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Construction and Properties of a Mutant of Herpes Simplex Virus Type 1 with Glycoprotein H Coding Sequences Deleted

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A mutant of herpes simplex virus type 1 (HSV-1) in which glycoprotein H (gH) coding sequences were deleted and replaced by the *Escherichia coli lacZ* gene under the control of the human cytomegalovirus IE-1 gene promoter was constructed. The mutant was propagated in Vero cells which contained multiple copies of the HSV-1 gH gene under the control of the HSV-1 gD promoter and which therefore provide gH in *trans* following HSV-1 infection. Phenotypically gH-negative virions were obtained by a single growth cycle in Vero cells. These virions were noninfectious, as judged by plaque assay and by expression of β-galactosidase following high-multiplicity infection, but partial recovery of infectivity was achieved by using the fusogenic agent polyethylene glycol. Adsorption of gH-negative virions to cells blocked the adsorption of superinfecting wild-type virus, a result in contrast to that obtained with gD-negative virions (D. C. Johnson and M. W. Ligas, J. Virol. 62:4605–4612, 1988). The simplest conclusion is that gH is required for membrane fusion but not for receptor binding, a conclusion consistent with the conservation of gH in all herpesviruses.

The diversity of the herpesviruses is amply illustrated by the variety of the surface glycoproteins specified by different members of the group. All herpesviruses specify multiple surface glycoproteins, but only two, represented by glycoprotein B (gB) and gH of herpes simplex virus (HSV), are found in herpesviruses of all subgroups (2, 8, 9, 20, 22, 36, 41). gB and its homologs have been studied extensively. The amino acid sequence is relatively well conserved among different herpesviruses, and studies with temperature-sensitive mutants, antibody-resistant mutants, and gB null mutants show that gB functions in viral entry into the host cell (4, 5, 10, 23, 33, 44). These studies all suggest that gB operates in membrane fusion rather than virus adsorption, a view supported by the fact that mutations in gB can influence the syncytial phenotype (3, 5); however, studies of the interaction of viral glycoproteins with the cell surface, done with HSV and pseudorabies virus, suggest that gB may also be involved in the adsorption process (27, 30, 38). The current view is that gB is certainly required for membrane fusion but may additionally participate in virus adsorption to the plasma membrane.

By contrast, gH has been the subject of few studies. The facts that a temperature-sensitive (*ts*) mutation has been identified in HSV type 1 (HSV-1) gH (7, 11) and that antibody to gH efficiently neutralizes virion infectivity in the absence of complement (1) suggest that gH is essential for virion infectivity. In addition, gH antibodies will inhibit membrane fusion by syncytial virus strains (20), and virus particles treated with anti-gH antibodies will bind to cells but will not penetrate (17). Data for gH of other herpesviruses are very limited. The amino acid sequences of gH homologs are poorly conserved, homology being limited to the C-terminal portion of the extracellular domain (19), and the view that gH homologs have conserved function is based on the observations that antibodies against these homologs have similar biological effects on their respective viruses (28, 42, 48).

Thus, the available data suggest that gH, like gB, is required for virion entry into the host cell, that its function is common to all herpesviruses, and that gH operates in membrane fusion, but the data are few and the evidence is indirect. In an attempt to obtain more direct evidence, we decided to produce an HSV-1 mutant in which the gH coding sequences were deleted. This article describes the construction and properties of such a mutant.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown in Glasgow's modified Eagle's medium (GMEM) containing 10% newborn calf serum. BHK-21 cells and BU-BHK cells (TK^-, bromodeoxyuridine-resistant BHK cells) were maintained in the same medium supplemented with 10% tryptose phosphate broth. VD60 cells, a cell line derived from Vero and able to supply HSV-1 gD in *trans* (32), were a gift from D. C. Johnson (McMaster University, Hamilton, Ontario, Canada) and were maintained in GMEM lacking histidine and supplemented with 1.0 mM histidinol and 7% fetal calf serum.

The following viruses were used: HSV-2 strain 25766; HSV-1 strains HFEM, SC16, KOS, and *ts*Q26 (a *ts* mutant derived from KOS, containing a lesion in the gH coding sequence) (7, 11). TKDM21 is a thymidine kinase deletion mutant derived from SC16 (12). All virus stocks were grown at a multiplicity of infection (MOI) of 0.01 in BHK-21 cells and were assayed in BHK-21 cells. FgD-β is a gD-negative virus (32) and was a gift from D. C. Johnson. The virus was propagated and assayed in VD60 cells. Phenotypically gD-negative virions were obtained by a single passage at an MOI of 3 in Vero cells. Unadsorbed input virus was removed by adding neutralizing monoclonal antibody LP2 (39) at a final concentration of 30 μg/ml for 1 h at 37°C at the end of the adsorption period.

Plasmids. pAF2 contains the HSV-1 (HFEM) gH coding

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sequence within the vector sp64T. This vector provides flanking sequences corresponding to the 5′ and 3′ untranslated sequences of the *Xenopus* β-globulin transcript. The construction of pAF2 has been described (21). Plasmid pgDBr (13, 14) contains the HSV-1 gD promoter (−392 to +11). pSV2Neo (47) contains the G418 resistance marker under the control of the simian virus 40 early promoter. Plasmid pMV10 is derived from pUC18 and contains a *Hin*dIII insert composed of the *Escherichia coli lacZ* coding sequence flanked by the cytomegalovirus (CMV) IE-1 promoter and poly(A) addition site. Flanking sequences on the 5′ side correspond to nucleotides −299 to +69 with respect to the IE-1 transcript start site and on the 3′ side correspond to nucleotides +2757 to +3053. The construction of pMV10 will be described elsewhere.

Transfection. Transfection of Vero cells or BHK-21 cells was performed on subconfluent monolayers by the modified calcium phosphate procedure of Chen and Okayama (6). When the objective was to produce a stably transfected cell line, the cells were grown in 25-cm^2 flasks and transfected with 15 μg of plasmid carrying the selectable marker (pSV2Neo) and 15 μg of nonselected plasmid. The transfected cells were harvested after 48 h and replated in medium containing G418 (400 μg/ml). Surviving colonies were harvested and cloned by limiting dilution in the presence of G418.

When the objective was to construct recombinant viruses carrying the *lacZ* gene, similar monolayers were transfected with 20 μg of HSV-1-infected cell DNA plus 5 μg of plasmid DNA carrying the desired sequences for recombination. After 3 days, the cells, which exhibited extensive cytopathic effect, were harvested and sonicated. The number of progeny and the proportion of recombinant β-galactosidase-positive virus were then measured as follows. Dilutions of virus were plated on cell monolayers, and the cells were fixed after 2 days with 0.5% glutaraldehyde in phosphate-buffered saline for 15 min at room temperature. After being fixed, the cells were permeabilized by incubation on ice for 10 min in 2 mM $MgCl_2$–0.01% sodium deoxycholate–0.02% Nonidet P-40. This mixture was then removed and replaced with the same solution containing 5 mM potassium ferricyanide-ferrocyanide plus 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (1 mg/ml). Plates were counterstained with phenol red, and "blue" and "white" plaques were counted. Appropriate dilutions of transfection progeny were then plated, and after 3 days, recombinant plaques were detected by using an overlay of 1% low-melting-temperature agarose containing X-Gal (300 μg/ml). Blue plaques were picked, subjected to two further rounds of plating and plaque picking, and finally cloned by limiting dilution.

Construction of HSV-1 Δ5Z. For the competition binding experiments described in the Results section, it was convenient to use a virus which expressed β-galactosidase but was of wild-type phenotype in vitro. The Us5 coding sequence has been shown to be dispensable in vitro (50) and was therefore used as an insertion site for the *lacZ* gene. An *Acc*I fragment (nucleotides Us 3704 to 6591 [37]) containing the Us5 gene was cloned into the *Acc*I site of pBR322 (nucleotides 651 to 2244), and a synthetic polylinker was inserted into the *Sst*I site within the Us5 gene (nucleotide Us 5337). The *Hin*dIII insert of plasmid pMV10 was then excised, end repaired, gel purified, and inserted into the *Eco*RV site of the polylinker. A plasmid was chosen in which the reading direction of the *lacZ* insert corresponded to the reading direction of the Us5 gene. This plasmid was then cotransfected into BHK cells with HSV-1 (SC16) DNA, and a recombinant, β-galactosidase-positive virus was selected and cloned from the transfection progeny as described above. The genotype of the virus was confirmed by Southern blotting and found to contain the expected *lacZ* coding sequences, driven by the CMV IE-1 promoter, within Us5. This virus plaqued normally and had particle-infectivity ratios indistinguishable from those of the parent virus.

Enzyme assays. Monolayers of 5×10^4 infected cells were washed once with 100 mM sodium phosphate–10 mM KCl–10 mM $MgCl_2$–50 mM 2-mercaptoethanol and lysed in 200 μl of the same buffer containing 1% Triton X-100. Lysates were stored at −70°C. β-Galactosidase assays were performed by addition of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) to thawed lysate to a final concentration of 6 mM. The increase in A_{420} was measured over a period of 2 h at room temperature. Thymidine kinase assays were performed as described by Klemperer et al. (29).

Other methods. HSV-1 and recombinant virus derived from it were purified from the tissue culture medium and the cytoplasm of infected cells as described previously (11). Particle numbers were estimated by comparison with latex beads (approximately 250 nm in diameter) of known concentration by the "loop drop" method (49). All particle numbers refer to enveloped particles. The methods used for Southern blotting and Western immunoblotting have been described previously (11).

RESULTS

Rationale for generating a gH⁻ mutant of HSV-1. Our objective was to engineer an HSV-1 mutant in which gH coding sequences were replaced by *lacZ* coding sequences under the control of a constitutive promoter, the IE-1 promoter of human CMV, generating a virus that could be identified by virtue of its ability to synthesize β-galactosidase. Since previous data indicate that gH is required for virion infectivity (11), we predicted that propogation of a deletion mutant would require a cell line capable of supplying gH in *trans*. Although a cell line constitutively expressing gH has been described (16), our own attempts to express gH have suggested that constitutive expression of gH at adequate levels would be cytotoxic (21). Our strategy was therefore to establish a cell line containing a gH gene under the control of an HSV-1-specific promoter so that gH would be expressed only after infection with HSV. However, since gH is a late gene (24, 46), the gH promoter itself is unlikely to be suitable for this purpose because a late promoter resident in the cell genome is likely to be subject to shutdown by HSV-1 infection. This problem was avoided by introducing a gH coding sequence into cells under the control of the gD-1 promoter. A similar strategy has been used to achieve inducible expression of HSV gC (43).

Generation of a cell line inducible for gH-1 expression. Plasmid pAF2 (see Materials and Methods) was digested with *Hin*dIII and partially digested with *Eco*RI to generate a 3.8-kb fragment which contained the gH-coding sequence of HSV-1 strain HFEM flanked by 5′ and 3′ untranslated sequences of the *Xenopus* β-globin transcript. This fragment was ligated with *Hin*dIII- and *Eco*RI-digested pgDBr (13, 14) to place the gD-1 promoter (bases −392 to +11) upstream of the gH-coding sequences. The resulting plasmid was named pgDBrgH (see Fig. 2).

Subconfluent monolayers of Vero cells were cotransfected with pgDBrgH and pSV2Neo (47), and after 2 days, the cells were harvested, transferred to 96-well microtiter trays, and

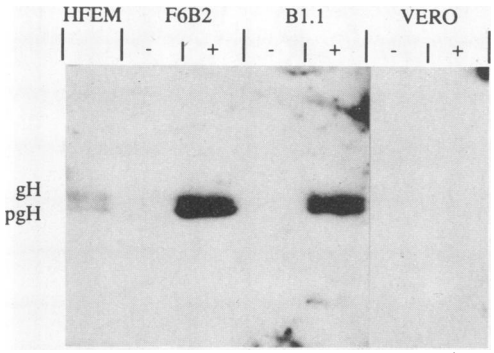

FIG. 1. Induction of gH-1 synthesis by HSV-2 infection of Vero cells transfected with the HSV-1 gH gene. Vero cells or cloned cell lines transfected with the gH-1 gene as described in the text were infected with HSV-2 at an MOI of 5 (+) or left uninfected (−). After 16 h, the cells were harvested, the lysates were subjected to SDS-PAGE, and the products were transferred to nitrocellulose. HSV-1 gH was detected by using antiserum to a TrypE-gH fusion protein. The track marked HFEM contained a lysate of Vero cells infected with HSV-1 (HFEM) at an MOI of 5. All tracks contain a lysate derived from approximately 5×10^5 cells. pgH, high mannose precursor of gH (11).

grown in medium containing G418. After 15 days, surviving colonies were harvested, plated in duplicate 24-well trays, and grown to confluence ($\sim 2 \times 10^5$ cells per well) in medium containing G418. One set of cultures was then infected with HSV-2 at an MOI of 5, and after 16 h, the infected cells were harvested, lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the products were transferred to nitrocellulose. Lysates containing HSV-1-specific gH were identified by using antiserum to a TrypE-gH fusion protein (11). Among 136 cultures, 5 were found to synthesize gH-1 following infection with HSV-2, and these cultures were cloned by limiting dilution. Cloned cells were again subjected to HSV-2 infection and Western blotting with TrypE-gH antiserum.

The results obtained with two clones from different cultures are shown in Fig. 1. Both clones produced gH-1 in response to HSV-2 infection, but neither contained detectable gH when uninfected, and both clones produced substantially more gH-1 than equivalent numbers of HSV-1-infected Vero cells. No gH was detected in HSV-2-infected Vero cells, confirming the specificity of the antiserum for HSV-1 gH. DNA was extracted from both cell lines and subjected to *Eco*RI digestion. The digestion products were examined by Southern hybridization with a probe composed of gH coding sequences flanked by β-globin untranslated transcript sequences (from pAF2). The results (Fig. 2) indicate a high copy number of gH coding sequences, considerably in excess of 10 copies per cell and probably close to 100 copies per cell. The majority of the hybridization signal was associated with fragments of 3.7 and 2.9 kb, corresponding to the fragments generated by *Eco*RI digestion of the transfection plasmid pgDBRgH, implying that the transfected cells contain large head-to-tail concatemers of the input plasmid. This was not investigated further. The F6B2 cell line was used in all subsequent work and is hereafter called F6. The high levels of gH made upon infection of the F6 cell line (by comparison with HSV-1 infection of Vero cells) probably reflect both the high DNA copy number and the fact that the gene is driven by the relatively strong gD promoter.

The cell line F6 appeared to have the required properties

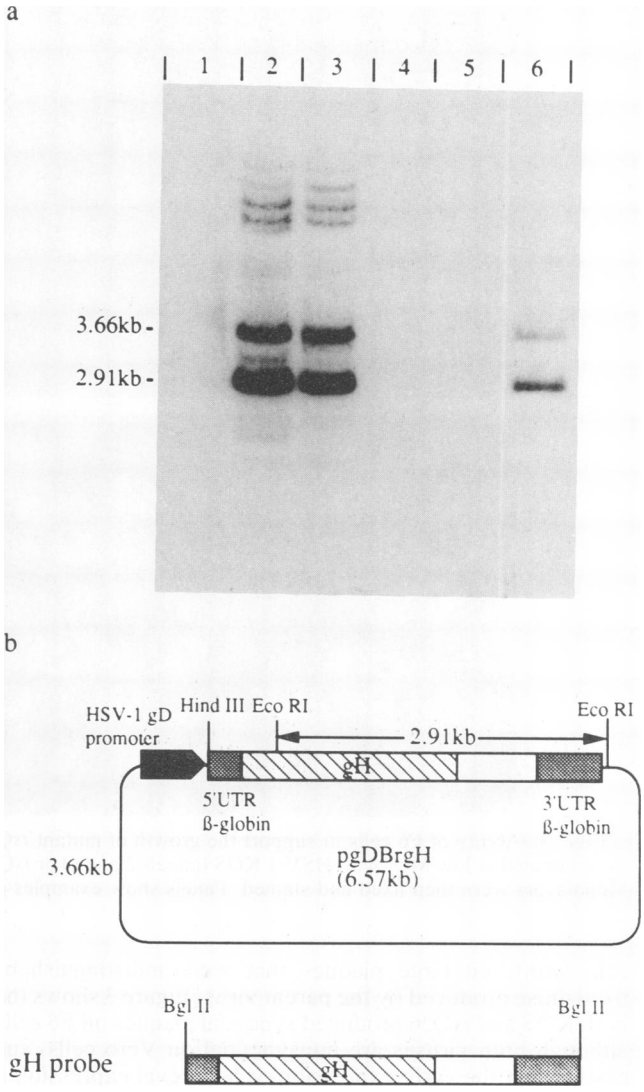

FIG. 2. DNA was extracted from Vero cells and from transfected cell lines. The DNA was digested with *Eco*RI and subjected to gel electrophoresis and Southern blotting. The filters were hybridized with the *Bgl*II fragment derived from pAF2. (a) Track 1, Vero cells; track 2, F6 cells; track 3, B1.1 cells; tracks 4 and 5, DNA from neomycin-resistant clones that did not yield gH-1 on infection with HSV-2. Track 6 is a positive control containing Vero cell DNA plus pgDBrgH DNA equivalent to approximately 10 copies per cell. The exposure time for track 6 was eight times longer than for the remaining tracks. (b) Positions of the *Eco*RI sites in pgDBrgH, the plasmid used for transfection, and the region covered by the probe.

for supplying gH in *trans* to a gH-deficient HSV-1 mutant. However, it was conceivable that the very high level of expression of gH achieved following infection and the fact that expression of gH from the gD promoter would occur earlier in the growth cycle than in a standard HSV infection might be incompatible with efficient HSV replication. To reassure ourselves on this point before proceeding with production of a null mutant, the *ts* mutant *ts*Q26, which is nonpermissive at 39°C by virtue of a mutation in gH, was plated at 39°C on Vero cells or F6 cells. The parental virus, HSV-1 strain KOS, was also plated as a control. *ts*Q26 produced minute plaques on Vero cells at 39°C but in F6

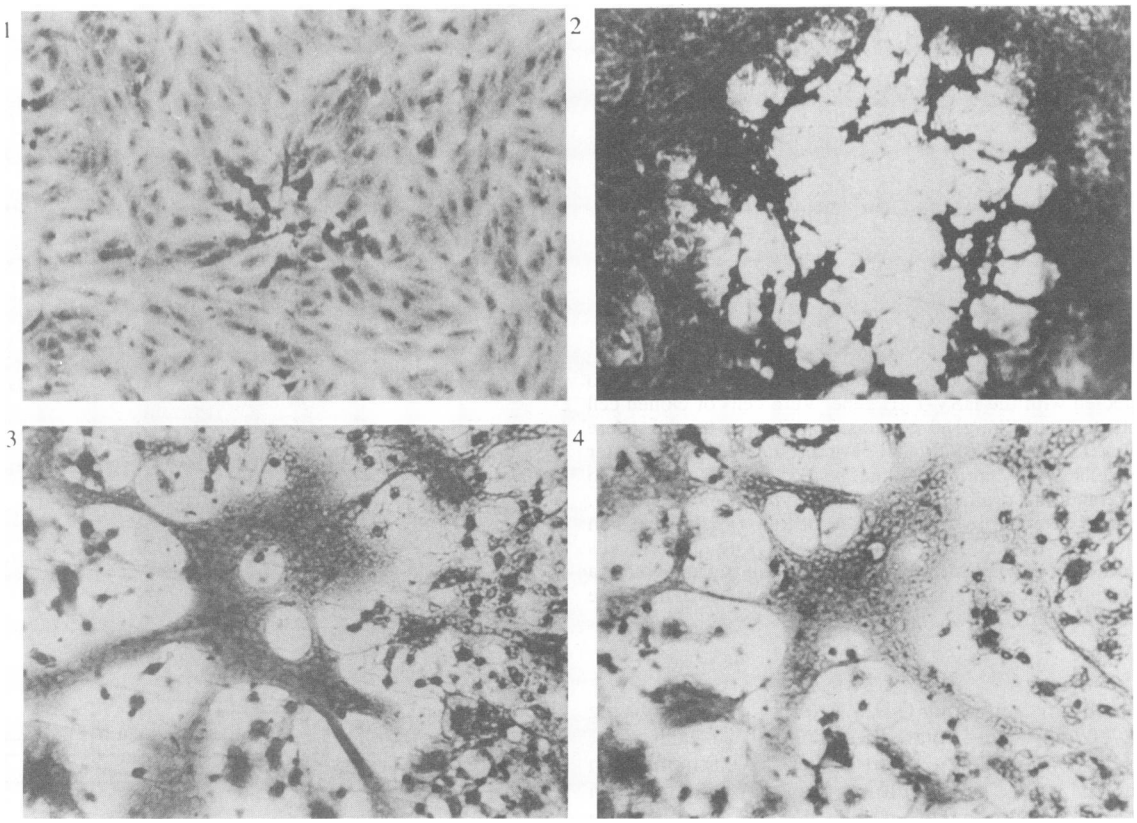

FIG. 3. Ability of F6 cells to support the growth of mutant *ts*Q26. Monolayers of Vero cells (panels 1 and 2) or F6 cells (panels 3 and 4) were infected at low MOI with HSV-1 KOS (panels 2 and 4) or *ts*Q26 (panels 1 and 3), and the monolayers were incubated for 2 days at 39°C. Monolayers were then fixed and stained. Panels show examples of individual plaques.

cells produced large plaques that were indistinguishable from those produced by the parent virus. Figure 3 shows that both KOS and *ts*Q26 produced syncytial plaques on F6 cells, although both viruses are nonsyncytial on Vero cells, suggesting that the early expression or high-level expression of gH is capable of conferring the syncytial phenotype.

Production of gH null mutant. The *Bgl*II m fragment of HSV-1 HFEM (bases 41429 to 47860 [35]) was cloned into the *Bam*HI site of pAT153, and the majority of the gH-coding sequences were removed by digestion with *Pvu*II (bases 44960 to 46070) and religation to form pATΔgH. A 3.8-kb *lacZ* cassette was obtained from pMV10 by *Hin*dIII digestion and gel purification. This fragment, which contains the *lacZ* gene flanked by the promoter and poly(A) addition site of the CMV IE-1 gene, was end-repaired with Klenow fragment and inserted into the *Pvu*II site of pATΔgH to generate pJAM2. Restriction analysis of pJAM2 indicated that the *lacZ* gene was oriented in the same direction as the interrupted gH gene. Since the CMV IE-1 promoter (bases −299 to +69 in pMV10) contains translation termination codons in all reading frames, a transcript derived from the gH promoter in pJAM2 would be translated to give the first 104 residues (including the signal peptide) of gH and would terminate soon thereafter in the CMV IE-1 promoter sequence.

BHK-21 cells were cotransfected with HSV-1 strain SC16 DNA and pJAM2 DNA. After two days, when the monolayers exhibited extensive cytopathic effect, the cultures were harvested, sonicated, and replated on monolayers of F6 cells. After 2 days, the monolayers were overlaid with 1% agarose in medium containing X-Gal, and β-galactosidase-positive plaques were picked and subjected to two further rounds of plaque picking, followed by cloning by limiting dilution. Southern hybridization of one cloned virus confirmed that the gH coding sequences had been replaced by the *lacZ* cassette (Fig. 4), and this virus, named SCgHZ, was used in subsequent experiments. This virus failed to form plaques on Vero cells (the relative plaquing efficiency on Vero cells compared with that on F6 cells was less than 10^{-5}). SCgHZ is derived from strain SC16, whereas the complementary cell line, F6, was constructed by using a gH coding sequence derived from strain HFEM because convenient cloned DNA was available. SCgHZ grown in F6 cells will not, therefore, be phenotypically identical to the parent virus, SC16. However, we do not think that this raises problems because we have found no electrophoretic or antigenic difference between the gHs of HFEM and SC16 and the two proteins differ in amino acid sequence at only three positions (18).

Properties of SCgHZ. In order to prepare SCgHZ virions lacking gH, 10^8 Vero cells were infected with SCgHZ at an MOI of 3, and after 1 h for adsorption, nonadsorbed virus was neutralized with antibody LP2 (ascitic fluid diluted 1:200 in medium). The antibody was replaced with fresh medium after 30 min, and the cells were harvested and sonicated after 24 h. Electron microscopic examination revealed that the numbers of virus particles and the proportions of enveloped and naked particles were similar to those in a stock of wild-type SC16 virus made in parallel in Vero cells under the same conditions. To find whether phenotypically gH-nega-

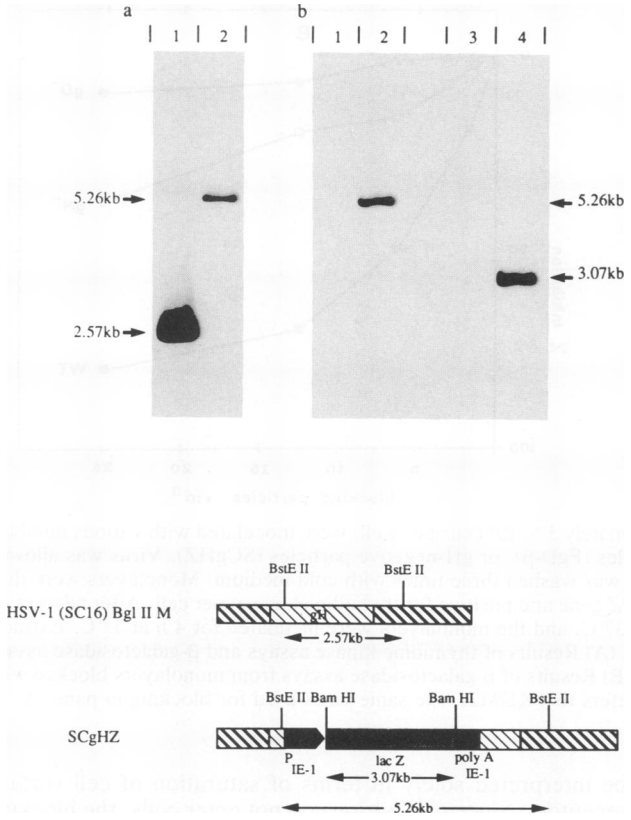

FIG. 4. DNA samples from F6 cells infected with HSV-1 SCgHZ (lanes 2 and 4) or from Vero cells infected with HSV-1 SC16 were digested with *Bst*EII (lanes 1 and 2) or with *Bam*HI (lanes 3 and 4) and subjected to agarose gel electrophoresis and Southern transfer to nitrocellulose. The filters were hybridized with ^{32}P-labeled probes comprising (a) the 2.57-kb *Bst*EII subfragment of an HSV-1 *Bgl*II-m clone or (b) the 3.07-kb *lacZ* coding sequence derived by *Bam*HI digestion of pMV10. Positions of the relevant restriction sites in HSV-1 DNA and in SCgHZ are shown below. poly A, poly(A) addition site; P_{IE-1}, CMV IE-1 promoter.

tive virions were capable of initiating infection, stocks of wild-type virus (SC16), SCgHZ grown on F6 cells, and SCgHZ grown on Vero cells were assayed on monolayers of F6 cells, and the results were expressed as PFU per 10^9 enveloped virus particles. Table 1 shows that the particle/PFU ratios of wild-type virus and SCgHZ virus grown on F6 cells were indistinguishable. Thus, the F6 cell line compensates fully for the genetic defect in SCgHZ. Phenotypically gH-negative virions (SCgHZ virus grown on Vero cells) yielded no plaques at the highest concentration used, confirming that gH is absolutely required for virion infectivity (11). When the monolayers were treated with 50% (wt/wt) polyethylene glycol (PEG) after virus adsorption as described by Sarmiento et al. (44), the numbers of plaques formed by wild-type virus or by SCgHZ (gH^+) virus were unchanged. However, this treatment resulted in the partial recovery of infectivity by gH-negative virions. This result can be interpreted to mean that gH-negative virions will bind to the cell surface but that membrane fusion, a process that can be achieved artificially with PEG, fails to occur. This interpretation is consistent with that of Fuller et al. (17), who found that virions neutralized with anti-gH antibody bound to the cell surface but failed to penetrate. The results obtained by using PEG fusion should be interpreted with caution because the recovery of infectivity is low. Nevertheless, the results with gH-negative virions are quantitatively similar to those obtained with gB-negative and gD-negative virions (5, 32), implying that all three types of virions attach equally well to the cell surface.

TABLE 1. Rescue of infectivity of gH-negative virions by using PEG

Virus (cells[a])	gH genotype	gH phenotype	PFU/10^9 particles on F6 cells[b]	
			Without PEG	With PEG
HSV-1 SC16 (Vero)	gH^+	gH^+	8.5×10^6	7.6×10^6
SCgHZ (F6)	gH^-	gH^+	9.2×10^6	5.5×10^6
SCgHZ (Vero)	gH^-	gH^-	$<10^c$	3.2×10^4

[a] Cell type used for virus propagation.
[b] Particle counts were performed on crude virus stocks, and serial dilutions were plated on monolayers (5×10^7 cells) of F6 cells. Particle numbers refer to enveloped particles.
[c] An inoculum comprising approximately 10^8 enveloped particles yielded no plaques.

The nature of the interaction of a mutant virus with the cell surface can be examined by determining whether adsorption of mutant virions will block infection by wild-type virions. This rationale has been used by Johnson and Ligas (26) to show that gD-negative virions, despite their ability to bind, will not block entry by superinfecting virus, a result which they interpreted to mean that gD binds to a saturable receptor. We therefore performed similar experiments with gH-negative virions. Different concentrations of blocking virions (wild type, gD negative, and gH negative) were added to monolayers of BHK-21 cells in 96-well trays and allowed to adsorb for 2 h at 4°C. Nonabsorbed virus was removed by washing with three changes of medium, and wild-type virus was added at an MOI of 5. After a further 2 h at 4°C, nonadsorbed virus was removed by washing and the temperature was raised to 37°C to allow penetration. In these experiments, the wild-type virus (i.e., gD^+ gH^+) used for blocking was a thymidine kinase deletion mutant (TKDM21). The superinfecting virus was SC16Δ5Z, which is thymidine kinase positive and carries a *lacZ* gene driven by the CMV IE-1 promoter. Infection by superinfecting virus could therefore be measured by thymidine kinase and β-galactosidase activities. Note that both the gD-negative and gH-negative blocking virions also contain *lacZ* genes, but in preliminary experiments these viruses induced no β-galactosidase activity even at the highest concentrations used for blocking, either in the absence of a superinfecting wild-type virus or following infection by a *lacZ*-negative wild-type virus. Thus, gD^- and gH^- viruses are entirely unable to enter cells and cannot be "helped" to enter cells by wild-type virions.

Initial experiments were performed with TK^- β-Gal^- (TKDM21) blocking virions and TK^+ β-gal^+ (SC16Δ5Z) superinfecting virions. Cultures were harvested 4 h after the temperature was raised to 37°C and assayed for thymidine kinase and β-galactosidase. It is apparent from Fig. 5A that different results are obtained by assaying different activities. When infection by superinfecting virus is measured by thymidine kinase activity, the observed effect of blocking virions is greater than that observed when β-galactosidase activity is measured. Thymidine kinase activity induced by superinfecting virus is virtually abolished by 10^4 blocking particles, a result close to that obtained by Johnson and

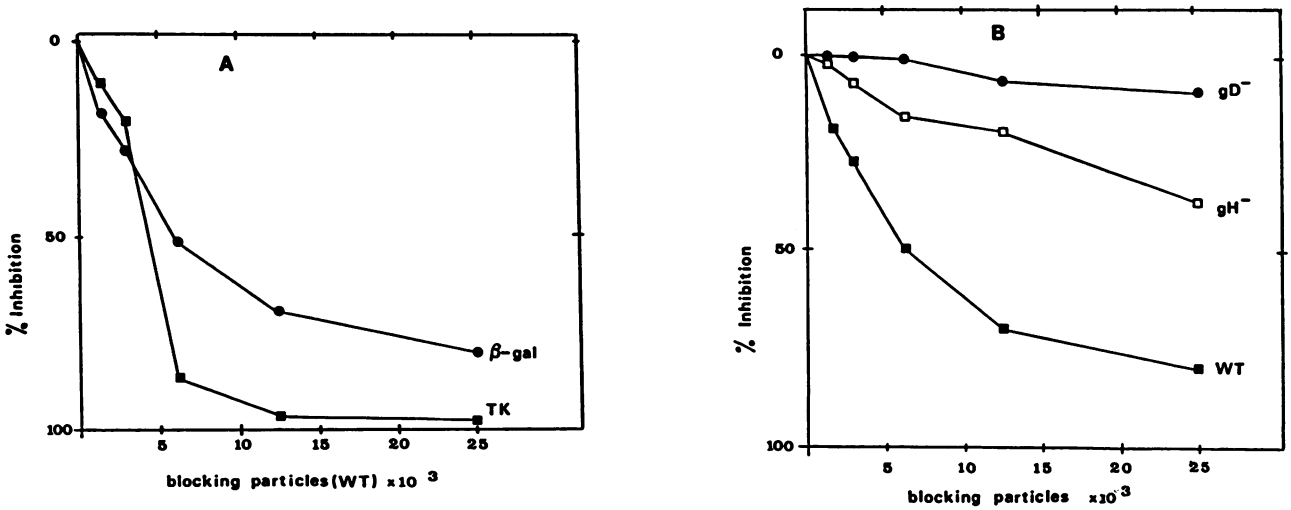

FIG. 5. Monolayers of BU-BHK (TK^-) cells in 96-well trays (approximately 5×10^4 cells per well) were inoculated with various numbers of purified TKDM21 particles (TK^- wild type [WT]), gD-negative particles (FgD-β), or gH-negative particles (SCgHZ). Virus was allowed to adsorb for 2 h at 4°C, the inoculum was removed, and the monolayer was washed three times with cold medium. Monolayers were then inoculated with 10 PFU of HSV-1 Δ5Z (wild-type virus expressing the *lacZ* gene and positive for thymidine kinase) per cell. After adsorption for 2 h at 4°C, the inoculum was removed and replaced with medium at 37°C, and the monolayers were incubated for 4 h at 37°C. Extracts were then prepared and assayed for thymidine kinase or β-galactosidase. (A) Results of thymidine kinase assays and β-galactosidase assays from monolayers blocked with TKDM21 and infected with HSV-1 Δ5Z. (B) Results of β-galactosidase assays from monolayers blocked with different particle types and infected with HSV-1 Δ5Z. In panel B, WT refers to TKDM21, the same virus used for blocking in panel A.

Ligas (26). Considerably larger numbers of blocking particles are required to achieve equal reduction of induced β-galactosidase activity. One obvious interpretation of these results is that, since thymidine kinase is synthesized as a delayed early protein, its synthesis will be sensitive to factors in addition to those which affect cell surface blocking. For example, since TKDM21 blocking particles enter cells when the temperature is raised, competition for virus-specific transcription factors may occur. Whatever the reason, it seemed that β-galactosidase was the more relevant assay to use in these experiments because this gene was controlled by the CMV IE-1 promoter: it should therefore be expressed at immediate-early times and independently of virus-specific transcription factors.

The relative abilities of gD^+ gH^+ virions (TKDM21), gD^- virions, and gH^- virions to block superinfecting virus is shown in Fig. 5B. We found, as reported by Johnson and Ligas (26), that gD^- virions fail to block superinfecting virus, while wild-type virus does so efficiently. Johnson and Ligas interpreted this result to indicate that gD binds to a saturable receptor, and this view is supported by the fact that soluble gD will also block infection (25). However, it is apparent that gH^- virions block superinfection much less efficiently than wild-type virus. A number of explanations are possible: for example, it is conceivable that gD is absolutely required for saturation of a cell surface receptor but that this interaction is less efficient in the absence of gH. A much simpler explanation is that the blocking effects of normal virions involve multiple mechanisms. The designs of the experiments described here and of those performed by Johnson and Ligas (26) inevitably result in the entry of the blocking (wild-type) virions when the culture temperature is raised. These virions will then compete in all processes required to initiate infection: transport of the nucleocapsid to the nucleus, entry into the nucleus, and transcription of the viral genome. This reasoning implies that, in experiments of this type, the "blocking" effects of wild-type virions cannot be interpreted solely in terms of saturation of cell surface receptors. Since gH^- virions do not enter cells, the blocking effect of these virions is presumably due to effects at the cell surface alone.

DISCUSSION

It has been assumed for several years that gH of HSV-1 performs an essential function in vitro because mutations within the gH coding sequence have been shown to be lethal or conditionally lethal (7, 11, 34). In this article, we formally confirm this assumption by showing that deletion of gH coding sequences is lethal and can be fully compensated for by providing gH in *trans*. Previous data have implied that gH is required for virion infectivity (11), and the results presented here confirm this view. Virions lacking gH cannot initiate plaque formation in gH-positive cells, and even when used at an MOI of 10^5 enveloped particles per cell, no virus entry was detected, as judged by expression of the *lacZ* gene resident in gH^- virions. Whether gH functions in processes other than virion infectivity is uncertain. Infection of Vero cells with genotypically gH^- virions resulted in the production of progeny virions in numbers similar to those observed in parallel wild-type infections, and the proportion of enveloped particles was unaltered. However, a detailed biochemical analysis of the gH^- virions has not been done. The ability of gH antibodies to inhibit virus spread (1, 20) suggests that gH is involved in interactions between the plasma membranes of infected and uninfected cells, and the behavior of different antibody-resistant mutants implies that these functions of gH, in virion infectivity and intercellular transmission, may not be identical (18).

gH is conserved among members of all the herpesvirus subgroups, and in the case of Epstein-Barr virus, we can be confident that gp85 (the homolog of HSV-1 gH) does not function as a receptor-binding protein because this function is performed by the Epstein-Barr virus gp350 molecule in

binding to CR2 on B cells (15, 40). A priori, then, we would predict that gH of HSV-1 is not responsible for receptor binding, though this prediction assumes a common function for gH in all herpesviruses. Our results support this view. First, the defect in gH-negative virions can be overcome in part by achieving membrane fusion with PEG, and second, gH^- virions will block adsorption of superinfecting wild-type virions. Both results imply that gH^- virions bind to cells but fail to enter and hence that gH operates in membrane fusion. Nevertheless, these results should be interpreted with caution. Induction of membrane fusion with PEG rescues only a small fraction of infectivity, and similar results have been attained with gB^- and gD^- virions (5, 32), implying that none of the known essential glycoproteins of HSV-1 is required for adsorption and that all three are required for membrane fusion. WuDunn and Spear (51) have shown that the adsorption of HSV-1 virions to cell surfaces is mediated by interactions with cell surface heparin sulfate. It seems unlikely that gB, gD, or gH is involved in this interaction, because virions lacking each of these glycoproteins will adsorb to cells. Pseudorabies virus also adsorbs to heparin sulfate, and in this case the protein involved is apparently gpIII, a dispensable glycoprotein and the homolog of HSV-1 gC (38, 45). It is uncertain whether HSV-1 gC is a heparin-binding protein, but it has been noted that gC^- virions adsorb more slowly than wild-type virions (31). Johnson et al. (25) take the view that the accumulation of virus particles at the cell surface by interaction with heparin sulfate is followed by the interaction of the virus with a saturable receptor in the plasma membrane and that gD is absolutely required for this indispensable step (but not for heparin binding) because gD^- virions will bind to cells but will not block superinfecting wild-type virus (26) and because soluble gD will block infection (25). We confirm the result of Johnson and Ligas (26) that gD^- virions will not block entry of superinfecting virus, whereas we find that gH^- virions will not enter cells but will block adsorption of wild-type virus. The simplest interpretation is that gH functions after gD in the entry process, presumably in membrane fusion. However, this interpretation must be qualified by the fact that gH^- virions blocked superinfection less efficiently than gH^+ virions. We consider that this is best explained by the fact that wild-type virions can interfere with infection by competing virus particles at several points in the infection process in addition to receptor binding, but this is difficult to quantify, and at present we cannot rule out the possibility that gH is involved in receptor binding.

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REFERENCES

1. **Buckmaster, E. A., U. Gompels, and A. C. Minson.** 1984. Characterisation and physical mapping of an HSV-1 glycoprotein of approximately 115×10^3 M.W. Virology **139**:408–413.
2. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence specifying the glycoprotein gB of herpes simplex virus type 1. Virology **133**:301–314.
3. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence of a region of the herpes simplex type 1 glycoprotein B gene: mutations affecting rate of virus entry and cell fusion. Virology **137**:185–190.
4. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B in viral entry and cell fusion. J. Virol. **62**:2596–2604.
5. **Cai, W., S. Person, S. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker insertion, nonsense, and restriction site deletion mutants of the gB glycoprotein of herpes simplex virus type 1. J. Virol. **61**:714–721.
6. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
7. **Chu, C.-T., D. S. Parris, R. A. F. Dixon, F. E. Farber, and P. A. Schaffer.** 1979. Hydroxylamine mutagenesis of HSV DNA and DNA fragments: introduction of mutations into selected regions of the viral genome. Virology **98**:168–181.
8. **Cranage, M. P., T. Kouzarides, A. T. Bankier, S. Satchwell, K. Weston, P. Tomlinson, B. Barrell, H. Hart, S. E. Bell, A. C. Minson, and G. L. Smith.** 1986. Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralising antibodies via its expression in a recombinant vaccinia virus. EMBO J. **5**:3057–3063.
9. **Cranage, M. P., G. L. Smith, S. E. Bell, H. Hart, C. Brown, A. T. Bankier, P. Tomlinson, B. G. Barrell, and A. C. Minson.** 1988. Identification and expression of a human cytomegalovirus glycoprotein with homology to the Epstein-Barr virus BXLF2 product, varicella zoster virus gpIII, and herpes simplex virus type 1 glycoprotein H. J. Virol. **62**:1416–1422.
10. **DeLuca, N. A., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 affecting virus entry, cell fusion and production of glycoprotein B. Virology **122**:411–423.
11. **Desai, P. J., P. A. Schaffer, and A. C. Minson.** 1988. Excretion of non-infectious particles lacking glycoprotein H by a temperature sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity. J. Gen. Virol. **69**:1147–1156.
12. **Efstathiou, S., S. Kemp, G. Darby, and A. C. Minson.** 1989. The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. J. Gen. Virol. **70**:869–879.
13. **Everett, R. D.** 1983. DNA sequence elements required for regulated expression of the HSV-1 glycoprotein D gene lie within 83 bp of the RNA capsites. Nucleic Acids Res. **11**:6647–6666.
14. **Everett, R. D.** 1984. A detailed analysis of an HSV-1 early promoter: sequence involved in transactivation by viral immediate early gene products are not early gene specific. Nucleic Acids Res. **12**:3037–3056.
15. **Fingeroth, J. D., J. J. Weiss, T. F. Tedder, J. L. Strominger, P. A. Biro, and D. T. Fearon.** 1984. Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. Proc. Natl. Acad. Sci. USA **811**:4510–4514.
16. **Foà-Tomasi, L., E. Avitabile, A. Boscaro, R. Brandimarti, R. Gualandri, R. Manservigi, F. Dall'Olio, F. Serafini-Cessi, and G. Campadelli-Fuime.** 1991. Herpes simplex virus (HSV) glycoprotein H is partially processed in a cell line that expresses the glycoprotein and fully processed in cells infected with deletion or ts mutants in the known HSV glycoproteins. Virology **180**:474–482.
17. **Fuller, A. O., R. E. Santos, and P. G. Spear.** 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. J. Virol. **63**:3435–3443.
18. **Gompels, U. A., A. L. Carss, C. Saxby, D. C. Hancock, A. Forrester, and A. C. Minson.** 1991. Characterization and sequence analyses of antibody-selected antigenic variants of herpes simplex virus show a conformationally complex epitope on glycoprotein H. J. Virol. **65**:2393–2401.
19. **Gompels, U., M. A. Craxton, and R. W. Honess.** 1988. Conservation of the gH gene in herpesviruses: nucleotide sequence of the gH gene from herpesvirus saimiri. J. Gen. Virol. **69**:2819–2829.
20. **Gompels, U., and A. C. Minson.** 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology **153**:230–247.
21. **Gompels, U. A., and A. C. Minson.** 1989. Antigenic properties

and cellular localization of herpes simplex virus glycoprotein H synthesized in a mammalian cell experimental system. J. Virol. **63**:4744–4755.

22. **Heineman, T., M. Gong, J. Sample, and E. Kieff.** 1988. Identification of the Epstein-Barr virus gp85 gene. J. Virol. **62**:1101–1107.
23. **Highlander, S. L., D. J. Dorney, P. J. Gage, J. C. Holland, W. Cai, S. Person, M. Levine, and J. C. Glorioso.** 1989. Identification of *mar* mutations in herpes simplex virus type 1 glycoprotein B which alter antigenic structure and function in virus penetration. J. Virol. **63**:730–738.
24. **Holland, L. E., R. M. Sandri-Goldin, A. L. Goldin, J. C. Glorioso, and M. Levine.** 1984. Transcriptional and genetic analyses of herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. J. Virol. **49**:947–959.
25. **Johnson, D. C., R. C. Burke, and T. Gregory.** 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. J. Virol. **64**:2569–2576.
26. **Johnson, D. C., and M. W. Ligas.** 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. **62**:4605–4612.
27. **Johnson, D. C., M. Wittels, and P. G. Spear.** 1984. Binding to cells of virosomes containing herpes simplex virus type 1 glycoproteins and evidence for fusion. J. Virol. **52**:238–247.
28. **Keller, P. M., A. J. Davison, R. S. Lowe, M. W. Rieman, and R. W. Ellis.** 1987. Identification and sequence of the gene encoding gpIII, a major glycoprotein of varicella zoster virus. Virology **157**:526–533.
29. **Klemperer, H. G., G. R. Haynes, W. I. H. Sheddon, and D. H. Watson.** 1967. A virus-specific thymidine kinase in BHK-21 cells infected with herpes simplex virus. Virology **31**:120–128.
30. **Kuhn, J. E., M. D. Kramer, W. Willenbacher, H. Weiland, E. U. Lorentzen, and R. W. Braun.** 1990. Identification of herpes simplex virus type 1 glycoproteins interacting with the cell surface. J. Virol. **64**:2491–2497.
31. **Langeland, N., A. M. Oyan, H. S. Marsden, A. Cross, J. C. Glorioso, L. S. Moore, and L. Haars.** 1990. Localization on the herpes simplex virus type 1 genome of a region encoding proteins involved in adsorption to the cellular receptor. J. Virol. **64**:1271–1277.
32. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequence is replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486–1494.
33. **Little, S. P., J. T. Joffre, 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.
34. **MacLean, A. R., and S. H. Brown.** 1987. Generation of a herpes simplex virus type 1 devoid of XbaI sites. J. Gen. Virol. **68**:1165–1171.
35. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the Long Unique Region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
36. **McGeoch, D. J., and A. J. Davison.** 1986. DNA sequence of the herpes simplex virus type 1 gene encoding glycoprotein H and identification of homologues in the genomes of varicella zoster virus and Epstein-Barr virus. Nucleic Acids Res. **14**:111–114.
37. **McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon.** 1985. Sequence determination and genetic content of the short unique region of the genome of herpes simplex virus type 1. J. Mol. Biol. **181**:1–13.
38. **Mettenleiter, T. C., L. Zsak, F. Zuckermann, N. Sugg, H. Kern, and T. Ben-Porat.** 1990. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J. Virol. **64**:278–286.
39. **Minson, A. C., T. C. Hodgman, P. Digard, D. C. Hancock, S. E. Bell, and E. A. Buckmaster.** 1986. An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralization. J. Gen. Virol. **67**:1001–1013.
40. **Nemerov, G. R., R. Wolfert, M. E. McNaughton, and N. R. Cooper.** 1985. Identification and characterization of the Epstein-Barr virus receptor on human B lymphocytes and its relationship to the C3d complement receptor (CR2). J. Virol. **55**:347–351.
41. **Pellet, P. E., M. D. Biggin, B. Barrell, and B. Roizman.** 1985. Epstein-Barr virus may encode a protein showing significant amino acid and predicted secondary-structure homology with glycoprotein B of herpes simplex virus type 1. J. Virol. **56**:807–813.
42. **Rasmussen, L. E., R. M. Nelson, D. C. Kelsall, and T. C. Merigan.** 1984. Murine monoclonal antibody to single protein neutralizes the infectivity of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81**:876–880.
43. **Rosenthal, K. L., J. R. Smiley, S. South, and D. C. Johnson.** 1987. Cells expressing herpes simplex virus glycoprotein C, but not gB, gD, or gE, are recognized by murine virus-specific cytotoxic T lymphocytes. J. Virol. **61**:2438–2447.
44. **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.
45. **Sawitzky, D., H. Hampl, and K.-O. Habermehl.** 1990. Comparison of heparin sensitive attachment of pseudorabies virus (PRV) and herpes simplex virus type 1 and identification of the heparin binding PRV glycoprotein. J. Gen. Virol. **71**:1221–1225.
46. **Sharp, J. A., M. J. Wagner, and W. C. Summers.** 1983. Transcription of herpes simplex virus genes in vivo: overlaps of a late promoter with the 3′ end of the early thymidine kinase gene. J. Virol. **45**:10–17.
47. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
48. **Strnad, B. C., T. Schuster, R. Klein, R. F. Hopkins, T. Witmer, R. H. Neubauer, and H. Rabin.** 1982. Production and characterization of monoclonal antibodies to the Epstein-Barr virus membrane antigens. J. Virol. **44**:258–264.
49. **Watson, D. H., W. C. Russell, and P. W. Wildy.** 1963. Electron microscopy particle counts on herpes virus using the phosphotungstate negative staining technique. Virology **19**:250–260.
50. **Weber, P. C., M. Levine, and J. C. Glorioso.** 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn*5* mutagenesis. Science **236**:576–579.
51. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. **63**:52–58. Debbie Nelson aac-dec91 1525-186