JOURNAL OF VIROLOGY, Apr. 1987, p. 945–954
0022-538X/87/040945-10$02.00/0

Vol. 61, No. 4

A Bicistronic Epstein-Barr Virus mRNA Encodes Two Nuclear Proteins in Latently Infected, Growth-Transformed Lymphocytes

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Received 3 September 1986/Accepted 3 December 1986

EBNA2 is a nuclear protein expressed in all cells latently infected with and growth transformed by Epstein-Barr virus (EBV) infection (K. Hennessy and E. Kieff, Science 227:1230–1240, 1985). The nucleotide sequence of the EBNA2 mRNA (J. Sample, M. Hummel, D. Braun, M. Birkenbach, and E. Kieff, Proc. Natl. Acad. Sci. USA 83:5096–5100, 1986) revealed that it begins with a 924-base open reading frame that has an unusual potential translational initiation site (CAAATGG). This open reading frame is followed by 138 nucleotides with only one highly unlikely translational initiation site (TACATGC), which would translate a pentapeptide before the next stop codon. The last part of the mRNA is the open reading frame which encodes EBNA2. In this paper, we demonstrate that the 924-base open reading frame translates a 40-kilodalton protein in vitro or in murine cells transfected with the EBNA2 cDNA under control of the murine leukemia virus long terminal repeat. A protein of identical size was detected in EBV-transformed, latently infected human lymphocyte nuclei by using antibody specific for the leader open reading frame expressed in bacteria. Therefore, this is a rare example of a mRNA which translates two proteins from nonoverlapping open reading frames. Since the protein encoded by the leader of the EBNA mRNA is expressed in all nuclei of a latently infected cell line, it was designated EBNA-LP. EBNA-LP localizes to small intranuclear particles and differs in this respect from EBNA1, EBNA2, or EBNA3. EBNA-LP is not expressed in an EBV-transformed marmoset lymphocyte cell line (B95-8) or in one EBV-infected Burkitt tumor cell line (Raji) but is expressed in three other Burkitt tumor cell lines (Namalwa, P3HR-1, and Daudi).

Accumulated evidence indicates that Epstein-Barr virus (EBV) is an etiologic agent of nasopharyngeal carcinoma and of some B-cell lymphomas (11, 55, 64, 65, 72). EBV infection of human lymphocytes results in cell proliferation (28, 53). The virus genome persists in proliferating cells as a multicopy episome (49) or as a linear molecule integrated into cell DNA (50). The 170-kilobase-pair EBV genome has been completely cloned (13, 56) and sequenced (2). The genome has unique (U), terminally repetitive (TR), and internally repetitive (IR) DNA elements organized in the format TR-U1-IR1-U2-IR2-U3-IR3-U4-IR4-U5-TR (15, 41). Characteristically, a highly restricted set of virus genes are expressed in infected lymphocytes and there is little or no virus replication (15, 41). The genes expressed in latent infection are of interest because of their potential role in maintaining latent infection and cell proliferation.

Most of the virus genes expressed in latently infected growth-transformed lymphocytes have been identified, and the primary amino acid sequences of the proteins they encode have been revealed from the nucleotide sequences of exons of the viral latent-cycle mRNAs (15, 41). Only one gene is transcribed from right to left from the prototype viral genome in latent infection (18, 43, 44, 66, 67). This gene maps in U5 (18, 43, 44, 67) and encodes a cytoplasmic and plasma membrane protein (29, 48) which can significantly enhance the tumorigenic properties of continuous rodent cell lines (68). Three widely separated regions of the other EBV DNA strand encode exons of three mRNAs expressed in latent infection (26, 30, 31, 33, 34, 37, 66, 67). 3′ to these exons are signals for polyadenylation, and DNAs 3′ to the exons do not hybridize to the same mRNA, so that each exon is probably the end of a separate mRNA. These exons encode three unrelated EB nuclear proteins (EBNAs), which are expressed in almost all latently infected cells. EBNA1, encoded by a U3-IR3-U4 exon (26, 31, 32), functions in *trans* with a putative origin region (ori P) to permit episome maintenance and perhaps transcriptional activation (57, 58, 70, 71). EBNA1 has little effect on the growth of primary or continuous rodent cells (16). EBNA2, encoded by a U2 exon, enhances the ability of Rat-1 cells to grow in low serum (16). EBNA3, probably a family of three related proteins encoded by U3 exons, has no known function (34).

The EBNA1 and EBNA2 (and possibly EBNA3) mRNAs have unusually long leaders preceding the exons which encode EBNA (15, 41, 45). Recent cDNA cloning of mRNAs from mostly latently infected cultures revealed that EBNA2 and possibly EBNA1 mRNAs begin in IR1 downstream of the IR1 promoter (60). The EBNA2 mRNA (possibly also EBNA1 and EBNA3 latent mRNAs) begins with two unique IR1 exons and includes two repeating IR1 exons before splicing into three U2 exons, the third of which encodes EBNA2 (60). A weak potential initiation codon is created by the first splice (60). Only if this intiation codon were used would the long open reading frame at the beginning of the EBNA2 mRNA be translated. If it were translated, the EBNA2 mRNA (and possibly also EBNA1 and EBNA3) would be bicistronic. We now demonstrate that the EBNA2 mRNA is bicistronic and that the leader of the EBNA2 mRNA encodes a new nuclear protein present in all latently infected cells. We designate this nuclear protein encoded by the EBNA2 mRNA leader to be EBNA-LP.

MATERIALS AND METHODS

Procaryotic expression of EBNA-LP. A procaryotic fusion protein expression vector, pTL1, having the complete *lacZ* gene (8) was constructed by inserting the *Fsp*I-*Bam*HI fragment of *lacZ* from pMC1871 (Pharmacia Fine Chemicals) into the *Fsp*I site of pUC18 (8, 69). T4 DNA polymerases

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were used to remove four nucleotides at the polylinker *Pst*I site so that insertion of a foreign open reading frame is required for beta-galactosidase expression from the resulting plasmid, pTL2. The recombinant was confirmed by *Fsp*I digestion and by sequence of the polylinker with forward and reverse sequencing primers (52, 61). A 936-base-pair *Eco*RI-*Tth*III-I fragment which contains the open reading frame for the putative leader protein was isolated from T65 (60) (Fig. 1). The termini were randomized by BAL 31 nuclease digestion and then repaired with T4 DNA polymerase. DNA fragments of approximately 850 to 950 base pairs were isolated and ligated into *Sma*I-cut, phosphatase-treated pTL2, the pUC18 derivative which can be used as a beta-galactosidase fusion protein expression vector similar to pMC1513 (8). After transformation of *Escherichia coli* JM109 (69), clones were screened for EBV DNA, for expression of a beta-galactosidase fusion protein, and for reactivity with an EBV immune human serum. EBV inserts in the recombinant plasmids were sequenced by the dideoxy method (61) with forward and reverse sequencing primers (Bethesda Research Laboratories).

Purification of EBNA-LP specific antibodies. *E. coli* containing plasmids which express EBV LP open reading frame-*lacZ* gene fusions were grown in LB medium to 0.8 optical density units. Beta-galactosidase expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co.). The fusion protein was purified by substrate analog affinity chromatography (21, 34). Briefly, cell pellets (43 g) were suspended in approximately 100 ml of breaking buffer (0.2 M Tris hydrochloride [pH 7.6], 0.25 M NaCl, 0.01 M magnesium acetate 10 mM 2-mercaptoethanol, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.1% deoxycholate) and lysed by addition of 0.3 mg of lysozyme per ml for 30 min at 4°C. After the mixture was frozen and thawed twice and sonicated for 15 min on ice, the viscosity was reduced by addition of DNase to 5 μg/ml. The lysate was centrifuged at 70,000 × *g* for 1 h at 4°C. Saturated ammonium sulfate was added to a final concentration of 40% (vol/vol). The ammonium sulfate precipitate was suspended in and dialyzed against column buffer (0.25 M Tris hydrochloride [pH 7.6], 10 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% Triton X-100). The clarified solution was passed through a column containing 20 ml of *p*-aminophenyl-β-D-thiogalactopyranoside-agarose (Sigma). The column was washed with 200 ml of column buffer and then with 200 ml of column buffer without Triton X-100. Fusion protein was eluted from the column with 0.1 M sodium borate (pH 10) and precipitated with ammonium sulfate. The eluted protein was dialyzed against column buffer and subjected to a second round of the affinity column purification. Purified protein (3 mg) was coupled to 1.5 ml tresyl-activated Sepharose 4B (Pharmacia) in coupling buffer (0.1 M $NaHCO_3$, 0.15 M NaCl, 0.1% sodium dodecyl sulfate) as described by the manufacturer. An EBV-immune human serum was passed through a beta-galactosidase–Sepharose 4B column to remove any anti-beta-galactosidase activity and then was passed through the EBV LP beta-galactosidase fusion protein affinity column (34). Antibodies bound to the fusion protein affinity matrix were eluted with successive applications of 3 M $MgCl_2$, 1 M NH_4OH, 1 M NH_4OH–10% dioxane, 0.1 M glycine (pH 2.5), and 0.1 M glycine (pH 2.5)–10% dioxane. The eluates were dialyzed against phsophate-buffered saline, pooled, concentrated, and then dialyzed again. The antibody was purified further by a second round of fusion protein affinity chromatography.

Eucaryotic expression of EBNA-LP. After the ends were repaired with T4 DNA polymerase, EBV EBNA2 mRNA cDNA (T65) was cloned into the *Bam*HI site of expression vector pZip-neo-SV(X)1 (9, 12) to yield pZip-T65. A second plasmid, pZip-LP, which has the complete coding region for the EBNA2 mRNA leader protein and only the first 116 amino acids of the EBNA2 coding region, was constructed by throwing the EBNA2 gene out of frame at the single *Bam*HI site. For construction of pZip-LP, pZip-T65 was digested with *Bam*HI, treated with T4 DNA polymerase, and reclosed with T4 DNA ligase.

The recombinant plasmids were linearized with *Sal*I and transfected into NIH 3T3 or Rat-1 (20) cells by calcium precipitation (23). Transfected foci were selected with media containing 500 μg of G418 (GIBCO Laboratories) per ml and cloned in glass cylinders. Cells were screened for protein expression by immunoblot. EBV-infected Burkitt tumor cell lines (Namalwa, Raji, Daudi, or P3HR-1 [35]), EBV-infected and growth-transformed cord blood cells (IB4), or non-EBV-infected Burkitt tumor (Louckes) cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

In vitro transcription and translation. The EBNA2 mRNA cDNA which contains both LP and EBNA2 open reading frames (60), the *Eco*RI-to-*Fnu*DII fragment of the cDNA which contains the LP open reading frame, or the *Fnu*DII-to-*Eco*RI fragment of the cDNA which contains the EBNA2 open reading frame, was cloned into the polylinker of pSP64 or pSP65 (51) to yield pS65, pSLP, or pSE2, respectively (Fig. 1). The pS65, pSLP, and pSE2 DNAs were in vitro transcribed and translated with SP6 RNA polymerase (51) and reticulocyte lysate (Promega Biotec). Briefly, the plasmids were linearized with the restriction enzyme *Pvu*II, phenol and chloroform extracted, and ethanol precipitated. DNA (5 μg) was then transcribed in 40 mM Tris hydrochloride (pH 7.5)–6 mM $MgCl_2$–2 mM spermidine–10 mM NaCl–10 mM dithiothreitol, 1 U of RNasin per μl–0.5 mM each ATP, CTP, GTP, and UTP–50 U of Riboprobe SP6 RNA polymerase (Promega Biotec)–5 mM m7G(5′)ppp(5′)G (Pharmacia) in 100 μl at 37°C for 1 h. DNAse (5 U; Promega Biotec) was then added for 15 min. The RNA was purified and determined to be full length on a formamide-formaldehyde denaturing agarose gel. RNA (1 μg) was then translated by using a reticulocyte lysate system (Promega Biotec) at 30°C for 1 h.

Immunofluorescent staining. Lymphocytes were dried onto microwell slides, and fibroblasts were grown overnight on an eight-chamber microscope slide (Labtek). The slides were fixed in −20°C methanol and stained with human sera or LP-affinity purified human antibody by using a biotin-streptavidin-fluorescein isothiocyanate system (34) (Bethesda Research Laboratories). Slides were photographed onto Ektachrome ASA400 slide film with a Zeiss photomicroscope equipped with epifluorescence.

Electrophoresis and immunoblotting. Cell, nucleus, or cytoplasm protein samples were prepared by being boiled in an equal volume of sample buffer (6% sodium dodecyl sulfate, 2 mM 2-mercaptoethanol, 140 mM Tris hydrochloride [pH 7.0], 2% glycerol, 0.07% bromphenol blue). The protein samples were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, transferred electrophoretically to nitrocellulose (7), and stained with 10% Ponceau S (Sigma) to determine the migration of the size markers. (The size markers used were the Bio-Rad high- and low-molecular-weight sets.) The immunoblots then were blocked in milk buffer (5% nonfat dry milk, 0.1% sodium azide in phosphate-buffered saline) for 30 to 60 min at 37°C and reacted for 2 h at 22°C with either human serum, affinity-purified antibody,

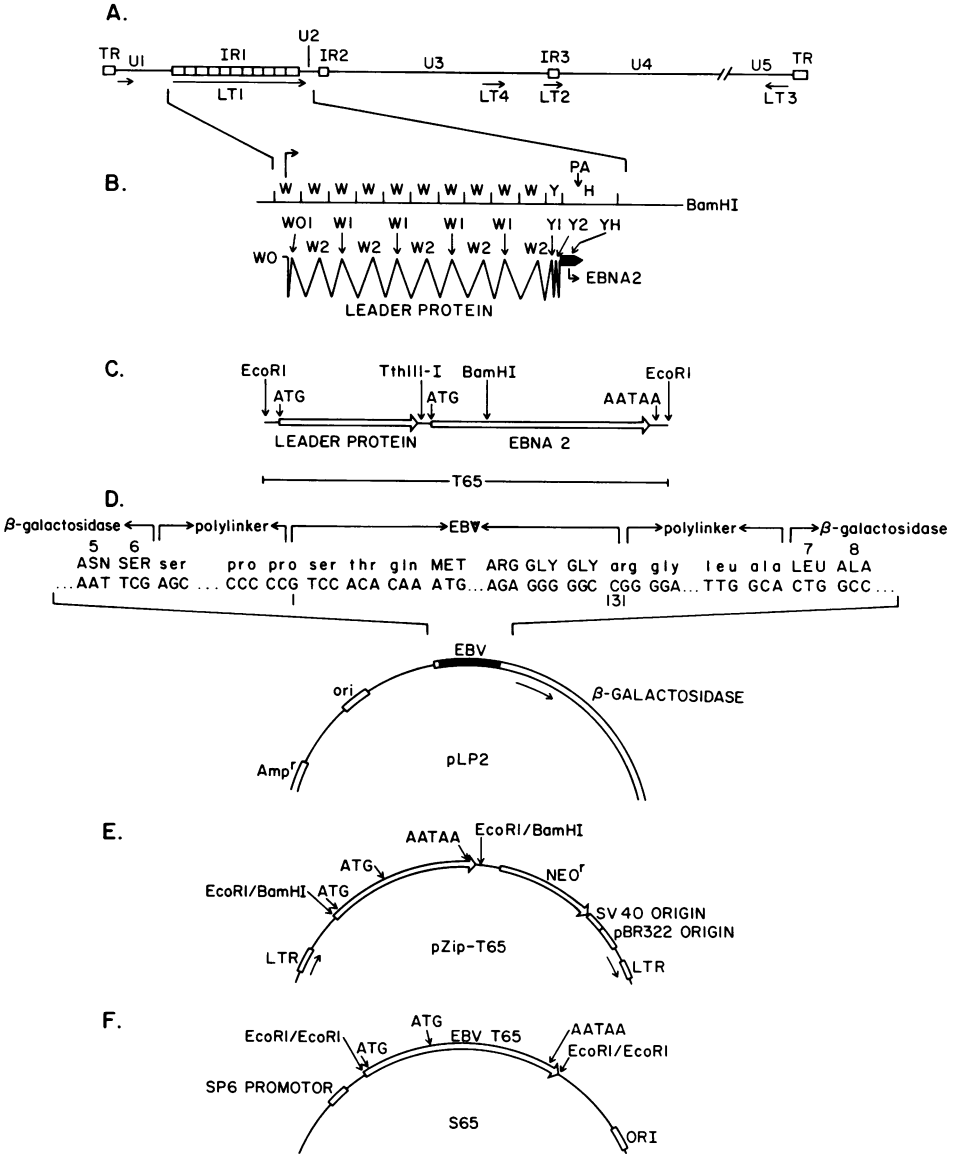

FIG. 1. Expression of the bicistronic EBV EBNA2 cDNA. (A) A schematic diagram of the EBV B95-8 and IB4 genomes is shown with major internal (IR) and terminal (TR) repeats and largely unique (U) DNA domains (15, 41). Viral genes expressed in latent growth-transforming infection (LT) are indicated. (B) Structure of the EBV EBNA2 mRNA cDNA (T65 clone) containing the potential leader protein open reading frames (60). The exons of leader protein open reading frames are derived from the *Bam*HI W (IR1) and Y fragments, and the EBNA2 open reading frame exon is derived from the *Bam*HI Y and H fragments (16). (C) A schematic of the T65 EBNA2 cDNA clone showing the initiation codons, (ATG), polyadenylation signal (AATAA), restriction endonuclease sites, and terminal synthetic *Eco*RI linkers. The *Fnu*dII site used in subcloning parts of T65 is 30 bases 5′ to the EBNA2 initiation codon. (D) The recombinant procaryotic expression vector pLP2. A fragment of the leader protein cDNA was inserted into the *Sma*I site in the polylinker of a pUC18 plasmid which had been modified to contain the complete *lacZ* gene as described in Materials and Methods. The sequence of the gene fusion was determined by using forward and reverse pUC sequencing primers. Superscripts for the protein sequence refer to the codon number in beta-galactosidase (8), and subscripts refer to the sequence number in T65 cDNA (60). (E) The recombinant eucaryotic expression vector pZip-T65. The important features of the parent expression vector pZip-neo-SV(X)1 (9) are the murine leukemia virus long terminal repeat, the *Bam*HI cloning site and the neomycin resistance gene (Neo^r). (F) The recombinant pS65 plasmid for in vitro translation. The T65 EBV cDNA was inserted into a linker site downstream from the SP6 promoter of pSP65 (51) and used to synthesize RNA for in vitro translation as described in Materials and Methods. Similar constructs of the *Eco*RI-to-*Fnu*dII or *Fnu*dII-to-*Eco*RI sites of T65 inserted into pSP65 or pSP64 are designated pSLP and pSE2, respectively.

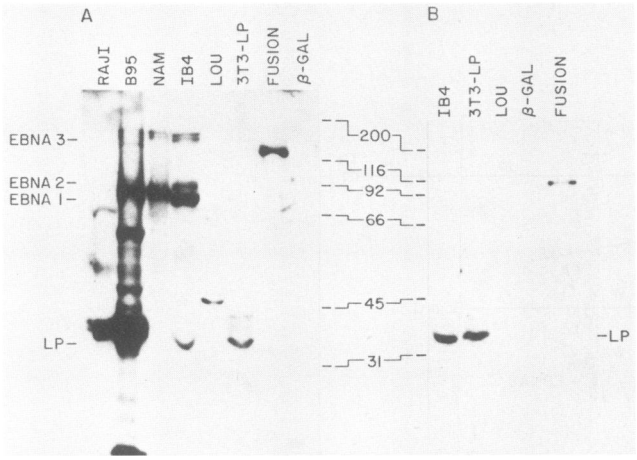

FIG. 2. Affinity-purified antibodies to the PLP-2 fusion protein specifically react with a 40-kDa protein in EBV latently infected lymphocytes. Protein samples from IB4 or Namalwa cells (EBV latently infected lymphocytes), B95-8 or Raji induced cells (productively infected lymphocytes), Louckes cells (EBV-negative lymphocytes), and 3T3-LP cells (NIH 3T3 cells transfected with pZip-LP), as well as 1 μg of beta-galactosidase (β-GAL) and 0.2 μg of purified fusion protein were run on 8.5% discontinuous sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose. The resulting immunoblots were reacted with an EBV-immune human serum (A) or PLP-2 fusion affinity-purified antibodies from the EBV-immune human serum (B). The numbers between panels A and B represent the sizes (in kilodaltons) of the molecular mass markers.

or an LMP-specific murine monoclonal antibody S12, diluted in milk buffer. Blots were then washed in milk buffer, reacted with ^{125}I-labeled protein A (Amersham Corp.) for 30 min at 22°C or with rabbit anti-mouse immunoglobulin G (Cappel Laboratories) followed by ^{125}I-protein A, and washed again extensively in milk buffer.

RESULTS

Procaryotic expression of putative LP. The part of the EBNA2 mRNA cDNA which contains the putative LP open reading frame was subcloned into pTL2, a pUC18-derived beta-galactosidase fusion protein expression vector (Fig. 1). The vector differs from pUC18 in that (i) it contains the entire *lacZ* gene and (ii) four nucleotides have been deleted from the polylinker interrupting the *lacZ* open reading frame just after its initiation codon. Thus, beta-galactosidase is not expressed 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 beta-galactosidase fusion protein. Target DNAs were treated with BAL 31 so that 1/9 of the recombinants with inserts in the correct orientation should be translated into fusion proteins. Colonies expressing beta-galactosidase are readily identified as red colonies on McConkey agar. With a pUC background, the plasmid copy number is high and fusion protein expression can be repressed or induced.

In the first series of experiments, the transformed *E. coli* cells were directly plated on McConkey agar, and 50 high-level beta-galactosidase-expressing EBV DNA-positive clones were identified. Most were making stable fusion proteins by immunoblot analysis with anti-beta-galactosidase antibody. Almost all had inserts which were opposite in orientation to the putative LP open reading frame. These fusion proteins did not react with EBV immune human sera. One clone, which reacted with EBV immune human sera, contained a nearly full-size insert in the correct orientation but expressed little fusion protein. When sequenced, the clone was found to have an amber stop codon at the 3′ junction of insert and vector which was partially suppressed in the *E. coli* JM109 host (69). These data indicated that the opposite orientation of the LP open reading frame is probably not expressed in human EBV infection and that there is strong selection against expression of the correct-orientation LP open reading frame in *E. coli*. In subsequent experiments, bacteria containing recombinant plasmids were initially identified and maintained under conditions of Plac repression. One clone was obtained which gave a mottled appearance on McConkey plates and expressed small amounts of a 143-kilodalton (kDa) fusion protein that reacted with most EBV immune human sera. The nucleotide sequence of the clone indicated that it was the first 725 nucleotides of the EBNA2 mRNA cDNA (60) inserted in the correct orientation giving an in-frame gene fusion. After serial subcloning, a stable subclone was isolated which expressed an immunoreactive 121-kDa fusion protein as 1 to 2% of the total cell protein. The nucleotide sequence of this clone revealed that repetitions of the repeating exon subunits of the leader sequence were deleted and it now consisted of the first 131 nucleotides completely in frame with beta-galactosidase (Fig. 1).

The clone was grown under conditions of Plac repression and induced, and the fusion protein was purified by two cycles of substrate affinity chromatography. The purified fusion protein (or beta-galactosidase) was coupled to tresyl-activated Sepharose. After removal of beta-galactosidase antibody with the beta-galactosidase coupled sepharose, the LP-specific antibody in the immune human serum was adsorbed onto the fusion protein-coupled Sepharose and eluted. The eluted sera were markedly enriched for antibody to a 40-kDa protein in latently infected IB4 cells (Fig. 2). No similar protein was detected in the non-EBV-infected B lymphoblastoid cell line (Louckes line) with the LP-specific antibody. Notably, almost all of the reactivity of the human serum for the 40-kDa protein was removed by the epitopes contained in the LP-beta-galactosidase fusion protein (data not shown). The fusion protein-purified antibody still contained some antibody to EBNA1, EBNA2, and EBNA3 (data not shown). However, after a second round of purification on the fusion protein-Sepharose column, the antibody reacted only with the 40-kDa protein in IB4 cells (comparison of IB4 lanes in Fig. 2A and B). Thus, the putative LP open reading frame encodes at least part of a protein in latently infected IB4 cells.

The 40-kDa protein detected in IB4 cells is fully encoded by the EBNA2 mRNA leader sequence. Two series of experiments were undertaken to prove that the putative LP open reading frame fully encodes the 40-kDa protein. First, the entire EBNA2 mRNA cDNA from IB4 cells, the LP open reading frame part of the cDNA, or the EBNA2 open reading frame part of the EBNA cDNA was transcribed in vitro with SP6 polymerase and excess methyl GTP. The RNAs were then translated in vitro. Both RNAs containing the LP open reading frame translated 40-kDa proteins, indistinguishable in size from the IB4 40-kDa protein (Fig. 3; lanes S65 and SLP). The 40-kDa protein was readily detected by immunoblots (Fig. 3A) and by [^{14}C]proline label (Fig. 3C). Although there is a methionine codon at the beginning of the LP open reading frame, no methionine was detected in the in vitro translation product (Fig. 3B). Probably, a cleavage

occurred between the methionine initiation codon and the attached glycine soon after synthesis (19). EBNA2 was translated about 5 to 10% as efficiently from the in vitro-transcribed bicistronic LP EBNA2 RNA as it was from the RNA in which the EBNA2 initiation codon is only 71 bases from the cap site (Fig. 3B and C; compare lanes SE2 and S65). Also, the in vitro-translated EBNA2 was 80 kDa, while EBNA2 in EBV-infected IB4 cells or in murine cells expressing the EBNA2 open reading frame was 85 kDa (Fig. 3A). The smaller size of EBNA2 transcribed and translated in vitro was not an artifact of deletion within the DNA template or during transcription, since the DNA template for in vitro transcription yielded the same restriction endonuclease fragments as did the T65 EBNA2 cDNA clone from which it was made, the size of the transcript was correct on denaturing gels, and transcription and translation of independently derived clones yielded identical results.

In a second series of experiments, the cDNA was inserted into pZip-neo-SV(X)1, a vector which provides for transcription starting at a cap site in the vector but not for initiation of translation (9, 12, 16, 27, 62) (Fig. 1). Cell transfected with pZip containing the entire T65 EBNA2 mRNA cDNA clone expressed predominantly the 40-kDa LP protein but also small amounts of the 85-kDa EBNA2 (Fig. 4). Thus, LP transcribed and translated in vitro or expressed in murine cells in vivo from the EBNA2 mRNA cDNA clone is identical in size to LP in lymphocytes. Further, EBNA2 transcribed and translated in vitro from the EBNA2 open reading frame part of the cDNA is 80 kDa, while EBNA2 expressed from the cDNA in a heterologous vector in vivo or from EBV in vivo is 85 kDa (Fig. 3 and 4). EBNA2 therefore undergoes significant posttranslational modification. Since EBNA2 is modified in murine cells transfected with recombinant vectors containing only the EBNA2 open reading frame, posttranslational modification of EBNA2 is not dependent on expression of other EBV proteins or on cell type.

LP is a nuclear protein characteristic of latently infected cells. After treatment of cells with nonionic detergent, most of the latent infection membrane protein was extracted with the detergent-soluble cytoplasmic fraction (Fig. 5). LP, however, partitioned with the nuclear fraction (Fig. 5). Even under conditions when a substantial part of EBNA1 had leaked into the cytoplasm, LP remained with the nuclear fraction. In indirect immunofluorescence microscopy, the doubly affinity-purified LP-specific antibody reacted with small spots of nuclear antigen in almost every IB4 cell (Fig. 6). A similar distribution of antigen was seen in LP- expressing rodent cells, indicating that the intranuclear LP distribution is not dependent on other EB nuclear proteins or proteins distinct to human lymphocytes (Fig. 6). Although in murine cell nuclei, LP was associated with intranuclear granules as it was in EBV-infected lymphocytes, the granularity was more uniformly distributed through the nucleus and LP also was diffusely distributed (Fig. 6). The specificity of the affinity-purified serum for LP in fluorescence microscopy was confirmed by demonstrating that rodent cell lines expressing EBNA1, EBNA2, or EBNA3 exhibited bright nuclear fluorescence with the EBV immune human serum but did not react at all with doubly affinity-purified human antibody (16, 34) (Fig. 6). Since these data indicate that LP is an EBV-encoded nuclear protein expressed in every cell in a latently infected cell culture, the protein is similar to other EBNA proteins and is therefore designated EBNA-LP.

EBNA-LP encoded by different EBV isolates. EBV genes

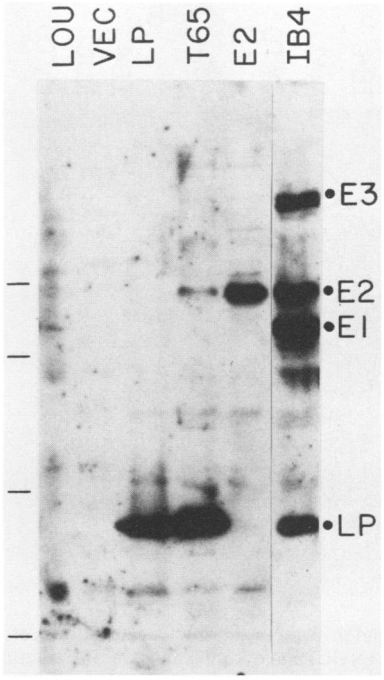

FIG. 4. Expression of both leader protein and EBNA2 in rodent cells detected by immunoblot. NIH 3T3 cells were transfected with pZip-LP (LP), which contains an intact leader protein open reading frame but has a frameshifted EBNA2 reading frame, with pZip-T65, which contains the entire EBNA2 mRNA cDNA having the LP and EBNA2 open reading frames, with pZip-U2-1 (EB2), which contains the EBNA2 open reading frame only, or with pZip-neo-SV(X)1 (VEC) as a negative control. IB4 cells (IB4), a latently EBV-infected cell line, express the EBV-encoded proteins EBNA1 (E1), EBNA2 (E2), EBNA3 (E3), and leader protein (LP). Louckes cells (LOU) are an EBV-negative lymphoblastoid line. Molecular mass markers represented by dashes are 92, 66, 45, and 31 kDa.

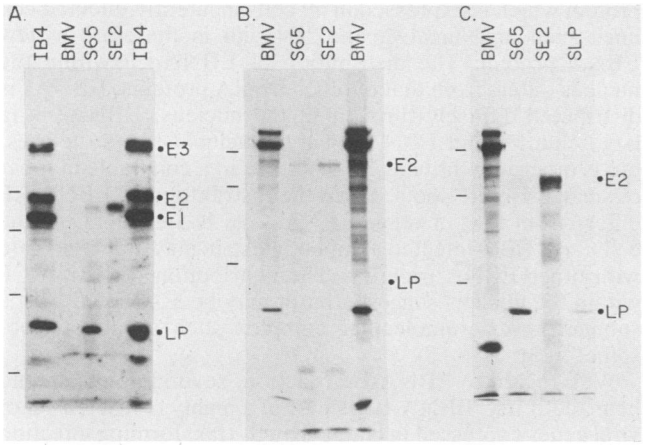

FIG. 3. In vitro translation of leader protein and EBNA2 detected by immunoblotting (A) and autoradiography (B and C). RNA was synthesized from pS65, which contains the full EBNA2 cDNA including the LP open reading frame, from pSE2, which contains EBNA2 open reading frame, or with pSLP, which contains the leader protein open reading frame only, and translated in vitro in the presence of [^{14}C]proline (panel C), [^{35}S]methionine (panel B), or no label (panel A). Translation products were visualized by autoradiography (panels B and C) or by immunoblotting (panel A) with pooled EBV-immune sera. Molecular mass markers, represented by dashes, are 92, 66, 45, and 31 kDa.

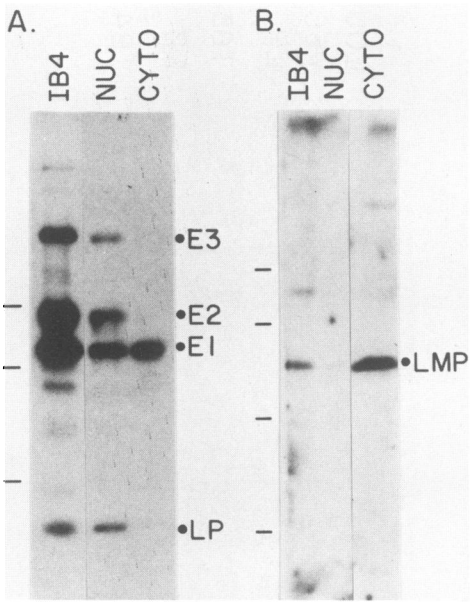

FIG. 5. Leader protein in nuclear fractions of EBV-infected cells. Nuclear (NUC) and cytoplasmic (CYTO) fractions of IB4 cells were prepared as described in Materials and Methods and immunoblotted with either pooled EBV-immune sera (A) or S12 (B), a monoclonal antibody that detects the latent membrane protein (LMP) encoded by EBV. Whole-cell extracts of IB4 cells (IB4) served as a positive control. Molecular mass markers in panel A are 92, 66, and 45 kDa and in panel B are 92, 66, 45, and 31 kDa.

are frequently polymorphic because of variation in repeat sequence domains (26, 29–34). Since LP contains repeating exons from IR1, LP might vary in size among cells infected with different EBV isolates. Some EBV isolates such as P3HR-1 or Daudi not only have fewer IR1 repeats but also are deleted for the U2 domain (6, 25, 39, 40, 42, 56). If these isolates make an LP protein, it would have to terminate in U3 rather than in U2, where LP normally terminates (10, 13–15, 60). Analysis of LP in immunoblots of cells infected with different EBV isolates indicates that LP in the tightly latently infected Namalwa Burkitt tumor cell line (54) is 68 kDa (Fig. 7). No LP cross-reactive protein was detected in Raji or B95-8 cells or in such cells treated with phorbol esters to induce EBV replication. Surprisingly, P3HR-1 and Daudi cells express stable LP cross-reactive proteins of 27 and 30 kDa, respectively. Minor cross-reactive proteins of 34 and 48 kDa were detected in P3HR-1 cells, and proteins of 34 and 62 kDa were detected in Daudi cells. As expected for a protein associated with latent infection, the amount of LP expressed in P3HR-1 or Daudi cell cultures did not increase when virus replication cycle gene expression was induced with phorbol ester and butyrate (data not shown). Immunofluorescence microscopy showed that the P3HR-1 and Daudi LP cross-reactive proteins localized to the cell nucleus (data not shown). These data suggest that the signal for nuclear localization (59) of LP is in the part of LP encoded by IR1-derived exons. Almost all P3HR-1 cells contained a similar distribution of antigen to that in IB4 cells (i.e., LP localized to small, discrete intranuclear granules).

DISCUSSION

The data presented in this paper demonstrate that the open reading frame in the leader of the EBV EBNA2 mRNA is translated in vitro, in murine cells, and in latently infected growth-transformed lymphocytes. This is a remarkable finding for the following reasons. (i) LP is expressed in vivo from an open reading frame which has a single methionine codon, CAAATGG, that differs from most other animal cell initiation codons because it has a pyrimidine in the −3 position and purines in the −2 and −1 positions (45, 46, 47; M. Kozak, Adv. Virus Res., in press). Despite the unusual intiation codon, in vitro-synthesized LP mRNA with its initiation codon 26 bases from its cap site was translated as efficiently as was EBNA 2 (SE2) mRNA, which has its initiation codon, ATCATGC, 71 bases from its cap site. (ii) Since the LP open reading frame starts and stops before the start of the EBNA2 open reading frame and there is no other EBNA2 mRNA in latently infected growth-transformed lymphocytes (66), LP and EBNA2 must be translated from the same mRNA in vivo. Since initial ribosome binding to the EBNA2 translational initiation site, which is more than 1 kilobase from the LP-EBNA2 mRNA cap site, would be highly inefficient, translation probably begins at the LP translational initiation site, stops at the end of the LP open reading frame, and reinitiates at the EBNA2 translational initiation site (45; Kozak, in press). Thus, LP and EBNA 2 are likely to be translated by one ribosome from one mRNA molecule. Aside from a simian virus 40 mRNA (38) and two Rous sarcoma virus mRNAs (24, 36) which encode short peptides at their 5′ end, cauliflower mosaic virus (17), is the only previous example of a single eucaryotic cell mRNA encoding two proteins in nonoverlapping reading frames. Most eucaryotic mRNAs are monocistronic (Kozak, in press). (iii) The protein predicted from the nucleotide sequence of the LP open reading frame is highly unusual. It consists mostly of four perfect repeats of a 44-amino-acid peptide interrupted by three perfect repeats of a 22-amino-acid peptide. The protein consists of 18% arginine, 9% glutamic acid, and 17% proline and is strongly basic with 33 net positively charged amino acids.

The data also indicate that the protein encoded by the EBNA2 mRNA leader open reading frame is a nuclear protein which is expressed in all cells in latently infected cell lines such as Namalwa and IB4 and is therefore a new EBNA protein. The distribution of EBNA-LP within the nucleus differs from that of other EBNA proteins. EBNA1 is distributed diffusely throughout the nucleus, EBNA2 is in large clumps, and EBNA3 spares nucleoluslike structures, but is otherwise diffuse. EBNA-LP is in a countable number of small, discrete spots. Since the distribution of EBNA-LP differs from that of other EBNAs and is similar in murine cells and EBV-infected lymphocytes, it may not associate with other EBNA proteins. The distribution of EBNA-LP within the nucleus suggests that it may be associated with a subnuclear macromolecular complex such as a "spliceosome" (22).

With regard to EBNA-LP function, several points should be made. First, EBNA-LP is part of a highly restricted set of viral genes expressed in latent growth-transforming infection and is therefore likely to play a role in maintaining latency or in inducing cell proliferation (15, 41). Second, the coupling of the EBNA-LP open reading frame to that of EBNA2 (and possibly also EBNA1 and EBNA3) in the same mRNA suggests a necessity for coordinate expression of EBNA-LP and EBNA2 (and possibly LP and EBNA1 or EBNA3). The purpose of coordinate expression could be that EBNA LP regulates EBNA expression or could be to ensure stoichiometric quantities of EBNA LP and other EBNAs so that they could function coordinately. Although EBNA LP is

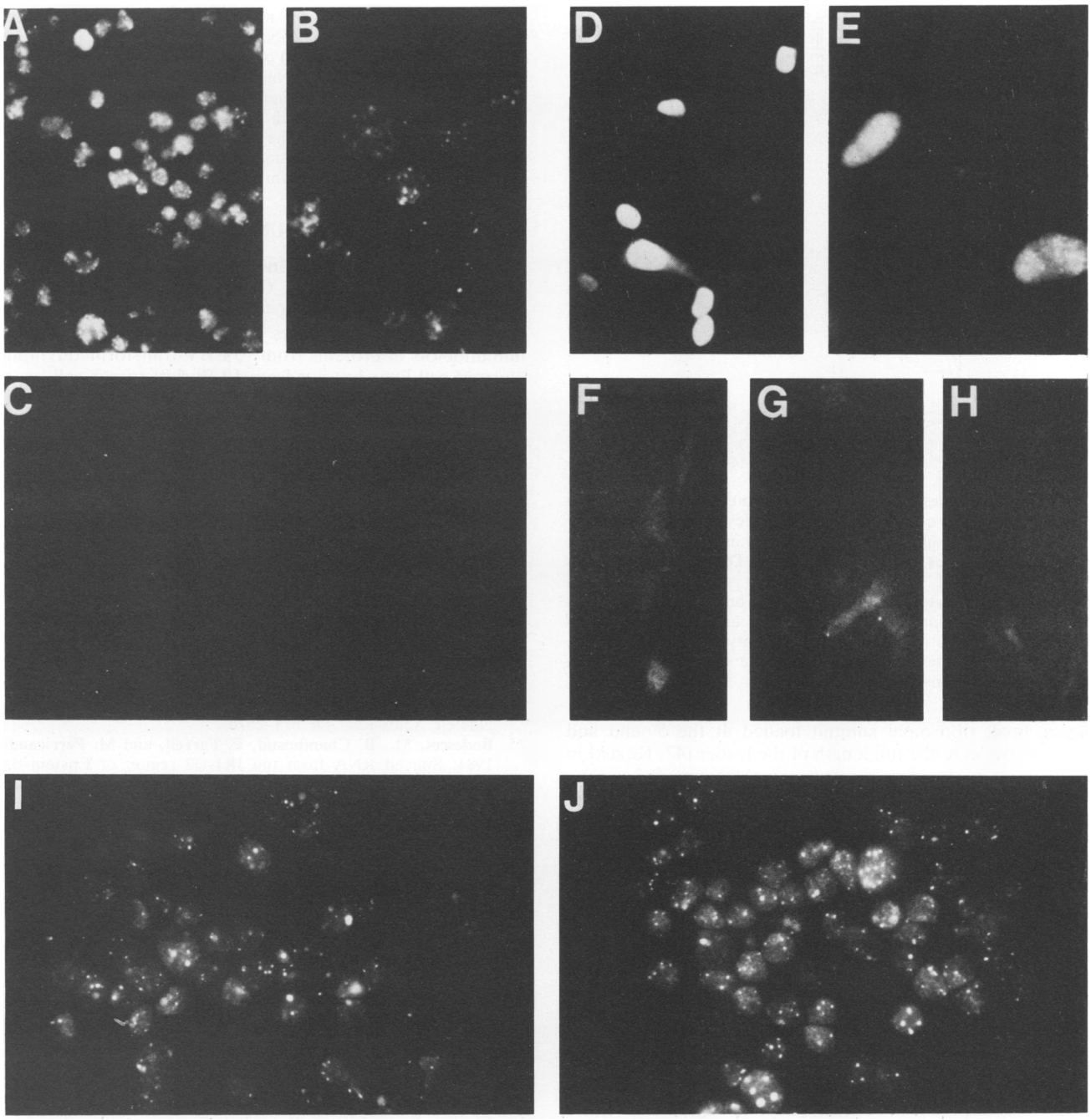

FIG. 6. Immunofluorescent staining for LP reveals a nuclear protein in all latently infected cells. Affinity-purified LP specific antibody was used to stain latently IB4 cells (A, B, I, and J), EBV-negative Louckes cells (C), or Rat-1 cells transfected with either pZip-LP (D and E), pZip EBNA1 (F), pZip-EBNA2 (G), or pZip EBNA3 (H) (16, 34). Cells were photographed under a magnification of ×360 (panels A, C, D, F, G, and H) or ×760 (panels B, E, I, and J). Panels F, G, and H are overexposed to reveal the cells.

distributed differently within the nucleus from the other EBNAs, it could interact with another EBNA or act in concert with another EBNA in the same macromolecular process. With regard to a possible role in nuclear regulation of EBNA2 expression, it is unlikely that the EBNA-LP particles are replication or transcriptional sites, since EBNA-LP associates with similar particles irrespective of the presence of the EBV genome in the cell. More likely, these will be particles involved in RNA processing such as spliceosomes. Differential polyadenylation and splicing are important in ensuring adequate representation of the various EBNA proteins, since all of their primary transcripts originate from the same region of the EBV genome and possibly from the same promoter (60). Lastly, although EBNA-LP is predominantly a nuclear protein, a cotranslational effect on expression of its mRNA is also possible. Almost certainly, the presence of a translatable open reading frame 5′ to the EBNA2 open reading frame has a *cis* effecton the translation of EBNA2, since a complete ribosome moving along an mRNA is likely to be more stably associated with the mRNA

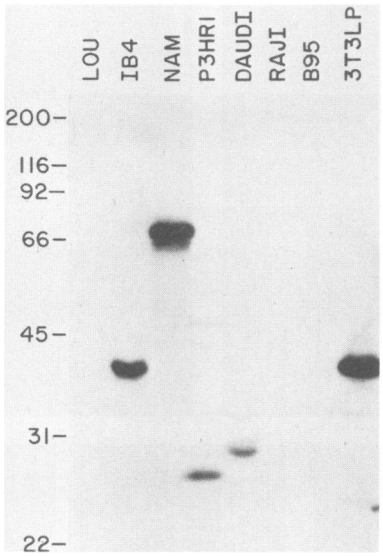

FIG. 7. Differences in sizes of leader protein in different EBV-infected lymphocyte cell lines. Protein samples prepared from EBV latently infected lymphocytes (IB4 and Namalwa), semilatently infected lymphocytes (uninduced P3HR-1, Daudi, Raji, or B95), 3T3-LP cells (NIH 3T3 cells transfected with pZip-LP), and uninfected lymphocytes (Louckes) were run on 8.5% discontinuous sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose. The blot was reacted with affinity-purified LP-specific antibody. The numbers to the left indicate the sizes of protein markers in kilodaltons.

than a small ribosomal subunit loaded at the 5′ end and having to traverse the full length of the leader (47; Kozak, in press).

A partial cDNA clone was derived from Raji cells and consists of the IR1 repeating exons and the U2 exons of LP spliced to an acceptor site near the EBNA2 mRNA polyadenylation site (5). This suggests that a monocistronic LP-specific RNA may be transcribed in Raji cells. We did not detect LP in Raji cells. Raji cells differ from other predominantly latently infected cell lines in that there is extensive transcription of the EBV genome in Raji cells and some cells express early productive-cycle viral antigens (44). Recently, cDNA cloning of an mRNA from B95-8 cells which are partially productively infected revealed an EBNA3 mRNA cDNA which begins in U1 and splices into the repeating exons which encode LP before splicing into two U3 exons which probably encode the entire EBNA3 protein (4). Upstream of the open reading frame of the repeating LP exons in this cDNA clone there are in-frame stop codons and no initiation codon. Thus, B95-8 cells contain an EBNA3 mRNA which is transcribed from a U1 promoter. Although this mRNA contains part of the LP open reading frame, it cannot encode LP. The failure to detect LP cross-reactive proteins in Raji or B95-8 cells suggests that LP is translated only from mRNAs which are transcribed from the IR1 promoter and that such RNAs may not be found in Raji or B95-8 cells. Since B95-8 and Raji cells express EBNA1, EBNA2, and EBNA3, expression of LP does not appear to be uniformly linked to expression of these other EBNA proteins at all stages of EBV infection.

ACKNOWLEDGMENTS

The advice of M. Kozak, R. Mulligan, and G. Khoury and the gifts of 3T3 cells from R. Lowe, Rat-1 cells from R. Weinberg through M. Villareal, and LMP monoclonal antibody from K. Mann and D. Thorley-Lawson contributed to these studies.

This research was supported by Public Health Service grants CA 19264 and CA 17281 from the National Institutes of Health and ACS MV32 from the American Cancer Society. F.W. and L.P. are recipients of National Research Service Awards AI 07099 and CA 09241, respectively. D.B. is the recipient of a Pfizer Postdoctoral Fellowship Award. S.S. is partially supported by a Summer fellowship from the College of the University of Chicago.

ADDENDUM IN PROOF

In a recent report (J. Dillner, B. Kallin, H. Alexander, I. Ernberg, M. Uno, Y. Ono, G. Klein, and R. Lerner, Proc. Natl. Acad. Sci. USA, **83**:6441–6645, 1986), rabbit antisera to three synthetic peptides of EBNA-LP identified it in immunoblots of proteins from 10 EBV-transformed lymphoblastoid cell lines but not from 10 Burkitt tumor cell lines.

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