*Proc. Natl. Acad. Sci. USA*
Vol. 82, pp. 5944–5948, September 1985
Medical Sciences

# A third viral nuclear protein in lymphoblasts immortalized by Epstein–Barr virus

(herpesvirus/cancer/rheumatoid arthritis/anti-nuclear antibodies)

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*Communicated by Josef Fried, May 10, 1985*

ABSTRACT **Most sera from patients with rheumatoid arthritis as well as some sera from normal Epstein–Barr virus (EBV)-infected people detect a 140-kDa protein on immunoblots of EBV-infected lymphoblasts. The 140-kDa protein is a nuclear protein characteristic of latent EBV infection. Sera reactive with this protein identify a distinctive globular nuclear antigen. Although the 140-kDa protein is encoded by EBV, it is not encoded by genes that encode the two previously described EBV nuclear antigens (EBNA) or the latent-infection membrane protein. The 140-kDa protein is therefore designated EBNA3. The EBV genes, including the gene encoding EBNA3, that are characteristically expressed in latent infection are likely to play a role in the maintenance of persistent latent viral infection or in the cell proliferation caused by virus infection.**

Epstein–Barr virus (EBV) transforms human B lymphocytes to a state of continuous proliferation (1). The 170-kilobase (kb) virus genome (2, 3) persists in the infected lymphoblast (4, 5) by forming a circular episome (6) or by integrating into cell DNA (7). Despite the presence of the complete virus genome, there is little or no virus replication. However, most EBV-infected cell lines continuously give rise to a small number of cells that are partially or fully permissive for expression of virus replicative functions. Cells permissive for virus replication produce large amounts of replicative cycle RNA and proteins, complicating the identification of viral gene products in latently infected cells (8–10). Some EBV-infected cell lines, such as Namalwa and IB4, consistently maintain the virus genome in a latent state (8, 9, 11–13). In such cell lines, only a few virus genes are transcribed. Identification of virus genes expressed in latently infected transformed cells is a key step to evaluating the role of each gene in latency and transformation. Two virus-associated (VA)-like RNAs (14, 15) and three messenger RNAs (16–19) are characteristically transcribed from separate regions of the EBV genome in latently infected proliferating cells. The three messenger RNAs have been shown to encode two nuclear proteins and a membrane protein by demonstrating that antibody to proteins expressed from each of the nucleotide sequences reacts with a specific protein in EBV-infected lymphocytes (16–23). These antisera and subsequent rabbit antisera to synthetic peptides (24) or murine monoclonal antibodies (25) permit the identification of each of the three viral proteins.

We now report a third nuclear protein in latently infected IB4 and Namalwa cell lines. This protein was detected in all EBV-infected cells but was not found in non-EBV-infected proliferating B lymphocytes. Some EBV-immune human sera react with the protein, while nonimmune sera do not. The size of the protein varies in cells infected with different EBV isolates, indicating genetic polymorphism, as has been noted with other EBV proteins (22, 23, 26). This protein is antigenically distinct and exhibits different polymorphism than any of the previously detected nuclear or membrane proteins.

## MATERIALS AND METHODS

**Cell Lines.** All cell lines were maintained in RPMI 1640 medium supplemented with either 10% newborn calf or fetal calf serum. Namalwa is a Burkitt tumor cell line which is nonpermissive for virus replication (11, 12). IB4 clone D, II 100-1, and I 100-1 are human placental cord blood lymphocytes transformed with the B95-8 viral strain (13). BIF-AG876 or BIF-B95-8 are cells infected with B95-8 or AG876 EBV strains, respectively. Raji, W91, Lamont, and P3HR-1 are cell lines that are partially permissive of EBV replication.

**Preparation of Protein Samples, Electrophoresis, and Immunoblotting.** Nuclei were isolated by breaking cells in lysis buffer (10 mM Tris·HCl, pH 7.5/1 mM phenylmenthylsulfonyl fluoride/2% sucrose/10 mM NaCl/3 mM $Mg(OAc)_2$/0.7% Nonidet P-40) with a Dounce homogenizer and centrifugation for 10 min at 500 × *g*. Cytoplasmic and nuclear fractions were solubilized with sample buffer and boiled (20). Total cell protein samples were prepared by boiling $10^7$ cells in 0.4 ml of sample buffer. Protein concentrations of each sample were assayed by dot-blotting successive 1:1 dilutions of the sample onto nitrocellulose and staining the sheet with amido black (27).

The conditions for electrophoresis are those of Laemmli (28). Proteins were separated on either 6.5% or 6% 3-mm-thick slab $NaDodSO_4$/polyacrylamide gels. Transfer of the proteins to nitrocellulose was typically carried out at 8°C with a field strength of 70 V for 18 hr (27). The blot was then rinsed in phosphate-buffered saline and treated in one of two ways: (*i*) the blot was divided into smaller pieces for immunostaining, with one piece from each gel being stained with amido black to locate the size markers; or (*ii*) the entire blot was stained with ponceau S as described by the manufacturer (Sigma), the migration of size markers was noted, the sheet was divided into smaller units, and the stain was removed by washing in phosphate-buffered saline prior to blocking. Blocking and all subsequent reactions and washing were done in blocking buffer [5% (wt/vol) low-fat dry milk/0.05% (vol/vol) Tween 20/10 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% sodium azide]. After blocking, blots were reacted with sera, usually at a dilution of 1:30, for 2 hr at 22°C followed by 18 hr at 4°C. They were washed and treated with $I^{125}$-labeled protein A (Amersham) and washed again. When the monoclonal antibody was used, an additional reaction with rabbit anti-mouse IgG and a washing step were inserted after the initial antiserum wash. Size markers were the high molecular weight set (Bio-Rad), which includes myosin (200

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Abbreviations: VA, virus-associated; EBNA, Epstein–Barr nuclear antigen; EBV, Epstein–Barr virus; LMP, latent cell membrane protein; kb, kilobase(s).

kDa), beta-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

**Fluorescence Microscopy.** Cells were fixed to a microwell slide with absolute methanol. Diluted WC serum (1:10), a human serum from a patient with chronic persistent EBV infection, was added to the wells, incubated for 60 min at 37°C, and enhanced by incubation with human complement followed by fluorescein isothiocyanate-labeled goat anti-human C3 (third component of complement; Cappel Laboratories, Cochranville, PA) (29). Similar results are obtained with WC serum when the biotin–strepavidin system (described below) is used. Rabbit antiserum against EBV nuclear antigen 2 (EBNA2) (1:20 dilution) was added to methanol-fixed slides and treated with a goat affinity-purified biotinylated Fab fragment of anti-rabbit IgG and fluorescein isothiocyanate-labeled strepavidin (Bethesda Research Laboratories) as suggested by the manufacturer.

**RNA, cDNA, Blotting, and Hybridization.** RNA was extracted from IB4 cell cytoplasm by the guanidine isothiocyanate/cesium chloride procedure (30). Polyadenylylated RNA was selected by oligo(dT) column chromatography (31). cDNA ($2 \times 10^8$ cpm) was synthesized from 20 $\mu$g of polyadenylylated RNA by using [$\alpha$-$^{32}$P]dCTP (400 Ci/mm; 1 Ci = 37 GBq) and was hybridized to Southern blots of digested EBV recombinant plasmid DNAs (2, 13, 14). The *Bam*HI fragment E of EBV DNA was cut with *Bgl* I. Each of the fragments was cloned into pSP64, as was the rightward *Ava* I–*Hin*dIII fragment of the *Bam*HI–*Hin*dIII fragment of *Bam*HI fragment K (17, 32). RNA was synthesized from recombinant EBV DNA in pSP64 by using SP6 polymerase obtained from Promega Biotec (Madison, WI) (33). EBV RNA ($1 \times 10^9$ cpm) was hybridized to blots of IB4 polyadenylylated RNA. Blots were washed with ribonuclease A before autoradiographic exposure for 1 or 2 days.

## RESULTS

**Immune Human Sera Detect a 140-kDa Protein.** Most humans have been infected with EBV and have antibody to the EBV nuclear antigen (EBNA) complex (for review, see ref. 34). These sera usually react with a 65- to 85-kDa protein designated EBNA1, which is encoded by the EBV DNA U3–IR3–U4 domain (20, 26, 35). Some human sera react with a 75- to 85-kDa nuclear protein designated EBNA2, encoded by the EBV U2 domain (20, 21, 23). No human sera have as yet been identified that react on immunoblots with the 60-kDa latent cell membrane protein (LMP) (22).

A human serum (WC) reacts with EBNA2 (20, 21) but not EBNA1 (20, 21, 36) and has high-titer reactivity with early or late EBV replicative-cycle proteins (10). This serum strongly reacted with a 140-kDa protein in latently and partially permissively infected cell lines (Fig. 1 *A* and *C*). WC serum also reacted with early viral replicative antigen components of 45–50 and 135 kDa in P3HR-1, W91, and Lamont cells. A small fraction of the cells in these lines are partially permissive of virus replication and account for this reactivity (data not shown). However, it is evident from Fig. 1 that none of these early antigen proteins were detected in IB4 or Namalwa cells, whereas the 140-kDa protein was detected. A pool of high-EBNA-titer human sera reacted with the 140-kDa protein and substantially less with virus early antigens than did the WC serum (Fig. 1*D*).

Sera from patients with rheumatoid arthritis have been reported to react with a 92-kDa protein similar to EBNA2 (37). We tested the sera from eight patients with rheumatoid arthritis, and all were found to react with EBNA1 and EBNA2 (data not shown). Seven also reacted with the 140-kDa protein (as an example, see Fig. 1*F*). Sera DR (Fig. 1*E*) and MC (Fig. 2*A*) in a previous study (37) reacted with "92-" and "110/115-" kDa proteins, which now are shown to be EBNA2 and the 140-kDa protein, respectively. The lower molecular weight bands visualized with DR and VC sera in Fig. 1 *E* and *F* are due to sample degradation because they were not observed with other samples and the same sera. The 140-kDa protein was not found in Loucks or BJAB lymphoblastoid cell lines, neither of which are infected with EBV. Also, five EBV-seronegative human sera and seven high-titer EBNA-seropositive human sera from nonrheumatoid patients did not react with the 140-kDa protein in EBV-infected cells (Figs. 1*B*, 3 *B* and *C*, and data not shown).

The size of the 140-kDa protein was slightly different among cell lines infected with different EBV isolates. For example, the W91, Lamont, and P3HR-1 proteins were 140 kDa; Namalwa and IB4 were 142 kDa; and Raji was 139 kDa.

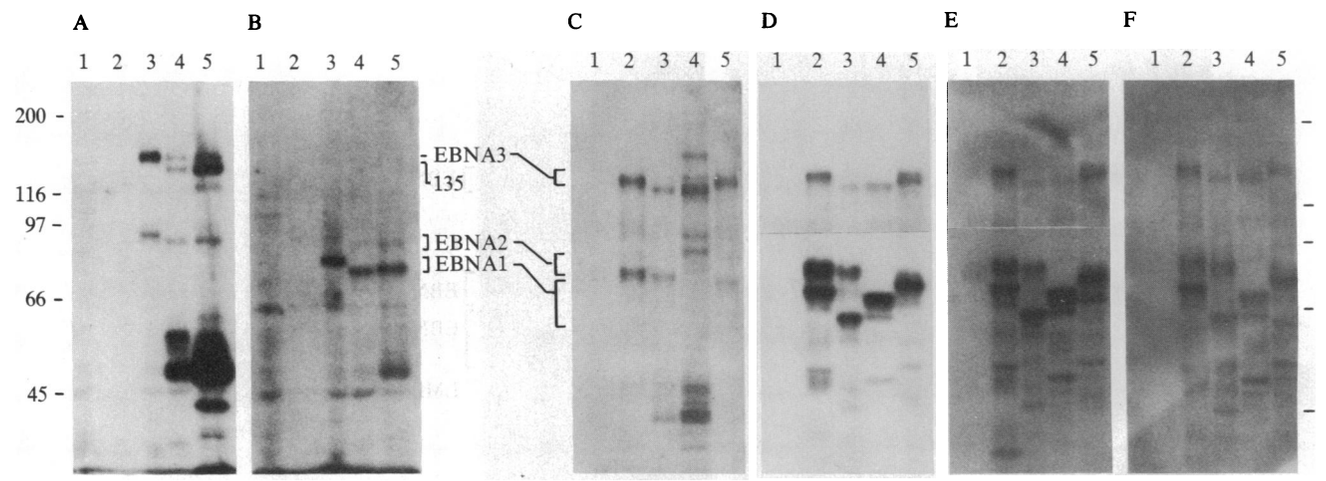


FIG. 1. Human sera recognize an antigen of about 140 kDa in all EBV-infected cells. Nuclear proteins from EBV-infected or uninfected (Loukes, BJAB) human B-lymphocyte cultures were separated by NaDodSO$_4$/PAGE, transferred to nitrocellulose, and treated with the indicated human sera: WC (*A* and *C*), TU334 (*B*), DR (*E*), and VC (*F*) are EBV immune human sera from patients with chronic persistent EBV infection, Burkitt tumors, normal latent infection, and rheumatoid arthritis, respectively. Blot *D* was treated with pooled EBNA-reactive human sera. Blots *D* and *E* are shown as composite exposures with the EBNA1 and EBNA2 bands exposed for shorter times than the EBNA3 bands. Size markers (in kDa) described in *Materials and Methods* are indicated (–) along the side of each series (*A–B* or *C–F*) of blots. Lanes in *A* and *B*: 1, Loukes; 2, BJAB; 3, IB4; 4, Lamont; 5, W91. Lanes in *C–F*: 1, Loukes; 2, IB4; 3, Raji; 4, P3HR-1; 5, Namalwa.

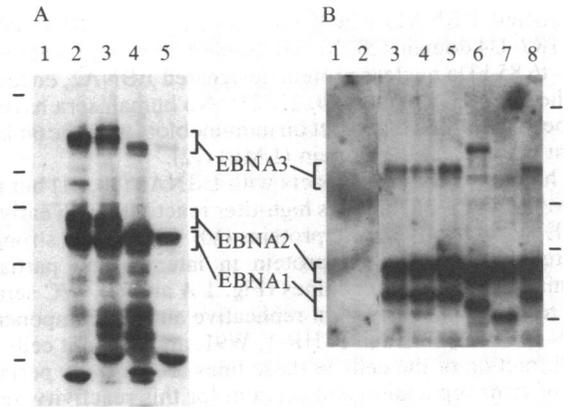


FIG. 2. Variation in antigen size is determined by EBV. Immunoblots of total (*A*) or nuclear (*B*) proteins from each of the indicated cell lines were incubated with human EBNA-reactive sera. (*A*) Human MC serum. (*B*) Pooled EBNA. Protein size markers are shown (−) along the side of each blot as in Fig. 1. Lanes in *A*: 1, Loukes; 2, IB4; 3, BIF/B95-8; 4, BIF/AG876; 5, AG876. Lanes in *B*: 1, BJAB; 2, Loukes; 3, I 100-1; 4, II 100-1; 5 and 8, IB4; 6, Lamont; 7, Raji.

To confirm that the size of the protein is determined by the EBV genome, cells transformed by B95-8 virus were compared with each other and with cells infected with other EBV isolates (Fig. 2). Three independently derived cell lines infected by B95-8 virus—I 100-1, II 100-1, and IB4—had a 142-kDa protein, while cells infected with other EBVs differed. Notably, cells of the same individual infected with B95-8 or AG876 EBV by A. Rickinson, as part of another study, had proteins characteristic of the input virus, 142 kDa for B95-8 and 140 kDa for AG876. Thus, the protein is EBV-encoded.

The 140-kDa protein is not related to the EBNA1 or EBNA2 proteins or to the EBV-encoded LMP because (*i*) human antisera with high-titer reactivity with EBNA2 (Fig. 3*B*) or EBNA1 (Fig. 3*C*), monospecific rabbit antisera to EBNA2 (ref. 22 and data not shown), and monoclonal antisera against EBNA1 (25) or LMP (Fig. 3*E*) did not react with the 140-kDa protein; (*ii*) the size of the protein varied in a unique pattern among cell lines infected with different EBV isolates distinct from the variation of EBNA1, EBNA2, or LMP; and (*iii*) P3HR-1 cells were deleted for EBNA2 (20, 22) but had a 140-kDa protein (Fig. 1).

**Nuclear Localization of the 140-kDa Protein.** To determine the intracellular locale of the 140-kDa protein, cells were lysed in nonionic detergent so as to separate nuclear and cytoplasmic fractions. Although there was some leakage of EBNA1 into the cytoplasm, EBNA2 and the 140-kDa protein remained tightly associated with the nuclear fraction (Fig. 4). Since WC serum does not react with EBNA1 on immunoblots (20, 21) or by immunofluorescence (36) and since P3HR-1 cells lack EBNA2 (21), WC sera is a specific probe for the 140-kDa protein in "latently infected" P3HR-1 cells. Bright discrete spots of fluorescence were seen in the nuclei of all P3HR-1 cells with WC serum (Fig. 4 *E* and *I*). This pattern is distinctive from that seen with EBNA1- or EBNA2-reactive sera (ref. 20; Fig. 4 *F* and *H*). A similar pattern also was observed in IB4 cells. As expected (21), rabbit serum against EBNA2 gave no reactivity with P3HR-1 cells (Fig. 4*G*). In Raji and IB4 cells, the rabbit anti-EBNA2 antiserum gave diffuse lacey fluorescence (Fig. 4*F*) similar to that obtained with anti-EBNA1 or anti-EBNA2 specific human antisera (20).

**Region of EBV Genome that May Encode the 140-kDa Protein.** The most abundant RNAs in latently infected IB4 cells are 2.8-, 3.7-, and 3.0-kb RNAs, which encode, respectively, the 60-kDa LMP (60 RNA copies per cell) and the EBNA1 and EBNA2 proteins (3–5 RNA copies per cell). Two less-abundant polyadenylylated cytoplasmic RNAs of 2.0 and 2.3 kb are transcribed from the left end of the EBV genome in IB4 cells (16) but are too small to encode the 140-kDa protein. cDNA made from IB4 polyadenylylated cytoplasmic RNA hybridizes to EBV DNA fragments that encode the previously characterized RNAs and, to a lesser extent, to the EBV DNA *Bam*HI fragment E (13). This previous observation was reconfirmed (Fig. 5*B*). *Bam*HI fragment E could encode the 140-kDa protein or, alternatively, might encode part of the 5′ untranslated leader of the

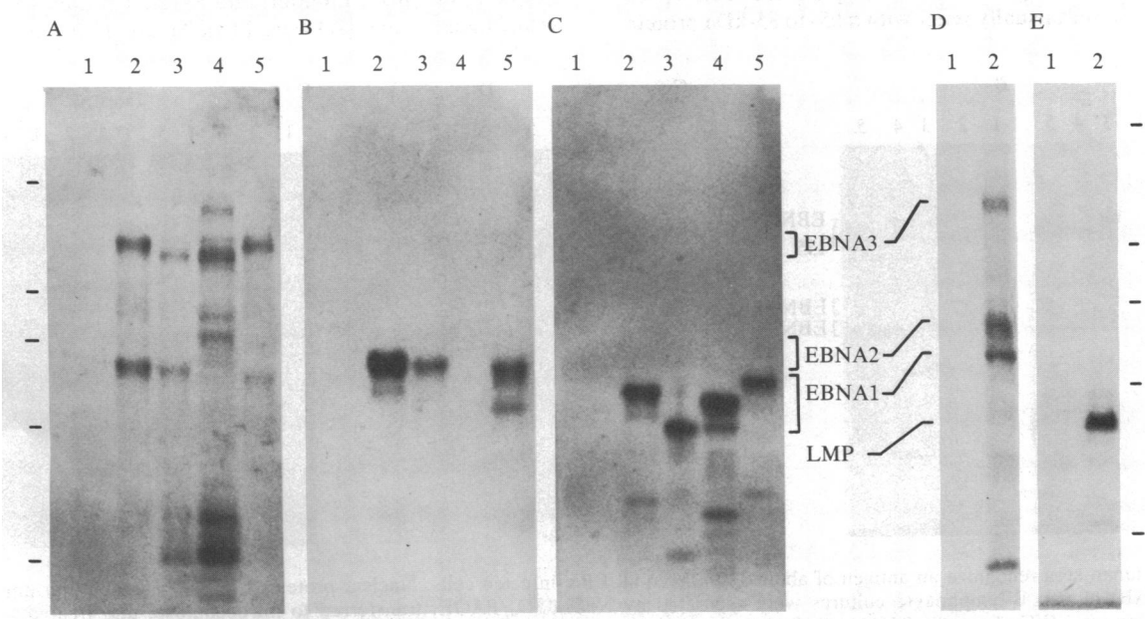


FIG. 3. The 140-kDa protein is antigenically distinct from the previously described viral proteins characteristic of latent virus infection. Immunoblots of nuclear proteins (prepared with those used in Fig. 1 *C–F*) were treated with human sera WC (*A*), 125 (*B*), TU457 (*C*), and VC (*D*) or with mouse S12 monoclonal antibody against the LMP (*E*). Size markers are indicated as in Fig. 1 (−) along the side of the blots. TU457 and 125 are EBV-immune human sera from non-rheumatoid-arthritic patients.

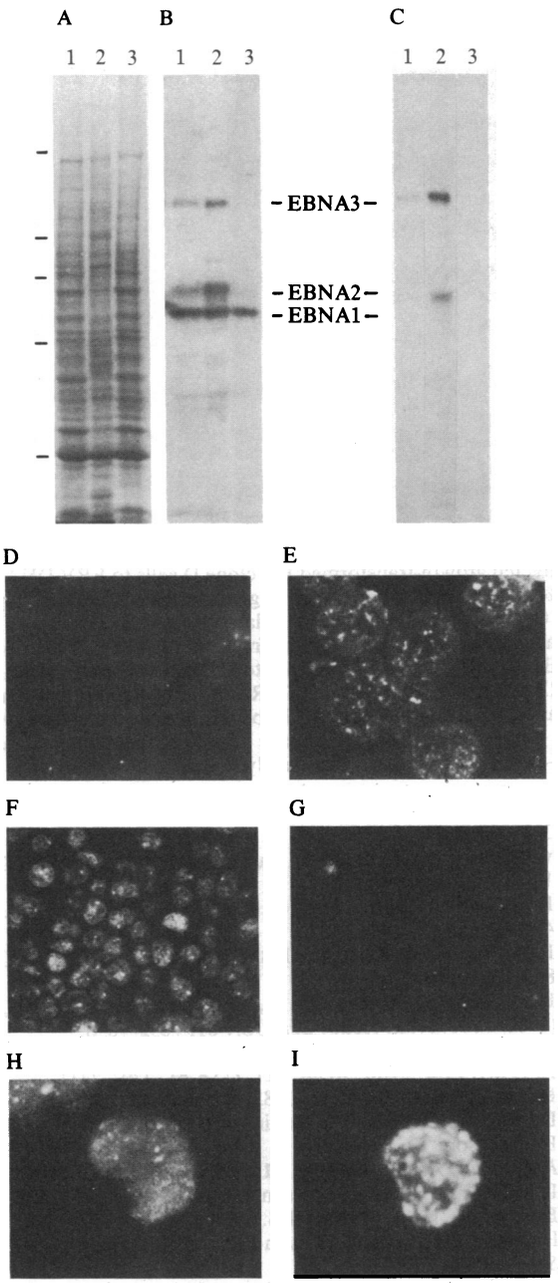


FIG. 4. The 140-kDa antigen is a nuclear protein. (*A–C*) IB4 cellular proteins were separated into nuclear (lanes 2) or cytoplasmic (lanes 3) fractions, and samples from each fraction were electrophoresed along with cellular protein (lanes 1). Similar blots were stained with either amido black (*A*) or human serum VC (*B*) or WC (*C*). The fractionations and blot shown in *C* were done independently of those shown in *A* and *B*. Size markers are indicated as in Fig. 1 (−) along the side of the stained blot. The nuclear location of EBNA3 is also demonstrated by immunostaining of EBNA2-negative P3HR-1 cells with EBNA1-negative WC sera (*E* and *I*). This reactivity is distinctively different from that of an EBNA1 serum (*H*). No reaction is seen with the same serum on the EBV-negative cell line Loukes (*D*). Rabbit EBNA2-specific sera give a typical EBNA staining of Raji cells (*F*) but not P3HR-1 cells (*G*). (*D* and *E* ×720; *F* and *G* ×288; *H* and *I*, ×1440.)

EBNA1 message because *Bam*HI E is 5′ to the EBNA1 open reading frame. The left-most fragment of *Bam*HI E hybridized to a 4.5-kb RNA that is distinctly different in size from the 3.7-kb RNA previously shown to encode EBNA1 (Fig. 5*C*). This RNA is sufficiently large to encode the 140-kDa protein and does not hybridize to the *Bam*HI K fragment which encodes EBNA1.

## DISCUSSION

Sera from EBV-infected rheumatoid arthritis patients have been shown frequently to have a high level of reactivity with nuclear proteins in most EBV-infected cells that are larger than EBNA1 (37). These proteins were felt to be related to the EBNA2 protein because both were absent from P3HR-1 cells (37). Our data confirm the high prevalence of antibody against nuclear proteins larger than EBNA1 in sera from rheumatoid arthritis patients. Our data also significantly extend these previous observations by demonstrating that: (*i*) one of the larger nuclear proteins recognized by sera from rheumatoid arthritis patients is the EBNA2 protein; (*ii*) the largest protein recognized by sera from rheumatoid arthritis patients is the 140-kDa protein, which is present in all EBV-infected cell lines, including P3HR-1; (*iii*) the 140-kDa protein is not related to the previously described EBNA1, EBNA2, or LMPs because human, rabbit, or mouse monoclonal antibody specific for these proteins does not react with the 140-kDa protein; and (*iv*) the 140-kDa protein is virus-encoded because its size varies in cells infected with different EBV isolates but is identical in cells infected with the same EBV isolate.

Several lines of evidence indicate that the 140-kDa protein is expressed in latent infection. First, the protein is found in all EBV-infected cell lines irrespective of the fraction of cells that are permissive of virus replication. More importantly, the protein is expressed in IB4 and Namalwa cells, which are not known to express proteins or RNAs associated with early or late virus replication. Second, WC sera, which does not react with EBNA1 by immunofluorescence (36) or on immunoblots (20), reacts with a nuclear protein in all P3HR-1 cells, a cell line that does not contain the gene encoding EBNA2 (21) and that permits virus replication in only a small fraction of cells. The nuclear fluorescence is globular and distinctive from the diffuse nuclear fluorescence characteristic of EBNA1 and EBNA2. WC serum previously had been noted to react with a nuclear antigen distinct from EBNA1 (36). However, no new protein was identified; moreover, analyses of EBV RNA in latently infected cells (refs. 12–14, 16; Fig. 5) are inconsistent with the hypotheses presented in the previous study (36)—namely, that the nuclear antigen detected in latently infected cells with WC serum is identical to that induced in cells by transfection with the EBV DNA *Bam*HI M fragment. Third, a new RNA is demonstrated in latently infected cells that is sufficiently large to encode the 140-kDa protein.

Since the 140-kDa protein is expressed in the nuclei of latently infected cells, we propose that it be designated "EBNA3." EBNA3 is the fourth viral protein expressed in latently infected growth-transformed cells. Like EBNA1, EBNA2, and LMP, EBNA3 is likely to be important in the processes of growth transformation, viral genome maintenance, or gene regulation. The difference in nuclear fluorescence pattern of EBNA3-reactive serum from that of EBNA1 or EBNA2 suggests that EBNA3 may have a different site of action.

The presence of unusually high titers of antibody to EBNA2 and EBNA3 in sera of patients with rheumatoid arthritis raises again the question of whether EBV plays a role in the pathophysiology of this disease. Rheumatoid arthritis patients exhibit unusual responses to EBV and are defective in limiting the number of circulating EBV-infected cells (38, 39). The unusual antibody responsiveness of rheumatoid arthritis patients could be a consequence of this defect.

We thank T. Scully for human serum that allowed comparison of EBNA2 and the 92-kDa antigens, H. zur Hausen and G. Lenoir for

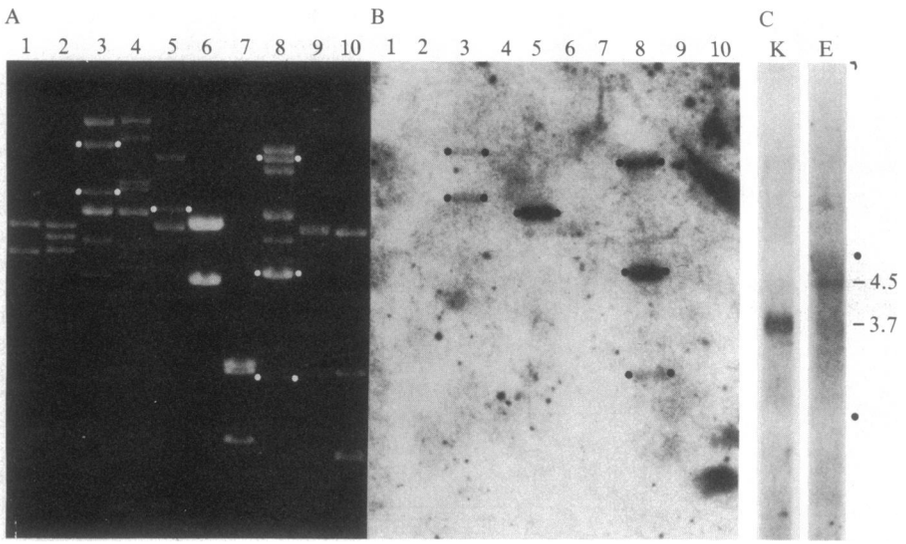


FIG. 5. Hybridization of cytoplasmic polyadenylylated RNA from latently infected growth-transformed IB4 clone D cells to EBV DNA. (*A*) Ethidium bromide-stained gel of recombinant EBV DNAs representative of 99% of the complexity of the EBV genome. *Bam*HI/*Eco*RI digests of EBV *Eco*RI fragments G2 (lanes 1); F (lanes 2); B and K (lanes 3); E, H, and C (lanes 4); D het (lanes 5); A (lanes 8); and G1 (lanes 9) which had been cloned into MUA3. The *Eco*RI B/K clone is from W91 DNA, which lacks the *Eco*RI site separating fragments B and K. Lanes 6 and 7 contain an *Eco*RI digest of EBV *Eco*RI fragment I and an *Ava* II digest of EBV *Eco*RI fragment J cloned into MUA3, respectively. Lane 8 also has a *Bam*HI/*Eco*RI digest of *Bam*HI fragments U and P cloned into pBR322. Lane 10 is a *Bam*HI/*Eco*RI digest of *Bam*HI fragment O cloned into pBR322. (*B*) A blot of the gel was hybridized with [$^{32}$P]cDNA, and an autoradiogram is shown. Hybridization is evident to *Bam*HI fragments K and E in lane 3, to *Eco*RI fragment D het in lane 5 and to *Bam*HI fragments V, X, and H in lane 8. (*C*) "Riboprobes" from the *Bam*HI fragments K and E were hybridized to blots of IB4 RNA, showing 3.7- and 4.5-kb RNAs, respectively.

EBNA sera, A. Rickenson for the BIF cell lines, T. Dambaugh for recombinant DNAs, M. Becker for sera from rheumatoid arthritis patients, K. Mann and D. Thorley-Lawson for the S12 monoclonal antibody, R. C. Schooley and M. Hirsch for WC serum, and Terri Reid for excellent technical assistance. This research was supported by Grants CA 17281 and CA 19264 from the United States Public Health Service and by MC 32J from the American Cancer Society. E.K. is a Faculty Research Awardee of the American Cancer Society. K.H. and S.F. are supported by National Research Service Awards GM 07183 and AI 07099, respectively.

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