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Epstein-Barr Virus Nuclear Antigen 3C Is a Powerful Repressor of Transcription when Tethered to DNA

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The expression of Epstein-Barr virus (EBV) nuclear antigen 3C (EBNA3C) is essential for the activation and immortalization of human B lymphocytes by EBV. EBNA3C consists of 992 amino acids and includes a potential bZIP motif and regions rich in acidic, proline, and glutamine residues. Thus, EBNA3C resembles several *trans* regulators of gene expression. It has recently been shown that a fragment of EBNA3C can activate reporter gene expression when fused to the DNA-binding domain of GAL4 (D. Marshall and C. Sample, J. Virol. 69:3624–3630, 1995). Although EBNA3C binds DNA, a specific site for EBNA3C binding has not been identified; to test the ability of full-length EBNA3C to regulate transcription, EBNA3C (amino acids 11 to 992) was fused to the DNA-binding domain of GAL4. We show that this fusion protein does not transactivate but rather is a potent repressor of reporter gene expression. Repression is dependent on the dose of GAL4-EBNA3C and on the presence of GAL4-binding sites within reporter plasmids. Repression is not restricted to B cells nor is it species or promoter specific. Repression is independent of the location of the GAL4-binding sites relative to the transcription start site. A fragment of EBNA3C (amino acids 280 to 525) which represses expression in a manner which is nearly identical to that of the full-length protein has been identified; this fragment is rich in acidic and proline residues. A second, less potent repressor region located C terminal to amino acids 280 to 525 has also been identified; this domain is rich in proline and glutamine residues. We also show binding of EBNA3C, in vitro, to the TATA-binding protein component of TFIID, and this suggests a mechanism by which EBNA3C may communicate with the basal transcription complex.

Epstein-Barr virus (EBV) infects and immortalizes B lymphocytes with exceptional efficiency (reviewed in reference 28). Although EBV has the capacity to code for more than 80 genes, only 11 of these are expressed during latent infection. Of these, only 6 have been shown to be essential for the immortalization of B cells: EBV nuclear antigens (EBNAs) 1, 2, 3A, 3C, and LP and the latent membrane protein 1 (LMP1) (reviewed in reference 28). Although little is understood about the exact function of these genes, it is known that EBNA1 is involved in the maintenance of the viral genome and that LMP1 is associated with cell activation and DNA synthesis in primary B cells. EBNA2 is a potent transactivator of both cellular and viral genes and is targeted to EBNA2-responsive promoters through an interaction with the cellular protein CBF1/RBP Jκ (13, 17, 31).

The focus of this study is EBNA3C, one of the EBNA3 family of proteins (EBNAs 3A, 3B, and 3C). EBNA3C is a large protein (992 amino acids [aa]) and has been shown to be essential for the immortalization of B lymphocytes (45). Although the precise function of EBNA3C is unknown, recent data have shown that an important role may be that of acting as an antagonist of EBNA2 function (23, 34, 39). This appears to be done by interfering with the EBNA2-CBF1 interaction (34, 39).

EBNA3C also has a probable role as a transcription factor (2, 37). It has been shown that EBNA3C can exert an effect on both cellular and viral genes. For example, single-gene transfer experiments have shown that the transfection of EBNA3C into an EBV-negative cell line results in an increase in CD21 expression (49). Not only is CD21 the EBV receptor but it is also a component of the cellular signal transduction pathway and is involved in cell cycle progression of B lymphocytes (5). We have also shown that the introduction of EBNA3C into the EBV-positive (but EBNA3C-negative) cell line Raji results in an increase of the viral LMP1 protein specifically in G_1 of the cell cycle (3).

These observations suggest that EBNA3C is a transcription factor, and indeed, an analysis of the EBNA3C amino acid sequence reveals features which resemble a DNA-binding domain (DBD), bZIP, and domains rich in acidic, proline, and glutamine residues which are candidates for regions that modulate transcription. In support of these observations, it has recently been shown that a proline-glutamine-rich fragment of EBNA3C (aa 724 to 826), when expressed as a fusion with the DBD of the yeast transactivator GAL4, will activate reporter gene expression in a variety of cell lines (34). These studies, however, have focused on the ability of a small fragment of EBNA3C to function as a transcriptional modulator. Therefore, we have investigated the ability of near-full-length EBNA3C to regulate transcription. Although EBNA3C encodes a potential bZIP motif and binds DNA-cellulose (20, 41), a specific EBNA3C-binding DNA sequence has not been demonstrated. Indeed, it is unclear whether EBNA3C binds DNA directly or does so through an interaction with a cellular or viral partner (41). Therefore, we have constructed a vector encoding an almost-full-length EBNA3C (aa 11 to 992) fused to the GAL4 DBD. We have found that this fusion molecule is a potent repressor of reporter gene expression when bound to DNA. This repression is neither cell type, species, nor promoter specific and is distance and position independent with respect to the location of the GAL4-binding sites. The repression maps mainly to aa 280 to 525, and this is a region rich in both proline and charged (acidic) residues. However, we have also identi-

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fied a second, less potent repressor region (aa 580 to 992), and this domain contains numerous proline and glutamine residues.

These data suggest that, in all respects, EBNA3C functions as a conventional transcriptional repressor and, taken with our demonstration that, in vitro, EBNA3C can bind to the TATA-binding protein (TBP) component of the basal transcription complex, provide a novel mechanism by which EBNA3C may function in the dual processes of cell immortalization and viral latency.

MATERIALS AND METHODS

Plasmids. The reporter plasmid pUASCAT contains five copies of the GAL4-binding site (UAS) upstream of the thymidine kinase promoter and has been described previously (50). The plasmid pBLCAT2 (32) is the parental plasmid of pUASCAT and differs only by lacking the UAS sites. The plasmid pGL2-(-536) GAL4 was derived from pGL2-(-536) (21) and contains the mouse B-*myb* promoter in which the E2F site has been replaced with a single copy of the high-affinity GAL4-binding site (51).

pG5LTR-HIV and pLTR-HIVG5 both encode the human immunodeficiency virus (HIV) long terminal repeat (LTR) upstream of chloramphenicol acetyltransferase (CAT) with the GAL4-binding sites either at position −700 relative to the transcription start site (pG5LTR-HIV) or positioned 3′ to the CAT gene (pLTR-HIVG5). Both plasmids are activated by pSV-Tat which encodes the HIV Tat transactivator under the control of the simian virus 40 promoter. pG5LTR-HIV, pLTR-HIVG5, and pSV-Tat have been described previously (36, 48).

Vectors used for the construction of GAL4 fusion plasmids are pMG147Spe (50) and pBS(RSV) GAL4-E2F1 (368-437) (19); both contain the DBD of GAL4 (aa 1 to 147) upstream of cloning sites. To clone into pBS(RSV) GAL4-E2F1(368-437), the E2F1 fragment was first excised with a *Bam*HI-*Xba*I digest and the purified backbone was ligated to a fragment with a 5′ *Bam*HI site and a 3′ *Xba*I site. Plasmids pGAL4-EBNA3C aa11-350 and pGAL4-EBNA3C aa280-525 were cloned into this vector by PCR amplification of the relevant region of EBNA3C. 5′ primers contained a *Bam*HI site and an ATG initiation codon to allow in vitro transcription and translation. 3′ primers included an *Xba*I site. pGAL4-EBNA3C aa580-992 was cloned into *Sal*I-digested pMG147Spe as a PCR product by using primers which contain *Sal*I sites. All EBNA3C-derived fragments (including one spanning EBNA3C aa 11 to 525) were also cloned into pBKCMV (Stratagene) to allow in vitro transcription and translation (see below).

pGAL4$_{1\text{-}147}$ was constructed by digesting pBS(RSV) GAL4-E2F1(368-437) with *Bam*HI and *Xba*I and ligating the backbone to an oligonucleotide which converts the *Bam*HI site into an *Xba*I site. pGAL4-EBNA3C aa11-992 was cloned by excising the EBNA3C sequence from pT36$^+$ (37) with a *Sac*I-*Hin*dIII digest. This was subcloned into a pBlueScript II (Stratagene) derivative which allowed the EBNA3C fragment to be cloned as an *Xba*I fragment into *Xba*I-digested pGAL4$_{1\text{-}147}$. This resulted in the loss of the first 10 aa from the EBNA3C sequence.

pGAL4-EBNA3C aa11-992 Δaa342-544 was constructed by first digesting T36$^+$ with *Aat*II and *Csp*45I, which excises the coding sequence for aa 342 to 544. The purified backbone was ligated to a short oligonucleotide which brings the EBNA3C sequence back into frame. This plasmid was designated T36$^+$ Δaa342-544. The plasmid was digested with *Eco*RV, and the purified fragment (which encompasses the deletion) was ligated into *Eco*RV-digested pGAL4-EBNA3C aa11-992. During this final step, a clone (pGAL4-EBNA3C aa11-992 × *Eco*RV) in which the *Eco*RV fragment is ligated into the vector in the reverse orientation was isolated. Therefore, this plasmid actually encodes aa 11 to 183 of the EBNA3C sequence but has an additional 8 aa (C terminal) before termination. All fusion proteins were analyzed by DNA sequencing to ensure that the correct reading frame was maintained.

pGEX-TFIID$_{1\text{-}163}$ (GST-TBP-N) and pGEX-TFIID$_{168\text{-}339}$ (GST-TBP-C) encode the amino-terminal (N) and carboxy-terminal (C) halves of TBP, respectively, and have been described elsewhere (14).

pGEMIE1 (14), pBS IE2 (7), T36$^+$ (37), and pSP64 BZLF1 (40) have all been described previously and were used for in vitro transcription and translation to yield HCMV IE1, HCMV IE2, EBNA3C, and EBV BZLF1 (also known as Zta or Z), respectively.

Cell culture. The EBV-negative human Burkitt lymphoma-derived cell line DG75 was grown at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. The mouse fibroblast line NIH 3T3 and the human osteosarcoma cell line U2OS were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics.

Transient transfections and assays. Approximately 10^7 DG75 cells in the exponential phase of growth were pelleted and resuspended in 150 μl of unsupplemented RPMI 1640 medium. Decanted medium was retained as conditioned medium. The cell suspension was added to a chilled cuvette containing the relevant DNA suspended in 50 μl of unsupplemented RPMI medium. The cuvettes were left on ice for 10 min before cells were electroporated with a Bio-Rad Gene Pulser (250 V, 960 μF). The cuvettes were placed in a 37°C incubator for 15 min, and the transfected cells were then resuspended in 10 ml of conditioned medium and incubated for 48 h.

NIH 3T3 cells and U2OS cells were transfected by calcium phosphate coprecipitation, essentially as described previously (12).

In all cases, 5 μg of reporter plasmid was transfected along with 2 μg of pSVβgal plasmid to act as an internal control for transfection efficiency. The indicated amount of effector plasmid was used, and the total amount of DNA in each transfection remained constant by making up the difference with empty vector (i.e., pMG147Spe or pGAL4$_{1\text{-}147}$). All cells were harvested 48 h after transfection, and lysates were prepared by four cycles of freeze-thaw. The β-galactosidase activity of each sample was determined by using *o*-nitrophenyl β-D-galactopyranoside as a colorimetric substrate (35).

For fast CAT assays, scintillation counting was used (43). Briefly, an aliquot of lysate was incubated with [^{14}C]acetyl coenzyme A and chloramphenicol, the reaction mixture was extracted with ethyl acetate, and the organic phase was added to the scintillation fluid prior to counting. All counts were normalized to

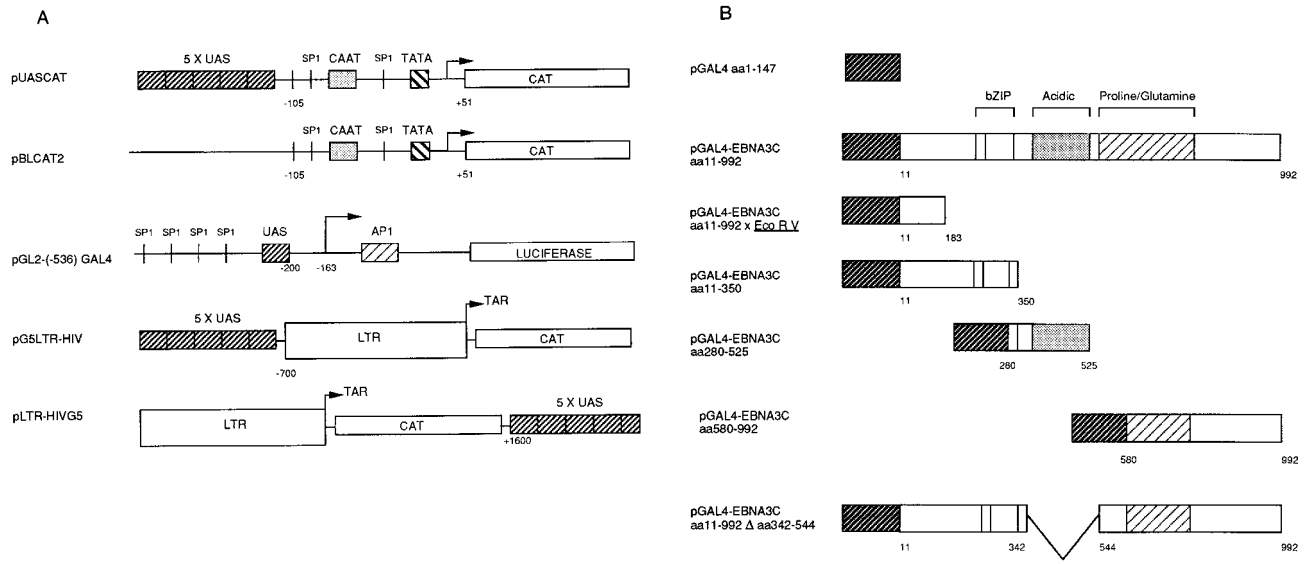
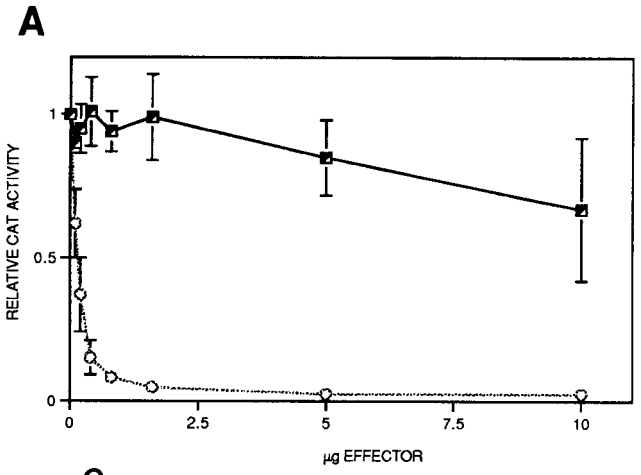
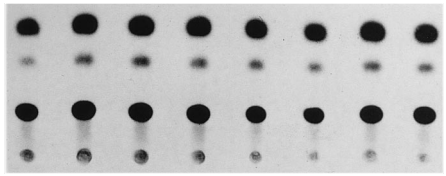
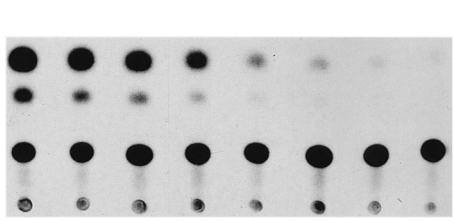


FIG. 1. (A) Reporter plasmids. UAS (stands for upstream activating sequence) indicates the position of the GAL4-binding site(s). TAR shows the position of the Tat-responsive sequence. (B) Effector plasmids used in this study (for details of construction, see Materials and Methods).





μg pGAL4 aa1-147	0	0.1	0.2	0.4	0.8	1.6	5.0	10.0
% Acetylation	22.1	25.2	27.2	24.8	30.5	30.2	30.8	26.5



μg pGAL4-EBNA3C aa11-992	0	0.1	0.2	0.4	0.8	1.6	5.0	10.0
% Acetylation	52.7	37.4	23.8	13.1	5.6	2.8	1.3	1.2

FIG. 2. GAL4-EBNA3C aa11-992 strongly represses transcription when bound to DNA. (A) The Burkitt lymphoma-derived cell line DG75 was transfected with 5 μg of the CAT reporter construct pUASCAT in which the GAL4-binding sites are located >100 bp upstream of the transcription start site. Two micrograms of pSVβgal was included in each transfection along with the indicated amount of effector plasmid. Lysates were prepared 48 h posttransfection, and β-galactosidase activity was determined as described in Materials and Methods. CAT activity was normalized to β-galactosidase activity and is expressed relative to background. Mean ± standard deviation values are shown and are the results from at least three independent transfections. GAL4-EBNA3C aa11-992 routinely represses reporter gene expression 20- to 40-fold. GAL4 aa1-147 does not significantly affect reporter gene expression. —◩—, pGAL4 aa1-147; ····○····, pGAL4-EBNA3C aa11-992. (B and C) CAT activity from representative experiments as determined by thin-layer chromatography. Normalized amounts of lysate were incubated in the presence of [^{14}C]chloramphenicol and run on silica gel plates in the presence of chloroform and methanol (95:5).

β-galactosidase activity. For CAT activity determined by thin-layer chromatography, normalized amounts of lysate were incubated in the presence of [^{14}C]chloramphenicol and the acetylated products were analyzed by ascending chromatography in the presence of chloroform and methanol (95:5). For quantification of acetylated species, spots were excised, added to the scintillant, and counted.

For luciferase assays, cells were washed in phosphate-buffered saline and harvested into Reporter Lysis Buffer (Promega). Half of the supernatant was assayed for β-galactosidase activity, and the other half was assayed for luciferase activity by using a Luciferase Assay system (Promega) and a luminometer (Berthold). Luciferase activity was normalized to β-galactosidase activity and is expressed as fold repression relative to background.

Purification of glutathione *S*-transferase (GST) fusion proteins. Using a method described previously (44), a 50-ml overnight culture of *Escherichia coli* transformed with the relevant pGEX plasmid was seeded into 500 ml of fresh 2× TY medium (16 g of tryptone [Difco] per liter, 10 g of yeast extract [Difco] per liter, 5 g of NaCl per liter, pH 7.4). The culture was grown for 1 h at 37°C before the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. The culture was transferred to room temperature and grown for a further 4 h. The cells were pelleted and resuspended in 5 ml of MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4) before being briefly sonicated (10 s). Triton X-100 was added to a final concentration of 1%, and cell debris was pelleted by centrifugation (12,000 × *g* for 10 min at 4°C). The supernatant was incubated with glutathione-Sepharose beads (50% [vol/vol] in MTPBS; Pharmacia) for 15 min at 4°C. The beads were washed four times with 15 ml of MTPBS, and purified protein was analyzed and quantified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The beads were stored at −70°C for a maximum of 3 weeks.

In vitro transcription and translation. Coupled transcription-translation reactions were carried out with the TNT system (Promega) according to the manufacturer's instructions. Plasmids pGEMIE1, pBS IE2, and pSP64 BZLF1 were in vitro transcribed and translated to yield HCMV IE1, HCMV IE2, and EBV BZLF1, respectively. Plasmids T36$^+$, T36$^+$ Δaa342-544, pBKCMV-EBNA3C aa11-525, pBKCMV-EBNA3C aa11-350, and pBKCMV-EBNA3C aa580-992 were in vitro transcribed and translated to yield EBNA3C, EBNA3C Δaa342-544, EBNA3C aa11-525, EBNA3C aa11-350, and EBNA3C aa580-992, respectively. Products were labelled with [^{35}S]methionine, and an aliquot was analyzed by SDS-PAGE with autoradiography.

GST fusion pull-down assays. By using the method of Hagemeier et al. (14), approximately 500 ng of GST-TBP-N or GST-TBP-C protein on beads was incubated with bovine serum albumin (1 mg/ml) for 10 min at room temperature. The beads were sedimented by centrifugation and resuspended in 200 μl of EBC buffer (140 mM NaCl, 100 mM NaF, 200 μM Na_3VO_4, 0.5% Nonidet P-40, 50 mM Tris-HCl [pH 8.0]). In vitro-synthesized protein (0.5 to 5 μl) was added to the beads and incubated for 1 h at 4°C. The beads were washed four times with 1 ml of NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris-HCl [pH 8.0]) with a 5-s vortex with each wash. The beads were boiled in SDS sample buffer, and bound proteins were resolved on SDS-polyacrylamide gels and visualized by autoradiography. For high-stringency binding assays, the sodium chloride concentration in the binding and wash buffers was increased to 250 mM.

RESULTS

GAL4-EBNA3C aa11-992 is a potent repressor of transcription. It has recently been reported that a region of EBNA3C (aa 724 to 826) when expressed as a fusion protein with the DBD of the yeast transactivator GAL4 will activate reporter gene expression (34). We wished to investigate the ability of the full-length EBNA3C protein to modulate transcription. However, a specific EBNA3C-binding DNA sequence has not been identified, and so we have constructed a vector in which all but the N-terminal 10 aa of EBNA3C are fused to the DBD of GAL4 (pGAL4-EBNA3C aa11-992; Fig. 1B). When this construct was cotransfected into the Burkitt lymphoma-derived cell line DG75 along with a reporter plasmid containing GAL4-binding sites (pUASCAT; Fig. 1A), GAL4-EBNA3C aa11-992 was shown to be a powerful repressor of reporter gene expression (Fig. 2A and B). Multiple transfections with different batches of reporter and effector plasmids have consistently resulted in 20- to 40-fold repression, and repression is observed with as little as 10 ng of pGAL4-EBNA3C aa11-992 (data not shown). In contrast, transfection of the DBD of GAL4 alone (pGAL4 aa1-147 or pMG147Spe) had no significant, reproducible effect on reporter gene expression (Fig. 2A and C and data not shown).

Repression is dependent on binding to DNA. In order to show that repression is not due to squelching (11), nonspecific cytotoxicity, or a posttranscriptional phenomenon, the parental plasmid of pUASCAT, pBLCAT2 (Fig. 1A), which lacks GAL4-binding sites, was used as a reporter construct. Trans-

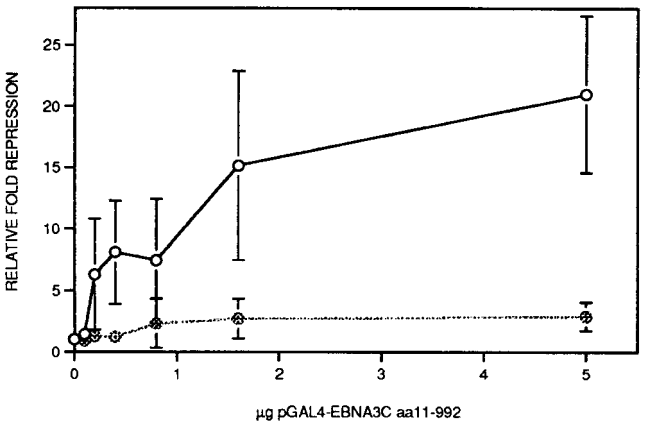


FIG. 3. GAL4-EBNA3C aa11-992 represses only when bound to DNA. DG75 cells were transfected with the indicated amount of pGAL4-EBNA3C aa11-992 and either 5 μg of pBLCAT2 which has no GAL4-binding sites or 5 μg of pUASCAT (pBLCAT2 containing five copies of the GAL4-binding site). Lysates were assayed as described in Materials and Methods. CAT activities are expressed as fold repression relative to background activity. —○—, pUASCAT reporter; ···⊕···, pBLCAT2 reporter.

fections clearly demonstrated that specific DNA binding is necessary for the repression exhibited by GAL4-EBNA3C aa11-992 (Fig. 3). Although a slight repressive effect (less than threefold) is sometimes observed with pBLCAT2, this is not significantly different from that occasionally obtained with the DBD of GAL4 alone (data not shown).

Repression is not restricted to human B lymphocytes. Repression is not restricted to the DG75 cell line but also occurs in other B-cell lines (data not shown). Similar transfections of pGAL4-EBNA3C aa11-992 into the human osteosarcoma cell line U2OS show that repression is not restricted to B lymphocytes (Fig. 4A), nor is repression restricted to human cells, as a significant level of repression is observed in the rodent fibroblast cell line NIH 3T3 (approximately sevenfold; Fig. 4B).

Repression is not promoter specific and is independent of the location and orientation of the GAL4-binding sites. The pUASCAT reporter plasmid is based on the herpes simplex virus thymidine kinase promoter, which includes a conventional TATA motif with the GAL4-binding sites (five copies) located approximately 100 bp upstream of the transcription start site. To determine whether EBNA3C-mediated repression is effective when the GAL4-EBNA3C fusion is bound to a promoter which lacks a TATA box, a plasmid in which the E2F-binding site in the murine B-*myb* promoter was replaced with a single high-affinity binding site for GAL4 was constructed (51). In this construct [pGL2-(-536) GAL4; Fig. 1A], the GAL4-binding site is located approximately 40 bp from the major cap site at position −163 relative to the start of the coding sequence. As before, GAL4-EBNA3C aa11-992 can efficiently repress reporter gene expression from this construct (Fig. 5A).

Because pGAL4-EBNA3C aa11-992 encodes such a large protein (>1,000 aa), it is conceivable (though unlikely) that repression is due to the fusion protein sterically blocking the promoter. Therefore, two constructs, pG5LTR-HIV and pLTR-HIVG5, in which the HIV LTR is positioned upstream of the CAT gene were obtained. These constructs have the GAL4-binding sites positioned either very far upstream of the transcription start site at position −700 (pG5LTR-HIV; Fig. 1A) or 3′ to the CAT gene (pLTR-HIVG5; Fig. 1A). To obtain a detectable level of activity from these constructs, it is necessary to activate them with the HIV Tat transactivator. These experiments have been performed with U2OS cells, since in our hands pSV-Tat had virtually no effect in human B cells (data not shown). GAL4-EBNA3C aa11-992 can repress Tat-mediated activation from both pG5LTR-HIV and pLTR-HIVG5 (Fig. 5B and C). The level of repression (five- to eightfold) is similar to that obtained with the pUASCAT reporter in U2OS cells. Therefore, our data suggest that by all accepted criteria, EBNA3C is a transcriptional repressor when bound to DNA.

Mapping of the repression domain of EBNA3C. In order to elucidate which region(s) of EBNA3C is responsible for repressor function, various vectors that contain the DBD of GAL4 fused to a series of overlapping fragments of EBNA3C have been constructed (Fig. 1B). These constructs have all been tested for their ability to repress CAT activity from the pUASCAT reporter. We have found that the N-terminal 350 aa are not necessary for repressor function (Fig. 6A and B). However, a fragment encompassing aa 280 to 525 was shown to repress CAT activity in a manner which is nearly identical to

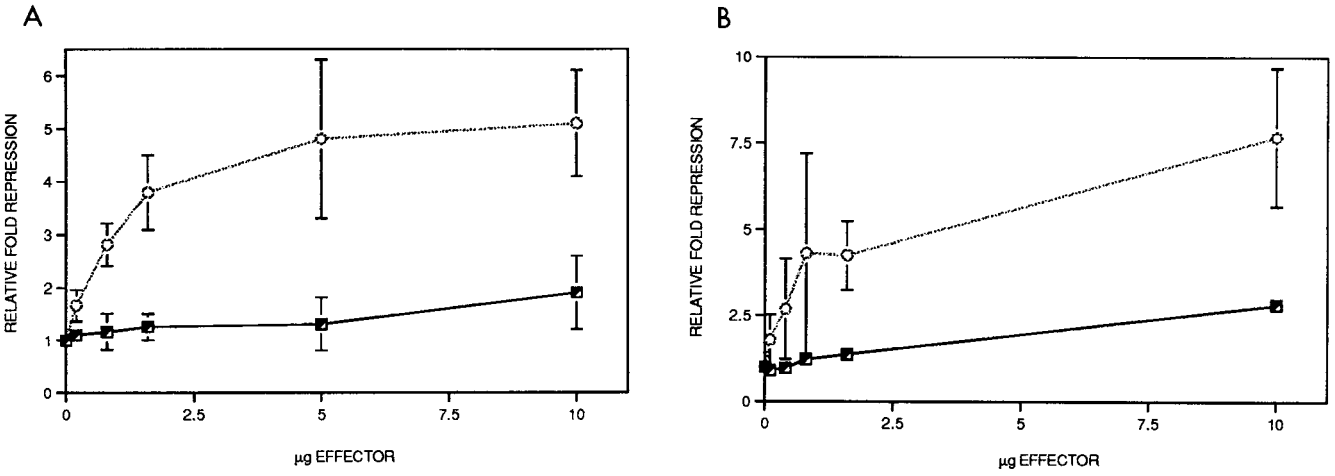


FIG. 4. Repression is neither cell type nor species specific. The human osteosarcoma cell line U2OS (A) and the mouse fibroblast cell line NIH 3T3 (B) were transfected with 5 μg of pUASCAT reporter plasmid and the indicated amount of effector plasmid. Lysates were prepared as described in Materials and Methods. ◩, pGAL4 aa1-147; ○, pGAL4-EBNA3C aa11-992.

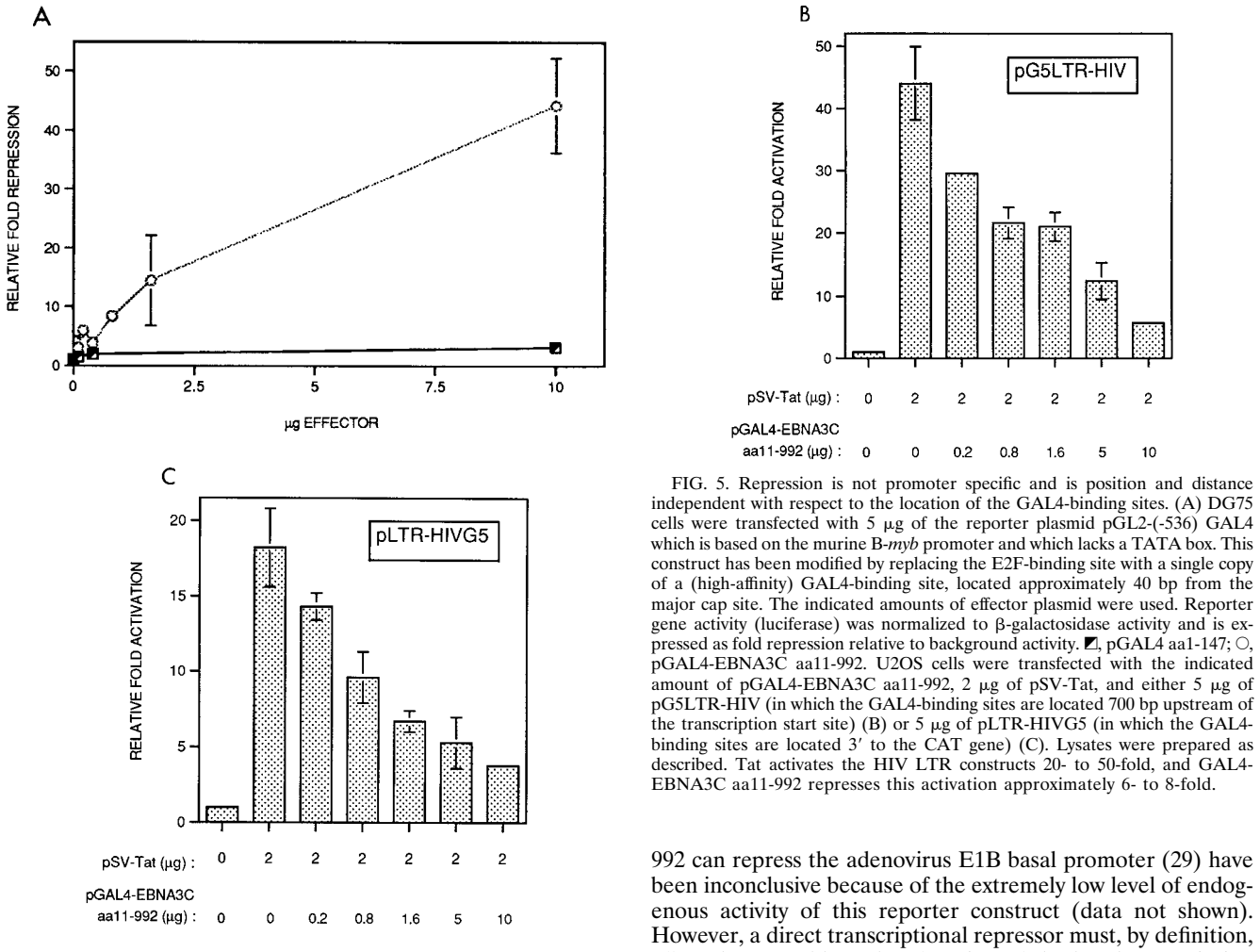


FIG. 5. Repression is not promoter specific and is position and distance independent with respect to the location of the GAL4-binding sites. (A) DG75 cells were transfected with 5 µg of the reporter plasmid pGL2-(-536) GAL4 which is based on the murine B-*myb* promoter and which lacks a TATA box. This construct has been modified by replacing the E2F-binding site with a single copy of a (high-affinity) GAL4-binding site, located approximately 40 bp from the major cap site. The indicated amounts of effector plasmid were used. Reporter gene activity (luciferase) was normalized to β-galactosidase activity and is expressed as fold repression relative to background activity. ◩, pGAL4 aa1-147; ○, pGAL4-EBNA3C aa11-992. U2OS cells were transfected with the indicated amount of pGAL4-EBNA3C aa11-992, 2 µg of pSV-Tat, and either 5 µg of pG5LTR-HIV (in which the GAL4-binding sites are located 700 bp upstream of the transcription start site) (B) or 5 µg of pLTR-HIVG5 (in which the GAL4-binding sites are located 3′ to the CAT gene) (C). Lysates were prepared as described. Tat activates the HIV LTR constructs 20- to 50-fold, and GAL4-EBNA3C aa11-992 represses this activation approximately 6- to 8-fold.

that of the full-length protein (typically a 12- to 18-fold repression; Fig. 6C). This fragment is rich in both charged and proline residues and therefore resembles the repressor domain of several well-defined transcriptional repressors. In addition, a proline-glutamine region of EBNA3C (aa 580 to 992) shows a moderate repressor activity (Fig. 6D). Consistent with these observations, we have found that deleting a fragment which includes aa 280 to 525 (pGAL4-EBNA3C aa11-992 Δaa342-544) reduces the repressive effect of GAL4-EBNA3C aa11-992 to a level similar to that obtained with aa 580 to 992 alone (Fig. 6E). These data are summarized in Table 1. All fragments which exhibit repressor activity do so in a manner identical to that of the full-length protein; for example, repression requires specific DNA binding (data not shown).

Mechanism of GAL4-EBNA3C-mediated repression. GAL4-EBNA3C aa11-992 can repress the activity of two reporter constructs, pUASCAT and pGL2-(-536) GAL4 (Fig. 2A, 2B, and 5A), which both exhibit a significant level of endogenous activity, presumably because of Sp1-mediated activation (Fig. 1A). However, GAL4-EBNA3C aa11-992 can also repress activation mediated by the HIV Tat transactivator (Fig. 5B and C). Therefore, it is unlikely that GAL4-EBNA3C aa11-992-mediated repression occurs as a result of the inhibition of specific activators, but instead, it is probable that GAL4-EBNA3C aa11-992 functions as a direct transcriptional repressor. Experiments to address whether GAL4-EBNA3C aa11-992 can repress the adenovirus E1B basal promoter (29) have been inconclusive because of the extremely low level of endogenous activity of this reporter construct (data not shown). However, a direct transcriptional repressor must, by definition, affect the assembly and/or function of the basal transcription apparatus. Therefore, we have investigated whether EBNA3C could interact with a component of the basal transcription complex. By using GST pull-down assays, we have found that in vitro-transcribed and -translated EBNA3C can bind to the conserved carboxy terminus (but not the N terminus) of TBP in a manner similar to that of the viral factors HCMV IE2 (reference 7 and references therein) and EBV BZLF1 (27) (Fig. 7A). This interaction is of an equal or greater affinity to that characterized between IE2 or BZLF1 and TBP. Furthermore, when the two halves of EBNA3C were in vitro translated and subjected to the same pull-down analysis, the N-terminal half of EBNA3C (aa 11 to 525), but not the C-terminal half (aa 580 to 992), bound to the conserved region of TBP. Specifically, the EBNA3C-TBP interaction appears to map to the N-terminal 350 residues of EBNA3C which contain the potential basic leucine zipper (bZIP) motif. These interactions are of high affinity and are not affected by increasing the salt concentration in the binding and wash buffers to 250 mM (Fig. 7B). Interestingly, EBNA3C aa580-992, which contains the EBNA3C activation domain (aa 724 to 826), does not appear to contact TBP in this assay (Fig. 7B, left panel).

DISCUSSION

EBNA3C is one of the small subset of EBV genes expressed in latently infected lymphoblastoid cell lines and has previously been shown to be essential for the immortalization of B lym-

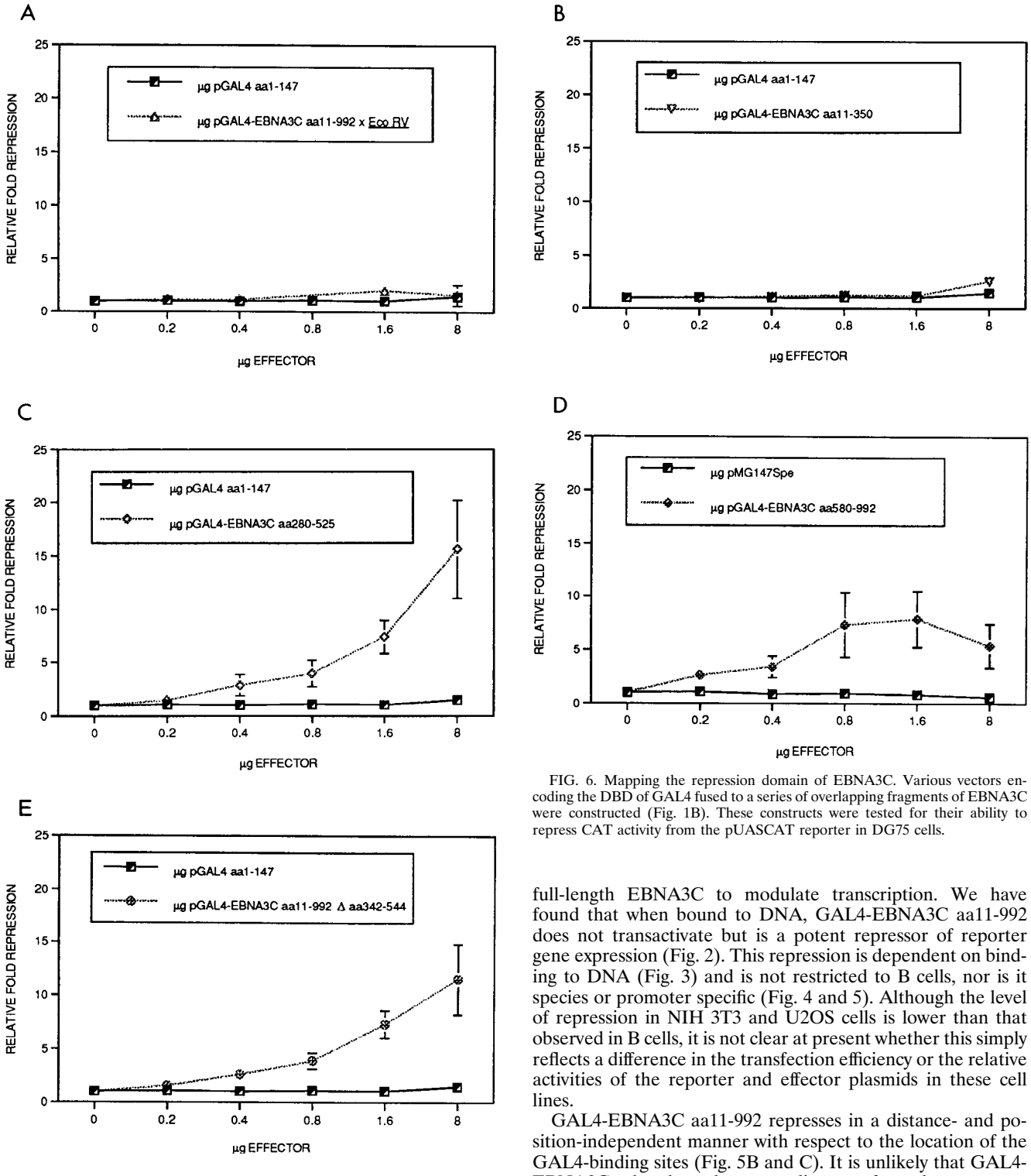


FIG. 6. Mapping the repression domain of EBNA3C. Various vectors encoding the DBD of GAL4 fused to a series of overlapping fragments of EBNA3C were constructed (Fig. 1B). These constructs were tested for their ability to repress CAT activity from the pUASCAT reporter in DG75 cells.

phocytes (45). The protein has structural motifs which suggest that it functions as a transcription factor (1, 37), and it has been shown to be capable of modulating the expression of both cellular and viral genes (2, 3, 49).

By using vectors encoding the GAL4 DBD fused to both the near-full-length EBNA3C and a series of overlapping fragments of EBNA3C, we have investigated the ability of near-full-length EBNA3C to modulate transcription. We have found that when bound to DNA, GAL4-EBNA3C aa11-992 does not transactivate but is a potent repressor of reporter gene expression (Fig. 2). This repression is dependent on binding to DNA (Fig. 3) and is not restricted to B cells, nor is it species or promoter specific (Fig. 4 and 5). Although the level of repression in NIH 3T3 and U2OS cells is lower than that observed in B cells, it is not clear at present whether this simply reflects a difference in the transfection efficiency or the relative activities of the reporter and effector plasmids in these cell lines.

GAL4-EBNA3C aa11-992 represses in a distance- and position-independent manner with respect to the location of the GAL4-binding sites (Fig. 5B and C). It is unlikely that GAL4-EBNA3C, when bound at great distances from the promoter, in this case >700 bp, would be capable of positioning a nucleosome precisely over the promoter and so prevent access by activators.

Our data show that GAL4-EBNA3C aa11-992 can repress activation mediated by both Sp1 and the HIV Tat transactivator (Fig. 2 and 5). These activators are radically different in terms of both structure and function, and so it is probable that repression is occurring at a more fundamental level than by

TABLE 1. Levels of repression obtained with various pGAL4-fusion proteins[a]

Fragment	Mean fold repression
pGAL4 aa1-147	1
pGAL4-EBNA3C aa11-992	23
pGAL4-EBNA3C aa11-992 × *Eco*RV	1
pGAL4-EBNA3C aa11-350	1
pGAL4-EBNA3C aa280-525	16
pGAL4-EBNA3C aa580-992	6
pGAL4-EBNA3C aa11-992 Δaa342-544	10

[a] Values were obtained from at least three independent transfections with multiple batches of reporter and effector plasmids.

merely interfering with specific activator function or quenching (reviewed in references 8, 24, and 38). In this respect, EBNA3C seems to differ from the *Drosophila* Krüppel protein, which is an activator-specific repressor (26).

Our data are consistent with EBNA3C functioning as a direct transcriptional repressor, acting at the level of basal transcription complex assembly and/or function. However, for technical reasons, it has not been possible to demonstrate EBNA3C-mediated repression of a basal promoter construct. We have addressed whether EBNA3C can interact with the basal transcription complex and have shown that, in vitro, EBNA3C can bind with high affinity to the TBP component of TFIID (Fig. 7), thus providing a mechanism by which EBNA3C could communicate with the basal transcription complex. It has been shown that an interaction with TBP is required for Even-skipped-mediated repression (4, 47). However, it is not yet clear to what extent TBP binding is involved in EBNA3C-mediated repression. Although binding to TBP seems to map to a different region from those that mediate repression (aa 11 to 350, compared with aa 280 to 525 and 580 to 992; Fig. 7B, 6C, and 6D, respectively), it could be that EBNA3C is similar to the repressor Dr1, which contains separable TBP-binding and repression domains (52).

In vitro assays have shown that a C-terminal fragment of EBNA3C (aa 580 to 992) which contains the activation domain does not bind to TBP (Fig. 7B). Therefore, it is possible that different regions of the EBNA3C molecule interact with different components of the basal transcription complex.

We have mapped the major repressor activity of EBNA3C to aa 280 to 525, although moderate repression is also observed C terminal to this region (aa 580 to 992). At present, it is unclear whether repression is due to a single repressor domain or one made up of subdomains. Deletion of aa 280 to 525 (as in pGAL4-EBNA3C aa11-992 Δaa342-544) results in a dramatically reduced repressing ability, thus illustrating the importance of these residues in repression. Repressors characterized in *Drosophila melanogaster*, such as Krüppel (Kr), Engrailed (En), and Even-skipped (Eve), all have repressor domains which are rich in alanine, proline, or glutamine residues (15, 16, 25). These same residues appear to be important for the mammalian repressors Dr1 (52) and the product of the Wilms tumor gene (WT1) (33). These repressors lack charged residues within the repressor domain; however, the E4BP4 repressor appears to contain numerous charged residues within the repressor region and therefore seems to be representative of a new class of transcriptional repressors (9). Analysis of the putative repressor domains of EBNA3C (aa 280 to 525 and 580 to 992) reveals a region equally rich in proline and charged (acidic) residues (aa 280 to 525) and one rich in proline and glutamine residues (aa 580 to 992). Therefore, the repressor domains of EBNA3C have similarities with both classes of transcriptional repressor.

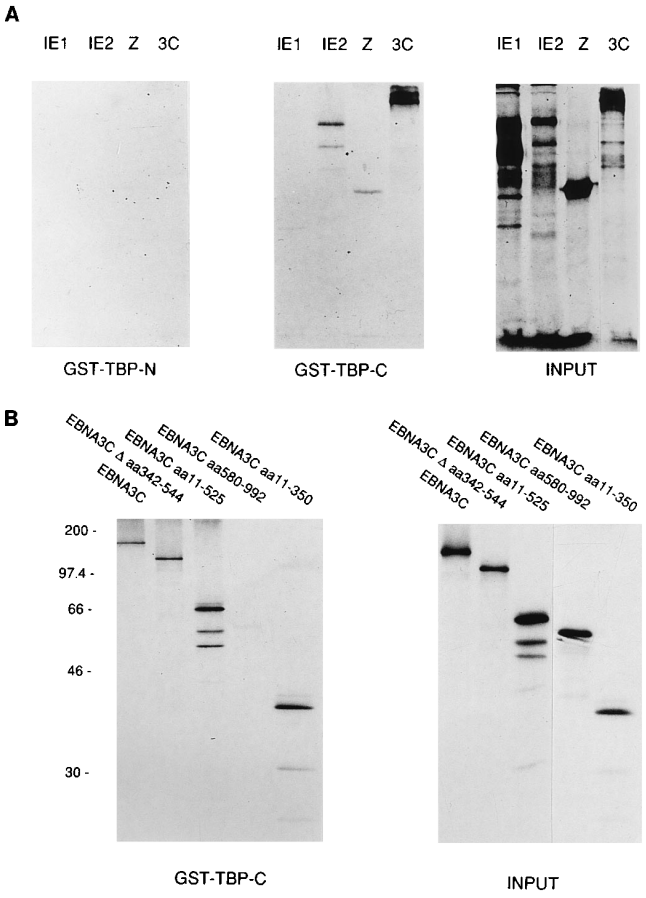


FIG. 7. In vitro, EBNA3C binds to the conserved carboxy terminus of the TBP, and this interaction requires the N-terminal region of EBNA3C. (A) GST pull-down experiments were conducted as follows. Five hundred nanograms of GST-TBP-N (left panel) or GST-TBP-C (middle panel) on beads was incubated with in vitro-synthesized ^{35}S-labelled protein in EBC binding buffer. Beads and protein were incubated for 1 h at 4°C, after which the beads were washed extensively. Proteins which remained bound to beads were resolved by SDS-PAGE (15% gel) and visualized by autoradiography. The test proteins used were HCMV IE1 (IE1) as a negative control, HCMV IE2 (IE2) and EBV BZLF1 (Z) as positive controls, and EBNA3C (3C). The amount of radiolabelled protein added to the reaction mixture is shown in the input panel. (B) EBNA3C and various EBNA3C-derived constructs were in vitro transcribed and translated in the presence of [^{35}S]methionine. Proteins were incubated in high-stringency binding buffer (250 mM NaCl) with either GST-TBP-N (data not shown) or GST-TBP-C (left panel). Beads and bound proteins were washed in high-stringency wash buffer (250 mM NaCl), resolved by SDS-PAGE (12.5% gel), and visualized by autoradiography. The input panel shows the amount of each protein added to the reaction mixture (shorter exposure). Molecular size markers (in kilodaltons) are shown on the left.

It has recently been shown that EBNA3C interacts with the cellular CBF1 protein (also known as RBP Jκ) (34, 39). CBF1 has been shown to function as a transcriptional repressor (10, 18), and so GAL4-EBNA3C-mediated repression may occur as a result of the binding and recruitment of CBF1 to DNA. However, recent data have shown that CBF1 interacts with the N-terminal region of EBNA3C (53). This region does not exhibit repressor activity (Fig. 6A and B). Although this does not rule out a role for CBF1 in EBNA3C-mediated repression, it does suggest that if repression is dependent on the presence of CBF1 in the GAL4-EBNA3C–DNA complex, there would have to be at least three CBF1-binding sites within the EBNA3C molecule (Fig. 6C and D).

We have also shown that EBNA3C binds to the retinoblas-

toma gene product, pRb, in vitro (data not shown). pRb has also been shown to act as a direct transcriptional repressor when tethered to DNA (6), and so it is just as feasible that EBNA3C-mediated repression is due to the presence of pRb (or one of the other pocket proteins) on DNA. We are currently investigating whether EBNA3C can repress independently or does so in conjunction with a corepressor (e.g., CBF1 or pRb).

Although we have characterized a repressor activity, this does not exclude the possibility that EBNA3C could also activate gene expression under different circumstances. Indeed, it has already been reported that EBNA3C encodes a potential activation domain (34). These observations are not incompatible and would instead reflect a dual role for EBNA3C in transcriptional modulation. Mechanisms for the control of switching between activation and repression could include differential phosphorylation. It is known that EBNA3C is a phosphoprotein (41) and contains numerous potential sites for casein kinase II and protein kinase C. Indeed, within the dominant repressor domain of EBNA3C (aa 280 to 525), we have noted two potential sites for casein kinase II-mediated phosphorylation. Because phosphorylation has been shown to affect functions as diverse as DNA binding (30) and transactivation potential (46), we have investigated whether activators of the protein kinase C pathway (such as 12-*O*-tetradecanoylphorbol-13-acetate) can affect EBNA3C-mediated repression. Preliminary experiments have shown no effect on repression (unpublished observations).

It is possible that EBNA3C interacts with various cofactors which could mediate the switch between repressor and activator function. For example, studies have shown that repression mediated by YY1 is relieved in the presence of E1a (42) and that a recently identified *Drosophila* protein, DSP1 (dorsal switch protein), can convert the transactivator proteins Dorsal and NF-κB from activators to repressors (22). It is also feasible that EBNA3C interacts with various cell cycle-regulated proteins, which could mask or expose the repressor domain in a cell cycle-dependent manner. This is a possible explanation for the observation that, in the presence of EBNA3C, LMP1 levels increase at a specific stage of the cell cycle (3).

In conclusion, we have identified a novel repressor function of EBNA3C. This repression is due to a region rich in both acidic and proline residues (aa 280 to 525) and to one rich in proline and glutamine residues (aa 580 to 992). Thus, these regions resemble the repressor domains of several well-defined transcriptional repressors. We are currently attempting to identify further the precise amino acids involved and to elucidate the exact mode of repression mediated by EBNA3C. It is tempting to speculate that, in vivo, there are specific EBNA3C-binding sites within the promoters of both cellular and viral genes to which EBNA3C could bind (either independently or in conjunction with a cellular or viral partner) and then strongly repress gene expression. Future studies will include an investigation into the significance of EBNA3C-mediated repression in the immortalization of B cells and in the maintenance of viral latency.

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