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# *Bam*HI E region of the Epstein–Barr virus genome encodes three transformation-associated nuclear proteins

(gene transfer/COS-1 cells/Epstein–Barr virus-encoded nuclear antigen expression)

ANNE RICKSTEN*, BENGT KALLIN†, HANNAH ALEXANDER‡, JOAKIM DILLNER†, ROBIN FÅHRAEUS†, GEORGE KLEIN†, RICHARD LERNER‡, AND LARS RYMO*

*Department of Medical Biochemistry, Gothenburg University, Box 33031, 40033 Gothenburg, Sweden; †Department of Tumor Biology, Karolinska Institute, Box 60400, 10401 Stockholm, Sweden; and ‡Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA

*Contributed by George Klein, October 14, 1987*

ABSTRACT **Recombinant vectors carrying DNA fragments from the *Bam*HI E region of the B95-8 Epstein–Barr virus (EBV) genome were transfected into COS-1 cells, and the transient expression of EBV-encoded nuclear antigens (EBNAs) was analyzed by using polyvalent human antisera and rabbit antibodies to synthetic peptides. Vector DNA containing two rightward open reading frames in the *Bam*HI E fragment, BERF2a and BERF2b, induced the expression of a nuclear antigen identical serologically and with respect to size to the larger of the two polypeptides previously designated as EBNA4 in B95-8 cells. An antigen corresponding to the smaller polypeptide was induced in cells transfected with constructs that contained two neighboring reading frames, BERF3 and BERF4. This antigen also reacted with a rabbit antiserum to the synthetic peptide 203, deduced from BERF4. Thus, the findings show that the two components of the EBNA4 doublet in B95-8 cells are encoded by separate genes. The antigen encoded by BERF2a and/or BERF2b has been designated as EBNA4 and the antigen encoded by BERF3 and/or BERF4 has been designated as EBNA6. Polyvalent human antisera detected EBNA4 and EBNA6 in 9 of 11 lymphoid cell lines carrying independent EBV isolates. In the remaining two lines, either EBNA4 or EBNA6 was not detectable.**

The Epstein–Barr virus (EBV)-determined nuclear antigen (EBNA) is a regularly detectable viral "footprint" in all EBV-transformed cells and is therefore believed to play an important role in growth transformation (1). The serologically defined EBNA has been dissected into several protein components. Five nuclear proteins present in latent infection have been identified, denoted EBNA1–EBNA5, and four of them have been localized on the genomic map. EBNA1 is encoded by a rightward open reading frame in the *Bam*HI K fragment, BKRF1 (2–5). EBNA2 is encoded by the BYRF1 reading frame in the *Bam*HI Y and H fragment region (3, 6–13). EBNA3 is largely encoded by the BERF1 reading frame in the *Bam*HI E fragment (14–16) and presumably the short BLRF3 frame in the *Bam*HI L fragment (17). The coding sequences for EBNA4, which was detected as a doublet of polypeptides in immunoblotting (15, 16), have not been previously identified. EBNA5 [alternative designation: leader protein, LP (12)] is encoded by several repeated exons in the *Bam*HI W and Y fragments (18, 19).

Some of the functions of EBNA1 have been identified (20–24). It binds to specific sites in the *oriP* region of the EBV genome. This binding is essential for the maintenance and replication of episomal viral DNA. It may also influence the transcription of the viral genes expressed in growth-transformed cells. EBNA2 may be involved in the initial phase of B-cell transformation. This has been deduced from the fact that the P3HR-1 substrain of EBV, which lacks a large part of EBNA2, does not stimulate DNA synthesis in resting B lymphocytes and cannot transform them into immortal lines (25–27). Recent work has shown that EBNA2-expressing transfected Rat-1 cells have acquired the ability to grow at low serum concentrations (11) and that the growth properties of EBV-transformed lymphoblastoid cell lines (LCL) are influenced by the EBNA2 subtype of the resident virus (28). The function of the other members of the EBNA family is unknown.

In the present report we show that the larger of the two polypeptides designated as EBNA4 in B95-8 cells (15) is encoded by a fragment of EBV DNA containing the BERF2a and BERF2b reading frames. We also show that the other EBNA4 polypeptide is an independently expressed protein, encoded by a neighboring segment of the viral genome that contains the BERF3 and BERF4 reading frames. This necessitates a change in nomenclature. We propose to retain the designation EBNA4 for the BERF2b-encoded polypeptide, mainly because its gene is adjacent to that of EBNA3. EBNA5 is a well-defined protein encoded by another region of the viral genome (18). This leaves the designation of EBNA6 for the BERF4-encoded polypeptide. We shall adhere to this nomenclature throughout. When referring to our previous definition of EBNA4, we shall mention it within quotation marks.

## MATERIALS AND METHODS

**Cell Culture and DNA Transfections.** Lymphoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. COS-1 cells were cultured in Iscove's modification of Dulbecco's medium (GIBCO) containing 10% fetal calf serum and antibiotics and were subcultured 20 hr before transfection. DNA transfections were performed by a modification of the DEAE-dextran technique as described earlier (13). EBNA staining of the cells was performed by anticomplement immunofluorescence (ACIF; ref. 13).

**Plasmid Constructions.** A library of cloned restriction endonuclease fragments of B95-8 EBV DNA was established earlier (29). All manipulations involved in the construction of recombinant plasmids were carried out by standard procedures (30) or under the conditions recommended by the enzyme manufacturer. Plasmid pBΔE1 was constructed by directional cloning of the larger *Eco*RI subfragment of the

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Abbreviations: EBV, Epstein–Barr virus; BL, Burkitt lymphoma(s); LCL, lymphoblastoid cell line(s); RA, rheumatoid arthritis; CBC, cord blood cell(s); BERF, *Bam*HI E rightward open reading frame; EBNA, EBV-encoded nuclear antigen; ACIF, anticomplement immunofluorescence.

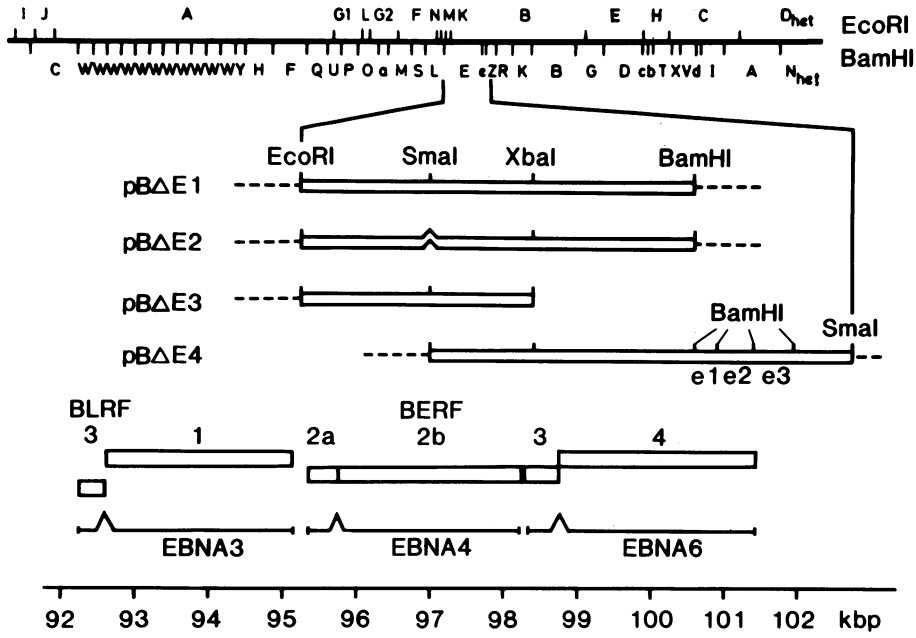


FIG. 1. Restriction fragment map of B95-8 EBV DNA and structure of plasmids used in transfection experiments. The unshaded area between double lines represents EBV DNA fragments in the recombinant vectors and broken lines are pSV2-gpt sequences. The major rightward open reading frames in the *Bam*HI E fragment region, BLRF3, BERF1, BERF2a, BERF2b, BERF3, and BERF4, are indicated by open boxes. The sequences suggested to encode EBNA3, EBNA4, and EBNA6, respectively, are indicated below the reading frames. The scale refers to the B95-8 EBV DNA sequence (33). kbp, Kilobase pairs.

*Bam*HI E fragment of B95-8 EBV DNA (nucleotides 95,239–100,613) between the *Eco*RI and *Bam*HI sites of the pSV2-gpt vector (31). The pBΔE2 plasmid was obtained by inserting an *Xho* I linker (C-C-T-C-G-A-G-G, New England Biolabs) into the unique *Sma* I site of the EBV DNA fragment in pBΔE1. Plasmid pBΔE3 was derived from pBΔE1 by deleting the *Xba* I–*Bam*HI subfragment of the *Bam*HI E fragment corresponding to nucleotides 98,398–100,613 of EBV DNA and replacing it with a *Bam*HI linker (C-G-G-A-T-C-C-G, New England Biolabs). Plasmid pBΔE4 was constructed by excising a *Sma* I subfragment (nucleotides 97,027–102,756) from the cloned *Eco*RI B fragment of B95-8 DNA and inserting it into the *Eco*RI site of the pSV2-gpt plasmid using *Eco*RI linkers (G-G-A-A-T-T-C-C, New England Biolabs). The orientation of the EBV DNA insert is the same as in the other recombinant plasmids.

**Production of Antisera and Immunoblotting.** Seven peptides were synthesized with sequences deduced from selected parts of the BERF1, BERF2b, BERF3, and BERF4 open reading frames (32). The peptides corresponded to the amino acids 706–720 (peptide 205) and 795–809 (peptide 204) of BERF1; 259–272 (peptide 207) and 628–643 (peptide 206) of BERF2b; 6–20 (peptide 209) of BERF3; and 621–633 (peptide 203) and 855–872 (peptide 202) of BERF4, respectively. The peptides were conjugated to keyhole limpet hemocyanin (Sigma) and the conjugates were used for immunization. Two rabbits were immunized with each peptide and sera were tested from three different bleedings for each animal.

Human sera were derived from healthy donors with or without antibodies to EBV antigens or from rheumatoid arthritis (RA) patients. RA sera with strong reactivity to EBNA3 and EBNA4 were pooled and used for most of the experiments. A panel of sera with known reactivity to EBNA3 and EBNA4 defined in an earlier study (15, 18) was used for the screening of the transfected cells.

$NaDodSO_4$/PAGE, electrophoretic transfer to nitrocellulose, and immunostaining were performed essentially as described (15).

## RESULTS

**Expression of EBNA4 in COS-1 Cells.** Recombinant vectors were constructed to identify gene products encoded by previously unassigned open reading frames within the *Bam*HI E fragment of B95-8 EBV DNA (see Fig. 1). The pBΔE1 plasmid contains the BERF2a, BERF2b, and BERF3 reading frames and the major part of BERF4. Transfection of pBΔE1 DNA into COS-1 cells induced a nuclear antigen in 20–50% of the cells that gave a brilliant staining in ACIF tests with the serum pool from RA patients but did not react with the sera of EBV-seronegative donors (Fig. 2*A*). The nuclear staining was somewhat more coarsely granular than the parallel polyvalent nuclear staining of Raji cells, but in

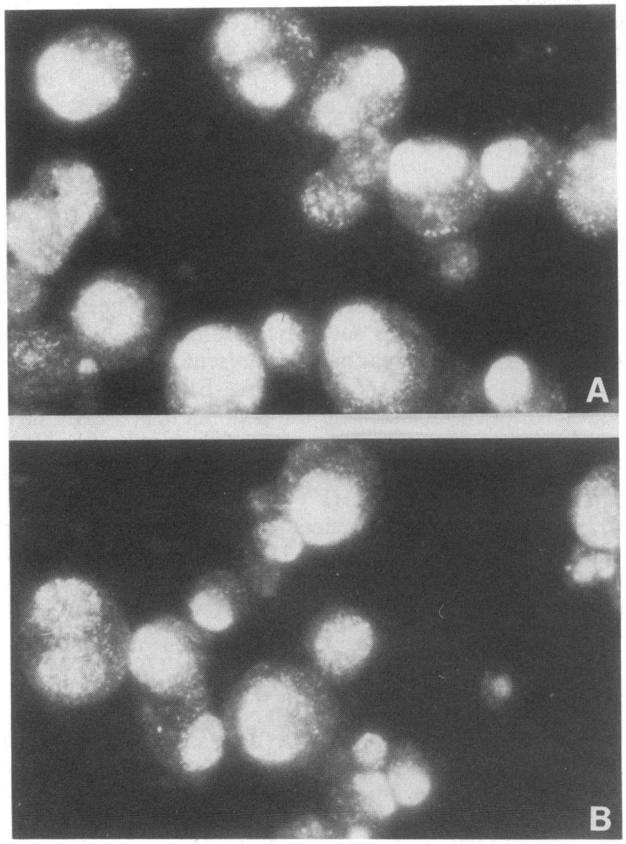


FIG. 2. Transient expression of nuclear antigens in COS-1 cells after transfection with the recombinant vector pBΔE3 (*A*) or pBΔE4 (*B*). Cells were stained by using the ACIF technique and a pool of human sera with high antibody titers to "EBNA4". (×300.)

many cells it was of comparable intensity. Sera lacking anti-EBNA antibodies did not produce nuclear fluorescence in these tests. $NaDodSO_4$/PAGE and immunoblotting analysis of transfected cell extracts demonstrated the presence of a 160-kDa polypeptide that reacted with the RA serum pool. It had a similar electrophoretic mobility as the slower of the two polypeptides in B95-8 cell extracts, previously defined as "EBNA4" (15).

A panel of human sera was used to identify the 160-kDa antigen expressed in pBΔE1-transfected cells. Of 13 sera that had antibodies to all EBNAs, including the double band originally described as "EBNA4" (15), all reacted with the 160-kDa antigen in the transfected cells. Ten sera with antibodies to one or more of the EBNAs, but not to "EBNA4", did not give any specific reaction with antigens in the transfected cells. Five sera that were negative for all EBV antibodies also failed to detect the 160-kDa antigen. This serological pattern, together with the comigration of the 160-kDa antigen in the transfected COS-1 cells with the larger of the two "EBNA4" antigens in B95-8 EBV-transformed cells, has prompted us to designate the 160-kDa protein as EBNA4.

Two other constructs were used for a more precise localization of the EBNA4 gene. The pBΔE2 plasmid, a derivative of pBΔE1, contains a short oligonucleotide, which includes an *Xho* I endonuclease recognition sequence, inserted into the *Sma* I site in the middle of the BERF2b reading frame. The insertion of the linker results in a translational frameshift and the introduction of stop codons ≈40 bp 3′ to the modification. COS-1 cells transfected with pBΔE2 DNA still expressed a nuclear antigen reactive with anti-EBNA antibody-containing sera in ACIF tests. However, $NaDodSO_4$/PAGE and immunoblotting analysis showed that the cells contained a 90-kDa polypeptide instead of the 160-kDa EBNA4 polypeptide found in cells transfected with the unmodified recombinant vector (Fig. 3). The results suggest that the 160-kDa EBNA4 is encoded by BERF2b. This was confirmed by transfection experiments with the pBΔE3 vector, which contains only the BERF2a and BERF2b reading frames. pBΔE3 DNA induced nuclear fluorescence and a full-length 160-kDa polypeptide in the cells (Fig. 3).

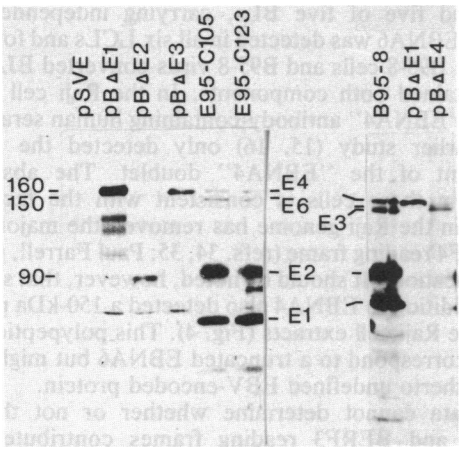


FIG. 3. Immunoblot analysis of viral proteins expressed in COS-1 cells transfected with EBV DNA-containing recombinant vectors. The designations of the vector constructs are as in Fig. 1. (*Left*) Immunoblot obtained with a human RA serum diluted 1:40, which under these conditions does not detect EBNA3 in B95-8 transformed cells. Molecular masses are given in kDa. (*Right*) Results obtained with a serum that detects EBNA3. EBNA polypeptides are indicated by the designations E1, E2, E3, E4, and E6, respectively.

**Expression of EBNA6 in COS-1 Cells.** These transfection experiments have proved that the BERF3 and BERF4 reading frames are not parts of the 160-kDa EBNA4 gene. However, an EBV transcript has been identified by cDNA cloning that contains sequences homologous to the right part of the *Bam*HI E fragment together with sequences corresponding to parts of the *Bam*HI W and Y fragments (33). This raised the question of whether the *Bam*HI E region may encode yet another nuclear polypeptide. We have constructed the pBΔE4 recombinant vector to test that possibility. The vector contains the BERF3 and BERF4 reading frames. Plasmid DNA was transfected into COS-1 cells and the transient expression of EBV-specific nuclear antigens was analyzed by ACIF, $NaDodSO_4$/PAGE, and immunoblotting techniques using the polyvalent RA serum pool with high titers of anti-"EBNA4" antibodies. A large fraction of the cells (30–70% in different experiments) expressed a nuclear antigen (Fig. 2*B*). A 150-kDa polypeptide was identified in the cell extracts with the same electrophoretic mobility as the faster of the two polypeptides previously designated "EBNA4". This polypeptide has been renamed EBNA6 (Fig. 3). The assignment of EBNA6 to the BERF4 reading frame was corroborated by our demonstration that an antiserum to the synthetic peptide 203, deduced from BERF4, reacted with the 150-kDa polypeptide on immunoblots (data not shown).

**Expression of EBNA3, EBNA4, and EBNA6 in Lymphoid Cell Lines.** Rabbit antibodies were raised against seven different peptides deduced from the BERF1, BERF2b, BERF3, and BERF4 reading frames. Three types of assays were performed to determine the specificity of the sera: (*i*) immunoblotting, (*ii*) immunoprecipitation from extracts of IB4 cells followed by the further identification of the precipitated product by immunoblotting with human antiserum, (*iii*) immunostaining of acetone-fixed cell smears. The antibodies were usually used in the form of crude sera and after affinity purification of peptide-specific antibody with immobilized peptide. Antisera to peptides 203 and 204 proved to contain antibodies to EBV-specific proteins.

Peptide 204 corresponds to the C-terminal part of the BERF1-encoded peptide. The specificity of the antibody to peptide 204 was indistinguishable from that of the human EBNA3-specific serum WC (15) and identified EBNA3 as a 130- to 140-kDa polypeptide in a number of different cell lines in immunoblotting and immunoprecipitation assays (Table 1 and additional data, not shown). The results suggest that BERF1 is the coding sequence of EBNA3 and confirm the observations made by Hennessy *et al.* (16).

Peptide 203 is derived from a sequence repeated twice in the right part of the BERF4 reading frame. The anti-203 antibody reacted with the larger of the two "EBNA4" polypeptides in EBV-transformed cells detected with the polyvalent human RA serum (Fig. 4 and Table 1). Some exceptions are noted below. This polypeptide is thus encoded by BERF4 and has been renamed EBNA6. The other component of the "EBNA4" doublet, which reacted with the polyvalent human antiserum but not with antibodies to peptide 203, is probably encoded by BERF2b, as suggested by the analogy with the findings on B95-8 cells. It is therefore designated as EBNA4. The cell lines analyzed included Burkitt lymphomas (BL) and LCL carrying EBV isolates from Africa, United States, and Sweden. The size and the relative amounts of EBNA4 and EBNA6 varied considerably between different cell lines. There were no obvious differences in the expression of EBNA3, EBNA4, and EBNA6 that could be related to the geographic or disease origin of the cells.

Unexpectedly, the anti-peptide 203 antiserum did not react with cell lines carrying the B95-8 EBV isolate, although the amino acid sequence of peptide 203 had been deduced

Table 1. Identification of EBNA4 and EBNA6 polypeptides in lymphoid cell lines by $NaDodSO_4$/PAGE and immunoblotting

| Cell line | Derivation of virus | Molecular size, kDa | | |
|---|---|---|---|---|
| | | EBNA3 | EBNA4 | EBNA6 |
| BJAB* | — | NBD | NBD | NBD |
| B95-8 | Am. IM | 145 | 160† | 150† |
| BJAB/B95-8‡ | Am. IM | 145 | 160† | 150† |
| P3HR-1 | Afr. BL | 132 | 155 | NBD |
| Namalwa | Afr. BL | 140 | 155 | 160 |
| BL60 | Afr. BL | 145 | 150 | 160 |
| Ak Caren | Afr. BL | 135 | 155 | 165 |
| Salim Mwalim | Afr. BL | 135 | 150 | 165 |
| CBC-Ek2 | Swe. IM | 135 | 160 | 180 |
| CBC-Ral-Sto | Afr. BL | 140 | NBD | 150 |
| CBC-M81-Sto | Am. BL | 140 | 160 | 150 |
| Cherry | Am. IM | 135 | 160 | 170 |
| CBC-M14 | Swe. IM | 125 | 160 | 175 |
| CBC-NAE5 | Afr. HD§ | 145 | 155 | 160 |

EBNA3 was identified with polyvalent human antisera that were characterized with regard to anti-EBNA antibodies in a previous study (15) and with the rabbit anti-peptide 204 antiserum. The EBNA4/EBNA6 doublet was identified with polyvalent human antisera, EBNA6 was defined as the component that reacts with rabbit anti-peptide 203 antibodies, and EBNA4 was defined as the one that does not. NBD, no band detected. Am., American; Afr., African; Swe., Swedish; CBC, cord blood cells; IM, infectious mononucleosis.

*EBV-negative BL.

†These polypeptides do not react with the antipeptide 203 antiserum but were defined as EBNA6 by a comparison with the polypeptides expressed in the transfected COS-1 cells.

‡BJAB cells converted to EBNA positivity by infection with B95-8 EBV.

§Throat washing of a healthy African donor (HD).

from the B95-8 virus genome. Furthermore, the peptide antiserum did react with transfected COS-1 cells expressing EBNA6. The finding may be attributed to some difference in the availability of the antigenic epitope to the antibodies in lymphoid cells as compared to COS-1 cells. Consequently, EBNA4 and EBNA6 detected in B95-8 cells by polyvalent human antiserum were also defined by their electrophoretic comigration with the corresponding polypeptides in the transfected COS-1 cells.

The peptide 203 antiserum did not detect EBV-specific polypeptides in Raji cell extracts. This was expected since a deletion in the Raji EBV genome is known to have removed the part of the BERF4 reading frame that encodes the epitope corresponding to peptide 203 (34, 35).

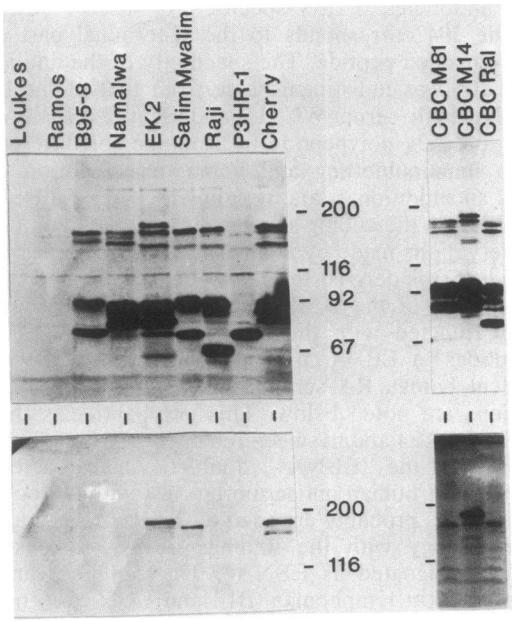


FIG. 4. Expression of EBV antigens in lymphoid cell lines. The EBNA polypeptides were detected by using polyvalent human antiserum (*Upper*) or antiserum to the synthetic peptide 203 (*Lower*). A faint but consistently detected band in Namalwa cell extracts is not well reproduced in the immunoblot shown in the lower left panel. Electrophoretic comigration of polypeptides was confirmed by differential immunostaining of blots of the same gel with human polyvalent and rabbit anti-peptide 203 antisera. Molecular masses are given in kDa.

## DISCUSSION

Two additional polypeptides belonging to the EBNA family were recently identified in EBV-transformed cells (15, 16). They were serologically distinct and of different size, 140 and 150–180 kDa. We have provisionally designated them as EBNA3 and "EBNA4." Like other EBNAs, EBNA3 and "EBNA4" vary in size between EBV-transformed cell lines that carry independent viral isolates and bind to DNA *in vitro*. In many lines, including the prototype B95-8 line, "EBNA4" appeared as two immunologically crossreactive polypeptide chains differing in apparent molecular mass by up to 20 kDa.

Our present findings show that the two components of the "EBNA4" doublet in B95-8 cells are encoded by separate open reading frames. For reasons mentioned in the Introduction, the polypeptide encoded wholly or partly by BERF2b has here been designated as EBNA4 and the polypeptide encoded by BERF4 has been designated as EBNA6. A seeming paradox is that the coding capacity of BERF2a and BERF2b or BERF3 and BERF4 is only about 1000 amino acids, whereas the apparent molecular sizes of EBNA4 and EBNA6 determined by $NaDodSO_4$/PAGE correspond to proteins of average composition containing about 1500 amino acid residues. Presumably, the discrepancies between the molecular weights predicted from the nucleotide sequence and the sizes deduced from the electrophoretic mobility reflect the unusual amino acid composition of these polypeptides, as has been observed for several other proline-rich proteins, including EBNA2 (11, 13).

Polyvalent human sera detected EBNA4 in five of six LCLs and five of five BLs, carrying independent viral isolates. EBNA6 was detected in all six LCLs and four of the five BLs. B95-8 cells and B95-8 virus-converted BJAB cells also contained both components. In the Raji cell line the panel of "EBNA4" antibody-containing human sera defined in an earlier study (15, 16) only detected the 160-kDa component of the "EBNA4" doublet. The absence of EBNA6 in these cells is consistent with the fact that a deletion in the Raji genome has removed the major part of the BERF4 reading frame (refs. 34, 35; Paul Farrell, personal communication). It should be noted, however, that some RA sera in addition to EBNA4 also detected a 150-kDa polypeptide in the Raji cell extracts (Fig. 4). This polypeptide is too large to correspond to a truncated EBNA6 but might represent a hitherto undefined EBV-encoded protein.

Our data cannot determine whether or not the short BERF2a and BERF3 reading frames contribute to the EBNA4 and EBNA6 genes. It might be relevant in this context that two cDNA clones containing exons from the *Bam*HI E region have been isolated from mRNA from EBV-transformed cells (17, 33). The T2 clone probably corresponds to an mRNA molecule that encodes EBNA3 (16). Our results suggest that the T4 clone may correspond to the EBNA6-encoding mRNA. Both cDNA molecules have a similar overall structure, suggesting that the mRNAs may have been cut from identical primary transcripts by alterna-

tive splicing. They have a common long 5′ leader sequence that contains exons derived from the *Bam*HI C and W fragments, followed by the unique reading frames. The T4 leader also contains exons from the *Bam*HI Y and U fragments. The T2 reading frame has been spliced together from a short exon that contains 344 nucleotides of BLRF3 and a long exon that includes the major part of BERF1. This gives a coding potential of 944 amino acids. The T4 clone represents an incomplete mRNA, containing 367 nucleotides from BERF3 and the first part of BERF4. The postulated EBNA4 gene has a strikingly similar organization. The BERF2a reading frame starts with a translation initiation codon and contains a splice donor consensus sequence close to its end (position 95,683). There are also several possible splice acceptor sites in the first part of BERF2b. It is likely that the transcription and processing of the mRNAs of the three proteins EBNA3, EBNA4, and EBNA6 follow a similar pattern and that BLRF3, BERF2a, and BERF3 are parts of the corresponding genes.

EBNA3, EBNA4, and EBNA6 are probably related products of viral evolution. There is a limited sequence homology between the BERF1, BERF2b, and BERF4 open reading frames (32). Significant homology can only be demonstrated within the first 600 bp of each frame corresponding mainly to hydrophobic and aromatic amino acid residues. The overall amino acid sequence homology between the three predicted polypeptides is not significant. However, the amino acid composition of the proteins is very similar and is characterized by a high content of proline and charged amino acids. Metzger *et al.* (36) have devised a method for the assessment of a possible relationship of two proteins based on their amino acid composition. The difference index obtained with this procedure varies from zero, when the proteins have the same composition, to 100 for proteins with no amino acid in common. Calculation of this index for the predicted proteins encoded by BERF1, BERF2b, and BERF4 yields values in the range of 7.3–8.8 as compared to a difference index for EBNA2A and EBNA2B (37, 38) of 6.8.

A number of proteins involved in nucleic acid binding or gene regulation have been identified that contain metal-binding domains (39–41). We have not found sequences similar to the metal-binding consensus motif (41) in any of the EBNA proteins. Still, EBNA1 has been shown to bind directly to specific EBV DNA sequences involved in regulatory processes (23). Whether other EBNAs operate in a similar manner remains to be established.

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