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Identification of the coding region for a second Epstein-Barr virus nuclear antigen (EBNA 2) by transfection of cloned DNA fragments

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Cell lines were established by co-transfection of cloned M-ABA Epstein-Barr virus (EBV) DNA fragments with plasmids conferring resistance to dominant selective markers. A baby hamster kidney cell line carrying the *Hind*III-I1 fragment exhibits a nuclear antigen of 82 000 daltons, serologically defined as EBV-determined nuclear antigen (EBNA) 1. Furthermore, a Rat-1 cell line transfected with DNA of the clone pM 780-28 containing three large internal repeats (*Bgl*II-U) and the adjacent *Bgl*II-C fragment expresses a nuclear antigen of 82 000 daltons which can be visualized only by a subset of anti EBNA-positive human sera. Sera recognizing the 82 000-dalton protein of the transfected cell line reacted with a protein of the same size in the non-producer line Raji, designated as EBNA 2. Conversely, sera without reactivity to the 82 000-dalton protein failed to react with EBNA 2 of Raji cells. P3HR-1 and Daudi cells with large deletions in *Bgl*II-U and -C are devoid of EBNA 2. The data presented provide evidence that a second EBNA protein is encoded by the region of the EBV genome which is deleted in the non-transforming P3HR-1 strain.

Key words: EBV-specific nuclear antigen (EBNA)/gene mapping/transfection/immunoblotting

Introduction

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is also associated with two human malignant tumors, Burkitt's lymphoma and nasopharyngeal carcinoma (for review, see Epstein and Achong, 1979). Furthermore, EBV has the ability to immortalize human B-lymphocytes by establishing a latent infection in which the majority of the viral genes is not expressed (Henle *et al.*, 1967). Immortalized B-cells, as well as tumor cells harbouring EBV genomes, express an EBV-associated nuclear antigen (EBNA) which can be detected by anti-complementary immunofluorescence (ACIF; Reedman and Klein, 1973). EBNA or EBNA-associated proteins are believed to play an important role in immortalization and maintenance of latency (Epstein and Achong, 1979). The analysis of the polypeptides serologically defined as EBNA progressed with the application of the immunoblotting technique. Strnad *et al.* (1984) were the first to demonstrate that EBNA (now designated EBNA 1) exhibits variations in the mol. wt. which are virus strain-specific. By transfection of cloned EBV DNA fragments, this protein was shown to be coded for by *Bam*HI K (Summers *et al.*, 1982). The differences in the mol. wt. of EBNA 1 in different virus isolates could be assigned to different numbers of repeats present in the coding region of this protein (Hennessy *et al.*, 1983; Fischer *et al.*, 1984).

Suggestive evidence for the existence of another EBNA protein with a mol. wt. of 81 000 was provided by Strnad *et al.* (1981) in their original report. Recently, Hennessy and Kieff (1983) confirmed that this is a viral antigen, which can be visualized in Raji cells only by some of the sera with anti-EBNA specificity. This EBNA 2 protein has not yet been assigned unequivocally to a region of the EBV genome. Based on transfection experiments Hennessy and Kieff (1983) suggested that the EBNA 2 protein might be encoded by sequences located at the right hand end of the genome, while, based on DNA sequence data and analysis of transcripts in latently infected cells, Fennewald *et al.* (1984) proposed that EBNA 2 might be coded for by the large internal repeats and the adjacent part of the long unique region.

Here we provide direct evidence using transfection of cloned EBV DNA fragments into recipient cells that EBNA 2 is encoded by sequences encompassing the large internal repeats and the adjacent part of the long unique region. This protein is absent from P3HR-1 and Daudi cells consistent with the fact that these viruses have large deletions at the boundary from the large internal repeats to the long unique region.

Results

A map showing the structural organization of M-ABA virus DNA and describing the origin of pM 780-28 and pM *Hind*III-I1 is given in Figure 1.

Characterization of EBNA-associated proteins

For the analysis of EBNA-associated proteins in various EBV genomes carrying cell lines, the cells were harvested in the logarithmic growth phase and the proteins analyzed by immunoblotting as described in Materials and methods. The analysis of the non-producer line Raji carrying the EBV genome with a series of EBNA-positive human sera revealed that one class of sera recognized one protein with a mol. wt. of 72 000 while another set of sera reacted with an additional protein of 82 000 daltons. Furthermore, some minor components with mol. wts. of ~ 60 000, 48 000 and 45 000 were identified by several sera (Figure 2, and data not shown). The results obtained with two representative sera are shown in Figure 2. The antibody titers against EBV antigens of these sera are listed in Table I.

None of the proteins detected by the EBNA-positive sera could be visualized by anti-EBNA-negative, anti-early antigen(EA)-positive human sera (Figure 2c) or in the lymphoma cell line BJA-B lacking the EBV genome (Figure 2a and b). Since the spontaneous rate of EA induction was very low (<0.1% of the cells), we conclude that the two major proteins identified in Raji cells with mol. wts. of 72 000 and 82 000 are EBNA proteins. This is in agreement with and confirms the observations made by Strnad *et al.* (1981) and Hennessy and Kieff (1983). Follow-

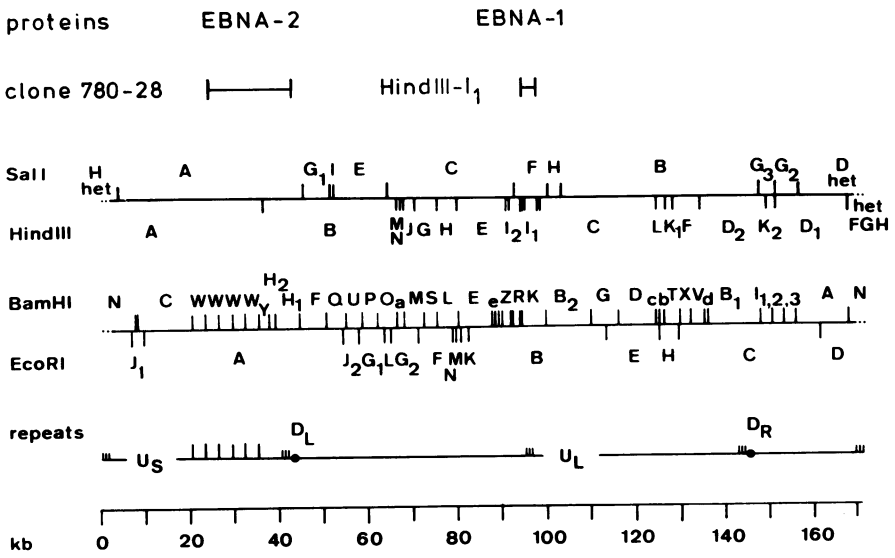

Fig. 1. Structural organization of the M-ABA EBV genome including the restriction sites for *Bam*HI, *Eco*RI, *Hind*III and *Sal*I. U_S and U_L: short and long unique regions. D_L and D_R: left and right duplicated region. The cloned fragment pM 780-28 contains three large internal repeats (*Bgl*II-U) and the adjacent *Bgl*II-C fragment (Polack *et al.*, 1984). The position of the cloned fragments pM 780-28 and pM *Hind*III-I1 used in this study is shown.

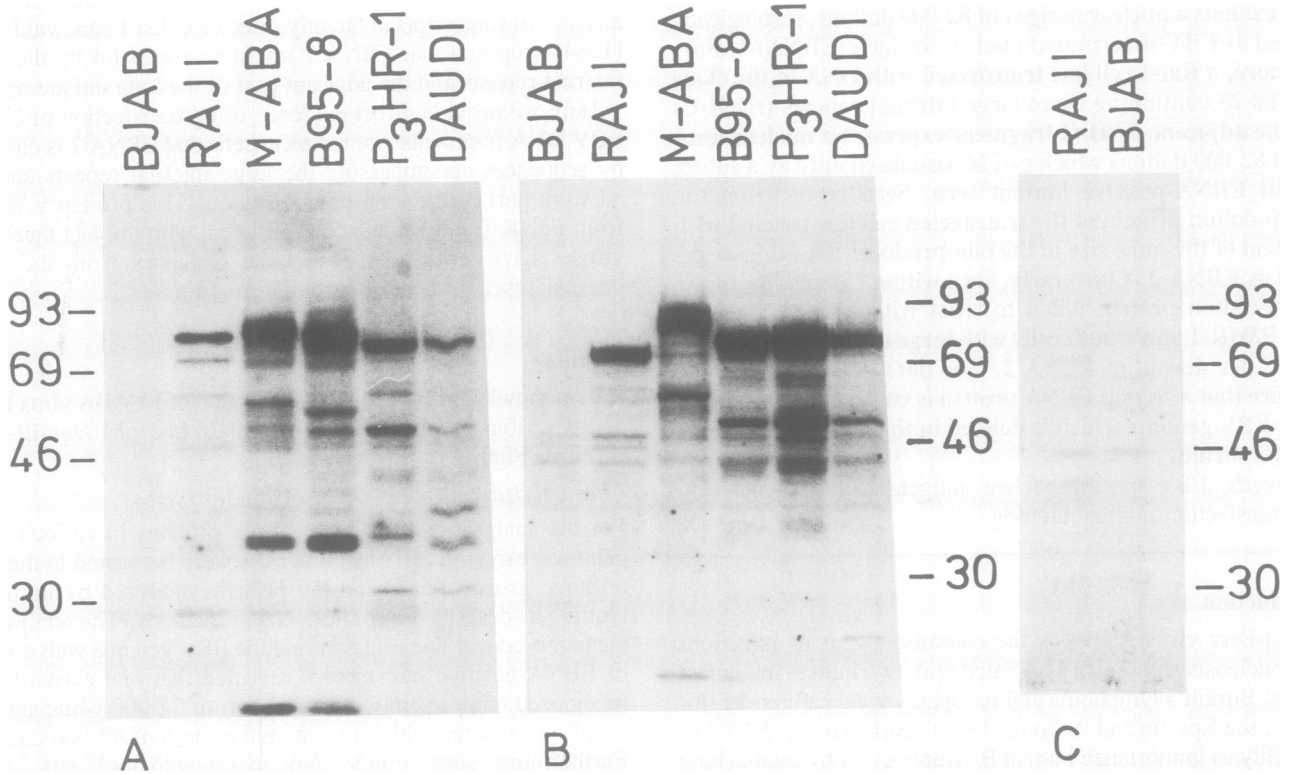

Fig. 2. Protein analysis by immunoblotting of EBV genome negative (BJA-B) and positive lymphoid cell lines (Raji, M-ABA, B95-8, P3HR-1 and Daudi) using an anti-EBNA 1 and -EBNA 2-positive serum (LyBe 83) shown in **(A)**, an anti-EBNA 1-positive, anti-EBNA 2-negative serum (no. 198) shown in **(B)**, and an anti-VCA- and anti-EA-positive, anti-EBNA-negative human serum (no. 5662) shown in **(C)**. The antibody titers of the sera against the various EBV-specific antigens are listed in Table I. The mol. wts. are given in kd.

ing the nomenclature of Hennessy and Kieff (1983) the 72 000 dalton protein is designated as EBNA 1 and the 82 000 dalton protein as EBNA 2. The analysis of the EBNA proteins in the virus producer lines M-ABA, B95-8, P3HR-1 and Daudi demonstrated that EBNA 1 varies in size between 72 000 daltons in Raji and 85 000 daltons in M-ABA cells, thus confirming and extending the earlier observation of Strnad *et al.* (1981), Hennessy *et al.* (1983) and Fisher *et al.* (1984).

The analysis of EBNA 2 in virus producer cell lines was more difficult than in Raji cells because all sera with anti-EBNA 2 specificity also had antibodies reacting against EA and virus capsid antigen (VCA) which revealed additional bands besides EBNA 2. In M-ABA and B95-8 cells, proteins of a mol. wt. of 80 000 −85 000 were recognized by the sera with high antibody titers against EBNA 2. Remarkably, sera with EBNA 2 specificity failed to react with proteins of this size in P3HR-1 and Daudi

cells. The additional bands visualized in the virus producer lines probably represent proteins induced in the lytic cycle which belong to the EA and VCA complex. Since a detailed analysis of the proteins of induced cells by immunoblotting is still lacking, the EA protein pattern seen by immunoprecipitation cannot be correlated yet to that observed by immunoblotting.

A cell line expressing M-ABA (EBV) EBNA 1

To be able to dissect the immune response against both EBNA proteins and to characterize both proteins separately, we attempted to establish cell lines which carry and express the genes for EBNA 1 and EBNA 2, respectively. EBNA 1 has been assigned to a 2.9-kb fragment overlapping between *Bam*HI-K and *Hind*III-I1 by Summers *et al.* (1982) and Fischer *et al.* (1984). To establish a control line expressing EBNA 1, DNA of the M-MABA EBV *Hind*III-I1 clone was co-transfected into baby hamster kidney (BHK) cells with the AG 60 plasmid conferring resistance to G-418. The transfected cells were grown in selective medium and then subjected to single cell cloning. ACIF revealed nuclear staining in >90% of the cell clones, which was only obtained with anti-EBNA-positive (Figure 3A) and not with anti-EBNA-negative human sera (data not shown). Southern blot analysis confirmed the presence of the M-ABA *Hind*III-I1 fragment of 3.5 kb in this cell line (Figure 4).

Characterization of the proteins showed that the BHK cells carrying *Hind*III-I1 express a protein with a mol. wt. of 82 000, which is only visualized by human sera with anti-EBNA antibodies (Figure 5A). Comparison of the EBNA protein in the transfected cell line with that of M-ABA cells revealed a slight difference in the mol. wt. (82 000 *versus* 85 000). Since a size heterogeneity in the cloned DNA fragment has been observed, this difference may reflect differences in the number of repeat units obtained upon cloning. In addition a set of 1–4 EBV-associated proteins could be recognized in *Hind*III-I1-transfected BHK cells using various anti-EBNA-positive human sera (Figure 5). The nature of these additional bands is going to be evaluated by further investigation.

A cell line expressing M-ABA (EBV) EBNA 2

In an attempt to map viral gene functions we have transfected the set of overlapping cosmid clones of M-ABA (EBV) (Polack

Table I. Antibody titers against EBV-specific antigens of the sera used for the experiments shown in Figures 2, 3 and 5

Sera	VCA[a]	EA[a]	EBNA[b]
LyBe (83)	640	640	320
198(84)	512	–	512
5662(84)	16 000	2000	16

[a]Determined by indirect immunofluorescence.
[b]Determined by ACIF.

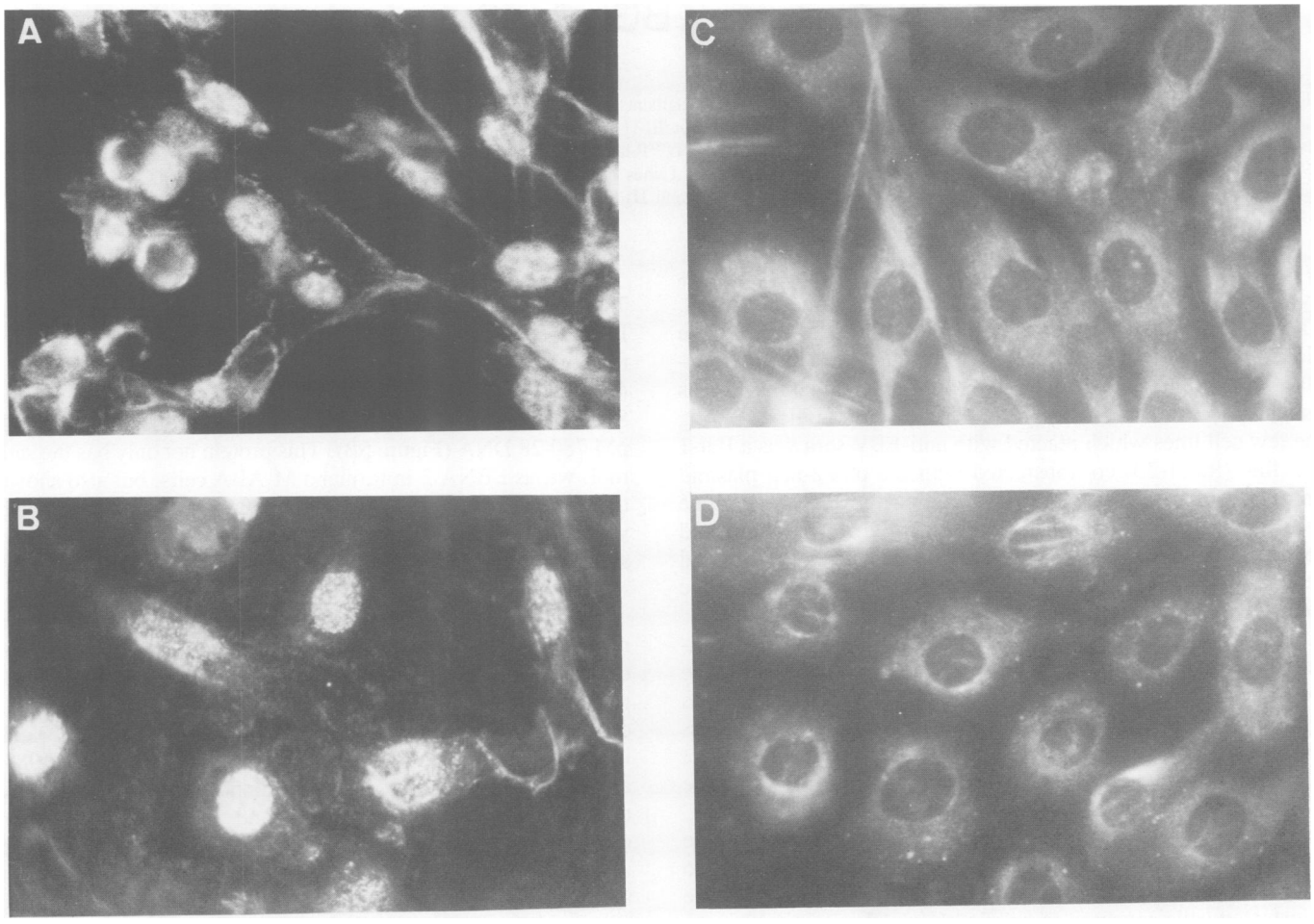

Fig. 3. Anti-complementary immunofluorescence analysis of BHK cells stably transfected by pM *Hind*III-I1 expressing EBNA 1 stained with serum 198 (84; **A**) and of Rat-1 cells carrying pM 780-28 and expressing EBNA 2 stained with serum LyBe (83; **B**) or with the anti-EA-R monoclonal antibody (**C**). Untransfected Rat-1 cells stained with serum LyBe (83) are shown in (**D**). The staining pattern of EBNA 2 was always more heterogeneous than that of EBNA 1. Sera are listed in Table I.

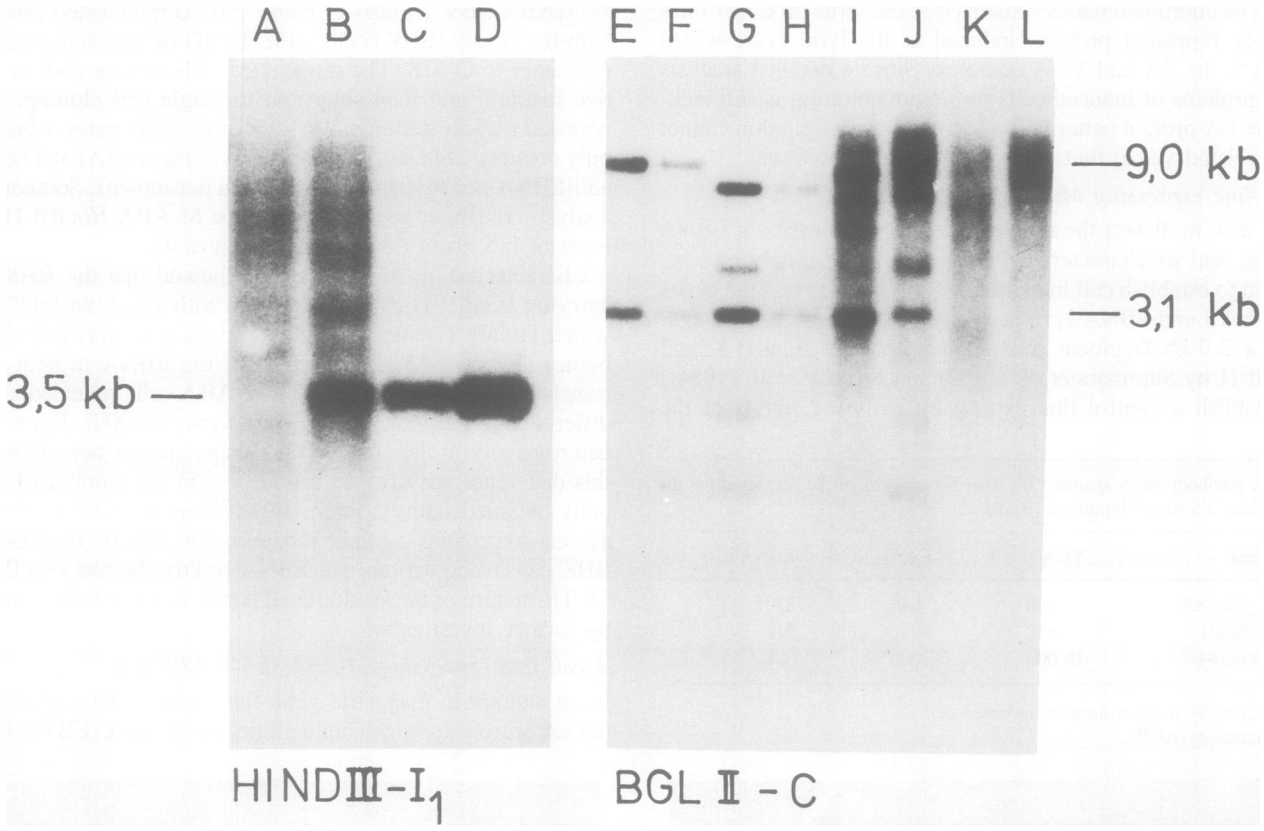

Fig. 4. Hybridization of nitrocellulose filters containing separated *Hind*III fragments of BHK cells carrying the AG 60 plasmid **(lane A)** and BHK cells carrying DNA of pAG 60 and pM *Hind*III-I1 **(lane B)** to the ^{32}P-labeled *Hind*III-I1 fragment. **Lanes C** and **D** show reconstitutions with one and five copies, respectively, of *Hind*III-digested pM *Hind*III-I1 DNA per cell. In **lanes E – L** ^{32}P-labeled *Bgl*II-C was hybridized to DNA of Rat-1 cells stably transfected by pM 780-28 and pSV2-neo digested with *Bgl*II **(lane I)** and *Bam*HI **(lane J)**. **Lanes K** and **L** contain *Bgl*II- **(K)** and *Bam*HI- **(L)** digested DNA of untransfected control rat cells. **Lanes E – G** show reconstitutions of one **(F** and **H)** and five **(E** and **G)** copies of *Bgl*II- **(E** and **F)** and *Bam*HI- **(G** and **H)** digested pM 780-28 DNA per cell.

et al., 1984) into primary rodent cells as well as into cell lines by co-transfection with a plasmid conferring resistance to G-418. The lines carrying EBV DNA fragments (two out of three) were cloned and then stained by indirect as well as anti-complementary immunofluorescence for the expression of viral antigens using a panel of human sera with different specificities. Among the few cell lines which reacted with anti-EBV sera was a Rat-1 cell line (Rat 1-20) co-transfected with the pSV2-neo plasmid and DNA of the M-ABA (EBV) clone pM 780-28. This clone was generated by partial digestion of cM Sal-A with *Bgl*II and carries three *Bgl*II-U fragments (three complete large internal repeats) and the adjacent *Bgl*II-C fragment which spans into the long unique region (Polack *et al.*, 1984).

ACIF revealed that only few human sera from an anti-EBNA-positive serum collection exhibited a nuclear staining in the majority of cells (Figure 3B). The staining pattern of the cells was more heterogeneous than that observed in *Hind*III-I1-transfected BHK cells (compare Figure 3A and B). Southern blot analysis confirmed that the cells indeed contain the DNA of the EBV DNA clone (Figure 4, lanes I and J). The labeled *Bgl*II-C fragment used for hybridization contains about two thirds of one large internal repeat unit and thus also hybridized efficiently to the 3.1-kb large internal repeat. The *Bgl*II-C fragment from the transfected cells appeared to be slightly smaller in size than the original fragment. The reason for this is not clear and may concern the presence of the *Not*I repeat cluster in this fragment (Freese *et al.*, 1983).

Sera found positive for EBNA on rat cells transfected by pM 780-28 (Rat 1-20) were negative on control cells without the EBV DNA fragment and reacted strongly with the 82 000-dalton protein of Raji, M-ABA and B95-8 cells identified as EBNA 2 (Figure 2). Furthermore, these sera recognized a protein of 82 000 daltons on immunoblots of Rat 1-20 cells carrying the pM 780-28 DNA (Figure 5B). This protein not only has the same mol. wt. as EBNA 2 in Raji and M-ABA cells, but also showed the same pattern of reactivity with human sera. It reacted strongly with sera with anti-EBNA 2 reactivity and failed to react with sera lacking anti-EBNA antibodies (data not shown) or containing only antibodies specific for EBNA 1 (Figure 5).

The clone pM 780-28 overlaps with *Bam*HI-H. *Bam*HI-H was reported to code for a 85 000-dalton protein of the early antigen complex (EA-R) by Glaser *et al.* (1983) which reacts with a monoclonal antibody described by Pearson *et al.* (1983). To exclude the possibility that the protein expressed in Rat 1-20 cells is the EA-R component described by Glaser *et al.* (1983), acetone-fixed Rat 1-20 cells were stained with the monoclonal antibody by anti-complementary and indirect immunofluorescence. As shown in Figure 3C no nuclear staining was observed with this antibody, while the cytoplasm of Rat 1-20 as well as untransfected Rat-1 cells exhibited an unspecific fluorescence (Figure 3C and D). In TPA-induced Raji and M-ABA cells intense cytoplasmic staining was observed (data not shown). By immunoblotting this antibody did not react with the 82 000-dalton protein from Rat 1-20 and uninduced Raji cells (data not shown).

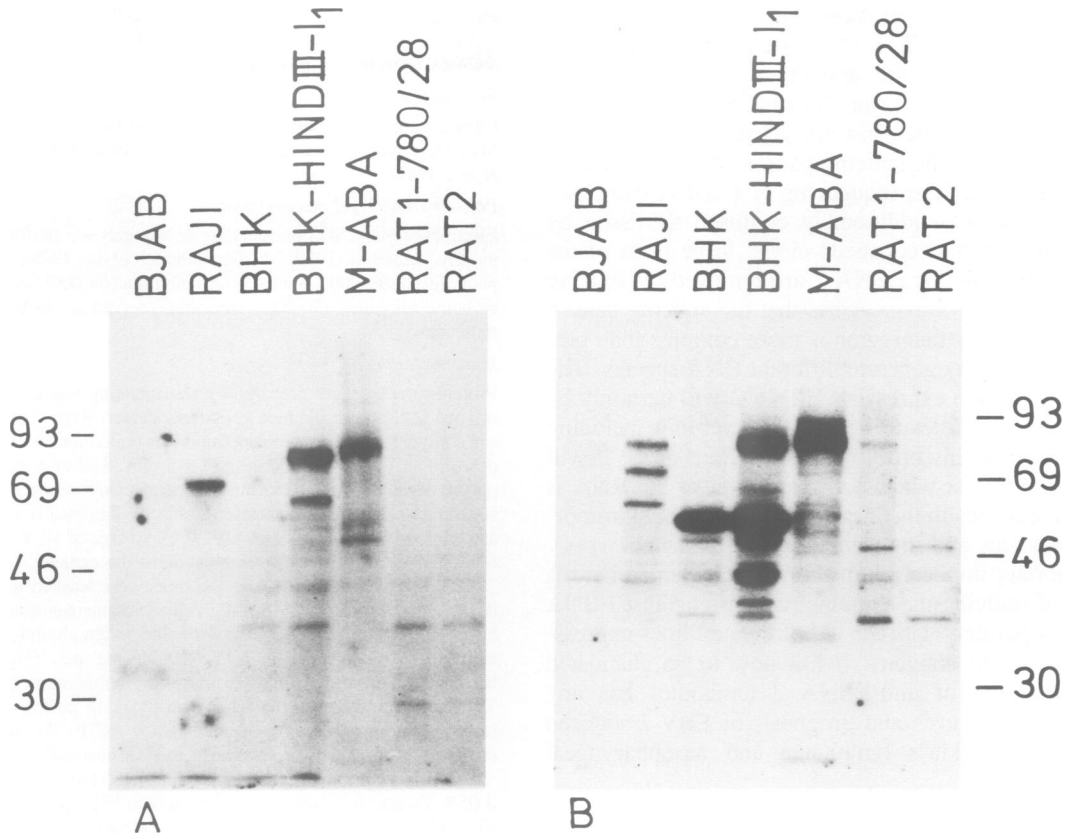

Fig. 5. Protein analysis by electrophoretic immunoblotting of EBV genome positive (Raji, M-ABA) and negative lymphoid cell lines (BJA-B) in comparison with BHK and Rat-1 cell lines carrying pM *Hind*III-I1 and pM 780-28 DNA, respectively, using an anti-EBNA 1-positive, anti-EBNA 2-negative serum (no. 198) shown in **(A)** and an anti-EBNA 1 and -EBNA 2-positive serum (LyBe 83) shown in **(B)**. Untransfected BHK and rat cells served as negative controls. Mol. wts. are given in kd.

We therefore conclude that the 82 000-dalton protein encoded by the region represented in pM 780-28 is the viral protein EBNA 2.

Discussion

In cells immortalized by EBV which carry the virus latently, three regions of the viral genome were shown to be transcribed by Kieff and co-workers (King *et al.*, 1980; van Santen *et al.*, 1981, 1983; Heller *et al.*, 1982). The first transcription unit comprises sequences located in the large internal repeats and the long unique region, the second is located in the middle of the genome and comprises small repeat structures, and the third is located at the right hand end of the viral genome.

At least three viral gene functions have been identified in cells immortalized by the virus. The first is a nuclear antigen visualized in all EBV genome-carrying cells by ACIF (Reedman and Klein, 1973). The second is the lymphocyte detected membrane antigen (LYDMA) which is recognized by T cells of individuals infected by EBV in an HLA-restricted fashion (Rickinson *et al.*, 1980). Evidence for the existence of more than one EBNA protein has been provided by Strnad *et al.* (1981) and Hennessy and Kieff (1983) who demonstrated that a protein with a mol. wt. of 82 000, designated as EBNA 2, is, in addition to EBNA 1, present in cells immortalized by EBV. Of these viral functions only EBNA 1 has so far been assigned unequivocally to a certain region of the EBV genome. By DNA transfer into recipient cells Summers *et al.* (1982) could show that *Bam*HI-K carrying the IR3 repeat cluster (Heller *et al.*, 1982) codes for the protein now called EBNA 1.

Here we have provided direct evidence, by using the same approach of stable gene transfer into recipient cells, that EBNA 2 is encoded by sequences located in the large internal repeats and the adjacent part of the long unique region. This antigen could, however, only be visualized by a subset of sera containing anti-EBNA antibodies and failed to react with anti-VCA-positive, anti-EA-positive, anti-EBNA-negative human sera. Immunoblot analysis of the proteins of the transfected rat cell line revealed a protein of 82 000 daltons which reacted only with those sera which stained positive for the nuclear antigen. Moreover, only those of the EBNA-positive sera reacted with a protein of the same size in Raji cells. We thus conclude that the protein with a mol. wt. of 82 000 is EBNA 2 and is encoded by sequences represented in the clone pM 780-28. This conclusion is in line with the finding that EBNA 2 is absent from P3HR-1 and Daudi cells. The viruses carried in both cell lines have large deletions in the region now identified as coding for EBNA 2 (Bornkamm *et al.*, 1982; Jones and Griffin, 1983). Since the P3HR-1 strain has lost its capacity to immortalize human B-lymphocytes, it is tempting to assume that EBNA 2 is somehow involved in the process of immortalization.

From the transfection experiments presented we do not know how the transcription unit of EBNA 2 is organized. The fact that the EBNA 2 proteins from Raji and the transfected rat cells have the same mol. wt. suggests that the coding region for EBNA 2 is entirely represented on the cloned fragment. The clone used for transfection (pM 780-28) contains three copies of the large internal repeat (*Bgl*II-U) and the adjacent *Bgl*II-C fragment. We also do not know whether the presence of several copies of the

repeat is important for the expression of EBNA 2. Attempts are in progress to test whether *Bgl*II-C by itself is also capable of mediating the expression of this antigen.

S1 mapping of the transcripts from Raji and EBV immortalized cells (van Santen *et al.*, 1983) has not yet fully elucidated the start of transcription and the splicing pattern of the RNA transcribed from this region. The sequencing of a cDNA clone isolated from Raji cells has additionally confused the issue by demonstrating that, in the sequenced clone, large parts of the region suspected to code for EBNA 2 are removed by splicing (Bodescot *et al.*, 1984). This suggests that the splicing pattern of the RNAs encoded by this region is more complex than previously assumed and may generate different RNA species. The availability of a cell line expressing EBNA 2 will certainly be useful to characterize in detail the transcription unit including the splicing pattern of this presumably important gene. It will also be interesting to test whether transcription of the RNA is initiated at identical sites in the large internal repeats in immortalized B-lymphocytes and the transfected rat cell line.

Another important implication of the work presented here is the possibility of studying the immune response against EBNA 1 and EBNA 2 separately with the aid of the cell lines expressing either one or both antigens. It has now to be elucidated whether the detection of anti-EBNA 2 antibodies has any significance in the diagnosis and prognosis of EBV-associated diseases including Burkitt's lymphoma and nasopharyngeal carcinoma.

Materials and methods

Cells

BJA-B, P3HR-1, Daudi, B95-8 and M-ABA cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% foetal calf serum, penicillin (100 international units/ml) and streptomycin (100 μg/ml). Monolayers of rodent cells (Rat-1, Rat-2, rat embryo fibroblasts and BHK) were cultivated in Eagle's minimum essential medium (E-MEM) with the same supplements as mentioned above. All cell lines were subcultured routinely once or twice weekly.

Determination of antibody titers against EBV antigens

Antibody titers against VCA and EA antibodies were determined by indirect immunofluorescence (Henle and Henle, 1966) on slides containing P3HR-1 cells and Raji cells induced by 12-O-tetradecanoylphorbol-13-acetate, respectively. Antibody titers against EBNA were determined on Raji cells by ACIF (Reedman and Klein, 1973).

EBNA staining

The EBNA was assayed on methanol-fixed cells by ACIF (Reedman and Klein, 1973).

Detection of DNA fragments

Restriction enzymes were purchased from Boehringer Mannheim, Bethesda Research Laboratories, New England Biolabs and Renner, Mutterstadt, FRG. DNA fragments were separated on horizontal agarose gels (0.4 – 1.0%). Lambda *Hind*III fragments were used as size markers.

Separated fragments were transferred to nitrocellulose filters as described by Southern (1975) with the modifications introduced by Wahl *et al.* (1979). Cloned EBV-DNA fragments, separated from the vector, were labeled with [^{32}P]dCTP by nick-translation (Amersham; 400 Ci/mmol; Rigby *et al.*, 1977). Hybridization and washing of the filters were carried out as described by Bornkamm *et al.* (1982). Filters were air-dried and exposed to Kodak Royal X-Omat AR films using intensifying screens.

Transfection

BHK cells were transfected by the calcium phosphate technique as described (Takaki *et al.*, 1984) using the AG60 plasmid conferring resistance to the antibiotic G-418 (Gibco) for co-transfection (Colbère-Garapin *et al.*, 1981). For rat cells, the protocol of Land *et al.* (1983) was used with pSV2-neo as the dominant selectable marker (Southern and Berg, 1982). Briefly, cultures of Rat-1 cell line were seeded at a density of 10^6 cells/100 mm Petri dishes in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum. Transfections were carried out using the calcium phosphate technique (Takaki *et al.*, 1984) using 75 μg Rat-1 carrier DNA, 10 μg plasmid DNA and 1 μg PSV2 neo-DNA for two dishes. After contact with the precipitate for 4 h, the cells were washed in PBS and fed with medium. After 24 h the transfected cells were pooled, and split in a ratio of 1:10. One day later the cells were subjected to G-418 selection (5000 μg/ml). Cultures were re-fed every 4 days. After 10 – 12 days, individual colonies were trypsinized and expanded.

Recombinant plasmids

Cloned viral DNA fragments were derived from the transforming virus strain M-ABA (Figure 1). A detailed description of the clones is given elsewhere (Polack *et al.*, 1984).

Polyacrylamide gel electrophoresis

Electrophoresis in SDS-polyacrylamide slab gels was performed as described previously (Laemmli, 1970; Mueller-Lantzsch *et al.*, 1979). ^{14}C-Methylated phosphorylase b (93 000), bovine serum albumin (69 000), ovalbumin (46 000) and carbonic anhydrase (30 000; Amersham) served as markers for mol. wt. determinations.

Immunoblotting

Protein samples were prepared by resuspending washed cells (2.5 x 10^7 cells) in 1 ml SDS-polyacrylamide gel sample buffer. After sonication for 15 s (Branson sonifier, setting 5) the suspension was boiled for 10 min and the debris spun down in an Eppendorf centrifuge for 5 min. 8 μl of a protein solution with an optical density of 0.8 at 280 nm were subjected to the gel. After electrophoretic separation proteins were transferred electrophoretically to nitrocellulose filters as described by Towbin *et al.* (1979). A voltage of 10 V was applied overnight for a distance of 2 cm from the anode to the cathode.

After electrophoretic transfer the blots were washed in fresh transfer buffer (0.025 M Tris, pH 8.5/0.192 M glycine/20% methanol) and soaked in blocking buffer (PBS/0.05% Tween 20/3% bovine serum albumin) for 2 x 1 h at room temperature to saturate additional protein binding sites. The filters were then incubated with human sera for 4 – 6 h at room temperature (or overnight at 4°C). The sera were diluted 1:50 – 1:200 in blocking buffer. Prior to their use for immunoblotting all sera had been tested by ACIF. To minimize the volume of the serum the incubation was performed in heat-sealed plastic bags.

The filters were then soaked for 1 h in five changes of washing buffer (PBS/0.05% Tween) followed by incubation with ^{125}I-labeled protein A (Amersham) (0.4 μCi/ml) diluted in blocking buffer at room temperature for 1 h.

After incubation with protein A, the nitrocellulose filters were washed in six changes of washing buffer for 2 h. The filters were then air-dried, wrapped in Saran Wrap and exposed to Kodak X-Omat AR films for 1 – 14 days with or without intensifying screens.

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