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Gene Mapping and Expression of Two Immunodominant Epstein-Barr Virus Capsid Proteins

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The genomic localization of two immunodominant genes encoding two proteins of the Epstein-Barr virus capsid antigen (VCA) complex, VCA-p18 and VCA-p40, has been identified. For that purpose, lambda gt11-based cDNA libraries were constructed from HH514.c16 cells induced for virus production. The libraries were screened with a monoclonal antibody, EBV.OT41A, directed against VCA-p40 or with affinity-purified human antibodies against VCA-p18. Sequencing of the inserts of positive plaques showed that VCA-p18 and VCA-p40 are encoded within open reading frames (ORFs) BFRF3 and BdRF1, respectively. Peptide scanning analysis of the predicted protein of ORF BdRF1 resulted in defining the epitope of monoclonal antibody EBV.OT41A at the C-terminal region. The dominant VCA-p18 reactivity of human sera can be completely inhibited by preadsorption with *Escherichia coli*-expressed BFRF3–β-galactosidase. Serum of a rabbit immunized with BFRF3–βgalactosidase reacts with a VCA-specific protein of 18 kDa. In addition, BFRF3–β-galactosidase affinity-purified antibodies react with VCA-p18 of virus-producing cells (HH514.c16). Complete inhibition of viral DNA polymerase activity by phosphonoacetic acid is associated with the absence of RNAs and protein products of both ORFs, indicating that VCA-p18 and VCA-p40 are true late antigens.

Epstein-Barr virus (EBV) is a ubiquitous member of the human herpesvirus family and is associated with a still increasing number of disease syndromes. EBV-specific diagnosis is based on the combination of different serological parameters. These parameters include the detection of antibodies of different classes against early antigens (EA), Epstein-Barr nuclear antigens (EBNA), viral capsid antigens (VCA), and membrane antigens.

Polypeptides belonging to the EBNA and EA complexes and their role in immunodiagnosis has been studied extensively (24, 29, 39). In contrast, only few studies have addressed in detail the human immune response to viral structural polypeptides. Studies of the structural polypeptides are hampered by the molecular complexity of the VCA complex, the polyspecificity of the human sera used to detect them, and the lack of an efficient virus production system. EBV virions may consist of some 30 polypeptides (9). Localization within the virion and a role in penetration, assembly, and budding have been suggested for only a few.

The viral capsid may be composed of at least seven proteins (7), of which the major capsid protein encoded by open reading frame (ORF) BcLF1 (37) and a protein of 36 kDa encoded by BGLF2 (5, 33) have been described in detail. Another VCA protein, gp110, is encoded within the BALF4 reading frame (34). This protein remains associated with cellular cytoplasmic and nuclear membranes and is not associated with the virion (15).

Recently, we have identified two new immunologically dominant VCA proteins with molecular sizes of 18 and 40 kDa (36). These two proteins are structurally associated with the viral capsid and are recognized by antibodies from almost all EBV carriers.

VCA-p18 may be identical to the 17-kDa protein described by Edson et al. (10) and the 22-kDa protein defined by Sculley et al. (31, 32) and may be part of the ZEBRA-associated protein complex (ZAP21) described by Katz et al. (18). The genomic localization of the ORFs encoding these proteins has not been described thus far.

In this report, we describe the identification of the ORFs on the EBV genome encoding the p18 and p40 proteins, using a monoclonal antibody (MAb) to VCA-p40 and VCA-p18 affinity-purified polyclonal human antibodies.

MATERIALS AND METHODS

Cell cultures and cell extracts. The P3HR1-derived cell line HH514.c16 (a gift of G. Miller, Yale University, New Haven, Conn.) was propagated as a suspension culture in roller bottles and induced for VCA and EA expression by using 20 ng of 12-tetradecanophorbol-13-acetate per ml and 3 mM sodium butyrate exactly as described previously (22). For the selective expression of EA antigens only, viral DNA polymerase was blocked by the addition of 0.5 mM phosphonoacetic acid to the induced cell culture.

MAbs. For the production of MAbs, BALB/c mice were immunized with the nuclear fraction of VCA-induced HH514 cells (38). Hybridomas were produced according to standard protocols, and supernatants were analyzed in standard EBV immunofluorescence tests and on immunoblot strips containing antigen extracts from VCA-induced HH514 cells as described by Middeldorp and Herbrink (22). One of the resulting hybridomas, EBV.OT41A, specifically reacted with a VCA polypeptide of 40 kDa (unpublished results).

Affinity purification of anti-p18 antibodies. Anti-p18 antibodies were purified from a human EBV-positive serum by the method of Robinson et al. (28), with some minor modifications. In short, following electrophoretic separation in 10% acrylamide gels (19), the proteins were blotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.), and the region corresponding to VCA-p18 was cut out and used as the affinity matrix. Nonspecific binding

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of antibodies to the strips was prevented by incubation overnight in blocking solution (5% dried milk powder–4% horse serum in phosphate-buffered saline, pH 7.4 [PBS]). Thereafter the strips were incubated with diluted human serum (1:25 in blocking solution) for 2 h. After three wash steps with PBS containing 0.05% Tween 20, the bound antibodies were eluted with 0.1 M glycine (pH 2.7) in two consecutive incubations. The eluate was neutralized with 1/20 volume of 1 M Tris-HCl (pH 9.0). Finally, the eluate was dialyzed against PBS and stored in aliquots at −20°C.

Rabbit immunization. A hyperimmune monospecific rabbit antiserum was prepared by immunizing a specific-pathogen-free rabbit with 0.45 mg of gel-purified BFRF3-βGal (a BFRF3–β-galactosidase [β-Gal] fusion protein) in Freund's complete adjuvant and then boosted two times (after 2 weeks each) with 0.45 mg of BFRF3-βGal in Freund's incomplete adjuvant.

RNA purification of HH514.c16 cells. Total RNA was isolated from induced HH514.c16 cells by the guanidinium-CsCl procedure as described by Maniatis and colleagues (21). Purification of poly(A)$^+$ RNA was performed by oligo(dT) chromatography (Pharmacia, Inc., Piscataway, N.Y.) as described by Ausubel et al. (1).

Northern (RNA) blot analysis. Total RNA (10 μg) was denatured by glyoxal (35) and run in agarose gels. After ethidium bromide staining, the separated RNA was vacuum blotted to nitrocellulose. After a 3-h prehybridization, the filter was hybridized overnight at 42°C with α-^{32}P-labeled (Amersham, Bristol, United Kingdom) randomly primed or nick-translated DNA probes. The hybridization solution consisted of 50% formamide, 5× SSPE, 5× Denhardt's solution (0.1% [wt/vol] polyvinylpyrrolidone, 0.1% [wt/vol] bovine serum albumin, 0.1% [wt/vol] Ficoll), 0.2 mg of sonicated herring sperm DNA per ml, and 0.5% sodium dodecyl sulfate (SDS). Subsequently, blots were washed and exposed to X-ray film (Eastman Kodak Co., Rochester, N.Y.), using intensifying screens.

Construction of a cDNA library in lambda gt11. Five micrograms of poly(A)$^+$-selected RNA was denatured with methylmercury hydroxide. The cDNA synthesis was primed with either oligo(dT) or hexanucleotides. Procedures for first- and second-strand synthesis were identical to those described by Gubler and Hoffman (16).

After *Eco*RI methylation, *Eco*RI linker addition, and *Eco*RI digestion, the modified cDNA was size selected by Sepharose CL4B (Pharmacia) chromatography as described by Maniatis et al. (21). cDNA varying in size from 0.5 to 3.0 kb was ligated into phosphatase-treated lambda gt11 arms and then subjected to in vitro packaging with use of the Packagene kit (Promega, San Diego, Calif.).

Immunological screening of the lambda gt11 library. A total of 10^4 recombinant phages of the oligo(dT)-primed library and 5×10^5 recombinants of the hexanucleotide-primed library were screened for immunological reactivity (21) with affinity-purified human anti-p18 antibodies or MAb EBV.OT41A. Immunoreactive plaques were detected with alkaline phosphatase-conjugated anti-human or anti-mouse immunoglobulin G as described by the manufacturer (Promega).

Nucleotide sequence analysis. Insert DNA of positive plaques was amplified by the polymerase chain reaction (PCR) technique, using primers of the lambda gt11 flanking sequences containing restriction sites at their 5′ ends (Table 1). After digestion with the appropriate restriction enzyme, the DNA fragment was subcloned in pGEM-4Z and sequenced from both sides, using a sequencing kit (Pharmacia, Uppsala, Sweden) which employs a modification of the method of Sanger et al. (30).

TABLE 1. Oligonucleotides used

Sequence	Description[a]
GGGGAATTCCGCACGCCGGCTGCCC ..+	primer BFRF3
CCAAGCTTCCCTGTTTCTTACGT .....−	primer BFRF3 fusion
CCAAGCTTCCCTACTACGTTTCT TACGT ..... −	primer BFRF3 nonfusion
CCGGAATTCCCTATCAGGTAACGCA ..+	primer BdRF1
CCAAGCTTCCAGCCACGCGTTTA .....−	primer BdRF1 fusion
CCAAGCTTCCCTACTAAGCCAC GCGTTTA ..... −	primer BdRF1 nonfusion
GGGGGATCCGGTGGCGACGACT CCTGGAGCCCG.....	Lambda gt11 forward *Bam*HI
GGGGAAGCTTTGACACCAGACCAA CTGGTAATG.....	Lambda gt11 reverse *Hin*dIII

[a] +, sense; −, antisense.

Cloning and expression of BFRF3 and BdRF1 in *Escherichia coli*. ORFs BFRF3 and BdRF1 were amplified by PCR, using virion DNA purified from HH514.c16 cells as a target. Primers of each set containing restriction sites at their 5′ ends (Table 1) were used for cloning the amplified fragment in the *Eco*RI-*Hin*dIII site of expression vector pMLB1113 (a derivative of PBR322) (40), which is located at the fifth codon of the 5′ end of the *lacZ* gene. Proteins expressed from these constructs consist of the first 5 amino acids of β-Gal followed by the recombinant protein linked at the C terminus to the remainder of β-Gal. Nonfusion proteins were constructed similarly, but with a stop codon inserted at the 3′ end of the insert.

***E. coli* expression of recombinant proteins.** Transformed *E. coli* cultures were induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). At 2 h postinduction, bacterial cells were collected by centrifugation, suspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, and analyzed by immunoblotting.

Peptide synthesis and immunoscreening (PEPSCAN). Peptides with a length of 12 amino acids and an overlap of 11 amino acids with the amino acid sequences of ORFs BFRF3 and BdRF1 were synthesized by automated solid-phase peptide synthesis onto chemically activated polyethylene pins as originally described by Geijsen et al. (13, 14). The immune reactivities of the human serum used for the isolation of VCA-p18-specific antibodies and for MAb EBV.OT41A were determined as described by Middeldorp and Meloen (23).

Western immunoblot analysis. After SDS-PAGE, proteins were transferred to nitrocellulose filters (0.2-μm pore size; Schleicher & Schuell, Den Bosch, The Netherlands). Nonspecific binding of antibodies to the filters was prevented by incubation for at least 1 h at room temperature with blocking buffer (4% dried milk powder–5% horse serum in Tris-buffered saline). Human sera were diluted to an appropriate dilution in blocking buffer and incubated for at least 1 h. Blots or strips were washed three times in Tris-buffered saline containing 0.05% Tween 20, and alkaline phosphatase-conjugated anti-human immunoglobulin antibody (Promega) was added. After further incubation and rinse steps, the blot was developed by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

2D gel electrophoresis. Sample preparation and two-dimensional (2D) gel electrophoresis were performed as described by Garrels (12), slightly modified as described by Berbers et

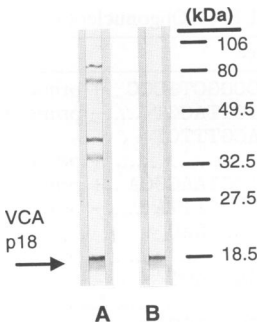

FIG. 1. Immunoblot analysis of the antibody responses of a human serum (A) and affinity-purified VCA-p18-specific immunoglobulins prepared from this serum (B). Antigens applied to the blot strips consist of polypeptides from HH514.c16 cells induced for expression of EA and VCA. Positions of molecular weight markers are shown at the right (12% polyacrylamide gel).

al. (3). Twenty-five micrograms of nuclear extracts from induced HH514.c16 cells was used for each 2D gel electrophoresis.

RESULTS

Characterization of human anti VCA-p18 antibodies and anti-p40 MAbs. For optimal screening of the expression library, it was necessary to use characterized immunological reagents. Human antibodies specific for VCA-p18 were immunoaffinity purified by binding to polyvinylidene difluoride membranes containing SDS-PAGE-separated HH514-derived VCA-p18. Figure 1 illustrates the immunological reactivity of human serum and the affinity-purified antibodies on blot strips containing HH514 VCA extract. The affinity purified antibodies react only with a protein band of 18 kDa.

MAb EBV.OT41A was obtained after immunization of a BALB/c mouse with the nuclear fraction of VCA-induced HH514.c16 cells. This MAb reacts with a structural protein of 40 kDa (Fig. 2A). To prove that the protein recognized by EBV.OT41A is the same as VCA-p40 recognized by sera of healthy EBV carriers, 2D immunoblots containing HH514.c16 antigens were prepared (Fig. 2B to D). The polypeptide identified with EBV.OT41A is detectable as four major spots at 40 kDa which differ only in their isoelectric points (some minor spots which probably represent precursor or breakdown products are also detectable). As a reference, a blot was double probed with MAb EBV.OT41A and R3 (Fig. 2C). MAb R3 is directed against the major EA-D antigen encoded in BMRF1 (6, 25). In accordance with published data (8), multiple spots were detectable. Comparison of this immunoblot with a blot double probed with MAb R3 and a polyclonal human serum with a strong reactivity against VCA-p40 (Fig. 2D) indicates that the VCA-p40 recognized by the human serum is identical to the protein recognized by EBV.OT41A.

Immunoscreening of the cDNA libraries. To detect the EBV genomic sequence encoding VCA-p18 and VCA-p40, oligo(dT)- and hexanucleotide-primed cDNA expression libraries were prepared in lambda gt11, using poly(A)-selected RNA from VCA-induced HH514.c16 cells as described in Materials and Methods. Transformants were immunologically screened by using affinity-purified human anti-p18 antibodies or EBV.OT41A. Two plaques reacting with the anti-p18 antibodies and one plaque positive with EBV.OT41A were identified. The cDNA insert sequence of the positive plaques was determined by PCR-based insert amplification followed by ligation into the sequencing vector pGEM-4Z. The resulting sequences were mapped on the EBV genome by using the computer program Microgenie (Beckman). The insert sequence of the plaques reacting with anti-p18 antibodies matched the 3′ end of ORF BFRF3, the oligo(dT)-primed cDNA insert from positions 61915 to 62088, and the random-primed cDNA insert from 61819 to 62046 (Fig. 3; positions according to Baer et al. [2]). These results suggest that VCA-p18 is encoded within BFRF3, located between positions 61507 and 62034 on the prototype B95-8 genome (2). The predicted molecular size of the protein encoded by BFRF3 is 18.1 kDa.

The cDNA insert of the plaque reacting with EBV.OT41A was mapped to the 3′ end of ORF BdRF1 at positions 149436 to 149749 (Fig. 3), indicating that VCA-p40 is encoded within BdRF1, located at positions 148707 to 149749 of the EBV genome. BdRF1 encodes a hypothetical protein with a molecular size of 36 kDa.

Confirmation of the binding epitope of EBV.OT41A. The PEPSCAN technique was used to confirm the binding specificity of EBV.OT41A with the putative protein encoded within ORF BdRF1. The immunological reactivity of EBV.OT41A with the individual overlapping peptides of the BdRF1-encoded protein is illustrated in Fig. 4. The MAb dominantly recognizes a peptide region localized at the C terminus of the protein (peptide 286-294; REDTNPQQPTTE GHHRGKKL). As expected, this epitope also is represented in the lambda transformant reacting with EBV.OT41A.

Preadsorption of human sera with *E. coli*-expressed fusion protein BFRF3-βGal inhibits the anti-VCA-p18 reactivity. An inhibition assay was carried out to confirm that the immunodominant VCA-p18 is encoded within BFRF3. For this purpose, the protein encoded by BFRF3 was expressed in *E. coli* as an insoluble fusion protein linked to the N terminus of β-Gal. BFRF3-βGal was purified by isotachoforesis from slices of SDS-polyacrylamide gels loaded with inclusion bodies. The inhibition assay was based on an overnight preadsorption of three human sera with increasing amounts of purified BFRF3-βGal and analysis of the anti-VCA-p18 activity on immunoblots. The results illustrate that it is possible to completely inhibit the anti-VCA-p18 reactivity of the sera on immunoblot strips with BFRF3-βGal (Fig. 5). However, preadsorption with BFRF3-βGal does not influence the reactivity of the sera with the other viral proteins (i.e., EBNA and VCA-p40), which indicates that the inhibition of the reactivity against VCA-p18 is specific. As a control, the sera also were preadsorbed with gel-purified *E. coli* β-Gal, which did not have any effect on the anti-VCA-p18 reactivity. This experiment further illustrates the specific inhibition of human polyclonal anti-VCA-p18 reactivity by the BFRF3 fusion protein. This finding confirms that VCA-p18 is encoded by BFRF3 and that a single protein species is responsible for the human antibody reactivity in the VCA-p18 region.

Rabbit serum raised against BFRF3-βGal reacts with a VCA protein of 18 kDa. A second experiment to confirm VCA-p18 as the product encoded by BFRF3 was done by immunization of a rabbit with gel-purified *E. coli*-expressed BFRF3-βGal. Antiserum of this rabbit was used to stain a blot containing antigens of EBV producer cell lines noninduced or induced for VCA or EA and an EBV-negative B-cell line (Fig. 6). The antiserum specifically reacts with a

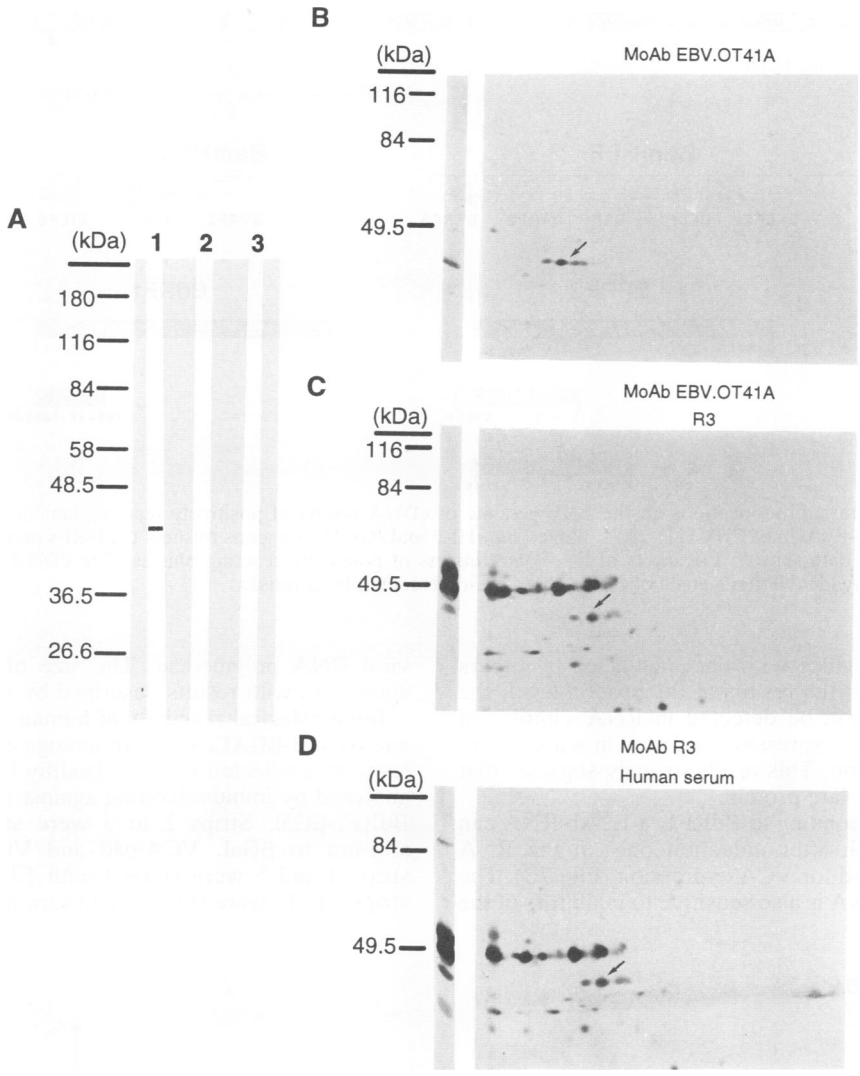

FIG. 2. Reactivity of MAb EBV.OT41A with VCA-p40 by 1D and 2D immunoblot analysis. (A) Immunoblot strips containing extracts of HH514 cells induced for expression of EA and VCA (strip 1), induced for expression of EA only (strip 2), or noninduced (strip 3), stained with EBV.OT41A. Immunoblots of a 2D gel containing HH514 (EA plus VCA) antigens were incubated with EBV.OT41A (B), EBV.OT41A plus R3 (C), or R3 plus a human serum with a strong reactivity against VCA-p40 (D). Positions of VCA-p40-related products are indicated by arrows. The strips at the left in panels B to D are blot strips containing HH514 (EA plus VCA) antigens separated by molecular weight only. Positions of molecular weight markers are shown at the left (12% polyacrylamide gel).

protein of 18 kDa only in extracts of EBV-producing cells induced for VCA expression.

Proteins encoded by RNAs of BFRF3 and BdRF1 are true late proteins. The VCA character of p18 and p40 was analyzed at the protein and RNA levels. For this purpose, HH514 cells were isolated at different times after induction for VCA or EA expression. As control, the expression of EA-related proteins in isolated cell extracts was tested by immunoblotting with MAb R3, which is directed against the major EA protein p47-52 (25), encoded by BMRF1 (Fig. 7A). As expected, a strongly reactive band was detected in cells induced for both early and late antigens. To study the expression of VCA-p18, human antibodies bound to nitrocellulose-immobilized BFRF3-βGal fusion protein were eluted by glycine and used to probe immunoblots containing the extracts expressing VCA and EA. Starting at 48 h after induction, a major protein band of 18 kDa and two additional minor protein bands of 20 and 17 kDa were detected (Fig. 7A) in antigen extracts of VCA-induced cells. There was no immunological reactivity detectable in the EA extracts at any time after induction, indicating that the expression product of BFRF3 belongs to the class of true late VCA. Figure 7A shows the analysis with EBV.OT41A. As with VCA-p18, a protein at 40 kDa is detectable in the extracts of VCA-induced cells only, starting at 48 h after induction. In the HH514 EA extracts, no protein band can be detected.

The expression of virus-related antigens was analyzed also at the RNA level (Fig. 7B), using a probe corresponding to ORF BMRF1. Two early transcripts of 2.6 and 3.5 kb can be detected in cells induced for VCA and EA expression, which is in agreement with the results of Pfitzner et al. (26). Analysis of BFRF3 at the RNA level resulted in the detection of an RNA of 0.8 kb after 48 h in RNA samples of cells induced for VCA expression (Fig. 7B). The size of the

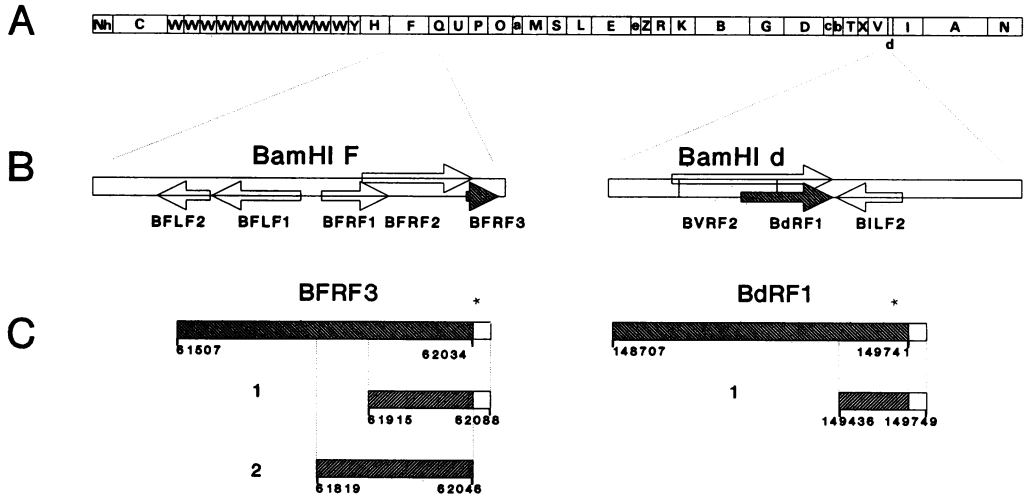

FIG. 3. Diagram illustrating the locations on the EBV genome of cDNA inserts of positively reacting lambda gt11 clones. (A) *Bam*HI restriction map of the B95-8 strain of EBV (11). (B) Enlarged *Bam*HI F and *Bam*HI d regions to show the ORFs predicted from the published B95-8 genomic sequence data (2). (C) Locations of the cDNA inserts of positively reacting phages. The cDNA inserts are indicated by numbers. Positions of polyadenylation signals of the mRNAs are represented by asterisks.

messenger is in accordance with data published by others (11). In agreement with the results at the protein level, the BFRF3-related RNA can be detected in RNA samples of cells induced for VCA expression and not in samples induced for EA expression. This result strongly suggests that BFRF3 encodes a true late protein.

With a probe corresponding to BdRF1, a 1.2-kb RNA can be detected from 48 h after induction only in the RNA samples of cells induced for VCA expression (Fig. 7B). The transcription of this RNA is also sensitive to inhibitors of the viral DNA polymerase. The size of the messenger is in agreement with results described by others (11).

Immunological reactivity of human sera with BdRF1-βGal and BFRF3-βGAL. The immunological reactivity of antibodies from a selected panel of healthy human individuals was analyzed by immunoblotting against both BFRF3-βGal and BdRF1-βGal. Strips 1 to 3 were stained with reference antisera to βGal, VCA-p40 and VCA-p18, respectively, strips 4 and 5 were stained with EBV-negative sera, and strips 6 to 15 were stained with sera from EBV-seropositive

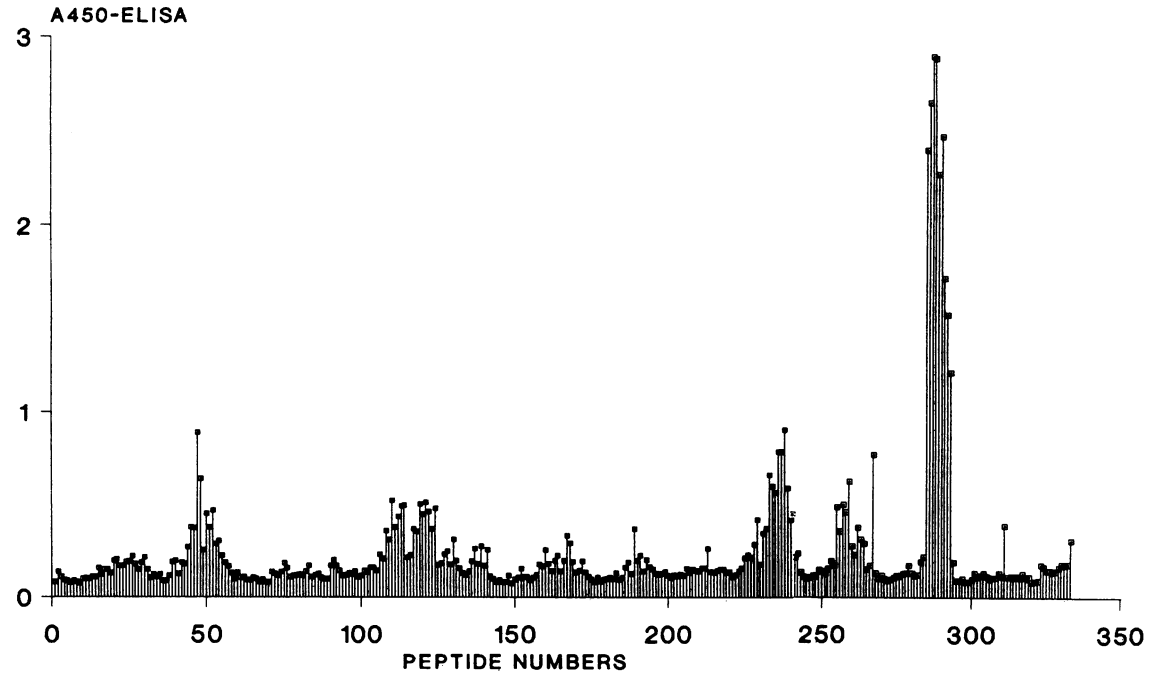

FIG. 4. PEPSCAN results of MAb EBV.OT41A reactive with VCA-p40, using overlapping peptides derived from the predicted sequences of BdRF1. Peptides were probed with protein A-purified EBV.OT41A at a concentration of 20 μg/ml. ELISA, enzyme-linked immunosorbent assay.

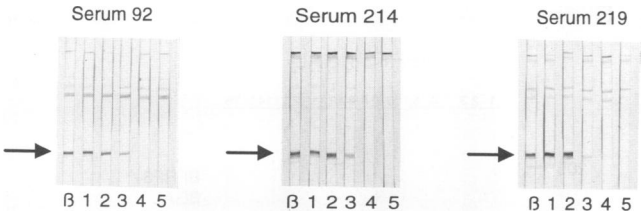

FIG. 5. Immunoadsorption of the VCA-p18 activities of human sera 92, 214, and 219. Sera were probed on blot strips containing antigen of HH514 cells induced for EA and VCA expression which were separated on a 12% polyacrylamide gel. Each serum was preadsorbed overnight with 10 μg of gel-purified β-Gal (lane β) or with 0, 0.01, 0.1, 0.5, and 1 μg of gel-purified BFRF3-βGal (lanes 1 to 5, respectively).

individuals (VCA^+ EA^-) with staining intensity different from that of VCA-p40 and VCA-p18. Strip 16 was incubated with serum from a patient with chronic EBV infection (VCA^+ EA^+). The immunological reaction of this serum panel with natural antigens of HH514.VCA cells is illustrated in Fig. 8A. Figure 8B illustrates the reactivity on blot strips containing *E. coli*-expressed BFRF3-βGal, and Fig. 8C shows the reactivity with BdRF1-βGal. Anti-*E. coli* (β-Gal) reactivity of the sera was tested on blot strips containing *E. coli* extracts expressing β-Gal only (Fig. 8D). No or only marginal reactivity was found.

The results show directly comparable intensities of immune staining of the human sera with viral p18 and p40 and the expression products encoded by BFRF3 and BdRF1, respectively.

DISCUSSION

The immunodominant VCA-p18 is encoded by ORF BFRF3. Screening of EBV-related cDNA expression libraries with affinity-purified human anti-VCA-p18 antibodies resulted in detection of ORF BFRF3 encoding VCA-p18. This finding is confirmed further by results of two experiments. First, the complete adsorption of VCA-p18 reactivity from human sera with purified *E. coli*-expressed BFRF3-βGal indicates that a single protein is responsible for the strong immunoreactivity in this region (Fig. 5). Second, antiserum of a rabbit which was immunized with purified BFRF3-βGal specifically reacts with an 18-kDa viral protein of the VCA complex. In addition, PEPSCAN analysis has shown that the human serum, used for the purification of the

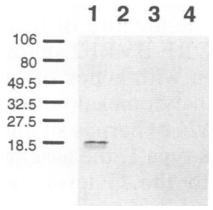

FIG. 6. Immunological response of a rabbit immunized with gel-purified BFRF3-βGal. The immunoblot contains nuclear antigen extracts of HH514 cells induced for EA and VCA expression (lane 1), induced for EA expression (lane 2), or noninduced (lane 3), separated on a 12% polyacrylamide gel. As a control, nuclear antigen extract of the EBV-negative RAMOS cell line was used (lane 4). Positions of molecular markers (in kilodaltons) are shown at the left.

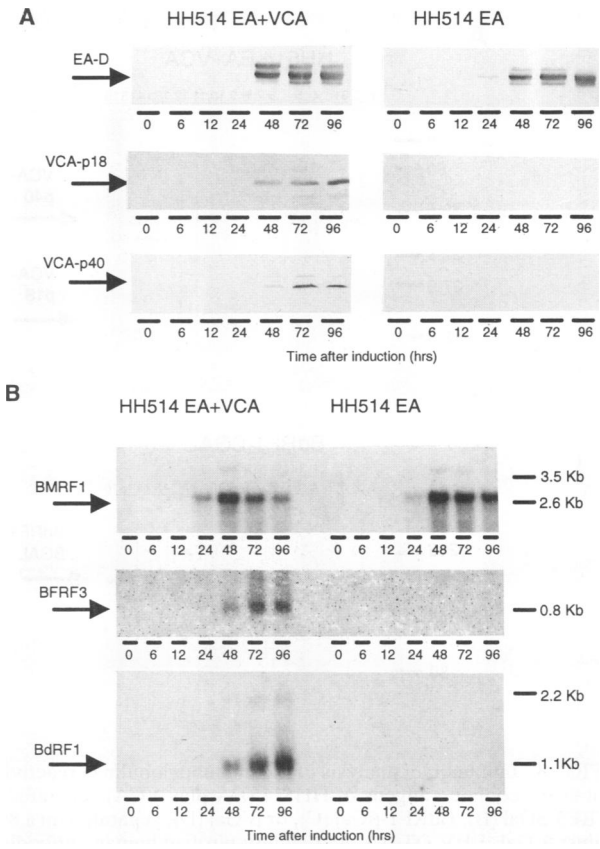

FIG. 7. (A) Immunoblots showing the time of appearance of VCA-p18 and VCA-p40 after induction for expression of lytic-phase proteins. Antigens applied to the blots are whole cell lysates of HH514 cells induced for expression of EA and VCA or EA only and isolated at different times after induction. Blots were incubated with MAb R3 against EA-D as a control for EA expression (EA-D), affinity-purified human anti-BFRF3 antibodies (VCA-p18), or EBV.OT41A (VCA-p40). Numbers below the immunoblots represents time of isolation after induction (12% polyacrylamide gel). (B) Autoradiographs of RNA blots hybridized with a probe specific for ORFs BMRF1, BFRF3, and BdRF1. Total RNA samples were prepared at various times after induction of EA and VCA or EA only. As probes for the nick translation fragment BMRF1 (79894 to 81106), fragment BFRF3 (61507 to 62034) digested with *BglI* and fragment BdRF1 (149436 to 149749) were used. Arrows at the right indicate the positions of the expected mRNA for each ORF (1.2% agarose gels).

antibodies against VCA-p18, recognizes three peptide regions localized at the C terminus of the protein of BFRF3 (data not shown). Finally, the directly comparable intensities of immune staining of human sera with viral p18 and the *E. coli* expression product encoded by BFRF3 (Fig. 8) further support the evidence that BFRF3 encodes VCA-p18.

ORF BdRF1 encodes VCA-p40. Screening of the cDNA expression library with MAb EBV.OT41A, which reacts with VCA-p40, resulted in the detection of ORF BdRF1. The specificity and epitope reactivity of MAb EBV.OT41A with the protein encoded by BdRF1 was confirmed by PEPSCAN analysis. No further studies were performed to confirm the specificity of MAb EBV.OT41A with the BdRF1 product, as it reacted on 2D blots with VCA-p40 identically to human serum antibodies (Fig. 2).

ORFs BdRF1 and BFRF3 encode true late expression prod-

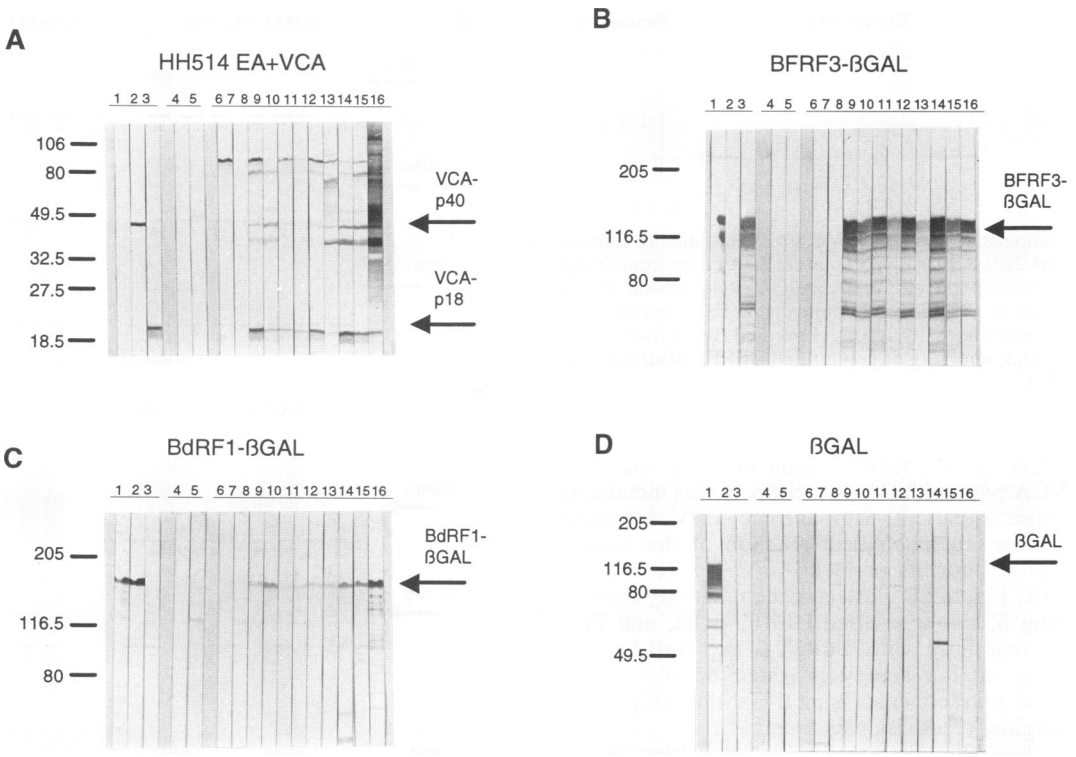

FIG. 8. Immunoblot analysis of the immunoglobulin G reactivity of a selected panel of human sera against BFRF3-βGal and BdRF1-βGal. Blot strips contain antigens of HH514 (EA plus VCA) separated on a 12% polyacrylamide gel (A) or protein extracts of *E. coli*-expressing BFRF3-βGal (B), BdRF1-βGal (C), or β-Gal (D), separated on a 8% polyacrylamide gel. As a control, strips 1 to 3 were incubated with a MAb against β-Gal, EBV.OT41A, and affinity-purified human antibodies against VCA-p18, respectively. Strip 4 and 5 were incubated with serum of two EBV-negative human individuals. Strips 6 to 16 were incubated with sera of EBV-positive individuals with different reactivities to VCA-p18 and VCA-p40. Positions of molecular weight markers (in kilodaltons) are shown at the left; positions of the reactive proteins of interest are indicated by arrows.

ucts. Immunoblot and Northern blot analyses demonstrate that the BFRF3 and BdRF1 genes encode proteins which have features of true late antigens (Fig. 7). Both proteins are associated with the capsid structure of the EBV virion (36). A 0.8-kb mRNA is transcribed from the BFRF3 gene and is translated into a protein with a predicted size of 18.1 kDa without potential N-linked glycosylation sites (17). The ORF BFRF3-specific purified human antiserum reacted with a major protein of 18 kDa and two minor proteins of 17 and 20 kDa, possibly indicating posttranslational modification of the VCA-p18 protein. The *Bam*HI F region of the EBV genome is transcribed in a very complex manner which has been studied extensively by Hudson et al. (17). They showed that BFRF3 is transcribed as an unspliced early messenger of 0.8 kb potentially encoding a basic protein of 18 kDa. The described characteristic of an early messenger contrasts with our results, which clearly demonstrate characteristics of a true late messenger. A possible explanation for this discrepancy could be the use of different conditions for blocking the viral DNA polymerase activity. This block is virtually 100% under the conditions used in our studies (22).

2D blot analysis indicates that VCA-p40 is expressed as a complex of proteins with different isoelectric points (Fig. 2B), suggesting posttranslational phosphate modifications similar to those described for the BMRF1-encoded protein (8). The 1.2-kb messenger of ORF BdRF1 (11) is translated into a protein without potential N-linked glycosylation sites with a predicted size of 36 kDa. The predicted size for the product of BdRF1 deviates somewhat from the size of 40 kDa determined in this study.

Farrel (11) reported that a protein encoded by BdRF1 is 100% identical to the C terminus of the early protein encoded within BVRF2. This finding suggests that a MAb directed against VCA-p40, like EBV.OT41A, also should react with a protein of 64 kDa (2) encoded within BVRF2 at early times after infection or in EA-specific extracts. In our studies, we detected a protein band of 40 kDa (and a faint band at 53 kDa) in VCA-induced cells only. Northern blot analysis shows, besides the 1.2-kb BdRF1 messenger, a messenger of 2.2 kb which may correspond to the messenger of BVRF2, which under our conditions also has the characteristics of a true late messenger. The identity of this larger mRNA was not analyzed further. ORF BVRF2 shares some homologies in genome organization with genes of other herpesviruses, such as UL80 of human cytomegalovirus, VZV33 of varicella-zoster virus, and UL26 of herpes simplex virus (4). In case of herpes simplex virus type 1, the assembly protein (ICP35: VP22a) corresponds to the C terminus of the predicted protein encoded by the UL26 gene (27). This genome organization is comparable to that of EBV with regard to the organization of BdRF1 and BVRF2. A recent study by Lau et al. (20), also using MAb EBV.OT41A, has indicated that the VCA-p40 protein selected by EBV.OT41A is derived from ORF BdRF1 and is expressed strongly in oral hairy leukoplakia lesions from AIDS patients. Computer comparison of published genomic sequences of other members of

the human herpesvirus family with the sequences of VCA-p18 and VCA-p40 did not reveal a homologous protein.

Are VCA-p18 and VCA-p40 important diagnostic markers? In the diagnosis of EBV-related diseases, the antibody activity (serum titer) against EBV VCA proteins is considered an important serological parameter (24). Immunoblot analysis with a panel human sera has shown that VCA-p18 and to a lesser extend VCA-p40 are the most relevant markers for the EBV VCA complex and for EBV seropositivity diagnostics (36). From this study, it was concluded that VCA-p18 can be considered to be a single dominant immunoreactive antigen for use in VCA diagnosis. The diagnostic value of VCA-p18 and VCA-p40 for serologic tests to detect diseases such as nasopharynx carcinoma, Burkitt's lymphoma, EBV-associated lymphoma, and oral hairy leukoplakia requires further evaluation.

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