Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1499–1503, March 1986
Microbiology

Four virally determined nuclear antigens are expressed in Epstein–Barr virus-transformed cells

(viral transformation/DNA-binding proteins)

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Contributed by Werner Henle, October 11, 1985

ABSTRACT **The expression of Epstein–Barr virus (EBV)-determined antigens associated with growth-transformation of B cells was studied by immunoblotting with human sera from healthy donors. Four antigens were detected in EBV-carrying cell lines and in B lymphocytes early after infection with the transforming B95-8 substrain of virus. They were not found in uninfected cells, nor could they be demonstrated with sera lacking antibodies to EBV antigens. All four antigens were nuclear. Each of them varied in size in the different cell lines. The two antigens with the lowest molecular weight were identified as EBV-determined nuclear antigens (EBNAs) 1 and 2. The two high molecular weight antigens (140–160 kDa and 150–180 kDa, respectively) were detected with 6 of 16 EBV antibody-positive sera. These proteins appeared to be antigenically unrelated to each other and to EBNAs 1 and 2 and were designated EBNAs 3 and 4. Like EBNAs 1 and 2, they bound to double- and single-stranded DNA *in vitro*.**

Epstein–Barr virus (EBV) immortalizes human B lymphocytes *in vitro* and *in vivo* (1). Only a limited part of the viral genome is expressed in virus-transformed cells (2). Two EBV-determined nuclear antigens, EBNA 1 and EBNA 2, have been identified in the virus-transformed cell. These antigens can be demonstrated by anti-complement immunofluorescence (ACIF) (3, 4) and by immunoblotting (5). EBNA 1 is encoded by the *Bam*HI K fragment (6–9). The coding gene for EBNA 2 was assigned to a 1.6-kilobase (kb) open reading frame in the *Bam*HI Y and H fragments (10, 11). A cell membrane antigen is encoded by the *Bam*HI N fragment (12). All three antigens are partly encoded by repeat sequences that vary in size among the different virus isolates. The antigens vary proportionally in size. In this paper, we describe two additional EBNAs in growth-transformed cells. They are tentatively designated EBNA 3 and EBNA 4.

MATERIALS AND METHODS

Cells and Virus. Human B lymphocytes were purified from tonsils as described (13). Cells (1×10^8) were washed in RPMI 1640 medium and suspended in 50 ml of virus-containing supernate from B95-8 cells. Cells were shaken for 1 hr at 25°C, pelleted, washed, and resuspended in medium containing 10% fetal calf serum. At 24-hr intervals, 2×10^7 cells were removed, washed, and lysed in electrophoresis sample buffer (11). Mock-infected cells were harvested immediately after "infection." EBV-transformed cord blood lines (designated CBC-SEB-xxx) were established by *in vitro* infection with EBV (B98-8) virus or with virus from throat washings from patients with infectious mononucleosis (IM) (see Table 2). EBV-transformed lymphoblastoid cell lines (LCLs) designated SPIM-xxx were established by spontaneous outgrowth from peripheral blood of patients with IM. Virus production in B95-8 cells was induced as described (14). At the time of harvest (24 hr), the culture contained 26% early antigen (EA)-positive cells and 19% viral capsid antigen (VCA)-positive cells.

Subcellular Fractionation, DNA Cellulose Chromatography, and Immunoblotting. Washed cells were lysed in Nonidet P-40 (NP-40) buffer as described (11). The nuclear pellet was washed once with the same buffer without NP-40, and the nuclei were collected by centrifugation. The nuclear wash and the NP-40 supernate were combined and designated cytoplasm (see Fig. 4). The nuclear pellet was sequentially washed with 0.15, 0.40, and 2.0 M NaCl (in 20 mM Tris·HCl, pH 7.5/2 mM $MgCl_2$). The resulting pellet was suspended in electrophoresis sample buffer.

Chromatography on single-stranded (ss) and double-stranded (ds) DNA-cellulose was as described (14). Briefly, the 0.4 M NaCl extract from 2×10^8 IB4 cells was diluted to 150 mM NaCl, and EDTA was added to a final concentration of 5 mM. The nuclear extract was loaded onto columns (2 ml) of ss or ds DNA-cellulose. Bound proteins were eluted stepwise with 5 ml of 0.25, 0.35, and 0.60 M NaCl. Electrophoresis and immunoblotting were performed with gels containing 9% acrylamide and 0.1% *N,N'*-methylenebisacrylamide or with 7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide as described (9, 11).

RESULTS

Screening of Human Sera by Immunoblotting. Blots of Raji and Ramos cells were incubated with the sera listed in Table 1. Seven of these sera were from donors lacking antibodies to EBV and 16 were from healthy seropositive donors. The serum WC is derived from a patient with chronic IM (15). Fifteen of the EBV-positive sera reacted with a 69-kDa protein of Raji cells that was identified as EBNA 1 with specific anti-peptide antisera (9) (Table 1). Eleven sera reacted with a 103-kDa polypeptide of the Raji extract that was classified as EBNA 2 on the basis of its reactivity with antibodies to synthetic peptides deduced from the nucleotide sequence of the *Bam*HI *YH* region (11). Seven of the EBNA 2 antibody-positive sera reacted with an additional 143-kDa polypeptide of the Raji extract. Six of these sera also reacted with a 180-kDa Raji polypeptide. These two high molecular weight polypeptides are hereafter referred to as EBNA 3 and EBNA 4, respectively. Fig. 1 shows blots with two EBV-

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Abbreviations: kb, kilobases; EBV, Epstein–Barr virus; EBNA, EBV-determined nuclear antigen; LCL, lymphoblastoid cell line; BL, Burkitt lymphoma; ss, single-stranded; ds, double-stranded; IM, infectious mononucleosis; ACIF, anti-complement immunofluorescence; EA, early antigen; VCA, viral capsid antigen.

Table 1. Human sera, reciprocal antibody titers to EBV antigens, and reactivity with the four EBNA polypeptides in immunoblotting against extracts of Raji and Ramos cells

	Antibody titer			Reaction with EBNA in immunoblotting			
Serum	VCA	EA	EBNA	EBNA 1	EBNA 2	EBNA 3	EBNA 4
LÄ-R	<10	<10	<2	−	−	−	−
LS	<10	<10	<2	−	−	−	−
KA	<10	<10	<2	−	−	−	−
RK	<10	<10	<2	−	−	−	−
JD	<10	<10	<2	−	−	−	−
IE	<10	<10	<2	−	−	−	−
CP	<10	<10	<2	−	−	−	−
AW	80	<10	20	+	−	−	−
GB	160	<10	10	−	+	−	−
BE-H	160	<10	20	+	−	−	−
MTB	160	<10	40	+	−	−	−
JR	160	<10	40	+	+	−	−
LE	160	10	80	+	−	−	−
KF	160	<10	160	+	+	+	+
AS	320	<10	40	+	+	−	−
ST	320	20	40	+	−	−	−
MH	320	10	80	+	+	+	−
RS	320	20	80	+	+	+	(+)*
WP	320	40	80	+	−	−	−
CN	320	20	160	+	+	+	(+)*
A-CS	320	10	160	+	+	+	+
EG	320	<10	320	+	+	+	+
BK	640	<10	160	+	+	+	+
WC†	20480	10240	10	−	+/−	+	−

*The sera RS and CN reacted weakly with EBNA 4.
†WC is as serum from a patient with chronic IM, whereas all other sera were from healthy donors.

negative sera, one EBNA 1-, 2-, and 3-positive serum (MH), and four sera that reacted with all four EBNAs. The WC serum reacted predominantly with EBNA 3 (Fig. 2*A*). The sera from healthy donors that detected EBNA 3 and/or EBNA 4 had anti-EBNA titers of 1:80 or higher by immunofluorescence (Table 1). Three of these sera had low antibody titers to EA and slightly elevated titers to VCA.

Expression of the High Molecular Weight Antigens in Different Cell Lines. The human donor KF serum was used for analysis of EBNA expression in a panel of 10 LCLs (Table 2; Fig. 2*B*). In each cell line, four or five prominent antigens were detected. EBNA 1 varied in molecular weight between 81 and 96 kDa. In one cell line, CBC-SEB-M123 (M123 in Fig. 2*B*), EBNA 1 comigrated with EBNA 2 that had an apparent molecular weight between 96 and 107 kDa. The third antigen, designated EBNA 3 in analogy with the Raji cell experiment (Fig. 1), varied in size between 136 and 157 kDa. In four of the cell lines (IB4, C123, C105, and M105), the high molecular weight antigens had a similar size, with a major 157-kDa polypeptide. The first three of these cell lines were established by B95-8 virus infection and were indistinguishable as to the mobility of EBNAs 1, 2, and 3. The other LCLs in Fig. 2*B* had EBNA 4 (166–180 kDa) as the dominating high molecular weight antigen. This antigen seemed to be substituted by two antigens (166 and 180 kDa) of lower intensity in

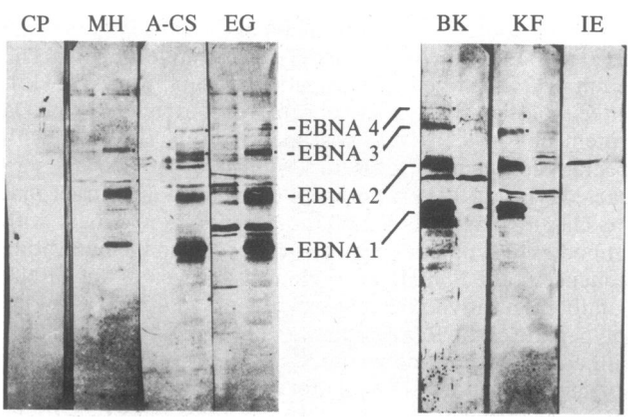

FIG. 1. Screening of sera with blots of extracts of Raji and Ramos cells. The antibody titers to EBV antigens are found in Table 1. (*Left*) Lanes with Raji extracts are to the right of those with Ramos extracts. (*Right*) Reverse orientation of lanes. The EBNA 3 and EBNA 4 bands are indicated with a small square to the right of the band.

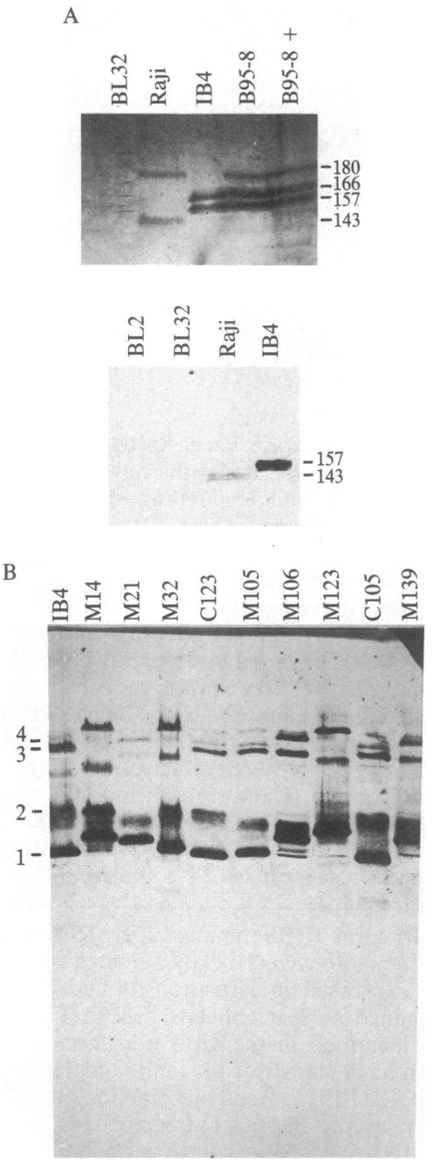

FIG. 2. (*A Upper*) Blot stained with an EBNA 3- and EBNA 4-positive serum. The lane labeled B95-8+ is cells treated with phorbol 12-myristate 13-acetate and butyrate to induce virus replication. (*A Lower*) Blot stained with WC serum (Table 1) that reacts with EBNA 3 but not with EBNA 4. Molecular size is shown in kDa. (*B*) Blot with extracts of 10 EBV-transformed LCLs stained with the KF serum. The position of EBNAs 1–4 in IB4 cell lines is indicated. The names of the different cell lines are abbreviated relative to those listed in Table 2. The lower portion of A and B has been removed.

Table 2. Detection of EBNA-associated polypeptides in LCL and cell lines established from BL

Cell line	EBV carrier state	EBNAs 1	2	3	4
	BL lines				
Loukes	−	−	−	−	−
Bjab	−	−	−	−	−
Ramos	−	−	−	−	−
U698	−	−	−	−	−
BL2	−	−	−	−	−
BL32	−	−	−	−	−
Salim Mwalim	+	+	+	+	+
Raji	+	+	+	+	+
Daudi*	+	+	−	+	+
Namalwa	+	+	+	+	+
Jijoye M13†	+	+	−	+	+
P3HR-1*	+	+	−	+	+
Naliaka*	+	+	−	+	+
	LCL				
CBC-SEB-M12	+	+	+	+	+
CBC-SEB-M13	+	+	+	+	+
CBC-SEB-M21	+	+	+	+	+
CBC-SEB-M31	+	+	+	+	+
CBC-SEB-M32	+	+	+	+	+
CBC-SEB-M105	+	+	+	+	+
CBC-SEB-M106	+	+	+	+	+
CBC-SEB-M123	+	+	+	+	+
CBC-SEB-M139	+	+	+	+	+
CBC-E95-C105‡	+	+	+	+	+
CBC-E95-C123‡	+	+	+	+	+
Spim-M17§	+	+	+	+	+
Spim-kk§	+	+	+	+	+
IB4‡	+	+	+	+	+

*Deleted EBNA 2 coding sequence.
†Variant EBNA 2 coding sequence.
‡Established by infection of B lymphocytes of cord blood with B95-8 virus.
§Established by spontaneous outgrowth from IM blood.

C123, M105, C105, and identical species were found in B95-8 cells (Fig. 2*A Upper*). In IB4 cells, as in M21 cells, EBNA 4 was identified as a 166-kDa polypeptide. The two polypeptides (180 and 166 kDa) in B95-8 cells and in the cell lines established by transformation with this virus were always detected together and are most likely antigenically related. The 166-kDa polypeptide in these cells might be a product of the 180-kDa antigen. In contrast to other B95-8-transformed cells, IB4 cells lacked the 180-kDa antigen (Fig. 2 *A* and *B*). In most other LCLs established by *in vitro* infection with IM virus or by spontaneous outgrowth from the blood of IM donors (Spim-M17 and Spim-kk), the largest antigen had a molecular weight close to 180 kDa, a pattern similar to that of M14. Induction of virus production in B95-8 cells did not detectably affect the level of EBNAs 3 and 4, nor was appearance of new antigens of similar sizes detected (Fig. 2*A Upper*).

In Burkitt lymphoma (BL) cell lines, the KF serum detected a similar size range of antigens as in the typical LCLs (Fig. 3). With three exceptions (Salim Mwalim, Raji, and Namalwa), expression of EBNA 3 and EBNA 4 was generally lower than in the LCLs. P3HR-1 and Jijoye cells had a similar size distribution of EBNAs 3 and 4 as observed in Namalwa and IB4 cells (Fig. 3). The apparent molecular weights obtained by electrophoresis on gels with low crosslinking (Figs. 1, 2, and 3*A*) was compared to that obtained with gels of normal crosslinking (Table 3). The apparent molecular weight of EBNA 4 of B95-8 cells as determined on

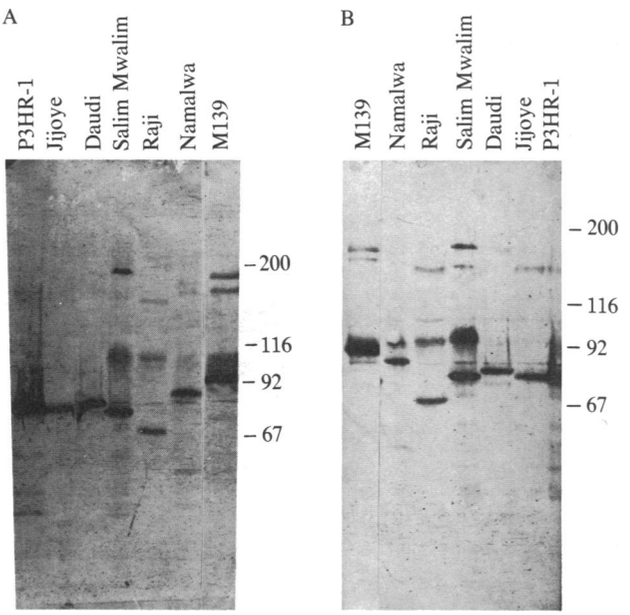

FIG. 3. Blots of extracts electrophoresed on 0.9% polyacrylamide (low crosslinking) (*A*) and on 7.5% polyacrylamide (normal crosslinking) (*B*). The mobility of molecular size markers (shown in kDa) in each experiment is indicated. The molecular mass of EBNA 1–4 in each cell line is found in Table 3. The bands indicated with bars on the left in *B* are artefacts.

gels with low crosslinking was 180 kDa, whereas gels with normal crosslinking yielded a value of 160 kDa. Similarly, the molecular weight determination for EBNA 3 varied proportionally depending on the gel system used (Table 3). The migration of EBNA 2 was significantly slower on gels with low crosslinking than on gels with normal crosslinking. This is clearly depicted with EBNA 1 and EBNA 2 of M139 cells where the two antigens migrate in a well-separated manner (Fig. 3*A*) but comigrate on gels with normal crosslinking (Fig. 3*B*). The EBNA 2 band was composed of three molecular species differing slightly with respect to their mobility (Fig. 3*B*).

Expression of EBNA-Associated Antigens in Human B Lymphocytes Infected with B95-8 Virus. In infected B cells, EBNA 2 and trace amounts of EBNAs 1 and 3 were detected on day 1 (Fig. 4). On day 2 the expression of EBNAs 1, 2, and 3 had increased to levels comparable to those of IB4 cells. On day 3, EBNA 4 appeared as two polypeptides (166 and 180

Table 3. Dependence of the apparent molecular masses for EBNAs 1–4 on the type of polyacrylamide gel used for separation

	Apparent molecular mass, kDa							
	EBNA 1		EBNA 2		EBNA 3		EBNA 4	
Cell line	A	B	A	B	A	B	A	B
B95-8	81	84	107	92	157	143	180 (166	158 148)
Raji	69	70	103	90	143	136	180	160
Salim Mwalim	76	81	105	92	146	138	180	158
P3HR-1	76	81	ND	ND	144	138	148	138
Daudi	81	84	ND	ND	160	140	170	150
Namalwa	79	76	100	88	140	138	150	145
Naliaka	76	81	ND	ND	144	136	148	138

A, low-crosslinking-type gel; B, normal-crosslinking-type gel; ND, not detected (gene deleted in P3HR-1, Daudi, and Naliaka cell lines).

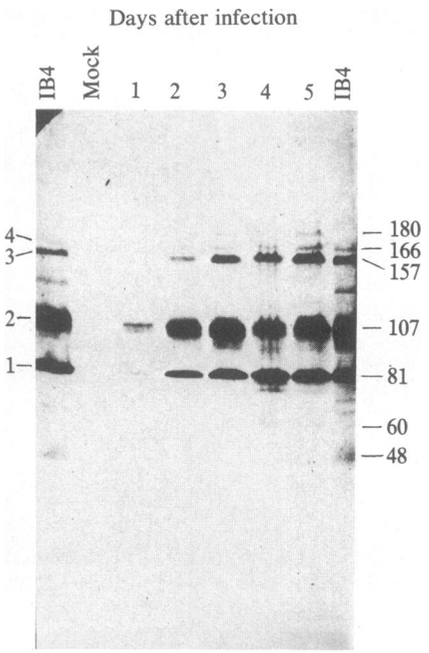

FIG. 4. The time course of infection of B cells from tonsils by B95-8 virus. Extracts of mock-infected and infected cells (4×10^6 cells) were separated on 9% polyacrylamide gels, and the blot was stained with the KF serum (Table 1). Extracts of the B95-8-transformed cell line IB4 (1×10^6 cells) were electrophoresed in parallel (left- and right-most lanes). The position of EBNAs 1–4 is indicated, and molecular sizes are shown in kDa.

kDa) as in B95-8 cells (Fig. 2*A*). The pattern of expression did not change qualitatively from day 3 on and was almost the same as that of the B95-8-transformed cells (Fig. 2*B*).

Subcellular Localization of the Four EBNA-Related Polypeptides. IB4 cells were fractionated into cytoplasm and nuclei. The nuclei were washed sequentially with increasing concentrations of NaCl. EBNAs 1, 2, and 3 were readily detected in whole-cell extracts, whereas EBNA 4 appeared only as a faint band (Fig. 5). In the nuclear fraction, EBNAs 2, 3, and 4 were enriched, whereas very little of these antigens was found in the cytoplasm. EBNA 1 was detected both in nuclei and in the cytoplasm. Sequential extraction of nuclear proteins with 0.15, 0.4, and 2 M NaCl revealed that 0.4 M NaCl was sufficient to solubilize most of EBNAs 2, 3, and 4; only trace amounts were found in the 2 M extract. After extraction with 2 M NaCl, EBNAs 2, 3, and 4 were readily detected in the pellet. EBNA 1 was detected in the 0.4 and 2.0 M NaCl extracts as well as in the residual pellet. The biphasic extraction profile of EBNAs 2, 3, and 4 was reproduced with Raji cells (not shown).

DNA-Cellulose Chromatography. *In vitro* binding to DNA was performed in 0.15 M NaCl, and bound proteins were eluted by stepwise increase of the NaCl concentration of the eluent. With ss DNA-cellulose (Fig. 6*A*), most of EBNA 3 was detected in the 0.25 M NaCl eluate. Trace amounts of EBNA 3 were found in the column flow-through. EBNA 1 and EBNA 2 were detected preferentially in the 0.35 M and 0.6 M ss DNA eluates, whereas EBNA 4 was detected in the 0.35 M NaCl eluate. The low molecular weight species of EBNA 2 were eluted preferentially with 0.25 M NaCl, whereas the major (and largest) component was dominant in the 0.6 M NaCl eluate. With ds DNA, the elution profile was similar but not identical (Fig. 6*B*). Most of EBNA 3 and small amounts of EBNA 1 failed to bind to ds DNA. The relative order of elution, EBNA 3, EBNA 4, EBNA 2, and EBNA 1, was the same as with ss DNA.

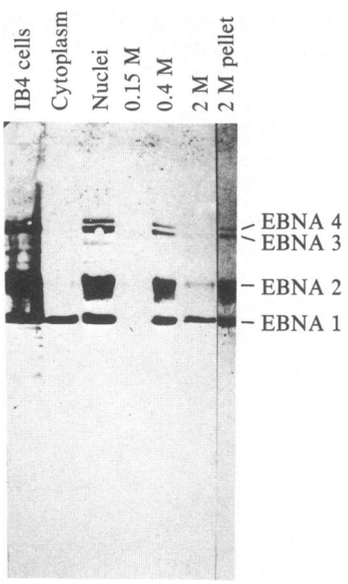

FIG. 5. Subcellular distribution of EBNAs 1–4 in IB4 cells and sequential extraction of the antigens from the nuclear fraction by increasing concentrations of NaCl. Lane designated 2 M pellet denotes the nuclear material insoluble in 2 M NaCl. Blots were stained with KF serum.

DISCUSSION

The following criteria were applied for the identification of EBNAs in cell extracts: (*i*) the antigen should be detected only with EBV-positive sera; (*ii*) it should only be expressed in EBV-carrying cells; (*iii*) it should be detectable in all lymphoblastoid cell lines that depend on EBV for their proliferation; and (*iv*) it should be expressed in B lymphocytes after infection with transforming EBV.

The first three criteria have been met for EBNAs 1 and 2 (5). Later it was shown that these two proteins are encoded by the virus (8, 9, 11, 12). Now we show that all four criteria are met by EBNAs 3 and 4 and, as expected, also by EBNAs 1 and 2. We found also that the size of EBNAs 3 and 4 is determined by the infecting virus. The molecular species of

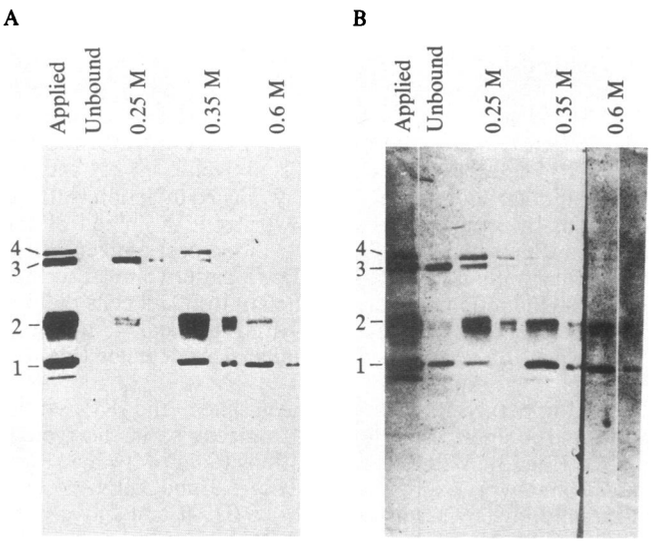

FIG. 6. (*A*) Chromatography of the 0.4 M NaCl extract of IB4 cells (Fig. 4) on ss DNA-cellulose. From each of the 0.6, 0.35, and 0.25 M NaCl eluates, 50- and 12.5-μl of solubilized proteins were electrophoresed. Blots were stained with KF serum. (*B*) Chromatography on ds DNA-cellulose. The analysis was performed as in *A*.

EBNAs 1, 2, 3, and 4 in B-cell lines transformed with B95-8 virus were the same as in the B95-8 cells themselves and in tonsillar B cells infected by B95-8 virus. In the primary infection, EBNA 2 appears slightly before EBNAs 1, 3, and 4. This finding is in line with the suggestion that the EBNA 2-coding region of the viral genome is involved with early activation-events leading to immortalization of B cells (2). EBNAs 1, 3, and 4 followed a similar pattern of appearance. Starting on day 3 of the primary infection, all four EBNA antigens were expressed to similar levels as in the virus-transformed cell lines.

BL-derived cell lines express less EBNA 3 and EBNA 4 than did the LCLs, as a rule. Also, many of the BL-derived lines contained no detectable EBNA 2. It is conceivable that EBNA 2 is not required for the proliferation of the malignant, tumorigenic BL cells or for the maintenance of the viral genomes. EBNAs 3 and 4 are compartmentalized in the cell and solubilized in a fashion nearly indistinguishable from EBNA 2 (11). Moreover, EBNAs 3 and 4 bind, like EBNAs 1 and 2, to ss and ds DNA *in vitro*. The conditions for binding were the same as were used for the purification of the viral DNA polymerase and the viral alkaline nuclease (14). Therefore, it is likely that EBNAs 3 and 4 also interact with DNA *in vivo* as suggested for EBNA 1 (16).

Several lines of evidence suggest that EBNAs 3 and 4 are neither interrelated nor related to EBNAs 1 and 2. First, there was no covariation in apparent molecular weight between the four antigens. EBNAs 3 and 4 were expressed in cell lines with deleted EBNA 2-coding sequences (P3HR-1, Daudi, and Naliaka). Second, sera raised against the Ala-Gly repeat of EBNA 1 (9) and against two domains of EBNA 2 (11) did not react with EBNA 3 or EBNA 4. Two sera (Table 1) reacted well with EBNA 3 but not at all with EBNA 4. Therefore, it is unlikely that EBNA 3 and EBNA 4 share antigenic determinants. Most of the experiments used gels with lower crosslinking than previously used (4, 9, 11). On this type of gel, the apparent molecular weights for EBNAs 3 and 4 varied between 140 and 157 kDa and between 150 and 190 kDa, respectively. On gels with normal crosslinking, the apparent molecular weights were about 10% lower (136–143 kDa for EBNA 3 and 150–160 kDa for EBNA 4). Although EBNAs 3 and 4 migrate close to major EA and late antigens (14), these antigens were not detected on immunoblots with the KF serum and extracts of virus-producing B95-8 cells (Fig. 2*A*). Since we observed no changes in the levels of EBNA 3 and EBNA 4 expression in cells ranging from <0.01% EA (Raji and IB4) to more than 20% EA (induced B95-8), we conclude that neither of these two antigens is related to the productive viral cycle. They are clearly associated with the latent infection of immortalized cells.

The size-variation of EBNA 4 seems to differ from that of EBNAs 1, 2, and 3. Under conditions where EBNAs 1, 2, and 3 migrated as single bands, EBNA 4 often appeared as two bands. This was well illustrated in B95-8 cells and in LCLs transformed by B95-8 virus, where EBNA 4 migrated as two bands of 166 and 180 kDa. It is possible that EBNA 4 undergoes posttranslational processing or that the mRNA for EBNA 4 has two alternative splicing patterns.

Recently, Sculley *et al.* (17) described multiple nuclear antigens in EBV-infected cells. Two of these antigens were assigned molecular weights of 110 and 115 kDa, respectively. Using a reference serum supplied by Sculley, we detected EBNAs 3 and 4 with blots of Raji and IB4 cells. The discrepancy in molecular weights could be due to the fact that Sculley *et al.* used no molecular weight markers larger than 92 kDa. The EBNA 3 and EBNA 4 herein reported are not accounted for by available transcription data (2). Sculley *et al.* (17) suggested that their high molecular weight nuclear antigens were associated with the putative transforming region of the viral genome. As we could detect EBNAs 3 and 4 in cell lines with deletions in the putative transforming sequences (18), we suggest that EBNAs 3 and 4 are encoded by sequences outside the previously known transcribed areas of the viral genome.

We are grateful to E. Kieff for the IB4 cell line, to J. L. Strominger and R. Schooley for gifts of WC serum, and T. Sculley for reference serum. We are also grateful to I. Jansson and G. Kjällström for purification of B cells and to K. Andersson for tissue culture. This study was supported by National Cancer Institute grants 5 R01, CA 28380-03, and 33324 and by grants from the Swedish Cancer Society. B.K. is a recipient of a fellowship from Concern Foundation and a fellowship from the Cancer Research Institute, Inc., New York.

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