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A Conserved Domain of the Epstein-Barr Virus Nuclear Antigens 3A and 3C Binds to a Discrete Domain of Jκ

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EBNA-3C can affect the LMP-1 promoter in both a positive and a negative manner through distinct DNA sequence elements. The viral transactivator EBNA-2 normally binds DNA indirectly via Jκ to activate transcription, but this activation is prevented in the presence of EBNA-3C. The DNA element recognized by Jκ is both required and sufficient for this inhibition. Jκ clones isolated in a yeast two-hybrid screen using EBNA-3C as bait allowed us to delineate the sequences of both proteins mediating the interaction. Two isoforms of Jκ that differ in exon 1, Jκ-1 and RBP-2N, interact with EBNA-3C, suggesting that exon 1 is not required for this interaction; indeed, clones with deletion of the N-terminal third of Jκ interacted as efficiently with EBNA-3C as full-length Jκ clones. A Jκ domain as small as 56 amino acids was sufficient to bind to EBNA-3C. A 74-amino-acid domain of EBNA-3C, conserved in all three EBNA-3 family members, was sufficient to interact with Jκ. A specific mutation in this conserved domain suppressed the ability of EBNA-3C to downregulate transcription. Accordingly, EBNA-3A was also able to interact with Jκ and downregulate Jκ-mediated transcription as efficiently as EBNA-3C. The ability of the EBNA-3 proteins to prevent Jκ from binding to DNA in vitro and suppress transactivation via Jκ DNA elements suggests that the EBNA-3 proteins act analogously to the *Drosophila* protein Hairless.

Epstein-Barr virus (EBV) infects human primary B lymphocytes in vitro, inducing cellular proliferation associated with the expression of six viral nuclear proteins (EBNAs) and three membrane proteins (LMPs) (for reviews, see references 28 and 34). Three of the EBNA proteins, EBNA-3A, -3B, and -3C, comprise a family of nuclear proteins that are arranged in tandem in the EBV genome and encoded by genes that are similar in structure (6, 24, 27, 37–39). These features have led to the proposal that the EBNA-3 genes might have arisen by gene duplication, although there is little overall homology between the protein sequences. Genetic studies using recombinant virus demonstrate that EBNA-3A and -3C are required for EBV-induced B-cell growth transformation in vitro, whereas EBNA-3B is dispensable (44, 45).

Expression of EBNA-3C in EBV-negative Burkitt lymphoma (BL) cell lines results in enhanced levels of the cellular protein CD21, receptor for both EBV and the C3d fragment of complement (47). Similarly, restoration of EBNA-3C expression in an EBV-positive BL cell line in which EBNA-3C has been deleted results in increased expression of the viral protein LMP-1 (1, 2). Since CD21 is associated with cellular proliferation (7, 8, 16, 36) and LMP-1 induces cellular changes associated with transformation (4, 46), the ability to regulate expression of these, and probably other, genes is likely to be central to the function of EBNA-3C in EBV-mediated transformation of primary B lymphocytes.

The fact that EBNA-3C increases gene expression and contains sequences homologous to a basic leucine zipper motif common to many transcription factors (11, 29) suggested that it may act as a transcription factor. Indeed, EBNA-3C has two effects on transcription. First, EBNA-3C can enhance the ability of EBNA-2 to transactivate the LMP-1 promoter (32). In agreement with this ability to activate transcription, we have defined a glutamine/proline-rich domain in the carboxyl terminus of EBNA-3C that activates transcription when fused to a heterologous DNA-binding domain. The enhancement of EBNA-2-mediated transactivation by EBNA-3C appears to be mediated via a DNA element distinct from Jκ binding sites because the enhancement persists even when Jκ binding sites have been mutated. Second, EBNA-3C can inhibit EBNA-2-mediated transactivation (30, 40, 32). EBNA-2 activates the LMP-1 promoter by binding indirectly to DNA through the cellular DNA-binding proteins Jκ and PU.1 (18, 21, 25, 31). The Jκ DNA-binding site is both required and sufficient for EBNA-3C to repress EBNA-2-mediated transactivation (32), and a direct interaction between EBNA-3C and Jκ proteins occurs in vitro (40, 32). This interaction prevents Jκ from binding to its cognate DNA sequence (40). EBNA-3B, but not EBNA-3A, has been reported to associate with Jκ in vitro but does not appear to affect the binding of Jκ to DNA (40).

Here, we focus on further characterization of the interaction between EBNA-3C and Jκ. A yeast two-hybrid screen allowed us to identify the region of EBNA-3C involved in the interaction; similarly, analysis of the Jκ cDNAs allowed us to determine that the N-terminal 144 amino acids were not required for interaction with EBNA-3C. Site-directed mutagenesis revealed that this interaction was mediated through a domain of EBNA-3C conserved in all EBNA-3 proteins. Consistent with this finding, EBNA-3A also associated with Jκ in vitro and downregulated EBNA-2-mediated transactivation through Jκ binding sites in vivo.

MATERIALS AND METHODS

Plasmid DNA. Vector pAS2, containing the yeast Gal4 DNA-binding domain coupled to a hemagglutinin (HA) antigenic epitope and a selectable marker allowing growth on tryptophan-deficient media, was provided by S. Elledge; this plasmid also contains the *CYH2* gene, which confers cycloheximide sensitivity to yeast strains (20). $E3C_1$ was constructed by inserting an *Nco*I-*Bgl*II fragment of

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the EBNA-3C cDNA encoding amino acids 181 to 438 into pAS2 in frame with the Gal4 DNA-binding domain; this EBNA-3C fragment encompasses the potential basic leucine zipper motif. $E3C_2$, encoding amino acids 181 to 365 of EBNA-3C, was constructed by inserting an *Nco*I-*Spe*I fragment of the EBNA-3C cDNA into pAS2. $E3C_3$, encoding amino acids 181 to 257 of EBNA-3C, was constructed by deleting a *Kpn*I-*Spe*I fragment of the EBNA-3C cDNA, which encodes the leucine repeats, from $E3C_2$. A cDNA library from EBV-transformed human peripheral B lymphocytes, in which cDNAs were fused to the Gal4 transactivation domain in pACT, was provided by S. Elledge; this plasmid contains a selectable marker allowing growth on leucine-deficient media. pAS2CyB expresses a fusion protein of the Gal4 DNA-binding domain and cyclophilin B. pACT-CAM expresses a fusion protein of the Gal4 transactivation domain and CAM, a protein which specifically interacts with cyclophilin B. Both plasmids were provided by R. Bram (9). pSG5-Jκ contains a full-length Jκ cDNA isolated from the yeast two-hybrid screen and subcloned into the mammalian expression vector pSG5 (Stratagene). Full-length and 5′-truncated Jκ cDNAs, isolated from the yeast two-hybrid screen, were subcloned into pGEX (Pharmacia) to form fusions with glutathione *S*-transferase (GST). Deletions of the 3′ end of the Jκ gene were generated by restriction endonuclease digestion. pSG5-EBNA-1, pSG5-EBNA-2, and pSG5-EBNA-3C have been described previously (32, 42). The cDNA for EBNA-3A, provided by J. Sample, contained upstream untranslated exons; the coding region was removed by restriction endonuclease digestion with *Pst*I and *Hin*dIII and subcloned into the expression vector pSG5 (Stratagene).

Construction of an EBNA-3C homology-minus mutation. A specific mutation in EBNA-3C was generated by using recombinant PCR. To generate 3C-hom$^-$, two overlapping primers (5′-GGCTGCAGCTGCGGCAGTTAACATGATG CT-3′ and 5′-GCAGCTGCAGCCCAAAATGCGGCACGAACT-3′) were used to replace the residues Thr-209, Phe-210, Gly-211, and Cys-212 with alanine residues. A fragment encompassing each of these mutated regions was then amplified with the primers 5′-GAGAGATTGGTACCAGAAGAGTCATACT CA-3′ and 5′-TAGATTCTTCGGTACCGCCTCTGC-3′, which contain *Kpn*I restriction endonuclease sites (underlined). The final recombinant PCR products were digested with *Kpn*I, and purified DNA fragments were ligated to pSG5-3C which had been digested with the same restriction endonuclease. This entire fragment was sequenced to verify mutations and ensure that no undesired mutations were generated during PCR.

Yeast two-hybrid system. Yeast transformation was performed by using lithium acetate (23). *Saccharomyces cerevisiae* Y190, containing the two Gal4-responsive reporter genes *HIS3* and the β-galactosidase gene, was transformed with $E3C_2$ and selected on tryptophan-deficient medium. Expression of the EBNA-3C–Gal4 fusion protein was confirmed by immunoblotting with an anti-HA monoclonal antibody (data not shown). Yeast transformants expressing the $E3C_2$ fusion protein were subsequently transformed with the cDNA library in plasmid pACT and selected on medium deficient in tryptophan, leucine, and histidine and containing either 50 or 20 mM 3-aminotriazole (3-AT). A filter lift assay was used to detect the expression of β-galactosidase; colonies that turned blue within 24 h were considered to be positive and were selected on medium containing cycloheximide (2.5 mg/ml) to eliminate the bait plasmid, $E3C_2$, from the otherwise cycloheximide-resistant host. Yeast strains containing only the cDNA library plasmid, or containing the cDNA library plasmid in the presence of the irrelevant protein cyclophilin B, were tested for β-galactosidase activity to eliminate false positives. Of 4×10^6 yeast transformants tested for growth in the presence of 50 mM 3-AT to select high-affinity interactions, 51 colonies grew in the absence of histidine; 21 of these colonies also activated the β-galactosidase reporter gene. Of 1.2×10^6 yeast transformants screened in the presence of 20 mM 3-AT to identify lower-affinity interactions, 24 colonies were positive for both histidine selection and β-galactosidase activity. These two groups of colonies were combined, and the cDNAs from 30 positive colonies dependent on the presence of EBNA-3C for activation of the two reporter genes were sequenced from both 5′ and 3′ ends. To quantitate β-galactosidase activity, yeast cells were grown in appropriate media to mid-log phase and lysed with glass beads, and β-galactosidase activity was assayed by using *o*-nitrophenyl-β-D-galactoside as a substrate.

In vitro transcription and translation. Capped RNA transcripts encoding EBNA-1, EBNA-2, EBNA-3C, and Jκ were generated from pSG5-derived expression vectors by in vitro transcription using T7 polymerase (mCAP kit; Stratagene). The mRNAs were then translated by using rabbit reticulocyte lysate (Promega) as suggested by the manufacturer in the presence of [^{35}S]methionine (DuPont).

GST fusion chromatography. GST fusion proteins were expressed in *E. coli* and purified on glutathione-Sepharose beads as described previously (43). Purified fusion proteins bound to beads were incubated with in vitro-translated proteins at 4°C for 30 min in 400 μl of NET-N (120 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 0.5% Nonidet P-40). Bound proteins were collected by centrifugation of the glutathione-Sepharose beads, washed five times in 1-ml volumes of NET-N, and recovered by boiling the beads in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer for analysis by SDS-PAGE and autoradiography.

Electrophoretic mobility shift assay (EMSA). Two overlapping double-stranded oligonucleotides encompassing a Jκ-binding site from the EBV *Bam*HI-C promoter (underlined), 5′-gatcGCCGTGGGAAAAAAT-3′, were labeled with [^{32}P]dCTP, using the Klenow fragment of DNA polymerase I. EBNA-1, EBNA-2, EBNA-3C, and Jκ were generated by in vitro translation, using unlabeled methionine, and preincubated on ice for 30 min in a 30-μl volume containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 60 mM KCl, 10% glycerol, 1 mM EDTA, and 1 mM dithiothreitol. ^{32}P-labeled oligonucleotide (10,000 cpm) was added, and incubation continued for an additional 30 min. DNA-protein complexes were analyzed on a 5% nondenaturing polyacrylamide gel in 1× Tris-borate-EDTA running buffer. For competition, a 100-fold excess of unlabeled oligonucleotide probe was included in the incubation. As a negative control, an oligonucleotide in which the Jκ binding site had been mutated, 5′-gatcGCCGgatccAAAAAAT-3′, was used.

Transfections and CAT assays. EBV-negative BL cell lines were transfected at a concentration of 8×10^6 cells in 250 μl of RPMI 1640. Reporter plasmid and expression vectors (5 μg of each) were introduced into cells by electroporation at 250 V and 960 μF; the amount of total DNA was 30 μg in all cases. Cells were harvested 36 to 48 h posttransfection. The human growth hormone reporter gene under the control of the cytomegalovirus immediate-early promoter, pCMV-hGH, was used as a transfection control for chloramphenicol acetyltransferase (CAT) reporter plasmids. CAT activity was determined by the standard two-phase partition method. CAT activity was calculated as the ratio of acetylated [^{14}C]chloramphenicol to the total acetylated and unacetylated [^{14}C]chloramphenicol. These values are then presented relative to that obtained with the expression vector containing no insert, which is set to 1. Levels of human growth hormone present in culture supernatants were quantitated by using a radioimmunoassay system as recommended by the manufacturer (Nichols Institute) and did not vary significantly. Immunofluorescence was used to confirm the expression of EBNA-2 and EBNA-3C in transfected cells. Following fixation in methanol-acetone (1:1) for 4 min at −20°C, EBNA-3C expression was detected with human EBV-positive serum followed by biotinylated goat anti-human secondary antibody and fluorescein isothiocyanate-conjugated streptavidin. A mouse monoclonal antibody to EBNA-2, PE2, followed by biotinylated rabbit anti-mouse antibody and fluorescein isothiocyanate-conjugated streptavidin, was used to detect EBNA-2.

Immunoprecipitations and immunoblotting. For immunoprecipitation from [^{35}S]methionine-labeled cells, EBV-negative cells were transfected with 10 μg of appropriate expression vectors. After 36 h, cells were washed in methionine- and cysteine-free medium (ICN) and preincubated in this medium for 30 min. Cells were collected by centrifugation and incubated in 3 ml of methionine- and cysteine-free medium containing 1 mCi of [^{35}S]methionine (ICN Translabel) for 4 h. Cells were extracted in 1 ml of 20 mM Tris-HCl (pH 8.0)–150 mM NaCl–0.5 mM EDTA–0.5% Nonidet P-40 for 30 min at 4°C. The extract was cleared by centrifugation for 10 min in a microcentrifuge. A 200-μl aliquot of extract was incubated with 1 ml of NET-N containing the appropriate antibody for 30 min at 4°C and then incubated for 30 min with 35 μl of a 1:1 suspension of protein A-Sepharose. SDS-PAGE sample buffer was added, and the sample was boiled for 3 min. Samples were analyzed by SDS-PAGE and autoradiography. For unlabeled cells, the samples were analyzed by immunoblotting onto Immobilon (Millipore) and processed for enhanced chemiluminescence analysis (ECL kit; Amersham) as recommended by the manufacturer.

RESULTS

Identification of the domain of EBNA-3C which specifically associates with Jκ. To identify proteins that interact with EBNA-3C, a portion of EBNA-3C encompassing the potential basic leucine zipper domain was used in a yeast two-hybrid screen (14, 15). Our initial experiments used construct $E3C_1$ (Fig. 1A), which contained amino acids 181 to 438 of EBNA-3C. The Gal4–EBNA-3C fusion protein $E3C_1$, however, activated expression of both Gal4-responsive reporter genes present in the yeast cells, allowing growth on histidine-deficient media and activating β-galactosidase activity alone. This finding suggested that this region of EBNA-3C contained sequences capable of acting as a transactivation domain, although we have been unable to demonstrate that this region of EBNA-3C possesses any ability to activate transcription in mammalian cells. A second probe, $E3C_2$, containing amino acids 181 to 365 of EBNA-3C, did not activate the yeast reporter genes alone and was used in a yeast two-hybrid screening of a cDNA library from EBV-transformed human B lymphocytes. Of the 30 cDNAs that encoded proteins interacting with EBNA-3C, 13 encoded the cellular DNA-binding protein Jκ.

Since the Jκ protein does not contain a leucine zipper, a third yeast two-hybrid bait, $E3C_3$ (Fig. 1A), was generated to determine whether the putative leucine zipper was required for

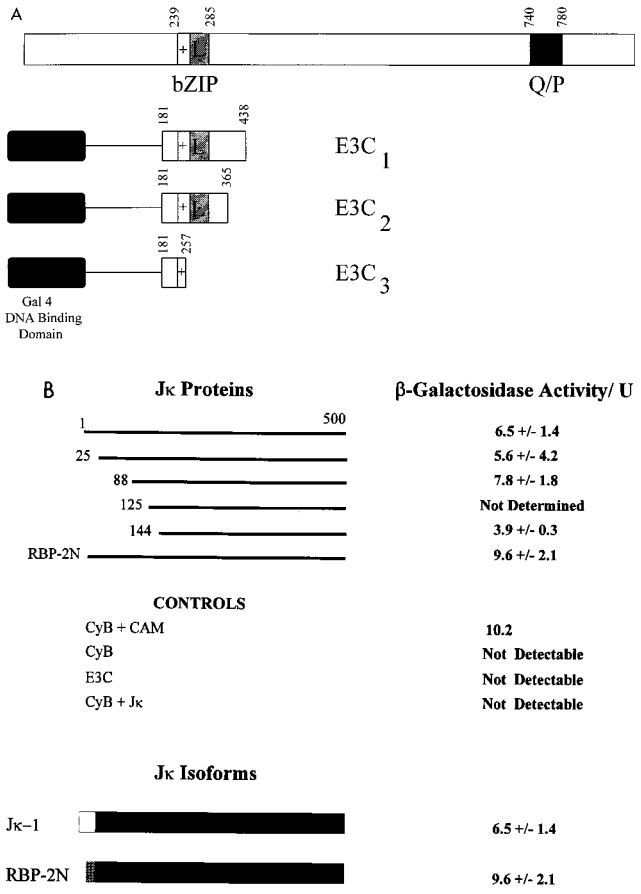

FIG. 1. Identification of the minimal EBNA-3C domain required for interaction with Jκ in *S. cerevisiae*. (A) EBNA-3C–Gal4 fusions used in the yeast two-hybrid system. The upper bar shows a schematic of the EBNA-3C protein noting the location of the potential basic leucine zipper (bZIP) motif and the putative transactivation domain (Q/P). The lower three bars indicate the residues of EBNA-3C fused to the Gal4 DNA-binding domain in relation to the leucine zipper motif (shaded region). (B) The amino terminus of Jκ is not required for interaction with EBNA-3C in *S. cerevisiae*. Jκ cDNAs isolated from the yeast two-hybrid screen were sequenced and found to have different 5′ ends but coterminal 3′ ends. Numbers refer to the amino acids of Jκ contained in each clone. The ability of each of these proteins to interact with $E3C_2$ was demonstrated by the ability to activate the Gal4-responsive β-galactosidase reporter gene. Assays were performed in triplicate, and results are presented as means with standard deviations. The results are expressed as units defined by Miller (35). As a positive control, cyclophilin B in the presence of CAM, which specifically binds cyclophilin B (CyB), was included. Negative controls included $E3C_2$ in the absence of Jκ, cyclophilin B, and Jκ plus cyclophilin B. Schematic representations of the Jκ-1 and RBP-2N proteins are shown below.

this interaction; this bait contained a smaller EBNA-3C fragment with deletion of the leucine zipper and half of the basic region but retaining sequences 181 to 257 of EBNA-3C. These sequences alone were sufficient to confer on yeast cells the ability to grow in selective media containing 50 mM 3-AT in the presence of Jκ (data not shown). This growth was equivalent to that of yeast cells expressing the EBNA-3C bait containing the potential leucine zipper in the presence of Jκ. The smaller construct $E3C_3$ was also able to activate β-galactosidase activity in yeast cells, although to a lesser extent than $E3C_2$ (data not shown). This result suggested that the domain located between amino acids 181 and 257 of EBNA-3C, and not the potential leucine zipper, mediated the interaction with Jκ.

The amino terminus of Jκ is not required for interaction with EBNA-3C. Analysis of the Jκ cDNAs isolated in the yeast two-hybrid screen produced two important pieces of information. First, the Jκ cDNA exists as four known alternatively spliced forms that vary in the first exon; sequence analysis of the Jκ cDNAs revealed that two of these alternatively spliced forms associated with EBNA-3C: RBP-2N, which has a unique exon 1 previously characterized as a mouse-specific exon encoding 7 amino acids (26), and Jκ-1, where exon 1 encodes 20 amino acids (3). Second, although all cDNAs were 3′ coterminal, analysis of the 5′ ends revealed that the cDNAs ranged from full length to various incomplete cDNAs, with the shortest initiating at nucleotide 509 of the Jκ cDNA (Fig. 1B), which allowed us to partially map the region of Jκ required for the interaction. The interaction of $E3C_2$ with the N-terminally deleted Jκ proteins encoded by these cDNAs was evaluated in yeast cells by a quantitative assay of β-galactosidase activity (Fig. 1B). Since all truncated Jκ proteins activated β-galactosidase activity in the presence of $E3C_2$ to comparable levels, roughly equivalent to that obtained with the full-length Jκ protein, we conclude that the smallest cDNA contains all sequences necessary for interaction with EBNA-3C. This smallest Jκ cDNA initiates at nucleotide 509, which corresponds to amino acid 144; therefore, amino acid residues 1 to 144 are not required for Jκ to associate with EBNA-3C.

Identification of the domain in Jκ mediating the association with EBNA-3C. Since the data obtained with *S. cerevisiae* had suggested that the domain of Jκ that bound to EBNA-3C was located carboxy terminal to amino acid 144, we examined the ability of a series of truncated GST-Jκ fusion proteins to bind to EBNA-3C (Fig. 2). Approximately 10% of the EBNA-3C bound to the GST-Jκ beads (data not shown; see Fig. 3 for an example). To delineate the N-terminal boundary of the Jκ domain that bound EBNA-3C, the amino terminus was further truncated from amino acids 1 to 178; removal of these amino acids resulted in loss of binding. Moreover, constructs that were further truncated to amino acid residue 280 showed no binding to EBNA-3C. Since our results from the yeast two-hybrid screen suggested that amino acids 1 to 144 were not required for association with EBNA-3C, these data suggest that the N-terminal boundary of the domain binding to EBNA-3C lies between amino acid residues 144 and 178 and are consistent with the fact that no cDNAs obtained in the yeast two-hybrid screen were truncated beyond amino acid 144.

To identify the C-terminal boundary of the interaction domain, we generated carboxyl-terminal deletions of GST-Jκ fusion proteins (Fig. 2). Truncation of the C terminus from amino acid residues 500 to 233, or additional truncation to

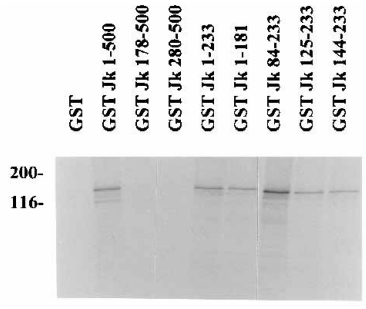

FIG. 2. Delineation of the Jκ domain required for interaction with EBNA-3C. Various regions of Jκ cDNA, encoding the amino acids listed above the lanes, were fused to GST. Fusion proteins bound to glutathione beads were incubated with ^{35}S-labeled EBNA-3C generated by in vitro translation. EBNA-3C was detected by autoradiography of SDS-polyacrylamide gels. Sizes are given in kilodaltons.

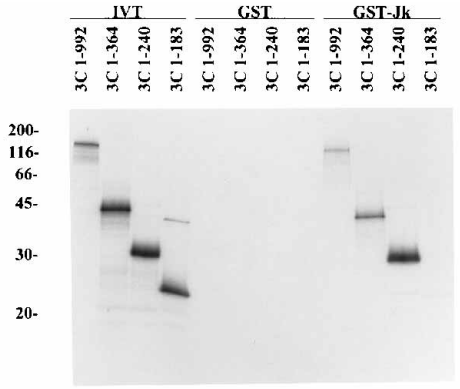

FIG. 3. A domain of EBNA-3C required for interaction with Jκ lies between amino acids 183 and 240. A plasmid containing the EBNA-3C cDNA was truncated by restriction endonuclease digestion; these templates were used for in vitro transcription and translation. The products obtained are shown under IVT (in vitro translation); numbers above the lanes represent the amino acids translated. The amount used represents 20% of the input used to bind to GST beads or GST-Jκ beads. Sizes are given in kilodaltons.

amino acid residue 181, resulted in binding similar to that obtained with full-length Jκ. This result suggested that the domain that efficiently bound to EBNA-3C lay between amino acid residues 144 and 181.

To determine whether a small region of Jκ encompassing the amino- and carboxy-terminal boundaries defined above was sufficient for interaction with EBNA-3C, we generated several small GST-Jκ fusion proteins extending only to amino acid residue 233 (Fig. 2). The GST-Jκ fusion protein containing amino acid residues 84 to 233 bound to EBNA-3C as efficiently as full-length GST-Jκ. A GST-Jκ fusion protein containing amino acid residues 125 to 233 produced similar results. A GST-Jκ fusion protein containing only 89 amino acids of Jκ encoding residues 144 to 233 also bound to EBNA-3C, though in some experiments this interaction was not as efficient as that obtained with full-length GST-Jκ. Therefore, these 89 amino acids represent the smallest domain tested responsible for interaction with EBNA-3C.

A region of EBNA-3C proximal to the amino terminus associates with Jκ. Since the yeast two-hybrid screen had indicated that amino acids 181 to 257 of EBNA-3C mediated the association with Jκ, we confirmed this observation by determining the ability of truncated EBNA-3C proteins to bind to GST-Jκ (Fig. 3). Full-length EBNA-3C (amino acids 1 to 992) and three carboxy-terminal truncated proteins were generated by in vitro translation (Fig. 3, IVT); none of these proteins bound to GST alone (Fig. 3, GST). Full-length EBNA-3C bound to GST-Jκ, and truncation to amino acid residue 364, or further to 240, led to increased binding (Fig. 3, GST-Jκ). This finding suggested that amino acids in the carboxy terminus not only are dispensable for association with Jκ but are in fact somewhat inhibitory. However, further truncation to amino acid 183 resulted in negligible binding. These data, together with the data obtained with *S. cerevisiae*, demonstrate that amino acids between 183 and 240 of EBNA-3C are both essential and sufficient for the ability to bind to Jκ.

To confirm that this small truncated EBNA-3C protein interacted with the same domain of Jκ, amino acids 1 to 240 of EBNA-3C were used to bind to various fragments of Jκ fused to GST (Fig. 4). Approximately 20% of the truncated EBNA-3C bound to full-length Jκ as well as Jκ truncated to amino acid 233 or 181, but further truncation to amino acid 154 or 125 led to a loss of binding. As demonstrated in Fig. 2, truncation of the amino terminus beyond amino acid 181 led to a loss of binding. The small fragments of Jκ extending from amino acids 84 to 233 or 125 to 233 bound to EBNA-3C, but as mentioned previously, binding to amino acids 144 to 233 was sometimes low as in this particular experiment. Two additional Jκ fragments used in this experiment demonstrated that amino acid residues 125 to 181, but not 181 to 233, bound to EBNA-3C. Thus, this small fragment of EBNA-3C bound equivalently to the same domain of Jκ as full-length EBNA-3C. In addition, amino acids 181 to 233, which can be inferred from Fig. 2 as being not required for binding to EBNA-3C, are indeed dispensable, reducing the number of amino acids of Jκ required for interaction with EBNA-3C to 56.

A conserved domain of EBNA-3C is important in the interaction with Jκ. Amino acids 182 to 231 of EBNA-3C are conserved in the other two EBNA-3 family members (Fig. 5A); this is the only significant homology between all three proteins. Since this homology is located in a region demonstrated to be essential for binding to Jκ, the central core of this conserved domain was mutated (Fig. 5A). To determine the effect of this mutation on the ability of EBNA-3C to repress EBNA-2-mediated transactivation, increasing amounts of wild-type EBNA-3C or EBNA-3C mutated in the conserved domain (3Chom$^-$) were transfected into cells together with EBNA-2 and a reporter gene controlled by oligomerized Jκ recognition elements. As shown in Fig. 5B, 1 μg of wild-type EBNA-3C expression vector drastically suppressed EBNA-2-mediated transcription, and 5 μg led to total inhibition. In contrast, addition of 5 μg of 3Chom$^-$ had little, if any, effect on EBNA-2-mediated transcription, suggesting that these four residues are critical for the suppression. Since EBNA-3C prevents Jκ from binding to its cognate DNA element (40), we performed EMSAs to examine the ability of Jκ to bind to DNA in the presence of the mutant protein 3Chom$^-$. All lanes except lane 1 contained in vitro-translated Jκ which formed a single complex on an oligonucleotide containing its cognate binding site (Fig. 6, lane 2). This complex was presumed to represent the specific binding of Jκ to its target sequence, since binding was inhibited by the addition of unlabeled oligonucleotide (Jκ DNA) but not by addition of a similar oligonucleotide with a mutation in the central GTGGGAA motif (Jκmt DNA). The binding specificity of this complex was further demonstrated when no competition for binding was observed in an assay using a portion of the

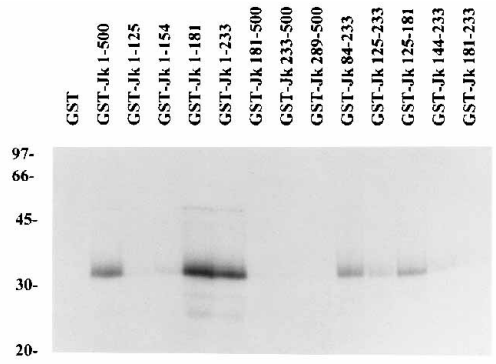

FIG. 4. EBNA-3C 1-240 associates with the same region of Jκ as full-length EBNA-3C. A plasmid containing the EBNA-3C cDNA was linearized by restriction endonuclease digestion and used for in vitro transcription and translation using [^{35}S]methionine. This protein was incubated with the GST-Jκ fusion proteins listed above the lanes. Bound protein was detected by autoradiography after SDS-PAGE. Sizes are given in kilodaltons.

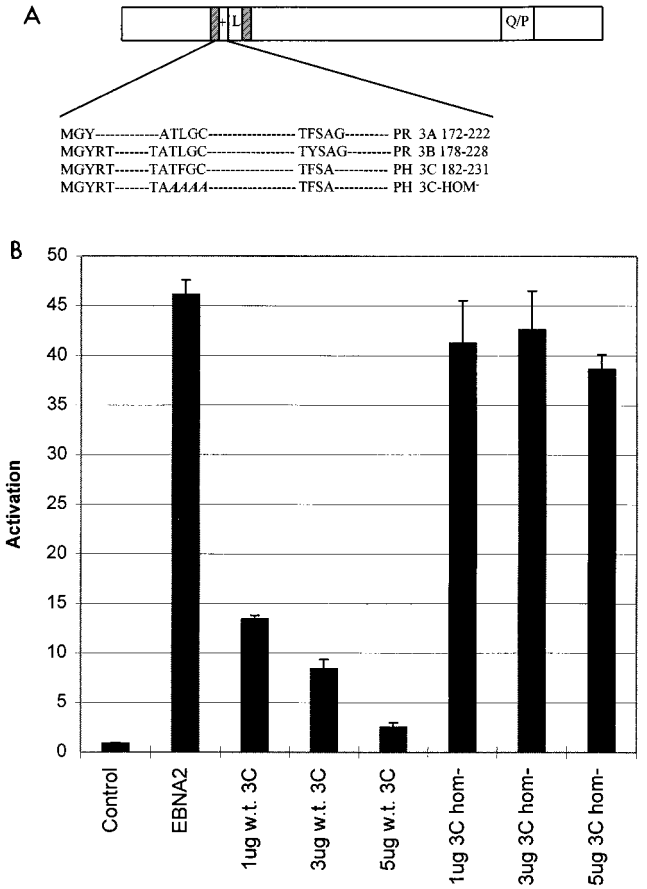

FIG. 5. The region of EBNA-3C required for interaction with Jκ contains sequences conserved among all EBNA-3 proteins. (A) Sequence alignment of EBNA-3A, -3B, and -3C demonstrates the homology between EBNA-3 family members. Site-specific mutagenesis generated a change in the central core of the conserved domain (3Chom$^-$). (B) A CAT reporter gene controlled by 10 copies of a Jκ recognition element was transfected into EBV-negative BL cells in the presence of an empty expression vector as a control, an EBNA-2 expression vector, or EBNA-2 in the presence of increasing amounts of either wild-type EBNA-3C (w.t. 3C) or the mutated EBNA-3C protein (3Chom$^-$). Data shown were obtained from duplicate transfections.

LMP-1 promoter containing a similar mutation at the Jκ sites (data not shown). Addition of increasing amounts of in vitro-translated EBNA-3C led to a loss of this complex as viewed by EMSA, and no new complexes indicative of an EBNA-3C–Jκ–DNA complex or an EBNA-3C–DNA complex were seen (lanes 6 and 7). Addition of comparable amounts of 3Chom$^-$ did not result in the loss of the Jκ-DNA complex (lanes 8 and 9). Immunoblotting verified that equivalent amounts of immunoreactive EBNA-3C and 3Chom$^-$ were added. As a negative control, addition of in vitro-translated EBNA-1 had no effect on the ability of Jκ to bind to DNA (lane 10). These data clearly show that mutation of the conserved residues diminished the ability of EBNA-3C to prevent Jκ from interacting with its target DNA sequence.

The conserved domain of EBNA-3A interacts with Jκ in vitro. If the interaction with Jκ is indeed mediated through the domain conserved in all EBNA-3 proteins, EBNA-3A and -3B should also be able to associate with Jκ through the conserved domain. Although a previous report showed that EBNA-3B but not EBNA-3A associates with Jκ (40), our data strongly suggested that the interaction of EBNA-3C was achieved via the conserved domain and therefore that the other EBNA-3

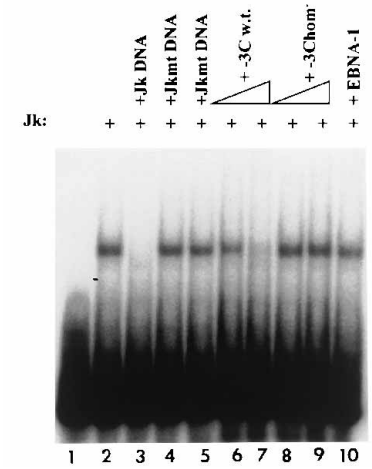

FIG. 6. EBNA-3C, mutated in the conserved motif, does not prevent Jκ from binding to its target sequence. EMSA was performed with an oligonucleotide encompassing a Jκ-binding site. All lanes, except lane 1, contained in vitro-translated Jκ. Specificity of binding was determined by competition with unlabeled oligonucleotide (Jκ DNA) or a similar oligonucleotide in which the central GTGGG core was mutated (Jκmt DNA). Increasing amounts of in vitro-translated wild-type EBNA-3C (-3C w.t.) or the mutated protein 3Chom$^-$ or a single amount of EBNA-1 was added where indicated.

proteins should also bind Jκ. Therefore, we reevaluated the ability of EBNA-3A to associate with Jκ. Truncated EBNA-3A proteins were generated by in vitro translation (Fig. 7, IVT) and did not bind to GST alone (Fig. 7, GST). Full-length EBNA-3A (amino acids 1 to 944) did not interact efficiently with GST-Jκ (data not shown). Truncation to amino acid 277 greatly enhanced the interaction with Jκ. EBNA-3A truncated immediately prior to the conserved domain (Fig. 7, EBNA-3A 1-223) interacted with Jκ, but deletion of the conserved domain resulted in a loss of binding (Fig. 7, EBNA-3A 1-172). In addition, binding of EBNA-3A to the small fragments of Jκ fused to GST was identical to that of EBNA-3C (data not shown). These data suggest that EBNA-3A, like EBNA-3C, binds to Jκ via the corresponding conserved domain.

EBNA-3A associates with Jκ in cell lysates. The weakness of the interaction between full-length EBNA-3A and Jκ in vitro led us to question its significance and whether any functional interaction would occur in vivo. Therefore, we examined the

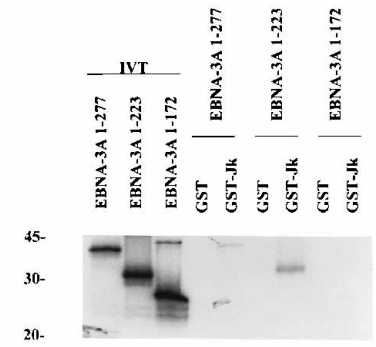

FIG. 7. The conserved domain of EBNA-3A is required for the interaction with Jκ. A plasmid containing the EBNA-3A cDNA was truncated by restriction endonuclease digestion; these templates were used for in vitro transcription and translation. The products obtained are shown under IVT (in vitro translation); this amount represents 10% of the amount used to bind to GST beads or GST-Jκ beads. Sizes are given in kilodaltons.

ability of EBNA-3A to bind to Jκ in vivo. EBV-negative BL cells were transfected with an HA-tagged Jκ expression vector plus either a control vector or a vector expressing either EBNA-3C or EBNA-3A, and proteins were labeled with [^{35}S]methionine. To determine whether EBNA-3A and -3C interacted with Jκ in vivo, proteins were immunoprecipitated with either an anti-HA monoclonal antibody, an anti-EBNA-3C monoclonal antibody, or an anti-EBV human serum recognizing both EBNA-3A and -3C. Immunoprecipitation with anti-HA from cells transfected only with the HA-tagged Jκ expression vector revealed a predominant protein band migrating at the appropriate molecular weight for Jκ (Fig. 8A; lower arrowhead). A protein of similar size is seen in HA immunoprecipitates from cells that were also transfected with either EBNA-3C or EBNA-3A. EBV-positive human serum and a monoclonal antibody recognizing EBNA-3C precipitate a higher-molecular-weight protein at the appropriate size for EBNA-3A or EBNA-3C(Fig. 8A, upper arrowhead). A similar EBNA-3-size protein is also present in the HA-Jκ immunoprecipitates only when EBNA-3A or -3C is coexpressed. Similarly, a protein comigrating with Jκ is immunoprecipitated with EBV-positive serum from cells expressing either EBNA-3C or EBNA-3A; since this protein is not detected in immunoprecipitates obtained by using EBV-positive serum from cells not transfected with Jκ, it is most likely Jκ associated with the EBNA proteins. In addition, a protein comigrating with Jκ is detected in immunoprecipitates obtained by using the EBNA-3C monoclonal antibody from lysates of cells expressing EBNA-3C but not from lysates of cells expressing EBNA-3A. These data suggest that EBNA-3A and -3C are both associated with Jκ in cellular extracts.

Since these results indicate only that proteins of the appropriate size are coimmunoprecipitated, duplicate immunoprecipitates from unlabeled cells were analyzed by immunoblotting for either EBNA-3C (Fig. 8B) or HA to detect Jκ (Fig. 8C). In cells cotransfected with EBNA-3C and Jκ expression vectors, EBNA-3C is detected not only in immunoprecipitates with the monoclonal antibody recognizing EBNA-3C and EBV-positive human serum but also in HA immunoprecipitates (Fig. 8B). Likewise, Jκ is detected in immunoprecipitates with EBV-positive human serum from cells transfected with EBNA-3C or EBNA-3A protein (Fig. 8C) and also in immunoprecipitates with the EBNA-3C monoclonal antibody from cells expressing EBNA-3C. Neither antibody immunoprecipitated Jκ from cells expressing only Jκ. The lower band present in the anti-HA immunoblots is the antibody added for immunoprecipitation. As a negative control, a monoclonal antibody recognizing EBNA-2 was included and did not immunoprecipitate either Jκ or the EBNA-3 proteins. These data suggest that EBNA-3A can interact with Jκ as efficiently as EBNA-3C in transfected cells. The fact that EBNA-3A interacts more efficiently with Jκ in vivo may suggest that EBNA-3A must undergo a posttranslational modification before it can efficiently bind to Jκ.

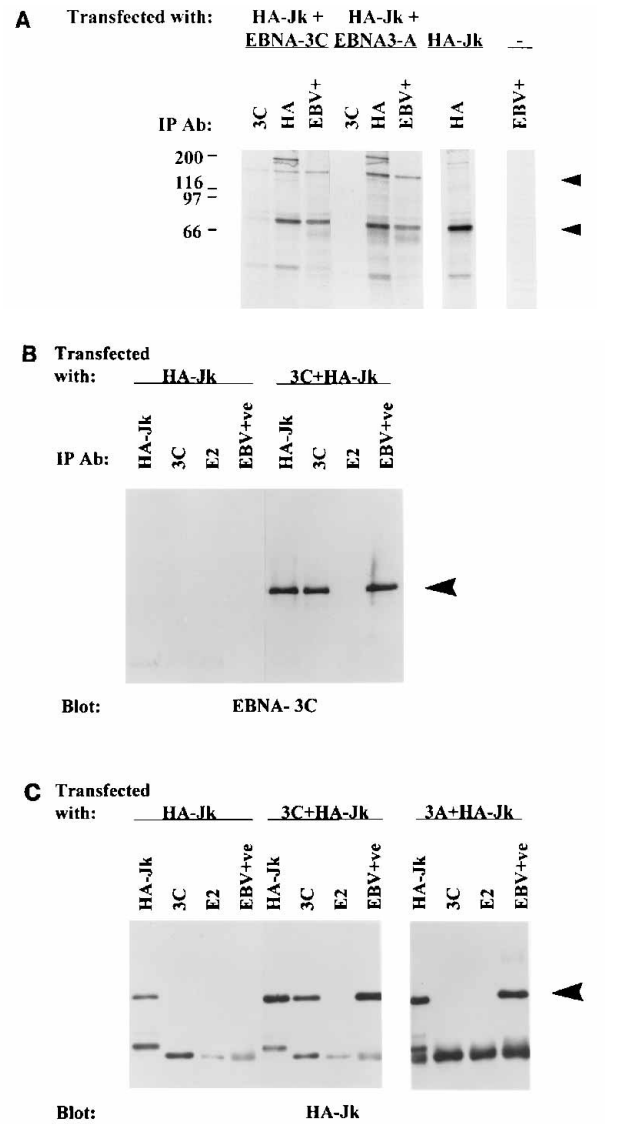

FIG. 8. EBNA-3A and -3C interact with Jκ efficiently in vivo, as determined by immunoprecipitation. EBV-negative BL (Louckes) cells were transfected with an HA-tagged Jκ expression vector with or without an EBNA-3C or EBNA-3A expression vector. Extracts were immunoprecipitated (IP) with an anti-HA monoclonal antibody (Ab) to precipitate Jκ, an anti-EBNA-3C monoclonal antibody (32a), or EBV-positive human serum. (A) Labeled proteins were detected by autoradiography. Sizes are given in kilodaltons. (B and C) Immunoprecipitates were divided in half and subjected to SDS-PAGE for immunoblot analysis with either anti-EBNA-3C (B) or anti-HA (C). The positions of EBNA-3C and Jκ are indicated by arrowheads.

EBNA-3A inhibits EBNA-2-mediated transactivation. To investigate whether the association of EBNA-3A with Jκ could also downregulate expression from Jκ target sequences in the presence of EBNA-2, we tested the effect of EBNA-3A on expression from a reporter gene under the control of multiple copies of the Jκ-binding site in the presence of EBNA-2. As shown in Fig. 9, EBNA-3A was able to completely inhibit the ability of EBNA-2 to activate expression via Jκ-binding sites. Since this promoter is controlled only by multiple copies of the Jκ-binding site, this finding suggested that full-length EBNA-3A may also prevent the binding of Jκ to its DNA recognition elements. As seen in Fig. 10, increasing amounts of EBNA-3A, like EBNA-3C, prevented Jκ from binding to its cognate DNA sequence. In contrast to EBNA-3C, this result could be obtained when nuclear extracts were used but not when in vitro-translated Jκ was used. Since we have shown that Jκ and EBNA-3A only weakly associate in vitro, this finding may reflect a modification of EBNA-3A by various kinases and other enzymes contained in the nuclear extract that would allow a more efficient interaction with Jκ. Neither EBNA-3A nor EBNA-3C bound to the DNA either in the presence or in the absence of Jκ. Thus, EBNA-3A acts identically to EBNA-3C: both proteins bind to Jκ, prevent Jκ from interacting with DNA, and consequently inhibit any transcription controlled by Jκ DNA-binding sites.

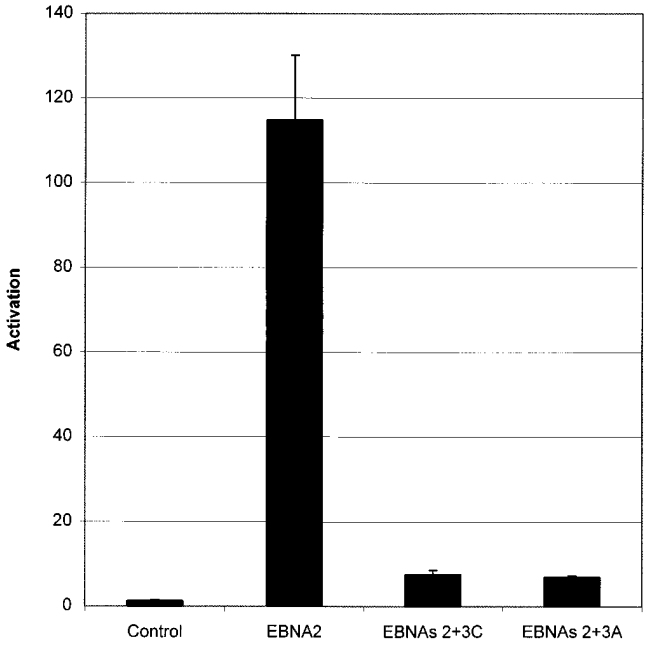

FIG. 9. EBNA-3A can suppress transactivation by EBNA-2 mediated through Jκ sites. A CAT reporter construct under the control of Jκ-binding sites was transfected into EBV-negative BL cells together with an empty expression vector, an EBNA-2 expression vector, both EBNA-2 and EBNA-3C expression vectors, or both EBNA-2 and EBNA-3A expression vectors. CAT activity was measured after 36 h. Data shown were obtained from duplicate transfections.

DISCUSSION

In this study, we have further characterized the association of EBNA-3C with the cellular DNA-binding protein Jκ, defining the domains of both proteins that mediate the interaction. The domain of EBNA-3C that binds to Jκ is conserved in all three EBNA-3 family members. These sequences are clearly sufficient to bind to Jκ in vitro, and a specific mutation in the conserved domain abolishes the ability of EBNA-3C to suppress EBNA-2-mediated transcription and to prevent Jκ from interacting with DNA. Moreover, EBNA-3A interacts with Jκ as efficiently as EBNA-3C in vivo and suppresses EBNA-2-mediated transcription to the same extent. Although we have not examined the ability of EBNA-3B to associate with Jκ, others have demonstrated that this interaction does occur in vitro (40). In that study, in contrast to our results, EBNA-3A was found not to associate with Jκ and not to prevent the binding of Jκ to DNA. This conclusion was based on two pieces of data. In contrast to EBNA-3C, a stable cell line expressing EBNA-3A did not exhibit reduced Jκ-DNA complexes detected by EMSA; immunoblotting of this cell line, however, reveals that much of the EBNA-3A protein appears to be degraded. The second finding was that in vitro-translated EBNA-3A did not bind to GST-Jκ. As shown in this report, the interaction of Jκ with EBNA-3A as measured in vitro is very weak. Interaction in vivo, however, seems to be equivalent to the interaction seen with EBNA-3C, which suggests that an event occurring in vivo such as posttranslational modification of the EBNA-3A protein stabilizes the interaction. This hypothesis is supported by our finding that in vitro-translated EBNA-3A could not prevent the formation of in vitro-translated Jκ-DNA complexes, whereas EBNA-3A could suppress the formation of Jκ-DNA complexes formed in nuclear extracts. This finding suggests that enzymes in the nuclear extract have modified EBNA-3A, allowing it to bind efficiently to Jκ. Computer analysis of the EBNA-3A protein sequence does not reveal any modifications that are likely to occur in the immediate vicinity of the conserved domain; however, numerous potential phosphorylation sites exist in the remainder of the molecule. Since EBNA-3A is 944 amino acids in length, one possibility is that such a modification may alter the three-dimensional structure of the molecule, making the conserved domain more accessible to Jκ. Similar reasoning may explain the finding that truncated EBNA-3 proteins associate more strongly with Jκ than full-length proteins.

All three EBNA-3 proteins are able to downregulate the ability of EBNA-2 to activate the terminal protein 1 promoter (30), a finding analogous to our results with the LMP-1 promoter (32, 40). Similar to the LMP-1 promoter, the major EBNA-2-responsive element of the terminal protein 1 promoter is a Jκ-binding site (33, 48), and we have demonstrated in this study that both EBNA-3A and EBNA-3C can suppress activation mediated solely through Jκ-binding sites. Therefore, it is most likely that the inhibition observed in that study reflects a loss of the ability of Jκ to bind DNA in the presence of any of the EBNA-3 proteins. The ability to prevent promoter targeting of Jκ therefore appears to be a common property of the EBNA-3 family and is consistent with our observation that a domain conserved in all three proteins binds to Jκ. The EBNA-3 family of genes is postulated to have arisen by gene duplication, a hypothesis supported by the presence of a conserved domain in all three proteins and the association of at least EBNA-3A and -3C with Jκ via this domain. Clearly, however, both EBNA-3A and EBNA-3C must also have unique functions because both are required for EBV-mediated immortalization of primary B lymphocytes (45). It is likely that further characterization of the cellular proteins which interact with EBNA-3C will allow us to identify such an additional function(s).

In *Drosophila melanogaster*, the homolog of Jκ, Suppressor of Hairless, acts as a transcription factor affecting the development of the peripheral nervous system (17) through promoters containing its recognition elements (10). A second *Drosophila* protein, Hairless, acts as a functional antagonist by inhibiting the binding of Suppressor of Hairless to its target DNA sequence (10). Both of these proteins are required for normal

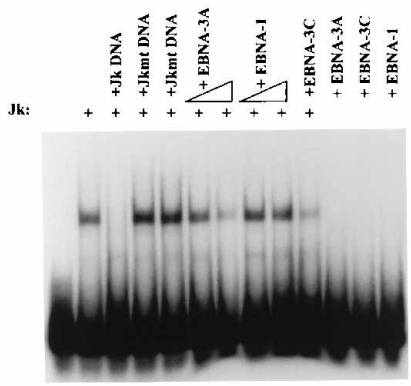

FIG. 10. EBNA-3A prevents Jκ from interacting with its target DNA. EMSA was performed with an oligonucleotide encompassing a Jκ-binding site. All lanes labeled + contained nuclear extract from the EBV-negative BL cell line BL2. Specificity of binding was determined by competition with unlabeled oligonucleotide (Jκ DNA) or a similar oligonucleotide in which the central GTGGG core was mutated (Jκmt DNA). Increasing amounts of in vitro-translated EBNA-3A and EBNA-1 (5 and 15 μl) or a fixed amount of EBNA-3C (10 μl) was added where indicated. Similar amounts of each protein were also added in the absence of Jκ.

development of the peripheral nervous system, with a perturbation of either protein resulting in abnormal development (5, 17). EBNA-3A and -3C, therefore, appear to act as functional homologs of Hairless by preventing Jκ, the Suppressor of Hairless homolog, from binding to DNA and thus preventing transactivation. There is, however, no notable homology between EBNA-3C and Hairless protein sequences. The regions of both Suppressor of Hairless and Jκ that interact with Hairless, however, have been identified (10) and are not the same as the region of Jκ that we have identified as binding to EBNA-3C in this study. The fact that mutation of widely separated amino acids of Jκ affects DNA binding (10, 12) is consistent with the ability of EBNA-3C and Hairless to interact with separate regions of Jκ with the same functional result. Thus, binding to EBNA-3C could either block the ability of Jκ to bind to DNA directly or alter the conformation of Jκ such that a separate domain can no longer bind to DNA. The region of Jκ that associates with EBNA-3C (amino acids 125 to 181) lies immediately adjacent to the domain reported to bind EBNA-2 (amino acids 179 to 361). Although the Jκ domain that binds EBNA-2 was not able to be more precisely defined, the fact that the EBNA-2 and EBNA-3 proteins bind to juxtaposed domains may indicate that EBNA-3 is more likely to affect the three-dimensional folding of Jκ.

What are the implications of preventing Jκ from binding to its target sequences in EBV-infected cells? Since even very low levels of cotransfected EBNA-3C suppressed transactivation by EBNA-2, this antagonistic binding to Jκ is also likely to occur in EBV-infected B cells. EBNA-2 and -3C may act to critically regulate proliferation of B cells in an antagonistic fashion similar to that of Hairless and Suppressor of Hairless. The function of such closely regulated expression might, for example, enable the cell to undergo continued proliferation while preventing differentiation or apoptosis. Computer searches for Jκ-binding sites reveal the presence of these sites in an extremely large and varied number of cellular promoters, indicating that by influencing the activity of Jκ, the activities of many promoters could possibly be affected. Growing evidence supports the likelihood that one normal function of Jκ in the cell is to act as a repressor of gene expression (13, 22). Given that promoter activity is a result of the sum of the activities of all proteins binding to that promoter, the exact effect of EBNA-3C would vary depending on the promoter. Thus, for a promoter with several strong positive elements, removal of a Jκ repressor protein would be expected to result in activation of this promoter; if, however, a promoter has few or weak positive elements, then little activation would be observed. If Jκ represented the major positive influence on a promoter, EBNA-3C would prevent promoter activity. Thus, on some promoters, both EBNA-2 and EBNA-3C could activate expression, whereas on other promoters, they may have an antagonistic effect. By acting in an opposing manner in EBV-transformed cells in which both proteins are normally present, promoter activity could be closely regulated. In the case of the LMP-1 promoter, for example, overexpression is cytotoxic (19) and may require a more critical regulation of protein levels. Alternatively, if EBNA-3C can bind to other promoter elements, Jκ itself may be targeted to promoters that do not contain its binding site; in this manner, EBNA-3C and Jκ could affect transcription from an even wider variety of promoters. Further analysis is required to investigate this possibility.

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