JOURNAL OF VIROLOGY, Nov. 1997, p. 8552–8562 Vol. 71, No. 11
0022-538X/97/$04.00+0

Epstein-Barr Virus EBNA3C Represses Cp, the Major Promoter for EBNA Expression, but Has No Effect on the Promoter of the Cell Gene CD21

STOYAN A. RADKOV,[1,2] MARK BAIN,[1,2]† PAUL J. FARRELL,[1,2] MICHELLE WEST,[3] MARTIN ROWE,[3,4] AND MARTIN J. ALLDAY[1,2]*

Department of Medical Microbiology[1] and Ludwig Institute for Cancer Research,[2] Imperial College School of Medicine at St Mary's, London W2 1PG, and CRC Institute for Cancer Studies, University of Birmingham B15 2TJ,[3] and Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XX,[4] United Kingdom

Received 21 May 1997/Accepted 30 July 1997

EBNA3C is a potent repressor of transcription when bound to DNA as a fusion with the DNA binding domain (DBD) of GAL4. A survey of promoters has revealed that the wild-type, unfused EBNA3C can specifically repress expression from reporter plasmids containing the Epstein-Barr virus Cp latency-associated promoter. Repression of Cp activity required amino acids 207 to 368, which encompasses a region resembling a basic DBD adjacent to a leucine zipper DNA binding motif and a site which binds to the cellular factor CBF1/RBP-Jκ. However, amino acids 207 to 368 are dispensable when the protein is bound to DNA as a fusion with the GAL4 DBD, thus implicating this region in DNA binding. Mutation of the CBF1/RBP-Jκ binding site in EBNA3C abrogated repression, strongly suggesting that CBF1/RBP-Jκ is necessary for targeting the viral protein to Cp. Consistent with this result, mutation of the EBNA2 response element (a CBF1/RBP-Jκ binding site) in Cp also prevented significant repression. In addition, amino acids 346 to 543, which were previously defined as important for the repressor activity of the GAL4-EBNA3C fusion proteins, also appear to be necessary for the repression of Cp. Since repression by these fusions was not observed in all cell types, it seems likely that EBNA3C either depends on a corepressor which may interact with amino acids 346 to 543 or is modified in a cell-specific manner in order to repress. These data are consistent with EBNA3C contributing to the regulation of EBNA expression in latently infected B cells through CBF1/RBP-Jκ and another factor, but this need not directly involve EBNA2. Finally, although it has been reported that EBNA3C can upregulate CD21 in some B cells, we were unable to demonstrate any effect of EBNA3C on reporter plasmids which contain the CD21 promoter.

In vitro, Epstein-Barr virus (EBV) can induce the continuous proliferation of a subset of resting human B cells. The resulting immortalized lymphoblastoid cell lines (LCLs) have a phenotype similar to that of activated B lymphoblasts and express nine latent viral proteins. Together these proteins are responsible for the activation of resting B cells which then enter the cell division cycle, induction of continuous proliferation, maintenance of the viral genome in its latent form, and perhaps prevention of terminal differentiation (recently reviewed in reference 18). In addition, EBV is the causative agent in the benign lymphoproliferation known as infectious mononucleosis and is associated with at least four types of human tumors: Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and lymphoma in immunocompromised individuals (reviewed in references 32 and 44).

The viral genes expressed in LCLs include six genes encoding antigenic proteins localized in the nucleus: EBNA1, -2, -3A, -3B, -3C, and -LP (the last four are also known as EBNA3, -4, -6, and -5, respectively). Additionally, there are three latent membrane proteins: LMP1, LMP2A, and LMP2B (also known as LMP, TP1, and TP2, respectively) (18). Three of the nuclear antigens, EBNA3A, -3B, and -3C, are considered to comprise a family which may have arisen by gene duplication since they share limited but significant amino acid homology, have the same gene structure (a short 5′ exon and a long 3′ exon), and are arranged tandemly in the EBV genome (1, 14, 28, 33, 38). Genetic studies using recombinant EBV have shown that EBNA3A and EBNA3C are essential for the immortalization of B cells whereas EBNA3B is dispensable (45, 46).

Experiments in which EBNA3C was stably expressed after transfection into EBV-negative lymphoma cells produced clones expressing high levels of the CD21 antigen (50). In similar experiments, EBNA3C was shown to increase the expression of the LMP1 gene in EBV-positive (but EBNA3C-negative) Raji cells arrested early in the G_1 phase of the cell division cycle (2, 3). EBNA3C in LCL lysates binds to DNA-cellulose in a nonspecific manner (17), and experiments using recombinant protein produced in the baculovirus expression system suggest that it may bind to DNA in association with a cellular or viral protein partner (37). However, binding to a specific nucleotide sequence has not been reported.

EBNA3C is a large polypeptide of 992 amino acids (aa) with a predicted molecular mass of ~110 kDa; in sodium dodecyl sulfate (SDS)-polyacrylamide gels, it appears even larger, migrating with an apparent molecular mass of about 160 kDa. Analysis of its predicted sequence has revealed features common to many viral and cellular transcription factors. These features include a region which resembles a basic DNA binding domain (DBD) adjacent to a potential leucine zipper motif (b-ZIP) and regions rich in acidic, proline, and glutamine residues which are good candidates for domains involved in the

* Corresponding author. Mailing address: Ludwig Institute for Cancer Research, Imperial College School of Medicine at St. Mary's, Norfolk Place, London W2 1PG, United Kingdom. Phone: (44) 171 724 5522, ext. 221. Fax: (44) 171 724 8586. E-mail: m.allday@ic.ac.uk.

† Present address: Department of Medicine, Addenbrooke's Hospital, Cambridge CB2 2QQ, United Kingdom.

modulation of transcription. In transient-transfection assays, EBNA3C can modulate the EBNA2-mediated activation of the LMP1 and LMP2A promoters (20, 24, 34). EBNA2 activation of these target genes is complex, involving interactions with various cellular transcription factors. These factors include CBF1 (also known as RBP-Jκ), PU1 family members, and proteins including a POU domain (11, 12, 15, 21, 22, 26, 41, 42, 48, 54, 55). The best characterized of these interactions involves the binding of EBNA2 to CBF1/RBP-Jκ. Binding of CBF1/RBP-Jκ to the DNA sequence 5′-GTGGGAA-3′ in the EBV latency-associated promoter, Cp, targets EBNA2 to DNA, which directs a strong activation domain in EBNA2 to the promoter and also interferes with the interaction between a corepressor that can also bind to CBF1/RBP-Jκ; the net result is strong activation of transcription (47, 54, 55).

The N-terminal 250 aa of EBNA3C also contain a binding site for CBF1/RBP-Jκ, which is located around aa 209 to 212 (35, 53) (Fig. 1B). The physical interaction between EBNA3C and CBF1/RBP-Jκ appears to inhibit the binding of the latter to DNA, both in vitro and in vivo (34, 49, 53). This protein-protein interaction, which prevents the association of the EBNA2-CBF1/RBP-Jκ complex with DNA, is thought to be responsible for the EBNA3C-mediated repression of EBNA2 activation shown on various natural and synthetic promoters which include CBF1/RBP-Jκ binding sites.

CBF1/RBP-Jκ belongs to a family of transcriptional regulators which includes the Suppressor of Hairless [Su(H)] in *Drosophila melanogaster*. In both *Drosophila* and mammalian cells, these proteins can be regulated by signalling from the Notch family of membrane receptors. A truncated form of murine Notch, consisting only of the intracellular domain (Notch IC), has been shown to be localized to the nucleus and function as an activated receptor. EBNA2 is thought to function in a similar manner to the human Notch IC, and since in *Drosophila*, the product of the *Hairless* gene regulates the function of Su(H) by inhibiting its binding to DNA, it has been suggested that EBNA3C mimics the mammalian equivalent of Hairless (13, 35, 53).

Another recent study in which a fragment of EBNA3C was fused to the DBD of the *Saccharomyces cerevisiae* transactivator GAL4 showed that aa 724 to 826 can activate transcription when bound to DNA (24). However, similar studies using GAL4 fusions with larger EBNA3C fragments, for example, aa 11 to 992 and 580 to 992, showed that when tethered to DNA, EBNA3C can be a very potent repressor of reporter gene expression (4, 49). In the context of larger fragments and the near-full-length protein, the activation domain is apparently masked. Deletion mapping revealed two domains which contribute to the repression; neither region of the protein has been reported as necessary for the physical interaction with CBF1/RBP-Jκ (4, 53). The identification of these domains suggests that EBNA3C also represses transcription by an additional, CBF1/RBP-Jκ-independent mechanism which may involve association with another cellular protein (or proteins).

We recently demonstrated that EBNA3C is an immortalizing protein in the same class as human papillomavirus type 16 E7 and adenovirus E1A (27). That is, it can cooperate in cotransfection assays with activated (Ha-)*ras* to immortalize and transform primary rodent fibroblasts, and it can also activate transcription through an E2F response element. Since EBNA3C can bind to the retinoblastoma protein (pRb) in vitro, in a manner which is dependent on the integrity of the pocket region of pRb, we have suggested that the EBNA3C-pRb interaction may also occur in vivo and contribute toward both transformation-immortalization and the repressor activity (4, 27).

This study assessed the action of EBNA3C on the EBV latency-associated promoter Cp, the promoter for its own RNA in most LCLs. The effect of EBNA3C on the CD21 promoter was also determined. The results show that EBNA3C can repress Cp activity whether or not Cp is transactivated by EBNA2 and that this appears to require CBF1/RBP-Jκ to target EBNA3C to DNA and also probably depends on interaction with a cofactor. Therefore, in the context of latent virus infection, EBNA3C has the capacity to negatively regulate its own expression and that of the other EBNAs. Surprisingly, however, although the chromosomal CD21 gene is apparently upregulated in some EBNA3C-expressing cells and the CD21 promoter is very efficiently transactivated by EBNA2, we were unable to detect any effect of EBNA3C on its activity in transient-transfection assays; EBNA3C did not activate or repress CD21 reporter plasmids.

MATERIALS AND METHODS

Plasmids. p-1425C-CAT includes the B95-8 virus C promoter region (nucleotides 9911 to 11336) linked to the *Bgl*II/*Bam*HI fragment of pSV0CAT and cloned into pUC18. Thus, this plasmid includes the chloramphenicol acetyltransferase (CAT) gene under the control of the Cp region (TATA box at position 11305) (36).

p-1425CE2RE-CAT includes nucleotides 9911 to 11336 with a five-nucleotide mutation of the EBNA2 response element (E2RE) (RBP-Jκ/CBF1 binding site), i.e., 5′-GTGAATTC-3′ in place of 5′-GTGGGAAA-3′, subcloned from pGL-E2RE (7). pUAS-CAT includes five copies of the GAL4-binding DNA sequence (5′-CTCCGCTCGGAGGACAGTA-3′) cloned into the vector pBLCAT2 (51). These sequences are located approximately 120 bp upstream of the transcription start site.

p-1235CD21-CAT includes the full-length CD21 promoter (nucleotides −1235 to +75) cloned upstream of the CAT gene in the pSP65CAT vector. Plasmid p-467CD21-CAT includes the CD21 promoter (nucleotides −467 to +75) cloned upstream of the CAT gene in the pSP65CAT vector (31). pCMV-EBNA3C includes a cDNA encoding EBNA3C cloned as a *Sma*I/*Hin*dIII fragment from pBSK-15 into the pBKCMV vector (27). pJ144-C1 (referred to hereafter as pSV-EBNA2) includes the *Bgl*II/*Not*I fragment of the *Bam*HI WYH region (nucleotides 44664 to 50628) of the B95-8 strain of EBV cloned into the pSV2-gpt vector. The cloned fragment includes the Wp region and the entire EBNA2 gene (8). pSV-β-gal includes the β-galactosidase gene under the control of the simian virus 40 early promoter (Promega). pBKCMV-EBNA3CΔ346-543 was generated by transferring the *Eco*RV fragment from pGal4-EBNA3Caa11-992Δ346-543 (4), which spans the deletion, into pBKCMV-EBNA3C.

The pSG5-EBNA3C and pSG5-EBNA3CΔ207-368 constructs are recombinants based on the pSG5 vector (Stratagene) and contain a wild-type EBNA3C cDNA and an EBNA3CΔbZIP cDNA with codons 207 to 368 deleted, respectively. The inserted genes are placed 3′ of a simian virus 40 promoter and a beta-globin intron. The EBNA3C cDNA was derived by PCR amplification of a B95.8 cDNA from the EBNA3C-pZip-neoSV plasmid (28) with primers designed to insert flanking *Sma*I sites. From this cDNA, the ΔbZIP cDNA was derived by removal of the *Hpa*I/*Spe*I restriction fragment, followed by blunt-end ligation to repair the gene; sequencing revealed that the nuclease blunting of the *Spe*I digest caused the removal of an additional 6 nucleotides so that the deletion spanned the coordinates 99061 to 99548, leading to an in-frame deletion removing codons 207 to 368. The *Sma*I restriction fragments containing the EBNA3C cDNA were blunt end ligated into the *Bgl*II site of the pSG5 vector.

pGAL4-EBNA3Caa11-992Δ207-368 was constructed by transferring the *Eco*RV fragment from pSG5-EBNA3CΔ207-368 which spans the deletion, into pGAL4-EBNA3Caa11-992.

pSG5-EBNA3C-Jκ (M) was constructed by recombinant PCR, essentially as described previously (53). Two overlapping primers, 5′-GGC**TGCAGC TGCGGCA**GTTAACATGATGCT-3′ and 5′-**GCAGCTGCAGCC**CAAAATG CGGCACGAACT-3′, were used to replace residues Thr-209 (T), Phe-210 (F), Gly-211 (G), and Cys-212 (C) with alanine (A) residues (codons in bold). A fragment which contained the mutated region was amplified with primers 5′-GAGAGATTGGTACCAGAAGAGTCATACTCA-3′ and 5′-TAGATTCT TCGGTACCGCCTCTGC-3′ which include *Kpn*I restriction endonuclease sites (underlined). The final recombinant PCR product was digested with *Kpn*I, and the purified fragment was ligated to pSG5-EBNA3C which had previously been digested with the same restriction endonuclease. Introduction of the mutations was confirmed by DNA sequence analysis of the entire fragment. Expression of the protein was demonstrated by in vitro transcription and translation and Western blotting of extracts from transiently transfected DG75 cells.

Cell culture. All B cells were grown in suspension and maintained in RPMI 1640 (Gibco BRL) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM L-glutamine (Gibco BRL), and 100 U of penicillin and streptomycin (Gibco BRL) per ml. Adherent cells were maintained in Dulbecco modified

Eagle medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM L-glutamine, and 100 U of penicillin and streptomycin per ml (all Gibco BRL). All cells were maintained at 37°C in a 10% CO_2 atmosphere.

Transient-transfection assays. B cells were split 1:3 with fresh growth medium 12 to 16 h before transfection. Approximately 10^7 cells were used per transfection. Cells were pelleted, and growth medium was aspirated and retained as conditioned medium. The cell pellet was resuspended in ice-cold unsupplemented RPMI 1640 (150 μl for DG75 cells), and the suspension was added to a chilled cuvette (0.4-cm gap; Bio-Rad) which contained the relevant DNA suspended in 50 μl of ice-cold unsupplemented RPMI 1640. The cell suspension was left on ice for 10 min and then electroporated with a Bio-Rad Gene Pulser set at a capacitance of 960 μF and 250 V. Transfected cells were placed at 37°C for 20 min before being resuspended in 10 ml of conditioned medium. Cells were harvested and extracts were prepared 40 to 48 h posttransfection. Adherent cells were transfected by the calcium phosphate coprecipitation method (10). Briefly, 2×10^5 to 5×10^5 cells were seeded onto 6-cm-diameter tissue culture dishes the evening before transfection. Thirty-two microliters of 2 M $CaCl_2$ was added to plasmid mixes, and each mix was made up to a final volume of 250 μl with double-distilled water. This solution was added, drop by drop, to 250 μl of 2× HBS (10) and incubated at room temperature for 20 to 30 min until a very fine white precipitate formed. During this incubation, the medium in each dish was replaced with fresh medium, and the precipitate was then added to the relevant dish. The precipitate was left on cells overnight and then removed by two washes with phosphate-buffered saline (PBS), and the cells were refed with 5 ml of fresh growth medium. Cells were harvested and lysates were prepared 24 h later. In all transfections, the total amount of input DNA was kept constant by including the appropriate empty vector. Every experiment was performed a minimum of three times with multiple batches of plasmid DNA and 2 μg of pSV-β-gal was included as a control in all transfections.

Transfected B cells were harvested by centrifugation (1,100 rpm for 5 min at 4°C), washed in 10 ml of ice-cold PBS, pelleted, and transferred to 1.5-ml tubes in 1 ml of PBS. Cells were pelleted and resuspended in 60 μl of cold 0.25 M Tris-HCl (pH 8.0) and then lysed by four cycles of freeze-thaw (5 min in dry ice-ethanol and 5 min in a 37°C water bath followed by a 10-s vortex). Cell debris was removed by centrifugation (14,000 rpm in an Eppendorf 5402 for 15 min at 4°C), and the supernatant was transferred to a fresh tube and stored at −70°C for a maximum of 4 weeks. Adherent cells were harvested after the medium was removed and the cells were washed with 5 ml of ice-cold PBS. They were then scraped into 1 ml of PBS and transferred to 1.5-ml tubes. Cells were pelleted (6,500 rpm in an Eppendorf 5402 for 30 s at 4°C) and resuspended in 60 μl of cold 0.25 M Tris-HCl (pH 8.0) and processed as described above for B cells. All extracts were normalized to β-galactosidase activity, as previously described (4), before thin-layer chromatography (TLC) assays were performed.

Liquid scintillation CAT assays with [^{14}C]acetyl coenzyme A and TLC CAT assays with [^{14}C]chloramphenicol have been described previously (4, 9, 19, 43). When necessary, extracts were diluted to ensure the assay was within the linear range (approximately 20 to 40% conversion for the TLC-based assays and 1,000 to 80,000 cpm for the liquid scintillation-based assays).

Western blotting. Protein gel electrophoresis and Western blotting were performed as described previously (3) with E3C.A10, a monoclonal antibody which recognizes the epitope WAPSV, i.e., aa 682 to 686 of the B95-8 EBNA3C protein (25), or a rabbit anti-CBF1/RBP-Jκ serum (16).

Immunofluorescence microscopy. Forty-eight hours after transfection with pBKCMVΔ346-543 in 3.5-cm-diameter tissue culture dishes, C33A cells were washed twice with ice-cold PBS and then fixed with acetone-methanol (1:1) for 5 min at −20°C. Cells were rehydrated for 10 to 15 min in PBS containing 20% normal rabbit serum at room temperature. Monoclonal antibody E3C.A10 (neat tissue culture supernatant) was added to the dishes, and they were incubated at 37°C for 60 min. After the cells were washed three times with ice-cold PBS, a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody was added and the dishes were again incubated at 37°C for 60 min. The cells were finally washed three times with ice-cold PBS, Citifluor was applied, and they were examined by fluorescence microscopy.

GST fusion pull-down assays. Five microliters of each in vitro-translated protein was incubated 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-Cl [pH 8.0]) with 1 mg of bovine serum albumin per ml and 5 μl of glutathione *S*-transferase (GST)-coated Sepharose beads. The reaction mixtures were incubated for 60 min rotating at 4°C to preclear. The beads were removed by centrifugation, and the supernatants were incubated with beads coated with 100 to 500 ng of GST-Jκ fusion protein or GST-coated beads as a control. The reaction mixtures were incubated for an additional 60 min at 4°C. The beads were collected by centrifugation and washed four times in 1 ml of NETN buffer (200 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris-Cl [pH 8.0]), with vigorous vortexing during each wash. The pellets of beads were boiled in 80 μl of SDS sample buffer to solubilize the proteins which were then resolved on an SDS-7.5% polyacrylamide gel and visualized by autoradiography. The coupled transcription-translation reactions were carried out with the TNT system (Promega) according to the manufacturer's instructions and as described previously (4). The GST fusion proteins were produced, purified, and quantified as described previously (4, 27).

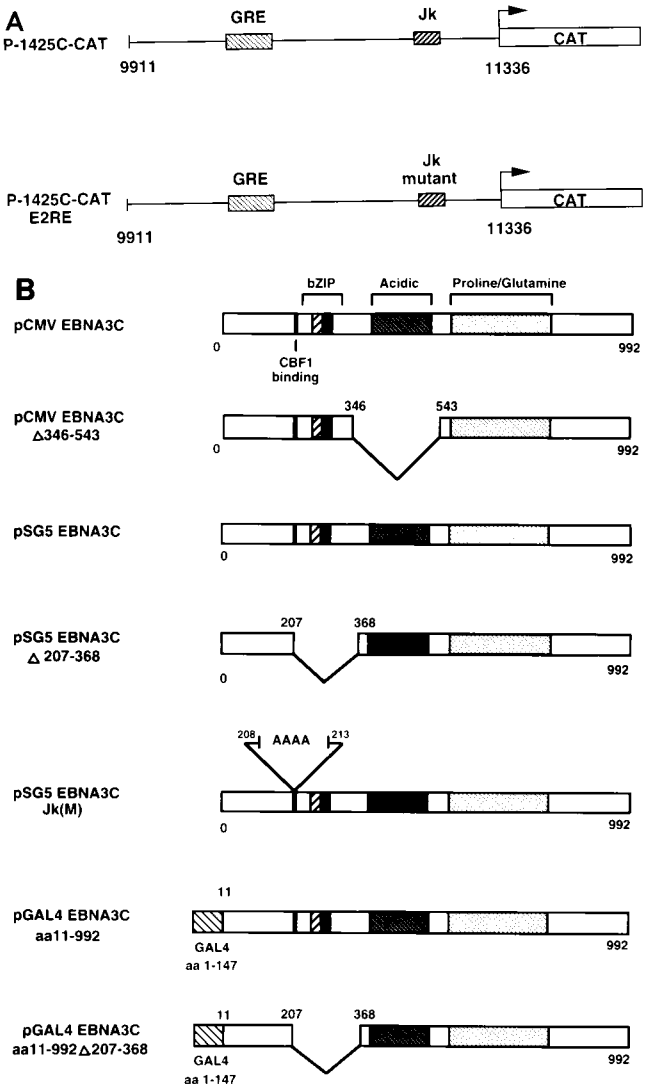


FIG. 1. (A) Cp reporter constructs. p-1425C-CAT includes a 1.425-kbp fragment of the C promoter upstream of the CAT gene. p-1425C-CAT-E2RE encodes the Cp fragment, containing a mutated CBF1/RBP-Jκ site (5′-GTGGG AAA-3′ mutated to 5′-GTGAATTC-3′), cloned upstream of the CAT gene. This mutated site no longer binds CBF1/RBP-Jκ in band shift assays (7, 21). The glucocorticoid response element (GRE), the CBF1/RBP-Jκ binding site (Jκ), and the transcription start site (↱) are indicated. The numbers are nucleotides. (B) Diagrammatic representation of the proteins encoded by the effector plasmids used in this study (for details of all constructions, see Materials and Methods). pBKCMV plasmids are labelled pCMV.

RESULTS

We and others previously showed that fusions of EBNA3C (aa 11 to 992) to the DBD of GAL4 could repress reporter gene expression from plasmids including GAL4 DNA-binding sites; repression was absolutely dependent on specific DNA binding. In order to determine whether wild-type EBNA3C could have a similar effect on any naturally occurring promoters, we surveyed various potential targets. Since it is quite common for regulators of gene expression to inhibit their own transcription, e.g., human cytomegalovirus IE2 (29, 30), the effect of EBNA3C on the EBNA promoter Cp was determined.

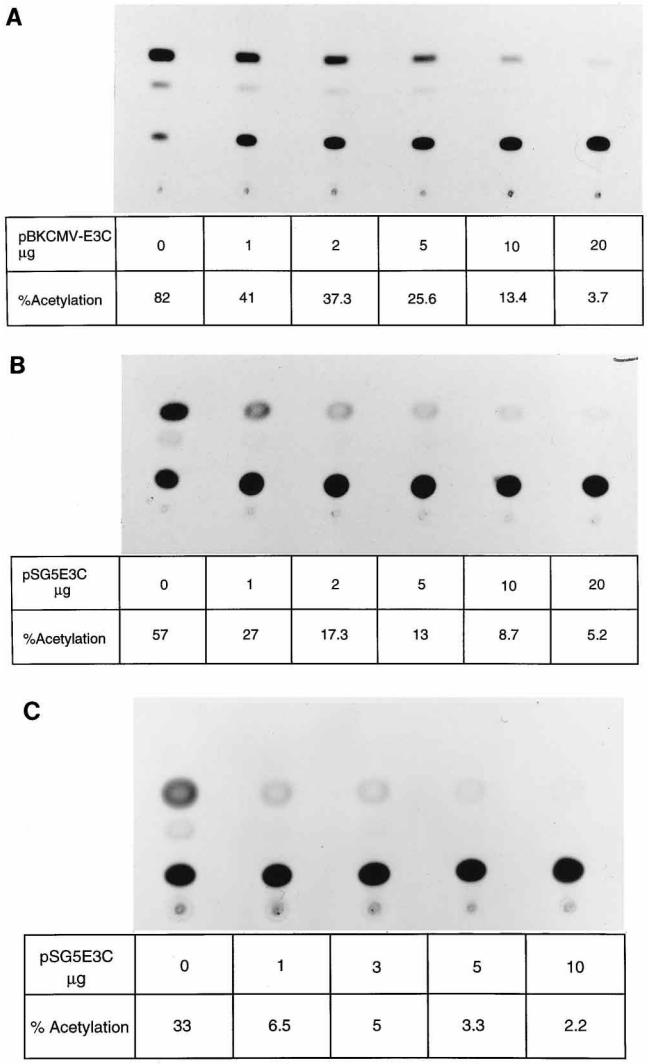


FIG. 2. EBNA3C can repress its own promoter, Cp. DG75 cells were transfected with 5 μg of p-1425C-CAT which includes the C promoter (Cp) upstream of the CAT gene. These and all subsequent transfections also included 2 μg of pSV-β-gal in addition to the indicated amount of pBKCMV-EBNA3C (pBKCMV-E3C) (A) or pSG5-EBNA3C (pSG5E3C) (B and C). Lysates were prepared 48 h after transfection and, after normalization to β-galactosidase activity, analyzed by TLC. (C) A similar series of transfections performed in EBNA2-positive Raji BL cells.

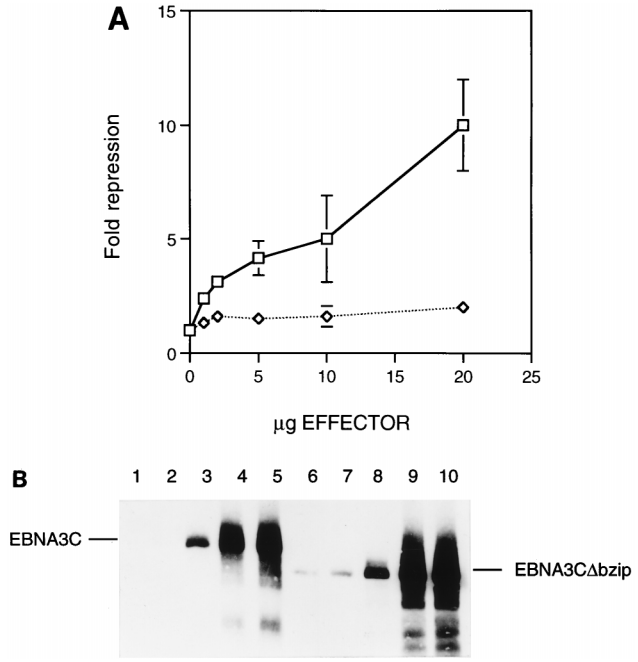


FIG. 3. Repression of Cp requires EBNA3C aa 207 to 368 which include a potential b-ZIP motif and a CBF1/RBP-Jκ binding site. The activity of pSG5-EBNA3CΔ207-368 was compared with that of the wild-type protein. (A) DG75 cells were transfected with 5 μg of p-1425C-CAT with the indicated amount of pSG5-EBNA3CΔ207-368 (◇) or pSG5-EBNA3C (□). The plotted values are the mean and standard deviation values for three experiments analyzed by the nonchromatographic liquid scintillation method after normalization to β-galactosidase activity (43). (B) The results of Western blot analysis show that the mutant and wild-type proteins are expressed at similar levels. Lanes 1 to 5, lysates from cells transfected with 1, 2, 5, 10, and 20 μg of pSG5-EBNA3C, respectively; lanes 6 to 10, similar lysates from cells transfected with pSG5-Δ207-368 (labelled EBNA3CΔbzip).

Expression from p-1425C-CAT (Cp) is repressed by EBNA3C. In an initial series of experiments, the Cp reporter plasmid p-1425C-CAT (Fig. 1A) was cotransfected with increasing amounts of the EBNA3C expression vector pBKCMV-EBNA3C or pSG5-EBNA3C (Fig. 1B) into the Burkitt's lymphoma-derived cell line DG75. These cells sustain a reasonably high level of basal activity from Cp in the absence of EBNA2 and are therefore particularly suitable for assaying repression. However, the promoter can still be activated ~10-fold by EBNA2 in these cells (unpublished data). Multiple titrations showed that wild-type EBNA3C expression represses CAT activity driven from Cp by up to 10- to 20-fold (Fig. 2A and B; see also Fig. 3, 4, and 7). The specificity of the repression to Cp was ensured by including pSV-β-gal (which is unaffected by EBNA3C) in all the transfections and calculating the CAT values only after normalizing to the β-galactosidase activity. The level of input DNA in this and all subsequent experiments was kept constant by the inclusion of control plasmids (pBKCMV or pSG5). Raji BL cells, which express all the EBNA proteins except EBNA3C (1), were also transfected in similar experiments in order to determine whether repression of Cp occurred in the presence of endogenous EBNA2 encoded by the Raji virus. The results (Fig. 2C) show that in these cells EBNA3C represses Cp as efficiently as in the DG75 EBNA2-negative background.

The region of EBNA3C which includes a potential b-ZIP motif and a CBF1/RBP-Jκ binding site is necessary for the repression of Cp. The region of EBNA3C between aa 207 and 368 includes two motifs which could be involved in targeting EBNA3C to Cp DNA. b-ZIP motifs (Fig. 1B and introduction) are frequently associated with specific DNA binding and in addition the residues flanking aa 209 to 212 of EBNA3C bind to the cellular DNA-binding factor CBF1/RBP-Jκ (53). A deletion mutant from which aa 207 to 368 were removed (pSG5-EBNA3CΔ207-368 [Fig. 1B]) was therefore constructed in order to remove these features from EBNA3C and test the hypothesis that it is necessary for the repression of Cp. This protein failed to repress Cp-driven reporter activity significantly, whereas wild-type EBNA3C, expressed from a similar expression vector, repressed expression from Cp by 10- to 15-fold. Transfections were repeated a minimum of three times, and the CAT activity was assayed by the liquid scintillation method (43). The results, graphically represented as the mean and standard deviation values in Fig. 3A, showed that

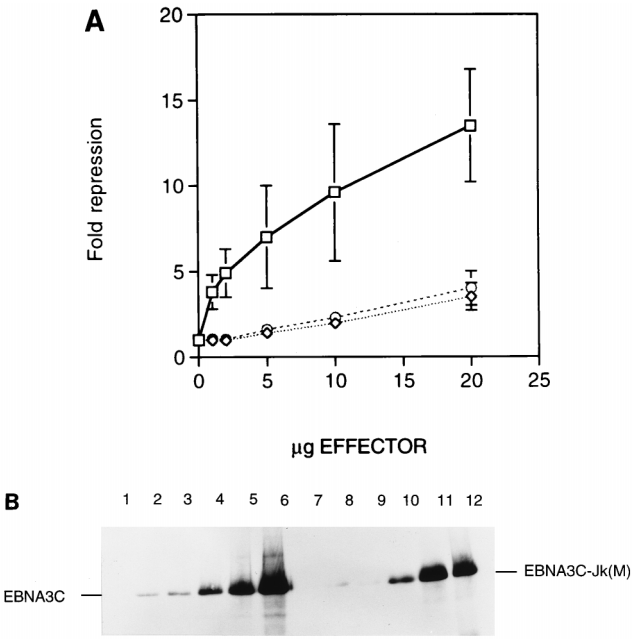


FIG. 4. (A) Site-specific mutation of both the CBF1/RBP-Jκ binding site in EBNA3C and the E2RE (CBF1/RBP-Jκ binding site) in Cp abrogates repression of Cp. DG75 cells were transfected with the amounts of pSG5-EBNA3C or pSG5-EBNA3C-Jκ(M) indicated and 5 μg of p-1425C-CAT or p-1425C-CAT-E2RE. Extracts were analyzed by the liquid scintillation method in order to directly compare the activities of mutant protein and mutant promoter with wild-type activities. The mean and standard deviation values of three titration experiments calculated after normalization to β-galactosidase activity are shown. Symbols: □, pSG5-EBNA3C plus wild-type Cp; ◇, pSG5-EBNA3C-Jκ(M) plus wild-type Cp; ○, pSG5-EBNA3C plus p-1425C-CAT-E2RE. (B) Western blot showing expression of EBNA3C-Jκ(M). Lanes 2 to 6, extracts from cells transfected with 1, 2, 5, 10, and 20 μg of pSG5-EBNA3C, respectively; lanes 8 to 12, extracts from cells similarly transfected with pSG5-EBNA3C-Jκ(M); lanes 1 and 7, extracts from untransfected cells.

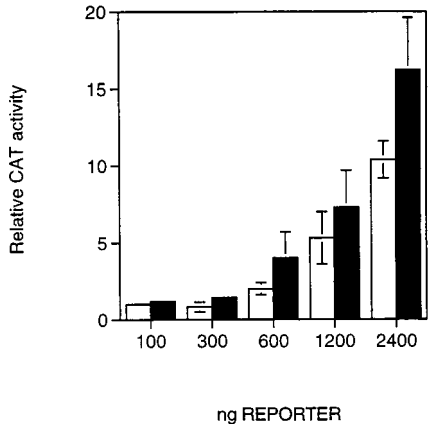


FIG. 5. The basal activities of Cp and Cp with a mutant E2RE are very similar. Parallel transfections of DG75 cells were performed with the amounts of plasmid DNA indicated. CAT activity was determined by the liquid scintillation assay, and the mean and standard deviation values for three separate titration experiments are shown. p-1425C-CAT (white bars) and p-1425C-CAT-E2RE (black bars). After corrections for β-galactosidase activity were made, the CAT activity from 100 ng of wild-type promoter plasmid was given the value of one, and all other values are shown relative to this value.

this deletion mutant is unable to repress Cp activity, although after transfection it is expressed at a level similar to that of wild-type EBNA3C (for example, Fig. 3B).

Repression of Cp requires the CBF1/RBP-Jκ binding site in EBNA3C and the E2RE in Cp. Zhao and colleagues identified a short sequence of amino acids surrounding aa 209 to 212 which is both necessary and sufficient for the functional interaction between EBNA3C and CBF1/RBP-Jκ (53). This site is located near the potential b-ZIP and was also deleted in Δ207-368. An expression vector was therefore constructed in which aa 209 to 212 were replaced with alanine residues in a manner similar to that described previously (53). The results of cotransfections with p-1425-CpCAT demonstrated a requirement for these amino acids in the repression of Cp (Fig. 4A). Both mutant and wild-type protein were expressed at similar levels after transfection (Fig. 4B).

Because the site in EBNA3C necessary for binding to CBF1/RBP-Jκ is required for repression of wild-type Cp, the reciprocal experiment with a mutant promoter and wild-type protein was performed. A Cp promoter in which the E2RE or RBP-Jκ binding site was mutated (so that it no longer binds CBF1/RBP-Jκ) was subcloned from pGL-E2RE (7) upstream of the CAT gene. The results of cotransfecting this with pSG5-EBNA3C are also shown in Fig. 4A. The data are consistent with a model in which CBF1/RBP-Jκ bound to its cognate site in Cp targets EBNA3C to the promoter. That is, the mutation reduced the level of repression by EBNA3C to a maximum of threefold. This is consistent with the observation that the same mutation significantly reduced EBNA2-mediated transactivation of Cp.

Cp and Cp with a mutated E2RE have similar levels of basal activity. An alternative interpretation of the experiments described in the previous two sections was that, in DG75 cells, Cp activity is mediated by a cellular activation pathway (for instance, Notch IC [see introduction]) acting through CBF1/RBP-Jκ and that EBNA3C, by interfering with CBF1/RBP-Jκ binding to DNA apparently induces repression. In order to establish whether E2RE does mediate activation through CBF1/RBP-Jκ in these cells, the p-1425C-CAT and p-1425C-CAT-E2RE reporters were transfected, in parallel, into DG75 cells. The results of multiple experiments using various batches of plasmid failed to show a significant difference in the basal activity of these two promoters in DG75 (Fig. 5). It therefore seems very unlikely that EBNA3C represses by destabilizing an

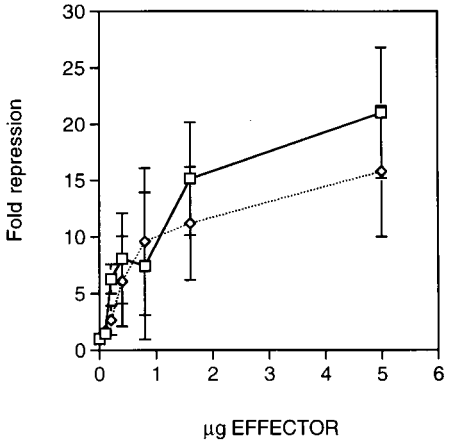
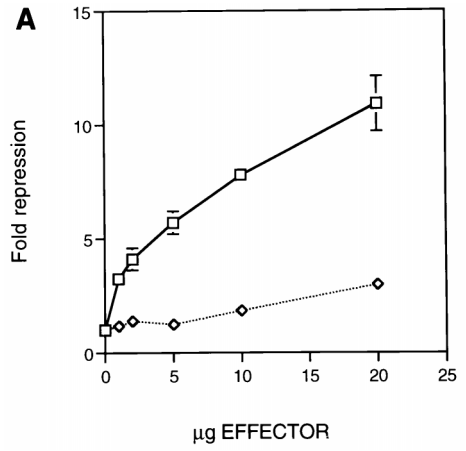
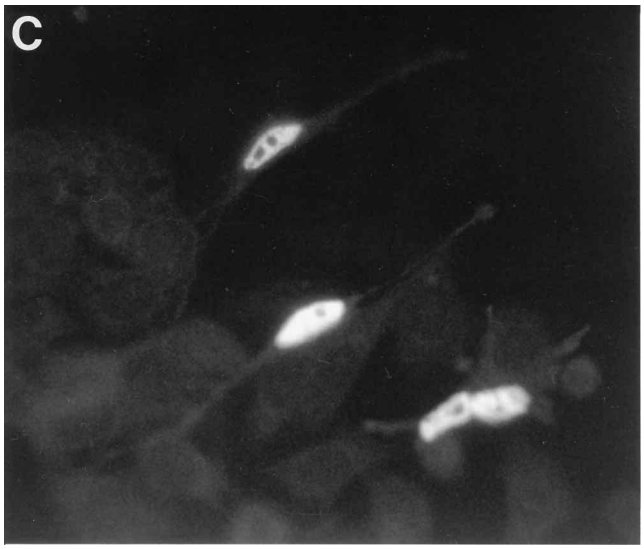
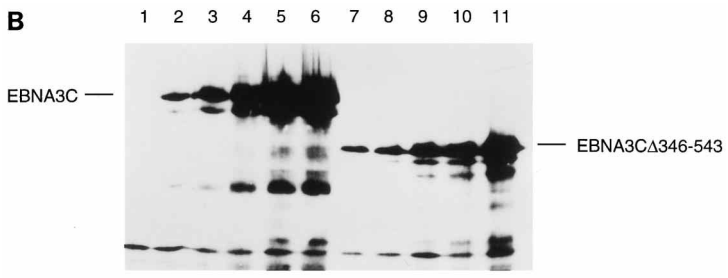


FIG. 6. A GAL4 DBD can functionally substitute for aa 207 to 368 of EBNA3C. DG75 cells were transfected with 5 μg of pUAS-CAT along with the number of micrograms of pGAL4-E3CΔbZip (pGAL4EBNA3Caa11-992Δ207-368) indicated. Multiple transfections of DG75 with pUAS-CAT along with either mutant (Δ207-368) or wild-type pGAL4-EBNA3C are compared. Symbols: □, pGAL4-EBNA3Caa11-992; ◇, pGAL4-EBNA3CΔ207-368.







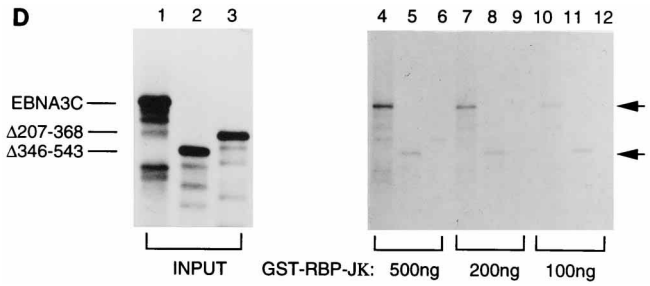


FIG. 7. EBNA3C requires aa 346 to 543 to repress Cp. (A) DG75 cells were transfected with 5 μg of p-1425C-CAT and the indicated amounts of pBKCMV-EBNA3CΔ346-543 (◇) and pBKCMV-EBNA3C (□). The plotted values are the mean and standard deviation values for three titration experiments analyzed by the liquid scintillation method after normalization to β-galactosidase activity. (B) Western blot analysis of EBNA3C expression 48 h after transfection into DG75 cells. Lane 1, lysate from untransfected DG75 cells; lanes 2 to 6, lysates from cells transfected with 1, 2, 5, 10, and 20 μg of pCMV-EBNA3C, respectively; lanes 7 to 11, lysates from cells similarly transfected with pCMV-EBNA3CΔ346-543. (C) Immunofluorescence staining of C33A cells transfected with 5 μg of pCMV-EBNA3CΔ346-543, illustrating the nuclear distribution of the mutant protein. (D) GST pull-down experiment showing that EBNA3CΔ346-543 retains the ability to bind to GST-RBP-Jκ fusion protein. Approximately equal amounts of ^{35}S-labelled EBNA3C (lanes 1, 4, 7, and 10), EBNA3CΔ346-543 (lanes 2, 5, 8, and 11), and EBNA3CΔ207-368 (lanes 3, 6, 9, and 12) were incubated separately with the amounts of GST-CBF1/RBP-Jκ indicated. The amount of labelled protein included in each binding reaction is shown below lanes 4 to 12.

activating complex which includes CBF1/RBP-Jκ on the wild-type promoter. The failure to repress the mutant promoter described above must therefore be a consequence of EBNA3C not being efficiently targeted to Cp DNA.

Further evidence for a model in which EBNA3C represses transcription by specifically associating with Cp comes indirectly from an experiment in which the deletion of aa 207 to 368 was functionally replaced by a GAL4 DBD. Cotransfection of pGAL4-EBNA3Caa11-992Δ207-368 with pUAS-CAT showed that this fusion polypeptide represses almost as well as a wild-type GAL4-EBNA3C fusion (Fig. 6). These results confirm that aa 207 to 368 are necessary to direct EBNA3C to DNA and are therefore consistent with CBF1/RBP-Jκ playing a central role in targeting EBNA3C to Cp.

Repression of Cp requires the domain located between aa 346 to 543 which is also required for repression by EBNA3C-GAL4 DBD fusions. When GAL4-EBNA3C was targeted to a variety of promoters which were engineered to include five binding sites for GAL4, it repressed reporter gene activity 15- to 40-fold (see above and reference 4). Analysis of GAL4 fusions containing smaller fragments of EBNA3C revealed two independent regions which contribute to repressor activity. The more potent of these domains is highly acidic (aa 280 to 525), and when linked to the GAL4 DBD, it repressed almost as well as the near-full-length polypeptide (aa 11 to 992) (4). Deletion of this region significantly reduced the degree of repression but did not abolish it completely. To analyze further the contribution of this highly acidic region of EBNA3C to the repression of Cp, cotransfections were performed with the plasmid pBKCMV-EBNA3CΔ346-543 in which this major repression domain had largely been deleted (Fig. 1B). Whereas in parallel transfections pCMV-driven wild-type EBNA3C repressed Cp activity ~10-fold, the deletion mutant had no significant effect on reporter gene activity driven by Cp (Fig. 7A). Western blot analysis of extracts made from cells after transfection showed that both proteins were expressed at similar levels (Fig. 7B for example). The region of EBNA3C which includes aa 346 to 543 appears to be a major requirement for repression of Cp, which is consistent with it including an important repression domain. The deleted region of the protein also includes the amino acid sequence KKPRKLP which was identified as a potential nuclear localization signal (28). However, these amino acids are not essential to target EBNA3C to the nucleus since immunofluorescence staining of cells 48 h after transfection with pBKCMV-EBNA3CΔ346-543 showed EBNA3C distributed in nuclei (Fig. 7C). Previous reports suggest that EBNA3CΔ346-543 should still bind to CBF1/RBP-Jκ (35, 53), which was confirmed by GST pull-down experiments (Fig. 7D). Since we found that at high concentrations of GST-RBP-Jκ fusion protein there was significant nonspecific bind-

ing to various in vitro-translated polypeptides (unpublished data), a titration was performed and within the limitations of this assay, it can be seen that Δ346-543 binds to RBP-Jκ as efficiently as wild-type EBNA3C. In contrast and as predicted, the Δ207-368 mutant (53), which lacks the characterized CBF1/RBP-Jκ binding site, is unable to bind at the same concentrations of fusion protein. None of the in vitro-translated polypeptides bound to wild-type GST or a GST fusion containing the N terminus of TATA-binding protein (data not shown and reference 4). These results imply that repression of Cp is dependent on more than the simple recruitment of EBNA3C by CBF1/RBP-Jκ. We hypothesize that aa 346 to 543 are necessary for the interaction between EBNA3C and another cofactor in transcriptional repression.

Repression by EBNA3C tethered to DNA can be cell type specific, which suggests that it might require a corepressor. It has been suggested that GAL4-EBNA3C represses transcription by recruiting CBF1/RBP-Jκ to DNA (49). However, the results presented above and elsewhere (4) indicate that this is unlikely and that in these fusions, GAL4-DBD fulfils the role of CBF1/RBP-Jκ in specifically localizing EBNA3C to DNA. We and others previously showed that repression by GAL4-EBNA3C is not restricted to human B cells but was also seen in human osteosarcoma cells, murine fibroblasts, and human carcinoma cells (4, 49). However, two cell lines have subsequently been identified in which the herpes simplex virus thymidine kinase promoter including five copies of UAS (pUAS-CAT) functions but is not repressed by GAL4-EBNA3C. SAOS-2 is a cell line derived from a human osteosarcoma which supports measurable basal activity from pUAS-CAT. Cotransfection of increasing amounts of GAL4-EBNA3C (up to 20 μg) had no detectable effect on CAT activity (Fig. 8A). Similar cotransfections using the cervical cancer-derived cell line C33A also showed no repression (Fig. 8B). The level of fusion protein in the transfected cells was judged by Western blotting to be equivalent to that in transfected DG75 cells, and nuclear localization was demonstrated (data not shown). These data suggest that SAOS-2 and C33A cells might lack a corepressor which interacts with GAL4-EBNA3C. Alternatively, repression by EBNA3C may depend on posttranslational modification, such as phosphorylation, which may differ in these cells. Lack of repression is not due to the absence of CBF1/RBP-Jκ, since it is abundantly expressed in both lines (Fig. 8C). Unfortunately, the basal activity of Cp (p-1425C-CAT) in SAOS-2 and C33A cells was too low for repression of this promoter to be measured, so a direct assessment of Cp repression could not be determined in these cells.

EBNA3C has no detectable effect on the CD21 promoter. It has been reported that both EBNA2 and EBNA3C stimulate increased expression of the CD21 antigen on the surfaces of some Burkitt's lymphoma-derived B cells (6, 50). It has been proposed that the EBNA3C-mediated activation of CD21 may occur as a result of EBNA3C binding to CBF1/RBP-Jκ and inhibiting its DNA binding activity (34). Since CBF1/RBP-Jκ can act as a transcriptional repressor when bound to DNA (13, 55), displacement of CBF1/RBP-Jκ from its cognate site by EBNA3C would result in derepression, that is, activation of promoters bearing CBF1/RBP-Jκ binding sites. An analysis of the CD21 promoter DNA sequence reveals two potential CBF1/RBP-Jκ binding sites (6 of 7 matches of the core 5′-GTGGGAA-3′ sequence. However, it has not yet been reported whether CBF1/RBP-Jκ binds to the CD21 promoter.

We examined the effect of EBNA2 and EBNA3C on reporter constructs including up to 1235 bp upstream of the CD21 transcriptional initiation site (Fig. 9). A shorter promoter (−467 CD21-CAT) was included in the study because it includes the potential CBF1/RBP-Jκ binding sites, but lacking an upstream negative element, it has a moderately high basal activity in B cells (12a, 31).

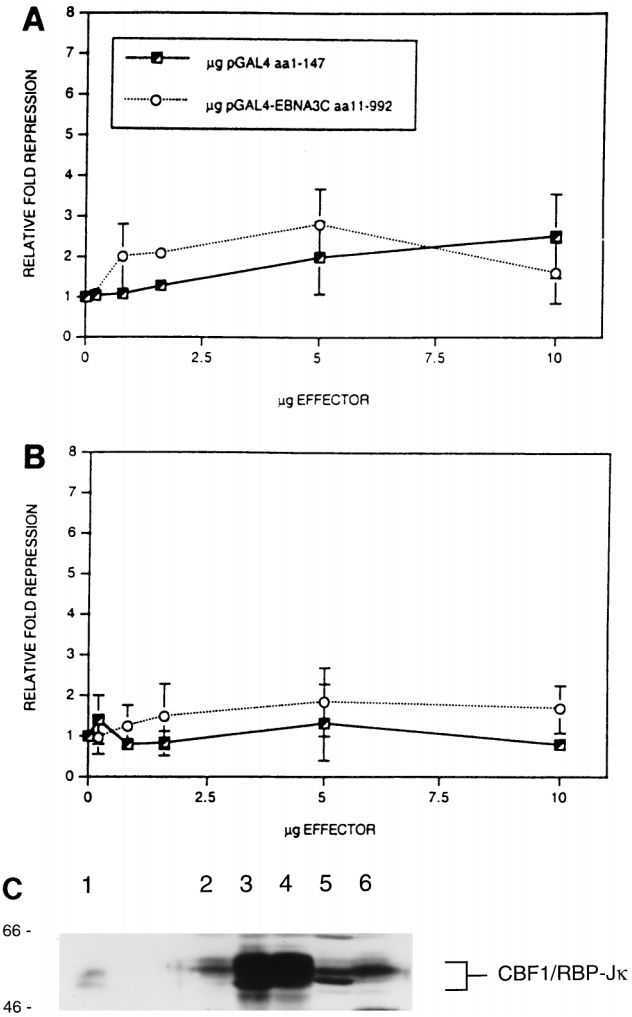


FIG. 8. Identification of two cell lines which do not support the transcriptional repression mediated by GAL4-EBNA3Caa11-992. SAOS-2 or C33A cells were transfected with 5 μg of pUAS-CAT reporter plasmid and the indicated amounts of pGAL4-EBNA3Caa11-992. Titration experiments were performed in parallel with transfections using pGAL4aa1-147 as a negative control. Normalized CAT activities were expressed as fold repression relative to the background level, which was arbitrarily set at 1. The plotted values are the mean and standard deviation values for three separate experiments in SAOS-2 (A) and C33A (B) cells. (C) CBF1/RBP-Jκ status of various cell lines. Approximately 10^6 cells of each type were resuspended in SDS sample buffer, sonicated, boiled, and clarified, and proteins were separated on an SDS–10% polyacrylamide gel. Following transfer to nitrocellulose, the filter was incubated with a rabbit polyclonal antibody which recognizes human CBF1/RBP-Jκ (isoform I). The position of CBF1/RBP-Jκ as a polypeptide of approximately 60 kDa is indicated and is associated with a slightly faster migrating species, which is consistent with previous observations (16). The positions of molecular mass markers (in kilodaltons) are shown to the left. Lane 1, in vitro-transcribed and -translated CBF1/RBP-Jκ, which was used as a marker; lane 2, DG75 cells; lane 3, NIH 3T3 cells; lane 4, U2OS cells; lane 5, C33-A cells; lane 6, SAOS-2 cells.

Cotransfections of p-1235CD21-CAT into DG75 cells with increasing amounts of pSV-EBNA2 induced CAT activity from the basal level as much as 27-fold (Fig. 10A); this suggests there might be legitimate CBF1/RBP-Jκ binding sites in the promoter. A similar titration performed with p-467CD21-CAT produced ~17-fold activation. However, when these CD21

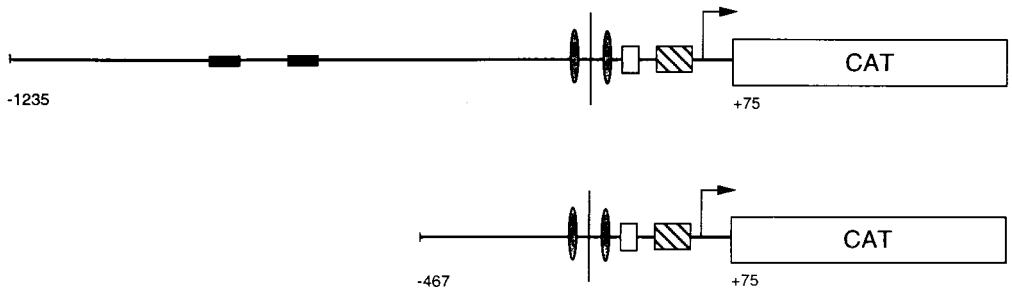


FIG. 9. CD21 promoter constructs. A full-length CD21 promoter construct (p-1235CD21-CAT) (top) and a 467-bp truncation (p-467CD21-CAT) (31) (bottom) are shown. The TATA box (▨), an AP1-like squence (□), an Sp1 site (|), and sequences which are identical to those found in the CD23 promoter (■) are shown. Two potential CBF1/RBP-Jκ binding sites (six of seven matches of the core consensus sequence 5′-GTGGGAA-3′) (◖ ◗) are indicated. Numbers are the nucleotide positions relative to the transcription start site (↱).

promoter constructs were each cotransfected with pBKCMV-EBNA3C, there was neither an increase nor decrease in CAT activity (Fig. 10B). In all the experiments with the CD21 promoter, the basal activity was low enough for transactivation to be easily detected (for example, Fig. 10A). The activity of the longer promoter construction (p-1235CD21-CAT) was so low that TLC assays would not reveal repression, but even at this low level of activity, the more-sensitive liquid scintillation assays (43) would have revealed severalfold repression if it occurred. The basal activity from p-467 was sufficiently high to register up to 10-fold repression in either assay system, but no activity was detected in this study (Fig. 10B). In these experiments the level of EBNA3C, as judged by Western blotting, was equivalent to that in similar experiments in which Cp was repressed (data not shown).

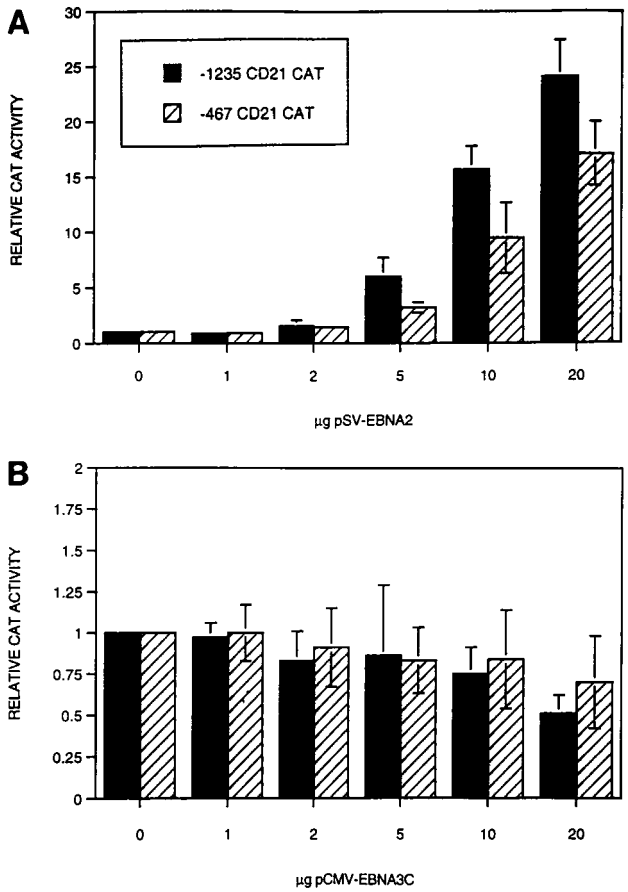


FIG. 10. Effect of EBNA2 and EBNA3C on the CD21 promoter. (A) DG75 cells were transfected with 5 μg of p-1235CD21-CAT or p-467CD21-CAT and the amounts of pSV-EBNA2 indicated. (B) DG75 cells were transfected with 5 μg of p-1235CD21-CAT or p-467CD21-CAT and the amounts of pBKCMV-EBNA3C indicated. Multiple experiments showed that EBNA2 activates but EBNA3C has no effect on p-1235CD21-CAT or p-467CD21-CAT: in each case, the mean and standard deviation values are shown for the results, after normalization to β-galactosidase activity, of three independent titration experiments analyzed by the liquid scintillation method.

DISCUSSION

EBNA3C specifically represses Cp. In this study, we have shown EBNA3C represses expression from reporter plasmids which include the major promoter for EBNA expression, Cp. This is the first demonstration that EBNA3C can specifically regulate an EBV promoter independently of EBNA2 activation. Several lines of evidence indicate that this is specific to Cp. The demonstration in earlier reports that GAL4-EBNA3C repressed the herpes simplex virus thymidine kinase promoter, mouse B-*myb* promoter, and the human immunodeficiency virus long terminal repeat only when GAL4 binding sites were present in the reporter, showed that GAL4-EBNA3C(11-992) does not repress by nonspecific cytotoxicity, squelching, or posttranslational phenomena (4, 49). Similarly, in the present study, several CD21 promoter-CAT plasmids, the E2RE-Cp mutant and also the pSV-β-gal control vector were all unaffected by full-length EBNA3C (Fig. 9 and unpublished data). Expression from other EBV-derived promoters such as BZLF1 (−221) and LMP1(−512) was not significantly affected when they were cotransfected with EBNA3C expression vectors (24, 34; also data not shown), and the human B-*myb* promoter is weakly transactivated by EBNA3C (27). All these data testify to the specificity of EBNA3C-mediated repression to Cp.

Repression requires CBF1/RBP-Jκ as a bridge to promoter DNA and probably involves another cofactor. It is well established that both in vitro and in vivo EBNA3C and CBF1/RBP-Jκ can interact. Furthermore, it has been proposed that EBNA3C can abolish EBNA2-mediated transactivation by inhibiting the binding of the EBNA2-CBF1/RBP-Jκ complex to its cognate binding site on DNA (24, 34, 49, 53). Deletion and mutation analyses of EBNA3C have shown that amino acids between positions 183 to 240 are both essential and sufficient for the binding to CBF1/RBP-Jκ and inhibition of EBNA2 transactivation; substitution of aa 209 to 212 with alanine residues abolishes the interaction (53).

Our current data convincingly show that both the CBF1/RBP-Jκ binding site in EBNA3C (aa 209 to 212) and the

interaction between CBF1/RBP-Jκ and the E2RE in Cp are required but are not sufficient for EBNA3C repression of Cp reporter gene expression. This is consistent with a physical interaction between the two proteins targeting EBNA3C to the E2RE in Cp. This model is supported by the data showing that EBNA3C can also repress transcription if it is tethered to various promoters through a GAL4-DBD (4, 49). However, an alternative interpretation of this repression phenomenon must be considered. In vitro, EBNA3C can disrupt CBF1/RBP-Jκ complexes with DNA, so it is possible that in vivo EBNA3C destabilizes the interaction of CBF1/RBP-Jκ with the E2RE in Cp. In order to cause repression by this mechanism, CBF1/RBP-Jκ would have to be functioning as an activator of Cp in DG75 cells. Although mammalian activation pathways like Notch exist and could be active in DG75 cells, for a number of reasons we think this is an unlikely explanation for the repression of Cp seen here. First, a mutant of EBNA3C (Δ346-543), which retains the RBP-Jκ binding site at aa 209 to 212 and binds to CBF1/RBP-Jκ in vitro as efficiently as the wild-type protein, does not repress Cp. This suggests that more than just the interaction with RBP-Jκ is required. Second, if CBF1/RBP-Jκ were activating Cp in DG75, then the mutant p-1425C-CAT E2RE promoter would have significantly lower activity in DG75 cells than the wild-type promoter. This is not the case however; a careful comparison showed that there is very little difference between the basal activities of the two promoters in these cells. If anything, the mutant is slightly more active. This is consistent with other reports of comparisons between reporter plasmids including wild-type CBF1/RBP-Jκ binding sites and their mutant counterparts in various cells, including DG75 (7, 47). Consequently, while our data suggest that EBNA3C physically interacts with CBF1/RBP-Jκ in vivo, they are not entirely consistent with previous models which proposed that EBNA3C repression (of EBNA2 transactivation) is due to EBNA3C inhibiting the binding of CBF1/RBP-Jκ to DNA rather than forming a complex with it. Such models were based largely on the observation that the introduction of EBNA3C into electromobility shift assays (EMSAs) disrupted CBF1/RBP-Jκ complexes with DNA. We think the most likely explanation for the discrepancy between these data and our current model is that EMSAs do not always accurately reflect in vivo interactions. This is certainly the case for EBNA2, since although it is widely accepted that CBF1/RBP-Jκ recruits EBNA2 to DNA in vivo, under some conditions EBNA2 disrupts the binding of CBF1/RBP-Jκ to DNA in EMSAs (49).

Because aa 346 to 543 are essential for the repression of Cp and encompass the region which, when linked to the GAL4 DBD, represses almost as well as wild-type EBNA3C (4), we suggest that it makes contact with a cellular protein which mediates repression. Since such a molecule may be absent or unavailable for binding in C33A and SAOS-2 cells, this corepressor is unlikely to be an essential component of the transcription machinery. It is also unlikely to be CBF1/RBP-Jκ, since this is abundantly expressed in these cells. We previously showed that EBNA3C can bind to pRb in vitro (27) and both C33A and SAOS-2 cells lack a functional pRb (39), so it is very tempting to speculate that pRb might be such a corepressor. It may also be significant that GAL4-EBNA3C represses in H1299 and U20S cells which, like C33A and SAOS-2, are derived from a human carcinoma and an osteosarcoma, respectively, but both have a normal pRb (4, 23, 40; unpublished data). The Rb protein has been identified as a transcriptional repressor when targeted to DNA (5, 52), but our attempts to rescue EBNA3C-mediated repression in C33A cells by titrating in pCMVpRb have been unsuccessful (our unpublished data). It therefore seems unlikely that pRb is involved directly. However, we need to understand how pRb and its associated cyclin-dependent kinases are regulated (or deregulated) in these various tumor-derived cell lines before we can fully assess the significance of these results.

EBNA3C has no direct effect on the CD21 promoter. When EBNA3C was stably expressed in the EBV-negative lymphoma line BJAB, there was an increase in the level of the CD21 antigen on the cells' surface; this led to the speculation that EBNA3C may regulate the CD21 gene (50). We tested this by cotransfecting plasmids containing the CD21 promoter (31) with EBNA3C expression vectors. Although the CD21 promoter (nucleotide −1235) and the truncated version (−467) are both responsive to EBNA2, we were unable to see any effect produced by the expression of EBNA3C under the same experimental conditions in which it represses Cp. This result was rather unexpected, since the stable-transfection experiments had suggested that the CD21 promoter might be activated by EBNA3C (50). Clearly this is not the case for the CD21 promoter-reporter plasmids used in this study. The p-1235-CAT plasmid was originally defined as including the region upstream of the CD21 transcriptional initiation site which conferred B-cell-specific activity to the reporter gene. It was also found to be structurally similar to CD23, another cellular promoter which is regulated by EBV (31). Our results suggest that EBNA3C does not directly activate CD21. However, at this stage we cannot exclude the existence of an EBNA3C-responsive element not included in p-1235-CAT; such an element could be located further upstream than nucleotide -1235 or possibly in a CD21 intron.

Although the original prediction was that EBNA3C might transactivate the CD21 promoter, in the light of the results with Cp, we should consider why EBNA3C does not repress the CD21 reporters in DG75 cells. There are two potential binding sites for CBF1/RBP-Jκ located in both p-1235CD21-CAT and p-467CD21-CAT, and both of these reporters are efficiently transactivated by EBNA2. If EBNA2 were acting through these sites, then EBNA3C might be expected to repress the CD21 reporters in a similar manner to that of Cp. Since it does not, at least two explanations should be considered. First, the potential E2REs may not be legitimate CBF1/RBP-Jκ binding sites and EBNA2 may transactivate this promoter by alternative mechanisms, as is largely the case for LMP1 (41, 42). A second, more intriguing possibility, is that EBNA2 does in fact act through these sites and that EBNA3C can discriminate between the sites in the CD21 promoter and those in Cp. It will not be possible to distinguish between these scenarios until much more is known about the regulation of CD21 by EBV. These experiments are in progress but are beyond the scope of this investigation.

How does EBNA3C repress gene expression and what is likely to be its significance in the life cycle of EBV? To summarize, we have shown that EBNA3C specifically represses Cp, even when Cp is not activated by EBNA2 and the data are consistent with EBNA3C being targeted to Cp by CBF1/RBP-Jκ. The precise nature of the interaction between EBNA3C and Cp remains to be determined. It is still unclear, for instance, whether additional proteins are involved, whether EBNA3C makes direct contact with DNA, or whether its function is modulated by posttranslational modification. The role, if any, of the potential b-ZIP has yet to be established.

Since Cp is the site of initiation for EBNA mRNA synthesis in most LCLs, these data are consistent with EBNA3C negatively regulating its own expression and also the expression of the other EBNA species. It is likely therefore that EBNA3C cooperates with the other EBNAs in a complex regulatory

feedback loop involving *trans*-activators and -repressors; this ensures tight control of Cp activity, thus maintaining the very low level of EBNA-specific mRNA consistently observed in LCLs (18, 28). Finally, there may be some specificity to the action of EBNA3C which could be determined by the sequences which flank CBF1/RBP-Jκ binding sites in different promoters. Consequently, it is possible that EBNA3C also affects the expression of a subset of unidentified cell genes which include CBF1/RBP-Jκ binding sites in their regulatory elements, which may contribute to the role of EBNA3C in growth transformation by EBV. We are currently addressing these issues.

ACKNOWLEDGMENTS

Stoyan A. Radkov and Mark Bain contributed equally to this study.

This research was partly funded by the Royal Society, London, United Kingdom, which provided a University Research Fellowship to M.J.A., and by the Wellcome Trust, which provided a project grant to M.J.A.

We thank Michael Holers (St. Louis, Mo.) for the CD21 plasmids, Lars Rymo (Gothenburg, Sweden) for the EBNA2 expression plasmid, Diane Hayward (Baltimore, Md.) for the GST-CBF1/RBP-Jκ plasmid, and Erle Robertson (Boston, Mass.) for the RBP-Jκ antiserum. We are also very grateful to Gareth Inman for helpful comments on the manuscript and Gillian Parker for excellent technical assistance.

REFERENCES

1. **Allday, M. J., D. H. Crawford, and B. E. Griffin.** 1988. Prediction and demonstration of a novel Epstein-Barr virus nuclear antigen. Nucleic Acids Res. **16:**4353–4367.
2. **Allday, M. J., D. H. Crawford, and J. A. Thomas.** 1993. Epstein-Barr virus (EBV) nuclear antigen 6 induces expression of the EBV latent membrane protein and an activated phenotype in Raji cells. J. Gen. Virol. **74:**361–369.
3. **Allday, M. J., and P. J. Farrell.** 1994. Epstein-Barr virus nuclear antigen EBNA3C/6 expression maintains the level of latent membrane protein 1 in G_1-arrested cells. J. Virol. **68:**3491–3498.
4. **Bain, M., R. J. Watson, P. J. Farrell, and M. J. Allday.** 1996. Epstein-Barr virus nuclear antigen 3C is a powerful repressor of transcription when tethered to DNA. J. Virol. **70:**2481–2489.
5. **Bremner, R., B. L. Cohen, M. Sopta, P. A. Hamel, C. J. Ingles, B. L. Gallie, and R. A. Phillips.** 1995. Direct transcriptional repression by pRB and its reversal by specific cyclins. Mol. Cell. Biol. **15:**3256–3265.
6. **Cordier, M., A. Calender, M. Billaud, U. Zimber, G. Rousselet, O. Pavlish, J. Banchereau, T. Tursz, G. Bornkamm, and G. M. Lenoir.** 1990. Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. J. Virol. **64:**1002–1013.
7. **Evans, T. J., P. J. Farrell, and S. Swaminathan.** 1996. Molecular genetic analysis of Epstein-Barr virus Cp promoter function. J. Virol. **70:**1695–1705.
8. **Fahraeus, R., A. Jansson, A. Sjoblom, T. Nilsson, G. Klein, and L. Rymo.** 1993. Cell phenotype-dependent control of Epstein-Barr virus latent membrane protein 1 gene regulatory sequences. Virology **195:**71–80.
9. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
10. **Graham, F. L., and A. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
11. **Grossman, S. R., E. Johannsen, X. Tong, R. Yalamanchili, and E. Kieff.** 1994. The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the J kappa recombination signal binding protein. Proc. Natl. Acad. Sci. USA **91:**7568–7572.
12. **Henkel, T., P. D. Ling, S. D. Hayward, and M. G. Peterson.** 1994. Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J kappa. Science **265:**92–95.

12a. **Holers, V. M.** Personal communication.

13. **Hsieh, J. J.-D., T. Henkel, P. Salmon, E. Robey, M. G. Peterson, and S. D. Hayward.** 1996. Truncated mammalian Notch1 activates CBF1/RBP-Jκ-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. Mol. Cell. Biol. **16:**952–959.
14. **Joab, I., D. T. Rowe, M. Bodescot, J. C. Nicolas, P. J. Farrell, and M. Perricaudet.** 1987. Mapping of the gene coding for Epstein-Barr virus-determined nuclear antigen EBNA3 and its transient overexpression in a human cell line by using an adenovirus expression vector. J. Virol. **61:**3340–3344.
15. **Johannsen, E., E. Koh, G. Mosialos, X. Tong, E. Kieff, and S. R. Grossman.** 1995. Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by Jκ and PU.1. J. Virol. **69:**253–262.
16. **Johannsen, E., C. L. Miller, S. R. Grossman, and E. Kieff.** 1996. EBNA-2 and EBNA-3C extensively and mutually exclusively associate with RBPJκ in Epstein-Barr virus-transformed lymphocytes. J. Virol. **70:**4179–4183.
17. **Kallin, B., J. Dillner, I. Ernberg, B. Ehlin-Henriksson, A. Rosen, W. Henle, G. Henle, and G. Klein.** 1986. Four virally determined nuclear antigens are expressed in Epstein-Barr virus-transformed cells. Proc. Natl. Acad. Sci. USA **83:**1499–1503.
18. **Kieff, E.** 1996. Epstein-Barr virus and its replication, p. 2343–2396. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology. Lippincott-Raven, Philadelphia, Pa.
19. **Kingston, R. E., and J. Sheen.** 1995. Isotopic assays for reporter gene activity, p. 9.7.1–9.7.11. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
20. **Le Roux, A., B. Kerdiles, D. Walls, J. F. Dedieu, and M. Perricaudet.** 1994. The Epstein-Barr virus determined nuclear antigens EBNA-3A, -3B, and -3C repress EBNA-2-mediated transactivation of the viral terminal protein 1 gene promoter. Virology **205:**596–602.
21. **Ling, P. D., J. J. Hsieh, I. K. Ruf, D. R. Rawlins, and S. D. Hayward.** 1994. EBNA-2 upregulation of Epstein-Barr virus latency promoters and the cellular CD23 promoter utilizes a common targeting intermediate, CBF1. J. Virol. **68:**5375–5383.
22. **Ling, P. D., D. R. Rawlins, and S. D. Hayward.** 1993. The Epstein-Barr virus immortalizing protein EBNA-2 is targeted to DNA by a cellular enhancer-binding protein. Proc. Natl. Acad. Sci. USA **90:**9237–9241.
23. **Lukas, J., D. Parry, L. Aagaard, D. J. Mann, J. Bartkova, M. Strauss, G. Peters, and J. Bartek.** 1995. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature **375:**503–506.
24. **Marshall, D., and C. Sample.** 1995. Epstein-Barr virus nuclear antigen 3C is a transcriptional regulator. J. Virol. **69:**3624–3630.
25. **Maunders, M. J., L. Petti, and M. Rowe.** 1994. Precipitation of the Epstein-Barr virus protein EBNA 2 by an EBNA 3c-specific monoclonal antibody. J. Gen. Virol. **75:**769–778.
26. **Meitinger, C., L. J. Strobl, G. Marschall, G. W. Bornkamm, and U. Zimber-Strobl.** 1994. Crucial sequences within the Epstein-Barr virus TP1 promoter for EBNA2-mediated transactivation and interaction of EBNA2 with its responsive element. J. Virol. **68:**7497–7506.
27. **Parker, G. A., T. Crook, M. Bain, E. A. Sara, P. J. Farrell, and M. J. Allday.** 1996. Epstein-Barr virus nuclear antigen (EBNA) 3C is an immortalizing oncoprotein with similar properties to adenovirus E1A and papillomavirus E7. Oncogene **13:**2541–2549.
28. **Petti, L., J. Sample, F. Wang, and E. Kieff.** 1988. A fifth Epstein-Barr virus nuclear protein (EBNA3C) is expressed in latently infected growth-transformed lymphocytes. J. Virol. **62:**1330–1338.
29. **Pizzorno, M. C., and G. S. Hayward.** 1990. The IE2 gene products of human cytomegalovirus specifically down-regulate expression from the major immediate-early promoter through a target sequence located near the cap site. J. Virol. **64:**6154–6165.
30. **Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward.** 1988. *trans*-Activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. J. Virol. **62:**1167–1179.
31. **Rayhel, E. J., M. H. Dehoff, and V. M. Holers.** 1991. Characterization of the human complement receptor 2 (CR2, CD21) promoter reveals sequences shared with regulatory regions of other developmentally restricted B cell proteins. J. Immunol. **146:**2021–2026.
32. **Rickinson, A. B., and E. Kieff.** 1996. Epstein-Barr virus, p. 2397–2446. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology. Lippincott-Raven, Philadelphia, Pa.
33. **Ricksten, A., B. Kallin, H. Alexander, J. Dillner, R. Fahraeus, G. Klein, R. Lerner, and L. Rymo.** 1988. BamHI E region of the Epstein-Barr virus genome encodes three transformation-associated nuclear proteins. Proc. Natl. Acad. Sci. USA **85:**995–999.
34. **Robertson, E. S., S. Grossman, E. Johannsen, C. Miller, J. Lin, B. Tomkinson, and E. Kieff.** 1995. Epstein-Barr virus nuclear protein 3C modulates transcription through interaction with the sequence-specific DNA-binding protein Jκ. J. Virol. **69:**3108–3116.
35. **Robertson, E. S., J. Lin, and E. Kieff.** 1996. The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJκ. J. Virol. **70:**3068–3074.
36. **Rooney, C. M., M. Brimmell, M. Buschle, G. Allan, P. J. Farrell, and J. L. Kolman.** 1992. Host cell and EBNA-2 regulation of Epstein-Barr virus latent-cycle promoter activity in B lymphocytes. J. Virol. **66:**496–504.
37. **Sample, C., and B. Parker.** 1994. Biochemical characterization of Epstein-Barr virus nuclear antigen 3A and 3C proteins. Virology **205:**534–539.
38. **Sample, J., L. Young, B. Martin, T. Chatman, E. Kieff, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. J. Virol. **64:**4084–4092.
39. **Scheffner, M., K. Munger, J. C. Byrne, and P. M. Howley.** 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc. Natl. Acad. Sci. USA **88:**5523–5527.

40. **Shimizu, E., A. Coxon, G. A. Otterson, S. M. Steinberg, R. A. Kratzke, Y. W. Kim, J. Fedorko, H. Oie, B. E. Johnson, J. L. Mulshine, J. D. Minna, A. F. Gazdar, and F. J. Kaye.** 1994. RB protein status and clinical correlation from 171 cell lines representing lung cancer, extrapulmonary small cell carcinoma, and mesothelioma. Oncogene **9:**2441–2448.
41. **Sjoblom, A., A. Jansson, W. Yang, S. Lain, T. Nilsson, and L. Rymo.** 1995. PU box-binding transcription factors and a POU domain protein cooperate in the Epstein-Barr virus (EBV) nuclear antigen 2-induced transactivation of the EBV latent membrane protein 1 promoter. J. Gen. Virol. **76:**2679–2692.
42. **Sjoblom, A., A. Nerstedt, A. Jansson, and L. Rymo.** 1995. Domains of the Epstein-Barr virus nuclear antigen 2 (EBNA2) involved in the transactivation of the latent membrane protein 1 and the EBNA Cp promoters. J. Gen. Virol. **76:**2669–2678.
43. **Sleigh, M. J.** 1986. Non chromatographic assay for expression of the chloramphenicol acetyl transferase gene in eukaryotic cells. Anal. Biochem. **156:** 251–256.
44. **Thomas, J. A., M. J. Allday, and D. H. Crawford.** 1991. Epstein-Barr virus-associated lymphoproliferative disorders in immunocompromised individuals. Adv. Cancer Res. **57:**329–380.
45. **Tomkinson, B., and E. Kieff.** 1992. Use of second-site homologous recombination to demonstrate that Epstein-Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation in vitro. J. Virol. **66:**2893–2903.
46. **Tomkinson, B., E. Robertson, and E. Kieff.** 1993. Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. J. Virol. **67:**2014–2025.
47. **Waltzer, L., P. Y. Bourillot, A. Sergeant, and E. Manet.** 1995. RBP-J kappa repression activity is mediated by a co-repressor and antagonized by the Epstein-Barr virus transcription factor EBNA2. Nucleic Acids Res **23:**4939–4945.
48. **Waltzer, L., F. Logeat, C. Brou, A. Israel, A. Sergeant, and E. Manet.** 1994. The human J kappa recombination signal sequence binding protein (RBP-J kappa) targets the Epstein-Barr virus EBNA2 protein to its DNA responsive elements. EMBO J. **13:**5633–5638.
49. **Waltzer, L., M. Perricaudet, A. Sergeant, and E. Manet.** 1996. Epstein-Barr virus EBNA3A and EBNA3C proteins both repress RBP-Jκ–EBNA2-activated transcription by inhibiting the binding of RBP-Jκ to DNA. J. Virol. **70:**5909–5915.
50. **Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. J. Virol. **64:**2309–2318.
51. **Watson, R. J., C. Robinson, and E. W. Lam.** 1993. Transcription regulation by murine B-myb is distinct from that by c-myb. Nucleic Acids Res. **21:**267–272.
52. **Weintraub, S. J., K. N. Chow, R. X. Luo, S. H. Zhang, S. He, and D. C. Dean.** 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature **375:**812–815.
53. **Zhao, B., D. R. Marshall, and C. E. Sample.** 1996. A conserved domain of the Epstein-Barr virus nuclear antigens 3A and 3C binds to a discrete domain of Jκ. J. Virol. **70:**4228–4236.
54. **Zimber-Strobl, U., E. Kremmer, F. Grasser, G. Marschall, G. Laux, and G. W. Bornkamm.** 1993. The Epstein-Barr virus nuclear antigen 2 interacts with an EBNA2 responsive cis-element of the terminal protein 1 gene promoter. EMBO J. **12:**167–175.
55. **Zimber-Strobl, U., L. J. Strobl, C. Meitinger, R. Hinrichs, T. Sakai, T. Furukawa, T. Honjo, and G. W. Bornkamm.** 1994. Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-J kappa, the homologue of Drosophila Suppressor of Hairless. EMBO J. **13:**4973–4982.