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Identification and Characterization of an Epstein-Barr Virus Early Antigen That Is Encoded by the *Not*I Repeats

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The Epstein-Barr virus (EBV) genome is characterized by two regions carrying partially homologous clusters of short tandem repeats (*Not*I and *Pst*I repeats) flanked by 1,044 and 1,045 base pairs with almost perfect homology (D_L and D_R, left and right duplications, respectively). Both repetitive regions are transcribed into poly(A)$^+$ mRNA after induction of the productive EBV cycle with the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate and contain open reading frames. To identify the potential protein encoded by the *Not*I repeat open reading frame (BHLF1), two repeat units of EBV strain M-ABA were expressed using the tryptophan-regulated *Escherichia coli* expression vector pATH11. Rabbit antisera generated against the resulting fusion protein reacted specifically with a protein varying in molecular size between 70,000 and 90,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, found after 12-*O*-tetradecanoyl-phorbol-13-acetate or *n*-butyrate induction in various cell lines harboring EBV. In immunofluorescence tests with the BHLF1-specific antiserum, an immunofluorescence with EA-D specificity could be observed. In addition, the BHLF1 protein is exhibiting polyanion-binding activity with a maximum for single-stranded DNA. Furthermore, the fusion protein is recognized by a number of human EBV-positive sera.

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, causes infectious mononucleosis, a self-limiting lymphoproliferative disease (14). It is also associated with two human malignant tumors, Burkitt's lymphoma and nasopharyngeal carcinoma (for a review, see reference 7). Furthermore, EBV has the ability to immortalize human B lymphocytes by establishing a latent infection in which the majority of viral genes are not expressed (13).

In most EBV-infected cell lines a productive viral cycle can be chemically induced, for example, by treatment with the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (37, 38) or with *n*-butyric acid (23). The EBV-encoded early antigens (EA) are synthesized before replication of the viral genome, followed by synthesis of the late antigens and production of virions. Phosphonoacetic acid (PAA) inhibits herpesviral DNA polymerases (20). It can be used for the identification of components of the EBV-encoded EA complex (10).

The EBV genome isolated from viral particles is a linear double-stranded DNA (dsDNA) molecule of about 175 kilobase pairs (kbp) (1). The EBV prototype M-ABA (2, 4) is characterized by two almost perfect homologous regions of 1,044 and 1,045 bp (19). These regions are separated by more than 100 kbp on the viral genome (27) and are called duplication left (D_L) and duplication right (D_R). D_L and D_R have recently been shown to contain the origins of replication of the lytic cycle (oriLyt) of EBV (11). Soon after induction of EBV-infected cells, two very abundant transcripts are started from D_L and D_R. Both RNAs are transcribed from right to left across the regions adjacent to D_L and D_R. The transcripts are polyadenylated and can be purified from the polyribosomal fraction of the cytoplasm (N. Mueller-Lantzsch, unpublished results). Furthermore, they contain open reading frames (ORFs) consisting of short tandem repeats: the *Not*I repeats (125-bp repeat) on the D_L transcript and the *Pst*I repeats (102-bp repeat) on the D_R transcript (12, 15). Variations among different EBV strains in the lengths of these transcripts are due to differing numbers of repeat units (8). The ORF encompassing the *Not*I repeats is also called BHLF1 (1) and contains up to 17 repeat units (24). BHLF1 is adjacent to the ORF encoding the latent gene product EBNA2. Both ORFs are deleted in the genome of the nonimmortalizing EBV strain P3HR1 (3, 17). These genes are therefore good candidates in the search of functions responsible for the immortalizing potential of EBV. Early after infection of tonsillar B lymphocytes with EBV strain B95-8, the BHLF1 ORF is transcribed together with the messages for the latent EBV products in the initial phase of immortalization. In this system BHLF1 is transcribed in the presence of cycloheximide and has been assigned an immediate early gene (35).

We identified and characterized the protein encoded by BHLF1. This protein is variable in molecular weight in different EBV-positive cell lines probably due to various numbers of *Not*I repeats. It is expressed in vivo. This BHLF1-encoded protein belongs to subgroup EA-D of EBV-encoded antigens and exhibits in vitro binding activities comparable to those of the polyanions dsDNA-cellulose and phosphocellulose. Furthermore, this protein appears to have stronger binding to single-stranded DNA (ssDNA)–cellulose.

MATERIALS AND METHODS

Cells and reagents. The origin of BJAB, BL41, Jijoye, P3HR1, B95-8, M-ABA, BL72, BL74, and QIMR-WIL is described elsewhere (9, 26, 36). Cells were maintained in RPMI 1640 medium (GIBCO Laboratories) supplemented with 10% fetal calf serum, penicillin (40 IU/ml), streptomycin (50 μg/ml), nystatin (10 IU/ml), and neomycin sulfate (10 μg/ml). Cells were subcultured routinely once or twice weekly. For EBV induction, TPA (dissolved in dimethyl sulfoxide) was added to the cell culture at a concentration of 20 ng/ml or *n*-butyric acid was added at a concentration of 3 mM (37) or both were added.

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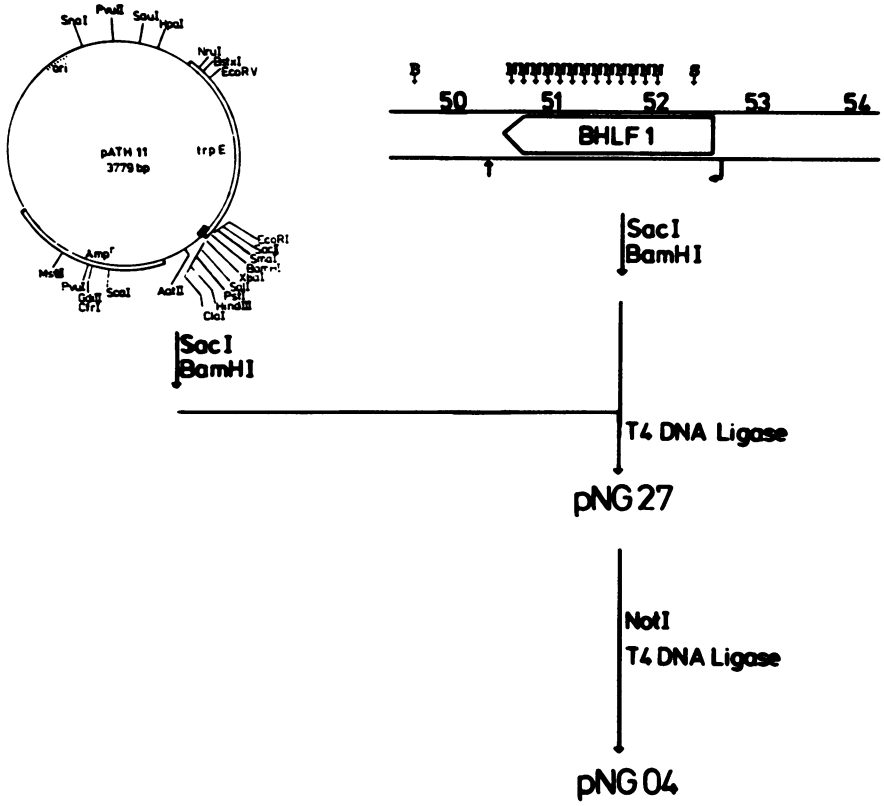

FIG. 1. Cloning strategy for ligation of parts of the BHLF1 reading frame into expression vector pATH11 as described in Materials and Methods. The position of BHLF1 on the B95-8 DNA (1) is indicated by numbers (kilobase pairs), and the sites of restriction enzymes are indicated by arrows. N, *Not*I; S, *Sac*I; B, *Bam*HI.

For inhibition of the EBV-specific DNA polymerase, PAA was added together with the inducers to the cell culture at a concentration of 100 μg/ml.

Tunicamycin was added to the cell culture at a concentration of 2.5 μg/ml.

Equal amounts of protein were used for polyacrylamide gel electrophoresis (PAGE) after estimation of the protein concentration by optical density at 280 nm or by the method of Lowry et al. (22).

Immunofluorescence. EA fluorescence was assayed by indirect immunofluorescence as previously described (9) with the use of specific rabbit antisera and fluorescein isothiocyanate-labeled goat anti-rabbit sera.

Procaryotic expression of BHLF1. The 2.7-kbp *Bam*HI-*Sac*I fragment of M-ABA DNA clone 760-22 (25) encompasses more than 90% of the *Not*I repeat ORF (BHLF1, coordinates 52557 to 50578 of B95-8 DNA) (1). The fragment was isolated and ligated into the polylinker of the tryptophan-regulated *Escherichia coli* expression vector pATH11 (T. J. Koerner, unpublished data). The resulting construct, pNG27, encodes a fusion protein containing the amino-terminal moiety (37 kilodaltons [kDa]) of the anthranilate synthetase (TrpE) of *E. coli* and an additional 70 kDa of the BHLF1-encoded protein (Fig. 1). Digestion of pNG27 with *Not*I and religation resulted in construct pNG04. This construct encodes 145 BHLF1-specific amino acids in addition to the TrpE moiety (Fig. 1). Production of the fusion proteins was induced as described previously (32). Bacteria were harvested and prepared in sodium dodecyl sulfate (SDS)-PAGE Laemmli buffer (18).

The 2.1-kbp *Nco*I fragment of M-ABA DNA clone 760-22 (25) contains the entire BHLF1 reading frame. The fragment was isolated and ligated into the procaryotic expression vector pJLA602 (29) behind the strong promoters P_R and P_L of bacteriophage lambda. *E. coli* bacteria of strain SG21063 (23a) were transformed with construct pJNG21 containing the insert in the desired orientation. Induction of the non-fused BHLF1-specific protein was achieved by shifting the incubation temperature to 42°C. Bacterial extracts were analyzed by SDS-PAGE and immunoblotting.

Purification of fusion proteins and preparation and testing of antisera. Fusion proteins were purified by SDS-PAGE. After electrophoresis, gels were stained in "aqueous Coomassie" (0.6% Coomassie blue in 100 mM Tris hydrochloride [pH 7.4]–20% methanol) (34). Fusion protein bands were excised, and the protein was eluted with elution buffer (50 mM NH_4HCO_3, 5% mercaptoethanol). After lyophilization the protein was dissolved in phosphate-buffered saline and used for immunization (approximately 300 μg of fusion protein per rabbit) as described previously (33). To control the specificity, a sample of immune serum was preincubated with 50 μg of purified TrpE protein or pNG04-encoded fusion protein and subsequently tested in an immunoblot.

DNA-binding experiments. To test DNA-binding activity, cell extracts were applied to cellulose, thymus dsDNA-cellulose, thymus ssDNA-cellulose, or phosphocellulose columns (Sigma Chemical Co.) (30). To avoid possible denaturation, all experiments were carried out immediately after preparation of the extracts. One milliliter of each extract (ca. 5×10^6 cells) was passed over 1.1-ml columns. The columns had been preequilibrated with equilibration buffer (10 mM NaH_2PO_4 [pH 7.5], 0.14 M NaCl, 1 mM

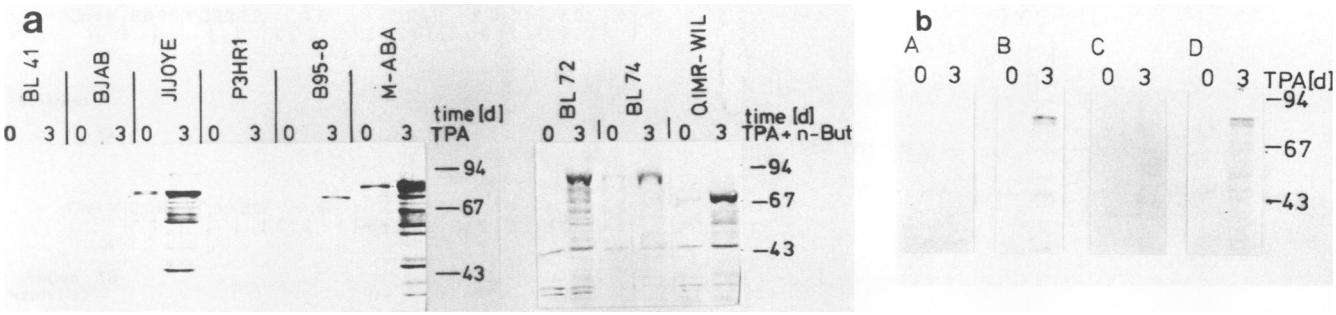

FIG. 2. (a) Protein analysis by immunoblotting of EBV genome-negative (BL41 and BJAB) and -positive (Jijoye, P3HR1, B95-8, M-ABA, BL72, BL74, and QIMR-WIL) lymphoid cell lines, using rabbit anti-pNG04 serum. The extracts had been prepared from uninduced cells ($t = 0$) or from cells treated for 3 days ($t = 3$) with TPA or TPA and *n*-butyrate, as indicated. (b) Protein analysis by immunoblotting of Jijoye cells, uninduced ($t = 0$) or treated with TPA for 3 days ($t = 3$), using preimmune serum (A) or anti-pNG04 immune serum, preincubated with purified TrpE protein (B), preincubated with purified pNG04 fusion protein (C), and without preincubation (D). The apparent molecular weights (in thousands) are estimated by comparison with comigrating marker proteins.

EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5% Nonidet P-40, 1 μg of aprotinin per ml). After the columns were washed with 8 volumes of equilibration buffer, elution of bound proteins was performed by applying stepwise a series of equilibration buffers containing increasing concentrations of NaCl (0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 M). Samples of each fraction were precipitated in 20% trichloroacetic acid and analyzed by SDS-PAGE and immunoblotting.

RESULTS

Procaryotic expression of BHLF1. To generate a polyclonal monospecific serum specific for the putative protein encoded by BHLF1, TrpE fusion proteins (pNG27 and pNG04 [Fig. 1]) were constructed. pNG27 encodes a fusion protein with a size of more than 100 kDa. After 2 h of induction, this fusion protein was expressed in *E. coli* C600 in low amounts and was identified only in immunoblots by using a rabbit anti-TrpE serum (data not shown). On the other hand, the pNG04-encoded fusion protein (57 kDa) was stably expressed in *E. coli* C600, as judged by staining of the gels with Coomassie blue and by analyzing the fusion protein with an anti-TrpE serum in immunoblots (data not shown). The fusion protein encoded by pNG04 was purified and used for immunization of rabbits.

Identification of the BHLF1-encoded protein. In cell extracts of EBV-harboring lymphoblastoid cell lines (Jijoye, B95-8, M-ABA, BL72, BL74, and QIMR-WIL), rabbit anti-pNG04 serum reacts with proteins varying in apparent molecular size between 70,000 and 90,000 and, especially after chemical induction, with their degradation products (Fig. 2a). No proteins could be specifically detected in EBV-negative cell lines (BL41 and BJAB) or in a cell line infected by BHLF1-deleted EBV strain P3HR1 (Fig. 2a).

The BHLF1 protein, detected before induction in producer lines, results from spontaneous activation of the viral cycle since only a few cells express the protein (see immunofluorescence data). Furthermore, a lymphoblastoid cell line, obtained by in vitro immortalization of cord blood lymphocytes with M-ABA virus, is not inducible for the synthesis of antigens of the productive cycle. This cell line, called M-ABA CBL, is negative for the BHLF1-encoded protein, too (data not shown).

Preincubation of immune serum with purified pNG04-encoded fusion protein abolishes the specific reaction with extracts of EBV-positive cell lines, while preincubation of immune serum with purified TrpE does not. Preimmune serum shows no reactivity against cellular proteins (Fig. 2b).

To prove further the specificity of rabbit anti-pNG04 fusion protein serum, this serum was tested on extracts of *E. coli* SG21063 transformed with pJNG21. This pJNG21 construct contains the complete M-ABA BHLF1 reading frame under the control of the strong bacteriophage lambda promoters P_R and P_L. Anti-pNG04 immune serum recognizes specifically the BHLF1-encoded gene product expressed as a nonfused protein after 2 or 4 h of induction (Fig. 3). Even in this protease Lon-mutated strain of *E. coli*, a rapid degradation of the BHLF1-encoded gene product could be observed. Anti-pNG04 immune serum also reacts with a number of proteins with an apparent molecular weight of between 50,000 and 60,000 in the extracts of bacteria transformed by vector pJLA602 alone or by construct pJNG21. This background reactivity could be due to bacterial proteins copurified with the fusion protein before immunization since preimmune serum shows almost no reaction against bacterial proteins (data not shown).

Induction of the BHLF1-encoded protein. To study the nature of the BHLF1-encoded protein, various cell lines

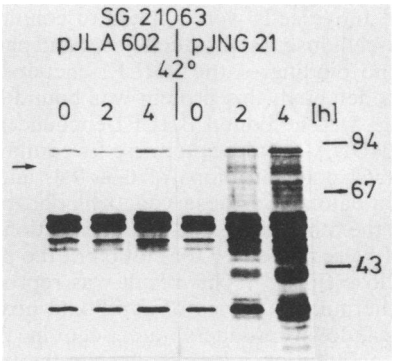

FIG. 3. Analysis of the specificity of rabbit anti-pNG04 serum. Extracts of *E. coli* SG21063, transformed with expression vector pJLA602 or construct pJNG21 and induced for expression for 2 and 4 h, are analyzed by immunoblotting, using rabbit anti-pNG04 serum. The pJNG21 construct contains the complete M-ABA BHLF1 reading frame without fusion to a bacterial gene, as described in Materials and Methods. The position of the pJNG21 protein with an apparent molecular size of 85,000 is indicated by an arrow.

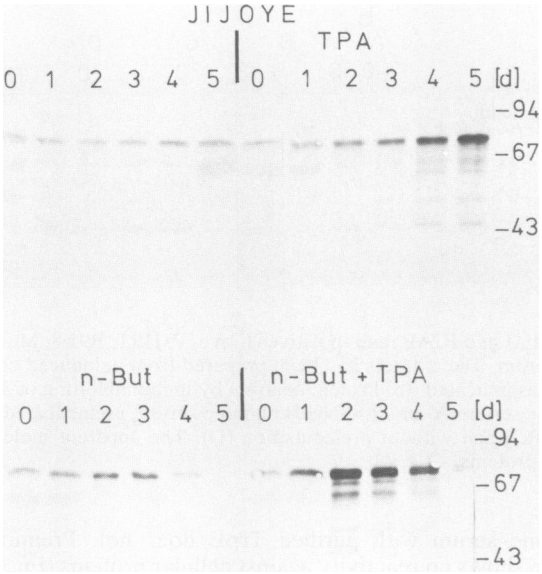

FIG. 4. Induction (0 to 5 days) of the BHLF1-encoded protein by treatment with TPA or *n*-butyric acid (n-But) or both in Jijoye cells and analysis of equal amounts of protein by immunoblotting, using rabbit anti-pNG04 serum.

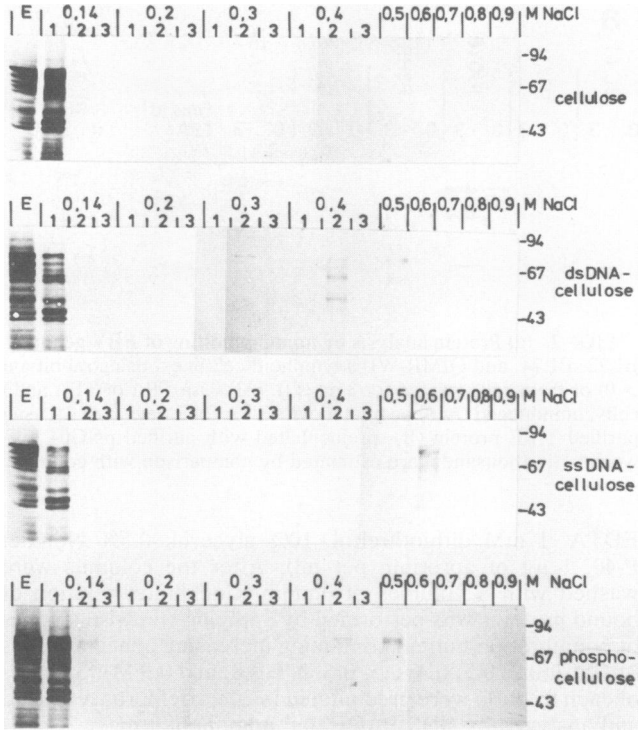

FIG. 5. Immunoblot analysis of Jijoye cell extracts eluted by stepwise increasing NaCl concentrations from cellulose, dsDNA-cellulose, ssDNA-cellulose, and phosphocellulose columns, using rabbit anti-pNG04 serum. E, Cell extract. The numbers on top describe the fractions eluted from the columns.

were treated with EBV-inducing substances and with a viral DNA synthesis inhibitor. The BHLF1-encoded protein is inducible in cells by TPA and, slightly, by *n*-butyric acid. Maximal induction is achieved by a combination of these two inducers (Fig. 4). The induction is resistant to PAA treatment of the cells while, in contrast, the induction of the EBV-encoded late protein gp350/220 is sensitive to PAA treatment, as judged by analyzing the same cell extracts in immunoblots with a rabbit serum specific for these two glycoproteins (data not shown). The results obtained with the EBV-inducing substances (TPA and *n*-butyric acid), as well as with PAA, suggest that this protein belongs to the EA complex of EBV.

DNA-binding experiments. The BHLF1-encoded protein was tested for in vitro DNA-binding activity. Extracts of TPA-induced Jijoye cells were applied to columns of cellulose, dsDNA-cellulose, ssDNA-cellulose, and phosphocellulose. While no binding of the BHLF1-encoded protein to cellulose was detected, this protein was bound by dsDNA-cellulose (Fig. 5). The bound BHLF1-encoded protein was eluted from dsDNA-cellulose stepwise by equilibration buffers, using NaCl concentrations of 0.3, 0.4, and 0.5 M. A similar elution pattern was observed using phosphocellulose columns. On the other hand, NaCl concentrations of 0.5 and mainly 0.6 M were necessary for elution of the protein from ssDNA-cellulose (Fig. 5). This result was reproducibly observed. Furthermore, a series of additional protein bands, probably breakdown products, are seen in the extract, run-through, and elution fractions, despite the use of protease inhibitors (aprotinin and phenylmethylsulfonyl fluoride). Potential cross-reacting cellular or viral proteins, visible in immunoblots only after chromatographic enrichment, could be excluded by performing the same experiments with extracts of cells infected by BHLF1-deleted EBV strain P3HR1 (data not shown).

In vivo phosphorylation of Jijoye cells with $^{32}P_i$, followed by immunoprecipitation with rabbit anti-pNG04 serum, PAGE, and autoradiography, did not give any indication of this posttranslational modification of the BHLF1-encoded protein (data not shown). The successful immunoprecipitation of the BHLF1 protein from Jijoye cells was confirmed by immunoblotting. Furthermore, the phosphorylation of EA phosphoprotein pp58/50 could be observed under the same experimental procedure (28).

The BHLF1 protein contains a potential site for N-linked terminal glycosylation (position 105, Asn-Pro-Thr). Inhibi-

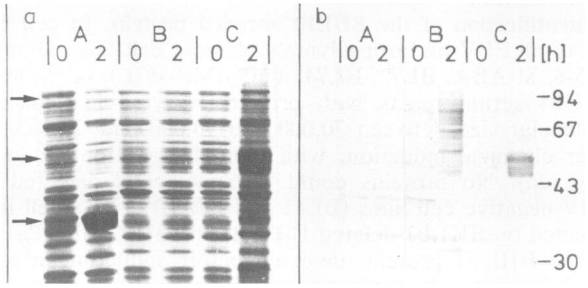

FIG. 6. Analysis of extracts of *E. coli* C600 transformed with expression vector pATH11 (A), construct pNG27 (B), or construct pNG04 (C). The extracts had been prepared from bacteria either uninduced ($t = 0$) or induced for expression of TrpE proteins for 2 h ($t = 2$). (a) Coomassie blue staining of SDS-PAGE; (b) immunoblot analysis, using human EBV-positive serum. This human serum exhibits an EA titer of 128. The arrows indicate the position of the truncated TrpE protein (37 kDa), the pNG27 recombinant protein (100 kDa), or the pNG04-encoded fusion protein (57 kDa). The apparent molecular weights are estimated by comparison with comigrating marker proteins.

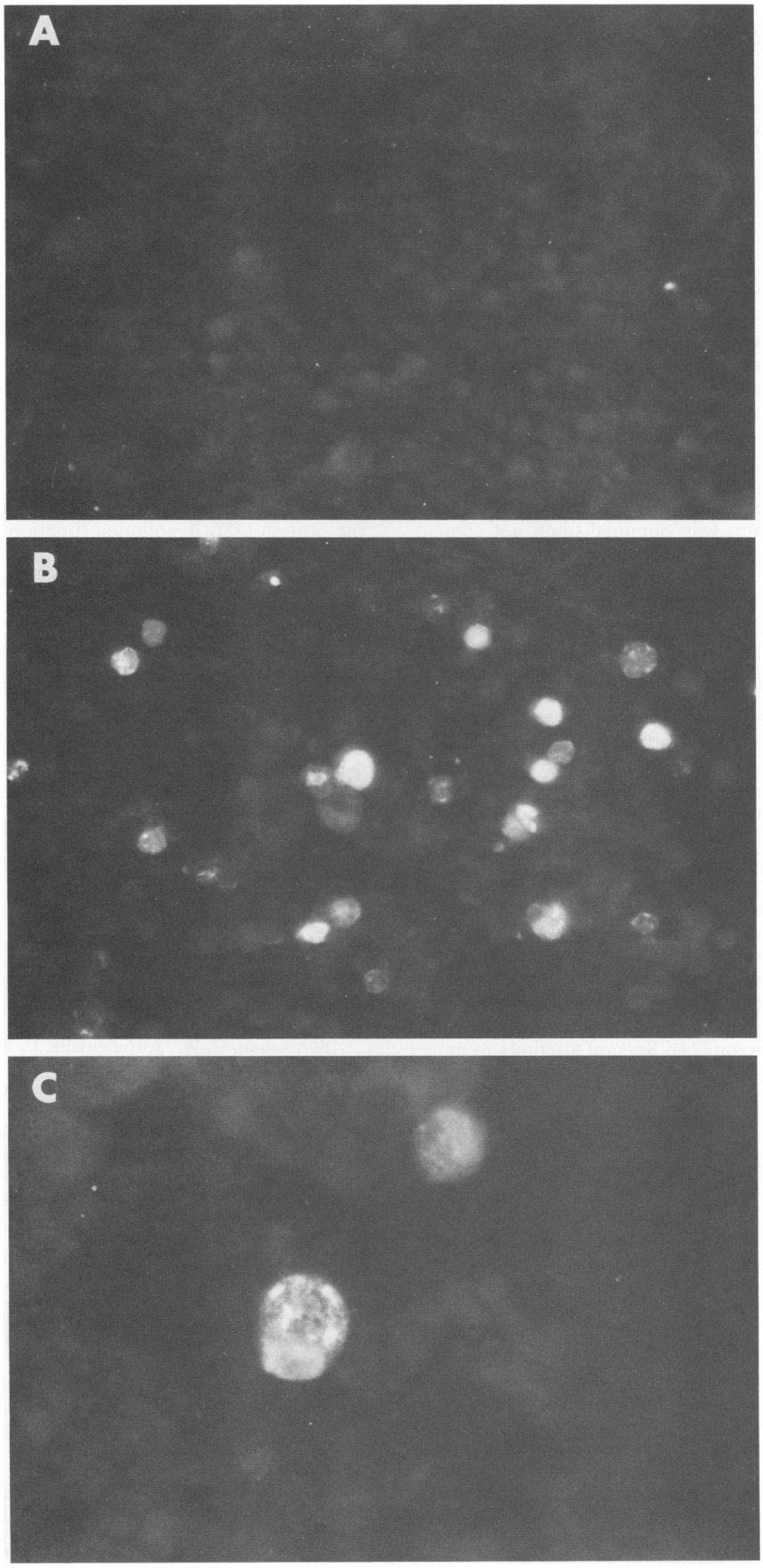

FIG. 7. Immunofluorescence analysis of Jijoye cells treated for 3 days with TPA and fixed with acetone (A and B) or methanol (C). Immunofluorescence was obtained using preimmune serum (A) or rabbit anti-pNG04 serum (B and C). Magnifications: (A and B) ×1,520; (C) ×3,800.

tion of N-linked glycosylation with tunicamycin, followed by SDS-PAGE and immunoblotting, revealed no shift in size of the BHLF1 protein, while the tunicamycin effect was demonstrated with viral glycoprotein gp350/220 (data not shown).

Reaction of human sera. A panel of 40 human sera (30 EA positive, 7 EA negative, and 3 EBV negative), was tested by immunoblotting for recognition of the fusion proteins encoded by pNG27 and pNG04. Only three human EA-positive sera with EA titers of 64, 128, and 512, obtained from patients with different symptoms, react specifically with the fusion proteins. A representative result is shown in Fig. 6b. The relatively strong reaction of the human serum with the pNG27-encoded fusion protein, compared with the low expression of the protein, as judged from the Coomassie blue gel is probably due to the repetition of the epitopes. In addition, a reaction of the same serum with the BHLF1-encoded protein immunoprecipitated from Jijoye cells, using rabbit anti-pNG04 serum, was obtained (data not shown). All of these results indicate the in vivo expression of the BHLF1 protein.

Immunofluorescence analysis. Immunofluorescence was carried out on Jijoye cells with rabbit anti-pNG04 serum. Specific immunofluorescence is limited to 1% of noninduced cells, increasing to 10 to 20% after 3 days of induction by TPA (Fig. 7B). The immunofluorescence pattern is granular, spread over the whole cell, and resistant to methanol fixation (Fig. 7C). With respect to these criteria, the BHLF1-encoded protein belongs to the EA-D subgroup of EBV-encoded EA. No immunofluorescence is detected using rabbit preimmune serum (Fig. 7A).

DISCUSSION

BHLF1-carrying transcripts are started from D_L and are found to be the most abundant EBV-specific transcripts in the early phase of the lytic cycle of EBV. BHLF1 represents a quite remarkable ORF of EBV because it is composed almost entirely of tandem repeats with a size of 125 bp each (*Not*I repeats). There is strong evidence that BHLF1 RNA is transcribed without splicing (24). Several attempts to detect the putative BHLF1-encoded protein have been reported (16, 24, 31). The lack of success may in part be due to the nature of this protein. The sequence of the BHLF1-encoded protein contains only one methionine residue (encoded by the start codon). Since it is well known that the activity which removes N-terminal amino acids is also present in reticulocyte lysates, the efficient labeling of the BHLF1-encoded protein, using [^{35}S]methionine, is inefficient. Furthermore, screening with human sera may also be problematic since we found only a few human sera with anti-BHLF1 reactivity. Using a polyclonal monospecific rabbit serum, we were able to identify the protein encoded by BHLF1. During preparation of this manuscript, our data were confirmed by Lieberman and colleagues, identifying the BHLF1 protein in immunofluorescence tests with an anti-peptide serum (21).

The variation in molecular size of this repetitive protein (125-amino-acid repeat) in cell lines infected by different EBV strains (Fig. 2a) is not surprising since it is well known that EBV strains vary with respect to the number of their *Not*I repeats (5, 15). We found a good correlation between the length of the D_L RNA (8) and the molecular size of the BHLF1-encoded protein of the same EBV strain, as demonstrated for BHLF1-encoded proteins from B95-8, M-ABA, and QIMR-WIL. So far, there are no indications for the occurrence of posttranslational modification of the BHLF1-encoded protein either by phosphorylation or by N-linked glycosylation. Induction kinetics, using inducers of the EBV lytic cycle (TPA and *n*-butyrate), resistance to PAA, and immunofluorescence data, provide evidence that this protein belongs to the EA-D complex.

Regions in D_L and D_R have recently been identified as the origins of DNA replication of the lytic cycle (oriLyt) of EBV (11). Transcriptional activation of regions adjacent to origins of DNA replication is quite common in the eucaryotic system (6).

The *Not*I repeat protein has a hypothetical pI of 12.4. Because of the basic nature of this protein, the binding to polyanion columns is not surprising. The question of whether the relatively strong binding to ssDNA-cellulose has functional implications or not needs to be elucidated in further experiments. In this context it is of interest that Lieberman and colleagues found a nucleolar localization of the protein (21). A potential function in gene regulation, as suggested by Yamamoto et al. (35), or a hypothetical involvement of the protein in DNA replication has to be studied in further experiments.

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