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Epstein-Barr Virus Nuclear Protein LP Stimulates EBNA-2 Acidic Domain-Mediated Transcriptional Activation

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Epstein-Barr virus (EBV) nuclear proteins EBNA-LP and EBNA-2 are the first two proteins expressed in latent infection of primary B lymphocytes. EBNA-2 is essential for lymphocyte transformation, and EBNA-LP is at least critical. While EBNA-2 activates specific viral and cellular promoters, EBNA-LP's role has been obscure. We now show that EBNA-LP stimulates EBNA-2 activation of the LMP1 promoter and of the LMP1/LMP2B bidirectional transcriptional regulatory element. EBNA-LP alone has only a negative effect. EBNA-LP also stimulates EBNA-2 activation of a multimerized regulatory element from the BamC EBNA promoter. Since both viral regulatory elements can bind the EBNA-2-associated cell protein RBPJκ, consensus RBPJκ binding sites were positioned upstream of the herpes simplex virus type 1 thymidine kinase promoter and were found to be sufficient for EBNA-LP and EBNA-2 coactivation. EBNA-LP strongly stimulated activation of an adenovirus E1b promoter with upstream Gal4 binding sites by a Gal4 DNA binding domain/EBNA-2 acidic domain fusion protein, indicating that EBNA-LP coactivation requires only the EBNA-2 acidic domain to be localized near a promoter. The EBNA-LP stimulatory activity resides in the amino-terminal 66-amino-acid repeat domain. The carboxyl-terminal unique 45 amino acids appear to regulate EBNA-LP's effects. The first 11 amino acids of the 45 have a strong negative effect, while the last 10 are critical for the ability of the last 34 to relieve the negative effect. These results indicate that EBNA-LP's critical role in EBV-mediated cell growth transformation is in stimulating (and probably regulating) EBNA-2-mediated transcriptional activation.

Epstein-Barr virus (EBV) infects resting human B lymphocytes and causes their proliferation (for reviews, see references 24 and 38). In infected B lymphocytes, the first EBV promoter is within the 10 tandem 3-kbp internal direct repeats (2, 42, 51). The promoter in the most 5′ internal repeat enables transcription of the downstream repeats and of the 3′ unique sequence DNA. The transcript is extensively spliced into a 27-bp first exon, five alternating 66- and 132-bp exons derived from the 10 copies of the 3-kbp repeat, and 33-, 122-, and 647-bp exons derived from the unique DNA. The 3′ exon encodes a latent-infection nuclear protein, EBNA-2. Depending on alternative splicing between the first and second exons, which can generate an initiation codon, the 5′ exons encode the long untranslated leader of the EBNA-2 mRNA or another nuclear protein called leader protein or EBNA-LP (11, 13). The latter mRNA is efficiently translated into EBNA-LP and inefficiently reinitiates at the EBNA-2 open reading frame, while the former mRNA is efficiently translated into EBNA-2 (19, 52). The linkage between EBNA-LP and EBNA-2 expression in the same initial transcript suggests that their functions need to be tightly coordinated.

EBNA-2 is essential for EBV-mediated B-lymphocyte growth transformation (9, 17). Since the domains which are essential for B-lymphocyte growth transformation are also essential for activation of transcription of specific viral and cellular genes, EBNA-2's principal role in transformation is likely to be in transcriptional regulation (6–9, 52–56). EBNA-2 upregulates transcription through a domain which interacts with cellular, sequence-specific DNA binding proteins (16, 18, 22, 27, 31, 44, 58, 59) and through an acidic domain (EBNA-2AcD) which interacts with basal and activated transcription factors and with a novel coactivator (7, 8, 30, 47–49).

The role of EBNA-LP in EBV-induced B-cell transformation is uncertain. EBNA-LP has an effect on cell growth (3, 33) and binds weakly to pRB and p53 in vitro (45) but does not affect pRB or p53 transcriptional regulation (20). EBNA-LP localizes to PML bodies and associates with hsp 70 (25, 34, 37, 46). Introduction of a stop codon between the repeating and 3′ unique exons of the EBNA-LP open reading frame decreases the efficiency of primary B-lymphocyte growth transformation by 90%, indicating that EBNA-LP has a critical or essential role in transformation (33). In one experiment with primary B lymphocytes, cyclin D2 transcription increased in response to transfection with EBNA-LP and EBNA-2 expression vectors, while EBNA-LP or EBNA-2 alone had less effect (43). Although early attempts to demonstrate an effect of EBNA-LP on EBNA-2 activation of the LMP1 promoter were not successful (55), difficulties in pursuing the cyclin D2 effects, a clearer understanding of EBNA-2 activation of the bidirectional LMP1/LMP2B promoter regulatory element (22, 27, 44, 50), and the need for an assay of EBNA-LP's relevant effects led us to reexamine the possible effect of EBNA-LP on EBNA-2 activation of the LMP1 promoter.

MATERIALS AND METHODS

Cells and cell culture. BJAB is an EBV-negative B-lymphoma cell line. IB4 is an EBV-transformed B-lymphoblastoid cell line. Cells were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated fetal calf serum (HyClone) and gentamicin.

Plasmids. pSG-EBNA-LP was generated by cloning the EBNA-LP cDNA derived from type 1 EBV (42, 52) into the pSG5 expression vector (Stratagene) under control of the simian virus 40 (SV40) early promoter. pSG-EBNA-2 was generated by cloning the 1.8-kbp *Bst*UI-*Dra*I fragment encompassing the entire

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CAT reporter plasmid: promoter-regulatory region		promoter	n	EBNA-2 10 ug	LP 10 ug	EBNA-2 10 ug plus LP (ug): 1	2	5	10	20	50
				fold activation							
LMP1	-512 [ ] +40	-	2	2.1	0.1				3.7		
LMP1	-234 [ ] +40	-	3	5.0	0.7				12	3.0	
LMP1	+40 [ ] -234	tk	8	6.0	0.8	33	80	200	250	240	46
LMP1	-236 [ ] -145	tk	2	1.5	0.5		2.8		1.2	0.9	
LMP1	-145 [ ] -236	tk	3	1.6	0.2	5.3	3.9	1.0	0.1	0.1	
BamCp	-380 [ ] -330	tk	3	2.0	0.6	1.7	1.6	1.0	0.9	0.6	
BamCp	(-430 [ ] -330) x8	E1b	3	14	1.0	180	240	270	210	120	56
Synthetic	(RBPJK site) x5	tk	3	25	1.0	80	81	83	68	45	

FIG. 1. Summary of the effects of pSG-EBNA-LP on pSG-EBNA-2-mediated activation of various promoters in BJAB B-lymphoma cells. CAT reporter plasmids were transfected into BJAB cells along with the indicated amounts of pSG-EBNA-2 and/or pSG-EBNA-LP. The total amount of transfected DNA in each sample was equalized with pSG5 vector DNA. Fold activation relative to that with pSG5 is indicated. These are representative results of *n* independent experiments.

EBNA-2-coding region of EBV strain W91 (8) into pSG5. Plasmid Gal4-EBNA-2AcD is the transactivating EBNA-2AcD (amino acids 426 to 462) fused to the carboxyl terminus of the Gal4 DNA binding domain (7). Plasmid Gal4-VP16 contains the herpesvirus transactivating VP16 acidic domain (VP16AcD) (amino acids 412 to 490) fused to the carboxyl terminus of the Gal4 DNA binding domain (41).

EBNA-LP mutants pSG-LPd10 and pSG-LPd34 were made by cloning PCR-amplified fragments of EBNA-LP into the *Sfi*I site of the EBNA-LP cDNA in pSG-EBNA-LP. The 3′ primer incorporated a stop codon after the appropriate codon. Full-length EBNA-LP with a 3′ untranslated region identical to those of the mutant EBNA-LP constructs was used as a control for transfections with mutant EBNA-LP. To generate pSG-LPd45, which lacks the 45 carboxyl-terminal codons, an *Xba*I linker (Stratagene) was inserted into the *Sfi*I site of the EBNA-LP cDNA in pSG-EBNA-LP. This results in three extra codons encoding PLV before the termination of translation of EBNA-LPd45. EBNA-LPd45/14 has 14 extra codons encoding PGDLIKAELVYCSL before the termination of translation.

pSG-LPHT was constructed by replacing the EBNA-LP stop codon with a *Bgl*II site. The *Bam*HI-*Eco*RI fragment of pBS$^+$MER/G525R, which contains the modified ligand binding domain of the murine estrogen receptor (HT), which binds only 4-hydroxytamoxifen (4OHT) (32) (generously provided by T. Littlewood), was cloned into the *Bgl*II site. The approximately 2-kbp fragment from the resultant plasmid that contained EBNA-LP fused with HT was inserted into the pSG5 vector to generate pSG-LPHT.

p-512/+40LMP1CAT and p-234/+40LMP1CAT contain the indicated LMP1 promoter sequences cloned into the promoterless chloramphenicol acetyltransferase (CAT) reporter plasmid pCAT-3M (50). p+40/-234tkLMP1CAT, p−145/-236tkLMP1CAT, and p-236/145tkLMP1CAT were derived from pBLCAT2, which contains the herpes simplex virus type 1 (HSV-1) thymidine kinase (tk) promoter upstream of CAT (50). pCptkCAT has the sequence −331 to −380 from the EBV BamC promoter (21) in pBLCAT2 (56). Plasmid pBamCp8 has eight copies of the EBNA-2-responsive −330 to −430 sequence from the BamC promoter and was kindly provided by P. Ling and D. Hayward (30). Plasmid pJK5tkCAT, which was kindly provided by E. Johannsen and E. Robertson, has five copies of the RBPJκ binding site (5′gatcgactcgtgggaaaatgggc3′) in pBLCAT2 and will be fully described elsewhere (40a). Plasmid pG4tkCAT contains five copies of the Gal4 binding site in pBLCAT2 (7, 29). Plasmid pG4E1bCAT contains five copies of the Gal4 binding site upstream of the minimal adenovirus E1B promoter and a CAT reporter (29) (kindly provided by J. Lillie and M. Green).

All plasmid constructions that were derived by PCR amplification or from synthetic oligonucleotides were verified by sequencing.

Transfections and reporter assays. Fifteen million (1.5×10^7) BJAB cells in log-phase growth were transfected with 10 μg of reporter plasmid, 10 μg of EBNA-2 expression plasmid, and various amounts of pSG-EBNA-LP DNA. To compensate for the maximum amount of pSG-EBNA-LP DNA in each experiment, an appropriate amount of vector plasmid DNA was added to transfection mixtures which had less pSG-EBNA-LP DNA. The mixture of cells and DNA was suspended in final volume of 0.4 ml of RPMI 1640 medium and electroporated with a Bio-Rad Gene Pulser at 200 V and 960 μF. Two micrograms of CMV-β-gal plasmid was also cotransfected, being used as an internal control for transfection efficiency. Plasmid DNAs were purified twice on CsCl gradients. Transfected cells were harvested 20 h after electroporation. Each sample was divided into two portions; one was used for the CAT assay, and the other was used for Western blotting to ensure appropriate expression of EBNA-2 and EBNA-LP in each sample.

For the CAT assay, cell extracts were prepared by three cycles of freeze-thawing and assayed as previously described (50). Percent acetylation was calculated by using ImageQuant software and a PhosphorImager (Molecular Dynamics). Fold activation activity was relative to that of control expression vector pSG5.

Immunoblots. Whole-cell lysates were prepared by solubilization in sodium dodecyl sulfate (SDS) sample buffer and boiling for 10 min. Equal amounts of each sample were separated on Laemmli 8 or 10% polyacrylamide-SDS gels. Proteins were transferred onto nitrocellulose filters (Schleicher & Schuell) and detected with monoclonal antibody JF186 (for EBNA-LP) (13) or PE2 (for EBNA-2), peroxidase-conjugated anti-mouse immunoglobulin (Amersham), and a chemiluminescence detection kit (Pierce).

RESULTS

EBNA-LP potentiates EBNA-2 activation of the LMP1/LMP2B bidirectional promoter regulatory element. In non-EBV-infected BJAB B-lymphoma cells, transfection of an SV40 promoter-driven EBNA-2 expression plasmid (pSG5-EBNA-2) with −234/+40 and −512/+40 LMP1 promoter constructs resulted in five- and twofold-higher CAT reporter activity, respectively, than that with pSG5 vector-transfected controls (Fig. 1). pSG5-EBNA-LP had no positive effect and inhibited LMP1 promoter-mediated CAT expression. However, when 10 μg of pSG5-EBNA-LP was cotransfected with 10 μg of pSG5-EBNA-2, CAT reporter activity from the −234/+40 or −512/+40 LMP1 promoter was increased about twofold over CAT reporter activity with pSG5-EBNA-2 alone. Immunoblots for EBNA-2 indicated that the EBNA-LP effect was not due to increased EBNA-2 levels in the EBNA-LP-cotransfected cells. Thus, these data indicate that EBNA-LP increases EBNA-2 activation of the LMP1 promoter about twofold.

The LMP2B transcriptional start site is at position −266 and in the opposite direction relative to the LMP1 transcriptional start site, with the two promoters sharing a common regulatory element (16, 22, 27, 44, 50). When this element was positioned in the LMP2B orientation upstream of the HSV-1 tk promoter and assayed for EBNA-2 and/or EBNA-LP responsiveness (Fig. 1 and 2), EBNA-LP alone had a negative effect. EBNA-2 activated the +40/−234 LMP1-tk promoter sixfold. The effect of EBNA-LP on EBNA-2-activated CAT expression was much more than that with the native LMP1 promoter. Even 1 μg of pSG5-EBNA-LP increased CAT levels to more than five times

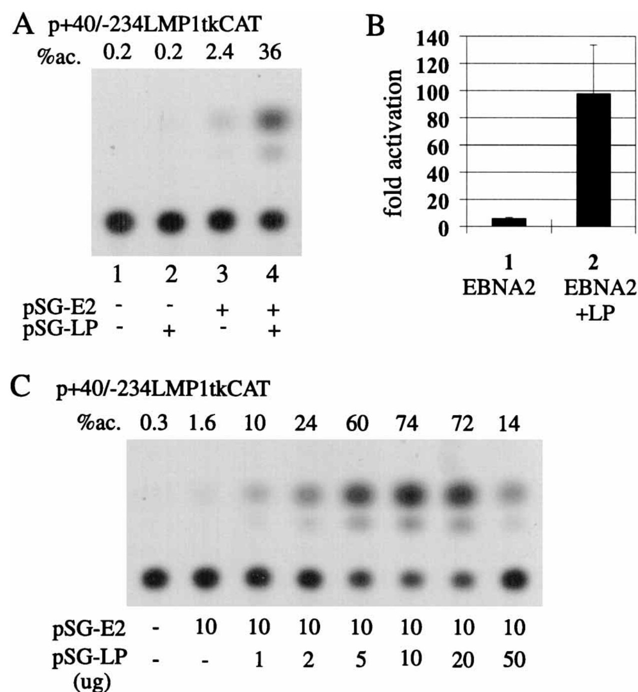

FIG. 2. EBNA-LP stimulates EBNA-2-mediated transactivation of the LMP1/LMP2B promoter regulatory element. The activating plasmid, pSG-EBNA-2, and the reporter plasmid, p+40/−234LMP1tkCAT, which has the EBNA-2-responsive LMP1/LMP2B element, were transfected into BJAB cells with or without pSG-EBNA-LP. (A) Representative CAT assay results among eight independent experiments. Lane 1, pSG5 vector control; lane 2, 10 μg of pSG-EBNA-LP alone; lane 3, 10 μg of pSG-EBNA-2 alone; lane 4, 10 μg each of pSG-EBNA-2 and pSG-EBNA-LP. Each transfection was balanced by addition of the appropriate amount of vector control DNA. The percent acetylation (%ac.) is shown above each lane. (B) Average fold activation activity among eight independent transfection experiments. Bar 1, mean result with EBNA-2 expression only; bar 2, mean result with both EBNA-2 and EBNA-LP expression. Fold activation is relative to that with the pSG5 vector control. Error bars indicate standard deviations. (C) EBNA-LP stimulation of EBNA-2-mediated transactivation of the LMP1 promoter regulatory region is dose dependent. BJAB cells were transfected with p+40/−234LMP1tkCAT along with pSG-EBNA-2 (pSG-E2) and/or pSG-EBNA-LP (pSG-LP). These results are representative of eight replicates.

that with 10 μg of pSG5-EBNA-2 alone. Increasing the amount of cotransfected pSG5-EBNA-LP to up to 10 μg resulted in a level of CAT more than 40 times that observed with EBNA-2 alone. Increasing the amount of cotransfected pSG5-EBNA-LP to 50 μg reproducibly resulted in less coactivation than with 2 to 20 μg, compatible with a partial squelching-like effect (Fig. 2C). A smaller and less EBNA-2-responsive piece of the LMP1/LMP2B regulatory sequence, −145/−236 LMP1 or −236/−145 LMP1, positioned upstream of the tk promoter, was only twofold EBNA-2 responsive and only fourfold EBNA-2 and EBNA-LP coactivated (Fig. 1).

Strong EBNA-LP coactivation with EBNA-2 requires only RBPJκ sites. Since the LMP1/LMP2B and BamC EBNA promoter (Cp) regulatory sequences both have RBPJκ sites which are important for EBNA-2 responsiveness (16, 18, 21, 22, 31, 56, 59), we tested whether a single or multimerized Cp element would also be coactivated by EBNA-2 and EBNA-LP (Fig. 1 and 3A). EBNA-2 transactivated the single Cp response element only twofold, and EBNA-LP had no effect. However, an eight-copy multimer of the −430/−330 Cp element upstream of the adenovirus E1b minimal promoter was activated 14-fold by EBNA-2 alone. EBNA-LP had no positive effect alone but coactivated up to 19 times over EBNA-2 alone (270-fold total activation when 5 to 10 μg of EBNA-LP expression plasmid was transfected along with 10 μg of EBNA-2 expression plasmid). Larger amounts of EBNA-LP expression plasmid had lesser coactivating effects.

To test whether RBPJκ sites alone are sufficient for EBNA-LP to coactivate with EBNA-2, five synthetic RBPJκ sites were positioned upstream of the HSV-1 tk promoter, and the construct was tested in BJAB cells with pSG5-EBNA-2 and/or pSG5-EBNA-LP (Fig. 1 and 3B). EBNA-2 resulted in a 25-fold activation. EBNA-LP alone had no effect, and EBNA-2 and EBNA-LP together activated the CAT reporter 80-fold. With either the multimerized Cp or multimerized RBPJκ element, even 1 μg of cotransfected pSG5-EBNA-LP produced near-maximal effects, and 20 to 50 μg resulted in a squelching-like effect.

Strong EBNA-LP coactivation with EBNA-2 requires only the EBNA-2AcD. EBNA-2 has two domains which are essential for transformation and for transcriptional activation. One domain associates with RBPJκ and also interacts with PU.1, another cellular, sequence-specific DNA binding protein (16, 18, 22, 27, 44, 54). The second domain (EBNA-2AcD) is an acidic activator, associates with a novel cellular nuclear protein (p100), and interacts with basal and activated transcription factors (47–49). To evaluate whether RBPJκ and the EBNA-2 domain that interacts with RBPJκ are essential for EBNA-LP stimulation of EBNA-2-mediated transcriptional activation, the EBNA-2AcD fused to the 3′ end of the Gal4 DNA binding domain was tested for coactivation with EBNA-LP in BJAB cells cotransfected with a reporter plasmid having five Gal4 binding sites upstream of either the HSV-1 tk or the adenovirus E1b promoter (Fig. 4A). Gal4-EBNA-2AcD activated CAT expression from the G4tk and G4E1b promoters six- and

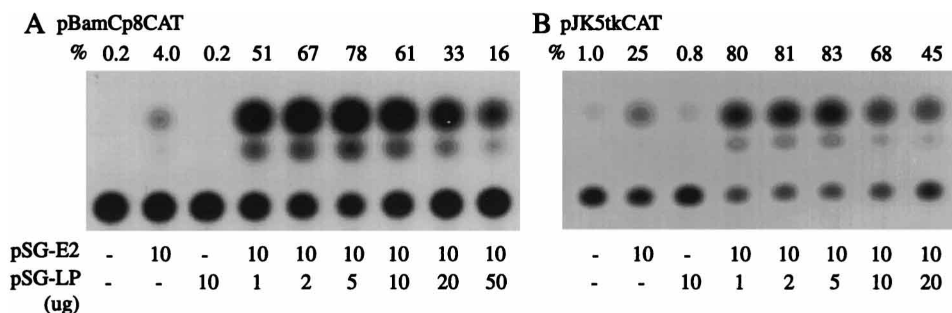

FIG. 3. EBNA-LP stimulates EBNA-2-mediated transactivation of the Cp regulatory element and requires only RBPJκ sites. pSG-EBNA-2 and CAT reporter plasmids which contain eight copies of the −330 to −430 Cp EBNA-2 response element upstream of the minimal E1b promoter (A) or five copies of an artificial RBPJκ site upstream of the tk promoter (B) were transfected into BJAB cells with or without pSG-EBNA-LP. These results are representative of six replicates.

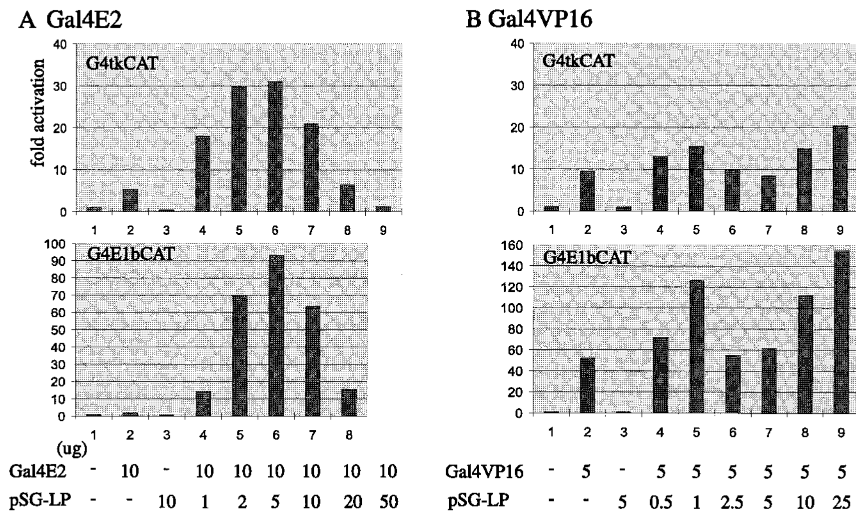

FIG. 4. The EBNA-2AcD is sufficient to confer EBNA-LP stimulation on a promoter, and EBNA-LP has substantially less effect on the VP16AcD. BJAB cells were transfected with an expression plasmid for the Gal4-EBNA-2AcD (A) or Gal4-VP16AcD (B), with the reporter plasmid pG4tkCAT, which contains five copies of the Gal4 binding site upstream of a tk promoter-driven CAT gene (upper panels), or with the reporter pG4E1bCAT, which contains five copies of the Gal4 binding site upstream of a minimal E1b promoter-driven CAT gene (lower panels), and with increasing amounts of EBNA-LP expression plasmid or vector control DNA.

threefold, respectively. As expected, EBNA-LP expression had no effect on CAT expression from either promoter in the absence of Gal4-EBNA-2AcD. In the presence of 10 μg of Gal4-EBNA-2AcD, increasing amounts of pSG5-EBNA-LP induced 5 times greater CAT expression (30-fold total) from the G4tk promoter than Gal4-EBNA-2AcD alone and 30 times greater CAT expression (90-fold total) from the G4E1b promoter. Maximal coactivation was achieved with 5 μg of pSG5-EBNA-LP and 10 μg of EBNA-2 expression plasmids. Less coactivation was observed with more than 5 μg of cotransfected pSG5-EBNA-LP. These results indicate that EBNA-LP can strongly coactivate promoters to which the EBNA-2AcD has been directed by fusion to the Gal4 DNA binding domain.

The effect of EBNA-LP is somewhat specific for the EBNA-2AcD (Fig. 4B). A fusion of Gal4 to the VP16AcD induced higher CAT levels than the Gal4-EBNA-2AcD fusion and was less EBNA-LP coactivated. Gal4-VP16AcD alone induced 10-fold activation of the G4tk promoter and 50-fold activation of the G4E1b promoter. Increasing amounts of cotransfected pSG5-EBNA-LP had reproducible waxing, waning, and then waxing stimulatory effects but never had more than a doubling effect on the G4tk promoter and a tripling effect on the G4E1b promoter. The more modest coactivating effects of EBNA-LP with Gal4-VP16AcD were not due to the high basal activity of Gal4-VP16AcD, since the EBNA-LP effect was not increased when cells were cotransfected with EBNA-LP and amounts of Gal4-VP16AcD expression plasmids that alone induced the same levels of activation as were induced by Gal4-EBNA-2AcD alone (data not shown).

Repeating domains of EBNA-LP are sufficient for coactivation with the EBNA-2AcD in transient-transfection assays, and the carboxyl-terminal unique domain has a regulatory role. The EBNA-LP cDNA used in this study encodes four 66-amino-acid repeats (the first with a methionine followed by 64 amino acids of the repeat) and the 45 carboxyl-terminal unique amino acids. To assess the role of these domains in EBNA-2AcD-mediated transcriptional coactivation, plasmid constructs which express full-length EBNA-LP (LP), EBNA-LP with the last 10 amino acids deleted (LPd10), EBNA-LP with the last 34 amino acids deleted (LPd34), or EBNA-LP with the last 45 amino acids deleted (LPd45) were transfected into BJAB cells with the Gal4-EBNA-2AcD expression vector and the G4tk promoter-CAT reporter vector (Fig. 5). As expected, Gal4-EBNA-2AcD alone induced twofold-higher levels of CAT, and each of the EBNA-LP expression constructs alone had no positive effect. Gal4-EBNA-2AcD with increasing amounts of cotransfected LP or LPd45 expression plasmid induced 18- to 20-times-higher CAT levels than Gal4-EBNA-2AcD alone. These data indicate that the repeating domains of EBNA-LP are sufficient for strong stimulation of EBNA-2AcD-mediated activation. Surprisingly, LPd10 and LPd34 were markedly deficient in EBNA-2AcD-mediated coactivation. The LPd10 deficiency was not due to instability or obvious posttranslational modification, since LPd10 was expressed in the same abundance as LP and the size was that expected for the 10-amino-acid deletion. LPd34 was less abundant in cells and was partially posttranslationally modified into a more slowly migrating species. This slowly migrating species is of substantial interest, since the mutation may have stabilized an evanescent component of EBNA-LP posttranslational processing. However, transfection of cells with sufficient LPd34 expression vector so that the level of unmodified LPd34 determined by Western blotting was near that of LP did not result in substantial coactivation. The effect was specific for the 11 additional EBNA-LPd34 amino acids versus EBNA-LPd45, since the in-frame fusion of 14 additional codons which encode an unrelated sequence to 3′ end of the EBNA-LPd45 open reading frame in EBNA-LPd45 (EBNA-LPd45/14) did not prevent EBNA-LPd45/14 from giving as strong coactivation as EBNA-LP or EBNA-LPd45 (data not shown). These data are compatible with a model in which the first 11 carboxyl-terminal EBNA-LP amino acids have a strong negative regulatory effect on coactivation by the repeating domains, while the last 34 carboxyl-terminal amino acids modulate the strong negative effect of the 11-amino-acid domain. The last 10 amino acids of EBNA-LP are critical to the modulatory effect of the last 34 amino acids, since EBNA-LPd10 did not stimulate CAT expression.

The importance of the carboxyl-terminal domain in affecting EBNA-LP function was underscored by a series of experiments initially designed to construct a 4OHT-responsive EBNA-LP

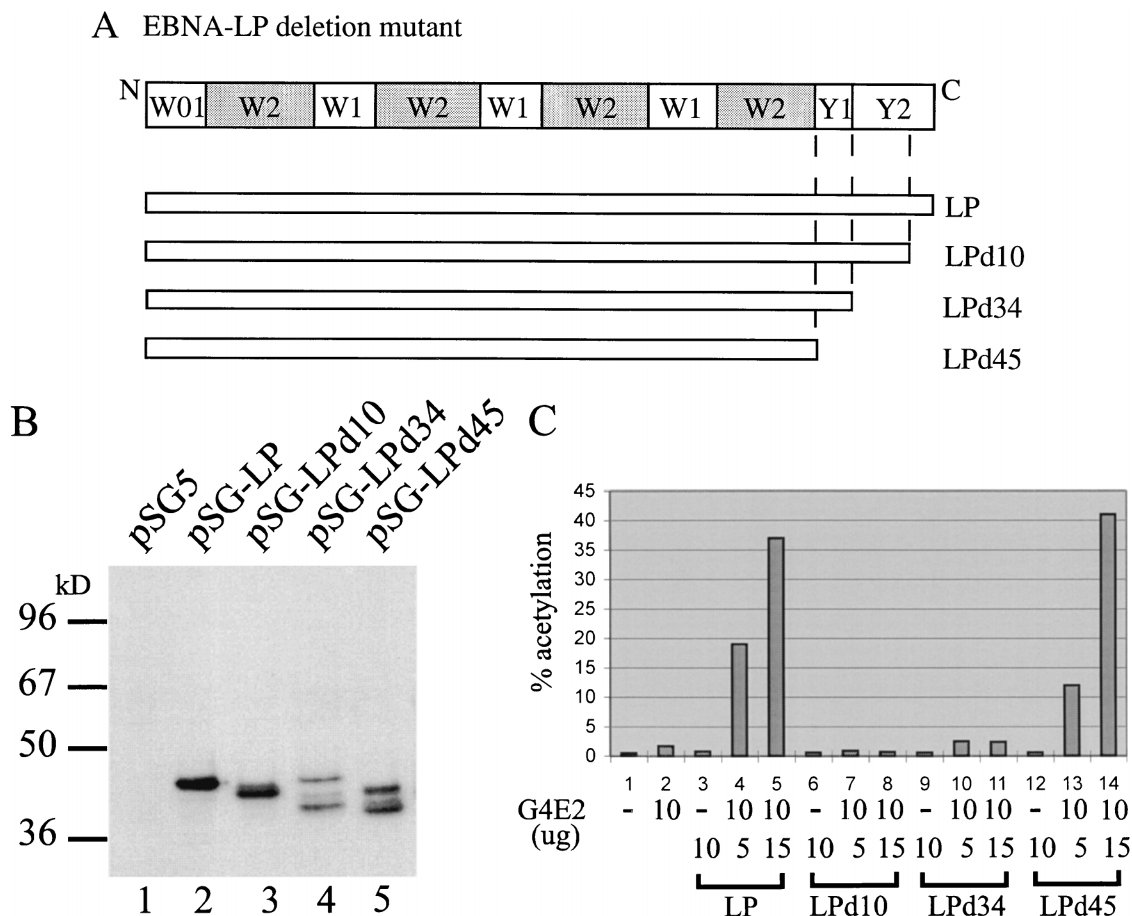

FIG. 5. The EBNA-LP repeat domain is sufficient for stimulating EBNA-2AcD-mediated activation, and the first 11 unique residues of the carboxyl terminus have a negative effect. (A) Map of EBNA-LP showing the exons derived from the EBV long internal repeat (W) and from the 3′ unique DNA (Y1 and Y2) and the deletion mutants. The W1 and W2 exons are 22 and 44 codons, respectively, and together encode 66-amino-acid repeats. The W01 splice adds an AT from the W0 exon and deletes the first 5 nucleotides from the first W1 exon, resulting in a methionine codon followed by the rest of the 20 W1 codons. Y1 is 11 codons and Y2 is 34 codons, followed by a nonsense codon. (B) Immunoblot of wild-type EBNA-LP (LP) and EBNA-LP with deletions of all 45 (LPd45), the last 34 (LPd34), or only the last 10 (LPd10) amino acids of the unique carboxyl terminus, expressed in BJAB cells and identified on the blot with the JF186 monoclonal antibody, which reacts with the repeat domain. BJAB cells were transfected with the pSG5 vector (5 μg) (lane 1), pSG-LP (5 μg) (lane 2), pSG-LPd10 (5 μg) (lane 3), pSG-LPd34 (15 μg) (lane 4), or pSG-LPd45 (5 μg) (lane 5). (C) Histogram of the effect of EBNA-LP or EBNA-LP deletion mutants on Gal4-EBNA-2AcD-mediated activation of pG4tkCAT. pG4tkCAT contains five Gal4 binding sites upstream of a tk promoter. BJAB cells were transfected with pG4tkCAT, with the control expression vector pSG-Gal4 (lanes 1, 3, 6, 9, and 12), which encodes the Gal4 DNA binding domain (amino acids 1 to 147), or with the activator plasmid pGal4-EBNA-2AcD (lanes 2, 4, 5, 7, 8, 10, 11, 13, and 14) and with pSG-LP (lanes 3, 4, and 5), pSG-LPd10 (lanes 6, 7, and 8), pSG-LPd34 (lanes 9, 10, and 11), or pSG-LPd45 (lanes 12, 13, and 14).

(Fig. 6) (32). The 4OHT-responsive estrogen receptor was fused to the carboxyl terminus of EBNA-LP (EBNA-LPHT). The EBNA-LPHT fusion protein was expressed at the appropriate levels in the presence or absence of 100 nM 4OHT, and the LMP1 promoter-driven CAT reporter expression was unaffected. However, the EBNA-LPHT fusion protein failed to stimulate EBNA-2 activation in the presence (or absence) of 4OHT. While EBNA-LP localized by immune microscopy to characteristic nuclear bodies as transfected cells reached saturation, EBNA-LPHT was diffuse in the nucleus at all times, in the presence (or absence) of 4OHT. Thus, EBNA-LP interfered with the function of the 4OHT-responsive estrogen receptor, and the 4OHT-responsive estrogen receptor altered EBNA-LP localization and blocked coactivation in the presence or absence of 4OHT.

DISCUSSION

In demonstrating that EBNA-LP stimulates transcriptional activation mediated by the EBNA-2AcD, these experiments have probably identified the central role of EBNA-LP in EBV-mediated primary B-lymphocyte growth transformation. EBNA-LP is now linked to EBNA-2 both in coexpression by the first EBV transcript in infected B lymphocytes and functionally by augmenting and regulating EBNA-2 activation of gene transcription in infected lymphocytes. This assessment is partially built on previous extensive genetic and biochemical analyses that correlate the domains of EBNA-2 which are essential for EBV-mediated primary B-lymphocyte growth transformation with the domains that mediate gene activation (6–8, 16, 18, 22, 27, 31, 47–49, 56, 57, 59). We have now shown that EBNA-LP augments EBNA-2 activation of the native LMP1 promoter and of the LMP1/LMP2B regulatory element. This element has a site which would bind RBPJκ and its associated EBNA-2 and a PU.1 site that can interact with EBNA-2 (22, 27, 44). Transactivation by EBNA-2 is dependent on both sites, suggesting that more than one EBNA-2 molecule may be involved (22). In fact, each of the EBNA-2 response elements that have been analyzed has more than one upstream RBPJκ site or depends on more than that site for EBNA-2 respon-

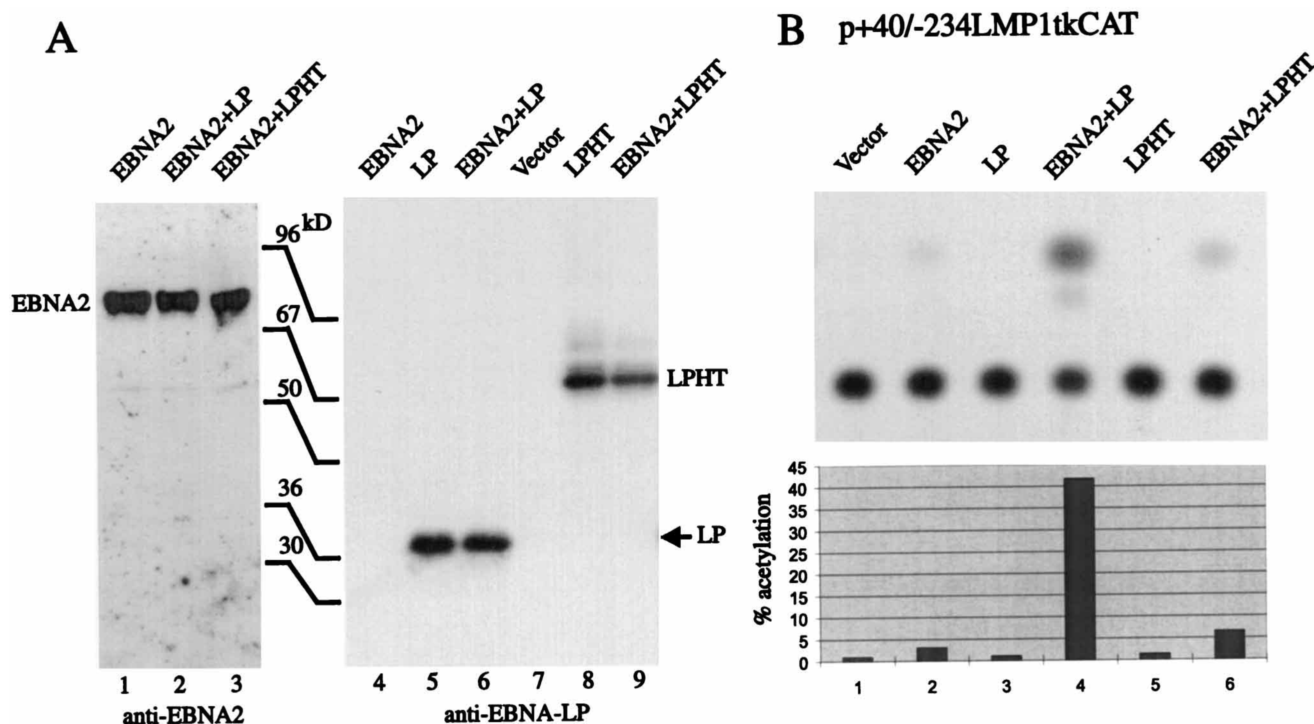

FIG. 6. EBNA-LP with a carboxyl-terminal 4-OHT-responsive estrogen receptor gene fusion (LPHT) has minimal effects on EBNA-2 activation of the LMP1 regulatory sequences upstream of the tk promoter. BJAB cells were transfected with the p+40/−234LMP1tkCAT reporter plasmid, with 10 μg of pSG-EBNA-2 or vector control, and with 10 μg of pSG-EBNA-LP (LP) or pSG-LPHT (LPHT). The total DNA in each sample was equalized with pSG5 vector DNA. (A) A whole-cell lysate of half of each sample was separated on a 10 or 8% polyacrylamide-SDS gel and transferred to a nitrocellulose filter, and proteins were detected by Western blotting with anti-EBNA-2 and anti-EBNA-LP monoclonal antibodies (PE2 and JF186, respectively). (B) CAT assay with the other half of each sample. Data are representative of three independent transfection experiments.

siveness, compatible with the notion that EBNA-2 transactivation requires more than a single molecule of EBNA-2 (16, 18, 21, 22, 27, 31, 59). Although EBNA-LP had no effect on the single Cp minimal response element in our experiments, the negative result may or may not be predictive of the role of EBNA-LP in augmenting EBNA-2 activation of the Cp in the context of the EBV genome in transformed B lymphocytes at various stages of infection and growth. Whether EBNA-LP will stimulate the full range of EBNA-2-responsive cellular promoters or whether the EBNA-LP effects will be proportional to the EBNA-2 effects or to the number of EBNA-2-interactive sites in the promoter regulatory element, as a strict interpretation of our data would suggest, is uncertain. Since the EBNA-LP effect requires only the presence of several EBNA-2AcDs near a promoter, some level of EBNA-LP stimulation could occur with any EBNA-2-responsive promoter.

Although these experiments have relied exclusively on reporter gene assays, the data indicate that the effect of EBNA-LP is at the level of transcription or initial RNA processing, since the effect requires only tethering of the EBNA-2AcD near a promoter. Minimal or no effects were observed with most cotransfected promoters that have upstream elements that lack the ability to directly or indirectly recruit the EBNA-2AcD, and for most promoters EBNA-LP alone had no activating effects or a negative effect. Even the related VP16AcD was at best two to three times as active in the presence of EBNA-LP as in its absence. Thus, EBNA-LP has substantial specificity for EBNA-2AcD-mediated activation. However, EBNA-LP is not completely specific for the EBNA-2AcD. EBNA-LP doubled or tripled activation by the VP16AcD, and in other experiments we have repeatedly observed 1.5- to 2-fold effects on the SV40 promoter in BJAB cells.

The partial specificity of EBNA-LP augmentation of EBNA-2AcD-mediated transcriptional activation versus VP16AcD-mediated activation correlates with the known similarities and differences between the two acidic domains. The EBNA-2AcD is similar to VP16AcD in up-regulating transcription when brought near promoters by linkage to sequence-specific DNA binding proteins, and part of the VP16AcD can substitute for part of the EBNA-2AcD in recombinant, transformation-competent EBV genomes (9). Further, the EBNA-2AcD is similar to the VP16AcD in interactions with TAF40, TFIIB, and TFIIH (47, 48). The EBNA-2AcD differs from the VP16AcD in having substantially less activity in most cell types, in interacting less with TBP (47), and in greater association with a novel coactivator, p100 (49). Much of the EBNA-2AcD in an EBV-transformed primary B lymphocyte is associated with p100 (49). p100 can interact with two components of TFIIE (49) and has a domain which is highly homologous to the negative regulatory domain of c-Myb (10).

In view of the greater effect of EBNA-LP on EBNA-2AcD-mediated transactivation, EBNA-LP may act by enhancing the interaction of the EBNA-2AcD with TBP or another basal or activation state transcription factor which then partially compensates for the relative weakness of the EBNA-2AcD in transcriptional activation. Given the relative specificity of p100 for the EBNA-2AcD, p100 is another candidate for EBNA-LP-mediated effects, perhaps affecting TFIIE, c-Myb (10), or another p100 interaction.

Our data map the critical EBNA-2 domain for EBNA-LP stimulation to the EBNA-2AcD and the critical EBNA-LP

domain to the 66-amino-acid repeats. The interaction between the EBNA-2AcD and the EBNA-LP repeat domain could be direct or highly indirect. EBNA-LP and EBNA-2 appear not to be part of the same complexes in extracts of EBV-transformed B lymphocytes (23). However, previous studies of the biochemical associations of EBNA-2 or EBNA-LP have used antibodies which interact with the EBNA-2AcD or with the EBNA-LP repeat domain and might therefore specifically select for mutually exclusive complexes (22).

The EBNA-LP effects demonstrated here are likely to be physiologically relevant to the role of EBNA-LP in EBV infection. The effects required levels of EBNA-LP expression which are similar to that in EBV-transformed B lymphocytes, as detected by immunoblot and immune fluorescence microscopy. The importance of precisely regulated expression from EBNA-2-responsive promoters is highlighted by the need for expression of LMP1 at an appropriate level, by the effect of cellular growth on LMP1 expression (24), by recent evidence that the three EBNA-3 proteins also participate in the regulation of promoters with RBPJκ sites (23, 28, 35, 39, 40), and by evidence that promoters with RBPJκ binding sites may be important in the effects of activated forms of Notch I in some human leukemias (4, 12, 14, 15, 36).

The findings that the 66-amino-acid repeats of EBNA-LP are sufficient for high-level stimulation of EBNA-2AcD-mediated transcriptional activation and that the carboxyl-terminal unique 45 residues modulate the activity of the repeat domain are of interest for at least two reasons. First, the significance of the repeat domain for primary B-lymphocyte growth transformation has not been evaluated as yet in EBV recombinant molecular genetic assays. EBV recombinants that express only the repeats (and not the carboxyl-terminal unique 45 residues) transform poorly, and transformed-cell outgrowth is inefficient (17, 33). The poor growth of cells transformed by recombinants which express only the repeats is likely to be related to the role of the last 45 amino acids in modulating transcriptional coactivation. If the sole role of the carboxyl-terminal sequence is in modulating coactivation by the repeat domain, as is indicated by the experiments reported here, a null mutation in EBNA-LP may be expected to have a more profound effect on transformation than previous mutations.

Second, EBNA-LP's effects on EBNA-2 may be regulated. EBNA-LP is extensively phosphorylated in EBV-transformed B lymphocytes, and the phosphorylated EBNA-LP is associated with the nuclear matrix (37). Phosphorylation and nuclear matrix association are likely to be inactivating steps in EBNA-LP processing. Consistent with this hypothesis is the recent finding that EBNA-LP is underphosphorylated in G_1/S-arrested cells and is hyperphosphorylated in G_2/M-arrested cells (26). Since EBV transforms G_0 cells and EBNA-2 and EBNA-LP must activate cell and virus gene expression in this context, phosphorylation during S, G_2, or M would be consistent with phosphorylation having an inactivating effect. Interestingly, the carboxyl-terminal 45 amino acids affect phosphorylation of the 44-amino-acid component of the 66-amino-acid EBNA-LP repeat by $p34^{cdc2}$ and by casein kinase II, in vitro (26). It is tempting to speculate that this may link EBNA-LP stimulation of EBNA-2-mediated activation to the cell cycle and that EBNA-LP may be a link between $p34^{cdc2}$ and proteins involved in transcription (5).

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ADDENDUM

In a paper submitted in parallel with this report, Nitsche et al. (35a) describe an augmenting effect of EBNA-LP on EBNA-2 activation of LMP1 and LMP1 mRNA expression after transfection of EBV-infected Burkitt's lymphoma cells that exhibit only EBNA-1-type latency prior to transfection. The EBNA-LP repeat domain is sufficient for this augmenting effect.

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