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Identification and expression of a nuclear antigen from the genomic region of the Jijoye strain of Epstein–Barr virus that is missing in its nonimmortalizing deletion mutant, P3HR-1

(gene transfer/cell transformation)

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ABSTRACT **An Epstein–Barr virus (EBV) deletion mutant, HR-1, cannot immortalize lymphocytes. HR-1 was derived from a virus strain, Jijoye, that is immortalization competent. Using human antiserum from certain patients with chronic active EBV infection, we have identified in Jijoye cells a protein of apparent mass of 78–80 kDa that is missing in cells with the HR-1 genome. A protein of identical size and antigenicity has been stably expressed in mouse LTK$^-$ cells by gene transfer with cloned Jijoye EBV DNA that encompasses the deletion in the HR-1 genome. The expressed product is a nuclear neoantigen. The polypeptide we have identified is likely to be essential in the immortalization process.**

Epstein–Barr virus (EBV) induces continuous growth of resting human B lymphocytes, a phenomenon termed immortalization (1–3). Since the process is so rapid and efficient, it is likely that one or more viral gene products are directly involved (4, 5).

A promising clue to the identify of at least one required viral gene is found in a laboratory strain of EBV, P3J-HR-1 (HR-1), that is immortalization deficient (6, 7). The HR-1 virus arose during single-cell cloning of a Burkitt lymphoma line, Jijoye, whose EBV is proficient at immortalization (8).

The nonimmortalizing HR-1 virus has a genomic deletion of ≈6.8 kilobase pairs by comparison to several laboratory strains of EBV that are immortalization competent (9–11). This deletion begins in one member of the first internal repeat of the viral genome (IR1, *Bam*HI fragment W) and extends through the adjacent unique sequences (U2, *Bam*HI fragments Y and H) (see Fig. 1). A number of restriction fragment polymorphisms distinguish the remainder of the HR-1 genome from other EBV strains. However, the principal polymorphism that differentiates the genome of the HR-1 from its parent, Jijoye, is the presence of the deletion in IR1 and U2 (11).

The importance of this deletion in the immortalization process is emphasized by analysis of a series of recombinant viruses between HR-1 and the EBV strain in Raji cells. Those recombinants that regained immortalizing ability all contained intact *Bam*HI fragments Y and H from the Raji genome (12).

In cells immortalized by EBV, in which the genome is latent and no viral particles are formed, at least three regions of the genome give rise to mRNAs and a fourth gives rise to the EBV-encoded small RNAs (13–17). Some of these expressed viral genes undoubtedly play a role in the immortalization process and in maintenance of EB viral plasmids.

The most abundant mRNA made in latency is a 2.8-kilobase RNA that hybridizes to sequences near the viral termini (13). On the basis of nucleotide sequence analysis it has been suggested that the product is a transmembrane protein, perhaps the lymphocyte-detected membrane antigen that is thought to be the target of cytotoxic T cells (18).

A second transcript, present during latency, is derived from a 2.0-kilobase-pair exon in the middle of the genome. By gene-transfer experiments it has been demonstrated that this region encodes a 78-kDa polypeptide nuclear antigen (Epstein–Barr nuclear antigen, EBNA) that binds to cellular metaphase chromosomes and is required to maintain the EBV plasmid origin of replication (oriP) (19–22).

The third mRNA found in immortalized cells comes from the region of the genome that is deleted in the HR-1 strain. A spliced transcript is homologous to *Bam*HI fragments W, Y, and H (see Fig. 1; also refs. 14, 23). The product of this important region of the Jijoye genome has not been identified. We have now defined the polypeptide that is missing in the nonimmortalizing virus and expressed it in eukaryotic cells using gene transfer techniques.

MATERIALS AND METHODS

Cells. Jijoye Burkitt lymphoma cells, which were obtained from the American Type Culture Collection, were cloned in semisoft agarose (25). Fifty clones were screened for viral DNA content by spot hybridization (26). Supernatants from each clone were tested for immortalization of human lymphocytes. Two clones, no. 5 and no. 44, were selected because they contained the largest amount of viral DNA and their supernatants were transformation competent. Human umbilical cord lymphocytes transformed by Jijoye clone 5 virus were also studied.

The parental P3J-HR-1 (HR-1) and three of its subclones, clones 13, 16, and 5, are all deficient in immortalization (27). Clones 13 and 16 are hyperresponsive to phorbol 12-myristate 13-acetate (superinducible). Clone 5 has heterogeneous, defective DNA; the other clones do not. Raji and BJAB are Burkitt lymphoma lines with and without the EBV genome. X50-7 are neonatal cells immortalized *in vitro* by EBV; they have a latent genome. SP and Broc are lymphoid lines established from the blood of patients with chronic EBV infection. LTK$^-$ mouse fibroblasts were a gift from W. Summers. COS-1 cells were used to express the EBNA from *Bam*HI fragment K (20).

Cloning Jijoye Viral DNA. Jijoye clone 5 cells were grown in the presence of 20 ng of phorbol 12-myristate 13-acetate

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Abbreviations: EBV, Epstein–Barr virus; EBNA, Epstein–Barr nuclear antigen; U, unique; IR, internal repeat.

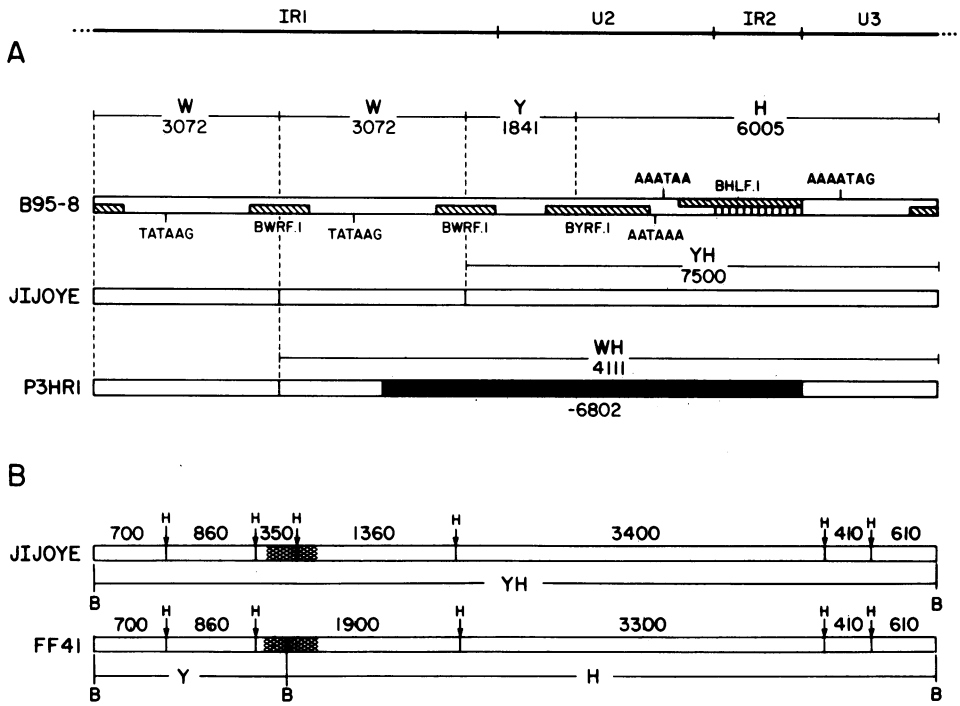

FIG. 1. Map of the region of EBV DNA that includes *Bam*HI fragments W, Y, and H. (*A*) Comparison of three virus strains, B95-8, Jijoye, and P3HR-1. IR1 and IR2 refer to IR sequences; U2 and U3 indicate U sequences (13). Vertical lines in the region of IR2 in the B95-8 sequence are the *Not* I repeats. Bars with hash marks indicate open reading frames (24) that are named according to the Cambridge convention (i.e., BWRF.1 is *Bam*HI W right frame 1). The solid black bar on the HR-1 map represents deleted DNA. (*B*) Map of *Bam*HI fragments Y and H in Jijoye and FF41. Numbers are sizes (base pairs) of the *Hin*cII (H) fragments. The cross-hatched region indicates nonhomology between Jijoye and FF41 based on Southern hybridization. Within this region Jijoye lacks a *Bam*HI site found in FF41 and contains an additional *Hin*cII site. The difference in size of the largest *Hin*cII fragment is likely due to an additional *Not* I repeat in the Jijoye strain.

per ml for 7 days. Intracellular DNA was prepared by the method of Wahl *et al.* from 10^7 cells (28). The DNA was centrifuged on a CsCl gradient and fractions of viral density were collected and pooled. The proportion of viral DNA in the preparation was estimated by comparison to purified plasmid DNA on a Southern blot. About 12% of the DNA preparation was viral. Intracellular Jijoye DNA was partially digested with *Bam*HI and the products were ligated into pBR322 that had been treated with calf intestine alkaline phosphatase. The plasmids were transfected into LE392. Bacterial colonies with viral inserts were first identified by hybridization with purified EB viral DNA (Life Sciences, St. Petersburg, FL). The colonies were then screened with a probe of *Bam*HI fragment H excised from a gel. Clones were identified that had Jijoye *Bam*HI fragments YH and WWYH. These are designated pJJ709-40 (YH) and pJJ709-43 (WWYH). Orientation of the inserts was determined by use of an asymmetric *Hin*dIII site in *Bam*HI fragment YH.

Gene Transfer. LTK$^-$ cells were cotransformed with Jijoye EBV DNA fragments in pBR322 and plasmid pXI containing the herpes simplex thymidine kinase gene by using the calcium technique. Selection was applied with MAGGT medium 24 hr after transfection (19, 29). In other experiments EBV DNA fragments were stably transferred into LTK$^-$ cells by means of cotransformation with pSV2-neo and selection with G418 (30).

Antigen Detection. LTK$^-$ cells growing on coverslips were fixed in acetone/methanol (2:1) and examined by anticomplement immunofluorescence. Antigens in various lymphoid cell lines and in the L cells were detected by immunoblotting. The procedures used have been described (20, 31, 32). The five sera with antibodies to EBNA but lacking antibody to the *Bam*HI K component are from patients with chronic active EBV infection (33). Patients 651 and 884 have acquired immunodeficiency syndrome; patients WC, SP, and Amor have chronic mononucleosis.

RESULTS

A 95-kDa Polypeptide Present in Raji Cells and Several Other Lymphoid Lines Is Absent in HR-1 and Jijoye Cells. An initial objective was to identify, by immunoblotting, viral polypeptides in lymphoblastoid cells latently infected by EBV. The strategy was to use highly reactive polyvalent human serum for this purpose. The most prominent polypeptide found in latently infected cells is that encoded by the leftmost 2.9 kilobase pairs of the *Bam*HI K fragment (20). This polypeptide (K), representing the EBNA that binds to metaphase chromosomes, is known to vary in size among different cell lines depending on the variable length of a repeat unit (IR3) in the EBV genome (20, 34). For example, the EBNA encoded by *Bam*HI K fragment has an apparent molecular mass of 65 kDa in Raji cells and 72 kDa in HR-1 cells, and in other strains it varies in apparent mass between 78 and 105 kDa (e.g., Broc in Fig. 2).

Many human sera that recognize K EBNA also identify a second polypeptide with an apparent mass of 95 kDa in Raji cells (20, 21, 35–37) (Fig. 2). This polypeptide was not seen in cells expressing the K EBNA as the result of gene transfer into COS-1 cells (Fig. 2*A*); thus, it is not likely to be encoded by the *Bam*HI K fragment. Furthermore, the 95-kDa polypeptide was invariant in size in a group of five cell lines in which the size of the K EBNA differed considerably (Fig. 2*B*). The 95-kDa polypeptide was seen in cells such as X50-7, which are tightly latent, and was not induced during viral replication in producer lines such as SP or FF41 (Fig. 2*B*).

The 95-kDa polypeptide was not detected in HR-1 cells or in three of its subclones, two of which contained and one of which lacked heterogeneous DNA (Figs. 2*B* and 3*A*). The polypeptide could not be induced in HR-1 cells by treatment with butyrate (Fig. 2*B*). The absence of the 95-kDa polypeptide in HR-1 cells raised the possibility that the protein might be encoded in the region of the EBV genome that is deleted in this virus strain (i.e., *Bam*HI fragments W, Y, and H). If this were true, then we would expect to see the 95-kDa polypeptide in Jijoye cells that are the parent of the HR-1 strain (11). To our surprise, however, the 95-kDa polypeptide was also missing in Jijoye cells, in a cellular subclone of Jijoye cells, and in human umbilical cord lymphocytes immortalized by Jijoye virus (Figs. 2*B* and 3*A*).

This perplexing finding might be explained by alterations in the coding region for such a protein in the Jijoye genome. There is a region of nonhomology between Jijoye and the prototype B95-8 virus that lies within the reading frame BYRF1 (Fig. 1*A*) (10, 24). Recently, Adldinger *et al.* showed by heteroduplex analysis that this region of Jijoye and

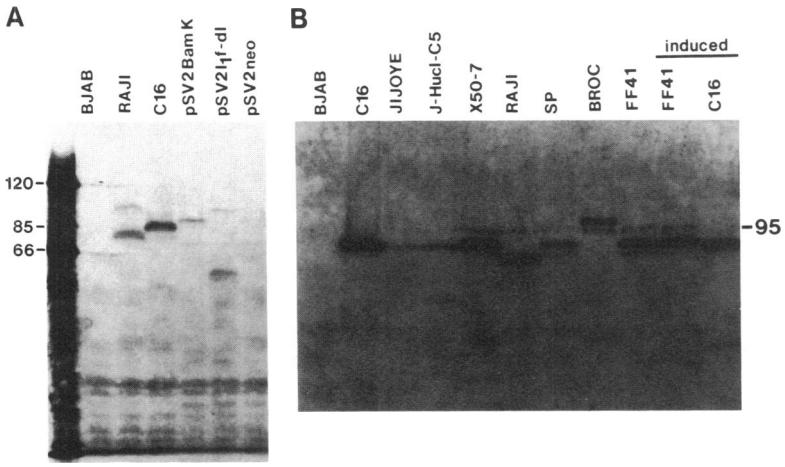

FIG. 2. Immunoblots identifying the *Bam*HI K EBNA and a second 95-kDa polypeptide in various cells. (*A*) The K EBNA varies in size in Raji and in HR-1 clone 16 (C16) cells. COS-1 cells were transfected with pSV2 *Bam*HI fragment K or with pSV2I$_1$f-dl, a plasmid that has a 600-base-pair deletion in the K EBNA gene. The 95-kDa polypeptide is seen only in Raji. (*B*) The 95-kDa polypeptide is seen in X50-7 (a cell line that is "tightly latent"), in Raji, and in two cell lines, SP and Broc, established from blood of patients with chronic EBV infection. It is also present in FF41, a virus-producer marmoset cell line, but its abundance is unaffected by induction of viral replication. However, the 95-kDa polypeptide is not present in Jijoye, in umbilical cord lymphocytes transformed by virus from clone 5 of Jijoye cells (J-HUCL-c5), or in clone 16 (C16) of HR-1 cells. The serum used on these two immunoblots, RM, was from a person convalescent from infectious mononucleosis.

M-ABA DNA is only partially homologous (38). By comparison of the cloned *Bam*HI YH fragment from Jijoye with the *Bam*HI Y and H fragments of strain FF41 we identified an additional *Hin*cII site in Jijoye DNA as well as loss of the *Bam*HI site separating fragments Y and H (Fig. 1*B*). We postulated that the polymorphic nature of the reading frame might give rise to a varient polypeptide that would comigrate with the K EBNA and thus escape detection with polyvalent antisera that also contain antibody to K.

Detection of a 78- to 80-kDa Polypeptide in Jijoye Cells That Is Missing in HR-1 Cells. To address the possibility of detecting two comigrating polypeptides we used EBNA-positive human sera that came from patients with chronic EBV infection who specifically fail to recognize the K EBNA (33). A serum from one such patient (Amor) without antibody to K EBNA reacted strongly with a doublet polypeptide of 78–80 kDa in Jijoye cells and in a subclone of Jijoye cells but failed to recognize a similarly sized polypeptide in HR-1 or any of its subclones (Fig. 3*C*). This serum recognized the 95-kDa polypeptide in Raji cells only weakly. A serum from another patient with chronic EBV infection, SP, identified the 95-kDa polypeptide in Raji and X50-7 cells but did not react with the 78–80-kDa doublet in Jijoye cells (Fig. 3*B*).

These findings indicated that indeed Jijoye cells contained a second polypeptide that comigrated on polyacrylamide gels with the K EBNA. Furthermore, they suggested that human

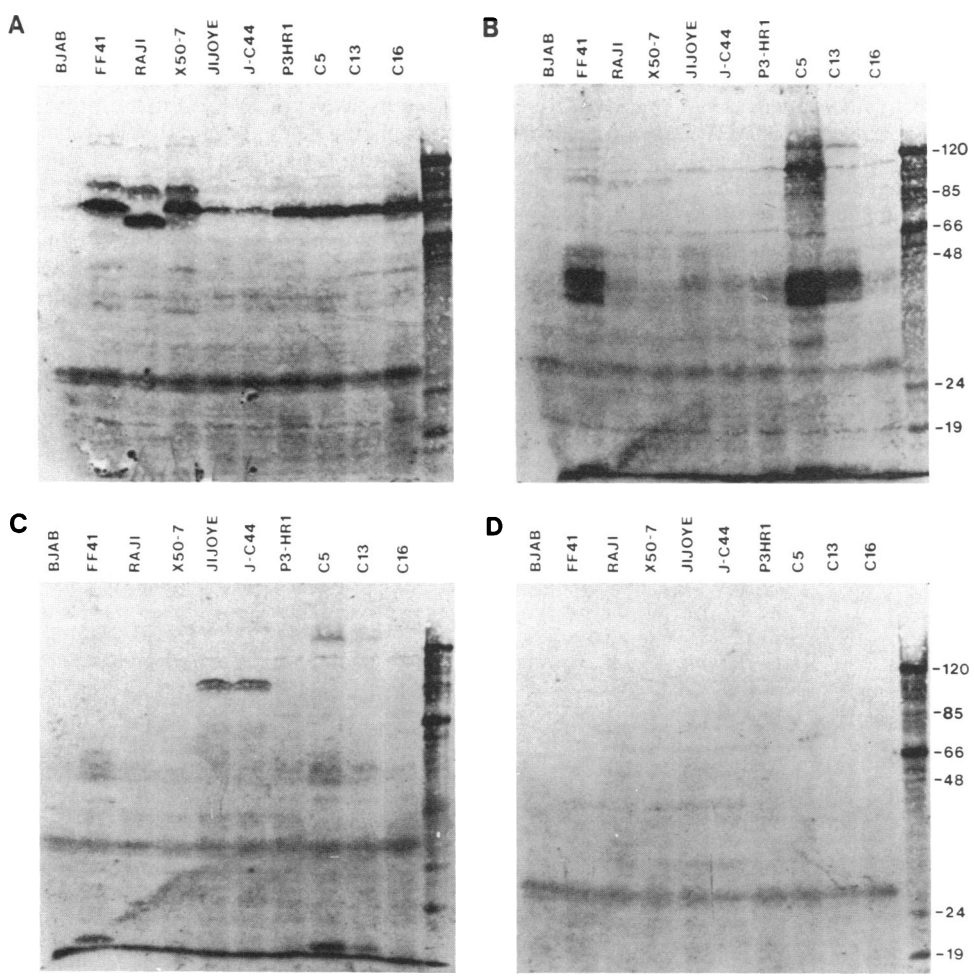

FIG. 3. Immunoblots showing that certain human sera recognize either a 78- to 80-kDa polypeptide in Jijoye cells or the 95 kDa polypeptide. Identical immunoblots were probed with RM (*A*), SP (*B*), Amor (*C*), or LH (*D*). RM identifies the K EBNA polypeptide as well as the 95-kDa protein (Fig. 2). Amor and SP, patients with chronic EBV infection, are nonreactive with the K polypeptide. LH is a healthy EBV-seronegative person. (*A*) Note that the 95-kDa polypeptide is seen in FF41, Raji, and X50-7 but not in Jijoye, clone 44 of Jijoye (J-C44), or P3HR-1 or its three subclones (C5, C13, or C16). (*B*) SP identifies the 95-kDa polypeptide in FF41, Raji, and X50-7 but not the K EBNA. SP also recognizes many replicative proteins in FF41 C5 and C13. (*C*) Amor recognizes a doublet 78- to 80-kDa protein in Jijoye cells and in subclone 44 of Jijoye. These proteins are not seen in any other cell line.

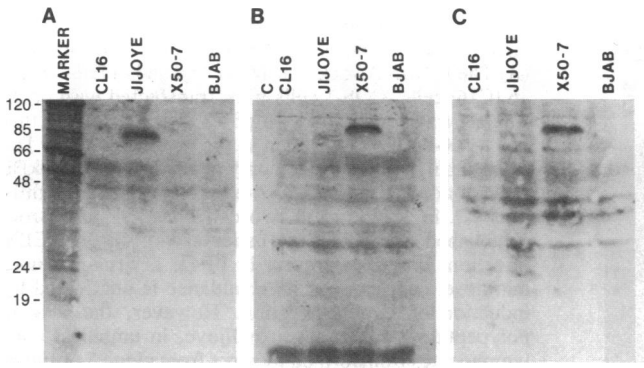

FIG. 4. Preferential recognition of the Jijoye 78- to 80-kDa protein or the 95-kDa polypeptide by three human sera that lack antibody to K EBNA. (*A*) WC serum from a patient with chronic EBV infection identifies the Jijoye protein. (*B* and *C*) 651 and 884 sera from patients with acquired immune deficiency syndrome identify the 95-kDa protein in X50-7 cells and only weakly react with the 78- to 80-kDa protein in Jijoye.

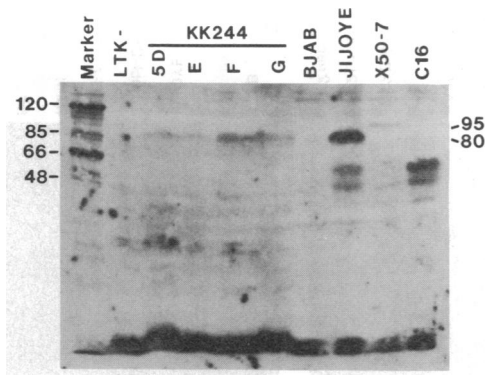

FIG. 6. Detection of the polypeptide expressed from Jijoye *Bam*HI fragments WWYH. The immunoblot was probed with the WC serum. Note that the polypeptide detected in clones D, E, F, and G is the same size as the 78- to 80-kDa polypeptide in Jijoye cells. The serum weakly detects the 95-kDa polypeptide in X50-7 cells.

serum preferentially reacted with either the 95-kDa polypeptide or the 78- to 80-kDa polypeptide in Jijoye cells. These hypotheses were born out by study of sera from three additional patients with chronic EBV infection who lacked antibodies to the K EBNA. One, WC, detected the Jijoye polypeptide strongly and detected the 95-kDa protein weakly (Fig. 4*A*); the other two, 651 and 884, reacted preferentially with the 95-kDa polypeptide and reacted weakly with the Jijoye 78- to 80-kDa protein (Fig. 4 *B* and *C*). None of these sera reacted with a corresponding polypeptide in the HR-1 cells.

Expression in LTK Cells of a Nuclear Antigen and 78- to 80-kDa Polypeptides from the Region of the Jijoye Genome That Is Deleted in the HR-1 Genome. The foregoing experiments provided indirect evidence that the 78- to 80-kDa protein found in Jijoye cells was encoded by the region that is deleted in the nonimmortalizing HR-1 strain. More direct evidence was obtained in parallel experiments in which we cloned *Bam*HI fragments of Jijoye viral DNA as partial digestion products in pBR322. Three plasmids were studied for antigen expression: (*i*) pJJ709-40, which contains Jijoye *Bam*HI fragment YH, (*ii*) pJJ709-43, which contains two copies of Jijoye *Bam*HI fragment W linked to *Bam*HI fragment YH (WWYH), and (*iii*) pKK253-35, which contains two copies of *Bam*HI fragment W. We looked for antigen in transient expression assays and in stable transformants selected by cotransformation with pSV2-neo or with pXI, a plasmid that contains the herpes simplex thymidine kinase gene.

In several trials the plasmid pJJ709-43 containing two copies of the first IR and the *Bam*HI YH fragment from Jijoye induced a nuclear antigen detected by anticomplement immunofluorescence 3 days after transfection of LTK^- cells. In parallel trials antigen was not seen when *Bam*HI WW and *Bam*HI YH fragments were used (data not shown). Stable LTK^+ cell transformants, established after selection with G418 or after selection for thymidine kinase gene, also expressed a nuclear antigen when the cotransforming cloned viral DNA was pJJ709-43. The antigen was nuclear in location and varied in intensity (Fig. 5). A number of human sera, including those with and without antibody to the K EBNA, detect the WWYH antigen by immunofluorescence (data not shown). Of the five human sera that lack antibody to the K EBNA, the two that react strongly with the Jijoye 78- to 80-kDa polypeptide (WC and Amor) also react with the WWYH antigen expressed in LTK cells. The titers by anticomplement immunofluorescence are 1:60 for WC and 1:80 for Amor. Only one of the three remaining sera, which all react preferentially with the 95-kDa polypeptide, detects the expressed antigen from Jijoye by immunofluorescence. Antigen was not seen in stable transformants containing Jijoye *Bam*HI fragment WW or YH. Furthermore, a cotransformant containing the deleted *Bam*HI WH fragment from HR-1 virus did not display antigen.

An LTK^+ cell line designated KK244-5, which was cotransformed with pXI and pJJ709-43, expressed nuclear antigen in about 25% of the cells. The cell line was cloned, and four individual cell clones (D, E, F, and G) that were 100% antigen positive by immnofluorescence were examined by the immunoblotting technique with the WC and Amor sera (Fig. 6). All four clones expressed a polypeptide of the same size as that found in Jijoye cells. This polypeptide was not seen in LTK^- cells. Thus, the product of the region of Jijoye virus that is deleted in HR-1 virus is a nuclear antigen with a mass of 78–80 kDa.

DISCUSSION

Characterization of the Jijoye Polypeptide. We have identified a polypeptide encoded by the region of the Jijoye virus

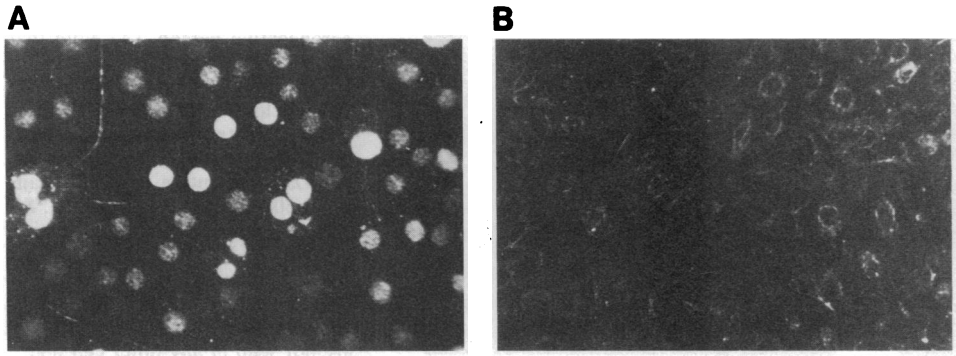

FIG. 5. Appearance of the nuclear antigen expressed in LTK^- cells after cotransformation with HSV thymidine kinase gene and pJJ709-43, a plasmid containing Jijoye *Bam*HI fragments WWYH. The cell line KK244-5 was allowed to react by anticomplement immunofluorescence with a 1:10 dilution of WC (*A*) or seronegative LH (*B*). Note that the antigen varies in intensity and is present in the nuclei of all of the cells.

genome that is deleted in its nonimmortalizing derivative, HR-1. The polypeptide is found in Jijoye cells, in a subclone therefrom, and in cells immortalized *in vitro* by Jijoye virus. It is not present in the HR-1 line or in several different HR-1 subclones and cannot be caused to appear in HR-1 cells by induction of viral replication. Furthermore, a polypeptide of the same size is expressed when the region of the Jijoye viral genome, which is deleted in HR-1, is cotransformed into LTK^- cells. DNA subfragments that do not encompass the entire deletion do not induce the 78- to 80-kDa polypeptide. Those human sera that recognize the polypeptide in Jijoye cells also detect the antigen and polypeptide expressed after gene transfer. Human sera that are nonreactive with the antigen expressed in the gene-transfer system react weakly or not at all with the 78- to 80-kDa Jijoye polypeptide. Thus, the product expressed after gene transfer appears authentic on the basis of size, antigenic characteristics, and cellular localization.

The size of the product made after gene transfer, which is similar to the size of the protein in Jijoye cells, can be accounted for by the utilization of only two open reading frames (24), one in the first repeat (BWRF1) and one that spans *Bam*HI fragments Y and H (BYRF1) (see Fig. 1). Alternatively, BYRF1 alone might be sufficient to account for a 78- to 80-kDa band on electrophoretic transfer blots of Jijoye extract. This reading frame would be translated into a proline-rich 55-kDa polypeptide that might migrate in an anomalous way on polyacrylamide gels.

Studies of transcripts from this region have suggested that small segments from each repeated BWRF1 are fused into a contiguous open reading frame that is used to code for protein (14, 23). Our gene-transfer experiments imply that this transcriptional plan may not be essential and that only two (and possibly only one) copies of the first IR are needed to make the protein.

Heterogeneity of the Product from *Bam*HI Fragments W, Y, and H. The product of this region of the EBV genome appears to be heterogeneous in size and in antigenicity (Figs. 3 and 4).

In these studies we have not directly proved by gene-transfer experiments that the 95-kDa polypeptide is encoded by the same region of the genome as the Jijoye 78- to 80-kDa polypeptide. A similar inference, about the relatedness of two differently sized EBNA polypeptides in the AG876 and B95-8 viruses, was recently drawn by Dambaugh *et al.* (39) on the basis of comparison of nucleotide sequences in the U2 region of these viruses.

Dambaugh *et al.* (39) were apparently unable to detect a second EBNA polypeptide in Jijoye cells. Our ability to recognize this polypeptide in Jijoye lymphocytes relied on the realization that the polypeptide might comigrate with the EBNA encoded by the *Bam*HI K fragment. It depended, therefore, on the availability of human serum that selectively recognized the Jijoye 78- to 80-kDa polypeptide and lacked antibody to the EBNA encoded by the *Bam*HI K fragment (Figs. 3 and 4). Our experiments show further that patients with chronic active EBV infection who selectively fail to recognize the K EBNA do make antibodies to a second nuclear antigen expressed during latency.

That human serum preferentially recognizes different size classes of polypeptide from *Bam*HI W, Y, and H fragments indicates that the product from this region is structurally heterogeneous. It is somewhat surprising that a product of diverse structure may be essential to immortalization. Obviously, certain domains of the protein may be constant in all immortalizing viruses.

The antigenic and structural diversity of the products of the region that is deleted in HR-1 virus raises the intriguing possibility that variation in this product is in some way responsible for the diversity of the biologic outcome of EBV infections.

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