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Prediction and demonstration of a novel Epstein-Barr virus nuclear antigen

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ABSTRACT

The protein sequence predicted by the Epstein Barr virus (EBV) BERF4 open reading frame includes a tetrapeptide, Lys-Arg-Pro-Arg (KRPR), shown for other proteins to be a component of a signal for rapid nuclear localization. A subgenomic fragment of EBV DNA containing BERF4 has been incorporated into an expression vector, transfected onto primate cells and the nuclear distribution of the resulting protein established by immunofluorescence using EBV positive human sera. These sera contained high titres of antibodies to a fusion protein, produced in E. coli, consisting of β-galactosidase and the C-terminal 167 amino acids of BERF4. Immunoaffinity purified antibodies reactive with the EBV component of the fusion show the molecular weight of this antigen in EBV immortalized B-cell lines to be about 160 kD. The demonstration that BERF4 contains an exon encoding a nuclear protein identifies a new EBNA gene (EBNA-6) and suggests that KRPR is a signal sequence common to a number of viral and cellular nuclear polypeptides which bind to nucleic acids and may therefore be of predictive value in identifying karyophilic proteins.

INTRODUCTION

Epstein Barr virus (EBV) has the ability to transform resting human B-lymphocytes in vitro into continuously growing lymphoblastoid cell lines (LCLs). The 176kbp EBV genome is generally maintained in these cells as multiple copies of an extrachromosomal circular episome. Viral gene expression from this is highly restricted and the cells are described as "immortalized" or "latently infected" by EBV (reviewed, ref. 1).

An EBV associated nuclear antigen complex (EBNA) was first demonstrated in latently infected cells by anti-complement immunofluorescence (2) and it has gradually emerged that viral gene expression in these cells is predominantly derived from a family of genes encoding nuclear proteins, all designated EBNAs (3, 4, 5). To date, only one other EBV protein (the latent membrane protein, LMP) has been demonstrated in LCLs (6). Thus it is generally assumed that the EBNAs play a major rôle in the processes of establishing and

maintaining the continuous growth of LCLs and also in controlling EBV gene expression in the proliferating cell population.

A group of relatively high molecular weight (68-200kD) nuclear antigens (EBNAs 1-4) has been revealed by immunoblotting protein extracts from latently infected cells with suitable EBV positive human sera (7, 8). Three of these antigens, EBNAs 1, 2 and 3, have been characterised and mapped on the EBV genome by DNA-mediated gene transfer experiments and using antibodies to synthetic peptides and fusion proteins (Fig. 1A). EBNA 1 maps to the BamHI K restriction fragment (9, 10), EBNA 2 (of which two variants, A and B, exist) is encoded by exons in the BamHI Y and H fragments (11) and EBNA 3 has been localized to the BamHI E region (4). EBNA 4 has not yet been definitively mapped but it probably is also localized to BamHI E (L. Rymo, pers. comm.). An additional nuclear protein with a variable molecular weight (41-70kD) has also been identified and designated EBNA-LP (3) or EBNA 5 (12). This is encoded by repeated exons mapping to the BamHI WY region. EBNA 1 transactivates a putative EBV origin of DNA replication and enhancer and is thought to be necessary for the maintenance of the extrachromosomal form of the EBV genome in latently infected cells (13). EBNA 2 probably plays a key function in the initiation of B-cell immortalization (14). The rôles of the remaining EBNAs, however, have still to be defined.

Here we report that the EBV BERF4 open reading frame (see ref. 15 and Fig. 1A and B) contains an exon encoding a further member of the high molecular weight family of EBNAs. The rationale for investigating this gene was originally based on results from an analysis and comparison of the amino acid sequences of known viral nuclear antigens. The short amino acid sequence Lys-Arg-Pro-Arg (KRPR), thought to be part of a signal necessary for the rapid transport to the nucleus of polyoma virus large T antigen (16) and adenovirus Ela proteins (17), is also present in EBNA 1 and both variants of EBNA 2. An analysis of other EBV open reading frames revealed KRPR also in BERF4. A further search of all the available computer data bases showed that this short sequence was not common and was apparently restricted in eukaryotes to proteins known (or suspected) to have a nuclear distribution. Cloned subgenomic fragments of EBV containing all or part of BERF4 have been used here to construct mammalian and bacterial expression vectors. These have been utilized to confirm that an EBNA, which we call EBNA 6, is encoded within an EBV fragment containing the BERF3 and 4 open reading frames.

MATERIALS AND METHODS

Plasmid construction

Plasmid pSVE3/4 was constructed by cloning the EBV SmaI fragment, which includes the open reading frames BERF3 and 4, from an EcoRI B clone of EBV DNA (24) into the SmaI site of the polylinker in the plasmid vector "Bluescribe" (Stratagene Inc.) (Fig. 1C). The EBV fragment, plus a few flanking base pairs, was then excised as an EcoRI/HindIII fragment and ligated to the EcoRI/HindIII fragment of pSV2CAT (25) which contains the SV40 early region origin and promoter.

The plasmid pUR290e_2 (Fig. 1C) was constructed by subcloning the 507bp e_2 fragment of EBV from pBR322 into the BamHI site of the β-galactosidase fusion vector pUR290 (18). Restriction enzyme digestions and ligations were performed under standard conditions and plasmid DNA for transfection was purified by caesium chloride gradient centrifugation.

Induction and purification of the β-galactosidase/BERF4 fusion protein (e_2-fusion)

Log phase cultures of the E.coli K12 strain TG2 (F', RecA, T. Gibson, pers. comm.) containing the plasmid pUR290e_2 or pUR290 were induced to synthesise a fusion protein or β-galactosidase by the addition of isopropyl thiogalactoside (IPTG, Sigma) and shaking gently at room temperature for four hours. (This decreased temperature was found to increase the stability of the fusion protein and four hours was found to be an optimum time for induction).

Protein extracts were prepared and partially purified on p-amino-phenyl-β-D-thiogalactoside (APTG) agarose columns (Sigma) as described by Koenen et al., 1985)(26). After dialysis against phosphate buffered saline (PBS), protein samples were divided into aliquots and stored at -20°C until used.

Cell culture and transfection

Cos7 cells (27) were grown on plastic dishes in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% foetal calf serum (fcs) and antibiotics. IB4 (28), B95-8 (29), Ramos (30), Raji (31), Daudi (32) and P3HR1 (33) cells were grown as suspension cultures in RPMI 1640 with 10% fcs and antibiotics. Cos7 cells were transfected with 10µg of pSVE3/4 DNA (or 10µg of sonicated salmon sperm DNA in controls) by the calcium phosphate precipitation procedure (19).

Sera and antibodies

EBV positive serum, RT, was from a normal healthy donor. EBV positive

serum, 062 (a gift from Professor M. Ng, Hong Kong) was from a patient with nasopharyngeal carcinoma. The anti-β-galactosidase murine monoclonal antibody was a gift from Dr. J. Burke, Sussex.

Antibodies reactive with the e_2-fusion product were eluted from a stained strip of fusion protein blotted onto nitrocellulose essentially as described previously (34, 35). Approximately 500μg of e_2-fusion protein was electrophoresed through a 7.5% SDS-polyacrylamide gel and electro-transferred to nitrocellulose. After probing with the RT serum and staining as for other western blots (see below) the bands corresponding to the fusion were excised. The specific antibodies were eluted from strips using 5 mM glycine-HCl, pH 2.3 (300μl per cm of nitrocellulose) and immediately neutralised by making the solution 50 mM with respect to Na_2HPO_4.

Immunofluorescence

Forty-eight hours post-transfection (see above) petri dishes containing adherent Cos7 cells were washed twice in PBS and fixed in cold methanol: acetone (1:1). Staining was carried out by a routine indirect technique using serum 062 and rabbit anti human Ig fluorescein conjugate (1:10) (Dakopatts, Mercia Brocades) as the second layer. A negative control serum was included with each batch.

Protein analysis

Both SDS-PAGE and western blotting experiments were performed as described previously (34, 36), with minor modifications. Nitrocellulose filters were blocked, and sera were diluted in 5% powdered milk in PBS-Tween. Filters were then incubated with first antibodies overnight at 4°C. Second antibodies used were: Goat anti-human Ig peroxidase conjugate (Nordic Immunology) and goat anti-mouse IgG peroxidase conjugate (Dakopatts, Mercia Brocades).

RESULTS

Identification of anti-BERF4 antisera

The recombinant plasmid, designated pUR290e_2 (Fig. 1C), which encodes a fusion protein consisting of β-galactosidase and 167 amino acids corresponding to the C-terminal end of BERF4, was constructed as described in the Materials and Methods section. The correct orientation of the inserted fragment and continuous open reading frame from β-galactosidase to BERF4 were confirmed by restriction mapping and sequencing through the BamHI junction (data not shown). The e_2-fusion protein was partially purified after extraction by substrate analogue affinity chromatography and was then used to

screen, by western immunoblotting, EBV positive human sera from a variety of sources. Only a small number (10-20%) of these sera clearly recognised the fusion protein on western blots. (A more detailed serological study is in progress and will be reported subsequently.) Two sera, RT (from a normal EBV seropositive individual) and 062 (from a patient with nasopharygneal carcinoma, NPC), were found to have particularly high titres of antibody to the EBV component of the fusion protein and these were therefore used in the remainder of the present study. Fig. 2A shows the partially purified protein separated by SDS-PAGE (10%) and stained with Coomassie blue dye (lane 1). Lane 2 shows β-galactosidase (encoded by the pUR290 vector with no insert) similarly separated and stained. These results show that in addition to a fusion product (f), of a size predicted from the complete amino acid sequence, there exists a second major species (f'), only slightly larger than the unmodified β-galactosidase (see also immunoblot in Fig. 2B). This presumably results from an inherent instability of the fusion in E.coli as has been reported for other hybrid polypeptides of this type (18). Fig. 2B (lane 1) shows the fusion products separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with an anti-β -galactosidase monoclonal antibody. The fusion protein similarly separated, transferred to nitrocellulose and probed with the human serum 062 is shown in Fig. 2B (lane 2). The β -galactosidase synthesised in E.coli carrying the pUR290 plasmid with no insert was similarly analysed as a control (Fig. 2B, lane 3). It can be seen that the antibodies in the human serum directed against the fusion protein are specific for the EBV component (that is the C-terminus of BERF4) and do not bind to the unmodified β -galactosidase.

DNA-mediated gene transfer of BERF4

A recombinant expression vector, designated pSVE3/4 (Fig. 1C), which encodes the EBV open reading frames BERF3 and 4 under the transcriptional control of the SV40 early promoter was constructed as described in the Materials and Methods section. Forty-eight hours after calcium phosphate transfection with pSVE3/4 DNA or salmon sperm DNA onto Cos7 cells, they were fixed and stained by indirect immunofluorescence (IF). The results show that in the cells transfected with pSVE3/4, IF using serum 062 produced only nuclear staining (Fig. 3A). The fluorescent staining had a distinctly punctate appearance and nucleoli were unstained. The observed perinuclear staining suggests a possible association with the nuclear envelope in addition to chromatin association. Cos7 cells transfected with salmon sperm

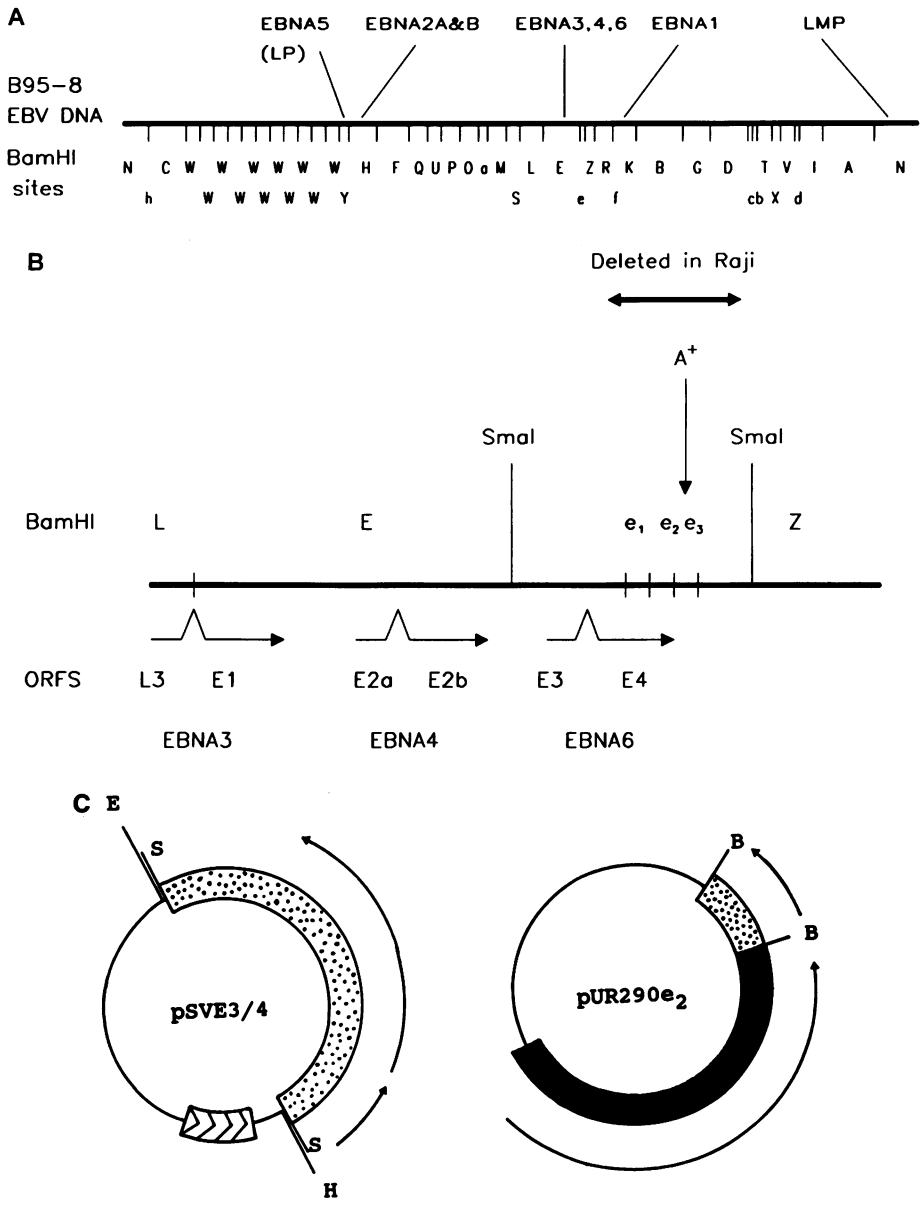

Figure 1:
EBV and plasmid restriction maps. (A) The physical map of the B95-8 strain of EBV DNA showing the BamHI restriction sites and location of the exons encoding the known or proposed latent proteins EBNAs 1-6 (EBNA 5 is also known as Leader Protein, LP) and latent membrane protein (LMP). (B) The

BamHI L - Z sub-region of EBV showing the six open reading frames (ORFs) probably encoding EBNA3, EBNA4 and EBNA6 (that is Bam L Right Frame 3, BLRF3, and Bam E Right Frames, BERF, 1, 2a, 2b, 3 and 4; see ref. 15 and text). Also shown are the polyadenylation site (A+) at the 3' end of BERF4, the Sma I restriction fragment present in pSVE3/4 (see below) and the region deleted from Raji EBV DNA (20). (C) Structure of the recombinants pSVE3/4 and pUR290e$_2$: ⊡ represents the EBV DNA sequences encompassed in a SmaI fragment in pSVE 3/4 and the Bam HIe$_2$ fragment in pUR290e$_2$; ⊠ represents the SV40 early region derived from pSV2CAT, the direction of transcription being indicated by > signs; ■ the β -galactosidase gene. The thin lines represent other sequences derived from pSV2CAT and pUR290, respectively. Arrows indicate the direction of open reading frames. Restriction enzyme sites are E(EcoRI), H(Hind III), B (BamHI), S(SmaI); see text for details of construction.

DNA gave no fluorescent staining above background levels (Fig. 3B) and an EBV negative serum elicited no fluorescence (not shown). The frequency of stained cells (app. 0.5-1%) was of the order expected from the transfection protocol used (our unpublished observations; ref. 19). Transient expression of EBNA6 in Cos7 cells did not produce sufficient protein for western immunoblot analysis. Further, attempts to establish cell lines stably expressing this antigen have not so far been successful. Preliminary experiments with rodent fibroblasts (Balbc 3T3) suggest that high level expression of EBNA6 was toxic to the cells.

EBNA6 in EBV infected B-cells

Both sera 062 and RT were used to probe western immunoblots of EBV infected B-cells. Each serum recognises, in addition to EBNAs 1 and 2, a high molecular weight doublet in EBV positive IB4 cells (Fig. 3C, lane 1 [*]; Fig. 4B, lane 1) and a similar doublet in virus producing B95-8 cells (Fig. 4A, lane 4) but not in the EBV negative B-cell line Ramos (Figs. 3C lane 2 and 4A lane 3). To determine whether one or both of these species corresponds to the nuclear antigen demonstrated by gene transfer, monospecific antibodies were required. As neither serum was available in large quantities and no monospecific animal sera are available, a microimmunoaffinity purification technique was employed to produce antibodies specific against the BERF4 encoded protein (see Materials and Methods and Fig. 4A, lane 1). These antibodies were then used to probe a western immunoblot of IB4. The data show that the antibodies recognise a protein species of approximately 160kD in the tightly latent IB4 cell line (Fig. 4A, lane 2). This corresponds to one of the proteins in the high molecular weight group of EBNAs normally recognised by RT and 062 sera in the virus producer line, B95-8 (Fig. 4A, lane 4) as well as in IB4 (Fig. 3C, lane 1 and Fig. 4B, lane 1); this species is absent from the EBV negative line Ramos (Fig. 3C, lane 2

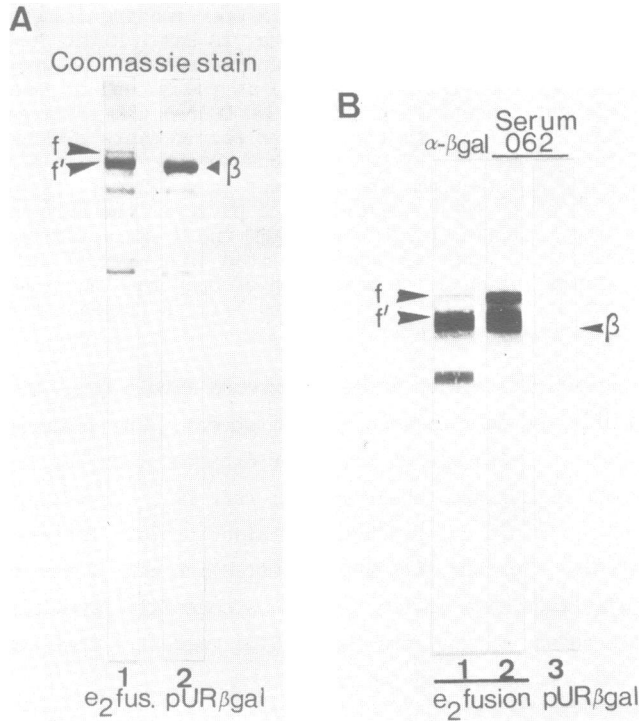

Figure 2:
e_2-β-galactosidase fusion protein. (A) Coomassie blue dye stained 10% SDS-polyacrylamide gel showing partially purified e_2-fusion protein (lane 1) and unmodified β-galactosidase (lane 2). (B) e_2-fusion protein (lanes 1 and 2) and β-galactosidase (lane 3) separated on 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose. The blots were probed with an anti-β-galactosidase murine monoclonal antibody (lane 1) and the EBV positive human serum 062 diluted 1/100 (lanes 2 and 3). f indicates the position of the largest species of e_2-fusion protein and f' the major product probably resulting from proteolytic degradation; β shows the position of unmodified β-galactosidase.

and Fig. 4A, lane 3). We have designated this antigen EBNA 6. The affinity purified antibodies also bind to a protein band 200kD (Fig. 4A, lane 2). This corresponds to a similar band revealed using the polyclonal RT serum (Fig. 4B, lane 1) and may be a complex related to the 160kD species.

Serum RT was also used to immunoblot three other EBV infected B-cell lines, each derived from a Burkitt's lymphoma. High molecular weight, EBV specific proteins were detected in all three lines (Fig. 4B, lanes 2, 3, 4). These show virus strain specific size variation as has been found for most of the EBV nuclear proteins (8, 10, 11, 12, 34, and see EBNA1 in Fig. 4B). In

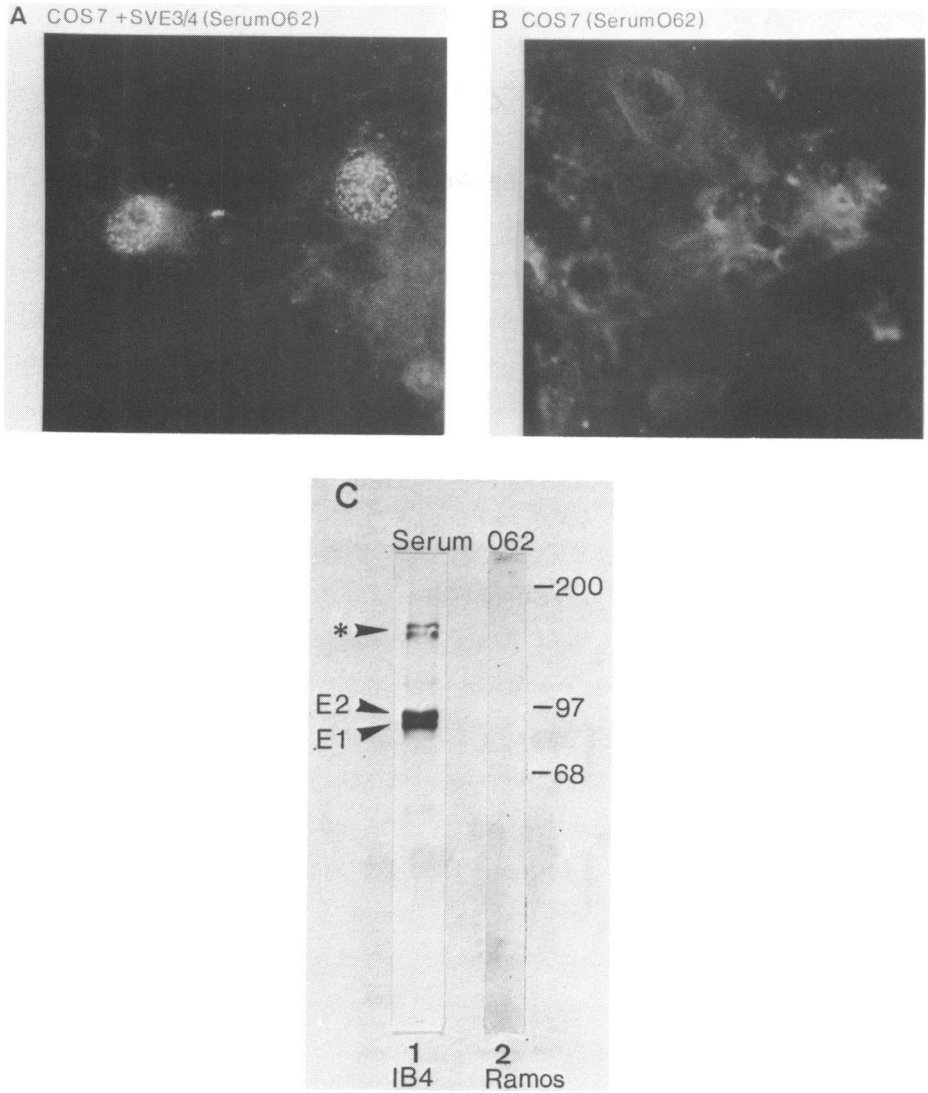

Figure 3:
Expression of BERF4/EBNA6. (A) Transient expression of EBNA6 in Cos7 cells after transfection with pSVE3/4. Cells were stained by indirect immunofluorescence using EBV positive human serum 062. (B) Cells transfected with salmon sperm DNA and similarly stained. (C) SDS-solubilised proteins from the EBV positive B-cell line, IB4 (lane 1) and EBV negative Ramos separated on a 5-15% SDS- polyacrylamide gel, transferred to nitrocellulose and probed with serum 062. EBNAs (E) 1 and 2 are indicated (arrow). Putative EBNA6 species are indicated (*).

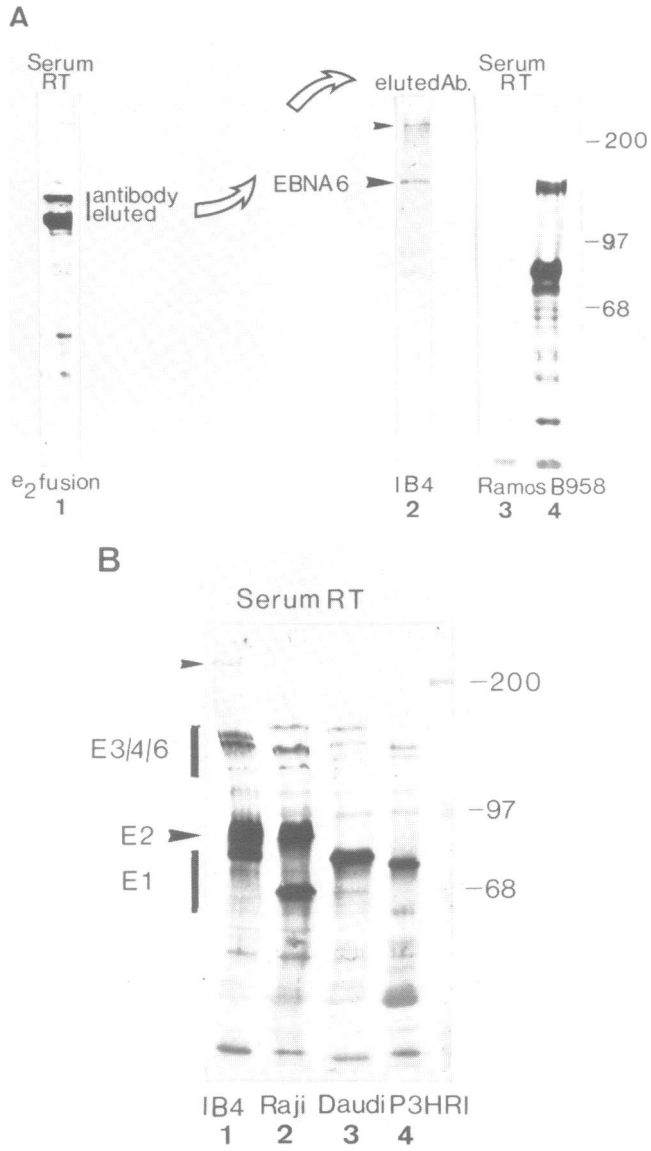

Figure 4:
Demonstration of EBNA6 in B-cell lines. (A) Partially purified e_2-fusion protein separated on a 7.5% SDS-polyacrylamide gel was transferred to nitrocellulose and probed with serum RT diluted 1/50 (lane 1). Antibodies were eluted from the immobilized fusion as shown (details in text) and used to probe an IB4 extract similarly separated and blotted onto nitrocellulose (lane 2). Extracts of EBV negative Ramos (lane 3) and the virus producer line B95-8 (lane 4) were similarly separated, transferred to nitrocellulose and probed with RT serum diluted 1/50 as controls. (B) Extracts from EBV

positive lines IB4 (lane 1), Raji (lane 2), Daudi (lane 3), P3HR1 (lane 4), separated on a 7.5% gel, transferred to nitrocellulose and similarly probed with RT serum. The position of EBNAs (E) 1, 2, 3, 4 and 6 are indicated and the relative mobilities of molecular weight standards (BRL, prestained) are shown in kD.

each line there appears to be a doublet or triplet between approximately 150-180kD. We assume that in addition to EBNA6 these bands correspond to EBNAs 3 and 4 (4, 8 and L. Rymo, pers. comm.). Definitive identification of the relative mobility of EBNA6 in each line will require larger quantities of monospecific sera; these are in preparation. The presence of this group of proteins in the Raji cell line (Fig. 4B, lane 2) was unexpected. A deletion in Raji virus DNA (20, P. Farrell pers. comm, and Fig. 1B) removes most of the BERF4 open reading frame and the polyadenylation signal also thought to be used in the expression of BERF2b. However, three EBV specific proteins (150-180kD) appear in the immunoblotted extract probed with RT serum (Fig. 4B, lane 2) and also serum 062 (not shown). The sequence analysis suggests that an EBNA gene is deleted in Raji (Fig. 1B) yet the cells apparently produce a full complement of these latent proteins. Monospecific reagents, when available, should resolve this paradox.

DISCUSSION

The EBNA family

We have demonstrated here that the polypeptide encoded by the EBV BERF4 open reading frame is a nuclear protein, that it is synthesised in the tightly latent lymphoblastoid cell line, IB4, as well as the virus producer line B95-8 and is recognised by some EBNA positive human sera. It is absent from the EBV negative B-cell line Ramos. By previously accepted criteria (see ref. 8) this identifies the antigen as an "EBNA". Thus the number of EBV encoded nuclear antigens found in latent LCLs is now six. Further studies, using mammalian expression vectors, are in progress to determine the effect of EBNA6 on different cell types and its rôle in cellular immortalization and EBV latency.

This investigation was originally stimulated by a comparison of amino acid sequences common to known viral nuclear antigens (Table I). However, two other observations subsequently supported our view that BERF4 would encode an EBNA. First, a cDNA clone was isolated which includes part of a continuous open reading frame produced by splicing BERF3 to BERF4 and this was found to have a 5' leader sequence (comprised of exons from BamHI W and Y) similar to

Table 1: Nuclear Localisation Signals - confirmed or predicted

Protein	Sequence	Nuclear	ANA†	Ref
Viral				
Polyoma Large T Ag	PVSRKRPRPAG	+	+	16
Adenovirus 5/2 E1a	NLSCKRPRP-COOH	+	-	17
Adenovirus 12 E1a	DLSVKRPRCN-COOH	+	-	37
Adenovirus 2 polypeptide VI precursor	GRGEKRPRPDR	+	?	38
EBNA1	GRGEKRPRSPS	+	+	39
EBNA2A	QGPSKRPRPSIQ-COOH	+	+	39
EBNA2B	GGPSKRPRTSTQ-COOH	+	+	39
BERF4/EBNA6	ATTPKRPRVEE	+	+	8,15
HIV2 env polyprotein	QPINKRPRQAW	?	?	40
HSV1 DNA polymerase	PSPAKRPRETP	+	+	41
HSV1/2 exonuclease	DSPPKRPRPNS	+	+	42
VZV orf 59	KKTRKRPRGLP	?	?	43
Eukaryotic-Homeotic Proteins				
Drosophila engrailed	TNDEKRPRTAF	+	+	44
Drosophila inverted	SPEDKRPRTAF	+	+	44
Honeybee E60	RPEEKRPRTAF	+	+	44
Honeybee E30	GPEEKRPRTAF	+	+	44
Mouse En1	KEDKRPRTAF	+	+	44
Mouse En2	KEDKRPRTAF	+	+	44
Eukaryotic-Other Proteins				
30S Ribosomal protein S8	KNKQKRPRSRT	+	+	45
Prokaryotic				
Gene 278 protein filamentous phage Pf3	EPGAKRPRGDR	N/A‡	+	46
Plasmids R100 and R1 RepA4 protein	LITEKRPRPGP	N/A	+	47
Phage 434 Cro protein	AGVKRPRFLF	N/A	+	48

† Associated with Nucleic Acids. ‡ Not applicable

those in mRNAs for EBNAs 1 and 2 (21). Secondly, BERF4 appears to be the sixth of a group of tandemly arranged open reading frames in the EBV genome (see Fig. 1B). Analysis of further cDNA clones has revealed that another pair, BLRF3 and BERF1, can be spliced together (5) and it has recently been shown that BERF1 encodes a large part of the protein designated EBNA3 (4).

Thus, it would appear that not only are all the EBNA molecules related by their nuclear distribution but they may also be linked, in LCLs, as a gene

family in which the coding exons for the different proteins are differentially spliced from very large primary transcripts.

EBNA Nomenclature

Because the EBNAs have been identified and characterised independently in different laboratories over several years, their nomenclature has not been systematised. The exons encoding EBNAs 1 (from BamHIK), 2A and B (from BamHI Y and H) and 3 (from BamHI L and E) have been identified and clearly defined and there is general agreement with respect to these. Another high molecular weight protein seen on western blots is probably the predicted EBNA 4 encoded by BERF2a/b; a cloned fragment of the EBV genome containing the BERF2a/b open reading frames has been shown by experiments similar to those described here to encode a nuclear antigen (L. Rymo, pers. comm.). The nuclear antigen encoded by the BamHI WY repeated exons has variously been called EBNA leader protein (EBNA-LP) (3), EBNA5 (12) and EBNA IV (22). Here we have identified the product of the open reading frame BERF4 as a nuclear antigen, which to avoid further confusion, we have designated EBNA6.

KRPR and nuclear localization

It has been established that KRPR(P) can be a signal sequence which, if it is located at the C-terminal end of a polypeptide (for example, in adenovirus Ela or a recombinant galactokinase), can promote rapid transport of that protein into the nucleus. A search of all the predicted open reading frames of EBV (15) revealed that KRPR is also present in three known EBV nuclear antigens (EBNA1, EBNA2A and EBNA2B). In this report we have shown that the other EBV reading frame containing this sequence (BERF4) also encodes a nuclear antigen. A more extensive search of the available protein sequences (PIR database release 13) reveals that this short sequence is not common (22 proteins only identified) and that 17 of 19 eukaryotic proteins which include it are known to have a nuclear distribution. Most (14/19) are also known or suspected to bind to nucleic acids, as are the three prokaryotic proteins containing KRPR (see Table I). KRPR is clearly not an absolute requirement for nuclear localization because many known nuclear proteins (including EBNA3) do not include this sequence (for review, see ref. 23). Also there is no definitive proof that this signal is always involved in nuclear localization; for instance, in addition to the sequence, its location in the polypeptide may be critical or it may only be recognised by factors in certain cell types (17). However, this sequence has been useful in identifying a nuclear antigen and the observation that 17 of 19 proteins

containing it are nuclear provides circumstantial but very persuasive evidence that it may have some predictive value, as shown here.

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