JOURNAL OF VIROLOGY, June 1987, p. 1796–1807
0022-538X/87/061796-12$02.00/0

Vol. 61, No. 6

Expression of the Epstein-Barr Virus gp350/220 Gene in Rodent and Primate Cells

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Received 6 November 1986/Accepted 18 February 1987

The gene encoding the Epstein-Barr virus envelope glycoproteins gp350 and gp220 was inserted downstream of the cytomegalovirus immediate-early, Moloney murine leukemia virus, mouse mammary tumor virus, or varicella-zoster virus gpI promoters in vectors containing selectable markers. Host cell and recombinant vector systems were defined which enabled the isolation of rodent or primate cell clones which expressed gp350/220 in substantial quantities. Continued expression of gp350/220 required maintenance of cells under positive selection for linked markers and periodic cloning. gp350/220 expressed in various host cells varied slightly in electrophoretic mobility, probably reflecting differences in glycosylation. Insertion of a stop codon into the gp350/220 open reading frame, upstream of the putative membrane anchor sequence, resulted in efficient secretion of truncated gp350 and gp220 from rat pituitary (GH3) cells. gp350/220 expressed in mammalian cells is highly immunogenic and elicits virus-neutralizing antibodies when administered to mice.

Epstein-Barr virus (EBV) infection is prevalent in all human populations. The manifestations of primary infection in normal humans range from insignificant illness to infectious mononucleosis (25). In immune-deficient humans, EBV can cause fatal lymphoproliferative disease (8). Persistent or latent EBV infection is an important etiologic factor in Burkitt's lymphoma in Africans and in nasopharyngeal carcinoma among ethnic southern Chinese (13). Because of the associated morbidity and mortality, prevention of EBV infection is desirable. Prevention of infection by vaccination would likely require immunization with those viral antigens which are displayed on the outer surface of the virus and of virus-infected cells.

Like other herpesviruses, EBV is surrounded by a lipid envelope. Glycoproteins of 350, 220, and 85 kilodaltons (kDa) have been identified on the outer surface of the EBV envelope (15–18, 26, 38–40, 43, 45–47, 50–52, 56–58). These glycoproteins are also on the outer surface of infected cells in which the virus is replicating. Because of their location, these glycoproteins are likely to be important in the development of immunity to EBV infection. Monoclonal or polyclonal monospecific antisera against gp350 react with gp220 and neutralize viral infectivity, indicating that these two glycoproteins have common epitopes which can generate a neutralizing antibody response (26, 38, 51, 57, 58). Absorption of EBV-immune human sera with gp350 prepared from EBV-infected cells substantially reduces their neutralizing potency, suggesting that a considerable fraction of the human neutralizing antibody response is directed against gp350 (58). gp350 and gp220 also appear to be target membrane antigens for antibody-dependent cellular cytotoxicity (47). Moreover, in some experiments, immunization with gp350 protected cotton-top tamarins against EBV-induced lymphoma (20, 21).

Both gp350 and gp220 are encoded by the same EBV DNA open reading frame (2, 3, 27). While gp350 is encoded by the continuous open reading frame, gp220 is encoded by two exons of the same frame. Some features of the proteins which are predicted from the gp350/220 mRNA nucleotide sequences (2) are as follows. (i) The primary translation product of gp350 is 907 amino acids, while that of gp220 is 710 amino acids. (ii) The first 18 amino acids common to both proteins are likely to be a cleavable signal peptide (61). (iii) At 26 amino acids away from the carboxy terminus of both proteins, there is a single hydrophobic domain likely to be a transmembrane anchor. (iv) The 850 amino acids of gp350 and the 653 amino acids of gp220 between the putative signal and anchor sequences are serine and threonine rich and contain 36 (gp350) or 25 (gp220) asparagine-X-serine or -X-threonine sequences, respectively, which are potential signals for N-linked glycosylation. These regions are therefore likely to be on the outer surface of the membrane. In fact, peptide-specific antisera demonstrated that this region is displayed on the outer surface of the virus and of virus-infected cells (2). The gp350 and gp220 primary translation products have anomalous electrophoretic mobilities in polyacrylamide gels run in the presence of sodium dodecyl sulfate (SDS) and appear to be larger than expected. Even so, the protein core still accounts for less than half of the apparent 350- and 220-kDa sizes of the fully glycosylated glycoproteins (2, 18, 27, 39, 52). gp350 is extensively modified with N-linked and O-linked glycosyl side chains since treatment with *N*-glycanase to remove complex and high-mannose N-linked sugars results in an apparent size of 230 kDa (L. Schultz, J. Tanner, K. Hofman, E. Emini, J. Condra, R. Jones, E. Kieff, and R. Ellis, submitted for publication), while the apparent size of the completely unglycosylated gp350 precursor is 135 kDa (27).

The unusually extensive glycosylation of gp350 and gp220 is likely to influence structure and immunogenicity. When a significant portion of the gp350 protein was expressed in and purified from *Escherichia coli*, an antibody response could be induced in rabbits which neutralized EBV (2). However, multiple injections of substantial quantities of protein were

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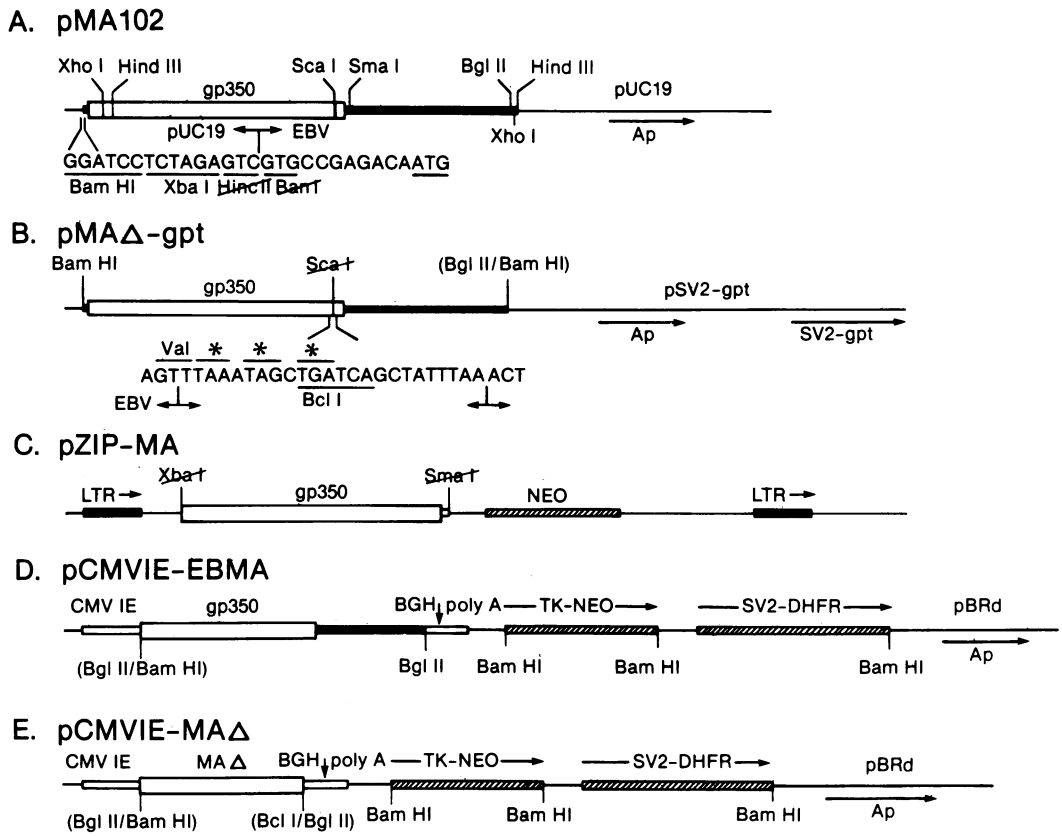

FIG. 1. Structure of recombinant DNA clones. (A) An EBV DNA fragment beginning 10 bp upstream of the gp350 open reading frame (represented by the open box) and ending 2 kbp downstream of the gp350 mRNA polyadenylation site (represented by thick solid line) was inserted into the polylinker of pUC19 to create pMA102. Restriction endonuclease sites destroyed during the cloning are indicated by a slash. (B) pMAΔ-gpt is pSV2-gpt containing the EBV gp350 DNA fragment of pMA102. An oligonucleotide containing a *Bcl*I site and stop codons (*) in all three frames was inserted into a *Sca*I site located at the beginning of the coding region for the hydrophobic transmembrane domain. (C) pZIP-MA is pZIP-neo-SV(X)1 containing the EBV gp350 DNA fragment of pMA102 inserted into the *Bam*HI site of the expression vector. The MuLV LTR drives the expression of both the gp350 gene and the neomycin (G418) phosphotransferase mRNAs. (D) pCMVIE-EBMA is the EBV gp350 DNA fragment of pMA102 inserted into a *Bgl*II site downstream of the CMV IE transcriptional promoter in pCMVIE-AK1-DHFR. This expression vector contains two selectable marker genes, the neomycin (G418) resistance gene and the DHFR gene. (E) pCMVIE-MAΔ contains the EBV gp350 DNA sequence derived from pMAΔ-gpt in the same vector as panel D above.

required to elicit this response. The poor immunogenicity of the *E. coli*-expressed protein may be attributable to the absence of glycosylation. Since yeasts recognize signals for both N- and O-linked glycosylation, yeasts can be used to express glycosylated forms of gp350 (Schultz et al., submitted). However, the structure of the yeast glycosyl side chains may differ significantly from those of higher eucaryotic cells, and this could also affect the structure and immunologic identity of the glycoprotein (1, 36). Mammalian cells would be expected to express these glycoproteins in a form most similar to natural gp350 and gp220. Therefore, we undertook to express gp350/220 in rodent and primate cells so that we could study the properties of the mammalian-expressed glycoprotein.

MATERIALS AND METHODS

Plasmid constructions. The EBV gp350 coding domain was subcloned from the EBV DNA *Bam*HI L fragment (11) into pUC19 in two steps. First, the 340-base-pair (bp) *Ban*I-*Hin*dIII fragment coding for the N terminus was inserted between the pUC19 *Hin*cII and *Hin*dIII sites. Then a 4.2-kbp *Xho*I fragment, which begins at the *Xho*I site within the 340-bp *Ban*I-*Hin*dIII fragment and contains the rest of the gp350 coding region, was inserted into the cloned *Ban*I-*Hin*dIII fragment to recreate the complete coding sequence in pUC19 (pMA102, Fig. 1A). To construct the gp350 gene with a stop codon inserted before the carboxy-terminal transmembrane anchor sequence (pMAΔ-gpt), the 4.4-kbp *Bam*HI-*Bgl*II fragment of pMA102 containing the complete gp350 open reading frame was first inserted into the *Bam*HI site of pSV2-gpt (41) to create pMA-gpt. A synthetic oligonucleotide containing stop codons in three reading frames and a *Bcl*I site was then inserted into the *Sca*I site of the gp350 gene which interrupts the gene 8 bp after the beginning of the membrane anchor domain.

pZIP-MA (Fig. 1C) was constructed by ligating the 2.8-kbp *Xba*I-*Sma*I fragment of pMA102 into the *Bam*HI site of pZIP-neo-SV(X)1 (5) after target and vector DNAs were treated with T4 DNA polymerase. pCMVIE-EBMA (Fig. 1D) and pCMVIE-MAΔ (Fig. 1E) were constructed by inserting the 4.4-kbp *Bam*HI-*Bgl*II fragment of pMA102 and the 2.6-kbp *Bam*HI-*Bcl*I fragment of pMAΔ-gpt, respectively, into the *Bgl*II site of the expression vector pCMVIE-AK1-DHFR (M. Silberklang, J. Kopchick, S. Munshi, A. Lenny, T. Livelli, and R. Ellis, manuscript in preparation).

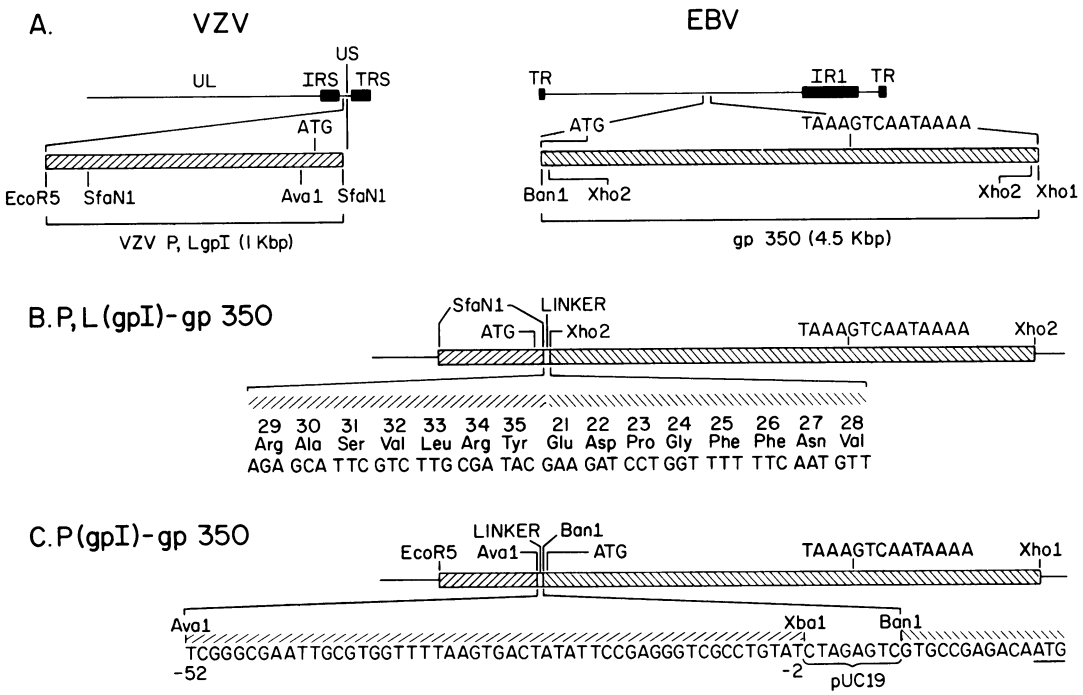

FIG. 2. Assembly of the VZV gpI promoter-EBV gp350 gene constructs. (A) Locations of the VZV gpI promoter and the EBV gp350 gene in their respective viral genomes. (B) Arrangement in the fusion construct of the VZV gpI promoter (P) and the leader sequence (L) of the VZV gpI protein up to codon 35, joined in frame to the gp350 sequence at codon 21. (C) Arrangement of the complete gp350 gene in the nonfusion construct juxtaposed with the VZV gpI promoter. The promoter segment extends from the *Eco*RV site right up to within 2 bp of the original VZV gpI ATG. As a consequence of the cloning protocol, the gp350 ATG is separated from the promoter segment by 19 bp.

This vector consists of plasmid pBRd (14), the mouse dihydrofolate reductase (DHFR) gene under simian virus 40 early promoter regulation (53), the Tn*5* aminoglycoside phosphotransferase gene under herpes simplex virus I thymidine kinase promoter regulation (9), and a unique *Bgl*II site for insertion of a gene between the major immediate-early (IE) promoter of cytomagalovirus (CMV) (44, 55) and the *Pvu*II-*Eco*RI translation termination and poly(A) signal region of the bovine growth hormone gene (64) (Fig. 1). A series of plasmids having CMV IE (55), murine leukemia virus long terminal repeat (MuLV LTR [49]), mouse mammary tumor virus (MMTV) LTR (32), or human metallothionein (29) promoters upstream of the gp350 open reading frame were constructed by inserting the respective promoter sequences at the *Bam*HI site of pMA-gpt.

Two types of varicella-zoster virus (VZV) gpI promoter-EBV gp350 recombinant plasmids were constructed (Fig. 2). VZV P,L(gpI)-gp350 is a VZV promoter-leader–EBV gp350 recombinant which consists of 890 bp of the VZV gpI promoter region, including the first 35 VZV gpI codons, fused in frame to EBV gp350 codon 21 (12) (Fig. 2B). VZV P(gpI)-gp350 is a recombinant which consists of 950 bp of the VZV gpI promoter region, extending to 2 bp before the VZV gpI translational initiation codon, fused to the EBV gp350 gene beginning 19 bp upstream of its translational initiation codon (Fig. 2C).

VZV P,L(gpI)-gp350 was constructed by inserting the T4 DNA polymerase-treated, 890-bp *Sfa*NI fragment from pSVG0-12 (a plasmid containing the *Sac*I G fragment of VZV DNA, which includes the VZV gpI gene [19]; gift of Paul Keller) into the *Hin*cII site of pUC19. The EBV *Bam*HI L-*Xho*II fragment containing the gp350/220 gene (Fig. 2A) was then inserted into the *Bam*HI site of the pUC19 polylinker so that the 21st codon of EBV gp350 was separated from the 33rd codon of VZV gpI by two codons of the pUC19 polylinker. These two codons were subsequently replaced by synthetic oligonucleotide linkers to restore VZV gpI codons 34 and 35 (Fig. 2B).

VZV P(gpI)-gp350 was constructed by ligating an 898-bp *Eco*RV-*Ava*I VZV gpI fragment to an *Ava*I-*Xba*I synthetic oligonucleotide. This restored 50 bp of VZV sequence downstream of the *Eco*RV-*Ava*I fragment so that the VZV sequence terminates at −2 bp from the VZV gpI translational initiation site. The resulting *Eco*RV-*Xba*I fragment was inserted into the *Xba*I site of pMA102 (described above and shown in Fig. 1) which places the VZV sequence 19 bp upstream of the complete gp350 gene. The recombination sites in these plasmids were verified by sequencing across the sites by the chain termination method (48) with EBV or VZV primers.

Cell culture, transfection, selection, and cell sorter analysis. Rat pituitary GH3 cells (54) were maintained in Dulbecco modified Eagle medium supplemented with 10% Nu-Serum (Collaborative Research, Inc., Waltham, Mass.). Mouse Ltk$^-$ APRT$^-$ cells (63) were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum. NIH 3T3 or Vero cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Chinese hamster ovary (CHO) cells (line DXB11, [59]) were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum.

For transfection, plasmids were prepared by equilibrium banding in CsCl-ethidium bromide gradients and were at least 90% covalently closed circular DNA. GH3 cells were transfected with 5 μg of plasmid DNA and 15 μg of carrier DNA by the calcium phosphate method (63). Selective

medium (Dulbecco modified Eagle medium containing 10% Nu-Serum and 200 μg of G418 [GIBCO Laboratories, Grand Island, N.Y.] per ml) was applied 24 h after removal of DNA. For transfection of Ltk$^-$ cells, 1 μg of plasmid DNA (and 19 μg of Ltk$^-$ DNA) was applied per plate for 20 to 24 h. Cells were exposed to 15% glycerol shock (34), fed with complete medium, and placed in medium with 400 μg of G418 per ml 24 h later. CHO cells were transfected with 5 μg of plasmid DNA and 15 μg of carrier DNA. After 4 h, cells were exposed to 15% glycerol shock for 2 min (34). Three days later, cells were fed with Ham's F12 medium lacking glycine, hypoxanthine, and thymidine and containing 10% dialyzed fetal bovine serum and 200 μg of G418 per ml. NIH 3T3 or Vero cells were transfected with 10 μg of plasmid DNA per T25 flask. DNA was left on the cells for 6 h. The cells were then fed with nonselective medium and passaged 2 days later. NIH 3T3 cells were placed in medium containing 500 μg of G418 per ml. Vero cells transfected with plasmids containing the gp350 gene downstream of the CMV IE, MuLV, or MMTV promoters in pSV2-gpt were selected by growth in hypoxanthine-aminopterin-thymidine medium supplemented with 250 μg of xanthine per ml and 33 μg of mycophenolic acid per ml (41). Plasmids containing the gp350 gene downstream of the VZV gpI promoter were cotransfected with pSV2-gpt. GH3 and Ltk$^-$ colonies were picked by cylinder cloning, expanded, and assayed for gp350 expression either by indirect immunofluorescence or by dot immunoblot. NIH 3T3 colonies were screened for gp350 expression by the in situ erythrocyte (RBC) rosetting assay (33). The colonies were first incubated with monoclonal antibody 2L10 specific for gp350 (gift of Gary Pearson). After washing, they were then incubated with RBCs coated with rabbit anti-mouse immunoglobulin G (IgG). By this sensitive assay, almost all cell colonies were found to be expressing gp350. About 1,000 colonies were pooled, and cells expressing the most surface gp350 were separated by cell sorting on a FACS II cell sorter (Becton Dickinson FACS Systems, Mountain View, Calif.). Briefly, lightly trypsinized cells were stained with 2L10 antibody and fluorescein-conjugated goat anti-mouse IgG. About 60% of the cells fluoresced above background. These cells were collected and cultured for 14 doublings. At a second round of sorting, the 15% most intensely fluorescent cells were collected, and clones were derived by plating at limiting dilution. Transient transfection of Vero and NIH 3T3 cells employed calcium phosphate as described above. Cells were assayed for gp350 expression by the RBC rosetting assay 2 or 3 days after transfection for transient expression.

Immunoblots and immunofluorescence. For dot-blot assay of secreted gp350, clarified culture medium was mixed with 1/10th volume of 1% CHAPS (3-[(3-cholamedopropyl)-dimethylammonio]-1-propane sulfonate [Serva, Heidelberg, Federal Republic of Germany]) in TBS (10 mM Tris [pH 7.6], 250 mM NaCl). An equal volume of 2× TBS with 40% methanol was then added. For assay of intracellular gp350, cells were suspended in an equal volume of 2× CHAPS lysis buffer (200 mM sodium HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] buffer [pH 7.5], 300 mM NaCl, 20 mM EDTA, 1% CHAPS). After 10 min at 25°C and a 5-min centrifugation in an Eppendorf microcentrifuge, supernatants were diluted with 4 volumes of 1× TBS–20% methanol. Samples (100 μl) were then applied to nitrocellulose which had been preequilibrated in 1× TBS–20% methanol in a 96-well dot-blot apparatus. Each well was washed with 500 μl of 1× TBS–20% methanol. The nitrocellulose was air dried for 1 h and then incubated for 2 h at 37°C in Blotto (28) (10% [wt/vol] nonfat dry milk, 0.9% [wt/vol] NaCl, 10 mM Tris, pH 7.5). High-titer EBV-immune human serum (VCA titer, 1:10,000; EA titer, 1:10,000 and EBNA titer, 1:160) was added to the Blotto and incubated at room temperature for 18 h (VCA is virus capsid antigen, EA is early antigen, and EBNA is EBV nuclear antigen). The filter was then washed twice in blot wash solution (50 mM Tris [pH 7.5], 250 mM NaCl, 3 mM EDTA, 0.05% Tween 20) for 1 h at 37°C and incubated for 2 h in affinity-purified ^{125}I-labeled protein A (2×10^5 cpm/ml; Amersham Corp., Arlington Heights, Ill.) in Blotto at 37°C. After two more 1-h washes at 37°C, filters were dried for 1 h and exposed to X-ray film. Known amounts of affinity-purified gp350 were used as standards in blot assays. (Because of its extensive glycosylation, gp350 protein determinations [by comparative staining of protein in acrylamide gels] are only approximate.) For immunoblot analysis (4), protein extracts were made from whole cells or medium by boiling in SDS-sample buffer (31). Protein samples were run on a 5 or 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and incubated with high-titer EBV-immune human serum or with BMA17 (a gift of Gary Pearson), a monoclonal antibody against the gp350 polypeptide expressed in *E. coli*, and rabbit anti-mouse IgG antibody (Cooper Biomedical, Inc., West Chester, Pa.). Blots were developed with ^{125}I-labeled protein A (Amersham). All incubations and washes were done in Blotto.

For analysis of fixed cells by indirect immunofluorescence, transfected cells were grown on a chamber slide (Miles Laboratories, Inc., Elkhart, Ind.), fixed in acetone or methanol at −20°C for 10 min, and incubated with 2L10 monoclonal antibody ascites fluid (1:200 dilution in phosphate-buffered saline and 5% goat serum), biotinylated goat anti-mouse IgG (1:200 dilution; Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and fluorescein-conjugated streptavidin (1:200 dilution; Bethesda Research Laboratories). Slides were observed and photographed with a Zeiss photomicroscope equipped with epifluorescence.

Nucleic acid isolation and blot hybridization. RNA was isolated from GH3 Δ19 cells by homogenization of cells in guanidine thiocyanate and centrifugation of the RNA through a CsCl cushion (6). Northern blotting was done as previously reported (60), except that a GeneScreen Plus (New England Nuclear Corp., Boston, Mass.) membrane was used. High-molecular-weight DNA was isolated from cells, and Southern blotting was carried out as described previously (24).

Immunization, immunoprecipitation, and virus neutralization. Swiss mice (obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were immunized with NIH 3T3 CL34 cells which express gp350. Each mouse was injected with 2×10^6 cells either in medium or emulsified in complete Freund adjuvant and subsequently with cells in medium or incomplete Freund adjuvant. After a third injection, antibody to gp350 was detectable by immunoprecipitation of gp350. For immunoprecipitation, the surface proteins of 2×10^7 induced B95-8 cells were radiolabeled with ^{125}I with lactoperoxidase (43), and the cells were lysed with 1 ml of a buffer containing 20 mM Tris (pH 7.4), 0.15 M NaCl, and 1% Nonidet P-40. Soluble extract (100 μl) was incubated with 10 μl of mouse serum and then with protein A-Sepharose. After three washes, the protein A-Sepharose was boiled in SDS-sample buffer and run on an SDS-polyacrylamide gel (31). For live-cell immunofluorescence, induced B95-8 cells were suspended for 1 h on ice in mouse serum diluted with phosphate-buffered saline and 5% goat

serum. After washing, cells were suspended for 60 min on ice in fluorescein-conjugated goat anti-mouse IgG diluted 1:100 with phosphate-buffered saline and 5% goat serum.

The activity of immune mouse serum in the neutralization of EBV (P3HR-1) infectivity was assayed by evaluating the reduction in infectivity for Raji cells (10). A 0.2-ml sample of the P3HR-1 culture supernatant was added to 0.1 ml of the mouse serum serially diluted in RPMI 1640 medium containing 10% fetal bovine serum and incubated for 1 h at 37°C. For complement-dependent neutralization, 0.1 ml of a 1:5 dilution of mouse complement (Cooper Biomedical) was added, and the virus was incubated for a further hour. Raji cells (2×10^5 in 0.5 ml) were added to the virus and, after 48 h, were assayed for early antigen expression with a human serum with a high antibody titer to EBV early antigens.

VZV infection. Vero cells (approximately 5×10^6 cells in a 25-cm^2 flask) were infected with VZV (Oka) when 70 to 80% confluent by removing culture fluid and overlaying the cells with an undiluted preparation of cell-free sonicated virus for 60 min at 37°C. Medium was added at the end of the incubation without removing the virus. The virus used for this purpose had been previously passaged in Vero cells, and its titer was 10^3 PFU/ml. Cells were harvested for gp350/220 assay when characteristic cytopathic effects and plaques appeared to involve most of the cell monolayer at 14 days after infection.

RESULTS

Expression of gp350. The complete gp350 open reading frame was inserted into pUC19, and the sequence at the cloning site was verified (Fig. 1). The open reading frame was then subcloned into eucaryotic expression vectors. One vector, pZIP-neo-SV(X)1 (5), has the MuLV promoter and the neomycin phosphotransferase gene to allow positive selection. A second, pCMVIE-AK1-DHFR, has a CMV IE promoter and DHFR and neomycin phosphotransferase genes to allow positive selection. pZIP-MA was used to transfect NIH 3T3 cells, while pCMVIE-EBMA was used to transfect CHO, GH3, or Ltk^- cells. After selection for the covalently linked positive selection marker, random colonies or colonies identified to be expressing gp350 by an in situ RBC rosette assay (with a gp350-specific monoclonal antibody) were cloned and further screened for gp350 expression by live-cell immunofluorescence and cell sorting or by fixed-cell immunofluorescence and dot blot. Clones differed considerably in uniformity and level of gp350 expression. In general, clones which expressed gp350/220 by fixed-cell immunofluorescence or dot blot had cells which were swollen and detaching from the monolayer. The abnormal cells were the most positive in fluorescence. The association of high-level gp350/220 expression and cytopathology was the basis for a working hypothesis that gp350/220 expression was toxic to cells.

CHO clones which expressed gp350 were the least uniform in fluorescence staining. Less than 10% of the cells in any clone scored above background. With passage in selective medium, the percentage of antigen-positive cells rapidly declined. Stable gp350-expressing clones could not be isolated even after selecting for cells expressing gp350 by the binding of gp350-specific antibody to live cells and sorting with a fluorescence-activated cell sorter (FACS). GH3 cell clones were slightly more stable, and L-cell clones were more stable than GH3. One relatively stable clone, L8, was expanded for further studies (see Fig. 4B).

To improve the likelihood of isolating stable clones expressing gp350, we followed a different strategy with NIH

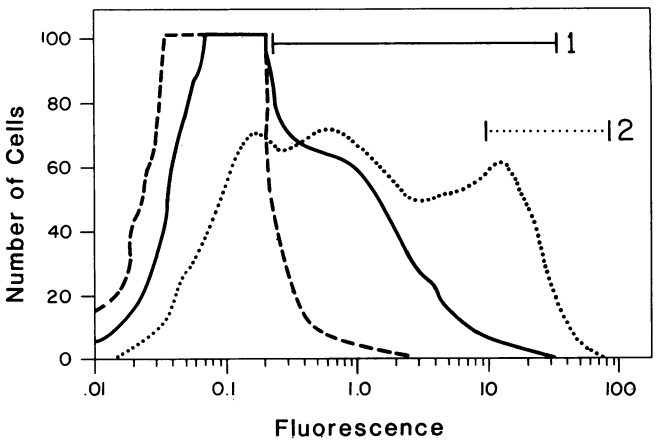

FIG. 3. FACS analysis of transfected NIH 3T3 cells stained with gp350-specific antibody. NIH 3T3 cells were transfected with pZIP-MA and selected for G418 resistance, and the resistant clones were pooled. The distribution of fluorescence intensity of pooled transfected cells stained with the anti-gp350 monoclonal antibody 2L10 and fluorescein-conjugated goat anti-mouse IgG antibody is indicated by the solid line. Cells staining above background (indicated by the solid bar, 1) were collected, cultured, and reanalyzed. The latter is represented by the dotted line. Cells exhibiting higher fluorescence (within the dotted bar, 2) were collected and cloned by limiting dilution. The dashed line represents background fluorescence of cells stained only with the fluorescein-conjugated antibody.

3T3 pZIP-MA transfectants. Approximately 1,000 G418-resistant colonies were pooled, incubated with gp350-specific monoclonal antibody, and subjected to two rounds of FACS cell sorting (Fig. 3). The cells which fluoresced above background (within solid bar of Fig. 3) were first sorted and recultured. In a second round of FACS analysis of the positive cells after 14 doublings in culture, most of the cells exhibited fluorescence above background. The 15% most intensely fluorescent cells (within dotted bar of Fig. 3) were sorted and cloned by limiting dilution. Several of these clones were expanded and analyzed for gp350 and gp220 expression. The apparent size and abundance of gp350 and gp220 relative to each other and relative to protein size markers such as myosin and thyroglobulin varied slightly among the many gels and immunoblots which were run, presumably as a consequence of anomalies attendant to the extensive glycosylation of the proteins. Most clones expressed more gp350 and gp220 than did EBV-infected B95-8 cells which had been induced to replicate EBV (for example, see Fig. 4A). The gp350 and gp220 glycoproteins expressed by NIH 3T3 or L cells were slightly smaller (by about 20 kDa) than gp350 and gp220 from B95-8 cells. NIH 3T3 cells expressed approximately one-third as much gp220 as gp350, while B95-8 cells expressed a lower ratio (about 1/6) of gp220 to gp350. By fluorescence microscopy, NIH 3T3 cell clones derived after two sorting cycles were more uniform in gp350 expression than unsorted colonies. Most cells expressed detectable antigen. Within cells, gp350 antigen was diffuse in the cytoplasm, with perinuclear accentuation (Fig. 5). There were also large and small patches of antigen in the endoplasmic reticulum and large patches through most of the plasma membrane. Thus, the distribution is similar to that observed in EBV-infected lymphocytes with gp350-specific monoclonal antibodies (26).

Vero may be a useful cell line for expression of gp350/220. As a continuous primate cell line, its glycosylating enzymes may be similar to those of human cells. Further, Vero has

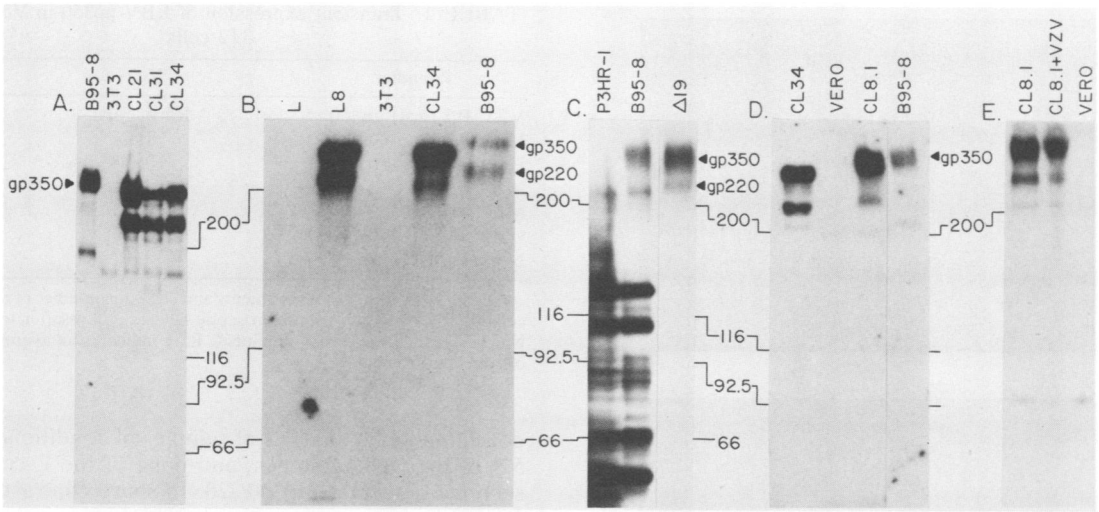

FIG. 4. Immunoblots of gp350/220 proteins synthesized by transfected mammalian cells. (A) Three clones (CL21, CL31, and CL34) of NIH 3T3 cells that were transfected with pZIP-MA and selected for high expression of gp350 by the FACS. The B95-8 lane contains membrane-enriched protein extracts of B95-8 lymphocytes induced for EBV replication. The 3T3 lane contains protein extracts of NIH 3T3 cells transfected with pZIP-neo vector only. (B) L cells, the L8 clone derived from L cells by transfection with pCMVIE-EBMA, NIH 3T3 cells, and CL34 cells. The B95-8 lane contains affinity-purified gp350/220. (C) The Δ19 lane contains affinity-purified gp350/220 from the tissue culture supernatant of the Δ19 clone of GH3 cells. The P3HR-1 and B95-8 lanes contain the whole-cell extracts of P3HR-1 and B95-8 cells induced for EBV replication. The immune human serum used to develop this immunoblot reacts with gp350/220 and many other EBV proteins in induced P3HR-1 or B95-8 cells. Although the mobilities shown are typical, the variability from gel to gel for the GH3 Δ19 proteins was somewhat greater than for the other proteins illustrated. (D) CL34 cells, Vero cells, CL8.1 cells [a Vero clone transfected with VZV P(gpI)-gp350 and expressing gp350/220], and induced B95-8. (E) Lane 1 shows the expression of gp350/220 in CL8.1 cells, and lane 2 shows the equivalent aliquot of the same cells after infection with VZV. Protein samples were electrophoresed in SDS-polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose filters were reacted with either the BMA17 monoclonal antibody and rabbit anti-mouse IgG (panels A, D, and E) or EBV-immune human serum (panels B and C), followed in both cases with ^{125}I-labeled protein A. The positions of proteins of known size (kilodaltons) (200, myosin; 116, galactosidase; 92.5, phosphorylase; 66, albumin) are indicated.

been used for production of poliovirus for human immunization (37). Vero also supports the replication of VZV, a live virus vector we planned to develop for EBV gp350/220 expression. Therefore, we sought to express gp350/220 in Vero cells using a variety of vectors. To evaluate the utility of various promoters in Vero cells, plasmids containing the gp350 gene under control of the MMTV, MuLV, human metallothionein, or CMV IE promoters in pSV2-gpt were transfected into Vero cells, and the cells were assayed for transient gp350 expression by the gp350 antibody-RBC rosetting assay. A VZV promoter which regulates the synthesis of the abundant VZV late glycoprotein, gpI, was also evaluated as a promoter for expression of EBV gp350/220 in Vero cells. VZV gpI and human metallothionein promoters yielded detectable expression of gp350 in Vero cells, while the MMTV, CMV IE, and MuLV promoters were less active or inactive (Table 1; Fig. 6). A different construct with the VZV gpI promoter, in which the VZV gpI leader peptide-coding sequence was recombined in frame with the gp350 open reading frame (Fig. 2), yielded very little expression of gp350 at the cell surface as assayed by the RBC rosetting assay. Transfection of these recombinant vectors into NIH 3T3 cells yielded similar results except for transfection of the vector with the MuLV promoter which expressed a gp350/220 in NIH 3T3 cells, although it was inactive in Vero cells. A second series of experiments was directed toward obtaining permanent clones of Vero cells expressing gp350. Vero cells were transfected with plasmids containing each of the promoters upstream of gp350 in pSV2-gpt or were cotransfected with either of the two VZV gpI promoter-gp350 constructs and pSV2-gpt. Clones obtained with the CMV IE promoter (15 of 15) or MMTV promoter (12 of 12) were negative for gp350 expression by the RBC rosetting assay, immunoblot, and immunofluorescence. One clone (1 of 23) obtained with the MuLV LTR vector was weakly positive by immunofluorescence analysis, but gp350 and gp220 expression were undetectable by immunoblot (data not shown). Of 50 clones of Vero cells obtained by cotransfection with pSV2-gpt and VZV P(gpI)-gp350, CL8.1 was found to express gp350 and gp220. This clone expressed more gp350 and gp220 than B95-8 cells induced to replicate EBV. Unlike the gp350/220 produced in rodent cells, gp350/220 electrophoretic mobility in Vero cells was identical to that of gp350 and gp220 obtained from induced B95-8 cells (Fig. 4D). The distribution of gp350/220 in Vero cells was also similar to that in B95-8 cells replicating EBV (Fig. 6C). Expression has been stable for the ensuing year that the clone has been passaged. Southern blot analysis of the VZV gpI promoter and gp350 DNA sequences in this cell line, using restriction endonucleases which cut upstream of the gpI promoter and within the gp350 gene in the recombinant plasmid that was transfected into these cells, demonstrated that these sequences remain connected in the same size fragment as in the transfected plasmid DNAs (data not shown). These experiments indicate that the VZV gpI promoter-EBV gp350/220 recombinant can function to express gp350/220. When this clone was infected with VZV, however, no increase in gp350 synthesis was observed, indicating that expression could not be substantially *trans*-activated by VZV infection (Fig. 4E).

Secretion of gp350. To avoid the putative toxic effects of membrane-bound gp350/220 and to facilitate purification, we attempted expression of a secreted form of gp350/220. The deduced amino acid sequence of gp350 has only one hydro-

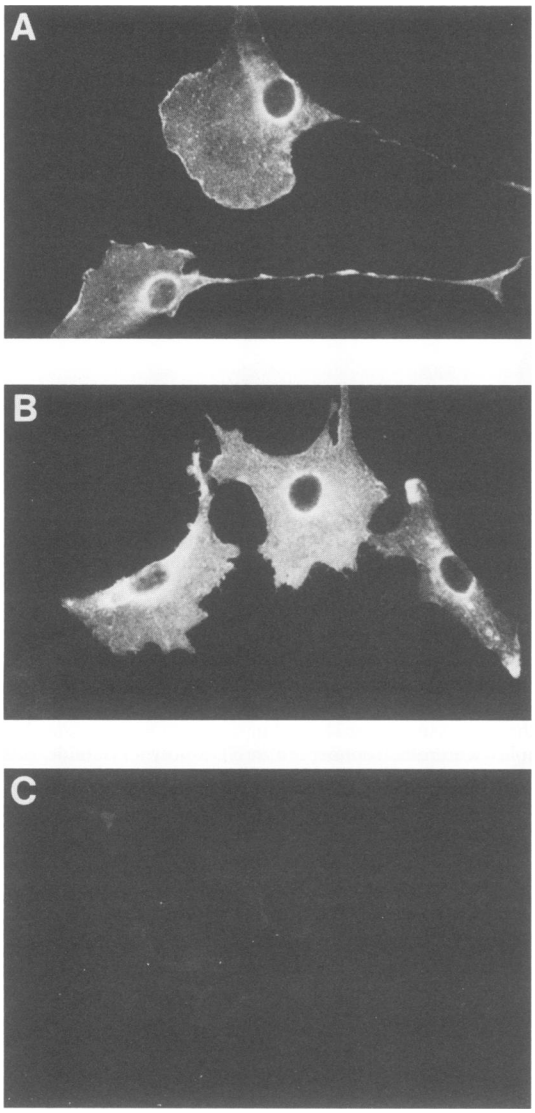

FIG. 5. Immunofluorescence of gp350-expressing NIH 3T3 CL34 cells. Acetone-fixed gp350-expressing NIH 3T3 CL34 cells (A and B) or cells transfected with vector only (C) were developed for immunofluorescence with a gp350-specific monoclonal antibody (2L10), biotinylated anti-mouse IgG, and fluorescein-conjugated streptavidin.

phobic domain which extends for more than 10 amino acids. This domain, near the carboxy terminus, consists of 23 hydrophobic amino acids which are predicted by Chou and Fasman (7) to assume an alpha-helical conformation similar to that of known transmembrane anchor domains. If this is the principal membrane anchor domain in gp350, introduction of a translational stop codon at the beginning of the coding sequence corresponding to this domain would be expected to result in complete translocation and secretion of a nearly full-sized gp350 molecule. Therefore, an oligonucleotide consisting of stop codons in all three reading frames was inserted into the third codon of the putative anchor sequence. The resulting truncated gp350 open reading frame was inserted into an expression vector to create pCMVIE-MAΔ (Fig. 1). This plasmid was transfected into CHO, GH3, or L cells, and G418-resistant colonies were isolated. Upon

TABLE 1. Transient expression of EBV gp350 in Vero and NIH 3T3 cells[a]

Promoter	Vero	NIH 3T3
VZV P,L(gpI)-gp350	Rare; rare	Rare; rare
VZV P(gpI)-gp350	8; 22	40; 46
Human metallothionein	10; 33	10; 67
MuLV LTR	Rare; rare	50; 20
MMTV LTR	2; rare	0; 14
CMV IE	0; rare	0; rare
None (pMA102)	0; 0	0; 0

[a] Results are from two experiments and show the number of rosette-forming cells in a low-power (×40) microscope field at 3 days after transfection, by which time cells were fully confluent. Rare indicates an average of less than one rosette per field.

dot-immunoblot analysis of samples of conditioned medium, 5% of the CHO colonies, and none of the L-cell colonies, secreted detectable gp350/220. In sharp contrast, almost all GH3 colonies were secreting gp350/220 antigen, and approximately 10% of these secreted large amounts. One clone (Δ19) was selected for subsequent analysis. The secreted gp350 and gp220 proteins were barely distinguishable in electrophoretic mobility from the membrane-bound gp350/220 expressed in rodent cells from vectors with the complete open reading frame (Fig. 4C). The productivity of the GH3 Δ19 cell line for gp350/220 was measured by dot-blot immunoassay (Fig. 7). A 1-ml portion of conditioned medium contained approximately 2.5 μg of gp350/220 as judged by comparative immunoreactivity against a purified gp350/220 standard. Surprisingly, when GH3 cells expressing gp350 were fixed and analyzed by indirect immunofluorescence microscopy with antibody to gp350/220, no intracellular antigen was detected (data not shown). The efficient secretion of gp350/220 from GH3 cells is consistent with their efficient secretion of growth hormone (54).

To prove that the gp220 protein expressed by rodent host cells carrying recombinant gp350 plasmids was made from a spliced mRNA similar to the B95-8 gp220 mRNA, we analyzed RNA from GH3 Δ19 cells expressing secreted glycoproteins by Northern blot analysis. The CMV IE promoter sequence provides a cap site and approximately 70 bp of 5′ untranslated leader upstream of the 2.8 kbp of EBV DNA encoding gp350 in the transfected plasmid. The end of the mRNA transcribed from this plasmid should include 115 bp of untranslated sequence contributed by the bovine growth hormone gene polyadenylation sequence (64). The size of the GH3 Δ19 gp350 mRNA was 3.0 kilobases (kb) (Fig. 8), in good agreement with the expected size of a nonspliced transcript of the recombinant construct (2,3). The second gp350-specific polyadenylated RNA in GH3 Δ19 cells was 2.4 kb (Fig. 8), also in good agreement with the expected size of the recombinant gp220 mRNA (2, 3, 27). The 2.4-kb GH3 Δ19 cell RNA must be spliced similarly to EBV gp220 mRNA, since a probe specific for the EBV gp220 mRNA intron hybridized to the 3.0-kb RNA but not to the 2.4-kb RNA (Fig. 8). In GH3 cells, the gp350 and gp220 RNAs were expressed in a ratio of 2:1 (as measured from densitometric scan of the Northern blot autoradiogram). This indicates that the transcript is more efficiently spliced in GH3 cells and presumably in other fibroblast cell lines used in this work than in EBV-infected B95-8 cells (27).

Immunogenicity of gp350/220 expressed in rodent cells. Mice were immunized with NIH 3T3 CL34 cells expressing gp350 and gp220 to determine whether these proteins had substantial immunogenicity. After three injections, the

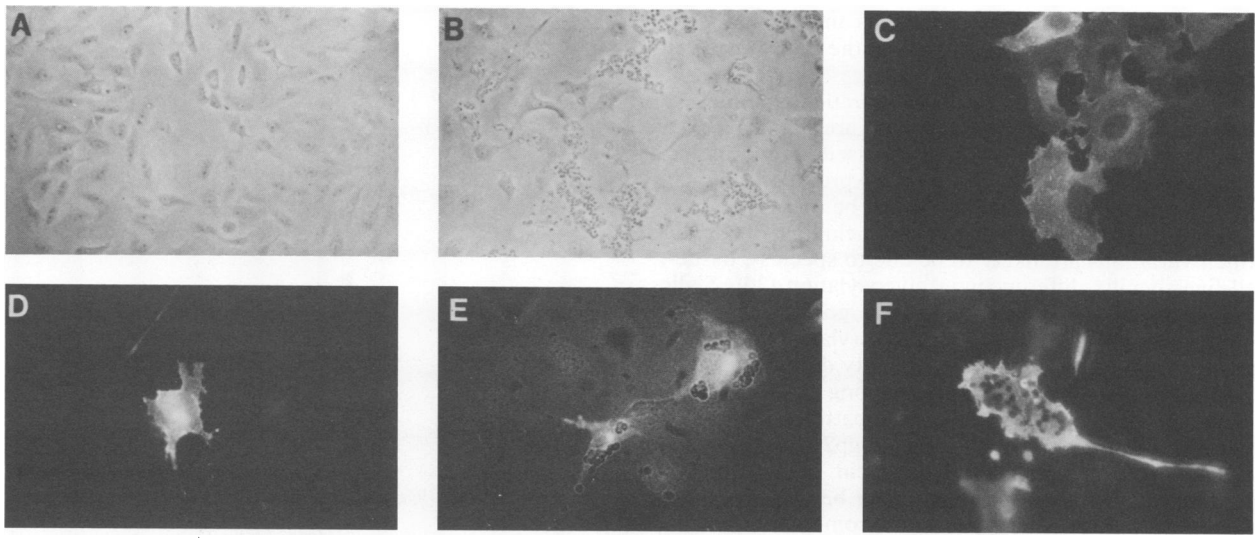

FIG. 6. Expression of EBV gp350 in Vero and NIH 3T3 cells with the VZV gpI and metallothionein promoters. (A and B) Transient expression of EBV gp350 after transfection of Vero cells is detected by an in situ RBC rosetting assay in which cells are reacted with an anti-gp350 monoclonal antibody (72A1) and then indicator RBCs coated with goat anti-mouse IgG antibody. (B) RBC rosettes are clearly visible on some Vero cells after transfection with VZV P(gpI)-gp350, while in panel A pMA102-transfected Vero cells show no rosettes. (C) Biotin-streptavidin-enhanced immunofluorescence of a methanol-fixed Vero cell clone, CL8.1, stably expressing gp350 after transfection with VZV P(gpI)-gp350 and selection with mycophenolic acid. (D, E, and F) Transient expression of gp350 detected by immunofluorescence of methanol-fixed cells. (D) Vero cell expressing gp350 after transfection with VZV P(gpI)-gp350. (E) gp350-expressing NIH 3T3 cells after transfection with VZV P(gpI)-gp350. (F) NIH 3T3 cells express gp350 under the control of the human metallothionein promoter. In panels E and F, gp350 was first detected on live cells by the monoclonal antibody-RBC rosetting assay before methanol fixation. In panels D, E, and F, immunofluorescence was carried out by reacting the fixed cells with a high-titered human serum and then with biotinylated goat anti-human IgG antibody followed by fluorescein-conjugated streptavidin.

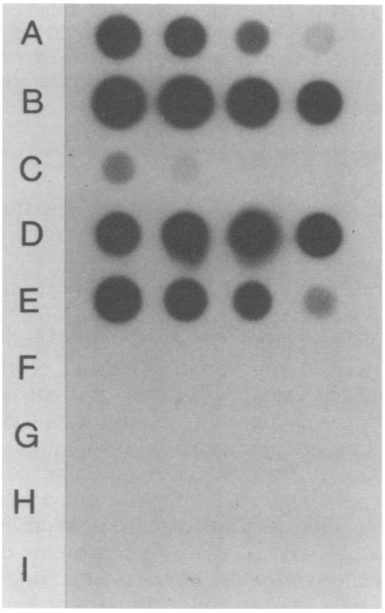

FIG. 7. Dot-blot estimation of gp350/220 production in several stably transfected cell clones expressing recombinant gp350/220. Dot-blot analysis was as follows. A, L8*; left to right, 10, 5, 2, 1 μg of protein; B, NIH 3T3 CL34*; left to right; 10, 5, 2, 1 μg of protein; C, Vero CL8.1*; left to right; 10, 5, 2, 1 μg of protein; D, GH3 Δ19, conditioned medium; left to right, 400, 200, 100, 50 μl; E, induced B95-8, affinity-purified gp350/220; left to right; 250, 125, 50, 25 ng of protein; F, Ltk$^-$ APRT$^-$* (control); left to right, 10, 5, 2, 1 μg of protein; G, NIH 3T3* (control); left to right, 10, 5, 2, 1 μg of protein; H, Vero* (control); left to right; 10, 5, 2, 1 μg of protein; I, GH3 (control), conditioned medium; left to right: 400, 200, 100, 50 μl. Note nonlinear response of the immunologic detection of gp350/220 at very high protein load, especially in rows B and D. *, CHAPS extract (Materials and Methods).

mouse serum contained antibody which specifically immunoprecipitated gp350 from extracts of B95-8 cells and specifically reacted with the surface of induced B95-8 cells in a live-cell immunofluorescence assay (Fig. 9). Mice immunized with cells suspended in Freund adjuvant had higher levels of antibody than mice immunized with cells suspended in medium (Fig. 9). The serum from mice immunized with gp350-expressing cells in adjuvant neutralized EBV at a 1:100 dilution without complement and at a 1:1,000 dilution in the presence of complement (Fig. 10).

DISCUSSION

We showed that the EBV gp350 and gp220 glycoproteins can be abundantly expressed in rodent or primate cells. These proteins are highly immunogenic in mice and induce the production of antibody which neutralizes EBV infectivity, particularly in the presence of complement. In previous experiments with rabbits, multiple injections of large amounts of an unglycosylated gp350 fusion protein, produced in *E. coli*, were necessary to attain a similar titer of neutralizing antibody (2). Subsequently, it was found that expression in *E. coli* of almost the entire gp350 protein, without fusion to beta-galactosidase, did not substantially enhance its immunogenicity in rabbits and mice (unpublished observations). These experiments, though not definitive, are consistent with the hypothesis that glycosylated forms of EBV gp350 and gp220 are superior immunogens in eliciting a neutralizing antibody response. Certainly, the

mammalian cell-expressed glycoproteins more closely resemble natural gp350 and gp220 than do the unglycosylated proteins expressed in *E. coli*.

It is noteworthy that the electrophoretic mobilities of gp350 and gp220 expressed in rodent cells are slightly greater than those of the Vero- or B95-8-expressed glycoproteins. The electrophoretic mobility of gp350 expressed in human cells has also been previously noted to be slightly greater than that of gp350 from tamarin cells, including B95-8 (17, 43). Such variations are likely to be due to species-, tissue-, or cell line-specific differences in glycosylation (30). Sialic acid content has an inverse effect on gp350 and gp220 electrophoretic mobilities in SDS-polyacrylamide gels (39). In any case, these electrophoretic mobility differences are a reminder that there are differences among mammals, primates, or even humans in glycosylation patterns (30). From this perspective, expression of gp350 or gp220 from a virus vector in which posttranslational protein modification is provided by the subject's own cells may be advantageous. gp350 has been expressed by using recombinant vaccinia virus (35). However, vaccinia virus has fundamental disadvantages as a vector because of the expression of numerous unnecessary vaccinia antigens as well as dermatological and neurological sequela associated with the vaccination procedure. Our studies of VZV promoter-EBV gp350 recombinants were undertaken with a long-range goal of examining the utility of VZV as a virus vector for gp350 expression (R. Lowe, P. Keller, B. Keech, A. Davison, Y. Whang, A. Morgan, E. Kieff, and R. Ellis, Proc. Natl. Acad. Sci. USA, in press). The experiments described here indicate that the VZV gpI promoter-gp350 coding sequence recombinant is functional in gp350 expression and can be used to construct live VZV-gp350 recombinants.

A frequent observation throughout our studies on mammalian expression of gp350/220 has been the rapid loss of expression, upon serial passage, by many clonal cell populations, especially those derived from the DHFR-deficient CHO line DXB11. Loss of gp350 and gp220 expression occurred despite uninterrupted maintenance of the cells in selective medium. For CHO clones, in particular, loss of expression of the membrane-anchored as well as the secreted forms of the glycoproteins occurred within 1 month of continuous passage in a medium dually selective for G418 resistance and DHFR expression, taking advantage of our dual-marker vector (Fig. 1D and E). Attempts to recover gp350 expression from declining clones, using FACS to segregate cells retaining expression or incubation of cells in 5-azacytidine or *n*-butyrate to reverse methylation (23), were to no avail (unpublished observations). During the latter experiments, it was observed that the FACS fraction of a clonal CHO cell population which contained greater than 90% intensely fluorescent cells exhibited less than 10% plating efficiency. This is consistent with the hypothesis that high gp350 expression is inimical to cell growth. Loss of expression is thus probably accelerated by the differential growth rate between expressor and nonexpressor cells. In any case, our results illustrate the utility of working with a variety of host cells and vectors which, in the present instance, has allowed the isolation of several useful clones expressing the membrane-anchored or secreted forms of gp350.

The abundant secretion of gp350 and gp220 from GH3 rat pituitary cells was a particularly fortuitous finding for two reasons. First, these cells continue to secrete protein when maintained in defined medium without serum (unpublished observations). Under these conditions, gp350 and gp220 are

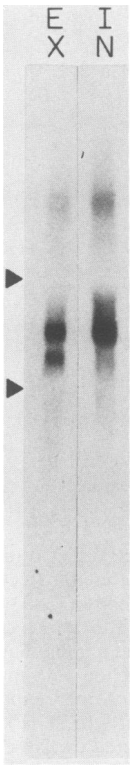

FIG. 8. Northern blots of the GH3 cell line expressing gp350 and gp220 mRNA. Total RNA from the GH3 Δ19 cell line, which secretes gp350 and gp220 proteins lacking a transmembrane anchor sequence, was separated by formaldehyde-agarose gel electrophoresis and transferred to a GeneScreen Plus membrane. The blots were hybridized with either an *Xba*I-*Sma*I fragment probe from pMA102 which includes the entire EBV gp350 DNA segment (EX) or a 0.58-kb *Nco*I-*Nde*I probe specific for the gp220 intron (IN). The positions of 28S (4.9-kb) and 18S (1.8-kb) RNAs which were internal size markers are indicated.

the largest proteins which are found in the extracellular fluid (unpublished observations), and their purification is relatively simple. A yield of 2.5 to 5 mg of gp350/220 per liter of medium is typically obtained (D. Lehman, personal communication). Second, the failure to detect gp350 or gp220 within the GH3 cells secreting these proteins implies a highly efficient posttranslational processing and secretion of this peptide sequence. The accumulation of a considerable fraction of membrane-anchored gp350 in the plasma membrane of each cell line also indicates efficient targeting of gp350 through the Golgi apparatus. The potential contribution of the various gp350 and gp220 peptide domains to their efficient plasma membrane translocation or secretion should be further evaluated. Herpesvirus glycoproteins differ in their targeting to cell membrane compartments, and these differences can be exploited to reveal important determinants of cellular localization.

The vectors employed in this study include promoters which are active in primate cells. Thus, these may be useful for achieving expression of gp350 in tamarin or human cell lines. Such cells expressing gp350 would be useful for evaluating the T-cell immune response to gp350. This may prove to be an important parameter in conferring host protection against EBV infection.

EBV adsorbs to lymphocytes by binding to CR2, the

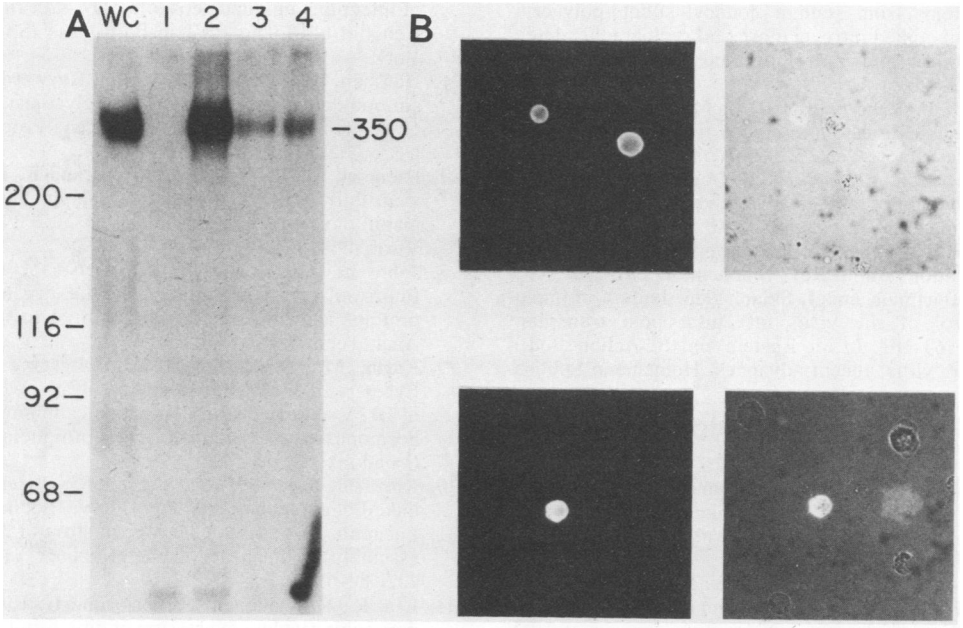

FIG. 9. Immunoprecipitation and immunofluorescence of B95-8 cells by immune mouse serum. (A) Sera from mice immunized with gp350/220-expressing NIH 3T3 CL34 cells were used to immunoprecipitate ^{125}I-labeled surface proteins of induced B95-8 cells. A high-titer EBV-immune human serum (WC) served as a positive control. Serum from a mouse immunized with NIH 3T3 cells transfected with pZIP-neo vector only (lane 1) did not react with the surface-labeled B95-8 cell extract, while sera from mice immunized with gp350-expressing NIH 3T3 CL34 cells administered either in medium without adjuvant (lanes 3 and 4) or in Freund adjuvant (lane 2) specifically immunoprecipitated gp350. (B) B95-8 cells were incubated with a 1:40 dilution of the mouse serum described in panel A, lane 2, and then with fluorescein-conjugated goat anti-mouse IgG. The left panel is a photomicrograph of a field under epifluorescence showing antigen on these cells induced to replicate EBV. The right panel is the same field with visual-light illumination showing the presence of latently infected cells which are not expressing gp350/220. Negative control sera gave no fluorescence and are not shown.

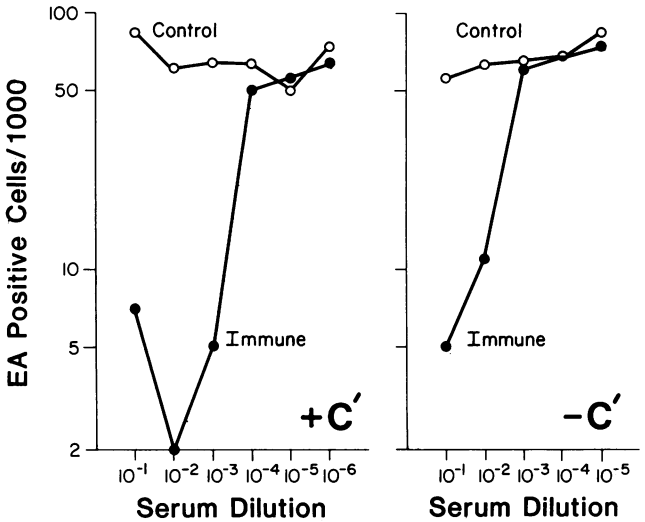

FIG. 10. Neutralization of EBV by immune mouse serum. Mouse sera were assayed for neutralizing activity in the presence or absence of complement (C'). P3HR-1 virus was incubated either with serum only or with serum and then complement and used to infect Raji cells. Early antigen (EA)-positive and -negative cells were counted after 48 h. Symbols: ●, immune mouse serum; ○, the serum from the mouse immunized with NIH 3T3 cells transfected with the vector alone.

complement C3d receptor (22, 42). Partially purified gp350 and gp220 reconstituted into liposomes interfere with EBV adsorption, suggesting that gp350 or closely associated molecules mediate EBV adsorption (62). The glycoproteins produced in rodent or primate cells should prove useful in evaluating the role of gp350 in receptor binding.

ACKNOWLEDGMENTS

We thank Jan Kai and Hans Schreiber for assistance in cell sorting, Tom Heineman for assistance in immunoprecipitations, Pamela Burke for synthesis of oligonucleotides, and Emilio Emini, Paul Keller, Robert Lowe, Gary Pearson, Marci Armstrong, and Jerome Tanner for materials, advice, or assistance.

This research was supported by Public Health Service grants CA 17281 and CA 19264 from the National Institutes of Health and by ACS MV32L from the American Cancer Society. Y.W. is supported by National Research Service award GM07281. A.J.M. was the recipient of a United Kingdom Medical Research Council travelling fellowship.

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