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A Fifth Epstein-Barr Virus Nuclear Protein (EBNA3C) Is Expressed in Latently Infected Growth-Transformed Lymphocytes

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Three distantly homologous neighboring long open reading frames in the Epstein-Barr virus (EBV) genome are preceded by short open reading frames. The leftmost short and long open reading frames encode EBNA3, a nuclear protein which is slightly smaller (145 kilodaltons [kDa]) than two other nuclear proteins (150 to 155 kDa) detected in Western blots (immunoblots) of latently infected cell protein (K. Hennessy, F. Wang, E. Woodland-Bushman, and E. Kieff, Proc. Natl. Acad. Sci. USA 83:5693–5697, 1986; I. Joab, D. T. Rowe, M. Bodescot, J.-C. Nicolas, P. J. Farrell, and M. Perricaudet, J. Virol. 61:3340–3344, 1987). We have demonstrated that the most rightward short (BERF3) and long (BERF4) open reading frames are spliced in frame at the 3′ end of a 5-kilobase latently infected cell RNA and that this RNA begins within or upstream of the EBV long internal repeat. EBV-immune human antibodies specific for the long open reading frame translation product identified a 155-kDa protein on Western blots of latently infected cell protein and specifically reacted with large nonnucleolar nuclear granules in every latently infected cell. Expression of the cDNA in BALB/c 3T3 cells resulted in translation of full-size EBNA3C but had no effect on cell morphology, contact inhibition, or serum independence.

Epstein-Barr virus (EBV) is the etiologic agent of infectious mononucleosis (26) and is associated with nasopharyngeal carcinoma and African Burkitt's lymphoma (11, 12, 14, 29, 54). The virus infects and establishes latency within B lymphocytes, causing cell proliferation (26, 35) and occasionally giving rise to a progeny-infected cell permissive for virus replication. In proliferating cells, the virus genome persists as a multicopy episome (31) or as a linear molecule integrated into cell DNA (32). A small fraction of the EBV genome is expressed during this largely latent infection (9, 26). The EBV genes expressed in the latent infection are of interest because they probably maintain cell proliferation or latent infection. One EBV gene expressed in latent infection encodes a cytoplasmic and plasma membrane protein, latent membrane protein (15, 18, 28, 30, 46, 47), which has broad transforming activities in continuous rodent cell lines (48). Another four genes encode EBV nuclear proteins (EBNAs) in latently infected cells. EBNA1 (17, 20, 21) binds to *oriP*, a putative replication origin, thereby permitting episome maintenance (39, 40, 52, 53). EBNA2 (22) enhances serum independence of rodent cells (10) and transactivates expression of CD23, a lymphocyte protein which may mediate B-cell activation (49). EBNA3 (19, 23) is an unrelated nuclear protein of unknown function. A fourth nuclear protein, EBNA-LP, is encoded by a 924-base open reading frame which is in the leader of the EBNA2 mRNA (and possibly the mRNAs of other EBNAs), making this mRNA an unusual example of a fully bicistronic eucaryotic mRNA (13, 42, 50).

EBNA3 is largely encoded by a long open reading frame of the EBV DNA *Bam*HI E fragment and by a short 5′ exon derived from *Bam*HI-L (4, 23, 24). *Bam*HI-E also contains two other long rightward open reading frames which are homologous to the EBNA3 long open reading frame, each of which is preceded by a short open reading frame, suggesting that these may be a family of EBNA3-like genes (1, 23). There is also direct evidence for a family of EBNA3 genes, since immunoblots with some EBV-immune human sera detect two proteins slightly larger than EBNA3 in latently infected lymphocytes (23, 25). We demonstrate in the present study that the most rightward short and long *Bam*HI-E open reading frames (BERFs), BERF3 and BERF4, in fact encode a nuclear protein characteristically expressed in each latently infected cell. We propose that this protein be named EBNA3C because of its relationship to EBNA3.

MATERIALS AND METHODS

Cells. IB4 and Namalwa are EBV-infected cell lines which can be grown under conditions in which EBV early-replication-cycle protein expression is not detectable (less than 1 in 10^5 cells positive by using an EBV-immune human serum containing a high titer of antibodies against EBV early antigens). Raji is an EBV-infected cell line in which 1 in 10^4 cells contains early antigen. B95-8, Daudi, P3HR1, and Jijoye are EBV-infected cell lines which are partially permissive for virus replication. Lymphocytes were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Procaryotic expression of BERF4. A 3,052-base-pair (bp) *Xba*I-*Nde*I fragment which contains the entire BERF4 open reading frame was subcloned from an EBV (W91) *Eco*RI B fragment clone, pNT126 (38), into the *Xba*I site of pUC19, generating the plasmid pUBXN-11 (Fig. 1). pUBXN-11 was then digested with *Sty*I, dividing the BERF4 open reading frame into 700-, 1,200-, and 500-bp fragments. These three fragments were briefly treated with BAL 31 nuclease and then with T4 DNA polymerase to randomize the ends relative to the translational open reading frames. The resulting pooled fragments were then ligated into *Sma*I-cut, phosphatase-treated pTL2 (50), a pUC18-derived plasmid which contains the entire β-galactosidase coding sequence shifted out of frame within the polylinker so that it can be used as a vector that expresses a β-galactosidase fusion protein and detects the open reading frame of the DNA insert, similar to

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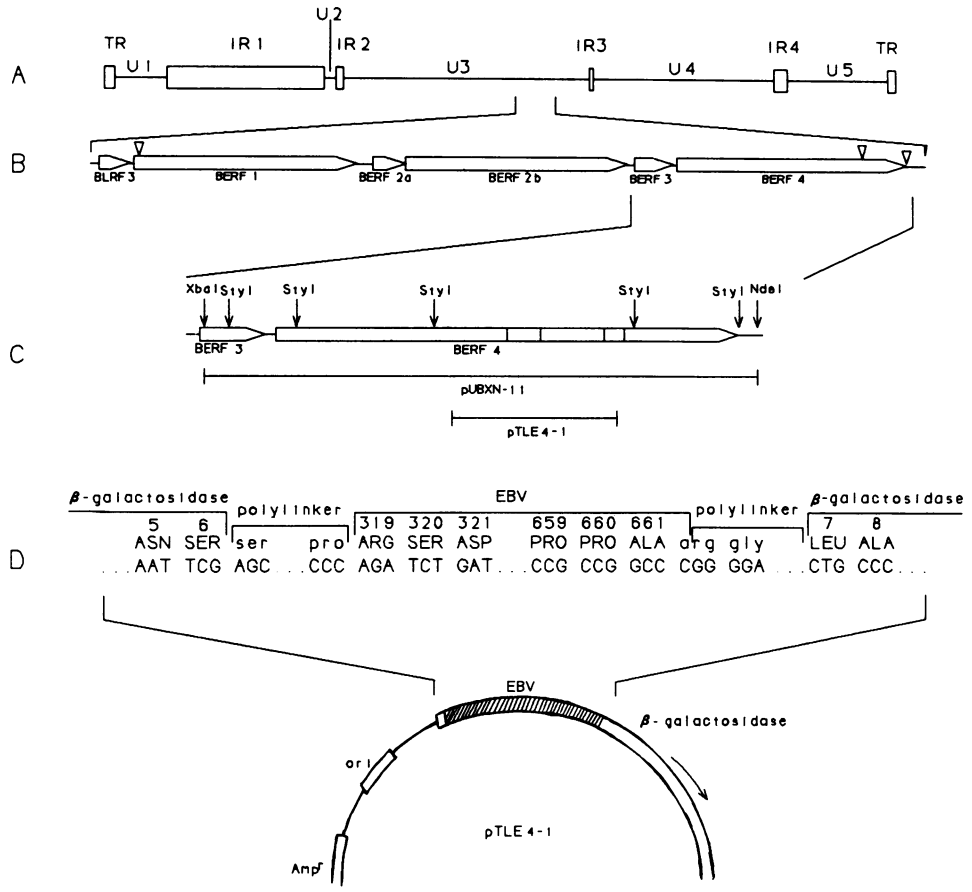

FIG. 1. Procaryotic expression of BERF4. (A) Schematic diagram of the EBV genome indicating major terminal (TR) and internal (IR) repeat and largely unique (U) DNA domains (9, 27). (B) The three *Bam*HI-E short and long open reading frames. Symbol: ▽, *Bam*HI site. (C) The 3-kb *Xba*I-*Nde*I *Eco*RI B fragment, including most of BERF3 and all of BERF4, which was cloned into pUC19 to generate pUBXN-11. The relevant restriction endonuclease sites, the minor repeat regions in BERF4 (□), and the segment which resulted in an immunoreactive fusion protein (pTLE4-1) are indicated. (D) A fragment of pUBXN-11, indicated in panel C, was cloned into the *Sma*I site of pTL2, a pUC18 plasmid which had been modified to contain the complete *lacZ* gene (50). The sequences at the recombination sites were determined as described in Materials and Methods. Superscripts refer to the codon number in either *lacZ* (6) or BERF4 (1).

pMC1513 (6). After transformation of *Escherichia coli* JM109 (51), β-galactosidase-positive clones were screened for expression of a β-galactosidase fusion protein and for reactivity with an EBV-immune human serum by immunoblot. The EBV insert in the recombinant plasmid from a clone which expressed a fusion protein reactive with human serum was sequenced by the dideoxy method with forward- and reverse-sequencing primers (43).

Purification of EBNA3C antibodies. The *E. coli* clone which expressed a stable EBV BERF4-β-galactosidase fusion protein was grown in LB medium to 0.65 optical density units, and expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co.). Cell pellets (38 g) were suspended in approximately 100 ml of breaking buffer (0.2 M Tris hydrochloride [pH 7.5], 0.25 M NaCl, 0.01 M magnesium acetate, 5% glycerol, 0.1% deoxycholate, 0.01 M 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) and lysed by adding 0.05 mg of lysozyme per ml for 40 min at 4°C. After being twice frozen and thawed, the cells were sonicated for 15 min and centrifuged at 70,000 × *g* for 1 h at 4°C. Saturated ammonium sulfate (40% [vol/vol]) was added. The resultant precipitate was suspended in and dialyzed against a solution of 20 mM Tris hydrochloride (pH 7.6), 20 mM NaCl, and 10 mM 2-mercaptoethanol. Sodium dodecyl sulfate (SDS) was added to a final concentration of 1%. After being heated at 95°C for 10 min and clarified, 2.4-ml aliquots were passed through a 200-ml Superose 6 (preparation grade; Pharmacia, Inc.) column. Fusion protein fractions were pooled, concentrated, and dialyzed against 6 M urea in 0.05 M Tris acetate (pH 7.1) to remove the SDS, against phosphate-buffered saline, and once against 0.1 M phosphate buffer (pH 7.0). Purified protein (approximately 3 mg) was coupled to 0.7 ml of Actigel A beads (Sterogene) in 0.1 M phosphate buffer (pH 7.0) in the presence of sodium cyanoborohydride (Aldrich Chemical Co., Inc.), as described by the manufacturer. Coupled beads were transferred to a small column and washed successively with 20 ml of 1 M NaCl, 50 ml of TBS buffer (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl), 3 ml of elution medium (Sterogene), and 50 ml of TBS. An EBV-immune serum then was passed through the fusion protein affinity column. The fusion protein column was washed extensively with TBS containing 0.1% bovine serum albumin and 0.3% Tween 20. Antibodies bound to the affinity matrix were eluted by washing the column with elution medium (Sterogene). The antibody was further purified by two more rounds of fusion protein affinity chromatography. The eluate was dialyzed against TBS (pH 7.5) and absorbed

against an EBV-negative cell (Louckes) lysate to remove potential reactivity with non-EBV-infected lymphocyte proteins.

Immunofluorescence staining. Lymphocytes were dried onto microwell slides. BALB/c 3T3 cells were grown on an eight-chamber microscope slide (Miles Scientific, Div. Miles Laboratories, Inc.). The slides were fixed in methanol at −20°C, blocked with 10% goat serum, stained with BERF4 affinity-purified antibody and incubated with fluorescein-conjugated goat anti-human immunoglobulin G (Jackson Laboratory). Slides were photographed under epifluorescence by using Ektachrome 160 film and a Zeiss photomicroscope.

Electrophoresis and immunoblotting. Protein samples were prepared by boiling cell pellets (2.5×10^7 cells) in 0.3 to 0.4 ml of sample buffer (6% SDS, 2 mM 2-mercaptoethanol, 140 mM Tris hydrochloride [pH 7.0], 2% glycerol, 0.07% bromophenol blue, 2 mM phenylmethylsulfonyl fluoride). Nuclei and cytoplasmic fractions were prepared by homogenizing cells (2.5×10^7) in a solution of 5 ml of 10 mM Tris hydrochloride (pH 7.5), 10 mM NaCl, 3 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride, 1.2% Nonidet P-40, and 1.5% sucrose at 4°C and by centrifuging nuclei to a pellet. Nuclei were suspended in 0.5 ml of 10 mM Tris hydrochloride (pH 7.5) plus 0.5 ml of protein sample buffer, boiled, and sonicated. The supernatant cytoplasmic fraction was concentrated and boiled in sample buffer. Protein samples were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie brilliant blue (0.03% [wt/vol] Coomassie blue, 25% [vol/vol] isopropanol, 10% [vol/vol] glacial acetic acid) or electrophoretically transferred to nitrocellulose and stained with 10% Ponceau S (Sigma) to localize size markers (Bio-Rad high-molecular-weight set). The immunoblots were then blocked in milk buffer (5% nonfat dry milk, 0.1% Tween 20, 0.03% sodium azide in phosphate-buffered saline) for 1 h at 37°C, reacted for 2 h at 22°C with whole human serum or affinity-purified antibody, washed in milk buffer, reacted with ^{125}I-labeled protein A (Amersham Corp.) for 30 min at room temperature, and washed again in milk buffer.

Isolation and characterization of cDNA clones. A cDNA library was constructed from the poly(A)$^+$ RNA of latently infected IB4 cells (42). The cDNA was ligated into the *Eco*RI restriction site of lambda gt10, packaged, and plated on *E. coli* C600 *hflA*. The library was screened with a ^{32}P-labeled (by nick translation) EBV genomic DNA fragment spanning the BERF4 open reading frame (1). The cDNA inserts from positive clones were recloned into the *Eco*RI restriction site of the Bluescript M13 phagemid vector (Stratagene Cloning Systems) for dideoxy sequencing analysis. Two extensively overlapping BERF4 cDNAs were isolated. One (T36) was 3,355 bp and extended from the 3′ terminus of the mRNA, indicated by a short poly(A) tract following a polyadenylation signal (AATAAA), across a splice from BERF4 to BERF3. This cDNA begins 16 bases after the first potential initiation codon of BERF3 (1). The second cDNA (T27) contained an additional 217 bp 5′ to the T36 cDNA but ended in BERF4 before the polyadenylation signal. The nucleotide coordinants of the T27 and T36 exons, revealed from their nucleotide sequences, are given in the legend to Fig. 6. ^{32}P-labeled T36 cDNA was hybridized to a Northern (RNA) blot of IB4 cell RNA.

Expression of EBNA3C in fibroblasts. An oligonucleotide was synthesized and ligated to the T36 cDNA by using the *Xba*I restriction site 9 bp 3′ (base 98391 of the EBV genome; 1) to the T36 5′ terminus. The oligonucleotide extends the cDNA to the splice acceptor site (revealed by the T27 cDNA), which is 7 bases 5′ to the likely initiator methionine codon (base 98371 of the genome; 1). The sequence of the recombinant oligonucleotide-T36 cDNA construct was confirmed. The construct was then cloned by blunt-end ligation into the *Bam*HI restriction site of the murine retrovirus expression vector pZip-neoSV(X)1 (7, 8). BALB/c 3T3 cells (obtained from the American Type Culture Collection) were transfected by calcium phosphate precipitation with EBV oligo-T36 cDNA-pZip-neoSV(X)1 recombinant plasmid or with parental plasmid DNA (16). Clones resistant to G418 (GIBCO Laboratories) were derived, expanded, and tested for EBNA expression and growth in medium supplemented with 10 or 1% fetal bovine serum.

RESULTS

Reaction of EBV-immune human serum with BERF4-encoded protein. The first objective of these experiments was to determine whether the EBNA3 homologous open reading frame, BERF4, encodes one of the putative EBNA proteins slightly larger than EBNA3. An antibody specific for the BERF4-encoded protein might identify the protein on Western blots (immunoblots) and within latently infected cells. To obtain a BERF4-specific antibody, large fragments of BERF4 were cloned into vector pTL2, which detects the open reading frame by expressing a β-galactosidase fusion protein. Twenty-three β-galactosidase-expressing colonies were identified and isolated. One of these, pTLE4-1, made a 180-kilodalton (kDa) fusion protein which was detected by immunoblot with an EBV-immune human serum. The 180-kDa fusion protein was present in small amounts; 170-, 155-, and 145-kDa immunoreactive proteins, probable protease cleavage products, were also evident. No immunoreactive proteins of a similar size were detected in Western blots of extracts from control *E. coli* expressing β-galactosidase. The nucleotide sequence of pTLE4-1 indicated that it contained BERF4 codon 319 (based on the EBV genomic sequence; 1) to codon 661 in frame with *lacZ* (Fig. 1D). Since the pTLE4-1 EBV DNA insert is 1,030 bp, pTLE4-1 should encode a fusion protein of at least 160 kDa. These results indicate that at least part of BERF4 is expressed in human EBV infection and that humans could be a source of BERF4-specific antisera.

Detection of a 155-kDa protein in extracts of latently infected lymphocytes by BERF4-specific antibody. The 145-, 155-, 170-, and 180-kDa fusion proteins were purified from *E. coli* lysates by Superose gel filtration chromatography and coupled to Actigel A. Reactive human antibodies were selectively adsorbed to and eluted from the fusion protein affinity matrix. The eluted antibodies specifically detected a 155-kDa protein in the tightly latently infected lymphoblastoid line IB4 (Fig. 2D). Reactivity with the 155-kDa IB4 protein was markedly increased by affinity chromatography (compare IB4 lanes in Fig. 2C and D with the IB4 lane in Fig. 2B probed with unpurified serum). The antibody did not react with a similar-size protein in the EBV-negative Burkitt's tumor B cell line (Louckes cells), indicating that the 155-kDa protein is dependent on EBV infection and is not B lymphocyte specific. The antibody recognized BERF4 determinants and did not react with β-galactosidase (Fig. 2D). Thus, at least part of the BERF4 open reading frame encodes a protein in latently infected IB4 cells.

Identification of the 155-kDa protein as a nuclear protein present in latently infected cells. The definitive identification of an EBV protein as an EBNA protein requires the dem-

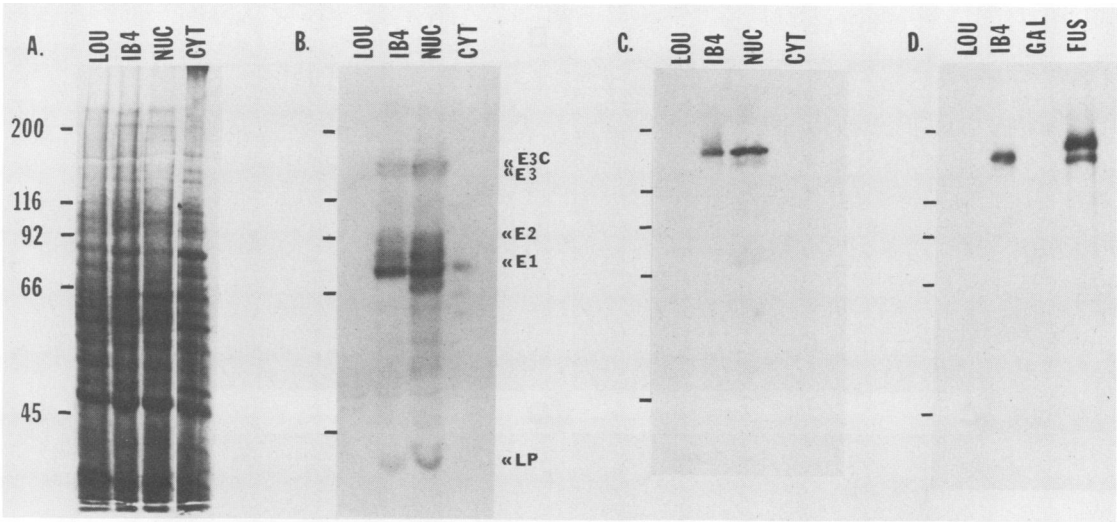

FIG. 2. Specific reaction of affinity-purified antibodies to the pTLE4-1 fusion protein with a 155-kDa protein in nuclear extracts of EBV-infected cells. Protein samples from IB4 whole cells (lymphocytes latently infected with EBV), IB4 nuclear (NUC) and cytoplasmic (CYT) fractions (prepared as described in Materials and Methods), Loukes cells (EBV-negative Burkitt's tumor lymphocytes [LOU]), 5 μg of β-galactosidase (GAL), or 0.04 μg of purified fusion protein (FUS) were run on discontinuous 7% polyacrylamide gels. Gels were either transferred to nitrocellulose or stained with Coomassie brilliant blue (A). Immunoblots were reacted with an EBV-immune human serum (B) or with BERF4 affinity-purified antibodies from the EBV-immune serum (C and D). The numbers to the left indicate the positions and sizes (in kilodaltons) of protein markers. EBNAs are marked on the right in panel B: E3C, EBNA3C; E3, EBNA3; E2, EBNA2; E1, EBNA1; LP, EBNA-LP.

onstration that the protein is expressed in latently infected cell nuclei. After treatment of IB4 cells with nonionic detergent, most of the EBNAs were in the nuclear fraction and only a small amount of EBNA1 was found in the cytoplasmic fraction (Fig. 2B). The 155-kDa protein notably partitioned to the nuclear fraction (Fig. 2C). An EBNA protein should also be present in most cells of a latently infected cell line and not just in the rare cell which may be partially permissive for virus replication and which may thereby express an abundant quantity of a productive infection-associated protein. In indirect immunofluorescence microscopy, the BERF4-specific antibody did indeed react with an antigen in the nucleus of almost every IB4 or Namalwa cell (Fig. 3A and B; data not shown). The fluorescence staining was particulate. The nucleoli were not stained. This was particularly noticeable in B95-8 cells (Fig. 3C). (Although 1 to 2% of these B95-8 cells expressed proteins associated with virus replication, most of the cells were latently infected.) These results show that the protein encoded by the BERF4 open reading frame is a nuclear antigen present in almost every cell of a culture latently infected with EBV and therefore should be referred to as an EBNA. We designated this EBNA3-related protein EBNA3C and will subsequently refer to the EBNA3 protein as EBNA3A.

Since the open reading frames encoding EBNA3A and EBNA3C are distantly homologous at their 5′ ends (23), an antibody against the BERF4 protein might cross-react with EBNA3A. To confirm that the BERF4-specific antibody reacted with EBNA3C and not EBNA3A, strip immunoblots from the same gel of an IB4 cell lysate were prepared and reacted with EBNA3A-specific antibody (Fig. 4, lane E3; 23), with BERF4-specific antibody (Fig. 4, lane E3C), or with three different EBV-immune human sera (Fig. 4). Clearly, EBNA3C detected by the BERF4-specific antibody on one strip blot was larger than the EBNA3A protein detected by the original EBNA3A (BERF1)-specific antibody on an adjacent strip (compare lanes E3C and E3 in Fig. 4). The EBNA3C-specific BERF4 antibody therefore does not cross-react with EBNA3A. EBV-immune human sera (RY, RS, or JT) varied in their relative reactivities with EBNA3A or EBNA3C proteins, although these sera had similar reactivities with EBNA1, EBNA2, and EBNA-LP (Fig. 4). The specificity of the BERF4 serum for EBNA3C was also evident in its immunostaining reactivity with BALB/c 3T3 cells expressing EBNA3C (see below) and in its lack of reactivity with BALB/c 3T3 cells expressing other EBNA proteins, including EBNA3A (data not shown).

EBNA3C encoded by other EBV isolates. Since EBNA3C is present in IB4 cells, one might expect it to be present in other cell lines latently infected with EBV. Immunoblots of lysates of cells infected with different EBV isolates revealed that a similar-size EBNA3C is present in the tightly latent Burkitt's tumor-derived cell line, Namalwa, as well as in the partially permissive Daudi and B95-8 cell lines (Fig. 5). EBNA3C in Namalwa and B95-8 cells is 155 kDa, as in IB4 cells, whereas EBNA3C in Daudi cells is approximately 160 kDa. Thus, some polymorphism in EBNA3C size is observed, probably due to the DNA repeats within BERF4. EBNA3C was not detected in blots of Raji cell proteins (Fig. 5). The Raji EBV strain contains a deletion which includes most of the BERF4 open reading frame (36), and therefore the absence of EBNA3C is expected. Surprisingly, EBNA3C also was not detected in P3HR1 or Jijoye cell extracts (Fig. 5). It is not known whether the P3HR1 and Jijoye cell lines have a deletion similar to that of the Raji cell line. Although Raji cells have EBNA3A, they did not stain with the BERF4-specific antibody in indirect immunofluorescence, providing further evidence that the antiserum is specific for EBNA3C (data not shown).

EBNA3C mRNA. RNA mapping and DNA sequence analyses of EBNA1, EBNA2, and EBNA3 cDNAs indicate that these mRNAs are complex transcripts which have IR1- and U2-derived exons near their beginnings but differ in their

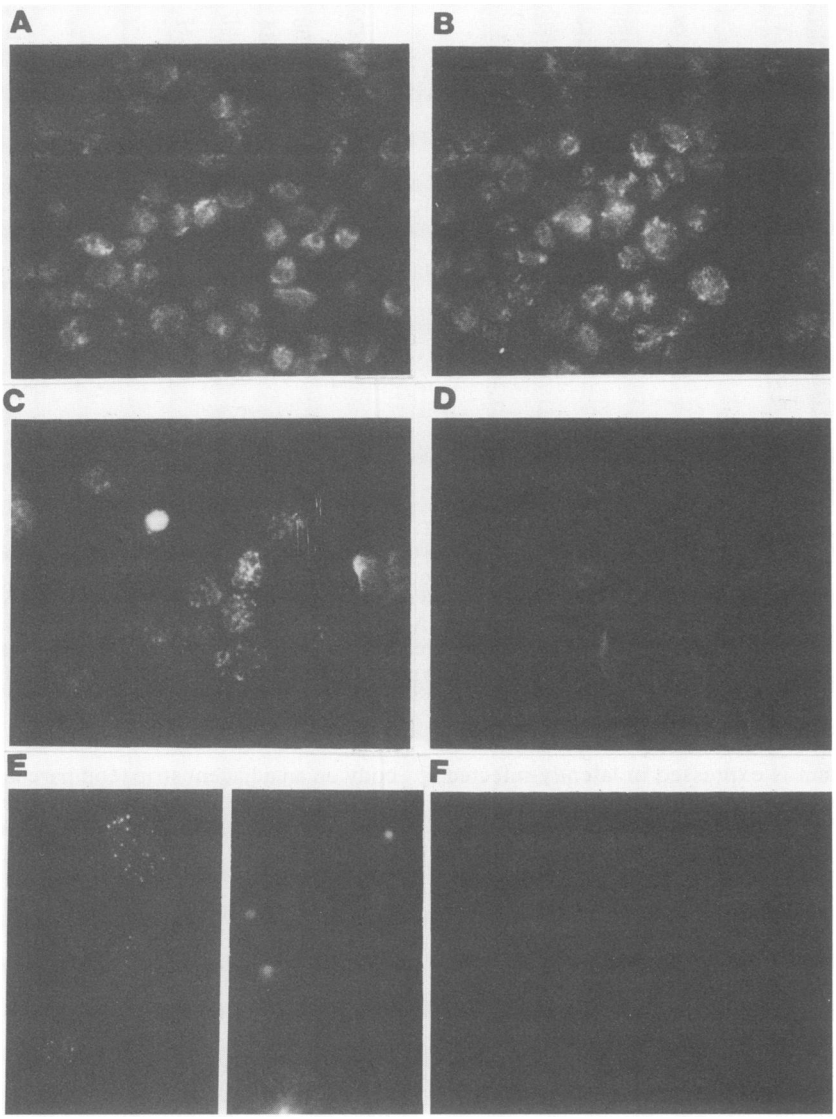

FIG. 3. Immunofluorescence staining for the BERF4 protein, showing that it is a nuclear antigen in all latently infected cells. Affinity-purified BERF4-specific antibody was used to stain latently infected IB4 cells (A and B), mostly latently infected B95-8 cells (C), EBV-negative Louckes cells (Burkitt's tumor B lymphocytes) (D), or BALB/c 3T3 cells transfected with either pZipE3C (E) or pZipneo (F). Magnification, ×365.

EBNA coding capacities because of alternative splicing of the U2 exons to other exons, the most 3′ of which encodes most or all of each of the EBNA proteins. A previous cDNA clone which contains part of the EBNA3C open reading frame and the previously derived EBNA3A cDNA clone were derived from partially productively infected B95-8 marmoset cell cultures (4, 5). We therefore undertook to determine whether latently infected human cell lines had similar mRNAs. Two overlapping EBNA3C cDNAs were isolated from a cDNA library made from latently infected IB4 cells (Fig. 6B). The first clone, T36, contains the complete mRNA 3′ end, as indicated by the presence of a short poly(A) tract at 19 bases downstream of a polyadenylation signal (AATAAA). This cDNA begins in BERF3, 16 bases after a potential translational initiation site, and is spliced in frame to BERF4. The second cDNA, T27, begins within the previously described Y3 exon, which is followed by a U exon, as in some other EBNA mRNAs (5, 42, 45). These are spliced to a BERF3 exon which begins 7 bases 5′ to the putative translational initiation site. The splice from BERF3 to BERF4 is identical to that of T36. (The complete nucleotide sequence coordinants of the T36 and T27 cDNA clones are given in the legend to Fig. 6.) Thus, the BERF3 and BERF4 exons appear to comprise the EBNA3C open reading frame (Fig. 6). Northern blot analysis of IB4 poly(A)$^+$ RNA, with T36 as a probe, indicated that a single RNA of approximately 5 kb is transcribed from this region of the genome during latent infection (Fig. 6C). If it is assumed that the EBNA3C mRNA contains all of the 5′ exons common to the other EBNA mRNAs, as suggested from these and previous cDNA data, then the predicted sizes of the EBNA3C mRNA and the detected transcript are in very good agreement.

Expression of EBNA3C in BALB/c 3T3 cells. The putative

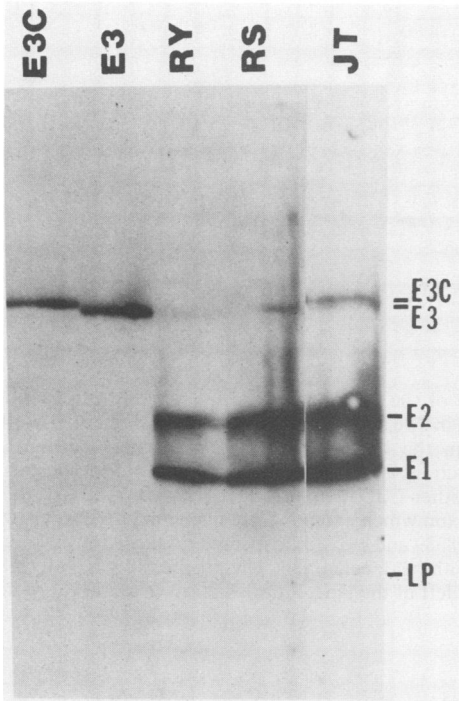

FIG. 4. Affinity-purified antibodies to EBNA3C do not cross-react with EBNA3A. An IB4 cell protein sample was run on one wide lane of a discontinuous SDS–6% polyacrylamide gel and transferred to nitrocellulose. The resultant blot was cut into strips, and each strip was separately reacted with affinity-purified BERF4-specific antibody (lane E3C), affinity-purified EBNA3A-specific antibody (lane E3) (23), or three different EBV-immune human sera (lanes RY, RS, and JT). EBNAs are indicated on the right. See the legend to Fig. 3 for abbreviations.

EBNA3C-encoding open reading frame which extends from the potential translational initiation site in the penultimate BERF3 exon through the BERF4 exon was recombined into the *gag* leader cloning site of pZip-neoSV(X)1 and transfected into BALB/c 3T3 cells. G418-resistant clones were selected. Clones transfected with the recombinant construct did not appear different from clones transfected with the parent vector. Individual cell morphology was unaltered. Cells remained fully contact inhibited and did not heap up. Four clones potentially expressing EBNA3C were isolated, expanded, and tested for EBNA3C expression by Western blot. Two clones expressed approximately the same EBNA3C amount as the lymphoblastoid cell line, IB4, latently infected with EBV. The size of the EBNA3C protein was identical to that in IB4 cells, confirming that the cDNA encodes all of EBNA3C (Fig. 7). Indirect immunofluorescence staining of the EBNA3C-expressing BALB/c 3T3 cells by using the fusion protein-specific antibody revealed particulate nuclear staining and nucleolar sparing (Fig. 3E) similar to that observed in EBV-infected lymphocytes (Fig. 3A to C). This antibody did not stain BALB/c 3T3 cells transfected with vector alone (Fig. 3F). The clones expressing EBNA3C were tested for the ability to grow in medium supplemented with 1% serum. They grew poorly in 1% serum, as did clones of cells transfected with the parent vector lacking EBNA3C-encoding DNA (data not shown).

DISCUSSION

The results of the present study demonstrate that the EBV BERF3 and BERF4 open reading frames are spliced together to position the favorable translational initiation site of BERF3 at the beginning of 971 translatable codons. The results further demonstrate that a 5-kb EBV mRNA partially encoded by BERF4 is expressed in tightly latently infected IB4 cells. On the basis of the cDNA sequence, the exons of this mRNA include exons from the EBV DNA *Bam*HI Y, *Bam*HI U (172 bases), and *Bam*HI E (367 bases and 2,984 bases) DNA fragments. The cDNA sequence begins in *Bam*HI-Y, within the previously characterized Y3 exon which is in the EBNA1 and EBNA3A mRNAs (5, 42; unpublished observations). Since the EBNA3C mRNA is 5 kb and the sequence 3′ to the Y3 exon totals 3,723 bases [if the poly(A) tract is assumed to have 200 bases], it is likely that the remainder of the RNA consists of repeating IR1 exons (W1 and W2) and the Y1 and Y2 exons, since these are known to contribute 1,013 bases to the EBNA2 mRNA (42) and are also part of the previously obtained BERF3-BERF4 cDNA from B95-8 cells (5). Since the EBNA3C mRNA has the repeating IR1 and unique Y exons, it could encode EBNA-LP within its leader. Whether the mRNA also encodes EBNA-LP and is bicistronic depends on whether the EBNA3C mRNA leader contains the unique W0 and W01 exons preceding the W1 and W2 exons, since the W0-W01 splice generates the only potential LP translational initiation codon (as in the EBNA2-LP) bicistronic mRNA (42, 50).

The experiments reported here also demonstrate that the BERF3 and BERF4 exons completely encode a new nuclear protein characteristically expressed in latent EBV infection. This new protein is designated EBNA3C on the basis of

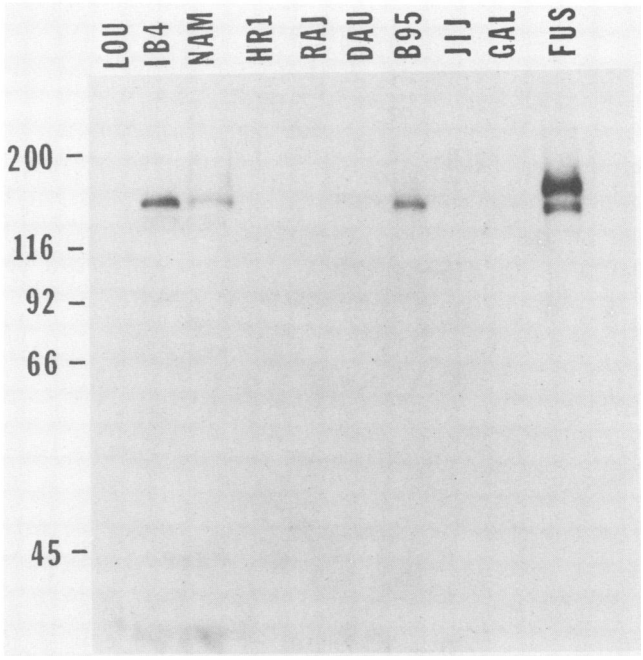

FIG. 5. The presence of EBNA3C in different EBV-infected lymphocyte lines. Protein samples prepared from lymphocytes latently infected with EBV (IB4 and Namalwa [NAM]), mostly latently infected lymphocytes (uninduced P3HR-1 [HR1], Raji [RAJ], Daudi [DAU], B95-8 [B95], and Jijoye [JIJ] cells), uninfected lymphocytes (Louckes [LOU]), 0.5 μg of β-galactosidase (GAL), and 0.04 μg of purified fusion protein (FUS) were run on a discontinuous SDS–7% polyacrylamide gel and transferred to nitrocellulose. The immunoblot was reacted with the affinity-purified BERF4-specific antibody. The numbers to the left indicate the sizes (in kilodaltons) of protein markers.

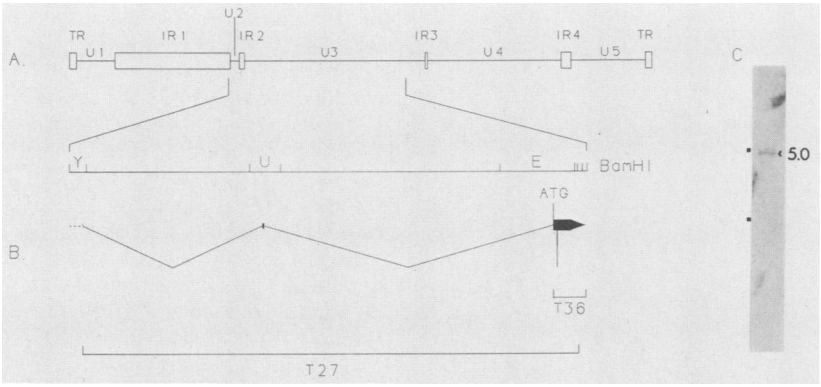

FIG. 6. EBNA3C mRNA. Below the EBV genome (A), the partial structure of the EBNA3C mRNA deduced from two overlapping cDNAs, T36 and T27, is shown (B). The parts of the EBNA3C mRNA represented in T36 and T27 cDNAs are indicated at the bottom. Exons are depicted by horizontal segments; introns are depicted by deflections from the horizontal. ATG is the start of translation. The EBNA3C-encoding open reading frame within the mRNA is shaded. All splice junctions conform to consensus sites (34). The partial *Bam*HI Y exon (Y3) and the U exon (U) are the same as those present in the mRNAs of other EBNAs (5, 42, 45). The two *Bam*HI-E (E) exons are the BERF3 exon which extends from nucleotides 98364 to 98730 and the BERF4 exon which extends from nucleotides 98805 to 101788 in the EBV sequence (1). The 5′ and 3′ coordinates of the beginning and end of T36 are 98389 and 101788, respectively; those for T27 are 48425 and 101016, respectively. (C) Northern blot of IB4 cell poly(A)$^+$ RNA (5 μg) probed with ^{32}P-labeled T36. The position to which the 28S and 18S rRNAs migrated in the 2.2 M formaldehyde–1.2% agarose gel are indicated to the left of the lane. Abbreviations in panel A are described in the legend to Fig. 1.

its relationship to the previously described EBNA3A. EBNA3A is encoded by a short *Bam*HI-L open reading frame (BLRF3) exon and the neighboring long BERF1 exon (23, 24). BLRF3-BERF1, BERF2A-BERF2B, and BERF3-BERF4 are similar short-long open-reading-frame pair motifs, and the long open reading frames are distantly homologous (1, 23). Since it is now evident that the BLRF3-BERF1 and BERF3-BERF4 exon pairs encode 145- and 155-kDa EBNA proteins, respectively, and a third nuclear protein, 165 kDa, is evident on Western blots of latently infected cell nuclear proteins (23; unpublished observations), it is likely that BERF2A-BERF2B encodes an EBNA protein which would be designated EBNA3B because of its map position between the DNA segments which encode EBNA3A and EBNA3C.

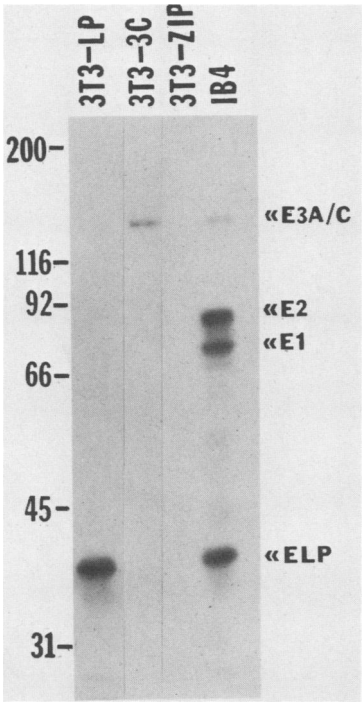

FIG. 7. BALB/c 3T3 clones expressing an EBNA3C after transfection with recombinant EBNA3C pZip-neoSV(X)1 DNA (lane 3T3-3C) which is the same size as the EBNA3C ordinarily expressed from the EBV genome in latent infection (lane IB4). 3T3 cells transfected with EBNA-LP recombinant pZip-neoSV(X)1 (3T3-LP) or with parental vector pZip-neoSV(X)1 (3T3-Zip) are shown for comparison. Positions and sizes (in kilodaltons) of proteins markers are indicated on the left. EBNAs are indicated on the right: E3A/C, EBNA3 or EBNA3C; E2, EBNA2; E1, EBNA1; ELP, EBNA-LP.

The sequence of EBNA3C (Fig. 8) is predicted from the cDNA and EBV genomic DNA sequences to have several remarkable features. First, it is composed of a high proportion of charged amino acids. Among the 992 amino acids are 77 arginines, 23 lysines, 31 histidines, 51 aspartic acids, and 53 glutamic acids. The abundance of lysine, arginine, and histidine suggests the possibility of direct binding to a polyanion such as DNA. However, no zinc-binding motifs exist within the sequence (3, 33). Second, there are several arginine- or lysine-rich domains, e.g., Arg-Arg-Arg-Arg-Arg-Arg-Arg, Arg-Arg-Tyr-Arg-Arg, Lys-Lys-Pro-Arg-Lys, Arg-Arg-Arg-Arg, or Arg-Arg-Glu-Lys-Arg, which resemble the nuclear localization signal of simian virus 40 T (Pro-Lys-Lys-Lys-Arg-Lys-Val) (44) or polyomavirus T (Pro-Lys-Lys-Ala-Arg-Glu-Asp and Val-Ser-Arg-Lys-Arg-Pro-Arg) (41) proteins. Third, there are two major repeating domains (Fig. 8) within the protein which could be sites for polyvalent interactions (37). The first repeat begins at amino acid 551 and includes six perfect repeats of the core element GPPAA. The second begins at amino acid 741 and consists of three near-perfect repeats of PQAPYQGYQEPPA.

Nothing is known as yet about EBNA3 functions. EBNA3A and EBNA3C are not homogeneously distributed through the latently infected cell nucleus. They appear to spare nucleolus-like structures. EBNA3A and EBNA3C appear to be associated with nuclear structures that are larger than EBNA2 or EBNA-LP. Possible functions could include regulation of transcription, of mRNA processing, or of EBV episome copy number. EBNA1 is required for

```
        10          20          30          40          50
MESFEGQGDS  RQSPDNERGD  NVQTTGEHDQ  DPGPGPPSSG  ASERLVPEES
        60          70          80          90         100
YSRDQQPWGQ  SRGDENRGWM  QRIRRRRRRR  AALSGHLLDT  EDNVPPWLPP
       110         120         130         140         150
HDITPYTARN  IRDAACRAVK  QSHLQALSNL  ILDSGLDTQH  ILCFVMAARQ
       160         170         180         190         200
RLQDIRRGPL  VAEGGVGWRH  WLLTSPSQSW  PMGYRTATLR  TLTPVPNRVG
       210         220         230         240         250
ADSIMLTATF  GCQNAARTLN  TFSATVWTPP  HAGPREQERY  AREAEVRFLR
       260         270         280         290         300
GKWQRRYRRI  YDLIELCGSL  HHIWQNLLQT  EENLLDFVRF  MGVMSSCNNP
       310         320         330         340         350
AVNYWFHKTI  GNFKPYYPWN  APPNENPYHA  RRGIKEHVIQ  NAFRKAQIQG
       360         370         380         390         400
LSMLATGGEP  RGDATSETSS  DEDTGRQGSD  VELESSDDEL  PYIDPNMEPV
       410         420         430         440         450
QQRPVMFVSR  VPAKKPRKLP  WPTPKTHPVK  RTNVKTSDRS  DKAEAQSTPE
       460         470         480         490         500
RPGPSEQSSV  TVEPAHPTPV  EMPMVILHQP  PPVPKPVPVK  PTPPPSRRRR
       510         520         530         540         550
GACVVYDDDV  IEVIDVETTE  DSSSVSQPNK  PHRKHQDGFQ  RSGRRQKRAA
       560         570         580         590         600
PPTVSPSDTG  PPAVGPPAAG  PPAAGPPAAG  PPAAGPPAAG  PPAAGPRILA
       610         620         630         640         650
PLSAGPPAAG  PHIVTPPSAR  PRIMAPPVVR  MFMRERQLPQ  STGRKPQCFW
       660         670         680         690         700
EMRAGREITQ  MQQEPSSHLQ  SATQPTTPRP  SWAPSVCALS  VMDAGKAQPI
       710         720         730         740         750
ESSHLSSMSP  TQPISHEEQP  RYEDPDAPLD  LSLHPDVAAQ  PAPQAPYQGY
       760         770         780         790         800
QEPPAPQAPY  QGYQEPPPPQ  APYQGYQEPP  AHGLQSSSYP  GYAGPWTPRS
       810         820         830         840         850
QHPCYRHPWA  PWSQDPVHGH  TQGPWDPRAP  HLPPQWDGSA  GHGQDQVSQF
       860         870         880         890         900
PHLQSETGPP  RLQLSLVPLV  SSSAPSWSSP  QPRAPIRPIP  TRFPPPPMPL
       910         920         930         940         950
QDSMAVGCDS  SGTACPSMPF  ASDYSQGAFT  PLDINATTPK  RPRVEESSHG
       960         970         980         990
PARCSQATAE  AQEILSDNSE  ISVFPKDAKQ  TDYDASTESE  LD
```

FIG. 8. Amino acid sequence of EBNA3C as predicted from the cDNA and genomic DNA sequences (1). The nucleotide sequence was translated (37), and amino acids were designated by using the one-letter code. The underlined sequences represent regions which contain repetitive elements (37).

episome persistence but may not be the sole EBV determinant of episome copy number. Even in the simpler papovaviruses, several viral nuclear proteins affect episome maintenance (2).

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