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Epstein-Barr Virus Nuclear Antigen 2 Transactivates the Long Terminal Repeat of Human Immunodeficiency Virus Type 1

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Human immunodeficiency virus type 1 (HIV-1)-infected subjects show a high incidence of Epstein-Barr virus (EBV) infection. This suggests that EBV may function as a cofactor that affects HIV-1 activation and may play a major role in the progression of AIDS. To test this hypothesis, we generated two EBV-negative human B-cell lines that stably express the EBNA2 gene of EBV. These EBNA2-positive cell lines were transiently transfected with plasmids that carry either the wild type or deletion mutants of the HIV-1 long terminal repeat (LTR) fused to the chloramphenicol acetyltransferase (CAT) gene. There was a consistently higher HIV-1 LTR activation in EBNA2-expressing cells than in control cells, which suggested that EBNA2 proteins could activate the HIV-1 promoter, possibly by inducing nuclear factors binding to HIV-1 *cis*-regulatory sequences. To test this possibility, we used CAT-based plasmids carrying deletions of the NF-κB (pNFA-CAT), Sp1 (pSpA-CAT), or TAR (pTAR-CAT) region of the HIV-1 LTR and retardation assays in which nuclear proteins from EBNA2-expressing cells were challenged with oligonucleotides encompassing the NF-κB or Sp1 region of the HIV-1 LTR. We found that both the NF-κB and the Sp1 sites of the HIV-1 LTR are necessary for EBNA2 transactivation and that increased expression resulted from the induction of NF-κB-like factors. Moreover, experiments with the TAR-deleted pTAR-CAT and with the *tat*-expressing pAR-TAT plasmids indicated that endogenous Tat-like proteins could participate in EBNA2-mediated activation of the HIV-1 LTR and that EBNA2 proteins can synergize with the viral *tat* transactivator. Transfection experiments with plasmids expressing the EBNA1, EBNA3, and EBNALP genes did not cause a significant HIV-1 LTR activation. Thus, it appears that among the latent EBV genes tested, EBNA2 was the only EBV gene active on the HIV-1 LTR. The transactivation function of EBNA2 was also observed in the HeLa epithelial cell line, which suggests that EBV and HIV-1 infection of non-B cells may result in HIV-1 promoter activation. Therefore, a specific gene product of EBV, EBNA2, can transactivate HIV-1 and possibly contribute to the clinical progression of AIDS.

Epstein-Barr virus (EBV) is a herpesvirus that preferentially infects B lymphocytes and induces immortalization, giving rise to long-term lymphoblastoid B-cell lines (LCL) (20). EBV is the causative agent of infectious mononucleosis, and it has been implicated in the development of endemic Burkitt lymphoma and in acquired immunodeficiency-associated non-Hodgkin lymphoma (NHL) (8, 9, 11). In fact, EBV cooperates with the deregulated expression of cellular genes in inducing a transformed phenotype in vitro (35). The EBV genome persists in infected cells either as an episome or as integrated viral DNA, with only a limited number of EBV genes being expressed in latently infected cells. These include genes coding for EBNA1, EBNA2, EBNA3, EBNALP, and latent membrane protein (LMP) (21). The blastic-like phenotype, together with the proliferation and immunoglobulin secretion induced by EBV, is similar to the phenotype induced by short-term antigen stimulation of normal B cells. This suggests that EBV may lead to B-cell immortalization by activating a set of host cellular genes transiently expressed during physiological B-cell activation. The EBNA2 gene product has been reported to activate the transcription of CD21, CD23, and c-*fgr* genes in human B cells (22, 38, 39). Moreover, EBNA2 protein expression could be crucial for EBV-mediated B-cell immortalization. In fact, the P3HR-1 strain of EBV, which carries a deletion of the *Bam*HI WYH region and does not therefore express EBNA2, is unable to immortalize B cells (2).

Patients with AIDS have a high incidence of EBV infection, with up to 96% showing serologic evidence of EBV (9). Moreover, a consistent percentage of AIDS-associated lymphomas harbor the EBV genome (11), and human immunodeficiency virus type 1 (HIV-1) can infect B cells as well as epithelial cells (3, 4, 6, 26, 31), which suggests that the two viruses can coexist in HIV-infected patients. Furthermore, HIV-1 and EBV infections have been reported in clones of EBV-infected B cells and in the cervical epithelium of patients with AIDS (3, 6, 26, 31). Thus, EBV may act as a cofactor that stimulates the expression of HIV-1, possibly through transactivation of the HIV-1 long terminal repeat (LTR), thus promoting a high level of HIV-1 replication and eventually reducing the clinical latency period of AIDS. To address this issue, we used several eukaryotic expression plasmids carrying EBNA1, EBNA2, EBNA3, and EBNALP coding sequences in cell transfection experiments to test whether the EBV genes modulate the expression of a reporter *cat* gene linked to the LTR sequences of HIV-1. By generating stable transfectants or by transient expression experiments, we show that the EBNA2 gene product of EBV transactivates the HIV-1 LTR sequence. Moreover, by using a set of HIV LTR chloramphenicol acetyltransferase (CAT)

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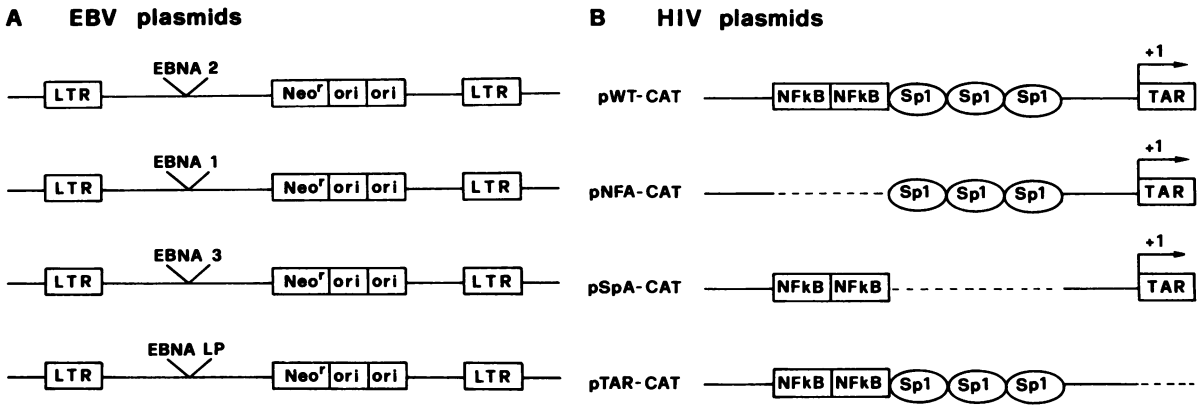

FIG. 1. Schematic representation of EBV eukaryotic expression plasmids (A) and HIV-1 LTR-CAT plasmids (B). The region of HIV-1 from nucleotide −108 to +80 is represented.

mutant plasmids and by DNA-protein-binding experiments, we also found that EBNA2-mediated transactivation requires intact κB sites of the HIV-1 LTR enhancer and that this transactivation is probably mediated by induction of NF-κB-like factors.

MATERIALS AND METHODS

Plasmids. The eukaryotic expression plasmids pZipSV neo(x) and pZipSVneo-EBNA1 (nucleotides 107830 to 110176), pZipSVneo-EBNA2 (nucleotides 95705 to 95398), pZipSVneo-EBNA3 (nucleotides 92705 to 95398), and pZipSVneo-EBNALP (cDNA clone T65) (37) were obtained from F. Wang and E. Kieff (Fig. 1A). The plasmids pILIC-CAT (hereafter referred to as pWT-CAT) carrying the wild-type HIV-1 LTR, pNFA-CAT carrying a deletion of the two NF-κB-binding sites, pSpA-CAT with a deletion of the three Sp1-binding sequences, pTAR-CAT which lacks the TAR region of HIV-1, and the *tat*-expressing pAR-TAT (12, 23) were kindly provided by A. Rabson (Fig. 1B).

Cells and transfection procedures. EBV-positive MC3 and CB33 cells were cultured as previously described (35). De Few and EW36 EBV-negative NHL cells were obtained from C. Gambacorti. Cells were cultured in Dulbecco modified Eagle medium (D-MEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Flow Laboratories, Milan, Italy), 3 mM glutamine, and 10 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2) (GIBCO Laboratories, Milan, Italy). Cells were transfected by electroporation essentially as previously described (35). Briefly, cells were washed and resuspended in 0.8 ml of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (D-PBS) at a concentration of 1.5×10^7/ml in the presence of 10 μg of intact pZipSVneo(x) or pZipSVneo-EBNA2 plasmids. Cells were subjected to a single electrical pulse (0.2 kV, 960 μF) with a Bio-Rad apparatus, recovered, and cultured for 48 h in D-MEM supplemented with 10% fetal calf serum before selection in 3 mg of G418 (Geneticin; approximate activity 50%; Sigma Chemical Co., Milan, Italy) per ml. Stable bulk cultures were obtained after 3 weeks and subjected to limiting dilution cultures (0.5 cells per well) to obtain subclones. Cells were routinely cultured in G418-supplemented medium. To analyze the integration of the transfected pZip SVneo plasmids, high-molecular-weight DNA was isolated, digested with *Sal*I or *Bam*HI enzymes, electrophoresed in 0.6% agarose gel, blotted on GeneScreen Plus filters (Du Pont, Milan, Italy), and analyzed with Southern blotting, using either a 1.4-kbp EBNA2 fragment isolated by *Bam*HI digestion of pZip-EBNA2 or a 1.1-kbp *Eco*RI *neo* fragment isolated from the pSV2-neo plasmid. Probes were ^{32}P-labeled to a specific activity of 0.8×10^9 to 1.0×10^9 cpm/μg by the random hexamer method. Hybridization was performed at 65°C in 1 M NaCl–10% dextran sulfate–1% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml. Washing was performed at 65°C as suggested by the manufacturer. Expression of EBNA2 proteins was examined by the immunoblot technique. Cell lysates (5×10^5 cells) were loaded over an 8% denaturing SDS-acrylamide gel and electrophoresed. Proteins were electrotransferred on cellulose membranes (Schleicher & Schuell, Inc., Dessel, Germany) and probed with the anti-EBNA2 PE2 monoclonal antibody (MAb) (43) that was purified over a protein A-Sepharose column (Pharmacia, Uppsala, Sweden). Detection was achieved by a peroxidase-conjugated sheep anti-mouse immunoglobulin G (Sigma) followed either by 3-3'-diaminobenzidine staining (Sigma) or by enhanced chemiluminescence (Pharmacia), according to the manufacturers' suggestions. Immunostaining was performed on cytocentrifuged smears by APAAP staining (Sigma) as suggested by the manufacturer.

For transient expression experiments, cells were transfected by electroporation at 10^7 cells in 0.8 ml of D-PBS as detailed above and cultured for 48 h in complete D-MEM. The amounts of transfected DNA were equalized either with the pZipSVneo(x) control plasmid or with pUC18 plasmid DNA. Moreover, in preliminary experiments, we found that cell transfections generated CAT activity that was linear in the range of 5 to 70 μg of pWT-CAT or pSV2-CAT per ml, after which it declined. Under these conditions, CAT activity in cellular extracts from duplicate transfections varied by less than 15%. Transfection efficiency was monitored by cotransfecting the cells with 5 μg of pnls-lacZ plasmid. β-galactosidase activity was assayed with 80 to 100 μg of protein cell extract as previously described (32). These experiments were performed in both pZipSVneo(x)- and pZipSVneo-EBNA2-transfected cells, and transfection efficiency was similar in the two cell systems (not shown). The transient expression experiments were performed at least four times with different plasmid preparations.

CAT assays. After 48 h of treatment, cells were harvested and washed once with PBS. Cell extracts were prepared by three cycles of freeze-thawing in 0.25 M Tris (pH 7.8), and CAT assays were performed as previously described (13). Proteins were measured in each cell extract with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.), and equal amounts of proteins were analyzed for each sample. Each assay contained 50 μg of cell extract, 20 μl of 4 mM acetyl coenzyme A (Boehringer GmbH, Mannheim, Germany), and 1 μl (0.5 μCi) of D-threo-[1.2-^{14}C]chloramphenicol (New England Nuclear, Boston, Mass.) in a final volume of 150 μl of 0.25 M Tris (pH 7.8). Reactions were incubated overnight at 37°C, extracted with ethyl acetate, dried, and spotted on Polygram Sil G silica gel plates (Macherey-Nagel, Duren, Germany). Plates were run in a thin-layer chromatography tank containing a 95:5 mixture of chloroform-methanol. After 16 h of autoradiography, the thin-layer chromatography plates were cut and samples were counted in a Beckman LS5000TD scintillation counter.

Electrophoretic mobility shift assays. Nuclear extracts and gel shift assays were performed as described elsewhere (7). After incubation, cells were harvested, washed once in cold PBS, and transferred to 1.7-ml microcentrifuge tubes for a second wash in cold PBS. The supernatant was removed, and the cell pellet was resuspended in lysing buffer (10 mM HEPES [pH 7.9], 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.2% (vol/vol) Nonidet P-40) for 5 min. Nuclei were collected by centrifugation (800 × *g*, 5 min), rinsed with Nonidet P-40-free lysing buffer, and resuspended in 150 μl of buffer containing 250 mM Tris-HCl (pH 7.8), 20% glycerol, 0.42 M NaCl, 60 mM KCl, 1 mM DTT, and 1 mM PMSF. Nuclei were then subjected to three cycles of freezing and thawing. The suspension was cleared by centrifugation (10,000 rpm, 15 min), and aliquots were immediately tested in gel-retardation assay or stored in liquid-phase N_2 until use. Oligonucleotide probes used included NF-κB 5′-ACAAGGGACTTTCCGCTGGGGACTTTCCAG-3′ and mutant NF-κB 5′-ACAACTCACTTTCCGCTGCTCACTT TCCAG-3′. The sequence of the Sp1 oligonucleotide encompassing the three Sp1 sites of the HIV-1 LTR was 5′-GGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGC-3′. Each oligonucleotide was annealed to its complementary strand and end labeled with [γ-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.) with polynucleotide kinase (New England Biolabs, Beverly, Mass.). Equal amounts of cell extracts were incubated in a reaction mixture consisting of 17 μl of buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 μg of poly(dI)-poly(dC) (Pharmacia), and 1 to 2 μl of extract (5 μg) for 5 min on ice. One microliter of γ-^{32}P-labeled double-stranded probe (0.2 ng, 40 × 10^3 to 60 × 10^3 cpm) was then added with or without a 100-fold excess of competitor wild-type or mutant oligonucleotide. The reactions were incubated at room temperature for 30 min and run on a 5% 30:1 acrylamide-bis-acrylamide gel in 0.5× 0.045 M Tris-borate–0.001 M EDTA. Gels were dried and autoradiographed.

RESULTS AND DISCUSSION

Expression of the HIV LTR-CAT plasmid in different B-cell lines was examined by transiently transfecting the pWT-CAT plasmid (Fig. 1B) into EW36 or De Few EBV-negative NHL cells and into EBV-positive MC3 or CB33 LCL. In these experiments, expression of the HIV-1 LTR in MC3 or CB33 cells was consistently higher than that observed in EBV-negative B-cell lines (Table 1), suggesting that latent EBV infection may result in a cellular environment that is efficient for HIV-1 expression, possibly mediated by the expression of latent EBV genes. Among these genes, EBNA2 induces the expression of CD23 and CD21 B-cell surface proteins (38, 39) and transcriptionally activates the LMP and CD23 genes by modulating 5′ upstream regulatory regions (40, 41).

TABLE 1. Constitutive expression of pWT-CAT in different B-cell lines[a]

Cell line	% Acetylation of [^{14}C]chloramphenicol[b] in:	
	EBV$^-$ NHL	EBV$^+$ LCL
EW36	0.50	
De Few	0.25	
MC3		37.50
CB33		42.00

[a] Cells were transfected with 10 μg of pWT-CAT plasmid by electroporation as detailed in Materials and Methods. CAT activities were assayed on cell extracts at 48 h posttransfection.

[b] Determined by scintillation counting of unacetylated and acetylated forms resolved by thin-layer chromatography.

To ascertain whether EBNA2 might induce a cell phenotype prone to HIV-1 expression, we transfected EW36 and De Few, two EBV-negative B-cell lines, with the pZipSV neo-EBNA2 plasmid (Fig. 1A) to obtain a constitutive synthesis of EBNA2 proteins. After electroporation with pZip SVneo-EBNA2 or pZipSVneo(x), stable transfectants of EW36 and De Few pZipSVneo-EBNA2 (hereafter referred to as EW36-EBNA2 and De Few-EBNA2) cells or EW36-pZipSVneo(x) and De Few-pZipSVneo(x) control cells were obtained after selection with G418 (3 mg/ml). Southern analysis of high-molecular-weight DNA of transfected cells with an EBNA2 or a *neo* probe revealed a random genomic integration of the transfected plasmids with an average of one copy per cell genome (data not shown). The protein product of the EBNA2 gene in the transfected cells was identified in immunoblots with the anti-EBNA2 PE2 MAb (43). As shown in Fig. 2a, the EW36-EBNA2 and De Few-EBNA2 cells showed a consistent level of EBNA2 protein. The correct subcellular localization of the expressed EBNA2 protein was tested by immunocytochemical analysis of the transfected cells with the PE2 MAb. Figure 2b, c, and d shows that the EBNA2 protein localized into the nuclei of the transfected cells in a pattern very similar to that obtained with MC3 LCL. These data indicated that the transfected EBNA2 gene efficiently expressed EBNA2 proteins of the expected molecular weight and with the correct nuclear localization and thus it was possibly able to function as a transactivator gene.

We next transiently transfected EW36-EBNA2, De Few-EBNA2, and the relative pZipSVneo(x) control cells with the pWT-CAT plasmid, which carries the wild-type HIV-1 LTR sequences linked to the bacterial *cat* gene. The level of HIV-1 LTR expression was assayed after 48 h by evaluating the CAT activity in cellular extracts. As shown in Fig. 3A, both the EBNA2-transfected EW36 and De Few cells efficiently expressed the transfected HIV-1 LTR-CAT plasmid to a greater extent than the pZipSVneo(x) control cells. This finding indicated that the EBNA2 protein could act as a transactivator of the HIV-1 promoter.

We then examined whether EBNA2 acts together with the viral *tat* transactivator (5). EW36-EBNA2 and De Few-

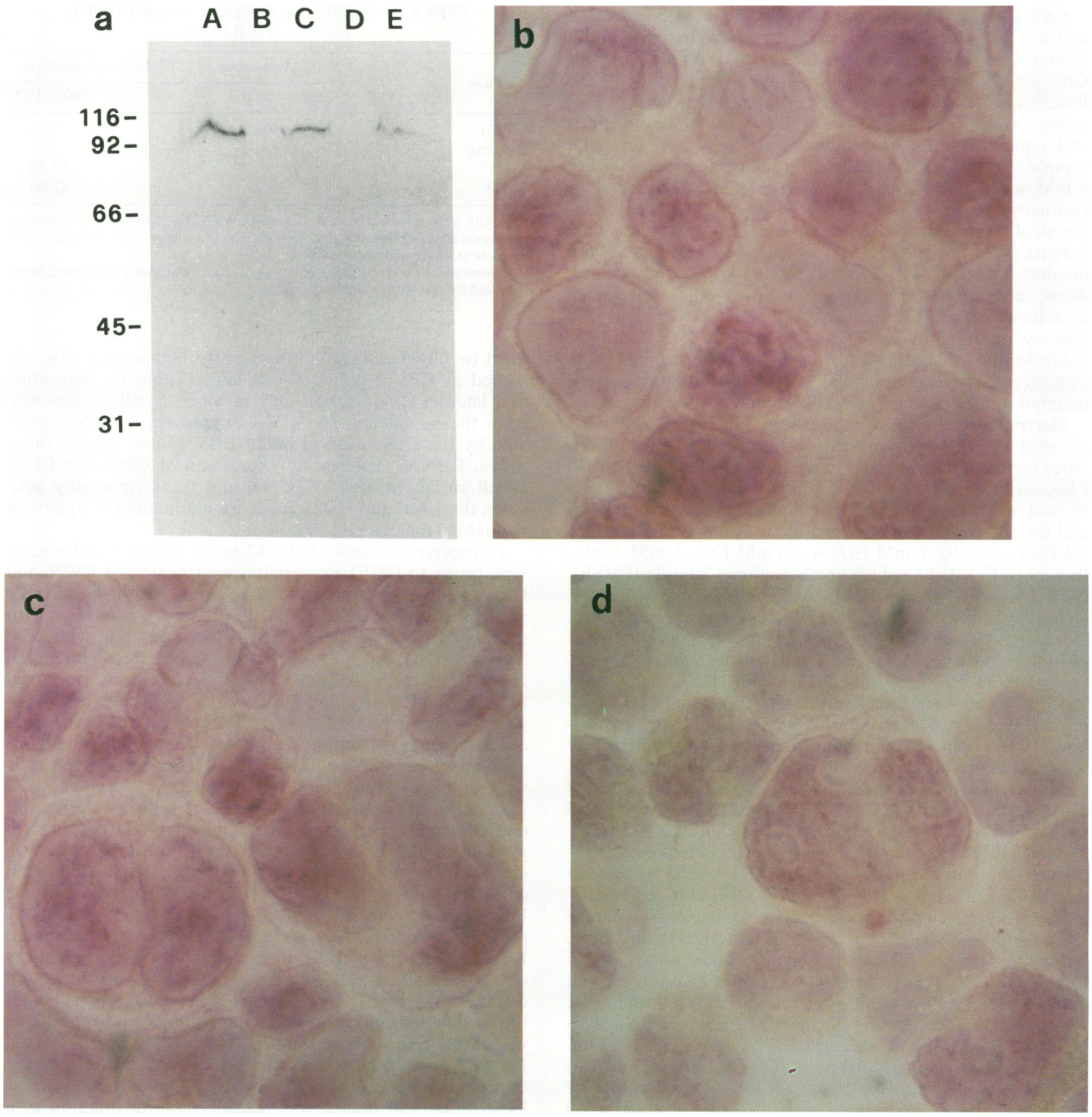

FIG. 2. Expression of EBNA2 proteins in transfected De Few and EW36 cells. (a) Immunoblots of De Few and EW36 cells stably transfected with pZipSVneo-EBNA2 or control pZipSVneo(x) plasmids. Cells (5×10^5) were lysed by being boiled in SDS-gel sample buffer. Proteins were separated on an 8% denaturing acrylamide gel, transferred onto nitrocellulose filters, probed with PE2 MAb (43), and then stained with peroxidase. Lanes: A, EBV-transformed MC3 cells; B, De Few-pZipSVneo(x) control cells; C, De Few-EBNA2 cells; D, EW36-pZipSVneo(x) cells; E: EW36-EBNA2 cells. (b, c, and d) Immunostaining of transfected De Few and EW36 cells with the anti-EBNA2 PE2 MAb. Cells (10^6) were cytocentrifuged and stained with the APAAP method. (b) EBV-positive MC3 cells; (c) De Few-EBNA2 cells; (d) EW36-EBNA2 cells. Magnification, ×1,060.

EBNA2 cells were transiently cotransfected with pWT-CAT and with the *tat*-expressing pAR-TAT vector. These experiments showed that EBNA2 actually synergizes with the HIV-1 *tat* gene, causing a more than additive increase in HIV-1 LTR-driven gene expression (Fig. 3B). Similar results were obtained when cell clones isolated from the transfected pZipSVneo(x) or pZipSVneo-EBNA2 bulk cultures were examined. For these experiments, De Few-EBNA2 and EW36-EBNA2 cells, and the relative control cells, were subjected to limiting dilution cultures (0.5 cell per well) to

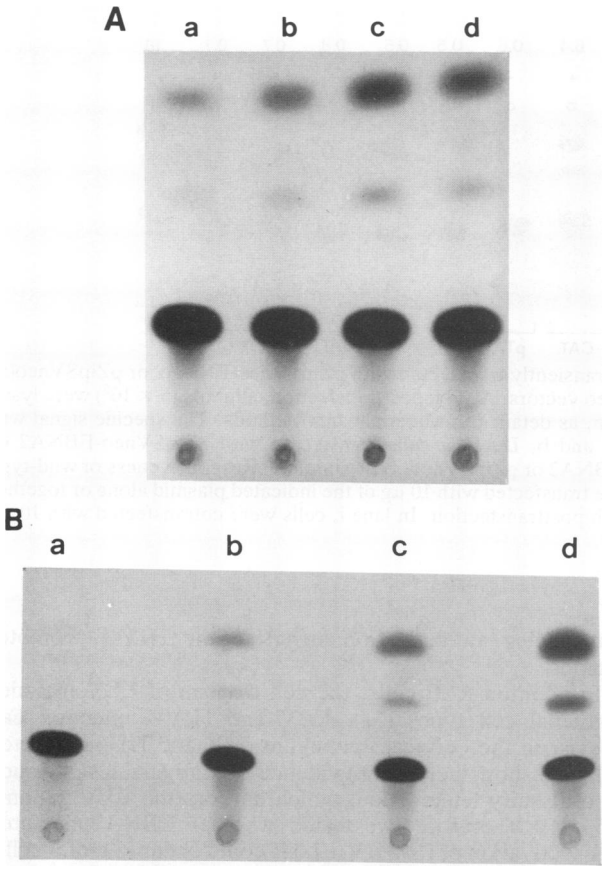

FIG. 3. (A) Constitutive expression of HIV-1 LTR in transfected De Few or EW36 cells. Cells were transfected by electroporation with 10 μg of pWT-CAT plasmid. CAT activity was assayed in cell lysates (50 μg) at 48 h posttransfection. Percent acetylation, shown in parenthesis, was calculated by directly counting the acetylated spots, and values are representative of several independent experiments with different plasmid preparations. Lanes: a and b, De Few-pZipSVneo(x) (0.2%) and EW36-pZipSVneo(x) (0.4%) control cells; c and d, De Few-EBNA2 (5.8%) and EW36-EBNA2 (4.9%) cells. (B) Synergistic activation of the HIV-1 LTR by EBNA2 and the *tat* gene product. Percent specific acetylation is reported in parenthesis. De Few cells were transiently transfected with 10 μg of pWT-CAT alone (lane a, 0.15%), 10 μg of pZipSVneo-EBNA2 (lane b, 1.8%), 5 μg of pAR-TAT (lane c, 3.1%), or 10 μg of pZipSVneo-EBNA2–5 μg of pAR-TAT (lane d, 12.5%).

isolate subclones. At least 30 subclones of EBNA2-expressing or De Few and EW36 control cells were individually examined for constitutive expression of the transiently transfected pWT-CAT plasmid. There were no significant differences in activation of the HIV-1 LTR among individual clones, and the levels of CAT expression were consistently higher in EBNA2-transfected cell clones than in the pZipSV-neo(x) control clones (not shown). These experiments indicated that stable expression of the EBNA2 gene in two EBV-negative B-cell lines resulted in a higher constitutive activation of the HIV-1 LTR promoter-enhancer regions, thus mimicking the high activation of the HIV-1 LTR observed in a variety of EBV-infected B cells isolated either by in vitro infection or from naturally EBV-infected patients with AIDS (data not shown).

The regulation of HIV-1 gene expression requires a complex interaction of cellular factors binding to transcriptionally regulatory sequences in the HIV-1 LTR. This includes a TATA box, three Sp1-binding sites, and a core enhancer region containing two κB sites (10, 18). In addition, a virus-encoded transactivator, *tat*, is required to bind a responsive region, TAR, located at +1 to +44 bp relative to the cap site (36). To identify the *cis*-regulatory sequences of the HIV-1 LTR target for EBNA2 activation, we performed cell transfection experiments in which the wild-type or mutant HIV-1 LTR plasmids carrying deletions of the κB enhancer region, Sp1 sites, or the TAR region were transiently cotransfected with the pZipSVneo(x) or pZipSVneo-EBNA2 vector in De Few or EW36 cells. After 48 h, cell aliquots from each transfection were analyzed for expression of EBNA2 protein by immunoblots (Fig. 4A) and cell extracts were tested for CAT activity. As shown in Fig. 4B, EBNA2-induced HIV-1 LTR activation was drastically diminished when plasmid pNFA-CAT, with the κB sites of the HIV-1 LTR deleted, or pTAR-CAT, with the TAR region deleted, were used. In the same experiments, deletions of the Sp1 sites also significantly affected transcriptional activation of the HIV-1 LTR by EBNA2. Similar results were obtained when EW36-EBNA2 and De Few-EBNA2 cells were transiently transfected with pWT-CAT and the relative deletion mutants (not shown). The lack of EBNA2-mediated transactivation of κB- or Sp1-deleted regions in pNFA-CAT and pSpA-CAT plasmids was not due to an intrinsic inability to function as promoter-enhancer. In fact, these plasmids were transactivated by the viral Tat protein, albeit with a lower efficiency (Table 2) than previously reported (23).

The results shown in Fig. 4B suggested that EBNA2 may activate the HIV-1 LTR by inducing NF-κB-like or Sp1-like binding factors. To address this issue, nuclear extracts from De Few-EBNA2 or EW36-EBNA2, and from De Few-pZipSVneo(x) or EW36-pZipSVneo(x) control cells were tested for the presence of specific NF-κB- or Sp1-binding activity in an electrophoretic mobility shift assay. These experiments showed that expression of the EBNA2 gene in stable transfectants resulted in an increase in the κB-binding factors (Fig. 5A). In the same experiments, no significant differences in Sp1-binding activity were observed between EBNA2-expressing cells and control cells (Fig. 5B). Similar results were obtained when subclones of EBNA2-expressing cells were tested and when De Few or EW36 cells were transiently transfected with pZipSVneo-EBNA2 or pZipSV neo(x) plasmid (data not shown). These findings indicate that EBNA2 may function by inducing active NF-κB-like transcription factors. These nuclear proteins share DNA-binding specificity with other cellular proteins, such as NF-κB and HIVEN 86A (10, 27), which bind to κB consensus sequences present in a set of cellular genes which includes the α subunit of the interleukin-2 (IL-2) receptor and IL-6 (10, 24, 27). We do not yet know whether the κB-binding factors induced by EBNA2 are identical to NF-κB or HIVEN 86A factors or whether they are members of a closely related family of DNA-binding cell factors. Deletion of the three Sp1 sites in the pSpA-CAT plasmid prevents the response to EBNA2 and is consistent with the finding that Sp1-binding *cis*-regulatory sequences are essential for activation of the HIV-1 LTR (18). In fact, deletion or inactivation by point mutations of the Sp1 site results in a marked reduction of HIV-1 LTR promoter activity and inducibility and greatly limits HIV-1 replication (29). The nonresponsiveness of the pTAR-CAT plasmid to EBNA2 (Fig. 4B) suggests that the TAR region of HIV-1 is necessary for EBNA2-mediated transactivation. This raises the possibility that EBNA2 may

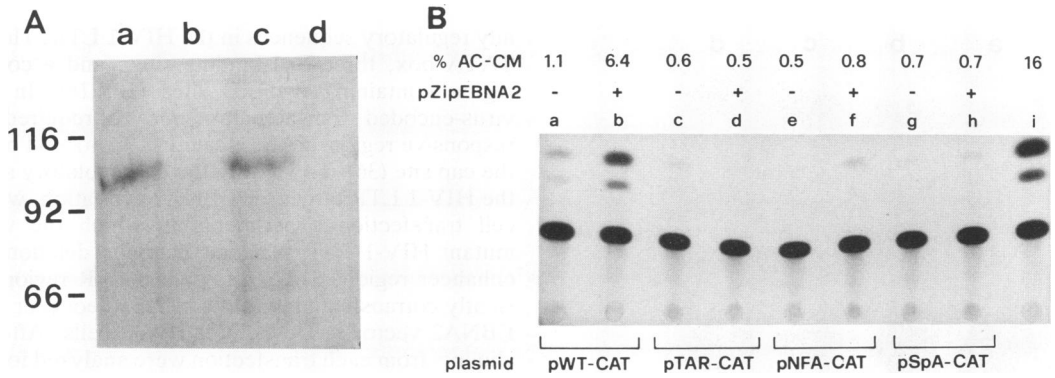

FIG. 4. (A) Expression of EBNA2 proteins in De Few and EW36 cells transiently transfected with pZipSVneo-EBNA2 or pZipSVneo(x) plasmid. Cells (5×10^6) were transiently transfected with 30 μg of pZipSVneo vectors. At 48 h posttransfection, aliquots (5×10^5) were lysed and cell proteins were tested for reactivity with the anti-EBNA2 PE2 MAb, as detailed in Materials and Methods. The specific signal was achieved by enhanced chemiluminescence in a 30-s exposure. Lanes: a and b, De Few cells transfected with pZipSVneo-EBNA2 or pZipSVneo(x) plasmid; c and d, EW36 cells transfected with pZipSVneo-EBNA2 or pZipSVneo(x) plasmid. (B) Responsiveness of wild-type or mutant HIV-1 LTR-CAT plasmids to the EBNA2 gene. De Few cells were transfected with 10 μg of the indicated plasmid alone or together with 30 μg of pZipSVneo-EBNA2 vector. CAT activity was assayed at 48 h posttransfection. In lane i, cells were cotransfected with 10 μg of pWT-CAT–5 μg of pAR-TAT.

function either by directly binding the TAR region or, more likely, by inducing endogenous TAR-binding proteins.

In addition to EBNA2, EBV expresses a set of genes, including EBNA1, EBNA3, and EBNALP, during the latent phase of chronic infection (21). To test whether expression of these EBV genes might result in activation of the HIV-1 LTR sequences, we cotransfected De Few cells with the pWT-CAT plasmid and with the pZipSVneo-EBNA1, pZip SVneo-EBNA3, pZipSVneo-EBNALP, or pZipSVneo-EBNA2 plasmid. The CAT activity in cell extracts after 48 h was considered a measure of possible activation of the HIV-1 LTR. As reported in Table 3, no activation of the HIV-1 LTR-CAT construct was observed as a consequence of EBNA1, EBNA3, or EBNALP gene transfection, whereas EBNA2 gene transfection resulted in increased activation of the HIV-1 LTR. Therefore, among the latent EBV genes tested, EBNA2 was the only one functional in transducing an activation signal to the HIV-1 promoter regions.

TABLE 2. Effects of *tat* expression on the activity of wild-type and mutant HIV-1 LTR-CAT plasmids[a]

Plasmid	Cell line			
	De Few		EW36	
	% Acetylation[b]	% Fold induction[c]	% Acetylation[b]	% Fold induction[c]
pZipSVneo(x)	0.40		0.25	
pZipSVneo(x) + pAR-TAT	0.30	0.75	0.20	1.25
pWT-CAT	0.50		0.40	
pWT-CAT + pAR-TAT	30.35	60.50	19.48	48.70
pNFA-CAT	0.20		0.18	
pNFA-CAT + pAR-TAT	3.12	15.60	3.40	18.90
pSpA-CAT	0.50		0.45	
pSpA-CAT + pAR-TAT	2.80	5.60	3.28	7.30
pTAR-CAT	0.45		0.30	
pTAR-CAT + pAR-TAT	0.38	0.85	0.27	0.90

[a] Cells were transfected with 10 μg of the indicated plasmids alone or with 5 μg of pAR-TAT plasmid.
[b] Determined by scintillation counting of unacetylated and acetylated spots.
[c] Fold induction of chloramphenicol acetylation induced by pAR-TAT cotransfection is expressed as the ratio of percentages acetylated in the presence or absence of pAR-TAT.

In addition to B cells, the cell tropism of EBV includes epithelial cell types (31). EBV and HIV-1 infection can coexist in the cervical epithelium (31), and HIV-1-infected subjects show increasing evidence of epithelial lesions such as oral hairy leukoplakia, which harbors the EBV genome (15, 44). Therefore, we tested whether EBNA2-mediated transactivation of the HIV-1 LTR could occur in HeLa cells, a cervical epithelial cell line. In these experiments, HeLa cells were transiently cotransfected with pWT-CAT in the presence of the pZipSVneo(x) or pZipSVneo-EBNA2 plasmid and the CAT levels were determined in cell lysates 48 h after transfection. As shown in Fig. 6, EBNA2 expression resulted in a significant transactivation of the HIV-1 LTR sequences, suggesting that the transactivating function of EBNA2 can be exerted in a non-B-cell phenotype. This may be of interest in the light of recent evidence that EBV can infect and activate lymphoid T cells (42).

Evidence is accumulating that EBV infection may play a pathogenic role in the development of AIDS-associated B-cell neoplasms and oral hairy leukoplakia lesions (11, 44). The cell tropism of HIV-1 and EBV is only partially known. Recent data suggest that HIV-1 can infect epithelial cells as well as EBV-transformed lymphoblastoid B cells (3, 4, 6, 26, 31), whereas EBV can infect T cells and contribute to the development of T-cell malignancies in severe immunocompromised patients (17, 42). This finding strongly suggests that EBV can coexist with HIV-1 at the cellular level and that it could modulate the clinical latency of HIV-1 infection, possibly by activating its cellular expression through induction of transactivating factors that interact with HIV-1 *cis*-regulatory sequences. To test this possibility, we examined the capacity of the EBNA1, EBNA2, EBNA3, and EBNALP latent genes of EBV to transactivate HIV-1 LTR regulatory sequences. Among the genes tested, EBNA2 was able to transactivate HIV-1 LTR sequences both in B cells (De Few and EW36) and in HeLa epithelial cells.

The molecular mechanisms of EBNA2 activation of HIV-1 expression are unknown. An EBNA2 gene product is a major transactivating factor of EBV. The EBNA2 proteins have a nuclear localization and induce the expression of

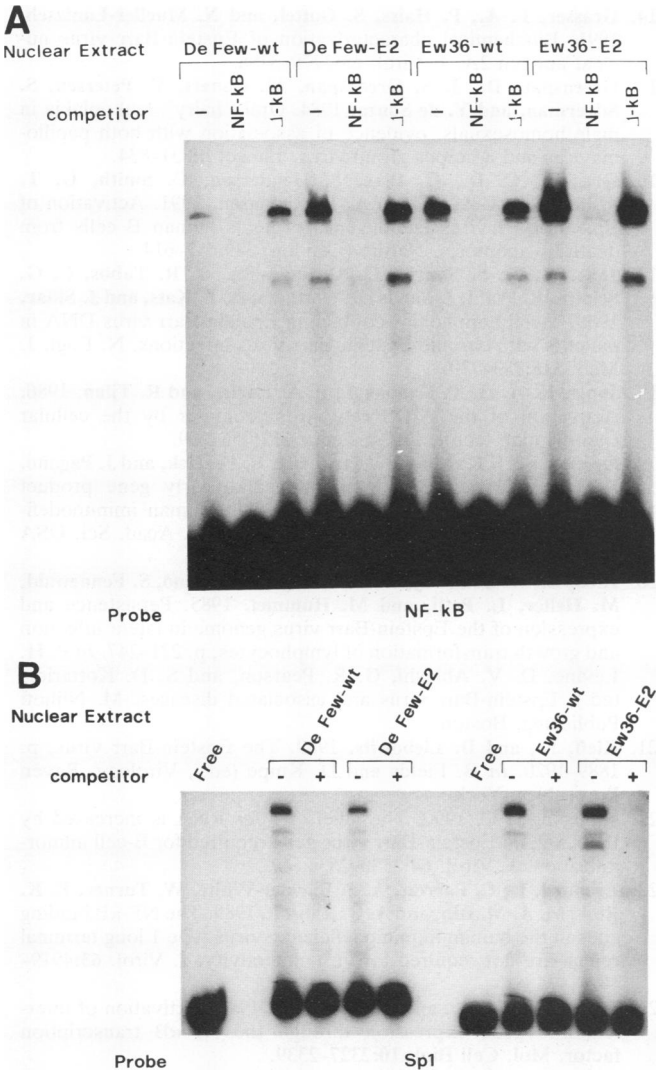

FIG. 5. Induction of nuclear factors binding to κB-like sequences of the HIV-1 LTR. Nuclear extracts were prepared by the method of Dignam et al. (7) and probed with ^{32}P-labeled double-stranded synthetic oligonucleotide corresponding to HIV-1 LTR NF-κB sites (A) or Sp1-binding sites (B). Competition experiments were performed by the addition of 100-fold molar concentrations of unlabeled wild-type or mutated oligonucleotide. pZipSVneo(x)- and pZipSV neo-EBNA2-transfected cells are referred to as wt and E2, respectively.

CD21, CD23, and c-*fgr* cellular proteins (22, 38, 39). In contrast to the immediate-early genes, such as BZLF-1 whose product activates HIV-1 expression (25), EBNA2 is constitutively expressed at high levels in circulating B cells that are latently infected with EBV and may act through the induction of host cellular genes. Indeed, EBNA2 expression is required for the immortalization of B cells, which results in continuous proliferation of lymphoblastoid-like cells (20). These EBV LCL produce cytokines such as IL-1 and IL-6 (33, 34, 37), which in turn could activate HIV-1 expression (28, 30). The ultimate mechanisms whereby EBNA2 exerts its effects on target DNA sequences are obscure. EBNA2 proteins do not bind directly to DNA sequences; however, they can associate in different forms of oligomerization and

TABLE 3. Transactivation of the HIV-1 LTR by latent EBV genes

Transactivating plasmid[a]	CAT activity[b]	
	% Acetylation	% Fold induction[c]
pZipSVneo(x)	0.36	
pZipSVneo-EBNA1	0.30	0.8
pZipSVneo-EBNA2	5.80	16.1
pZipSVneo-EBNA3	0.32	0.8
pZipSVneo-EBNALP	0.48	1.3

[a] EBV-negative De Few cells were transfected by electroporation with 30 μg of the indicated transactivating plasmid and with 10 μg of pWT-CAT plasmid.
[b] Determined at 48 h posttransfection by using 50 μg of cell extract.
[c] Expressed as the ratio of percentages acetylated in the presence or absence of pZipSVneo-EBNA plasmids.

possibly may interact with host cellular proteins (14). We found that the functional deletion of κB sites abolished EBNA2 transactivation, suggesting that EBNA2 may activate the HIV-1 promoter by inducing active NF-κB factors. This possibility was confirmed by in vitro binding of HIV-1 κB sequences to nuclear extracts of EBNA2-transfected cells. Because the nuclear translocation of active NF-κB complexes requires phosphorylation of the inhibitory protein IκB (1), our results suggest that EBNA2 may function by inducing posttranslational modifications of the host cellular protein, possibly by activating endogenous kinases. This hypothesis is now under investigation. Moreover, results with the *tat*-expressing plasmid and with the TAR-deleted HIV-1 CAT plasmid raise the possibility that EBNA2 can cooperate with cellular proteins to induce full complete transcription of HIV-1 genes. The level of EBNA2-induced transactivation of the HIV-1 LTR is probably underevaluated with respect to naturally occurring EBV infection. In fact, the EBNA2 gene product induces expression of the LMP gene of EBV and cooperates with LMP proteins in promoting complete induction of host cellular genes such as CD23 (39) and in protecting B cells from apoptosis (16). Indeed, we have recently obtained a synergistic higher HIV-1 LTR-driven CAT activity by transfecting De Few-EBNA2 or EW36-EBNA2 cells with the LMP-expressing pgpt-LMP plasmid (32a).

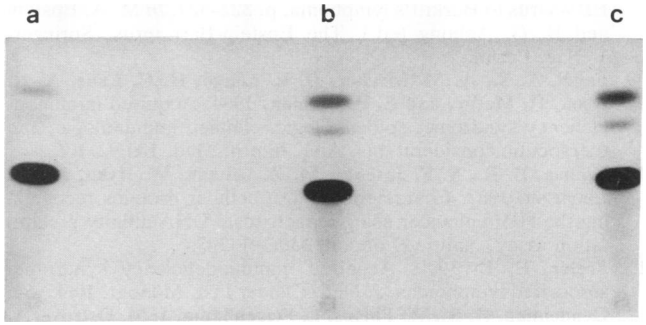

FIG. 6. Transactivation of HIV-1 LTR promoter sequences by EBNA2 in epithelial HeLa cells. CAT activities, shown in parentheses, were assayed in cell lysates at 48 h posttransfection. HeLa cells were transfected with 10 μg of pWT-CAT alone (lane a, 0.3%) or with 15 μg (lane b, 2.3%) and 30 μg (lane c, 6.4%) of pZipSVneo-EBNA2. The data are representative of five independent experiments.

Taken together, our data are consistent with previous reports on EBV-HIV-1 interactions (19, 25) and indicate that EBV infection may play a major role in the progression of AIDS.

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