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Antibodies against synthetic peptides react with the second Epstein-Barr virus-associated nuclear antigen

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Five peptides were synthesized on the basis of amino acid sequences predicted from the transformation-associated *Bam*HI WYH region of the genome of the Epstein-Barr virus (EBV). Antisera to two peptides deduced from a 1.6-kb open reading frame in the *Bam*HI H fragment identified an 87 000-dalton nuclear polypeptide that was present in EBV-carrying cell lines that expressed the second EBV-determined nuclear antigen (EBNA-2). This polypeptide was not detected in cell lines that carried EBV variants with a deleted *Bam*HI WYH region or in EBV-negative cell lines. Three peptides deduced from the 1.6-kb open reading frame reacted with human EBNA-positive sera, but not with EBNA-negative sera. Following affinity purification with the peptides, two of the corresponding human antibodies also reacted with the 87 000-dalton polypeptide.

Key words: transformation/Epstein-Barr virus/synthetic peptides/nuclear antigen

Introduction

Epstein-Barr virus (EBV) transforms human B-lymphocytes into continuously growing lymphoblastoid cell lines (Henle *et al.*, 1967). Transformation is regularly accompanied by the appearance of the EBV-determined nuclear antigen (EBNA), detected by anti-complement immunofluorescence (ACIF) in 100% of the cells in EBV-carrying cell lines (Reedman and Klein, 1973) and in the two EBV-associated human tumors, Burkitt's lymphoma (BL) (Reedman and Klein, 1973) and nasopharyngeal carcinoma (Klein *et al.*, 1974). A nuclear antigen can be induced by transfecting EBV-negative cells with the *Bam*HI K fragment of the viral genome (Summers *et al.*, 1982). The production of sequence-specific antisera conclusively showed that the *Bam*HI K fragment encodes a polypeptide that varies in size between 70 and 92 kd in different EBV-carrying cell lines (Hennessy and Kieff, 1983; Dillner *et al.*, 1984). This antigen is here referred to as EBNA-1.

The EBNA-1 coding *Bam*HI K fragment encodes one of the three major EBV transcripts expressed in latently infected, growth-transformed cell lines (reviewed by Kieff *et al.*, 1982). Another transcript is encoded by the *Bam*HI WYH region (van Santen *et al.*, 1981). EBV variants carried by two Burkitt's lymphoma lines, P3HR-1 and Daudi, have large deletions in this region (Raab-Traub *et al.*, 1978; Delius and Bornkamm, 1978; Jones *et al.*, 1984).

Absorption of EBNA-positive sera with P3HR-1 or Daudi cells removes the nuclear staining when the same lines are used as targets. However, some sera contain EBNA-reactive antibodies that are not removed by this absorption. They give a brilliant nuclear ACIF staining in 100% of the cells in lines that carry EBV substrains with an intact *Bam*HI WYH region (Dillner *et al.*, 1985). The corresponding fluorescence-detected nuclear antigen is referred to as EBNA-2. A nuclear antigen detectable with EBNA-2-positive sera can be induced accordingly by transfecting EBV-negative cells with the *Bam*HI WYH region (Rymo and Klein, 1985).

The EBV strain released by P3HR-1 cells is unable to transform (Miller *et al.*, 1974). This could be directly related to the deletion in the *Bam*HI WYH region. Transforming virus could be rescued from non-permissively infected Raji cells after superinfection with P3HR-1 virus (Fresen *et al.*, 1978; Yajima *et al.*, 1978). All transforming isolates had acquired the deleted region (Fresen *et al.*, 1980) presumably by recombination. Preliminary experiments indicate that the viral strain released by Daudi cells is also non-transforming (B.Griffin, personal communication; I. Ernberg, personal communication).

Immunoblotting with some EBV antibody-positive sera detected, in addition to EBNA-1, an 81-kd polypeptide in some EBV-carrying cell lines (Strnad *et al.*, 1981). This polypeptide was shown to be EBV-associated, nuclear and unrelated to EBNA-1 (Hennessy and Kieff, 1983). It is not detected in EBNA-2-negative BL lines such as P3HR-1 and Daudi (Sculley *et al.*, 1984; Dambaugh *et al.*, 1984; Dillner *et al.*, 1985) suggesting that it might be encoded or induced by the *Bam*HI WYH region.

The complete DNA sequence of EBV (173 kb) has been determined (Baer *et al.*, 1984). The most important features of the *Bam*HI WYH region are shown in Figure 1. A 3.0-kb major latent mRNA is probably transcribed from a canonical promoter in *Bam*HI W and seems to terminate at the poly(A) addition site indicated in Figure 1 (van Santen *et al.*, 1981). We synthesized peptides on the basis of amino acid sequences predicted from the two long open reading frames in the *Bam*HI WYH region in order to (i) develop monospecific and sequence-specific EBNA-2 reagents, (ii) confirm the assumption that the *Bam*HI WYH region may code for the EBNA-2-polypeptide detected by immunoblotting, and (iii) map the coding sequences of EBNA-2 within the *Bam*HI WYH region.

Results

Extraction of EBNA-1 and EBNA-2 from nuclei

Nuclear and cytoplasmic fractions were prepared from living B95-8 cells. The nuclei were sequentially extracted with 150 mM NaCl, 400 mM NaCl, 3 M urea and electrophoresis sample buffer. The fractions were analyzed for EBNA-1 and EBNA-2 content by immunoblotting with a human EBNA-1 and EBNA-2-positive serum (Dillner *et al.*, 1985). These antigens were identified as a 78- and 87-kd polypeptide, respectively, on the basis of their co-migration with polypeptides detected by antisera to synthetic peptides specific for EBNA-1 (Dillner *et al.*, 1984) or EBNA-2 (Figure 3). EBNA-1 showed a typical bimodal extrac-

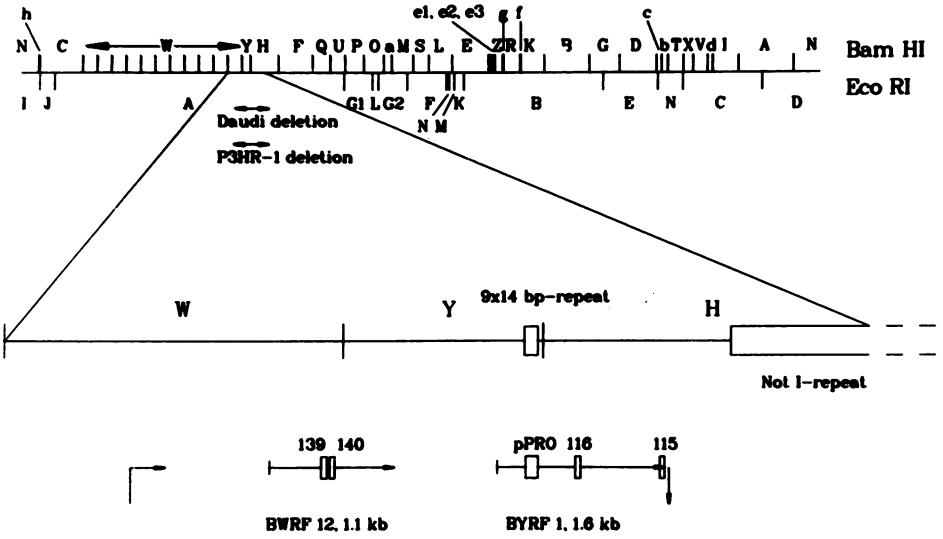


Fig. 1. Features of the *Bam*HI WYH region. Data shown are from the EBV sequence reported by Baer *et al.* (1984). (⟶) = open reading frame; kb = kilobase pairs; (⟶) = canonical promoter sequence; (↓) = canonical poly(A) addition site. The positions of the deduced peptide sequences are indicated by boxes on the open reading frames (see Table I). Repeat sequences are also indicated. The deletions of the P3HR-1 and Daudi viral substrains are indicated by horizontal lines.

tion pattern. A significant proportion of EBNA-1 was present in the cytoplasmic fraction. The major EBNA-1 band was seen in the 150 mM NaCl nuclear extract. No EBNA-1 could be detected in the further 400 mM NaCl and 3 M urea fractions, but a second peak of EBNA-1 was seen in the residual, SDS-solubilized fraction. EBNA-2 was not detected in the cytoplasmic fraction or in the nuclear 150 mM NaCl extract. A weak but distinct EBNA-2 band was seen in the 400 mM NaCl nuclear extract, but the protein could not be detected in the 3 M urea extract. The strongest EBNA-2 band was seen in the nuclear residual fraction (Figure 2).

Several lower mol. wt. proteins were also detected, notably a series of 48–57 kd polypeptides demonstrated in the 400 mM NaCl and 3 M urea extracts. These antigens are major early viral DNA-binding proteins (B.Kallin, unpublished data) encoded by the *Bam*HI M fragment, since they are also detected by a monoclonal antibody (R3) (Pearson *et al.*, 1983). The level of EA expression of the B95-8 cells before harvest was 0.5–1%.

A comparison with a parallel gel stained with Coomassie Brilliant Blue showed that most of the nuclear protein appeared in the 150 mM and 400 mM NaCl extracts (not shown). The SDS-solubilized fraction therefore represents partially purified EBNA-2. This fraction was used as the antigen for the immunoblotting experiments with the antibodies against the synthetic peptides.

Isolation of peptide-specific antibodies from immunized rabbit and human EBNA-positive sera

All peptides elicited an antibody response in rabbits (Table I). Peptide-specific antibodies could be purified by affinity chromatography with a yield of >90%, except for anti-peptide 116 antibodies where the maximum yield was 20%. Twenty-one human EBNA-positive sera, seven human EBV-negative sera and six human EBV-positive but EBNA-negative sera from early infectious mononucleosis patients were tested for reactivity against the different peptides in the direct-binding ELISA test. EBNA-positive sera were found that reacted with the three peptides deduced from the 1.6-kb BYRF1 open reading frame in the *Bam*HI Y-H fragments. EBNA-negative sera did not react with any of the peptides. Neither of the two peptides deduced from the BWRF12 reading frame in the *Bam*HI W-*Bam*HI Y fragment reacted with any sera tested. A 'mock' peptide that con-

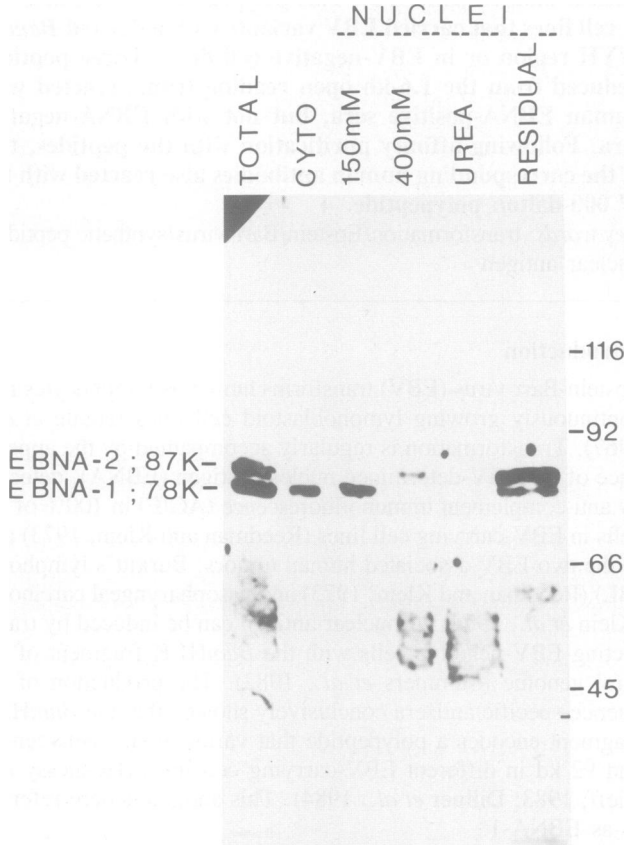


Fig. 2. Detection of EBNA-1 and EBNA-2 in subcellular fractions by immunoblotting. Nuclear and cytoplasmic fractions were prepared from living B95-8 cells. The nuclei were sequentially extracted with 150 mM NaCl, 400 mM NaCl, 3 M urea and the residue then dissolved in electrophoresis sample buffer. An amount of extract corresponding to 2 x 10^6 cells has been applied to each lane. Immunostaining was performed with a previously characterized EBNA-1 and EBNA-2-positive serum (see text), diluted 1:10. The 48–57 kd *Bam*HI M-encoded nuclear antigens, which are abundant early antigens, are seen in the 400 mM NaCl and 3 M urea fractions. Figures on the right indicate the position of the mol. wt. standards.

Table I. Synthetic peptides and anti-peptide antibodies

Peptide designation	Peptide sequence	Peptide sequence deduced from:	Rabbit anti-peptide titer Rabbit 1/Rabbit 2	Reactivity with the 87 000-dalton EBNA-2 polypeptide of rabbit anti-peptide sera	Proportion of human EBNA-positive sera with 1:10 or higher anti-peptide titer	Proportion of human EBNA-negative sera with 1:10 or higher anti-peptide titer	Reactivity with the 87 000-dalton EBNA-2 protein of human peptide-specific antibodies affinity purified on peptide column
115	Asp-Glu-Asp-Tyr-Val-Glu-Gly-Pro-Ser-Lys-Arg-Pro-Arg-Pro-Ser-Ile-Gln-Cys[a]	C terminus of BYRF1 (*Bam*HI H)	1:5120/1:1280	+	4/21	0/13	+
116	Pro-Pro-Arg-Pro Thr-Arg-Pro-Thr-Thr-Leu-Pro-Pro-Thr-Pro-Leu-Leu-Thr-Cys[a]	Internal sequence of BYRF1	1:10 240/1:5120	+	8/21	0/13	+
pPRO	(Pro) 20 amino acids	Near N terminus of BYRF1	NT	NT	9/21	0/13	–
139	Gln-Pro-Glu-Gly-Pro-Arg-Gln-Pro-Gly-Arg-Pro-Gln-Arg-Pro-Val-Pro-Arg-Pro-Cys[a]	Internal sequence of BWRF12 (*Bam*HI Y)	1:2560/1:2560	–	0/21	0/13	NT
140	Cys-Pro-Pro-Glu-Gly-Thr-Leu-Gly-Val-Pro-Ser-Pro-Pro-Leu-Gln	Internal sequence of BWRF12	1:2560/1:5120	–	0/21	0/13	NT
'115 mock'	Asp-Glu-Asp-Tyr-Val-Glu-Gly-Pro-Ser-Lys-PRO-Pro-Arg-Pro-Ser-Ile-Gln-Cys[a]	A one amino acid substitution compared with peptide 115	1:5120/1:1280 (of anti-115-rabbits)	NT	0/21	0/13	NT

[a]Not in the deduced amino acid sequence, but added to allow coupling to carrier.

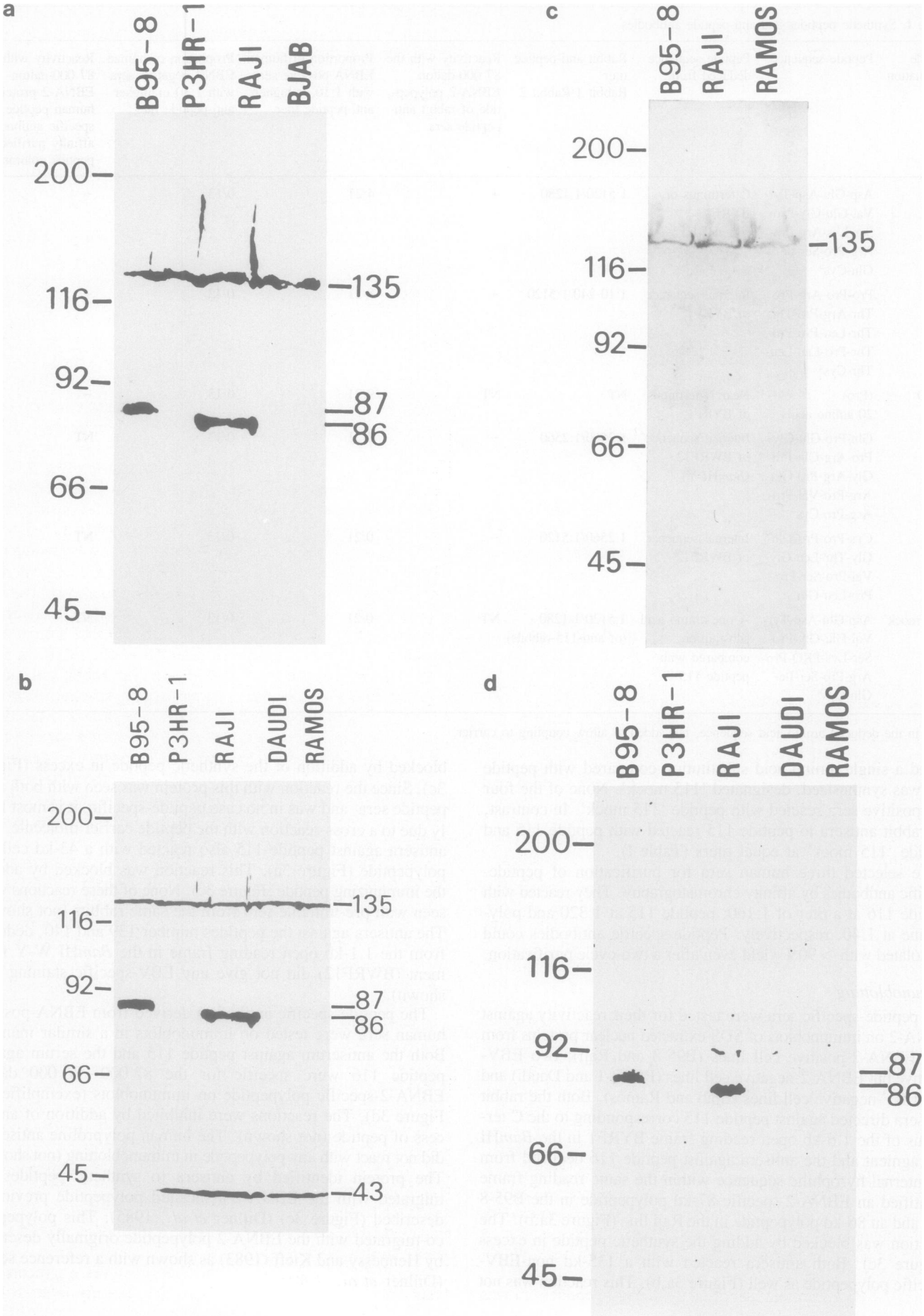
tained a single amino acid substitution compared with peptide 115 was synthesized, designated '115 mock'. None of the four 115-positive sera reacted with peptide '115 mock'. In contrast, the rabbit antisera to peptide 115 reacted with peptide 115 and peptide '115 mock' at equal titers (Table I).

We selected three human sera for purification of peptide-specific antibodies by affinity chromatography. They reacted with peptide 116 at a titer of 1:160, peptide 115 at 1:320 and polyproline at 1:40, respectively. Peptide-specific antibodies could be isolated with >90% yield even after a two-cycle purification.

Immunoblotting

The peptide-specific sera were tested for their reactivity against EBNA-2 on immunoblots of SDS-extracted nuclear proteins from two EBNA-2-positive cell lines (B95-8 and Raji), two EBV-positive but EBNA-2-negative cell lines (P3HR-1 and Daudi) and two EBV-negative cell lines (Bjab and Ramos). Both the rabbit antisera directed against peptide 115 corresponding to the C terminus of the 1.6-kb open reading frame BYRF1 in the *Bam*HI H fragment and the antisera against peptide 116 deduced from an internal hyrophilic sequence within the same reading frame identified an EBNA-2-specific 87-kd polypeptide in the B95-8 line and an 86-kd polypeptide in the Raji line (Figure 3a,b). The reaction was blocked by adding the synthetic peptide in excess (Figure 3c). Both antisera reacted with a 135-kd non-EBV-specific polypeptide as well (Figure 3a,b). This reaction was not blocked by addition of the synthetic peptide in excess (Figure 3c). Since the reaction with this protein was seen with both anti-peptide sera, and was in no case peptide-specific, it is most likely due to a cross-reaction with the peptide carrier molecule. The antisera against peptide 115 also reacted with a 43-kd cellular polypeptide (Figure 3a). This reaction was blocked by adding the immunizing peptide (Figure 3c). None of these reactions were seen with pre-immune sera from the same rabbits (not shown). The antisera against the peptides number 139 and 140, deduced from the 1.1-kb open reading frame in the *Bam*HI W-Y fragment (BWRF12) did not give any EBV-specific staining (not shown).

The peptide-specific antibodies derived from EBNA-positive human sera were tested on immunoblots in a similar manner. Both the antiserum against peptide 115 and the serum against peptide 116 were specific for the 87 000 – 86 000 dalton EBNA-2-specific polypeptide on immunoblots (exemplified in Figure 3d). The reactions were inhibited by addition of an excess of peptide (not shown). The human polyproline antiserum did not react with any polypeptide in immunoblotting (not shown). The protein identified by antisera to synthetic peptides co-migrated with the EBNA-2-associated polypeptide previously described (Figure 3e) (Dillner *et al.*, 1985). This polypeptide co-migrated with the EBNA-2 polypeptide originally described by Hennessy and Kieff (1983) as shown with a reference serum (Dillner *et al.*, 1985).


a
B95-8
P3HR-1
RAJI
BJAB
200
116
92
66
45
135
87
86
c
B95-8
RAJI
RAMOS
200
116
92
66
45
135
b
B95-8
P3HR-1
RAJI
DAUDI
RAMOS
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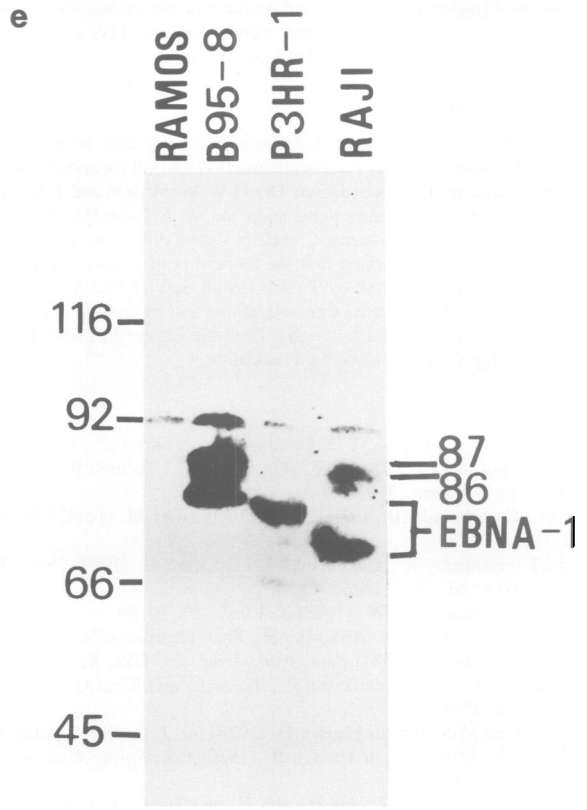

Fig. 3. Immunoblotting with antibodies against synthetic peptides. Raji and B95-8: EBV-carrying EBNA-2-positive cell lines; P3HR-1 and Daudi: EBV-positive but EBNA-2-negative cell lines that carry viral strains with deleted *Bam*HI WYH regions; Bjab and Ramos: EBV-negative cell lines. A nuclear residual pellet corresponding to 30 x 10^6 cells has been applied to each lane in **a**–**d**. In **e** 2 x 10^6 whole nuclei were applied to each lane. Figures on the left indicate the positions of the mol. wt. standards. **Blot a:** Rabbit affinity purified anti-peptide 116 serum, diluted 1:2. **Blot b:** Rabbit affinity purified anti-peptide 115 serum, diluted 1:2. **Blot c:** Same serum plus an excess of synthetic peptide 115. **Blot d:** Human serum affinity purified on a peptide 116 column, concentrated 5 ×. **Blot e:** Polyclonal human EBNA-positive serum, diluted 1:10.

Discussion

A second EBV-associated polypeptide has been detected by immunoblotting in the nuclear fraction of EBV-carrying, growth-transformed cells (Hennessy and Kieff, 1983; Sculley *et al.*, 1984; Dambaugh *et al.*, 1984). It could also be visualized by anti-complement immunofluorescence as a nuclear antigen (Dillner *et al.*, 1985). The antigen is not present in cells that carry EBV with a deleted *Bam*HI WYH region. Sculley *et al.* (1984) have studied the differential elution of EBNA-1 and EBNA-2 from nuclei. Both EBNA-1 and EBNA-2 were distributed in approximately equal amounts in the nuclei and the cytoplasm, and both antigens were detected in all fractions from the sequential salt extraction of nuclei. Our findings are not quite in line with this report. The difference may be due to the fact that Sculley *et al.* (1984) fractionated frozen cells, whereas we used living cells. Spelsberg *et al.* (1982) showed that EBNA was eluted from nuclei in two peaks, designated as class I and class II EBNA. EBNA was monitored by the complement fixation technique, which does not distinguish between EBNA-1 and EBNA-2. We could confirm this finding and have also shown that the two-peak elution is specifically valid for EBNA-1.

The 87 – 86 kd protein identified by the anti-peptide sera was identifed as EBNA-2 by the following criteria. (i) It was recognized in a peptide-specific fashion by two different human and rabbit antisera to synthetic peptides deduced from the DNA sequence of the EBNA-2-associated *Bam*HI WYH region. (ii) The subcellular localization of the protein was exclusively nuclear. (iii) The antigen is expressed in EBV-transformed, virus non-producer cells. The level of early antigen or viral capsid antigen expression of the Raji line was >0.01%. A major latently expressed mRNA has been mapped by the BYRF1 region (van Santen *et al.*, 1981). In contrast, no transcripts expressed during productive infection have been found which map to this region (Hummel and Kieff, 1982a, 1982b; reviewed by Kieff *et al.*, 1982). Furthermore, the protein is likely to correspond to the fluorescence-detected EBNA-2, which is exclusively nuclear and expressed in 100% of cells in latently infected cell lines containing intact *Bam*HI WYH regions (Dillner *et al.*, 1985). Notably, EBNA-2-positive sera also had high titers against the 86 – 87 kd protein. Using a reference serum we previously found that the 87 – 86 kd protein co-migrated with the 82 – 88 kd EBNA-2 polypeptide described by others in several latently infected cell lines (Hennessy and Kieff, 1983, 1985; Dambaugh *et al.*, 1984).

Human EBNA-positive sera were found that contained antibodies to all three BYRF1-derived peptides, indicating that epitopes of EBNA-2 are contained within the peptide sequences. Previously, we have found that human EBNA-positive sera regularly contain antibodies that react with a synthetic EBNA-1 peptide at approximately the same titers as the corresponding rabbit antisera (Dillner *et al.*, 1984). The human antibody titers against the EBNA-2 peptides were 20 – 100 times lower than the corresponding rabbit antisera. A regular presence of naturally occurring virus-specific anti-peptide antibodies of similar titer has been found in other systems (Tamura *et al.*, 1983). In spite of the low anti-peptide titer of the human sera, rabbit and human anti-peptide antibodies had a comparable reactivity against EBNA-2 on immunoblots. In the case of peptide 115 the reactivity of human sera appeared to be dependent on a specific conformation of the peptide, since it was abrogated by the substitution of a single amino acid in the peptide. Possibly, the cross-reaction between peptide 115 and EBNA-2 is due to a peptide configuration regularly recognized by the human, but only occasionally by the rabbit anti-115 antibodies.

The apparent 87-kd mol. wt. of EBNA-2 exceeds the maximum coding capacity of the BYRF1 reading frame which is 54 kd (Baer *et al.*, 1984). The BYRF1 reading frame was found to contain three possible splice acceptor sequences (Mount, 1982). Two are very close to the N terminus and one is immediately 3′ of the polyproline repeat. We did not find donor and acceptor sequences that would allow in-frame splicing within BYRF1. Although we did not succeed in staining the 87 000-dalton protein with human anti-polyproline sera, the EBV-specific reactivity of human sera to polyproline (Table I) suggests that the polyproline repeat is translated into protein. There is only one initiator codon (Kozak, 1981) in BYRF1 upstream of the polyproline repeat. If translation of EBNA-2 starts within BYRF1, the protein migrates anomalously slow on SDS gels. In view of the predicted 28% proline content of the protein, this is quite conceivable.

Our data thus show that a major part of EBNA-2 is encoded by BYRF1 and that the C terminus of EBNA-2 is at the 3′ end of BYRF1. The N terminus of EBNA-2 could be located at the initiator codon 5′ of the proline repeat or in another open reading frame further upstream.

A cDNA clone mapping to the *Bam*HI WYH fragments has been isolated from the EBNA-2-positive, latently infected Raji

cell line (Bodescot *et al.*, 1984). This transcript is extensively spliced and contains a long open reading frame assembled from several small open reading frames in the DNA sequence. The transcript ends at the poly(A) addition site 3′ of BYRF1. However, the long open reading frame ends shortly before BYRF1 and the transcript does not include the sequences corresponding to the EBNA-2-reactive peptide 116. Therefore, we conclude that two different mRNAs are transcribed from the *Bam*HI WYH region, one of which is translated into an 87 000-dalton nuclear antigen.

The availability of several monospecific human and rabbit synthetic peptide-derived antisera to the *Bam*HI WYH-coded EBNA-2 will be useful for the further characterization and isolation of this protein implicated to play an important role in the initiation of EBV transformation of normal human B-cells.

While this manuscript was in preparation Hennessy and Kieff (1985) arrived at similar conclusions, using a different approach. A hybrid protein between β-galactosidase and a part of the BYRF1 open reading frame, that includes the sequence of our peptide 116, was grown in bacteria, and used to immunize rabbits. Following pre-absorption with EBV-negative cells and β-galactosidase, the resulting serum was specific for the EBNA-2 polypeptide on immunoblots, showing that this part of BYRF1 codes for EBNA-2. An unpublished observation of S.Fennewald and E.Kieff was cited to show that the C terminus of EBNA-2 is positioned at the C terminus of BYRF1, the position of our peptide 115. Two human sera were found that reacted with the hybrid protein, in possible analogy with the reaction of human sera with our synthetic peptides.

Materials and methods

Cells

Cell lines were maintained on RPMI 1640 medium supplemented with 5% fetal calf serum, penicillin and streptomycin. The cultures were fed twice weekly. For origin of cell lines, see Ehlin-Henriksson and Klein (1984).

Production of synthetic peptides and anti-peptide antibodies

Peptides were synthesized and used for rabbit immunization as described (Sutcliffe *et al.*, 1980). Peptide-specific antibodies were isolated from the sera of immunized rabbits or from human EBNA-positive sera by affinity chromatography with AH-Sepharose (Pharmacia) coupled to the respective peptide. The column was washed with 50 column volumes of 20 mM Tris-HCl, pH 7.5, 2 M NaCl and bound antibodies were eluted with 3 M KSCN and dialyzed against 20 mM Tris-HCl, pH 7.5, 150 mM NaCl (PBS). The purification of the human anti-peptide antibodies was performed twice. In the case of rabbit anti-peptide 116 sera we used a combination of AH- and CH-Sepharose.

ELISA

Anti-peptide reactivity of sera was measured in a direct-binding ELISA test (Dillner *et al.*, 1984). The reaction was allowed to proceed for 12 h at 4°C. The titer was defined as the last dilution of serum to give an absorbance at 405 nm of 0.3 above background level (no serum).

Subcellular fractionation

2 x 10^8 cells were harvested 24 h after passage, washed twice in PBS and resuspended in 10 ml of 2% sucrose/10 mM Tris-HCl, pH 7.5/10 mM NaCl/3 mM $Mg(OAc)_2$/1 mM phenylmethylsulfonyl fluoride/0.7% NP-40 (nuclei isolation buffer). Cells were left on ice for 5 min, centrifuged and the nuclear pellet was washed in the same buffer without NP-40. After centrifugation the cells were sequentially extracted with nuclei isolation buffer without NP-40 containing 150 mM NaCl and the same buffer containing 400 mM NaCl. The pellet was further extracted with 3 M urea and finally the residual pellet was solubilized in electrophoresis sample buffer (Laemmli, 1970).

Gel electrophoresis and immunoblotting

Electrophoresis was performed on 7.5% polyacrylamide, 0.27% bisacrylamide (Figure 3a–d) or on 10% polyacrylamide, 0.27% bisacrylamide (Figure 2 and Figure 3e) gels as described (Laemmli, 1970). Whole nuclei (Figure 3e) were collected after the wash with nuclei isolation buffer without NP-40 and resuspended in electrophoresis sample buffer. A nuclear residual pellet (Figure 3a–d) was prepared as described in the previous paragraph. The proteins were transferred to nitrocellulose (Towbin *et al.*, 1977) and immunostained as described (Dillner *et al.*, 1984) except that the blocking and wash buffer was PBS supplemented with 6% (w/v) dry milk and 0.01% Antifoam A (Sigma).

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