Epstein-Barr Virus Glycoprotein Homologous to Herpes Simplex Virus gB

MICHAEL GONG, TADAMASA OOKA, TAKUMI MATSUO, AND ELLIOTT KIEFF*

Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637

Received 23 June 1986/Accepted 23 October 1986

The Epstein-Barr virus DNA open reading frame BALF4 (R. Baer, A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Stachwell, C. Sequin, P. S. Tuffnell, and B. G. Barrell, *Nature* [London] 310:207–211, 1984), which by nucleotide sequence comparison could encode a protein similar to herpes simplex virus gB (P. E. Pellett, M. D. Biggin, B. Barrell, and B. Roizman, *J. Virol.* 56:807–813, 1985), has now been shown to encode a 110-kilodalton glycoprotein. Late infectious cycle RNAs of 3.0 and 1.8 kilobases are transcribed from BALF4. Translation of these RNAs in vitro, transcription and translation of BALF4 in vitro, or metabolic labeling of cells in the presence of tunicamycin and immunoprecipitation with BALF4-specific sera results in identification of a 93-kilodalton precursor to gp110. Since *N*-glycosidase F only reduces the size of gp110 to 105 kilodaltons, gp110 probably has both *N*- and *O*-linked glycosylation. gp110 is an abundant glycoprotein in Epstein-Barr virus-infected cells. In infected lymphocytes and in 3T3 cells, in which the gene is expressed from a recombinant expression vector, most of the protein is cytoplasmic and perinuclear. In contrast to gB, gp110 was not detected in the infected-cell plasma membrane. In cells replicating Epstein-Barr virus, gp110 localized to the inner and outer nuclear membrane lamellae and to endoplasmic reticulum structures which sometimes contained enveloped virus. gp110 may play an important role in modifying infected intracellular membranes.

Epstein-Barr virus (EBV) causes infectious mononucleosis (27) and lymphoproliferative syndromes (22, 56) and is a factor in nasopharyngeal cancer (29, 42, 74). The virus is morphologically indistinguishable from other herpesviruses such as herpes simplex virus (HSV) and has similar features of its replicative intracellular development (for a review, see references 39 and 61). In vitro, the host range of EBV is restricted to B lymphocytes which are mostly nonpermissive for virus replication (28, 55). Because there is no cell fully permissive for virus replication in vitro, biochemical and genetic analysis of replication and of virus structural components has been retarded. In this setting, recombinant molecular cloning or comparative approaches are particularly useful. Consequently, EBV DNA was the first herpesvirus DNA to be cloned (15) and sequenced (2).

In contrast to EBV, HSV replicates well in a wide variety of continuous cell lines in vitro. Many features of HSV structure and replication have been delineated and are relevant to EBV. Most nonglycosylated EBV structural proteins are similar in size to those of HSV (16). At least six outer-envelope glycoproteins of HSV type 1 (HSV-1) have been identified (for a review, see reference 67). The genes encoding four of these glycoproteins, gB, gC, gD, and gE, have been cloned and sequenced (6, 23, 47, 54). HSV-1 gB is encoded by a single open reading frame (6, 54) which displayed a surprising degree of colinear homology to an open reading frame (BALF4 [2]) of EBV (53). While many potentially different EBV glycoproteins have been distinguished by size (for a review, see reference 72), only two glycoproteins, gp350/220 and gp85, are known to be distinct molecular species since specific antibodies to those proteins do not cross-react (3, 20, 30, 69). In consideration of the larger number of distinctly different HSV glycoproteins, the large number of EBV glycoproteins which could be different molecular species and the large number of EBV open

reading frames which could encode sites of *N*-linked glycosylation, it is likely that several important EBV glycoproteins remain to be identified. Among all of the potential EBV glycoproteins, only the gene encoding gp350/220 has been definitively identified (3).

Because HSV-1 gB is found on the outer surface of the virion and on the surface of virus-infected cells, it may play an important role in immunity to HSV. Some polyclonal antisera (18, 25) and monoclonal antibodies (32, 44) to gB-1 neutralize viral infectivity. Additionally, polyclonal antisera to gB-1 mediate immunocytolysis of HSV-1-infected cells (52). Furthermore, expression of gB on HSV-1-infected cell membranes may play an important role in natural killer cell recognition (4) as well as cell-mediated immunity (10). The localization of gB in the viral envelope also suggests it may be functionally important in viral infectivity. At the nonpermissive temperature, HSV-1 gB temperature-sensitive mutants adsorb to cells but do not penetrate. However, polyethylene glycol-induced fusion of the virus and cell membranes restores the infectivity of adsorbed, gB-negative, virus (46, 63).

gB is highly conserved among herpesviruses. Some HSV-1 gB antibodies cross-react with glycoproteins of HSV-2, varicella-zoster virus, bovine mammalitis virus, equine herpes virus 1, and B virus (19, 41, 50, 66). The importance of gB in HSV structure, penetration, and host immunity and the high degree of gB epitope or nucleotide sequence conservation among herpesviruses, including EBV, suggests that the putative EBV analog might encode an important glycoprotein. We therefore undertook to identify the protein encoded by the homologous EBV open reading frame BALF4.

MATERIALS AND METHODS

Cell lines. Lymphoblastoid cell lines were maintained in RPMI 1640 medium supplemented with 10% newborn calf serum. EBV replication was induced in 25 to 35% of the cells

* Corresponding author.

by adding phorbol ester and *n*-butyrate to the cultures 3 days before harvesting (69). To distinguish between viral genes whose expression is dependent on DNA replication and those which are not, viral DNA synthesis was inhibited by adding phosphonoacetic acid (PAA) (150 µg/ml) 4 h after induction. The induced, PAA-treated cells were harvested 48 h later. NIH 3T3 psi am 22b cells (14) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

EBV BALF4-*lacZ* gene fusion. The 2.3-kilobase (kb) *Mlu*I-*Xma*III fragment of *Bam*HI-A encoding most of leftward reading frame 4, BALF4 (2), was briefly digested with BAL 31 nuclease, and the ends were repaired with T4 DNA polymerase. The fragment was blunt-end ligated into *Sma*I-cut, calf intestinal alkaline phosphatase-treated pMC1513 *Escherichia coli* expression vector (7). The plasmid was transformed into *E. coli* MC1061, and beta-galactosidase-positive colonies were screened for stable fusion protein expression by immunoblotting with rabbit antibody to beta-galactosidase and EBV-immune human serum. Clone TO16 expressed a stable fusion protein of 185 kilodaltons (kDa). The junctions between EBV DNA and pMC1513 were sequenced by using the dideoxy chain termination method (62) and forward and reverse sequencing primers.

Fusion protein purification. Clone TO16 was grown in 12-liter batches in LB medium in the presence of 50 µg of ampicillin per ml. Cell pellets were stored at -80°C. The cells were ground with 2.5 weights of levigated alumina and suspended in 5 volumes of breaking buffer (0.2 M Tris hydrochloride [pH 7.6], 0.2 M NaCl, 0.01 M MgCl₂, 0.01 M beta-mercaptoethanol, 5% [vol/vol] glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF]) (48), and the alumina was removed by centrifugation at 4,000 × *g* for 10 min. A second pellet, prepared by centrifugation at 40,000 × *g* for 60 min, contained most of the cell debris and fusion protein. The pellet was extracted with phosphate-buffered saline (PBS) containing 1% (vol/vol) Nonidet P-40 (NP-40), 0.5% (wt/vol) sodium deoxycholate, 0.01 M beta-mercaptoethanol, and 1 mM PMSF. After centrifugation at 40,000 × *g* for 60 min, the pellet was suspended in 50 mM Tris hydrochloride (pH 7.5)-10 µg of RNase A per ml-10 µg of DNase I per ml-100 mM MgCl₂-1 mM PMSF and incubated at 37°C for 60 min. Sodium dodecyl sulfate (SDS) (5% [wt/vol]) and beta-mercaptoethanol (0.01 M) were added. The suspension was incubated at 72°C for 10 min and centrifuged again at 40,000 × *g* for 60 min. The supernatant was chromatographed on a Superose 6 column (16 mm by 1 m) by using an FPLC apparatus (Pharmacia, Inc.). The running buffer contained 20 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 0.1% (wt/vol) SDS, and 0.01 M beta-mercaptoethanol. Fractions were collected and assayed for fusion protein. Appropriate fractions were pooled and concentrated.

Rabbit antiserum preparation and purification. Purified fusion protein (100 µg) was dialyzed against PBS, emulsified with an equal volume of complete Freund adjuvant, and injected subcutaneously into a New Zealand White rabbit. The rabbit was then injected every 2 weeks with 200 µg of fusion protein emulsified with an equal volume of incomplete Freund adjuvant. Injection 9 was with 1 mg of fusion protein emulsified in a mixture of 100 µg of trehalose dimycolate, 100 µg of monophosphoryl lipid A (both from Ribi ImmunoChem Research), and squalene. Thereafter, the rabbit was similarly injected on a monthly basis and bled at 10 to 14 days after each injection. TO16 fusion protein-specific antibody was purified by coupling the fusion protein to tressyl-activated Sepharose 4B (Pharmacia). A 15-mg amount of

purified fusion protein was dialyzed against coupling buffer (0.1 M NaHCO₃ [pH 7.9], 0.15 M NaCl, 1 mM EDTA, 0.1% SDS), mixed with 1 ml of activated Sepharose 4B, and rotated at 22°C for 4 h. The pH of the mixture was increased to 9.0 with concentrated NaOH, and the suspension was rotated for 12 h at 22°C. The gel was deactivated by incubation with 1 M ethanolamine, pH 9.0, for 4 h at 22°C. Approximately 7 mg of fusion protein was coupled to the support matrix. An 8-mg amount of beta-galactosidase was similarly coupled to activated Sepharose. Immune rabbit serum was heat inactivated at 56°C for 30 min. Protease inhibitors, 0.5 µg of leupeptin per ml, 1 mM EDTA, 0.7 µg of pepstatin per ml, and 0.2 mM PMSF were added. After adding Tween 20 to 0.1%, the serum was cycled through the beta-galactosidase and fusion protein columns for 48 h at 4°C. Antibodies were eluted from the fusion protein column with 1 M NH₄OH (pH 11.5), 1 M NH₄OH (pH 11.5)-10% (vol/vol) dioxane, 0.1 M glycine hydrochloride (pH 2.5), 0.1 M glycine hydrochloride (pH 2.5)-10% dioxane, and 3 M MgCl₂. Each eluate was immediately neutralized and dialyzed against PBS. The elutions were combined and concentrated.

pZ110-5 construction and expression. The 3.5-kb *Tth*111I fragment of *Bam*HI-A, in which the first consensus translation start codon is the predicted translation initiation ATG of BALF4, was isolated. This fragment, which includes all of BALF4, was treated with T4 DNA polymerase and blunt-end ligated into *Bam*HI-cut, calf intestinal alkaline phosphatase- and T4 DNA polymerase-treated pZIP-NEO-SV(X)1 vector (9). NIH 3T3 psi am 22b cells (14) were transfected with 10 µg of plasmid DNA and 10 µg of salmon sperm DNA by the calcium phosphate method (26). Cells were split 1:10 and put into selective media containing 500 µg of G418 per ml. Resistant clones were examined for protein expression by immunofluorescence and by immunoblotting.

Immunofluorescence. Lymphocytes were dried onto a microwell slide, acetone fixed, and incubated with PBS with 10% normal goat serum (Cooper Biomedical, Inc.) for 30 min at 37°C. 3T3 cells were grown on eight-well microscope slides (Miles Laboratories, Inc.) and similarly processed. Diluted serum was added to the wells for 45 min at 37°C, followed by biotin-conjugated goat anti-rabbit or human immunoglobulin G (Bethesda Research Laboratories, Inc.) for 30 min at 37°C. Fluorescein isothiocyanate-conjugated streptavidin was used for visualization. Live cells for surface immunofluorescence were incubated on ice with preimmune or immune rabbit serum for 45 min, washed with cold saline containing 5% goat serum, and then incubated on ice for 45 min with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. The cells were observed by using a Zeiss universal photomicroscope equipped with epifluorescence.

Immunoelectron microscopy. Induced B95-8 cells were washed twice with RPMI 1640 medium, fixed for 2 h at 22°C in Karnovsky fixative (38), and made permeable with 70% ethanol for 30 min at 22°C. After blocking with 0.1 M glycine for 3 h followed by PBS with 20% normal goat serum for 30 min, cells were incubated overnight at 4°C with antibody diluted 1:20 in PBS with 10% normal goat serum. Cells were washed three times, incubated with 5 nm colloidal gold-labeled goat anti-rabbit antibodies (Janssen) diluted 1:2 in PBS with 10% normal goat serum for 30 min at 4°C, washed three times, and fixed with 2% glutaraldehyde in 0.1 M cacodylate for 1 h at 4°C. The cells were treated with 1% osmium tetroxide, embedded in Epon, sectioned, and viewed on a Siemens 102 electron microscope.

Virus neutralization. Virus neutralizing activity was assayed by the inhibition of virus-induced early antigen expression in a nonproducing cell line, Raji. Early-antigen-positive cells were detected by immunofluorescence by using an EBV-immune human serum. A 200- μ l volume of P3HR-1 supernatant (concentrated 10:1) was incubated for 1 h at 4°C with various dilutions of antibody. Rabbit complement (Cedarlane Laboratories) diluted 1:15 in RPMI 1640 medium was added and incubated for 30 min at 37°C. Raji cells (10^6) were added and incubated for 1 h at 37°C. The cells were diluted 1:5 in RPMI 1640 medium and incubated for 48 h at 37°C. Cell smears were prepared for immunofluorescence as described above.

RNA and in vitro translation. Induced B95-8 cytoplasmic RNAs were isolated by the guanidium thiocyanate-CsCl method (11). Polyadenylated RNAs were separated by oligo(dT)-cellulose chromatography (1). For RNA blots, cytoplasmic polyadenylated RNA was denatured with formamide and formaldehyde, size fractionated by electrophoresis on agarose formaldehyde gels, and transferred to Gene Screen Plus (New England Nuclear Corp.). DNA fragment-specific polyadenylated RNA was selected by using DNA bound to nitrocellulose filters. RNA was eluted in H_2O at 100°C and translated in vitro as previously described (33).

The 3.5-kb *Tth1111* fragment of *Bam*HI-A, used in the pZ110-5 construction, was treated with T4 DNA polymerase and blunt-end ligated into *Eco*RI-cut, calf intestinal alkaline phosphatase- and T4 DNA polymerase-treated pTZ19R vector, which contains the T7 RNA polymerase promoter (Pharmacia). After transformation into DH-5 cells, the plasmid was isolated, linearized, and transcribed as recommended in capping protocol 2 from Promega Biotec by using T7 RNA polymerase (Pharmacia). The RNA was translated with in vitro translation-grade [35 S]methionine (Amersham Corp.) and nuclease-treated rabbit reticulocyte lysate as described by the manufacturer (Promega Biotec).

Immunoblotting. Cells were solubilized by boiling in sample buffer. Proteins were separated on 6% SDS-polyacrylamide gels (45) and then electrophoretically transferred to nitrocellulose (5). The nitrocellulose blot was stained with Ponceau S (Sigma Chemical Co.) and washed with 5% acetic acid. The positions of molecular weight standards (Bio-Rad Laboratories) (which included myosin [200 kDa], beta-galactosidase [116 kDa], phosphorylase b [92 kDa], bovine serum albumin [66 kDa], and ovalbumin [45 kDa]) were noted. After blocking in 20 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–5% (wt/vol) low-fat dry milk–0.1% (vol/vol) Tween 20–0.05% (wt/vol) sodium azide for 30 min at 37°C, blots were reacted with diluted sera for 2 h at 22°C. The blots were then washed, incubated with 5×10^5 cpm of 125 I-labeled protein A (Amersham) per ml, washed, and exposed to XAR-5 film at –80°C.

Glucosamine labeling and immunoprecipitation. Cells were incubated for 18 h in medium supplemented with 10% dialyzed fetal calf serum and 50 μ Ci of [3 H]glucosamine (New England Nuclear) per ml. Cells were washed in cold PBS with 0.2 mM PMSF and lysed in PBS–0.5% NP-40 with protease inhibitors. The crude soluble extract was divided into aliquots, and antibody or serum was added and incubated for 2 h at 4°C. Protein A-Sepharose CL-4B (obtained from Sigma) was added and incubated for 30 min at 4°C. The immunoprecipitate was washed five times in 50 mM Tris hydrochloride (pH 8.3)–0.5 M NaCl–1% NP-40–0.1% Tween 20–0.1% (wt/vol) bovine serum albumin and boiled in sample buffer for SDS-polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue G, destained, and

treated with Amplify (Amersham) for 30 min before gel drying. The dried gel was exposed to XAR-5 film at –80°C.

Tunicamycin and N-glycanase treatment. Induced B95-8 cells were metabolically labeled with 100 μ Ci of [35 S]methionine (Amersham) per ml for 5 h in 90% methionine-free RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum. For tunicamycin treatment, cells were preincubated in medium supplemented with 5 μ g of tunicamycin (Boehringer Mannheim Biochemicals) per ml for 2 h and then incubated with the radiolabel in the presence of 5 μ g of tunicamycin per ml. Cells were washed in cold PBS with 0.2 mM PMSF and lysed in 10 mM Tris hydrochloride (pH 7.0)–150 mM NaCl–1 mM EDTA–1% deoxycholate–1% NP-40–0.1% SDS with protease inhibitors. The extracts were immunoprecipitated, and the precipitates were subjected to SDS-polyacrylamide gel electrophoresis. For N-glycanase treatment, before electrophoresis, the precipitate was boiled for 3 min in 0.5% SDS and 0.1 M beta-mercaptoethanol. The sample was diluted to 0.17% SDS–0.2 M sodium phosphate (pH 8.6)–10 mM 1,10-phenanthroline hydrate–1.25% NP-40–10 U of N-glycanase (Genzyme) per ml. The reaction mixture was incubated overnight at 37°C.

pZ110-5-transfected cells were similarly preincubated and metabolically labeled for 5 h in the presence of 5 μ g of tunicamycin per ml. Cells were washed, scraped, and then similarly lysed, immunoprecipitated, and electrophoresed.

RESULTS

Transcript and primary translation product of BALF4. BALF4 (Fig. 1) encodes 1.8- and 3.0-kb cytoplasmic polyadenylated RNAs in B95-8 cells induced to replicate EBV (Fig. 2). These RNAs were selected by hybridization to BALF4 DNA nitrocellulose filters and translated in vitro to yield a 93-kDa protein and several smaller proteins (Fig. 2B). The 93-kDa protein was specifically immunoprecipitated with EBV-immune human sera (Fig. 2C).

BALF4 encodes a 110-kDa protein in productively infected cells. To identify the protein encoded by BALF4 in cells replicating EBV, a BALF4-specific antiserum was derived. The antiserum was derived by fusing BALF4 into the 5' end of *lacZ* which was mobilized on a plasmid carrying ampicillin resistance (Fig. 1). Recombinant plasmids were transformed into *E. coli* and cells expressing stable, large, EBV BALF4 fusion proteins were identified by immunoblot by using a high-titer EBV-immune human sera. A clone, TO16, which expressed a 185-kDa immunoreactive fusion protein as approximately 1 to 2% of total cell protein, was the source of protein for purification and rabbit immunization. The part of BALF4 expressed as a fusion protein by TO16 begins with amino acid 83 and ends with amino acid 803. Thus, it includes 602 of the 685 amino acids predicted to be exposed on the outer surface of the virus envelope, 69 amino acids predicted to be membrane-spanning domains, and 49 amino acids predicted to be on the internal surface of the virus envelope. After seven subcutaneous injections with the TO16 fusion protein, a rabbit began to produce antibody which reacts with a 110-kDa protein in cells replicating the B95-8 EBV strain and a 108-kDa protein in cells replicating the P3HR-1 EBV strain (data not shown). No similar-size protein was detected in B lymphoblastoid cell lines which were latently infected with EBV, such as IB4 cells or in noninfected lymphoblastoid cell lines such as Louckes (data not shown). The specificity of the antiserum for the 110- or 108-kDa EBV protein was improved by selecting the BALF4-specific antibody by using affinity chromatography.

FIG. 1. Expression of the *Bam*HI-A leftward open reading frame 4 (BALF4, [2]). (A and B) Map of the EBV genome showing terminal repeat (TR), internal repeat (IR) and unique sequence (U) domains and the location of BALF4 in the *Bam*HI-EcoRI fragment of *Bam*HI-A. Potential promoter and polyadenylation signals and the fragments which were recombined to produce procaryotic (pTO16) or eucaryotic (pZ110-5) BALF4 expression vectors are also indicated. (C) The structure of the procaryotic expression plasmid pTO16. BALF4 was briefly treated with BAL 31 nuclease and ligated into the *Sma*I site of pMC1513, an expression vector which has a polylinker inserted into codon 7 of beta-galactosidase (7). The junctions between BALF4 and *lacZ* were sequenced, and the sequence indicates that the BALF4 insert is out of frame with the first seven codons of beta-galactosidase. Subscripts in the protein sequence indicate the codon number in BALF4. The first BALF4 ATG in frame with beta-galactosidase is underlined, and the cloning sites of pMC1513 are boxed. (D) Structure of the eucaryotic recombinant expression plasmid pZ110-5. BALF4 (black box) was cloned into the *Bam*HI site of pZIP-NEO-SV(X)1 (9). The open boxes indicate the murine leukemia virus long terminal repeat (LTR), the neomycin resistance (Neo) gene, the simian virus 40 origin (SVori), and the pBR322 origin (pBRori), all elements of the parent plasmid pZIP-NEO-SV(X)1. The part of the vector not shown is derived from pBR322.

Most of the beta-galactosidase antibodies were first removed by passage through a beta-galactosidase-Sepharose column. Antibodies then purified on a TO16 Sepharose column reacted specifically with the 110-kDa (or 108-kDa) EBV protein (Fig. 3). This is one of the most abundant proteins in B95-8 or P3HR-1 cells replicating EBV as detected in immunoblot by using an EBV-immune human serum (Fig. 3). The TO16-specific antibody also reacted with less abun-

dant (or less antigenic) 105-, 54-, and 50-kDa proteins in P3HR-1 but not B95-8 cells.

The TO16 fusion protein was smaller than would be expected based on the size of the BALF4 fragment (approximately 2.4 kb) which was ligated into the 5' end of *lacZ* and the known size of beta-galactosidase (116 kDa). The resultant fusion protein was expected to be over 200 kDa, while TO16 was only 185 kDa. The sequence of the junctions

between EBV and *lacZ* DNAs in pTO16 revealed (Fig. 1) that the 5' end of *lacZ* was fused to BALF4 so that the 5' end of BALF4 would be translated in an incorrect reading frame. However, 94 nucleotides after the beginning of the EBV DNA insert, there is a sequence of TGATG. The TGA would stop translation in the incorrect reading frame, and since the terminal nucleotide of the stop codon is the first nucleotide of a methionine codon in the correct reading frame, translation of BALF4 would likely be reinitiated by the methionine codon. The 3' junction of BALF4 and the rest of *lacZ* is in frame so that a 195-kDa EBV-beta-galactosidase fusion protein consisting of 79 kDa of BALF4 (from amino acid 83 to 803) and 116 kDa of beta-galactosidase is the anticipated product. This agrees well with the observed size of 185 kDa.

The 110-kDa protein is entirely encoded by BALF4. A DNA fragment beginning 144 base pairs 5' to BALF4 and ending 735 base pairs 3' to BALF4 was inserted into a recombinant Moloney murine leukemia virus expression vector pZIP-NEO-SV(X)1 (Fig. 1). Transcription of the resultant recombinant plasmid pZ110-5 should initiate 500 base pairs 5' to the site of insertion of BALF4. Translation is not known to initiate in the 500-base 5' leader (65). In fact, the first consensus eucaryotic translational initiation site in the mRNA which would be transcribed from pZ110-5 is the sequence ACCATGG at the beginning of BALF4. pZ110-5 was transfected into NIH 3T3 psi am 22b cells, and transfected cells were selected by using G418. Most clones of transfected cells expressed a 110-kDa protein (Fig. 3) identical in size to the BALF4 protein in B95-8 cells (the source of BALF4 for the pZ110-5 construct). This 110-kDa protein was detected in immunoblots by using EBV-immune human sera or the TO16-affinity-purified, TO16-immunized rabbit serum. The human sera also detected less abundant 54- and 50-kDa proteins in pZ110-5-transfected cells which were similar in size to proteins detected in P3HR-1 cells by

FIG. 2. BALF4 RNA and in vitro translation product. (A) A Northern blot of cytoplasmic polyadenylated RNAs from induced B95-8 cells was hybridized to the *BglII-StuI* fragment of *BamHI-A* which includes most of BALF4 (AL4). The positions of 18S and 28S rRNAs are indicated. Induced B95-8 cells were either treated (+) or not treated (–) with PAA (150 μ g/ml). (B) RNA from induced B95-8 cells was hybrid selected with DNA from BORF2 (OR2) or BALF4 (AL4) and translated in vitro. (C) The translated proteins were immunoprecipitated by using EBV-immune human serum (WC). BALF4-specific RNAs and BALF4- or BORF2-specific in vitro translation products are indicated (●). These proteins did not immunoprecipitate in parallel experiments with nonimmune human serum.

FIG. 3. Affinity-purified antibodies to the TO16 BALF4-*lacZ* fusion protein specifically react with a 110-kDa protein in immunoblots of proteins from induced B95-8 cells or with a 108-kDa protein from induced P3HR-1 cells. A 110-kDa protein is also detected in pZ110-5-transfected psi am 22b cells. Polyacrylamide gels (6%) were run and electrophoretically transferred to nitrocellulose for immunoblotting. Proteins from the following sources were tested: Louckes (an EBV-negative cell line) (LOU); induced B95-8 (B95); induced P3HR-1 (HRI); psi am 22b 3T3 cells transfected with pZip-NEO-SV(X)1 (3T3); psi am 22b transfected with pZ110-5 (C5); the host, MC1061, transformed with the nonrecombinant parental procaryotic expression vector pMC1513 (1513); and procaryotic fusion protein (TO 16). (A) EBV-immune human serum (WC), 1:100 dilution. (B) Affinity-purified rabbit antibody (Aff. Pur. Rab.), 1:20 dilution. The positions of gp110 or of TO 16 fusion protein are indicated (●). The positions of myosin, beta-galactosidase, phosphorylase b, or bovine serum albumin molecular weight standards are also indicated (–). Preimmune rabbit serum gave no specific bands (data not shown).

the TO16-specific serum. Since these proteins varied in abundance in different preparations and were not found in B95-8 cells, they could be degradation products of the 110-kDa protein. The TO16-specific sera also detected 66- and 61-kDa proteins in the transfected cells. No similar-size proteins were detected in cells transfected with pZIP-NEO-SV(X)1 (Fig. 3) or in immunoblots of pZ110-5-transfected cell protein by using nonimmune human or rabbit sera (data not shown).

Intracellular location of the BALF4 protein. EBV-immune human sera or affinity-purified TO16-specific antibody were used to localize the BALF4 protein in psi am 22b cells transfected with pZ110-5 (Fig. 4). Antigen was detected in the cytoplasm of almost all cells in varying amounts. Most of the antigen was perinuclear. In some cells, antigen was also diffusely cytoplasmic or in the nuclear membrane, or in intense perinuclear cytoplasmic patches. No difference was seen between the appearance of cells reacted with EBV-immune human sera or affinity-purified TO16-specific rabbit sera. No antigen was detected in cells of clones transfected with pZIP-NEO-SV(X)1 or in most cells of B95-8 cultures in which virus replication had not been induced. EBV-infected cells in which virus replication was induced exhibited similar patterns of antigen distribution as was seen in pZ110-5 transfected psi am 22b cells (Fig. 4). By using TO16-specific antisera, surface immunofluorescence on live or fixed productively infected B95-8 or P3HR-1 cells did not reveal any

FIG. 4. Distribution of BALF4 encoded protein in EBV-infected lymphocytes or transfected mouse fibroblasts. 3T3 psi am 22b cells transfected with pZ110-5 and cloned after selection by using G418 were incubated. (A) Incubation with 1:100 dilution of EBV-immune serum. (C) Incubation with 1:20 dilution of affinity-purified TO 16-specific rabbit antibody. (B and D) Induced B95-8 cells were incubated with 1:20 dilution of affinity-purified TO 16-specific rabbit antibodies. Preimmune sera gave no fluorescence. Louckes, an EBV-negative lymphoblastoid cell line, and psi am 22b cells transfected with pZIP-NEO-SV(X)1 also gave no fluorescence.

plasma membrane fluorescence, although intense plasma membrane fluorescence was seen with rabbit serum immune to a gp350-beta-galactosidase fusion protein (3; data not shown).

Immunoelectron microscopy of induced B95-8 cells, with affinity-purified TO16-specific antibodies, revealed that gp110 localized to both the inner and outer lamellae of the nuclear membranes of cells whose nuclei contained newly replicated nucleocapsids (Fig. 5), and intense labeling was also noted of some vesicles in the cytoplasm. Many of these vesicles were about twice the diameter of enveloped virus and some contained enveloped virus particles. Since the antibody was raised against a protein which consists of 602 amino acids predicted to be on the outside of the membrane and only 49 amino acids predicted to be on the inside of the membrane, it was quite surprising to find almost all of the antibody on the cytoplasm or nucleoplasm side of the

FIG. 5. Ultrastructural localization of gp110. Induced B95-8 cells were fixed in Karnovsky fixative, made permeable with ethanol, incubated with affinity-purified TO16-specific antibodies, labeled with 5 nm colloidal gold-labeled goat anti-rabbit antibodies and fixed with 2% glutaraldehyde. The left panel and lower right panel show the presence of gp110 in nuclear membranes. The upper right panel shows that gp110 is associated with cytoplasmic vesicles. The nucleus (N) and cytoplasm (C) are indicated. Bar, 100 nm.

FIG. 6. BALF4 encodes a 110-kDa glycoprotein (●). Induced P3HR-1 (HRI), pZ110-5 transfected psi am 22b (C5), or pZIP-NEO-SV(X)1-transfected psi am 22b (3T3) cells were labeled with [3 H]glucosamine for 16 h. Extracts were immunoprecipitated and run on 6% polyacrylamide gels with EBV-immune human serum (WC); preimmune rabbit serum (Pre); and affinity-purified, TO 16-specific rabbit antibodies (AFF).

membranes. No specific labeling was detected of plasma membranes or of enveloped virus, even when enveloped virus was between heavily labeled inner and outer nuclear membranes. Preimmune rabbit serum did not label infected cell membranes, and immune serum did not label membranes of latently infected cells that did not also contain viral nucleocapsids in the nucleus.

Virus neutralization. TO16-specific antiserum was unable to neutralize viral infectivity as determined by P3HR-1 superinfection of Raji cells. 72A1, a gp350 monoclonal antibody (30) (provided by A. J. Morgan), displayed neutralizing activity under similar conditions (data not shown).

BALF4 encodes a 110-kDa glycoprotein. Since BALF4 would encode a protein with several potential N-linked glycosylation sites and the BALF4 *in vitro* primary translation product is smaller than its *in vivo* product, the BALF4 protein is likely to be glycosylated. Three experiments were undertaken to investigate the extent of N-linked glycosylation. First, P3HR-1 cells were incubated with labeled glucosamine. Protein extracts were immunoprecipitated with TO16-specific sera and analyzed on acrylamide gels. The 108-kDa protein was labeled with glucosamine as well as a less abundant (or less antigenic) 77-kDa protein (Fig. 6). A considerable fraction of the glucosamine-labeled protein from the induced P3HR-1 cell extract which specifically immunoprecipitated with EBV-immune human sera was a gp108 (or gp110 from B95-8 cells) (Fig. 6 and data not shown). The TO16-specific antibodies also reacted with 110-, 77-, and 54-kDa glucosamine-labeled products in pZ110-5 expressing cells. These data confirm that the abundant 110-kDa protein detected on immunoblots with EBV-immune human sera (Fig. 3) is a glycoprotein. Second, cells were pulse labeled with methionine in the presence or absence of tunicamycin (Fig. 7). In the presence of

tunicamycin, the TO16 affinity-purified rabbit serum specifically immunoprecipitated a 93-kDa protein identical in size to the *in vitro* translation product of BALF4 RNA. Third, immunoprecipitates of EBV-infected cells with the TO16-specific serum were treated with *N*-glycanase (*N*-glycosidase F), which should remove N-linked sugars (71), and the size of gp110 was reduced to 105 kDa.

gp110 expression is dependent on viral DNA synthesis. PAA preferentially blocks EBV DNA synthesis rather than cell DNA synthesis. Treatment of induced B95-8 or P3HR-1 cells with PAA resulted in a substantial reduction in accumulation of the 3.0- and 1.8-kb RNAs of gp110 (or gp108 in P3HR-1), while the accumulation of other viral proteins, such as the p135 early protein, was unaffected by treatment with PAA (Fig. 2 and 8). These data indicate that gp110 expression is dependent on viral DNA synthesis and that gp110 is therefore a late viral gene.

DISCUSSION

These data indicate that the EBV BALF4 open reading frame encodes 3.0- and 1.8-kb RNAs and an abundant 108- to 110-kDa glycoprotein, now designated gp110. Expression of BALF4 is reduced when viral DNA synthesis is inhibited, indicating that gp110 is a "late" virus gene product. gp110 may have been previously noted. It had been reported that BALF4 encodes a late infection cycle cytoplasmic protein (12). Glycoproteins in the size of 105 to 130 kDa had been detected in extracts of EBV-infected lymphocytes which had been induced to productive infection and incubated with radioactive glucosamine (8, 20, 37, 40, 57, 58). Glycoproteins

FIG. 7. gp110 contains N-linked oligosaccharides. (A and B) Induced B95-8 cells were incubated with [³⁵S]methionine for 5 h, and extracts were immunoprecipitated. Antibodies used for immunoprecipitation were affinity-purified TO16-specific rabbit antibodies (AP Rab.) and preimmune rabbit serum (Pre). The immunoprecipitates were subjected to 6% polyacrylamide gel electrophoresis. (A) Radiolabeling occurred in the presence (+) or absence (-) of 5 μ g of tunicamycin per ml. (B) Immunoprecipitates of radiolabeled gp110 were treated (+) or not treated (-) with N-glycanase. (C) *In vitro* transcription-translation of BALF4. BALF4 was cloned into pTZ19R, a plasmid containing the T7 RNA polymerase promoter. RNA was transcribed and used for *in vitro* translation. Translation products were immunoprecipitated and run on a 7% polyacrylamide gel with B95-8 cells treated with tunicamycin (as in the second lane of panel A) (B95); pZ110-5-transfected psi am 22B cells treated with 5 μ g of tunicamycin per ml (C5); immunoprecipitates of the *in vitro* translation products, translated from T7 RNA polymerase-transcribed BALF4 (IVT); affinity-purified TO16-specific rabbit antiserum (Rab.); and EBV-immune human serum (WC). The positions of the molecular weight standards (-) and gp110 (●) are indicated.

FIG. 8. gp110 is a late viral protein. Beginning 4 h after induction, B95-8 (B95) or P3HR-1 (HR-1) cells were treated (+) or not treated (-) with PAA. Immunoblots were reacted with EBV-immune human serum (WC) (left panel) or affinity-purified TO16-specific rabbit serum (A. P. Rab.) (right panel). gp110 (●) the 135-kDa EA component (●●) are indicated. An immunoblot with an 85-kDa EA monoclonal, provided by G. Pearson, gave results similar to those of the 135-kDa EA (data not shown).

of similar size have also been found in preparations of purified virus (17). These previously detected glycoproteins could have been gp110 or protease products of other viral glycoproteins.

Several lines of evidence support the hypothesis that gp110 is translated from the 3-kb RNA and that the 3-kb RNA is an unspliced transcript of BALF4. First, the HSV-1 gB coding region has a similar configuration of transcriptional signals which specify initiation and polyadenylation of a nonspliced 3.4-kb mRNA which translates gB (31, 59). Second, of the two BALF4-encoded RNAs, the 3-kb mRNA is much more likely to translate the 93-kDa precursor to gp110 than is the 1.8-kb RNA, since a 1.8-kb mRNA would encode at most for 530 amino acids (allowing for a 200-base polyadenylation tract). Third, an *in vitro* promoter maps 48 bases upstream from a potential translational initiation site in BALF4 (2), and a consensus polyadenylation site is 41 bases downstream from the termination of BALF4. The putative promoter and polyadenylation sites demarcate a 2.7-kb DNA segment, appropriate to transcribe a 3-kb polyadenylated mRNA. Fourth, insertion of the BALF4 open reading frame into pZIP-NEO-SVX(1) resulted in the expression of a full-size gp110. Since pZIP-NEO-SVX(1) provides for initiation of transcription but not for initiation of translation and the potential translational initiation site in BALF4 would be the first likely translational initiation site in the recombinant transcript of pZ110-5, this site is likely to initiate translation of gp110. Fifth, transcription of BALF4 *in vitro* so that the potential translational initiation codon of BALF4 is the first initiation codon of the capped RNA and translation of the mRNA *in vitro* results in a protein the same size as the gp110 precursor.

gp110 is likely to have both N- and O-linked glycosylation

since treatment of B95-8 cells with tunicamycin results in a cross-reactive protein similar in size to the BALF4 *in vitro* translation product (93 kDa), while N-glycanase treatment of gp110 only reduced its size to 105 kDa. Most likely, N-glycanase is cleaving all N-linkages (71) and leaving O-linked sugars, whereas tunicamycin treatment is blocking all cotranslational or posttranslational modifications of gp110 by blocking formation of dolichol phosphate intermediates and thereby preventing transfer of the nascent polypeptide to the golgi (24, 70, 73). Further, it is known that HSV gB contains both N- and O-linked glycosylation (36, 43, 51, 64). Also, tunicamycin treatment of EBV-infected cells results in a 135-kDa cross-reactive putative precursor to EBV gp350, which is identical in size to the *in vitro* translation product of gp350 mRNA (34). gp350 contains both N- and O-linked oligosaccharides (21, 35, 49, 68). Alternatively, N-glycanase may incompletely cleave N-linked residues in gp110. It has been shown that a minimum of a di-N-acetylchitobiosyl core unit is necessary for N-glycanase cleavage (13). Thus, at least some N-linked conformations may not be recognized by (or be accessible to) the enzyme. Further experiments must be done to determine the structure of the modifications of gp110.

The important predicted common structural features of EBV BALF4 and HSV-1 gB which have been previously noted (53) include the following: (i) amino termini which are likely to be cleavable signal sequences for membrane translocation, (ii) a domain of approximately 650 amino acids containing four conserved asparagine-X-threonine or -serine potential sites for N-linked glycosylation and 10 conserved cysteine residues which is likely to be exposed on the outer surface of the virus or cell membrane, (iii) a domain of three potential membrane-spanning segments, and (iv) a 150-amino-acid carboxy terminus which is presumed to be on the opposite side of the membrane from the bulk of the protein. Analyses of the nucleotide sequence of monoclonal antibody-resistant mutants of HSV-1 gB indicated that these mutations cluster in the codons for amino acids 273 to 305 (54), consistent with the proposed model for membrane orientation of the gB domains.

Despite considerable experimental effort, we have not as yet succeeded in demonstrating that gp110 is similar to HSV-1 gB in its localization to the surface of virus or of virus-infected cells. Our TO16-specific polyclonal anti-gp110 antisera did not neutralize virus. In fact, a striking difference between HSV-1 gB and gp110 is that while HSV-1 gB is an important component of the virus envelope and of the plasma membrane of HSV-1 infected cells, we found little evidence for gp110 in the plasma membrane of EBV productively infected cells. Although gp110 contains 31 tyrosine residues, surface iodination of productively infected cells and immunoprecipitation with the monospecific rabbit or EBV-immune human sera resulted in very little immunoprecipitation of gp110 despite the substantial immunoprecipitation of surface-labeled gp350/220 and gp85 by the EBV-immune human serum. Further, immunofluorescence of live productively infected lymphocytes with the gp110-specific rabbit sera or gp350/220-specific rabbit sera revealed distinctive membrane fluorescence only with the latter. These results are compatible with the fixed cell fluorescence analyses (Fig. 4), which show little evidence for association of gp110 with the plasma membrane. An outstanding feature of this fixed cell fluorescence is the perinuclear localization of gp110. Immunoelectron microscopy (Fig. 5), revealed that gp110 is abundant in cytoplasmic vesicles as well as both the inner and outer nuclear membranes. The predicted inner

membrane domains of EBV gp110 and HSV-1 gB contain Arg-Lys-Arg-Arg or Arg-Arg-Arg-Arg sequences. Similar basic oligopeptides characterize nuclear proteins (60) and could anchor the carboxy-terminal domains of gp110 or gB in the nuclear matrix. Since herpesviruses are enveloped at the nuclear membrane and the immunoelectron microscopy indicates that there are discrete patches of gp110 in the inner and outer lamellae of the nuclear membrane, it is quite surprising that gp110 was not found to be associated with virus particles found between the inner and outer nuclear membranes. Thus, while the amino acid homology between HSV-1 gB and gp110 indicates a high degree of conservation, we find important quantitative differences in intracellular localization. The data therefore suggest that there are two functions of the HSV gB/EBV gp110 class of herpesvirus glycoproteins. One function readily evident with HSV-1 gB is as an important constituent of the virus outer envelope and of the cell plasma membranes. Another, evident in the case of EBV gp110 appears to be modification of internal infected cell membranes. Since gp110 is one of the most abundant viral proteins and many of the cytoplasmic gp110-associated vesicles appeared to contain virus particles, we propose that gp110 may be important in the reorganization of cell membranes to permit virus egress. The HSV-1 gB/EBV gp110 class of herpesvirus glycoproteins may only differ quantitatively in the extent to which they manifest their two phenotypes.

ACKNOWLEDGMENTS

Philip Pellett, Bernard Roizman, Patricia Spear, and Lena Claesson-Welsh provided advice at various stages of this research. Technical assistance was provided by Larvan Chamnankit (DNA sequencing), Shu Chou (electron microscopy), Kris Kalev (tissue culture), P. J. Morrison (manuscript preparation), and Teri Reid (tissue culture). Monoclonal antibodies were generously provided by G. Pearson (85-kDa EA) and A. J. Morgan (72A1).

T.O. and T.M. were visiting investigators supported by international fellowships from the North Atlantic Treaty Organization and the International Union Against Cancer, respectively. M.G. is supported by National Research Service award GM 07281. This research was supported by Public Health Service grants CA 19264 and CA 17281 from the National Cancer Institute and by grants from the American Cancer Society (MV32) and the Cancer Research Foundation.

LITERATURE CITED

1. Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* **69**:1408-1412.
2. Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Stachwell, C. Sequin, P. S. Tuffnell, and B. G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature (London)* **310**:207-211.
3. Beisel, C., J. Tanner, T. Matsuo, D. Thorley-Lawson, F. Kezdy, and E. Kieff. 1985. Two major outer envelope glycoproteins of Epstein-Barr virus are encoded by the same gene. *J. Virol.* **54**: 665-674.
4. Bishop, G. A., G. Kümel, S. A. Schwartz, and J. C. Glorioso. 1986. Specificity of human natural killer cells in limiting dilution culture for determinants of herpes simplex virus type 1 glycoproteins. *J. Virol.* **57**:294-300.
5. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodiumdodecylsulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**:192-203.
6. Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person. 1984. Nucleotide sequence specifying the glycoprotein gene, gB, of

- herpes simplex virus type 1. *Virology* 133:301-314.
7. Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou. 1983. Beta-galactosidase gene fusions for analyzing gene expression in *E. coli* and yeast. *Methods Enzymol.* 100:293-308.
 8. Casareale, D., W. Jones, T. Sairenji, and R. E. Humphreys. 1983. p105, an Epstein-Barr virus-induced, phosphonoacetic acid-insensitive glycoprotein target of the anti-Epstein-Barr virus immune response. *Infect. Immun.* 39:85-90.
 9. Cepko, C. L., B. E. Roberts, and R. C. Mulligan. 1984. Construction and applications of highly transmissible murine retrovirus shuttle vector. *Cell* 37:1053-1062.
 10. Chan, W. L., M. L. Lukig, and F. Y. Liew. 1985. Helper T cells induced by an immunopurified herpes simplex virus type 1 (HSV-1) 115 kilodalton glycoprotein (gB) protect mice against HSV-1 infection. *J. Exp. Med.* 162:1304-1318.
 11. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18: 5294-5299.
 12. Cho, M. S., L. Gissmann, and S. D. Hayward. 1984. Epstein-Barr virus (P3HR-1) defective DNA codes for components of both the early antigen and viral capsid antigen complexes. *Virology* 137:9-19.
 13. Chu, F. K. 1986. Requirements of cleavage of high mannose oligosaccharides in glycoproteins by peptide *N*-glycosidase F. *J. Biol. Chem.* 261:172-177.
 14. Cone, R., and R. Mulligan. 1985. High efficiency gene transfer into mammalian cells: generation of helper free recombinant retrovirus with broad mammalian host range. *Proc. Natl. Acad. Sci. USA* 81:6349-6353.
 15. Dambaugh, T., C. Beisel, M. Hummel, W. King, S. Fennewald, A. Cheung, M. Heller, N. Raab-Traub, and E. Kieff. 1980. EBV DNA VII: molecular cloning and detailed mapping of EBV (B95-8) DNA. *Proc. Natl. Acad. Sci. USA* 77:2999-3003.
 16. Dolyniuk, M., R. Pritchett, and E. Kieff. 1976. Proteins of Epstein-Barr virus. I. Analysis of the polypeptides of purified enveloped Epstein-Barr virus. *J. Virol.* 17:935-949.
 17. Dolyniuk, M., E. Wolff, and E. Kieff. 1976. Proteins of Epstein-Barr virus. II. Electrophoretic analysis of the polypeptides of the nucleocapsid and the glucosamine- and polysaccharide-containing components of enveloped virus. *J. Virol.* 18:289-297.
 18. Eberle, R., and R. J. Courtney. 1980. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. *J. Virol.* 35:902-917.
 19. Edson, C. M., B. A. Hosler, R. A. Respass, D. J. Waters, and D. A. Thorley-Lawson. 1985. Cross-reactivity between herpes simplex virus glycoprotein B and a 63,000-dalton varicella-zoster virus envelope glycoprotein. *J. Virol.* 56:333-336.
 20. Edson, C. M., and D. A. Thorley-Lawson. 1981. Epstein-Barr virus membrane antigens: characterization, distribution, and strain differences. *J. Virol.* 39:172-184.
 21. Edson, C. M., and D. A. Thorley-Lawson. 1983. Synthesis and processing of the three major envelope glycoproteins of Epstein-Barr virus. *J. Virol.* 46:547-556.
 22. Epstein, M., B. Achong, and Y. Barr. 1964. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet* i:702-703.
 23. Frink, R. J., R. Eisenberg, G. Cohen, and E. K. Wagner. 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. *J. Virol.* 45:634-647.
 24. Gibson, R., S. Kornfeld, and S. Schlesinger. 1980. A role of oligosaccharides in glycoprotein synthesis. *Trends Biochem. Sci.* 5:290-293.
 25. Glorioso, J., C. H. Schröder, G. Kumel, M. Szczesiul, and M. Levine. 1984. Immunogenicity of herpes simplex virus glycoproteins gC and gB and their role in protective immunity. *J. Virol.* 50:805-812.
 26. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay for infectivity of human adenovirus DNA. *Virology* 52:456-467.
 27. Henle, G., W. Henle, and V. Diehl. 1968. Relation of Burkitt tumor associated herpes-type virus to infectious mononucleosis. *Proc. Natl. Acad. Sci. USA* 59:94-101.
 28. Henle, W., B. Diehl, G. Kohn, H. zur Hausen, and G. Henle. 1967. Herpes virus type and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. *Science* 157:1065-1068.
 29. Henle, W., G. Henle, C. Ho, P. Burton, Y. Cachin, P. Clifford, A. De Schryver, G. de The, U. Diehl, and G. Klein. 1970. Antibodies to EB virus in nasopharyngeal carcinoma other head and neck neoplasms and control groups. *J. Natl. Cancer Inst.* 44:225-231.
 30. Hoffman, G., S. Lazarovitz, and S. Hayward. 1980. Monoclonal antibody against a 250,000 dalton glycoprotein of Epstein-Barr virus identifies a membrane antigen. *Proc. Natl. Acad. Sci. USA* 77:2979-2983.
 31. Holland, L. E., R. M. Sandri-Goldin, A. L. Goldin, J. C. Glorioso, and M. Levine. 1984. Transcriptional and genetic analyses of the herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. *J. Virol.* 49:947-959.
 32. Holland, T. C., S. D. Marlin, M. Levine, and J. Glorioso. 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. *J. Virol.* 45:672-682.
 33. Hummel, M., and E. Kieff. 1982. Mapping of polypeptides encoded by the Epstein-Barr virus genome in productive infection. *Proc. Natl. Acad. Sci. USA* 79:5698-5702.
 34. Hummel, M., D. Thorley-Lawson, and E. Kieff. 1984. An Epstein-Barr virus DNA fragment encodes messages for the two major envelope glycoproteins (gp350/300 and gp220/200). *J. Virol.* 49:413-417.
 35. Hutt-Fletcher, L. M., N. Balachandran, and P. A. LeBlanc. 1986. Modification of Epstein-Barr virus replication by tunicamycin. *J. Virol.* 57:117-123.
 36. Johnson, D. C., and P. G. Spear. 1983. O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the golgi apparatus. *Cell* 32:987-997.
 37. Kallin, B., J. Luka, and G. Klein. 1979. Immunochemical characterization of Epstein-Barr virus-associated early and late antigens in *n*-butyrate-treated P3HR-1 cells. *J. Virol.* 32:710-716.
 38. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* 27:137A-138A.
 39. Kieff, E., T. Dambaugh, M. Hummel, and M. Heller. 1983. Epstein-Barr virus transformation and replication, p. 133-182. *In* G. Klein (ed.), *Advances in viral oncology*, vol. 3. Raven Press, New York.
 40. Kishishita, M., J. Luka, B. Vroman, J. F. Poduslo, and G. R. Pearson. 1984. Production of monoclonal antibody to a late intracellular Epstein-Barr virus-induced antigen. *Virology* 133:363-375.
 41. Kitamura, K., J. Namazue, H. Campo-Vera, T. Ogino, and K. Yamanishi. 1986. Induction of neutralizing antibody against varicella zoster virus (VZV) by VZV gp3 and cross-reactivity between VZV gp3 and herpes simplex viruses gB. *Virology* 149:74-82.
 42. Klein, G., B. Giovannella, T. Lindahl, P. Fialko, S. Singh, and J. Stehlin. 1974. Direct evidence for the presence of Epstein-Barr virus DNA and nuclear antigen in malignant epithelial cells from patients with poorly differentiated carcinoma of the nasopharynx. *Proc. Natl. Acad. Sci. USA* 71:4737-4741.
 43. Kousoulas, K. G., D. J. Bzik, N. DeLuca, and S. Person. 1983. The effect of ammonium chloride and tunicamycin on the glycoprotein content and infectivity of herpes simplex virus type 1. *Virology* 125:468-474.
 44. Kousoulas, K. G., P. E. Pellett, L. Pereira, and B. Roizman. 1984. Mutations affecting conformation or sequence of neutralizing epitopes identified by reactivity of viable plaques segregate from syn and ts domains of HSV-1(F) gB gene. *Virology* 135:379-394.
 45. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
 46. Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer. 1981. A virion associated glycoprotein essential for infectivity of

- herpes simplex virus type 1. *Virology* **115**:149–160.
47. McGeech, D. J., A. Dolan, S. Donald, and F. J. Rixon. 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. *J. Mol. Biol.* **181**:1–13.
 48. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 49. Morgan, A. J., A. R. Smith, R. N. Barker, and M. A. Epstein. 1984. A structural investigation of the Epstein-Barr (EB) virus membrane antigen glycoprotein, gp340. *J. Gen. Virol.* **65**:397–404.
 50. Norrild, B., H. Ludwig, and R. Rott. 1978. Identification of a common antigen of herpes simplex virus, bovine herpes mamillitis virus, and B virus. *J. Virol.* **26**:712–717.
 51. Norrild, B., and B. Pedersen. 1982. Effect of tunicamycin on the synthesis of herpes simplex virus type 1 glycoproteins and their expression on the cell surface. *J. Virol.* **43**:395–402.
 52. Norrild, B., S. L. Shore, and A. J. Nahmias. 1979. Herpes simplex virus glycoproteins: participation of individual herpes simplex virus type 1 glycoprotein antigens in immunocytolysis and their correlation with previously identified glycopolypeptides. *J. Virol.* **32**:741–748.
 53. Pellett, P. E., M. D. Biggin, B. Barrell, and B. Roizman. 1985. Epstein-Barr virus genome may encode a protein showing significant amino acid and predicted secondary structure homology with glycoprotein B of herpes simplex virus 1. *J. Virol.* **56**:807–813.
 54. Pellett, P. E., K. G. Kousoulas, L. Pereira, and B. Roizman. 1985. Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. *J. Virol.* **53**:243–253.
 55. Pope, J., M. Horne, and W. Scott. 1968. Transformation of fetal human leukocytes in vitro by filtrates of a human leukemic cell line containing herpes like virus. *Int. J. Cancer* **3**:857–866.
 56. Purtilo, D. 1985. Association of Epstein-Barr virus and lymphoproliferative diseases in immune deficient persons, p. 3–18. *In* P. Levine, D. Ablashi, G. Pearson, and S. Kottaridis (ed.), *Epstein-Barr virus and associated diseases*. Martinus Nijhoff Publishers, Boston.
 57. Qualtiere, L. F., and G. R. Pearson. 1979. Epstein-Barr virus induced membrane antigens: immunochemical characterization of Triton X-100 solubilized viral membrane antigens from EBV superinfected Raji cells. *Int. J. Cancer* **23**:808–817.
 58. Qualtiere, L. F., and G. R. Pearson. 1980. Radioimmune precipitation study comparing the Epstein-Barr virus membrane antigens expressed on P3HR-1 virus superinfected Raji cells to those expressed of cells in a B-95 virus transformed producer culture activated with tumor promoting agent (TPA). *Virology* **102**:360–369.
 59. Rafield, L. F., and D. M. Knipe. 1984. Characterization of the major mRNAs transcribed from the genes for the glycoprotein B and DNA binding protein ICP8 of herpes simplex virus type 1. *J. Virol.* **49**:960–969.
 60. Richardson, W., B. Roberts, and A. Smith. 1986. Nuclear location signals in polyoma virus large-T. *Cell* **44**:77–85.
 61. Roizman, B., and E. Kieff. 1975. Herpes simplex and Epstein-Barr viruses in human cells and tissues: a study in contrasts, p. 241–322. *In* F. F. Becker (ed.), *Cancer: a comprehensive treatise*, vol. 2. Plenum Publishing Corp., New York.
 62. Sanger, F., A. Coulson, B. Barrell, J. Smith, and B. Roe. 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* **143**:161–178.
 63. Sarmiento, M., M. Haffey, and P. G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B₂) in virion infectivity. *J. Virol.* **29**:1149–1158.
 64. Serafini-Cessi, F., F. Dall'Olio, M. Scannavani, F. Costanzo, and G. Campdelli-Fiume. 1983. N-Acetylgalactosaminyltransferase activity involved in O-glycosylation of herpes simplex virus type 1 glycoproteins. *J. Virol.* **48**:325–329.
 65. Shinnick, T., R. Lerner, and J. Sutcliffe. 1981. Nucleotide sequence of Moloney murine leukemia virus. *Nature (London)* **293**:543–548.
 66. Snowden, B. W., P. R. Kinchington, K. L. Powell, and I. W. Halliburton. 1985. Antigenic and biochemical analysis of gB of herpes simplex virus type 1 and type 2 and of cross-reacting glycoproteins induced by bovine mamillitis virus and quine herpesvirus type 1. *J. Gen. Virol.* **66**:231–247.
 67. Spear, P. G. 1985. Glycoproteins specified by herpes simplex viruses, p. 315–356. *In* B. Roizman (ed.), *The herpesviruses*. Plenum Publishing Corp., New York.
 68. Strnad, B. C., M. R. Adams, and H. Rabin. 1983. Glycosylation pathways of two major Epstein-Barr virus membrane antigens. *Virology* **127**:168–176.
 69. Strnad, B. C., T. Schuster, R. Klein, R. F. Hopkins III, T. Witmur, R. H. Neubauer, and H. Rabin. 1982. Production and characterization of monoclonal antibodies against the Epstein-Barr virus membrane antigen. *J. Virol.* **41**:258–264.
 70. Struck, D. K., and W. J. Lennarz. 1980. The function of saccharide-lipids in synthesis of glycoproteins, p. 35–83. *In* W. J. Lennarz (ed.), *The biochemistry of glycoproteins and proteoglycans*. Plenum Publishing Corp., New York.
 71. Tarentino, A. L., C. M. Gomez, and T. H. Plummer. 1985. Deglycosylation of asparagine-linked glycans by peptide:N-glycosidase F. *Biochemistry* **24**:4665–4671.
 72. Thorley-Lawson, D. A., C. M. Edson, and K. Gellinger. 1982. Epstein-Barr virus antigens—a challenge to modern biochemistry. *Adv. Cancer Res.* **36**:295–348.
 73. Tkacz, J. S., and J. O. Lampen. 1975. Tunicamycin inhibition of polyisoprenyl N-actylglucosaminyl pyrophosphate formation in calf-liver microsomes. *Biochem. Biophys. Res. Commun.* **65**:248–257.
 74. Zeng, Y., and G. de The. 1985. Nasopharyngeal carcinoma: early detection and IgA related pre-NPC condition, achievements and perspectives, p. 151–163. *In* P. Levine, D. Ablashi, G. Pearson, and S. Kottaridis (ed.), *Epstein-Barr virus and associated diseases*. Martinus Nijhoff Publishing, Boston.