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Expression of the Epstein-Barr Virus Nuclear Protein 2 in Rodent Cells

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A 3.0-kilobase-pair Epstein-Barr virus (EBV) DNA segment necessary for lymphocyte immortalization encodes at least part of a nuclear protein (EBNA2) which is characteristically expressed in latently infected, immortalized cells. A 1.5-kilobase open reading frame within this DNA segment has now been inserted into a murine leukemia virus (MuLV)-derived expression vector (pZIP-NEO-SV(X)1) which provides for transcription of heterologous DNA but not for translational start. Transfection of the recombinant DNA into NIH 3T3 cells resulted in expression of a full-sized EBNA2 which localized to the cell nucleus. Significant new evidence is thereby provided that this 1.5 kilobase open reading frame includes a translational start site and encodes the entire EBNA2 protein. Transfection of the recombinant DNA into a helper cell line (psi am22b) providing amphotropic MuLV-packaging functions resulted in the release of a recombinant MuLV carrying the EBNA2 gene. This recombinant virus can infect rodent cells and convert them to stable EBNA2 expression. Rat-1 cells infected with the MuLV EBNA2 recombinant expressed EBNA2 and grew more rapidly in medium supplemented with 1 or 0.5% fetal calf serum than did Rat-1 cells infected with MuLV vector lacking EBNA2. The Rat-1 cells expressing EBNA2 remained contact inhibited, anchorage dependent, and nontumorigenic in nude mice. Different EBV isolates have one of at least two EBNA2 alleles. Despite divergence between the two alleles, a human serum recognized the prototype EBNA2 allele (EBNA2A) as well as the variant EBNA2B allele characteristic of some Burkitt tumor EBV isolates. The EBNA2B allele was also expressed from the MuLV-derived vector. The reproducible expression of EBNA2A or EBNA2B from these recombinant vectors will facilitate analysis of the EBNA2A and EBNA2B phenotypes.

A considerable body of evidence indicates that Epstein-Barr Virus (EBV) is an etiologic agent of nasopharyngeal carcinoma and African Burkitt's lymphoma (for a review, see references 11, 12, and 66). EBV infection of human B lymphocytes in vitro or in vivo results in perpetual cell proliferation and persistence of the virus genome in a latent state (22, 43; for a review, see reference 11). These infected lymphocytes can form tumors in newborn nude mouse skin, in adult nude mouse brain (41), in marmosets (38), or in immunocompromised patients (7, 45). Since EBV infection rapidly and efficiently immortalizes lymphocytes (21, 59), virus genes probably directly induce cell proliferation. Although the entire 172-kilobase-pair (kbp) virus genome persists in latently infected immortalized cells, only four genes are known to be expressed (for a review, see reference 11). These four genes encode three nuclear proteins, EBNA1, EBNA2, and EBNA3, and a membrane protein, LMP (10, 11, 14, 23–27, 61, 62). The role of these four genes in the persistence of the virus genome, latency, and cell growth transformation is only partly known. EBNA1 is essential for the maintenance of the virus genome as an episome (49, 64, 65). LMP seems important in the conversion of cells to a transformed phenotype (63). EBNA2 is likely to be directly or indirectly important in the initiation of or maintenance of cell immortalization since P3HR-1 and Daudi, two virus isolates which have deleted at least part of the DNA (3, 19, 31–33, 46; N. Raab-Traub, Ph.D. thesis, University of Chicago, Chicago, Ill., 1980) which encodes EBNA2 (10, 27), differ from other EBV isolates in their inability to immortalize lymphocytes (37). Further, immortalization-competent recombinant viruses formed by crosses between incompetent P3HR-1 EBV and the Raji EBV genome have all regained the specific DNA segment (56) which encodes at least part of EBNA2.

The coding and regulatory elements of the EBNA2 gene have not been completely defined because of the low abundance of its mRNA in latently infected cells (34, 61, 62). The mRNA is approximately 3 kilobases (kb) and is derived by complex splicing of nuclear transcripts encoded in part by the EBV IR1 (first internal repeat) and U2 (second unique) DNA domains (61, 62) (Fig. 1). Potential promoters for the transcription of the EBNA2 mRNA have been identified within IR1 or in the U1 domain 5′ to IR1 (2, 6, 61, 62). The EBNA2 mRNA ends in U2 with an exon whose 3′ end is 15 nucleotides after an AATAAA sequence since probes from the EBV genome 3′ to this site do not hybridize to the 3-kb RNA (10, 11, 27, 61, 62; S. Fennewald and E. Kieff, unpublished observations). This exon coincides with a 1,545-base-pair (bp) U2 open reading frame (2, 10). Rabbit antisera against a bacterial fusion protein encoded by a middle segment of this open reading frame recombined with the *lac*Z gene react specifically with EBNA2 in each latently infected cell, demonstrating that at least part of the EBV U2 reading frame encodes EBNA2 (27). Furthermore, differences between EBV isolates in the length of a DNA repeat located near the beginning of the open reading frame correlate with their EBNA2 size, suggesting that this part of the open reading frame also encodes EBNA2 (10). Based on these results and the sequence of the open reading frame, peptides which would be encoded by the middle or end of the

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open reading frame were synthesized and shown to induce antibody which react with EBNA2 (13). Finally, the sequence ATCATG located near the beginning of the open reading frame could be a site for the initiation of mRNA transcription (10, 35). A protein initiated at this site would have a predicted mass of 53 kilodaltons (kDa), while EBNA2 behaves in denaturing polyacrylamide gels as though it had a mass of 82 kda (10, 26). A 30-kbp EBV U1-IR1-U2-IR2-U3 or a 15-kbp IR1-U2-IR2-U3 DNA fragment cotransfected with a selectable marker into NIH 3T3 or BHK cells resulted in the expression of a nuclear antigen in some cells which was similar in size to EBNA2, providing additional evidence that EBNA2 is mostly encoded within this large EBV DNA segment (39, 52).

The objectives of the experiments described here were (i) to construct a recombinant eucaryotic vector for the reproducible expression of EBNA2 in a wide variety of cells, (ii) to determine whether the EBV U2 open reading frame encodes for all of EBNA2, (iii) to evaluate the effects of EBNA2 on cell growth, and (iv) to definitively identify the variant EBNA2 protein encoded by some of the Burkitt tumor EBV isolates which have a remarkably different EBV U2 open reading frame (1, 10, 33). A candidate variant EBNA2 protein has been identified with polyvalent EBV immune human sera (10, 27). The prototype EBNA2 is referred to as EBNA2A, and the variant is referred to as EBNA2B.

MATERIALS AND METHODS

Cell cultures. The AG876 (42) IB4 (34), P3HR-1 (28), and Jijoye (28) cell lines are EBV infected, while the Loukes cell line is EBV negative (61). BIF/B95-8 and BIF/AG876 are human lymphocytes infected with virus from the B95-8 (37) and AG876 (42) cell lines, respectively. Lymphocytes were maintained in culture in RPMI 1640 medium supplemented with 10% fetal calf serum. LTK^- cells (obtained from P. Spear, The University of Chicago), Rat-1 cells (obtained from M. Villareal, The University of Chicago), NIH 3T3 cells (obtained from R. Lowe, Merck Sharpe & Dohme, West Point, Pa.), and psi am22b cells (8) (obtained from R. Mulligan, Whitehead Institute) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Recombinant plasmids. Recombinant plasmids containing the IR1-U2 region of B95-8 EBV DNA (fragments 2 and 3 in Fig. 1A) inserted into the *Xba*I site of pSP64 (Promega Biotec) or inserted downstream of the herpes simplex virus type 1 ICP4 alpha promoter in pRB334 (44) were derived by subcloning of EBV DNA segments from a recombinant plasmid containing the 9.7-kb EBV fragment which extends from the last *Bgl*II site within the IR1 domain (*Bam*HI-W) to the first *Bgl*II site in U3 (*Bam*HI-H [9]). The common 3′ terminus for both fragments is the single *Aha*III cleavage site within the B95-8 U2 region (bp 3307 in reference 10). The 5′ ends of the 5.5-kb fragment 2 and the 5.3-kb fragment 3 are located at the single cleavage sites within IR1 for *Aha*III and *Sna*BI, respectively (2, 6). Fragment 4 was derived by isolation of a 1.87-kbp *Fnu*DII fragment from a clone of fragment 2 inserted in the antisense orientation in pRB3181 (30; T. Dambaugh, unpublished data). Fragment 4 consists of the U2 region of B95-8 EBV DNA from the *Fnu*DII cleavage site at bp 1468 to the *Aha*III site at bp 3307 (10) plus an additional 25 bp at the 3′ end derived from pUC13 and herpes simplex virus DNA sequences. The recombinant plasmid pZIP-U2-1 was constructed by inserting fragment 4 into the *Bam*HI site of pZIP-NEO-SV(X)1 (5) after the site was repaired with T4 DNA polymerase in the presence of excess nucleotides. A recombinant plasmid containing fragment 1 cloned into pSP64 (pSP-CWYH) was constructed by ligation of a 7.2-kbp *Eco*RI-*Sna*BI EBV DNA fragment to an *Eco*RI plus *Sna*BI digest of a recombinant clone of fragment 2 inserted in the relevant orientation at the *Xba*I site of pSP64. (The *Eco*RI site is from pSP64 and the *Sna*BI site is from EBV IR1.) The 7.2-kbp *Eco*RI-*Sna*BI fragment was isolated from a cosmid clone of the W91 EBV *Eco*RI A fragment (2, 46; Raab-Traub, Ph.D thesis) and consists of 4.9 kbp of the U1 region (including the entire *ori* P element) and 2.3 kbp from the first IR1 repeat. Fragment 1 was isolated from agarose gels as a 12.5-kbp *Eco*RI-*Sal*I fragment from the recombinant plasmid pSP-CWYH and inserted into the *Bam*HI site of pZIP-NEO-SV(X)1 after treatment of both the insert and the vector with T4 DNA polymerase plus excess nucleotides. An EBV AG876 U2 open reading frame pZIP-NEO-SV(X)1 recombinant expression vector was constructed by cloning an *Rsa*I partial digest from the appropriate *Sma*I fragment of the AG876 *Bam*HI D1 fragment (10, 46; Raab-Traub, Ph.D. thesis) into the *Bam*HI site of pZIP-NEO-SV(X)1. The EBV AG876 DNA fragment cloned into pZIP-NEO-SV(X)1 has its 5′ end 27 bp upstream of the *Fnu*DII site of fragment 4, described above. The orientation of the EBV DNA inserts relative to vector elements was defined by restriction endonuclease digestion of each recombinant construction.

Transfections and isolation of clones. NIH 3T3 (before passage 11), LTK^-, or psi am22b cells were transfected with 1 to 2 μg of uncut recombinant plasmid DNA in the presence of 20 μg of salmon sperm carrier DNA per ml by the calcium phosphate technique (17). Transfected LTK^- cells were placed under hypoxanthine-aminopterin-thymidine selection 48 h after transfection. NIH 3T3 or psi am22b cells transfected with pZIP-NEO-SV(X)1-derived plasmid DNAs were split 1:10 72 h after transfection and placed in selective media containing 400 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml. In all cases, transfections with plasmids containing the selectable marker but lacking EBV DNA inserts were done as controls. Single foci of resistant (TK^+ or $G418^r$) cells were isolated by using cloning cylinders or small circles of 3MM paper (Whatman, Inc., Clifton, N.J.) treated with 1× trypsin-versene. To generate NIH 3T3 cells displaying a transformed phenotype, NIH 3T3 cells were cotransfected with pZIP-NEO-SV(X)1 plasmid (to permit isolation of $G418^r$ foci) in the presence of a 10-fold excess of pEJ, a plasmid clone of the activated *ras* gene from the human EJ carcinoma cell line (60) (obtained from Ron Ellis, Merck Sharp & Dohme). NIH 3T3 cells expressing EJ *ras* were identified by either screening $G418^r$ cells for growth in low serum and loss of anchorage dependence or by growing freshly cotransfected NIH 3T3 cells in Dulbecco modified Eagle medium supplemented with 1% fetal calf serum. Rat-1 cells (15) (transfected before the eighth passage after receipt from R. Weinberg, Whitehead Institute) were infected with recombinant retrovirus encoding EBNA2 in the presence of 8 μg of polybrene per ml for 2.5 h with 1 ml of filtered (pore size, 0.22 μm) supernatant from established lines of transfected psi am22b cells (8) expressing EBNA2.

Immunoblotting. Nuclei were prepared by lysing washed cells in 2× sample buffer (27) consisting of 2% sucrose, 10 mM Tris hydrochloride (pH 7.5), 10 mM NaCl, 3 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride, and 0.7% Nonidet P-40. Samples were then boiled, electrophoresed on a sodium dodecyl sulfate-discontinuous poly-

acrylamide gel, and transferred to nitrocellulose (4, 27). The nitrocellulose blots were blocked with milk and immunostained with human sera (27). EBNA2-reactive human sera were preabsorbed with extracts of either Loukes or P3HR-1 cells. For absorption, 1 ml of packed cells was boiled for 10 min, sonicated, and added to 10 ml of diluted serum. After 18 h at 4°C, the serum was clarified by spinning at 100,000 × *g* for 30 min. Size markers were the high-molecular-mass set (Bio-Rad Laboratories, Richmond, Calif.), which includes myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (92 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

Assays for growth properties. Cells to be assayed for reduced serum-dependent growth were initially plated in Dulbecco modified medium supplemented with 10% fetal calf serum (Zetasera-D; AMF Biological and Diagnostic Products Co.) at a density of 1,000 cells per well in 96-well flat-bottomed microtiter plates (Costar). Six hours after plating, cells were washed three times and fed with medium supplemented with 10, 1.0, or 0.5% serum or no serum. Cultures were then incubated at 37°C with 5% CO_2. Replicate plates, including triplicate wells for each point, were labeled daily with 0.5 μCi of tritiated thymidine per well. After 16 h of labeling, the contents of each well, including the cell monolayer, were harvested onto filters by using an automated cell harvester (Skatron, Inc.) and trypsin. Cell-incorporated tritiated thymidine was determined by counting the contents of the filters in a scintillation counter. The geometric means of the counts per minute were calculated, and the growth of cells in various serum concentrations was expressed as the ratio of the counts per minute on the assay day to the counts per minute on day 2 (i.e., a labeling period of 32 to 48 h).

Anchorage dependence of cell growth was assayed in 0.3% agarose (Seaplaque; FMC Corp., Rockland, Maine) in medium with 10% fetal calf serum. Two milliliters of 0.6% agarose in complete medium was layered onto 60-mm petri dishes (Becton Dickinson Labware, Oxhard, Calif.). Trypsinized cells (5×10^4) were suspended in 0.3% agarose in complete medium and layered over the 0.6% agarose. Cultures were incubated in a humidified 5% CO_2 incubator at 37°C and fed with 2 drops of complete medium twice a week.

Nude mouse injections. Freshly trypsinized cells (5×10^5 to 2×10^6) were injected into the subcutaneous tissue of newborn nude mice (<5 days old). Mice were observed three times a week for 4 weeks.

RESULTS

An open reading frame encodes EBNA2. Because of the uncertainty surrounding the 5′ part of EBNA2 mRNA, four overlapping EBV DNA fragments were cloned into eucaryotic expression vectors. The four fragments terminated 30 bp downstream of the AATAAA sequence which was 3′ to the U2 open reading frame (Fig. 1). Fragment 1 began in U1, fragments 2 and 3 began in IR1, and fragment 4 began in U2 30 bp upstream of the potential translational start site within the open reading frame (Fig. 1). Two eucaryotic expression vectors which initiate transcription 5′ to their cloning site were used: pRB334 (44) and pZIP-NEO-SV(X)1 (5). pZIP-NEO-SV(X)1 contains a murine leukemia virus (MuLV) long terminal repeat, a capsite, and a 5′ untranslated RNA leader upstream of a cloning site, while pRB334 has a herpes simplex virus type 1 alpha four promoter and an mRNA cap site upstream of a cloning site. pZIP-NEO-SV(X)1 contains the Tn*5* gene that confers resistance to G418, and pRB334 contains the herpes simplex virus type 1 TK gene.

The first two recombinants tested for EBNA2 expression were derivatives of pRB334 which contained EBV DNA fragments (fragments 2 and 3 in Fig. 1A) beginning either 20 or 265 nucleotides 5′ to the CAAT sequence of a promoter in IR1 previously identified as a likely promoter for transcription of the EBNA2 RNA. Twelve clones of recombinant vector-transfected, TK^+-converted, LTK^- cells were selected for each construct. Each clone was tested for EBNA2 expression by immunoblot and was negative. Next, an EBV DNA fragment (fragment 1 in Fig. 1A) beginning near the center of U1 was cloned into pZIP-NEO-SV(X)1 and transfected into psi am22b. psi am22b is an NIH 3T3 cell line infected with a defective amphotropic MuLV which cannot be packaged into virus but will provide packaging for other RNAs if they contain the MuLV *cis* packaging site (8). Four recombinant EBV fragment 1 pZIP-NEO-SV(X)1-transfected, G418-resistant, psi am22b clones were derived and found to be negative for EBNA2 expression. The fourth recombinant construct tested was pZIP-NEO-SV(X)1, containing EBV DNA fragment 4, which begins 30 nucleotides 5′ to a potential translational start site in the U2 open reading frame. This recombinant (pZIP-U2-1) was transfected into psi am22b or NIH 3T3 cells, and G418-resistant clones were selected. Almost all clones were EBNA2 positive on immunoblot (Fig. 2). EBNA2 content of some clones was less than that of the latently EBV-infected cell line IB4, while other clones were similar to IB4 (Fig. 2). In the transfected cells, EBNA2 was 82 kDa, identical in size to IB4 EBNA2 (Fig. 2). (The recombinant EBV DNA fragment used to construct pZIP-U2-1 was derived from the B95-8 EBV strain, the same strain which was used to infect IB4 cells.)

EBNA2 translation from pZIP-U2-1 almost certainly initiated at the putative translational initiation site (Fig. 1D). The MuLV mRNA cap site is 569 nucleotides 5′ to the cloning site (5, 53). Translation of MuLV mRNAs is not known to initiate within this 569-nucleotide 5′ leader segment. Based on comparison with known eucaryotic translational initiation sites (35), the first likely eucaryotic translational start site in pZIP-U2-1 3′ to the MuLV capsite is the sequence ATCATG in the EBV U2 open reading frame (10, 27). Furthermore, each preceding ATG is followed almost immediately by a stop codon. Also, another gene expressed from a comparable MuLV vector has the same amino terminus as the natural protein (20). Thus, the regular expression of full-sized EBNA2 from pZIP-U2-1 provides strong evidence that the proposed initiation codon near the beginning of the EBV U2 open reading frame (10) (Fig. 1) is the authentic start for EBNA2 translation.

Since psi am22b cells will package some recombinant viral RNAs into replication-defective amphotropic MuLV, supernatant media from two pZIP-U2-1 transfected clones were assayed for virus (designated MuLV-U2-1) by using the conversion of Rat-1 cells to G418 resistance as an indicator. One milliliter of medium yielded 10 to 100 neomycin-resistant Rat-1 cell clones. Eight foci were isolated and expanded. Five of the eight cloned foci expressed EBNA2, some in amounts similar to the IB4 lymphocyte cell line (Fig. 2). EBNA2 expressed in Rat-1 cells was identical in size to authentic IB4 EBNA2.

Fluorescent microscopy of EBNA2-positive and -negative NIH 3T3, psi am22b, or Rat-1 clones with EBV-immune human or monospecific anti-EBNA2 rabbit serum (27) revealed that EBNA2 was located predominantly in the nucleus in EBNA2-expressing rodent cells (Fig. 3). Almost all cells of most clones contained a new nuclear antigen. No antigen was detected in control cells (Fig. 3). Although

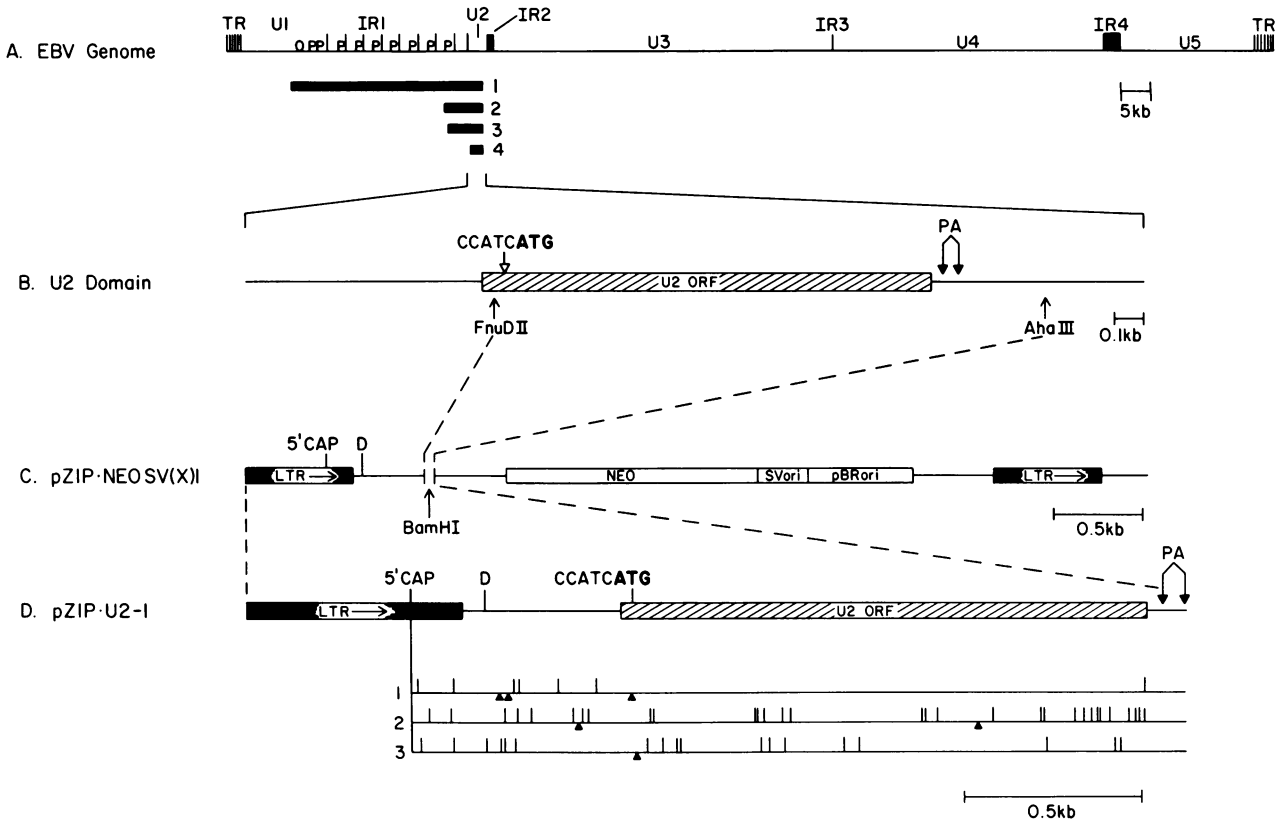

FIG. 1. EBV genome and construction of pZIP-U2-1. (A) Structure of the EBV genome, showing the location of the major internal repeats (IR1 through IR4), terminal repeats (TR), unique sequence domains (U1 through U5) (2, 11), potential transcriptional promoters for EBNA2 mRNA (P) (2, 6, 10, 62), and the *cis*-acting origin (O) of episomal EBV DNA replication, *ori* P (64, 65). Horizontal bars numbered 1 through 4 indicate the EBV DNA fragments which were cloned into the expression vectors pZIP-NEO-SV(X)1 (5) or pRB334 (44). The four fragments end at the *Aha*III site in U2 (10). Fragment 1 begins at the *Eco*RI site in U1 and contains only 1.6 copies of the IR1 repeat. Fragments 2 and 3 both contain the CAAT and TATA sequence of the putative promotor in IR1 and only one complete copy of IR1. Fragment 4 is described in detail below. (B) The 2.9-kb EBV (B95-8) DNA U2 domain, showing the location of the 1,545-bp open reading frame which coincides with the 3′ exon of the EBNA2 mRNA (10, 11, 27, 62). The sequence at the potential translational initiation site is indicated with the ATG codon in boldface. PA indicates the location of two polyadenylation signals, AATAAA and AATTAAA, downstream of the open reading frame. *Fnu*DII and *Aha*III sites indicate the 5′ and 3′ ends of fragment 4. The cleavage site for *Fnu*DII is located 30 bp upstream of the ATG codon. (C) Partial diagram of the eucaryotic expression vector pZIP-NEO-SV(X)1 (5, 53) showing the 5′ and 3′ Moloney MuLV long terminal repeats (LTR); the *Bam*HI site treated with T4 DNA polymerase for insertion of EBV DNA fragments; and *neo* (NEO), the Tn*5*-derived gene encoding resistance to G418. The 5′ cap site for initiating transcription of MuLV proviral DNA and the major 5′ RNA splice donor sequence, D, for formation of subgenomic mRNAs are also indicated. The normal 3′ splice acceptor site for MuLV subgenomic mRNA is located between the *Bam*HI site and the *neo* gene. (D) The 5′ transcriptional unit of the EBV DNA pZIP-NEO-SV(X)1 recombinant, pZIP-U2-1. Open reading frames are shown below the transcript, and termination codons are indicated by vertical lines. Each MuLV ATG and the first EBV ATG for each open reading frame (▲) are also indicated.

EBV-infected human lymphocytes show diffuse nuclear staining and some punctate accentuation with EBNA2-reactive sera (24), rodent cells expressing EBNA2 exhibited more globular nuclear staining and some diffuse nuclear staining (Fig. 3).

Properties of continuous rodent cell lines expressing EBNA2. The growth of NIH 3T3, psi am22b NIH 3T3, or Rat-1 clones which express EBNA2 was compared with that of clones converted to G418 resistance by vector alone. pZIP-U2-1-transfected or MuLV-U2-1-infected foci did not differ from pZIP-NEO-SV(X)1 transfected or infected foci in their initial outgrowth in selective media supplemented with 10% fetal calf serum. Nor was any difference evident on subsequent passage in 10% serum. EBNA2-positive and -negative cells were fully contact inhibited and morphologically indistinguishable. EBNA2-positive and -negative foci also did not differ in their lack of substantial growth in soft agar, although cells transformed with pEJ *ras* or the EBV LMP gene did grow into macroscopically visible foci in soft agar (Table 1). The EBNA2-positive NIH 3T3 or Rat-1 cell clones were also not tumorigenic in nude mice when assayed for tumor formation at 2 weeks after the injection of up to 10^6 cells. However, EBNA2-positive, MuLV-U2-1-infected, Rat-1 cell clones did grow better in medium supplemented with 1 or 0.5% serum than did control Rat-1 cell clones derived in parallel by infection with MuLV from pZIP-NEO-SV(X)1-transfected psi am22b cells (Fig. 4). In these latter experiments, four EBNA2-positive clones were compared with two EBNA2-negative clones. Cells were seeded at 1% of confluency in medium supplemented with 10% serum. After 6 h, cells were washed and maintained in medium supplemented with 10, 1, or 0.5% serum. Beginning 26 h later, thymidine uptake was monitored daily for 5 days. Although no significant difference was noted between the growth of EBNA2-expressing and control Rat-1 cells in 10% serum (as measured by the ratio of thymidine incorporation

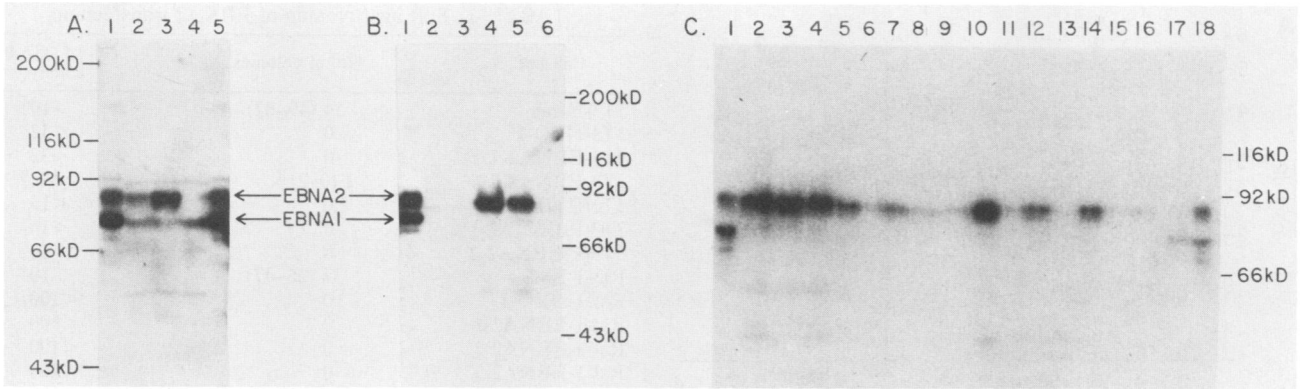

FIG. 2. Rodent fibroblasts transfected with pZIP-U2-1 plasmid or infected with recombinant retrovirus vector MuLV-U2-1 express full-sized EBNA2 in the cell nucleus. (A and B) Immunoblots of whole-cell protein extracts of NIH 3T3 cell clones obtained after transfection with pZIP-U2-1 (panel A, lanes 2 and 3; panel B, lanes 4 and 5) or with pZIP-NEO-SV(X)1 vector only (panel A, lane 4; panel B, lanes 2, 3, and 6). A nuclear protein extract from the EBV-transformed human lymphoblastoid cell line, IB4, is included (panel A, lanes 1 and 5; panel B, lane 1). The human sera used to detect EBNA2 on immunoblots also reacted with EBV EBNA1 protein in the IB4 protein extract. (C) Lanes 2 through 10 are immunoblots of protein extracts of G418-resistant Rat-1 cells derived by infection with recombinant retrovirus produced by the EBNA2-expressing psi am22B (7) clone 1 or clone 2 cell lines. Extracts from the following are shown: three different G418-resistant cloned lines (lanes 2 through 4) and a pool of 50 to 100 G418 foci (lane 5) resulting from infection with virus produced by psi am22b clone 2, and four other cloned lines (lanes 6 through 9) and a pool of drug-resistant foci (lane 10) resulting from infection with psi am22b clone 1 virus. Lanes 12 through 15 contain nuclear (lanes 13 and 15) and cytoplasmic (lanes 12 and 14) protein extracts from EBNA2-positive NIH 3T3 cell lines 2 and 1, respectively. Lanes 11, 16, and 17 contain, respectively, protein extracts from whole cells and nuclear and cytoplasmic fractions of an NIH 3T3 cell line transfected with vector only. Identical results were obtained with EBNA-negative Rat-1 cells. Lanes 1 and 18 contain nuclear extracts from the EBV-infected IB4 human lymphoblastoid cell line. Cell protein extracts were separated by discontinuous 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and then reacted with EBNA2- and EBNA1-reactive EBV immune human sera (10, 27). The human serum used (RB) was preabsorbed with extracts from EBV-negative human lymphoblastoid cells (Loukes) and also untransfected NIH 3T3 cells. Band sizes (in kilodaltons [kD]) are indicated beside each panel.

on day 3, 4, or 5 relative to day 2), and little difference in thymidine incorporation was apparent at day 2 (labeling from days 1 to 2) in 1 or 0.5% serum, the four EBNA2-positive Rat-1 cell clones differed from the two control clones in their growth over 6 days in medium supplemented with 1 or 0.5% serum (Fig. 4; similar data for two other EBNA2-expressing Rat-1 cells are not shown). For example, in 1% serum, the four EBNA2-positive Rat-1 cell clones incorporated 2.2- to

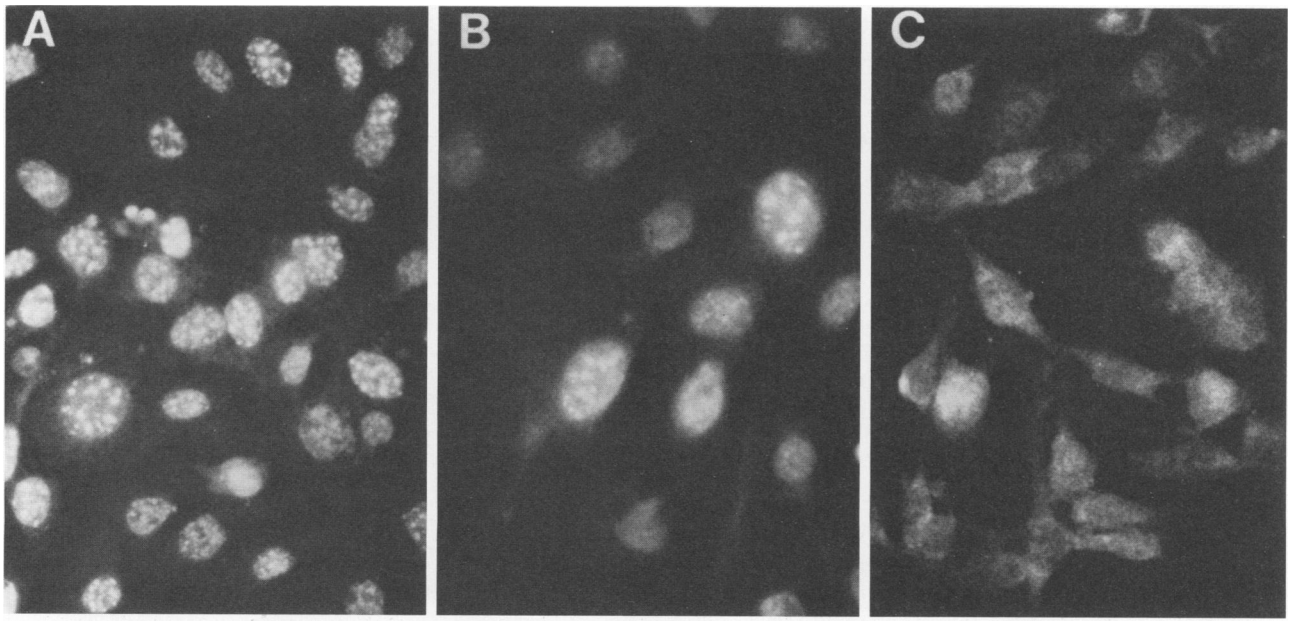

FIG. 3. NIH 3T3 cell clones transfected with pZIP-U2-1 (A and B) or pZIP-NEO-SV(X)1 (C) were reacted with EBNA2-positive human serum followed with biotinylated goat anti-human immunoglobulin G and with fluorescein-conjugated streptococcus avidin (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Bright globular nuclear fluorescence and some diffuse fluorescence was evident in EBNA2-expressing clones 1 and 2 (A and B, respectively). The photomicrograph of control cells (C) was exposed three times as long to demonstrate the absence of nuclear fluorescence at a level at which background cytoplasmic fluorescence was evident. Magnification, ×672.

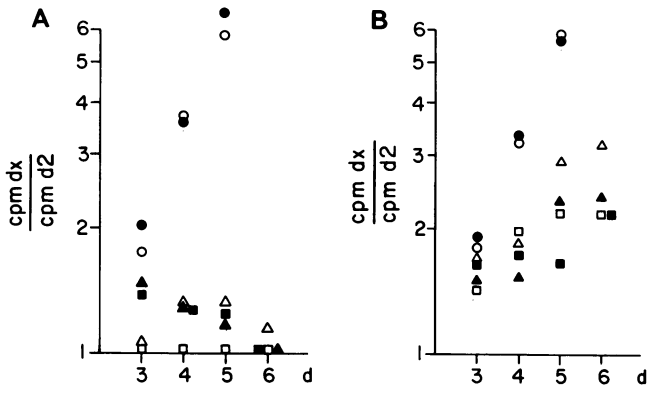

FIG. 4. Growth of Rat-1 cells expressing EBNA2 in low serum. Clones 1 (○, △, □) and 2 (●, ▲, ■) of control Rat-1 cells transfected with pZIP-NEO-SV(X)1 (A) or EBNA2-expressing pZIP-U2-1-transfected Rat-1 cells (B) were seeded at 1% confluency in medium supplemented with 10% fetal calf serum. After 6 h, cells were washed and replenished with medium supplemented with 10% (○, ●), 1% (△, ▲), or 0.5% (□, ■) fetal calf serum. Cells were then labeled with tritiated thymidine for 16 h before being harvested on day 2, 3, 4, 5, or 6. Data are expressed as the ratios of thymidine incorporation at days 3, 4, 5, and 6 relative to that observed at day 2.

TABLE 1. Soft agar cloning of EBNA2 transfectants

Cell line[a]	No. of colonies[b]	No. of cells per colony[c]
3T3-EJras	54 (49–61)	$>10^4$
3T3-NEO-1	0	<32
3T3-EBNA2-1	0	<32
3T3-EBNA2-2	1 (0–2)	<32
3T3(P)NEO-1	0	<16
3T3(P)EBNA2-1	0	<16
3T3(P)EBNA2-2	0	<16
Rat-1-LMP	37 (26–47)	$>10^4$
Rat-1-NEO-1	0	<100
Rat-1-EBNA2-1	0	<100
Rat-1-EBNA2-2	0	<100
Rat-1-EBNA2-3	0	<100
Rat-1-EBNA2-4	0	<100

[a] P in parentheses indicates psi am22b 3T3 cells. 3T3-EJras cells are NIH 3T3 cells transformed with pEJ *ras*; all other cell lines are NIH 3T3 cells transformed with the gene encoding the protein indicated.

[b] Number of colonies per 5,000 cells (the range is shown in parentheses) seeded in a 4-cm dish in 2 ml of Dulbecco modified Eagle medium plus 5% serum and 0.3% agarose.

[c] Approximate cell count at 4 weeks.

3.1-fold more thymidine at day 5 than at day 2 and, in 0.5% serum, incorporated 1.8- to 2.2-fold more thymidine at day 5 than at day 2. In 1% serum EBNA2-negative Rat-1 cells incorporated only 1.2- to 1.6-fold more thymidine at day 5 than at day 2. In 0.5% serum, their thymidine incorporation at day 5 was identical to that observed at day 2. EBNA2-positive Rat-1 cells tended to retain normal morphology in medium supplemented with 1 or 0.5% serum, while EBNA2-negative cells became larger and more circular.

An EBNA2 variant. A corollary of the accumulated evidence that the U2 open reading frame encodes EBNA2 is that EBV isolates which have a variant U2 open reading frame should encode a variant EBNA2 protein. B95-8, Lamont, and Cherry from the United States and Raji and W91 from African Burkitt tumor EBV isolates have U2 open reading frames (type A) which differ considerably from that of the AG876 and Jijoye (type B) African Burkitt tumor EBV isolates (1, 10, 11, 27, 33) (Fig. 5). Most EBNA2-reactive human sera from the United States, such as MC (Fig. 6B) or DR (Fig. 7), react more with the EBNA2 encoded by the type A U2 isolates than with the EBNA2 encoded by the type B U2 isolates (10). WC serum, from a patient with chronic mononucleosis, does not react with EBNA1 (24) and reacts preferentially with the AG876 and Jijoye type B EBNA2s, which are 75 kDa, slightly larger than the EBNA1 encoded by B95-8 virus (10) (Fig. 6A, cf. lanes BIF/AG876 and BIF/95-8; Fig. 7). This unusual reactivity of WC serum with the AG876 EBNA2B is not a consequence of unusual

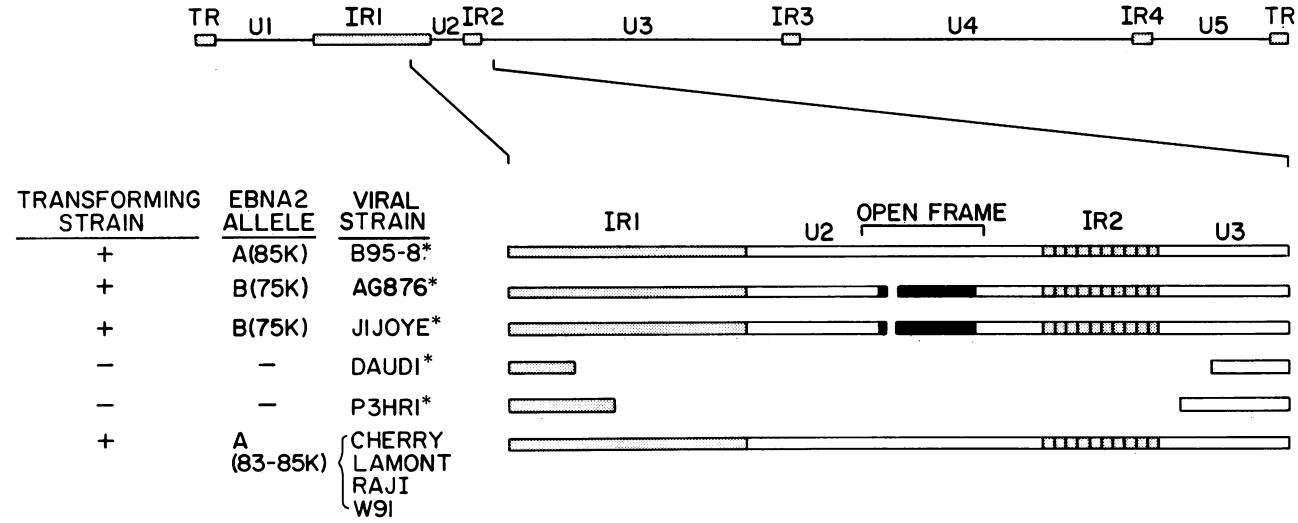

FIG. 5. Summary of allelic variation in EBNA2 and the second unique region (U2). Below the schematic of the EBV genome is an enlargement of the U2 domain from different viral isolates. The EBNA2 open reading frame is indicated by brackets. The AG876 and Jijoye isolates carry smaller EBNA2 open reading frames which exhibit remarkable sequence divergence from the B95-8 EBV U2 region (1, 10, 11, 33). In the Daudi and P3HR-1 isolates, the U2 region is deleted (1, 11, 19, 31–33, 46; Raab-Traub, Ph.D. thesis), and no EBNA2 has been detected in either cell line (10, 27). Symbols: ■, substitution; *, strain for which sequence analysis has been done; □, repeat sequence.

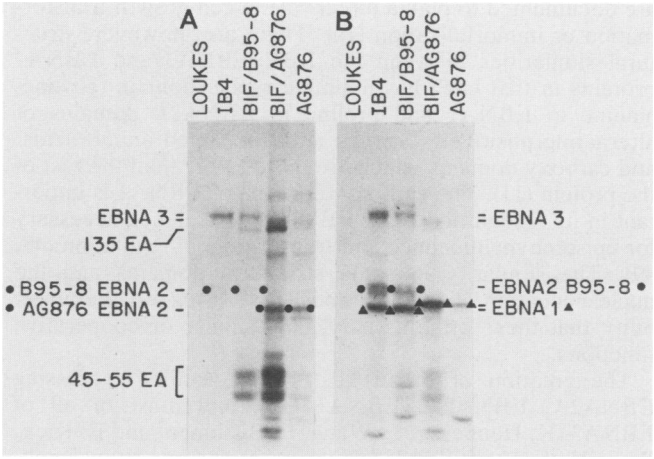

FIG. 6. WC serum reacts preferentially with the EBNA2B. The reactivities of WC (A) and MC (B) sera toward EBNA2A or EBNA2B are compared on Western blots. Note that no early antigens (EA) are present in the IB4 cell line. A longer exposure of panel B was previously presented (24). The Loukes cell line is included as a negative control. BIF/B95-8 and BIF/AG876 are human lymphoblastoid cell lines derived in vitro by infection of a cord blood lymphocyte preparation with B95-8 or AG876 EBV. IB4 is a latently infected, immortalized human lymphoblastoid line derived in vitro by infection with B95-8 EBV (37). AG876 is a lymphoblastoid cell line, derived from a Burkitt tumor biopsy, that produces extracellular AG876 EBV (42). The AG876, BIF/AG876, and BIF/B95-8 cell lines express 45- to 55-kDa early antigen proteins. Symbols: ●, position of EBNA2 (barely visible in the reproduction) and EBNA2B; ▲, position of EBNA1.

modification of the EBNA2B in AG876 cells since WC serum reacted preferentially with AG876 EBNA2B rather than B95-8 EBNA2A when these were expressed in lymphocytes of the same individual (Fig. 6, cf. lanes BIF/AG876 and BIF/B95-8). The unusual reactivity of WC serum with the AG876 EBNA2B could arise from the infection of this patient with a B-type virus or as a consequence of reactivity with a type-common EBNA2 epitope and abundant expression of EBNA2 by the AG876 virus. WC serum detected more EBNA2 in AG876 than in Jijoye (Fig. 7), although the size of Jijoye EBNA2 is identical to that of AG876, as is expected from the near identity of their U2 DNA sequences (1, 10; G. Bornkamm, personal communication).

Confirmation that the WC serum reacts with EBNA2B was obtained by absorbing the WC serum with P3HR-1 virus-infected lymphocytes and by expressing the AG876 U2 open reading frame in rodent cells. P3HR-1 is deleted for U2 and does not express an EBNA2 protein (27, 33), but otherwise it is similar to other EBV isolates (46; Raab-Traub, Ph.D. thesis). Absorption of WC serum with p3HR-1-infected cells removed reactivity with most other EBV proteins such as the 50- to 55-kDa proteins of the early antigen complex and increased the specific reactivity with type A and type B EBNA2 (Fig. 7). AG876 and Jijoye EBNA2B are 75 kDa, slightly larger than the B95-8 encoded EBNA1 and considerably smaller than the B95-8-encoded EBNA2A (10, 26) (Fig. 6 and 7). Frequently, on repeated analysis of an AG876 sample, a slightly smaller EBNA2B component equal in size to B95-8-encoded EBNA1 was evident (Fig. 6 and 7). Because this smaller protein was not present on initial analyses of some samples and because it reacts with the absorbed WC serum, it is likely to be a protease product of EBNA2B. Transfection of psi am22b NIH 3T3 cells with recombinant EBV AG876 U2-pZIP-NEO-SV(X)1 plasmid (pZIP-U2B) resulted in expression of 75- and 74-kDa EBNA2B proteins identical in size to the authentic AG876 EBNA2B, which is slightly larger than B95-8 EBNA1 (Fig. 6 through 8), and to the putative protease product, which is equal in size to B95-8 EBNA1 (Fig. 6 through 8). Since WC serum did not react with EBNA1 and since EBNA2 is 82 kda, no 75-to 74-kDa proteins were detected by WC serum in psi am22b NIH 3T3 cells expressing EBNA1 or EBNA2A (Fig. 8). WC serum reacted preferentially with EBNA2B expressed in psi am22b cells and had less reactivity with EBNA2A expressed in

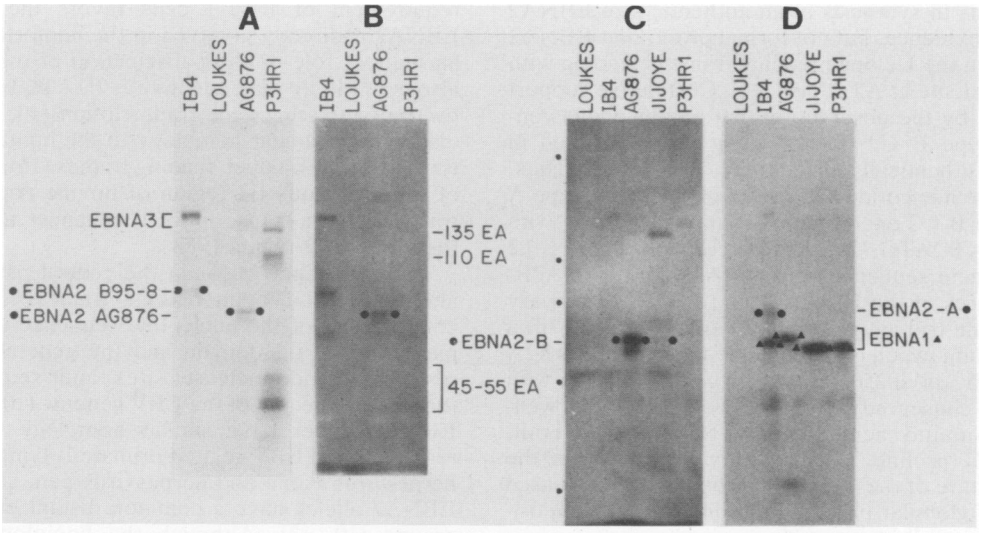

FIG. 7. EBNA2B is in AG876 and Jijoye cells but not in P3HR-1 cells. WC serum (B) and DR serum (C) were absorbed repeatedly with induced P3HR-1 lysate and compared with unabsorbed WC serum (A) and DR serum (D) at the same dilution on Western blots. Several early antigens (EA) are indicated. AG876 and Jijoye have the same size of EBNA2B identified by WC serum. DR serum reacts with the EBNA2A of IB4 cells but not with the EBNA2B of AG876 or Jijoye.

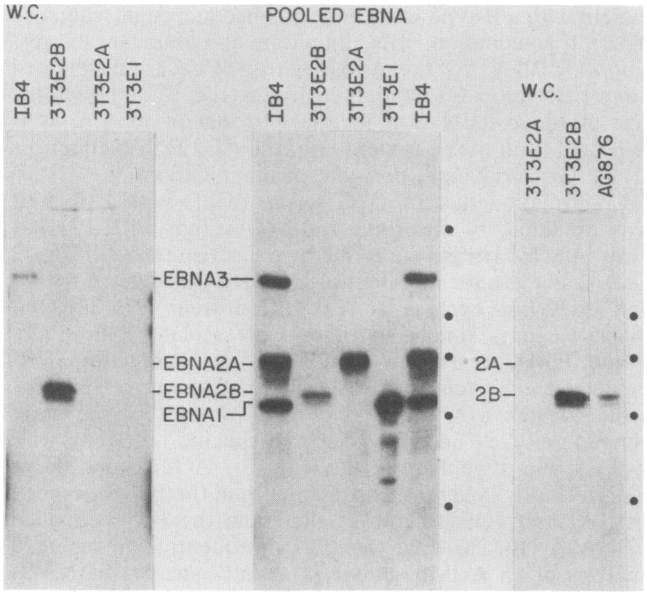

FIG. 8. The AG876 U2 open reading frame encodes EBNA2B. The AG876 U2 open reading frame was recombined into pZIP-NEO-SV(X)1 and transfected into psi am22B NIH 3T3 cells (left panel). EBNA2B identified with WC serum (left panel) is identical in size to AG876 EBNA2B (right panel). Pooled EBNA serum (middle panel) detected EBNA1, EBNA2A, and EBNA3 in an IB4 cell nuclear extract, EBNA2A in cells transfected with pZIP-U2-1, EBNA1 in cells transfected with pZIP-EBNA1, and EBNA2B in the pZIP-EBNA2B-transfected cells.

these cells (Fig. 8). EBNA2B expressed from the recombinant plasmid pZIP-U2B was identical in size to EBNA2B in AG876 cells (Fig. 8, right panel).

DISCUSSION

The finding that gene fusions between the EBV U2 open reading frame and an expression vector which provides for transcription and 5′ mRNA leader but not for the initiation of translation results in synthesis of an authentic size EBNA2 provides strong evidence, but not formal proof, that EBNA2 is translated from the U2 open reading frame beginning with the potential start site at ATCATG (10). Correlative support is also provided by the observation that an analogous construct from a type B U2 open reading frame resulted in expression of authentically sized type B EBNA2. Thus, translation of the nucleotide sequences of the B95-8 type A and AG876 type B U2 open reading frames, beginning with the conserved ATCATG (10), almost certainly defines the primary amino acid sequences of EBNA2A and EBNA2B. Prominent features of the EBNA2A and EBNA2B primary sequences include (i) highly conserved amino termini, (ii) a polyproline domain which is significantly longer in EBNA2A than in EBNA2B, (iii) a 250-amino acid central domain with less than 50% conserved amino acids, and (iv) a well-conserved 110-amino acid carboxy terminus. Overall, EBNA2A is 28% proline. Consequently, prediction of the secondary structure of these proteins from algorithms based on structures of globular proteins of more normal composition might be unusually inaccurate.

Notably, there was no significant homology between the proposed EBNA2 primary amino acid sequences and the sequences of a large number of other nuclear proteins which are documented to play a direct role in cell growth transformation or immortalization (10). There are, however, structural similarities between the EBV EBNA1 and EBNA2 proteins in that each has a simple repeat domain (glycine-alanine in EBNA1 and proline in EBNA2), domains of alternating positively charged and uncharged amino acids, and carboxy domains which are more acidic than the rest of the protein (11). The carboxy terminus of EBNA1 is important in its recognition of an EBV DNA sequence necessary for episome maintenance and transcriptional regulation (48, 49). The similarity in some structural domains and the nuclear location of EBNA2 and EBNA1 suggest the possibility that these proteins may have similar or cooperative functions.

The creation of broad host range vectors expressing EBNA2A, EBNA2B, EBNA1 (30), and most or all of EBNA3 (K. Hennessy, F. Wang, E. Bushman, and E. Kieff, Proc. Natl. Acad. Sci. USA, in press) has important implications for experiments delineating their biologic and chemical roles in EBV-induced cell transformation and persistent latent infection since the effects of these proteins can now be assayed in a wide variety of primary and continuous cells. Our data indicate that EBNA2 reduces the serum requirement for growth of Rat-1, a continuous Fisher Rat cell line (15). In this respect, EBNA2 is similar to polyomavirus large-T, simian virus 40 T, adenovirus E1A, and v-*myc* (29, 36, 47, 51). These other genes not only reduce the serum requirement of continuous cell lines but also immortalize primary rodent fibroblasts. Experiments to test whether EBNA2 can immortalize primary rodent fibroblasts and further tests of the effects of EBNA2 in reducing the serum or growth factor requirements of other cells in continuous culture are as yet inconclusive. Lymphocytes transformed by AG876 EBV which has the EBNA2B allele were recently reported to have a lower saturation density than lymphocytes transformed by B95-8 EBV which has the EBNA2A allele (A. Rickinson, 10th Int. Herpesvirus Workshop, p. 310, 1985). Like the serum effects demonstrated here for EBNA2A in Rat-1 cells, the different saturation density could reflect a differential effect of EBNA2A and EBNA2B on the cell requirement for growth factors.

While the demonstration that EBNA2 reduces the serum requirement of RAT-1 cells favors the hypothesis that EBNA2 is directly involved in the immortalization process, an indirect role or the participation of other viral genes is also possible. In fact, adenovirus E1A may immortalize cells by transactivating gene transcription (40, 58). EBNA2 has distant but colinear homology to the human papillomavirus type 8 E2 and E4 open reading frames (16), and one function of the analogous E2 region of bovine papillomavirus is to transactivate a transcriptional enhancer near the 5′ end of the virus early region (57).

The divergence between the central parts of the type A and type B U2 open reading frames contrasts with the conservation of the nucleotide sequence of noncoding segments in the U2 domain and the general conservation of restriction endonuclease sites and sequence homology throughout the rest of the EBV genome (10, 19, 33, 46). Both EBNA2 alleles have similar homology to the analogous regions of the EBV-related primate B lymphotropic viruses, herpesvirus Papio and herpesvirus pan, suggesting that the EBNA2 alleles have a common distant evolutionary background (10, 33). Although the homology to the human papillomavirus type 8 E2 and E4 open reading frames is modest, it is more significant than is found to published herpes simplex varicella-zoster or cytomegalovirus DNA

sequences. EBV and human papillomavirus share a common niche in epithelial cells (54, 55). Dual infection has been demonstrated (18). Thus, papillomaviruses could interact genetically with herpesviruses, including EBV.

Since this manuscript was submitted, a 13-kb Jijoye EBV IR1-U2-IR3-U3 DNA fragment was cotransfected into LTK$^-$ cells, resulting in a cell line containing a new 76-kDa nuclear protein reactive with WC serum (50). The protein was similar in size to a protein detected in Jijoye cells with WC serum (50) and described here as EBNA2B. By inference, these recently reported data extend the results of earlier cotransfection experiments which demonstrated that the analogous large EBV DNA fragments from type A EBV isolates induced expression in some cells of EBNA2 (39, 52), a protein characteristic of latent EBV infection (10, 27). Also, in another study, analyses of EBNA2 mRNA cDNA clones (J. Sample, M. Hummel, D. Braun, M. Birkenbach, and E. Kieff, Proc. Natl. Acad. Sci. USA, in press) revealed that (i) the promoter for transcription of EBNA2 mRNA is probably within IR1, (ii) EBNA2 mRNA is bicistronic, and (iii) EBNA2 is translated from the EBV U2 sequence, beginning with the translational start site indicated in Fig. 1.

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