the LMP1-specific monoclonal antibody S12. Molecular mass markers in kilodaltons are shown. Faint bands below the 60-kDa LMP1 represent degradation products.

or a complete EBNA-LP may be required for high-level LMP1 expression.

To evaluate the role of the EBNA-2 and EBNA-LP deletion, we studied the acute effect of EBNA-2 and EBNA-LP, alone or in combination, on LMP1 expression in P3HR-1-infected or Daudi lymphoma cells. The EBV type 1 or type 2 EBNA-2 open reading frames were expressed under control of the early SV40 promoter (SV-EB1NA2 or SV-EB2NA2) or under control of the MuLV LTR (LTR-EB1NA2 or LTR-EB2NA2). EBNA-LP was expressed from the bicistronic T65 cDNA (LTR-ELP) or the monocistronic EBNA-LP open reading frame (pZip-ELP) under control of the MuLV LTR. Although the T65 cDNA contains the EBNA-2 open reading frame after the EBNA-LP open reading frame, only EBNA-LP is expressed in detectable levels in B-lymphoma cells (47, 48).

Four days after transfection of SV-EB1NA2 or SV-EB2NA2 into BL30/P3HR-1 cells, immunofluorescent staining for EBNA-2 revealed speckled nuclear staining with nucleolar sparing characteristic of EBNA-2 in 2 to 8% of the cells (data not shown). There was an equal distribution of cells which stained less, equally, or more intensely than an EBV-infected lymphoblastoid cell line control. Immunofluorescent staining for LMP1 revealed bright membrane-staining cells when BL30/P3HR-1 cells were transfected with SV-EB1NA2 or SV-EB2NA2 but no bright membrane-staining cells in BL30/P3HR-1 cells transfected with vector control DNA (data not shown). The percentage of LMP1-staining cells was always slightly lower than the percentage of EBNA-2-positive cells, probably because of the easier discrimination of low-level nuclear fluorescence. Identical experiments with P3HR-1 or Daudi cells revealed similar results (data not shown).

LMP1 expression in transfected BL30/P3HR-1 and Daudi cells was also quantitatively assayed by sequential immunoprecipitation and immunoblotting. LMP1 was readily detectable in immunoprecipitates from BL30/P3HR-1 cells transfected with either SV-EB1NA2 (Fig. 3A, lane 3) or SV-EB2NA-2 (Fig. 3A, lane 4). Little or no LMP1 was detected in immunoprecipitates from vector control-transfected cells (Fig. 3A, lane 2) or an equivalent number of nontransfected BL30/P3HR-1 cells (Fig. 3A, lane 8). The 50- and 54-kilodalton (kDa) proteins are from the immunoprecipitating S12 monoclonal antibody, which is detected in the subsequent immunoblots by the secondary rabbit antiserum against mouse immunoglobulin. LMP1 immunoprecipitation from 5×10^4 IB4 cells mixed with nontransfected BL30/P3HR-1 cells was a control for the immunoprecipitation (Fig. 3A, lane 9). Recombinant MuLV EB1NA-2 or EB2NA-2 expression vectors also induced LMP1 expression 4 days after transfection into BL30/P3HR-1 cells (Fig. 3A, lanes 6 and 7). A similar vector containing the EBNA-LP and EBNA-2 bicistronic cDNA (LTR-ELP) did not induce LMP1 (Fig. 3A, lane 5). Acute EBNA-LP expression was not assayed in these experiments since BL30/P3HR-1 cells already express a truncated, serologically reactive EBNA-LP (49; unpublished observations). However, in control experiments in EBV-negative BJAB cells, the EBNA-LP expression vectors (LTR-ELP and pZip-ELP) acutely express, EBNA-LP levels comparable to those of an EBV-infected lymphoblastoid cell line by immunofluorescence with an affinity-purified monospecific human antiserum to EBNA-LP (49; data not shown). The acute transfection efficiency of the EBNA-LP and EBNA-2 vectors was also similar. Furthermore, these EBNA-LP expression vectors were previously shown to produce appropriate EBNA-LP levels after stable transfection into B-lymphoma cells (47, 48). Moreover, the recombinant constructs were similar to the EBNA-2 expression vectors which transactivated LMP1 expression in these transient assays. As was previously observed with stable transfection and expression of LTR-ELP (47, 48), acute LTR-ELP transfection resulted in EBNA-LP but no detectable EBNA-2 expression by immunofluorescence (data not shown). Thus, EBV type 1 or type 2 EBNA-2 alone induced LMP1 expression in BL30/P3HR-1 cells, while EBNA-LP had no effect. EB2NA-2 consistently induced as much or more LMP1 than did EB1NA-2, perhaps in part because of a slightly higher percentage of EB2NA-2 expression.

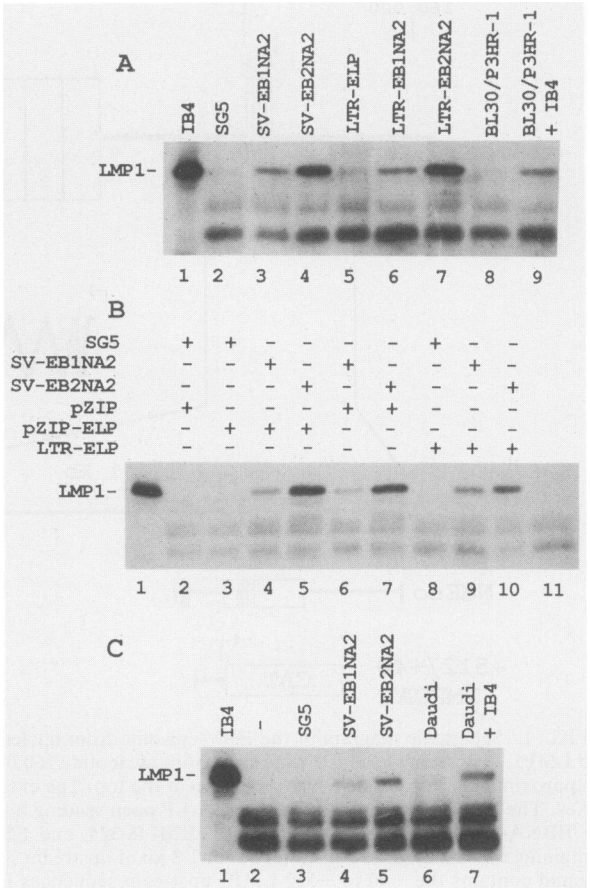

FIG. 3. LMP1 immunoprecipitations from EBNA-2- or EBNA-LP-transfected BL30/P3HR-1 cells (A and B) and Daudi cells (C). Protein extracts from 10^5 IB4 cells were loaded into lane 1 of each panel, and the remaining lanes all show LMP1 immunoprecipitations from cells transfected with the indicated plasmids. Immunoprecipitations from an equivalent number of untransfected cells (panel A, lane 8; panel B, lane 11; and panel C, lane 6) or untransfected cells plus 5×10^4 IB4 cells (panel A, lane 9; and panel C, lane 7) are also shown as controls. LMP1 is 60 kDa. The two smaller proteins come from the heavy chain of the S12 monoclonal antibody used for immunoprecipitation which is detected by the rabbit anti-mouse immunoglobulin secondary antibody used to probe the immunoblot.

Acute EBNA-2 transactivation of LMP1 could also be demonstrated in the Daudi cell line. Transfection of SV-EB1NA2 or SV-EB2NA2 into Daudi cells induced LMP1 which was slightly smaller than the B95-8 EBV LMP1 (Fig. 3C, lanes 4 and 5 versus lanes 1 and 7). The relatively low LMP1 induction is due in part to a 1 to 2% Daudi transfection efficiency as determined by EBNA-2 immunofluorescence.

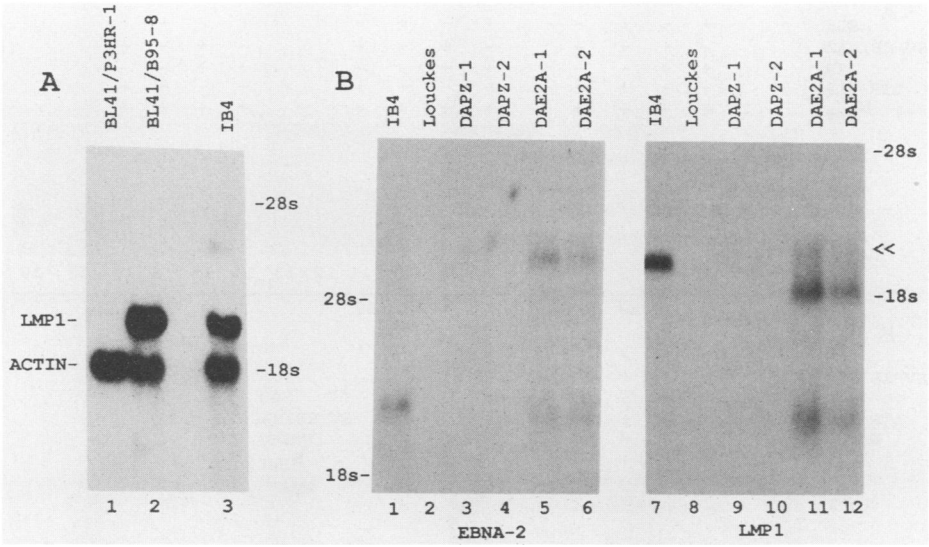

FIG. 4. Northern blots of P3HR-1- or B95-8-infected BL41 cells (A) or Daudi EB1NA2 transfectants (B). Cytoplasmic RNA (15 μg) was loaded into each lane. (A) Blot was hybridized with LMP1 and gamma-actin probes. (B) Two clones of vector control-transfected Daudi cells (DAPZ-1, -2) and two clones of pZip-EB1NA2-transfected Daudi cells (DAE2A-1, -2) were hybridized with a probe for EBNA-2 (left panel) or a 3.0-kb probe for LMP1 (right panel). The LMP1 probe includes the entire open reading frame and a large portion of the 3′ untranslated end. << indicates the Daudi LMP1 mRNA, which is the only mRNA detected with a probe specific for LMP1 derived for the 5′ exon of the LMP1 mRNA. Hybridization of this blot with an actin probe demonstrated comparable amounts of RNA in all lanes (not shown).

Since both P3HR-1 and Daudi EBV lack a complete EBNA-LP as well as EBNA-2, further experiments addressed whether a complete EBNA-LP might synergize with EBNA-2 to induce LMP1. As above, EBNA-LP (pZip-ELP or LTR-T65; Fig. 3B, lane 3 or 8, respectively) did not induce LMP1 in BL30/P3HR-1 cells, whereas both SV-EB1NA2 and SV-EB2NA2 (Fig. 3B, lanes 6 and 7) induced LMP1 expression. Cotransfection of pZip or LTR EBNA-LP expression vectors with an EBNA-2 expression vector did not increase LMP1 expression over that obtained with EBNA-2 alone (Fig. 3B, lanes 4, 5, 9, and 10 versus lanes 6 and 7). Thus, EBNA-LP does not transactivate LMP1 expression or modify the effect of EBNA-2 on LMP1 expression in the context of the P3HR-1 or Daudi EBV genomes.

Northern blot analysis of isogenic Burkitt's lymphoma cells infected with B95-8 or P3HR-1 EBV or Daudi cells stably transfected with pZip-EB1NA2 indicated that EBNA-2 induces LMP1 mRNA. The first evidence for an effect at the mRNA level was the observation that BL41/P3HR-1 cells did not express detectable LMP1 protein (Fig. 2, lane 7) or mRNA (Fig. 4A, lane 1), whereas BL41/B95-8 cells expressed high levels of both LMP1 protein (Fig. 2, lane 6) and mRNA (Fig. 4A, lane 2) (8). More directly, Daudi cells did not express LMP1 protein or mRNA (Fig. 2, lane 4, and Fig. 4B, lanes 9 and 10), while three clones derived by stable transfection of Daudi cells with pZip-EB1NA2 had detectable, albeit low, EBNA-2 levels (two clones shown in Fig. 4B, lanes 5 and 6) and LMP1 mRNA (Fig. 4B, lanes 11 and 12). No LMP1 protein induction was detected by immunoblot, probably owing to the low level of EBNA-2 protein expression (approximately 10% of IB4; data not shown). The EBNA-2-transfected Daudi cells had two messages that hybridized to an EBNA-2 probe (Fig. 4B, lanes 5 and 6). The 6.8-kb mRNA was the size expected for a 5′-to-3′ LTR transcript, while the 2.4-kb mRNA was the size expected for a transcript which is polyadenylated at the polyadenylation site of the EBNA-2 insert. The LMP probe detected 3.0-, 2.4-, and 1.0-kb mRNAs in the EBNA-2-expressing clones but not in the vector control clones (Fig. 4B, lanes 11 and 12 versus lanes 9 and 10). The 2.4- and 1.0-kb mRNAs likely represent productive cycle messages (18) which map 3′ to LMP1 since they did not hybridize to a probe specific for the 5′ part of the LMP1 mRNA. The 5′ LMP1 probe identifies a 3.0-kb RNA as the mRNA expressed from the Daudi LMP1 promoter (data not shown). These data suggest that EBNA-2 up regulates LMP1 mRNA and may also up regulate RNAs expressed from this part of the genome during virus replication.

EBNA-2 transactivation of LMP1 does not require repeating EBNA-LP domains or other EBV proteins. Daudi or P3HR-1-infected cells still express EBNA-1, EBNA-3A, and EBNA-3C as well as a truncated EBNA-LP, and these may be necessary for the transactivation of LMP1 by EBNA-2. We therefore evaluated the effect of EBNA-2 on LMP1 expression from a cotransfected EBV DNA fragment in EBV-negative B-lymphoma cells. The B95-8 EBV *Bam*HI N_{Het} fragment contains the entire LMP1 gene and approximately 5.4 kb of upstream sequences. Transfection of the *Bam*HI N_{Het} fragment into EBV-negative B-lymphoma BJAB or Louckes cells resulted in detectable LMP1 expression by immunoprecipitation (Fig. 5A, lanes 3 and 14). SV-EB1NA2 cotransfection induced more LMP1 expression from the *Bam*HI N_{Het} fragment than cotransfection with the pSG5 vector control DNA (Fig. 5A, lanes 4 and 15 versus lanes 3 and 14). A similar effect was evident in EBV-negative BL30 cells (Fig. 5A, lanes 8 and 9), although SV-EB1NA2-induced LMP1 expression was less because of a lower transfection efficiency in BL30 cells. In each of these cells, the levels of transactivation approximated that noted in transient transfection of the P3HR-1-infected B-lymphoma cells with EBNA-2 alone. Further, EBNA-LP alone (Fig. 5A, lanes 5, 10, and 16) or in combination with EBNA-2 (Fig. 5A, lanes 6, 11, and 17) did not affect LMP1 expression. Thus, other EBV genes were not required for the effect of EBNA-2 on LMP1 expression in EBV-negative B-lym-

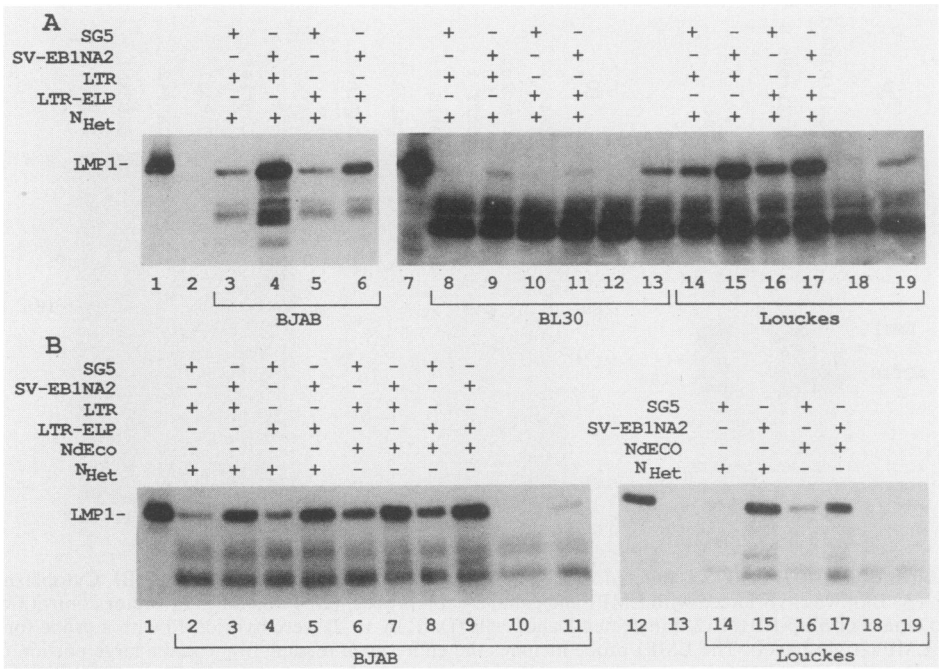

FIG. 5. LMP1 immunoprecipitations from EBV-negative B-lymphoma cells (BJAB, BL30, or Louckes) cotransfected with EBNA-2 or EBNA-LP and the *Bam*HI N_{Het} genomic LMP1 DNA. Protein extracts from 10^5 IB4 (LMP1-positive) cells are shown in panel A, lanes 1 and 7, and in panel B, lanes 1 and 12. Protein extracts from 10^5 BJAB or Louckes cells (LMP1 negative) are shown in panel A, lane 2, and panel B, lane 13. Immunoprecipitations from equivalent numbers of untransfected B-lymphoma cells (panel A, lanes 12 and 18; panel B, lanes 10 and 18) or untransfected cells plus 10^4 IB4 cells (panel A, lanes 13 and 19; panel B, lanes 11 and 19) are shown as controls. Plasmid DNAs transfected into the B-lymphoma cells analyzed in each lane are indicated by a + above the lane.

phoma cells, and EBNA-LP, specifically, had no effect either alone or in combination with EBNA-2.

EBNA-2-responsive element is within +40 to −512 relative to LMP1 cap site. A positive EBNA-2 effect on LMP1 expression can still be demonstrated if the *Bam*HI N_{Het} fragment is truncated so that only 1.3 kb of LMP1 upstream sequences remain (NdEco; Fig. 1). Cotransfection of NdEco with SV-EB1NA2 showed more than a twofold increase in LMP1 expression compared with NdEco plus vector control DNA in BJAB cells (Fig. 5B, lanes 7 and 9 versus lanes 6 and 8) and in Louckes cells (Fig. 5B, lane 17 versus lane 16). LMP1 expression from NdEco was consistently higher than from the longer *Bam*HI N_{Het} fragment by immunoprecipitation and immunofluorescence (Fig. 5B, lane 6 versus lane 2 and lane 16 versus lane 14; and data not shown). This effect may be due in part to the absence of inhibitory sequences between −1280 and −2350 defined by previous CAT analysis of LMP1 upstream sequences in EBV-infected cells (11) or due to increased transfection efficiency from the slightly smaller NdEco plasmid.

To further define the EBNA-2-responsive element, we studied the EBNA-2 responsiveness of the CAT gene (*cat*) under control of the −512 to +40 LMP1 upstream sequences (−512/+40 LMPCAT). These sequences contain an element that is more active in B95-8 EBV-infected cells than in P3HR-1 EBV-infected cells (11). Cotransfection of EB1NA-2-expressing plasmids versus vector control plasmids induced at least fourfold-increased CAT expression from −512/+40 LMPCAT (Fig. 6). The −512/+40 LMPCAT plasmid was responsive to cotransfection with SV-EB1NA2 or pZip-EB1NA2 as opposed to cotransfection with vector control DNA into BL30/P3HR-1 cells (Fig. 6A). EB1NA-2 had no significant effect on expression from the promoterless pCAT or SV40 promoter-driven pSV2CAT controls. Although slight increases in pSV2CAT activity were occasionally observed in EBNA-2 versus vector control cotransfections, slight decreases with EBNA-2 and pSV2CAT cotransfections were equally as frequent. In contrast, EBNA-2 consistently increased −512/+40 LMPCAT activity, by as much as 15-fold. The positive EBNA-2 effect on −512/+40 LMPCAT also did not require expression of other EBV genes since similar results could be obtained in the EBV-negative cell line Louckes (Fig. 6B).

EBNA-2 effect on LMP1 expression is human B lymphocyte specific. Similar experiments were performed in cell lines other than human B lymphomas to assess the species and tissue specificity of the EBNA-2 effect. No EBNA-2 enhancement of LMP1 expression was observed in a human T-cell-lymphotropic virus type I-transformed human T-cell line (MT-2), the HeLa cell line, or in two mouse B-cell lines (A20 and M12; data not shown). There was basal LMP1 expression from transfected *Bam*HI N_{Het} or NdEco in all cell lines as observed in the EBV-negative B-lymphoma cell lines and EBNA-2 expression as high as 30% in HeLa cells as positive controls for transfection. The lack of an EBNA-2 effect in these cell lines suggests that a human B-lymphocyte-specific factor is required for EBNA-2 transactivation of LMP1.

EBNA-2 mutants unable to transactivate LMP1 are growth transformation incompetent. Recent genetic experiments have begun to define EBNA-2 domains nonessential (amino acids 200 to 234) or essential (amino acids 19 to 115 or 148 to 324) for B-lymphocyte growth transformation (7; J. Cohen, unpublished data). Acute transfection of pZip-EB1NA2, pZip-EB2NA2, or pZip-E2d200-234 into BL30/P3HR-1 cells induced LMP1 expression (Fig. 7A, lanes 4, 5, 7, and 10), whereas the pZip-E2d19-115 or pZip-E2d148-324 deletion mutants did not (Fig. 7A, lanes 8 and 9). By EBNA-2

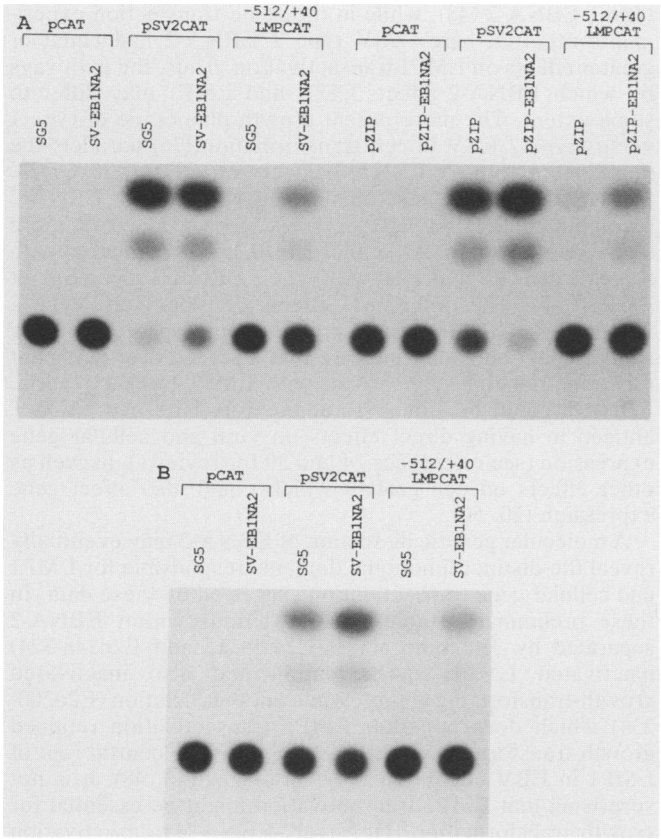

FIG. 6. CAT assays of LMP promoter activity with or without EBNA-2 in BL30/P3HR-1 (A) and Louckes (B) cells. Cells were cotransfected with vector control (SG5, pZip) or EBNA-2 (SV-EB1NA2, pZip-EB1NA2) without promoter (pCAT), with SV40 promoter (pSV2CAT), or with −512/+40 LMP1 promoter (−512/+40 LMPCAT) CAT reporter constructs indicated above each lane. The percent acetylation in panel A was 0.7 and 0.7% for pCAT, 96.6 and 82.5% for pSV2CAT, and 1.6 and 22.1% for −512/+40 LMPCAT when cotransfected with SG5 and SV-EB1NA2, respectively. When cotransfected with pZip and pZip-EB1NA2 in panel A, the percent acetylation was 0.9 and 1.3% for pCAT, 72.8 and 96% for pSV2CAT, and 3.0 and 13.5% for −512/+40 LMPCAT, respectively. In panel B, the percent acetylation was 0.7 and 1.0% for pCAT, 31.0 and 48.7% for pSV2CAT, and 3.1 and 15.9% for −512/+40 LMPCAT when cotransfected with SG5 and SV-EB1NA2, respectively.

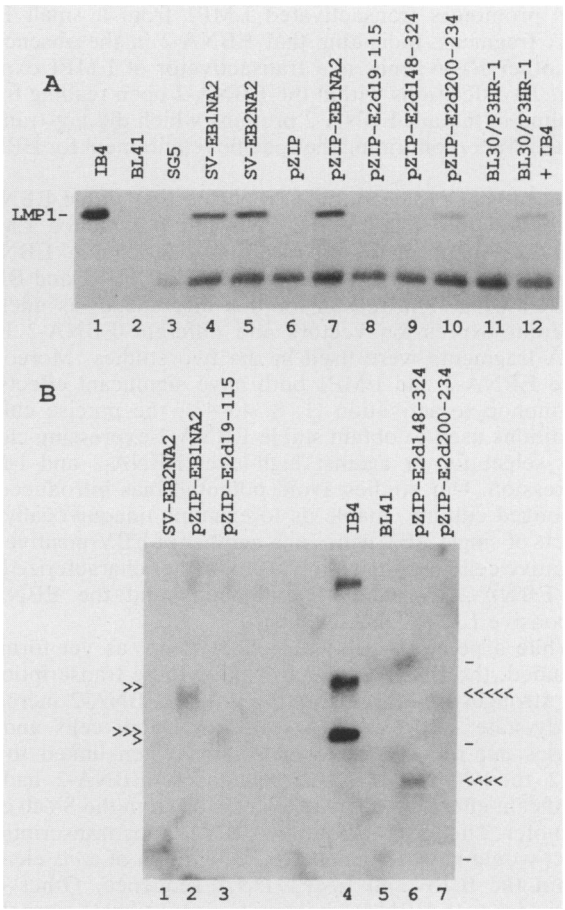

FIG. 7. LMP1 induction by EB1NA-2, EB2NA-2, or EB1NA-2 deletion mutants. LMP1 immunoprecipitated from transfected BL30/P3HR-1 cells is shown in an immunoblot in panel A, lanes 4 to 10. (B) EBNA-2 immunoprecipitations from the same samples. The EBNA-2 blot was developed with EBV-immune human sera which detect EBNAs. Protein extract from 10^5 IB4 cells shown in panel B, lane 4, expresses, from bottom to top, EBNA-1, EBNA-2, and the EBNA-3A and EBNA-3C doublet. Immunoprecipitation with an EBNA-2 monoclonal antibody detected comparable amounts of EBNA-2 in cells transfected with SV-EB2NA2 (indicated by >), pZip-EB1NA-2 (>>), pZip-E2d19-115 (>>>), pZip-E2d148-324 (<<<<), and pZip-E2d200-234 (<<<<<). Protein size markers shown by dashes are 92, 66, and 45 kDa.

immunofluorescence, there was comparable expression of EB1NA2, EB2NA2, EBNA-2d200-234, and EBNA-2d148-324 (data not shown). Although as many strongly positive pZip-E2d19-115 cells could be detected, lower-level expression was difficult to evaluate by immunofluorescent staining because of its diffuse nuclear pattern. To confirm that comparable levels were expressed from the EBNA-2 deletion mutant constructs, we immunoprecipitated EBNA-2 from the acute transfections sequentially after LMP1. Comparable levels of the 66-kDa EBNA-2d19-115, 56-kDa EBNA-2d148-324, and 80-kDa EBNA-2d200-234 were detected in immunoprecipitations from the acute transfections (Fig. 7B, lanes 3, 6, and 7). Thus, this limited deletion analysis is consistent with the hypothesis that EBNA-2 transactivation of LMP1 is essential for EBV growth transformation.

DISCUSSION

These experiments are the first demonstration that EBNA-2 is a necessary and sufficient transactivator of LMP1 expression in B lymphocytes. Previous experiments demonstrating normal LMP1 expression only after infection with EBNA-LP- and EBNA-2-expressing EBV strains were consistent with the hypothesis that EBNA-2 or EBNA-LP affects LMP1 expression (31). However, the relative contribution of EBNA-LP, EBNA-2, or other EBV genes to LMP1 expression from these nonisogenic EBV strains could not be distinguished. Molecular genetic recombinants restoring normal EBNA-LP and EBNA-2 genes to the P3HR-1 EBV strain more precisely implicated EBNA-LP or EBNA-2 in LMP1 expression (7) but did not allow for comparison with nonrecombinant viruses or discriminate between EBNA-LP and EBNA-2 effects. In the current experiments, the EBNA-2 open reading frame expressed under control of heterolo-

gous promoters transactivated LMP1 from a small EBV DNA fragment, indicating that EBNA-2 in the absence of any other EBV genes is a transactivator of LMP1 expression. Two deletions within the EBNA-2 open reading frame resulted in mutant EBNA-2 proteins which did not transactivate LMP1, confirming the specific requirement for EBNA-2.

Recently, Cordier et al. (8) reported that stable EBNA-2 expression in BL41/P3HR-1 cells did not induce LMP1, whereas Abbot et al. (1) reported that stable EBNA-2 expression induced LMP1 in both BL30/P3HR-1 and BL41/P3HR-1 cells. The reason for this discrepancy is unclear. Different expression vectors and different EBNA-2 EBV DNA fragments were used in the two studies. Moreover, since EBNA-2 and LMP1 both have significant effects on B-lymphocyte activation (1, 8, 46–48), the precise culture conditions used to obtain stable EBNA-2-expressing clones may select for or against high-level EBNA-2 and LMP1 expression. Our studies avoid potential bias introduced by prolonged culture, enable us to examine unequivocally the effects of single EBV genes in a number of EBV-positive and -negative cell lines, and allow us to further characterize both the EBNA-2 transactivating domains and the EBNA-2-responsive LMP1 DNA element.

While a posttranscriptional effect is not as yet formally excluded, the EBNA-2 effect is likely to be transcriptional. The strongest evidence for this is that EBNA-2 increases steady-state LMP1 mRNA levels in Daudi cells and increases *cat* reporter gene expression when linked to the −512 to +40 LMP1 DNA sequences. EBNA-2 had no significant effect on *cat* gene expression from the SV40 early promoter. Therefore, a putative EBNA-2 posttranscriptional effect would have to depend on recognition of a *cis* element within the 0 to +40 LMP1 DNA sequence. Other data consistent with EBNA-2 being a transcriptional transactivator include the following. (i) EBNA-2 is tightly localized to the cell nucleus (20). (ii) The tissue and species specificity of the effect of EBNA-2 on LMP1 is similar to that of other viral or cellular transcriptionally active factors. (iii) The low transcriptional rate of LMP1 compared with EBNA transcription even in EBNA-2-positive cells and the relatively high LMP1 mRNA levels in such cells (40) indicate that LMP1 expression could be stringently regulated at the transcriptional level. (iv) EBNA-2 also up regulates CD21 and CD23 cellular mRNAs (8, 47, 48). EBNA-2 can induce either the type a or type b CD23 mRNA, more compatible with an EBNA-2 effect on a CD23 transcriptional enhancer (48). Proof of an EBNA-2 transcriptional effect will require LMP1 nuclear runon assays in the presence or absence of EBNA-2. Further characterization and identification of the EBNA-2-responsive element within the −512 to +40 LMP1 DNA will also clarify whether the responsive element is part of the regulatory sequences or the transcript.

It will be important to determine whether EBNA-2 also transactivates the expression of two recently identified LMP genes, LMP2A and LMP2B (25). LMP2A and LMP2B initiate within 3.0 kilobase pairs and 250 base pairs of the LMP1 transcriptional initiation site, respectively, and are transcribed in the opposite direction from LMP1 (41). In fact, the LMP2B initiation site is within the −512/+40 LMP1 DNA shown to contain the EBNA-2-responsive element.

Although there are parallels between the effects of EBNA-2 on CD23 and LMP1, there is a major difference in the effects of the EBV type 1 and type 2 EBNA-2s on these two genes. In stable transfection experiments, EBV type 2 EBNA-2 has much less effect on CD23 and CD21 than EBV type 1 EBNA-2 (48), while in the acute transfection experiments reported here, EBV type 2 EBNA-2 had equal or greater effects on LMP1 transactivation. Thus, the pathways by which EBNA-2 affects CD23 and LMP1 may differ to some extent. The more potent growth phenotype of type 1 versus type 2 EBV in cell transformation (36) parallels the more potent type 1 EBNA-2 effects on CD21 and CD23. Indeed, B lymphocytes transformed with type 2 EBNA-2 recombinant P3HR-1 EBV express less CD23, grow more slowly, and yet express similar LMP1 levels compared with isogenic cells transformed with type 1 EBNA-2 recombinant P3HR-1 (7). The differential effects of type 1 and type 2 EBNA-2s on CD23 and cell growth phenotype versus their similar effect on LMP1 indicate that EBNA-2 has significant functions not fully concordant with LMP1 transactivation. EBNA-2 could be similar to adenovirus E1A and SV40 T antigen in having direct effects on viral and cellular gene expression (see references 24 and 30 for reviews), as well as other effects on cell growth which could also affect gene expression (10, 50).

A molecular genetic dissection of EBNA-2 may eventually reveal the distinct functional domains responsible for LMP1 and cellular gene transactivation suggested by these data. In these preliminary studies, two deletions within EBNA-2 separated by 33 amino acids (E2d19-115 and E2d148-324) inactivated LMP1 transactivation and also inactivated growth-transforming ability, while another deletion (E2d200-234) which does not alter LMP1 transactivation retained growth-transforming capacity. In light of the central role of LMP1 in EBV effects on B lymphocytes (46, 48), it is not surprising that LMP1 transactivation might be essential for growth transformation. However, EBNA-2 transactivation of LMP1 may not be sufficient for growth transformation, and we may also expect to define EBNA-2 domains which are necessary for growth transformation but are not necessary for LMP1 transactivation. These may define domains important for interaction with other cell transcriptional or growth-regulatory factors.

A major component of the action of EBNA-2 on B lymphocytes is mediated through LMP1, which is a broad effector of B-lymphocyte growth and gene expression. In three EBV-negative B-lymphoma cell lines, LMP1 induces homotypic adhesion associated with increased expression of cellular adhesion molecules, characteristic profiles of B-cell activation antigens, and vimentin expression (3, 46, 48). In at least one B-lymphoma cell line, LMP1 also raises the intracellular free calcium level and alters transforming growth factor beta responsiveness (46). Thus, the biological impact of EBNA-2 is amplified by its transactivation of the LMP1-mediated growth effects. The EBNA-2 effect can be further amplified by its synergistic interaction with LMP1 as seen with CD23 induction (48). CD23 is prominently up regulated in EBV infection. The type a CD23 mRNA is constitutively expressed in B lymphocytes, and the type b CD23 mRNA is preferentially turned on by LMP1. EBNA-2 can increase either CD23 mRNA form and acts synergistically when coexpressed with LMP1 to induce high-level CD23 expression. The effects of LMP1 require expression at the level normally found in latent EBV infection (46, 48). Thus, EBNA-2 regulation of LMP1 expression also underscores the central role for EBNA-2 in EBV-induced B-lymphocyte growth transformation.

Although EBNA-LP did not have any direct effects on LMP1 expression in these experiments, we cannot rule out an indirect role for EBNA-LP. Indeed, the linkage between EBNA-LP and EBNA-2 in bicistronic mRNAs suggests a

need for joint expression (39). Since we used heterologous promoters for these studies, it is possible that EBNA-LP, or other viral genes, affect LMP1 expression indirectly through modulation of EBNA-2 expression.

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