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# NOTES

## A Sixth Epstein-Barr Virus Nuclear Protein (EBNA3B) Is Expressed in Latently Infected Growth-Transformed Lymphocytes

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**In the Epstein-Barr virus *Bam*HI E genomic fragment, there are three distantly homologous long open reading frames, BERF1, BERF2b, and BERF4, each of which is preceded by a short open reading frame. The most leftward and most rightward short and long open reading frame pairs encode 145- and 155-kilodalton proteins in latently infected cells (EBNA3A and EBNA3C, respectively). In this report, we demonstrate that the middle long open reading frame, BERF2b, encodes part of a 165-kilodalton nuclear protein in every latently infected cell. Therefore, this protein is designated EBNA3B.**

Epstein-Barr virus (EBV) causes infectious mononucleosis (20) and is also involved in the etiology of nasopharyngeal carcinoma and some B-cell lymphomas (7, 8, 10, 22, 27, 39). Upon in vitro infection, the virus causes human B lymphocytes to continuously proliferate (20, 24). EBV infection is mostly latent with respect to virus replication; only an occasional progeny-infected lymphocyte is permissive for viral replication. Even in latently infected cells, however, a few genes, which perhaps function to maintain latency and cell growth transformation, are expressed (5, 20). Specifically, six EBV-encoded proteins have been identified and shown to be expressed in each cell of a predominantly latently infected cell line. One, LMP, is a cytoplasmic and plasma membrane protein (11, 12, 23, 33). The other five are nuclear proteins, called EBNAs (EBNA1 [14, 15, 29, 30, 37, 38], EBNA2 [6, 16, 34], EBNA3A [13, 17], EBNA3C [26], and, EBNA-LP [9, 31, 35]).

EBNA3A and EBNA3C have been mapped to the EBV genome *Bam*HI E DNA fragment. This fragment contains three distantly related long open reading frames, each preceded by a short open reading frame (see Fig. 1B). EBNA3A (145 kilodaltons [kDa]) is encoded by the most leftward short and long open reading frame motif, BLRF3-BERF1 (2, 3, 17, 18), while EBNA3C (155 kDa) is encoded by the most rightward one, BERF3-BERF4 (26). In both cases, the small open reading frame is spliced in frame to the larger one. Since EBNA3A and EBNA3C are encoded by two of three structurally similar and distantly homologous genes and are two of the three proteins similar in size detected on Western blots (immunoblots) of predominantly latently infected cell extracts (17, 19), it is likely that a third EBNA protein is encoded by the middle short and long open reading frame motif in *Bam*HI-E (BERF2a and BERF2b). The formal demonstration of EBNA3B existence requires a BERF2a- and 2b-specific probe which identifies a product in each latently infected cell. In these experiments, we expressed part of the BERF2b open reading frame in bacteria as an EBV-β-galactosidase fusion protein, used the fusion protein to affinity purify BERF2b-specific antibody from an EBV-immune human serum, and used the antibody to identify a 165-kDa protein in almost all latently infected cell nuclei.

**Procaryotic expression of BERF2b.** A 2,719-base-pair *Spe*I-*Xba*I fragment which contains the entire BERF2b open reading frame was isolated from an EBV *Bam*HI E fragment clone (Fig. 1), treated with BAL 31 nuclease to remove the sequence outside of the BERF2b open reading frame and to randomize the ends, and ligated into a β-galactosidase fusion protein expression vector, pMC1513 (4). The polylinker of this vector is inserted into codon 8 of the *lacZ* gene, shifting the reading frame such that translation of *lacZ* is prevented unless an open reading frame with the correct number of nucleotides at its 5′ and 3′ ends is inserted into the polylinker to create a β-galactosidase fusion protein. After transformation of *Escherichia coli* JM109 (36), 29 β-galactosidase-positive clones were identified on MacConkey agar plates. Immunoblot analysis using rabbit anti-β-galactosidase serum showed that 15 of these clones expressed a β-galactosidase fusion protein. One of these, pE2b-27, expressed a stable 190-kDa fusion protein reactive with a human EBV-immune serum upon immunoblotting. The EBV insert of pE2b-27 was subcloned into pUC18 and then sequenced by the dideoxy method (32) with forward- and reverse-sequencing primers. The nucleotide sequence revealed that pE2b-27 contained BERF2b codons 84 to 730 (on the basis of the EBV genomic sequence [1]) in frame with *lacZ* (Fig. 1D). With an EBV DNA insert of 1,942 base pairs, pE2b-27 should encode a 190-kDa fusion protein, as was observed.

**Purification of BERF2b-specific antibodies.** The 190-kDa pE2b-27 fusion protein was purified from bacterial lysates by Superose gel-filtration chromatography as described previously (26). Fractions containing the fusion protein were pooled, concentrated, and dialyzed against 6 M urea in 0.05 M Tris acetate, pH 7.8, to remove the sodium dodecyl sulfate, against phosphate-buffered saline, and then against 0.1 M phosphate buffer, pH 7.0. Purified protein (approximately 2 mg) was coupled to 1 ml of Actigel A beads (Sterogene). After passage of an EBV-immune human serum through a small fusion protein affinity column and extensive

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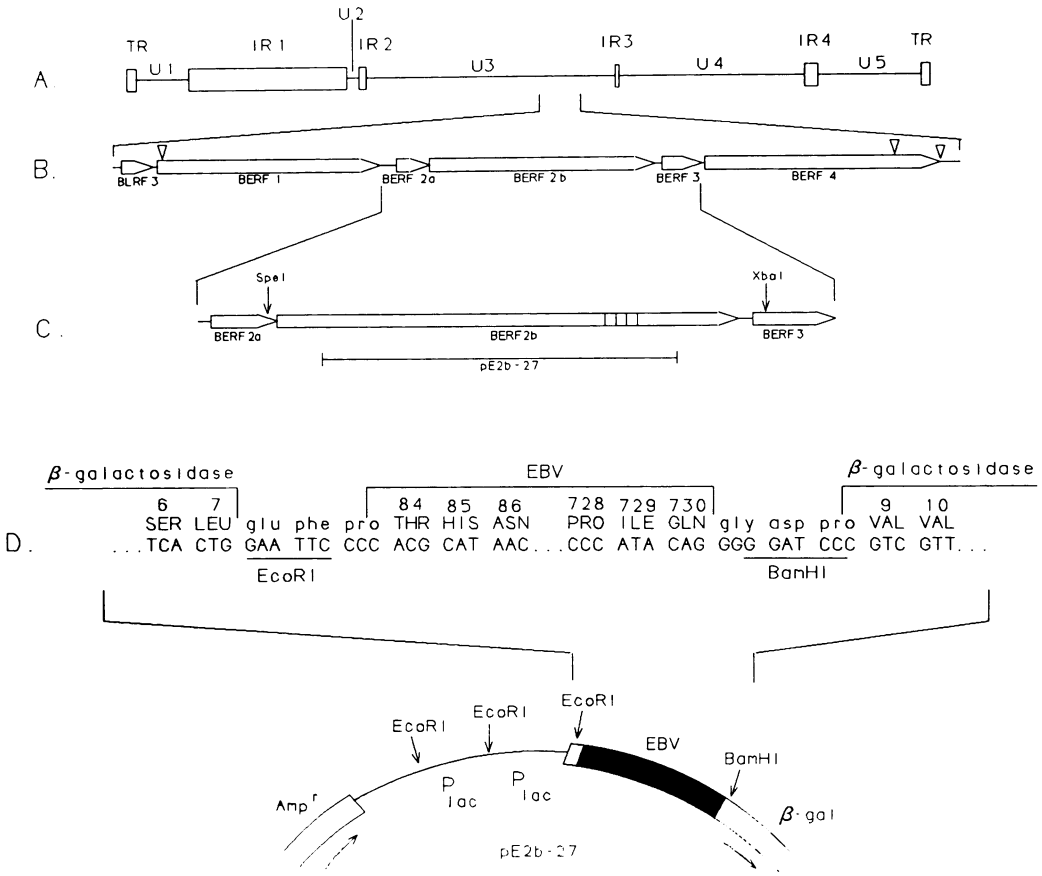


FIG. 1. Procaryotic expression of BERF2b. (A) Schematic diagram of the EBNA3 region within the EBV genome. The terminal (TR) and internal (IR) repeat, as well as largely unique (U) EBV DNA domains, are indicated (5, 21). (B) Schematic of the three *Bam*HI-E short and long open reading frame motifs. BLRF3 and BERF1 encode EBNA3A (17, 18), while BERF3 and BERF4 encode EBNA3C (26). ▽, *Bam*HI sites. (C) Diagram of the 2.7-kilobase *Bam*HI E *Spe*I-*Xba*I fragment, which was treated with BAL 31 and cloned into the β-galactosidase fusion protein expression plasmid pMC1513 to yield pE2b-27. The relevant restriction endonuclease sites and minor repeats (open boxes in BERF2b) and the segment which resulted in an immunoreactive fusion protein are indicated. (D) Schematic diagram of pE2b-27, which expressed a stable BERF2b–β-galactosidase fusion protein. The sequence at the recombination sites was determined by the dideoxy method (32). Numbers refer to the codon number in either *lacZ* (4) or BERF2b (1).

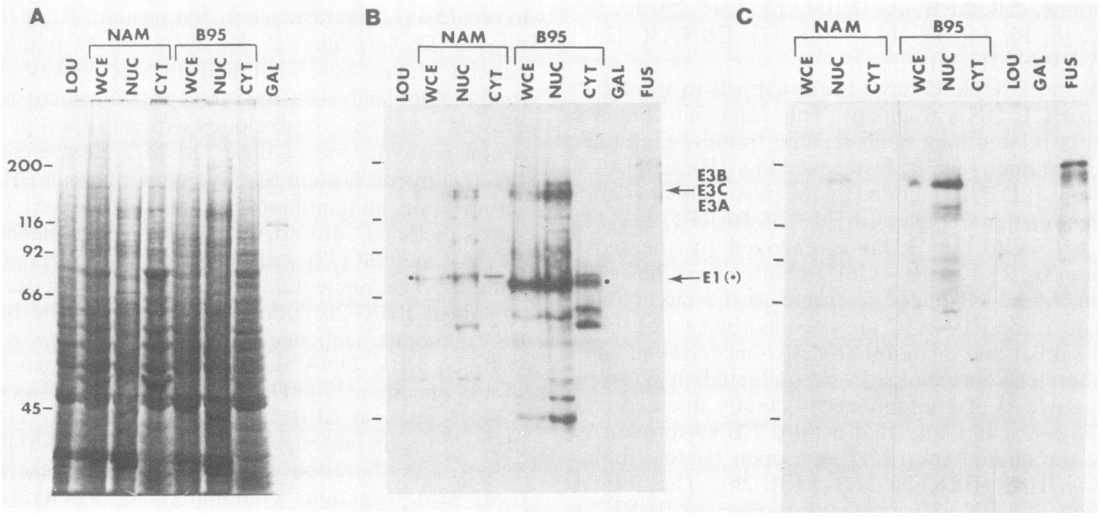


FIG. 2. Affinity-purified antibodies to the pE2b-27 fusion protein specifically react with a 165-kDa protein in nuclear extracts of EBV latently infected cells. Protein samples from Namalwa (EBV latently infected Burkitt tumor lymphocytes) and B95-8 (EBV semipermissively infected lymphocytes) whole-cell (WCE), nuclear (NUC), and cytoplasmic (CYT) extracts, Loucke cells (LOU) (EBV-negative Burkitt tumor lymphocytes, 0.5 μg of β-galactosidase (GAL), or 0.05 μg of purified fusion protein (FUS) were run on 7% discontinuous polyacrylamide gels. Approximately $0.5 \times 10^6$ to $1.0 \times 10^6$ cells were loaded in those gel lanes containing lymphocyte extracts. Gels were either transferred to nitrocellulose or stained with Coomassie brilliant blue (0.03% [wt/vol] Coomassie blue, 25% [vol/vol] isopropanol, 10% [vol/vol] glacial acetic acid) (A). Immunoblots were reacted with an EBV-immune human serum (B) or with BERF2b antibodies affinity purified from the EBV-immune serum (C). The numbers to the left indicate the sizes (in kilodaltons) of protein markers. EBV nuclear antigens expressed during latency (EBNAs) which are encoded by *Bam*HI-E open reading frames (E3A, E3B, E3C), as well as EBNA1 (E1), are marked on the right of panel B. Dots emphasize the locations of EBNA1 bands.

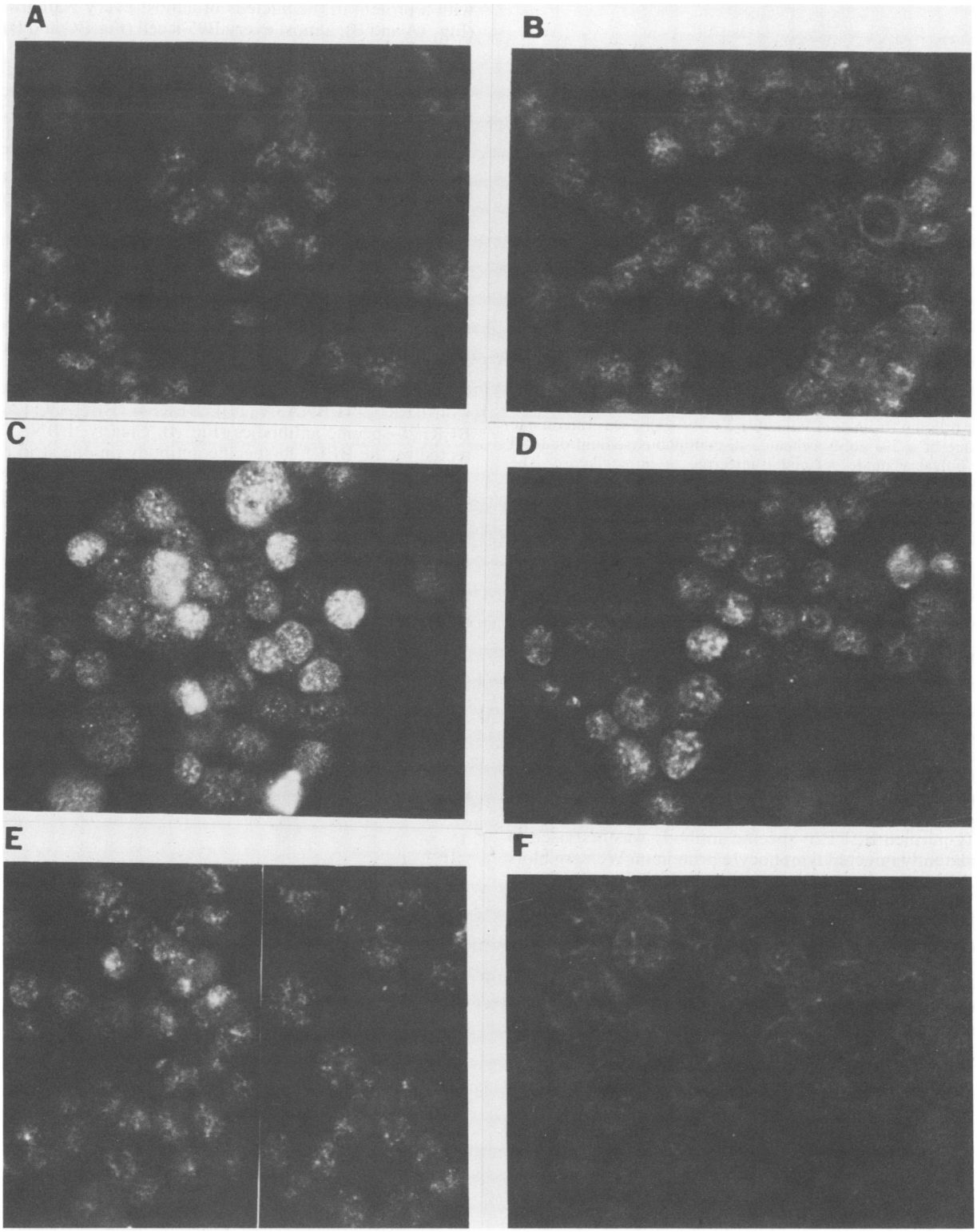


FIG. 3. Indirect immunofluorescence staining for the BERF2b protein, showing that it is a nuclear antigen in all latently infected cells. Affinity-purified BERF2b-specific antibody was used to stain latently infected Namalwa cells (A and B), mostly latently infected B95-8 (C and D) and Raji (E) cells, and EBV-negative Louckes lymphocytes (F) as previously described (26). Cells were photographed under epifluorescence and at magnification of ×500 by using Ektachrome 160 ASA film and a Zeiss photomicroscope.

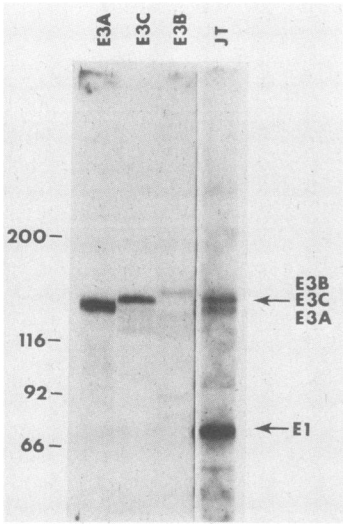


FIG. 4. Affinity-purified antibodies to EBNA3B do not cross-react with EBNA3A and EBNA3C. A protein sample of a Namalwa nuclear fraction (from approximately $2 \times 10^7$ cells) was run on one wide lane of a 5% polyacrylamide discontinuous sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose. The resultant blot was cut into strips, and each strip was separately reacted with affinity-purified EBNA3A antibody (E3A) (17), affinity-purified EBNA3C antibody (E3C) (26), affinity-purified BERF2b-specific antibody (E3B), or a human EBV-immune serum (JT). JT serum contained a small amount of EBNA3B-specific reactivity but was the serum used to derive affinity-purified EBNA3B-specific human antibody. Strips were placed back together to reconstruct the original blot. The numbers to the left indicate the sizes (in kilodaltons) of protein markers. The *Bam*HI-E encoded EBV nuclear antigens expressed during latency (EBNA3A, 3B, and 3C), as well as EBNA1, are marked on the right.

washing of the column to remove nonspecific antibody, antibody bound to the affinity matrix was eluted and further purified by another round of fusion protein affinity chromatography.

**Identification of EBNA3B in latently infected lymphocytes.** Affinity-purified BERF2b-specific antibody was first used to probe latently infected lymphocyte protein on Western blots (26). Affinity-purified BERF2b-specific antibody detected a 165-kDa protein in extracts of the tightly latently infected Namalwa Burkitt tumor lymphocyte line as well as in extracts of the partially permissively infected B95-8 lymphocyte line (Fig. 2C, lanes WCE; see Fig. 5A). Affinity-purified antibody reacted more strongly and more specifically with the 165-kDa protein than did the unpurified immune human serum (compare lanes WCE in Fig. 2C with lanes WCE in Fig. 2B). The antibody did not react with a protein of similar size in the EBV-negative Louckes Burkitt tumor B-cell line, confirming that the 165-kDa protein is EBV specific and not lymphocyte specific. Moreover, the antibody did not react with β-galactosidase (Fig. 2B and C) and therefore recognized only BERF2b determinants. Thus, at least part of BERF2b encodes a protein in latently infected lymphocytes.

Identification of an EBNA requires the demonstration of its expression in latently infected cell nuclei. After treatment of Namalwa cells with nonionic detergent (0.5% Nonidet P-40, as previously described [26]), most of the EBNAs were in the nuclear fraction and only a small amount of EBNA1 was in the cytoplasmic fraction (Fig. 2B). The 165-kDa protein partitioned to the nuclear fraction (Fig. 2C). A similar finding was observed when B95-8 cells were treated with nonionic detergent (Fig. 2C). By indirect-immunofluorescence microscopy, the BERF2b-specific antibody reacted with a protein in the nucleus of almost every Namalwa cell (Fig. 3A and B), almost every B95-8 cell (Fig. 3C and D), and almost every Raji cell (Fig. 3E). B95-8 cells tended to stain more brightly than Namalwa or Raji cells, even though the B95-8 cells were mostly latently infected. The fluorescence staining of all cells was similar to that of EBNA3C (26) in that it was particulate and in some cells spared nucleoluslike structures. Since the 165-kDa protein encoded by BERF2b is present in the nucleus of almost every cell of a tightly latently infected cell line, such as Namalwa, it is designated EBNA3B.

Since the open reading frames which encode EBNA3A, EBNA3B, and EBNA3C are distantly homologous at their 5′ ends (1, 17), an antibody against the BERF2b-encoded protein might cross-react with EBNA3A and EBNA3C. To confirm that the BERF2b-specific antibody actually recognizes EBNA3B and not EBNA3A or EBNA3C, an immunoblot of a Namalwa nuclear extract was cut into strips, each of which was reacted with an EBV-immune human serum or with BERF1- (EBNA3A [17]), BERF4- (EBNA3C [26]), or BERF2b-specific antibodies (Fig. 4). Clearly, EBNA3B detected by the BERF2b-specific antibody on one strip blot is larger than either EBNA3C detected by the EBNA3C (BERF4)-specific antibody or EBNA3A detected by the EBNA3A (BERF1)-specific antibody on adjacent strips. Thus, the EBNA3B antibody does not cross-react with EBNA3A or EBNA3C.

Since EBNA3B is present upon Western blotting in Namalwa and B95-8 cells, one might expect it to be present in Western blots of other EBV-infected cell lines. By using the

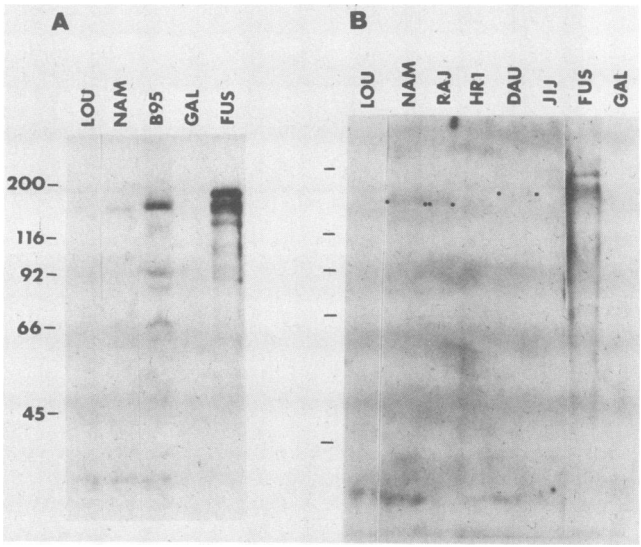


FIG. 5. Presence of EBNA3B in different EBV-infected lymphocyte lines. Protein samples (containing approximately $0.5 \times 10^6$ to $1.0 \times 10^6$ cells) prepared from EBV latently infected lymphocytes (Namalwa), mostly latently infected lymphocytes (uninduced P3HR1, Raji, Daudi, Jijoye, and B95-8), and uninfected lymphocytes (Louckes), as well as 0.5 μg of β-galactosidase (GAL) and 0.05 μg of purified fusion protein (FUS), were run on 7% polyacrylamide discontinuous sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose. Immunoblots in panels A and B were both reacted with the affinity-purified BERF2b-specific antibody. The numbers to the left indicate the sizes (in kilodaltons) of protein markers. In panel B, dots were placed to emphasize bands evident on X-ray film but which may not be evident on the photograph.

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        10          20          30          40          50
MKKAWLSRAQ  QADAGGASGS  EDPPDYGDQG  NVTQVGSEPI  SPEIGPFELS
        60          70          80          90         100
AASEDDPQSG  PVEENLDAAA  REEEEPHEQE  HNGGDDPLDV  HTRQPRFVDV
       110         120         130         140         150
NPTQAPVIQL  VHAVYDSMLQ  SDLRPLGSLF  LEQNLNIEEF  IWMCMTVRHR
       160         170         180         190         200
CQAIRKKPLP  IVKQRRWKLL  SSCRSWRMGY  RTHNLKVNSF  ESGGDNVHPV
       210         220         230         240         250
LVTATLGCDE  GTRHATTYSA  GIVQIPRISD  QNQKIETAFL  MARRARSLSA
       260         270         280         290         300
ERYTLFFDLV  SSGNTLYAIW  IGLGTKNRVS  FIEFVGWLCK  KDHTHIREWF
       310         320         330         340         350
RQCTGRPKAA  KPWLRAHPVA  IPYDDPLTNE  EIDLAYARGQ  AMNIEAPRLP
       360         370         380         390         400
DDPIIVEDDD  ESEEIEAESD  EEEDKSGMES  LKNIPQTLPY  NPTVYGRPAV
       410         420         430         440         450
FDRKSDAKST  KKCRAIVTDF  SVIKAIEEEH  RKKKAARTEQ  PRATPESQAP
       460         470         480         490         500
TVVLQRPPTQ  QEPGPVGPLS  VQARLEPWQP  LPGPQVTAVL  LHEESMQGVQ
       510         520         530         540         550
VHGSMLDLLE  KDDEVMEQRV  MATLLPPVPQ  QPRAGRRGPC  VFTGDLGIES
       560         570         580         590         600
DEPASTEPVH  DQLLPAPGPD  PLEIQPLTSP  TTSQLSSSAP  SCAQTPWPVV
       610         620         630         640         650
QPSQTPDDPT  KQSRPPETAA  PRQWPMPLRP  IPMRPLRMQP  IPFNHPVGPT
       660         670         680         690         700
PHQTPQVEIT  PYKPTWAQIG  HIPYQPTPTG  PATMLLRQWA  PATMQTPPRA
       710         720         730         740         750
PTPMSPPEVP  PVPRQRPRGA  PTPTPPPQVP  PVPRQRPRGA  PTPTPPPQVL
       760         770         780         790         800
PTPMQLALRA  PAGQQGPTKQ  ILRQLLTGGV  KKGRPSLKLQ  AALERQAAAG
       810         820         830         840         850
WQPSPGSGTS  DKIVQAPIFY  PPVLQPIQVM  GQGGSPTAMA  ASAVTQAPTE
       860         870         880         890         900
YTRERRGVGP  MPPTDIPPSK  RAKIEAYTEP  EMPHGGASHS  PVVILENVGQ
       910         920         930        938
GQQQTLECGG  TAKQERDMLG  LGDIAVSSPS  SSETSNDE
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FIG. 6. Amino acid sequence (in the one-letter code) of EBNA3B (28) predicted from the nucleotide sequence of BERF2a and BERF2b (1), assuming a similar splice, as has been demonstrated between BLRF3 and BERF1 and between BERF3 and BERF4. The underlined sequence indicates a repeat region within the protein.

BERF2b EBNA3B-specific antibody to probe immunoblots of lysates of cells infected with different EBV isolates, it was possible to detect an EBNA3B in abortively infected Raji and partially permissive Daudi cells (Fig. 5B) that was similar in size to the EBNA3B present in Namalwa (Fig. 5A and B) and B95-8 (Fig. 5A) cells. EBNA3B in Namalwa, B95-8, and Raji cells is 165 kDa, while EBNA3B in Daudi cells is approximately 170 kDa. (EBNA3C is also slightly larger in Daudi cells [26].) This polymorphism in EBNA3B size might be due to the DNA repeats within BERF2b. EBNA3B was not detected in lysates of the tightly latently EBV-infected lymphoblastoid line IB4 (data not shown) or of the partially permissively infected P3HR1 and Jijoye cell lines (Fig. 5B). It is not known whether EBNA3B actually is not expressed in these cell lines or whether it is produced in such a low amount that it cannot be detected by immunoblotting or immunofluorescence by using affinity-purified antibody.

These results demonstrate that BERF2b encodes at least part of a new nuclear protein expressed in EBV latently infected lymphocytes. Thus, we have definitively shown that, in addition to EBNA3A and EBNA3C, a third EBNA, designated EBNA3B, is encoded by the *Bam*HI E genomic fragment. Since EBNA3A (17, 18) and EBNA3C (26) are each encoded by a short and long exon pair (processed from BLRF3 and BERF1 and from BERF3 and BERF4, respectively), by analogy EBNA3B is likely to be encoded by BERF2a as well as by BERF2b (Fig. 1B). Assuming a BERF2a-2b splice motif similar to that demonstrated in BLRF3-BERF1 and BERF3-4, translation of BERF2a and BERF2b is predicted to yield the amino acid sequence shown in Fig. 6 (28). Comparison of the putative EBNA3B sequence with the predicted amino acid sequences for EBNA3A and EBNA3C revealed that the sequence is slightly longer, as would be expected from the slightly greater size of EBNA3B, and, although there is no homology among the primary sequences, there is some similarity in secondary structure (25). For example, the hydrophilicity profiles, as well as the charged amino acid distributions, show resemblances (25). All three also contain repeated domains at their carboxy termini which might serve as sites for polyvalent interactions, are similarly distributed throughout the latently infected cell nucleus, and are of comparable size. Thus, it is possible that EBNA3A, EBNA3B, and EBNA3C function coordinately in maintaining latency of EBV either through regulation of latent gene transcription or mRNA processing or through EBV DNA replication in the nucleus of the infected cell.

Jeff Sample provided useful advice.

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## ADDENDUM

In a recent report (A. Ricksten, B. Kallin, H. Alexander, J. Dillner, R. Fahraeus, G. Klein, R. Lerner, and L. Rymo, Proc. Natl. Acad. Sci. USA, **85**:995–999, 1988) COS-1 cells transfected with a DNA fragment including BERF2a and BERF2b expressed a 160-kDa nuclear protein by immunoblot and immunofluorescence analyses, using EBV-immune human sera. Since the protein reacted differentially with various EBV immune human sera in the same way as a 160-kDa protein previously detected in EBV-transformed lymphocytes, the authors concluded that the protein was a new EBNA. However, BERF2b peptide-specific antibodies could not be raised to demonstrate that the 160-kDa protein is expressed in most or all latently infected lymphocytes, a necessary criterion for EBNA designation.

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