Syncytium-Inducing Mutations Localize to Two Discrete Regions within the Cytoplasmic Domain of Herpes Simplex Virus Type 1 Glycoprotein B

PHILIP J. GAGE,^{1,2} M. LEVINE,² AND JOSEPH C. GLORIOSO^{3*}

Departments of Microbiology and Immunology¹ and Human Genetics,² University of Michigan Medical School, Ann Arbor, Michigan 48109-0618, and Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261³

Received 30 September 1992/Accepted 14 December 1992

Herpes simplex virus type 1 glycoprotein B (gB) is essential for virus entry, an event involving fusion of the virus envelope with the cell surface membrane, and virus-induced cell-cell fusion, resulting in polykaryocyte, or syncytium, formation. The experiments described in this report employed a random mutagenesis strategy to develop a more complete genetic map of mutations resulting in the *syn* mutant phenotype. The results indicate that *syn* mutations occur within two essential and highly conserved hydrophilic, α -helical regions of the gB cytoplasmic domain. Region I is immediately proximal to the transmembrane domain and includes residues R796 to E816/817. Region II is localized centrally in the cytoplasmic domain and includes residues A855 and R858. Positively charged residues were particularly affected in both regions, suggesting that charge interactions may be required to suppress the *syn* mutant phenotype. No *syn* mutations were identified within the transmembrane domain. A virus containing a rate of entry (*roe*) mutation at residue A851, either within or immediately proximal to *syn* region II, was isolated. Since *roe* mutations have also been discovered in the external domain of gB, it appears likely that the external and cytoplasmic domains cooperate in virus penetration. Moreover, the observation that both *roe* and *syn* mutations occur in the cytoplasmic domain further suggests that gB functions in an analogous manner in both membrane fusion events. It might be predicted from these observations that membrane fusion involves transduction of a fusion signal along the gB molecule through the transmembrane domain. Communication between the external and cytoplasmic domain may thus be required for gB-mediated membrane fusion events.

Herpes simplex virus (HSV) encodes at least 10 glycoproteins (5, 42, 43, 54, 67), whose various roles in the virus life cycle are still only partially understood. One of the best characterized is glycoprotein B (gB). The highly conserved gB gene is located between 0.348 and 0.366 map units on the viral genome (5, 7, 62). DNA sequence analysis of the gB gene predicts a 904-amino-acid polypeptide, including a 30-residue, cotranslationally cleaved signal sequence, an N-terminal extracellular domain, a large hydrophobic transmembrane domain postulated to include three membrane-spanning α -helices, and a large cytoplasmic C-terminal domain (7, 62). The functional gB molecule is an oligomer (11, 35) produced in high abundance in lytic infection and is readily detected in the host cell plasma membrane and the virion envelope.

The gB gene is highly conserved among herpesviruses; homologs have been found in all cases. Included among these are six human herpesviruses (6, 7, 15, 47, 55, 58, 60–62, 74, 77), pseudorabies virus (66), equine herpesviruses 1 and 4 (65, 76), bovine herpesvirus 1 (58, 77), feline herpesvirus (72), Marek's disease virus (68), and several simian herpesviruses (21). Thirteen homologs have been sequenced, and extensive conservation of both the deduced primary and predicted secondary structures has been observed, suggesting conservation of function(s) in their respective viruses. The recent demonstration that certain gB homologs can functionally substitute for one another during virus replication has strengthened this argument (56, 57).

HSV enters susceptible cells by fusion of the virion membrane envelope with the cellular plasma membrane (13, 17, 27). Glycoprotein B is one of four HSV-encoded proteins required for virus penetration (2, 12, 18, 25, 49, 70). Virions lacking gB bind to host cells but fail to penetrate and initiate infection (12, 70). Certain anti-gB monoclonal antibodies (MAbs) block virus penetration in a complement-independent manner (33, 59). In addition, MAbs recognizing two antigenic sites on the gB extracellular domain affect virus entry kinetics (33), while MAbs specific for a third antigenic site can select neutralization escape mutations which also alter the rate of entry (*roe*) of virus into cells (34). Other *roe* mutations have been identified for which altered antibody recognition of the mutant product has not been described (8, 16). These observations confirm the essential role of gB in virus penetration and identify particular regions within the gB external domain which are involved in virus entry and cell-to-cell spread.

Wild-type HSV elicits little (64) or no (23) cell-cell fusion, although syncytial (*syn*) mutants which induce polykaryocyte formation occur with considerable frequency (64). gB is essential for cell-cell fusion (10, 52), and *syn* mutations have been identified within the large 109-amino-acid (residues 796 to 904) cytoplasmic domain at positions A855 (24), R858 (8), and E816/L817 (10, 11). Truncation of the gB product after residue 863 has no apparent effect on gB function (41); however, chain termination after residue 851 results in a mutant protein which fails to complement a gB⁻ virus (10, 11) or function in cell-cell fusion (10). These data have provided the strongest evidence that the essential function of the gB cytoplasmic domain is related to its role in membrane

* Corresponding author.

fusion. Moreover, the observation that certain anti-gB MAbs have been reported to inhibit syncytium formation (59) suggests that the role of gB in membrane fusion likely involves some communication between the external and cytoplasmic domains. Other than the role of the cytoplasmic domain in syncytium formation, relatively little is known about the functional role of the conserved gB cytoplasmic domain.

HSV-induced membrane fusion during virus penetration and between adjacent cells is predicted to be analogous. This prediction is consistent with observations for several well-characterized virus-membrane fusion systems (78) and is supported by the observation that three virally encoded proteins in addition to gB are involved in both events (2, 10, 12, 18, 25, 26, 29, 35, 49, 52, 70). However, the roles of the individual proteins in each process are unknown, and their respective functional domains are poorly understood. A detailed map of the relevant functional domains on each protein is prerequisite to further understanding of both processes and the extent to which there is functional overlap.

In this report, a panel of recombinant viruses encoding missense mutations within the coding sequence for the gB transmembrane and cytoplasmic domains were used to map primary structures that affect membrane fusion and to search for mutations affecting virus penetration. The data demonstrated that mutations within two discrete regions of the gB cytoplasmic domain affect cell-cell fusion and that at least one of these regions also affects gB function in virus penetration.

MATERIALS AND METHODS

Cells and viruses. Vero cells and the D6 cell line were maintained in culture medium (CM) consisting of Eagle's minimal essential medium (MEM) (GIBCO BRL, Gaithersburg, Md.) supplemented with the nonessential amino acids, 5% newborn calf serum (GIBCO BRL), and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma Chemical Co., St. Louis, Mo.). D6 cell culture medium was additionally supplemented with 200 µg of the antibiotic G418 (Sigma Chemical Co.) per ml. The D6 cell line, which contains the complete HSV type 1 (HSV-1) gB gene and expresses gB upon infection with virus, has been described previously (12).

Escherichia coli DH5α and DH5αF'.IQ (GIBCO BRL) were used for double-stranded and single-stranded phagemid propagation, respectively. Transformed cells were grown on Luria broth or plate medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) supplemented with 75 µg of the antibiotic ampicillin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml.

The gB⁻ virus KΔT::lox1 (28) was propagated and titers were determined on the gB-complementing D6 cell line (12) as described previously. The wild-type HSV-1 strain KOS321 (37), the thymidine kinase-negative virus KΔT::108 (37), and the gB mutant temperature-sensitive (*ts*) HSV-1 strain *tsB5* (70) were propagated and titers were determined on Vero cells as described previously (37). The Cre-mediated recombinants derived from KΔT::lox1 in this report were isolated and initially propagated on D6 cells and subsequently propagated and titers were determined on Vero cells. For titrating and plaque assays, infected cultures were maintained under 0.5% methylcellulose in MEM plus 2% fetal calf serum for 2 to 3 days at 37°C, except where noted.

FIG. 1. pJG108 shuttle plasmid and bisulfite mutagenesis strategy. (A) pJG108 was derived previously (34) from pBLUESCRIPT (Stratagene) and contains the complete gB gene (0.345 to 0.370 map units) from HSV-1 strain KOS, a bacterial *lacZ* reporter gene under the control of the human cytomegalovirus immediate-early promoter (CMVIEP) element, and a bacteriophage P1 *loxP* site-specific recombination site. The positions of relevant components, the direction of transcription for gB and *lacZ*, and the polarity of the phage M13 origin are indicated. (B) Strategy for targeting sodium bisulfite mutagenesis within pJG108. The pJG120 deletion plasmid was constructed from pJG108 by deleting a 474-bp *Tth1111* (T) fragment and relegating with *NsiI* (N) linkers. The *Tth1111* sites are at nucleotides 2950 and 3424, numbering from the *XhoI* site at 0.372 map units as 1. By creating a gapped heteroduplex between *NsiI*-linearized pJG120 and single-stranded pJG108, the single-strand-specific bisulfite mutagenesis was limited to the gB coding sequence between the two *Tth1111* sites. This corresponds to amino acid residues 721 through 879, numbering from the initiation methionine as 1. EXT, TN, and CYT indicate the gB extracellular, transmembrane, and cytoplasmic domains, respectively.

Plasmids and plasmid constructions. Enzymes were purchased from GIBCO BRL, Boehringer Mannheim Biochemicals, or New England Biolabs (Beverly, Mass.), and reactions were performed as specified by the manufacturers. The pJG108 shuttle plasmid (Fig. 1A) has been described previously (28). pJG120 was constructed from pJG108 by deleting the 474-bp *Tth1111* fragment from within the gB coding sequences (Fig. 1B), removing the nonhomologous 1-bp overhang with mung bean nuclease, and religating with *NsiI* linkers (Stratagene, La Jolla, Calif.). pA2.1, pA5.6, pB1.4, pB1.8, pB2.8, pB10.1, pB11.6, p108.38, and p108.15 are all bisulfite-mutagenized derivatives of pJG108 which were recovered from the HSV genome as described below. pJG123,

pJG125, and pJG128 were constructed by exchanging restriction fragments from the multiple mutants pB10.1 and pB11.6 with the homologous restriction fragments from pJG108 to generate plasmids encoding only a single mutation (see Table 2). pJG123 (A843V) was constructed from pB10.1 by substitution of the 2.8-kb *XhoI-NheI* gB fragment with the homologous wild-type fragment from pJG108. pJG124 (R796C, A808V) and pJG125 (R870W) were constructed from pJG108 by substitution of the wild-type 2.8-kb *XhoI-NheI* gB fragment with the homologous fragment from pB11.6 and pB10.1, respectively. To construct pJG128 (A808V), a 220-bp *BstEII-PstI* gB fragment from pJG124 was ligated with the adjacent 749-bp *PstI-BstEII* gB fragment from pJG108 and redigested with *BstEII*; the 969-bp hybrid *BstEII* fragment was gel purified and religated in place of the homologous fragment into *BstEII*-digested pJG108. Each construct was confirmed by DNA sequence analysis of the relevant regions.

Sodium bisulfite mutagenesis. Single-stranded pJG108 phagemid DNA was isolated from phage particles produced after superinfection of a culture of DH5 α F'.IQ(pJG108) with the R408 helper phage (Stratagene). To limit the single-strand-specific bisulfite mutagenesis (31, 71) to the intended target area (Fig. 1B), *NsiI*-linearized, gel-purified pJG120 plasmid DNA was annealed to single-stranded pJG108 phagemid DNA to create a gapped-heteroduplex hybrid molecule which was entirely double stranded except for the region of the gB coding sequence between the two *Tth111I* sites. This hybrid molecule was treated with 3 M sodium bisulfite–2 mM hydroquinone at 37°C in the dark. After 5, 10, and 30 min, aliquots of the bisulfite reaction mixture were passed over a Sephadex G-50 resin bed (Sigma) to stop the bisulfite adduct addition reaction. Desalted samples were alkalized by addition of 0.1 volume of 1 M Tris-HCl (pH 9.5), and deamination was allowed to continue overnight at 37°C. Samples were precipitated with ethanol, the DNA was resuspended and incubated in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 2 U of DNA polymerase 1, 0.25 μ M each of the four deoxynucleoside triphosphates (dNTPs), and 2 U of T4 DNA ligase, and the reaction mixes were incubated for 15 min at 37°C to remove the nonhomologous *NsiI* linkers from the ends of the pJG120 protective fragment and heal the single-stranded gap of the heteroduplex molecule. Reaction mixtures were then transformed into competent DH5 α cells.

Site-directed mutagenesis. Site-specific mutations were engineered into pJG108 on restriction fragments by a two-step polymerase chain reaction (PCR)-based method (36). For each construct, an initial set of PCR amplification (step 1) products were generated which spanned the left and right halves of the restriction fragment and overlapped in the center across the desired mutation. Because the internal primers each contained the desired base substitution and were partially complementary to each other, each fragment contained the intended mutation within the small region of overlap. The overlapping fragments were then combined in a final PCR amplification (step 2) to generate a full-length mutant fragment spanning the restriction fragment and containing the site-directed mutation. This PCR product was restriction enzyme digested, gel purified, and ligated into pJG108, replacing the homologous wild-type fragment. Mutations created by this method were S769F, R848W, S872F, and T877I.

PCR reaction mixes contained template, reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, 0.01% [wt/vol] gelatin), 200 μ M each of the four dNTPs, 0.1

μ g of each primer, 10% formamide, and *Taq* DNA polymerase (kind gift of D. Engelke). For step 1 reactions, 25 ng of pJG108 was added to each reaction mix as the template. After gel purification in low-melting-point agarose (GIBCO BRL), step 1 reaction products were isolated as agarose slices, which were melted at 65°C and used directly in pairs to program the step 2 PCR reactions, with approximately 2 μ l of each fragment per reaction mix. PCR amplifications were run for 20 cycles with the following parameters: 94°C for 15 s; 54°C for 15 s; 71°C for 90 s. Step 2 reaction products were gel purified and restriction enzyme digested, and the appropriate restriction fragment was gel purified and ligated into pJG108 in place of the homologous fragment. Because the engineered mutation destroyed the 3' *BstEII* site in the gB gene, the fragment encoding the T877I missense mutation was cloned as an 1,104-bp *BstEII-SphI* fragment. The other three mutations were cloned as 969-bp *BstEII* fragments. Individual isolates were screened by restriction mapping and DNA sequence analysis.

The overlapping sense and antisense internal primer pairs used to introduce each mutation and the resulting plasmid are as follows (sense primer listed first): pJG129 (S769F), 5'-GGCGTCTCCTTCTTCATGTCC and 5'-GGTTGGACATGAAGAAGGAC; pJG130 (R848W), 5'-GAGATGATATGGTACATGGCC and 5'-CAGGGCCATGTACCAATATCA; pJG130 (S872F), 5'-AGCCGGCTGTTCAGCGGCATG and 5'-GACCTTGGCGCTGAACAGCC; and pJG132 (T877I), 5'-GCCAAGGTCATCGACATGGTC and 5'-TCGTGACCATGTCGATGACC. For each pair, the outside primers paired with the overlapping sense and antisense internal primer pairs were 5'-TTTCAGGCGCTTAGTCG and 5'-AGCTGACCTGTGAAACGAG, respectively.

Double-stranded DNA sequencing. Template DNA (2 to 3 μ g) was denatured in 0.2 M NaOH–0.2 mM EDTA at 37°C for 30 min. After ethanol precipitation, denatured template DNA was resuspended in 1 $\times$ Sequenase buffer plus 1.2 μ g of primer, heated to 65°C for 5 min, and allowed to cool slowly to room temperature. Labeling and extension-termination reactions were done as described in the Sequenase sequencing kit (U.S. Biochemicals, Cleveland, Ohio). Finished reaction mixtures were heated for 5 min at 80°C prior to denaturing polyacrylamide gel (6% acrylamide, 50% urea, 0.5 $\times$ TBE) electrophoresis. Gels were fixed in 10% methanol–10% glacial acetic acid, dried, and exposed to Kodak XAR-5 film.

In vivo Cre-mediated recombination reactions. Cell-free Cre recombination reactions were performed as described previously (1). Intermolecular Cre reaction mixes used to insert shuttle plasmid DNA into the viral genome contained KAT::lox1 viral DNA, a 20- to 40-fold molar excess of shuttle plasmid DNA, buffer (50 mM Tris-HCl [pH 7.5], 33 mM NaCl, 10 mM MgCl₂), 1.7% polyvinyl alcohol, and 40 ng of purified Cre protein (NEN Du Pont, Inc., Wilmington, Del.). After incubation for 30 to 40 min at 30°C, the reaction mixtures were heated to 65°C for 10 min to inactivate Cre and transfected into D6 cells as described by Graham and Van der Eb (30) and modified by Homa et al. (39). Transfection lysates were harvested when evidence of cytopathic effect was observed, generally 2 days after transfection. Where indicated, four shuttle plasmids were mixed together at equimolar ratios, and plasmid DNA from the mixed batches was used in Cre reactions with KAT::lox1 DNA (see below). Intramolecular Cre-mediated recombination reaction mixes used to recover inserted shuttle plasmids contained recombinant viral DNA but no polyvinyl alcohol or input plasmid

DNA and were transformed into competent *E. coli* DH5 α cells to propagate the rescued shuttle plasmids.

β -Gal activity assays. Viral plaques were assayed for β -galactosidase (β -Gal) activity by two methods. For isolation of viable virus, infected monolayers were rinsed with phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.1], 136 mM NaCl) and overlaid with CM supplemented with 0.5% low-melting-point agarose and 300 μ g of halogenated indolyl- β -D-galactoside (Bluo-gal; GIBCO BRL) per ml. Cultures were placed at 4°C for 1 h and then transferred to 37°C until a blue color developed (14). When viable virus was not required, monolayers were rinsed with PBS, fixed at room temperature for 5 min with 2% formaldehyde–0.3% glutaraldehyde in PBS, rinsed with PBS, and overlaid with a solution containing 0.1% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; Boehringer Mannheim Biochemicals), 5 mM K₄Fe(CN)₆ · 3H₂O, 5 mM K₃Fe(CN)₆, and 2 mM MgCl₂ in PBS. Monolayers were placed at 37°C until a blue color developed (30 to 60 min), rinsed with PBS, and counterstained with eosin (Richard-Allen Medical Industries, Richland, Mich.).

Isolation of recombinant viruses. D6 cell monolayers were infected at a low multiplicity of infection (≤ 0.001) with lysate stocks produced after transfection of intermolecular Cre recombination reaction mixtures and incubated at 37°C under methylcellulose until plaques formed. Monolayers were then rinsed with PBS and overlaid with Bluo-gal. β -Gal⁺ viruses were isolated as an agarose plug and dispersed in PBS by vortexing. For transfection lysates generated after Cre recombination reactions with a mixture of shuttle plasmids, at least 20 to 30 blue plaques were isolated to ensure that viruses containing each shuttle plasmid would be represented in the sample population. After preliminary assays (see below), selected viral isolates were subcloned on D6 cells by limiting dilution, with β -Gal activity as a marker. A virus isolate was considered pure when 100% of the subclones tested β -Gal⁺ through three successive rounds of limiting dilution.

IC assays. For infectious-center (IC) assays, replicate Vero cell monolayers were infected with KAT::108 or 108.15 (500 PFU per monolayer). At 4, 12, 24, and 48 h postinfection, the monolayers were trypsinized and serially diluted in PBS; aliquots of each dilution were used to inoculate suspensions of fresh Vero cells, and the mixed suspensions of infected and uninfected cells were deposited onto duplicate dishes. After the cells had formed monolayers, the CM was replaced with methylcellulose, and plaques were scored after 3 days of incubation at 37°C. Virus titers were calculated at each time point as the average for two dishes at each dilution. To account for potential variations in infection efficiency between the two viruses, results at each time point were standardized to the actual input PFU scored at 4 h postinfection.

Low-pH, citrate-restricted entry assay. Replicate Vero cell monolayers were infected with approximately 200 PFU of KOS321 or 108.15 per well at 4°C, at which temperature virions adsorb but do not penetrate (38). Following a 2-h adsorption period, monolayers were washed with PBS to remove unabsorbed virus, overlaid with CM, and shifted to 37°C to allow penetration of preadsorbed virus to proceed. At 0, 20, 40, 60, 90, 120, and 180 min after the temperature shift, medium was removed, and duplicate monolayers for each virus were rinsed with PBS, treated for 1 min with low-pH citrate-buffered saline (40 mM citric acid [pH 3.0], 10 mM KCl, 135 mM NaCl), and then promptly washed with PBS. Monolayers were overlaid with methylcellulose, and

plaques were scored after 3 days of incubation at 37°C. As the untreated control, duplicate sets of infected monolayers for each virus were not treated with citrate-buffered saline. The percent penetration for a virus at a given time point was calculated as the fraction of the average plaque number on the treated plates versus the average plaque number on the untreated plates, multiplied by 100.

RESULTS

Identification of *syn* mutations in gB. Our experimental approach was to use a random mutagenesis strategy to introduce missense mutations into the coding regions for the entire gB transmembrane domain and the essential portion of the cytoplasmic domain in order to develop a more complete genetic map of amino acid substitutions in these structures which might result in the formation of *syn* mutant plaques. Recently, we reported a novel method for the genetic analysis of gB based on the use of shuttle plasmids carrying mutagenized copies of the gB gene to introduce altered forms of gB into a gB deletion mutant genome (28). Both the recipient genome and the shuttle plasmids contained a recombination site, referred to as *loxP*, which is derived from bacteriophage P1 (1). This system was used to generate a large population of recombinant viruses, each containing an integrated shuttle plasmid potentially encoding a gB mutant. The shuttle plasmid was recombined into the recipient virus genome at the *loxP* site, located within the thymidine kinase (*tk*) locus, in a cell-free reaction mediated by the phage recombinase Cre. The Cre-*lox* reaction is very efficient, and recombinants have been found to represent as much as 40% of the plaques recovered after transfection of the recombinant viral DNA into appropriate cells. Recombinant viruses were screened to identify those containing shuttle plasmids encoding potential mutant gB polypeptides on the basis of expression of the *lacZ* reporter gene also contained on the shuttle plasmid. β -Gal⁺ plaques could be used for enrichment and purification of recombinants, and individual isolates were subsequently analyzed for relevant phenotypes. An important feature of this approach was that interesting mutant phenotypes could be traced to specific nucleotide changes by recovering the shuttle plasmids from the viral DNA extracted from plaque-purified isolates in subsequent Cre-mediated recombination reactions. After direct transformation of bacteria to prepare plasmid preparations, the mutant gB gene was analyzed by DNA sequencing. This overall strategy obviates the need for sorting through large numbers of precharacterized mutant gB plasmids in order to determine whether a specific mutation affects gB function.

As shown in Fig. 1A, the pJG108 shuttle plasmid (28) contained a complete wild-type gB gene derived from HSV-1 strain KOS, a *lacZ* reporter gene expressed from the human cytomegalovirus immediate-early promoter, and a *loxP* site-specific recombination site. The pJG108 gB coding sequences (plus strand) were mutagenized between two *Tth*111I sites (Fig. 1B) with the single-stranded DNA-specific chemical mutagen sodium bisulfite. This region spans 474 nucleotides or 158 codons and corresponds to amino acid residues 721 through 879 (Fig. 1B). Fifty-four of the 158 amino acid residues (34%) within the target region were susceptible to alteration by this strategy (see Fig. 3). DNA sequence analysis of DNA from a sample of the resulting transformants demonstrated that 36% of the plasmids contained nucleotide changes leading to one or more amino acid substitutions and that the mutations occurred randomly throughout the targeted region (data not shown).

FIG. 2. Plaque morphologies of representative *syn* mutants. Plaques from *syn* mutants A2.1, B2.8, and A5.6 are shown, representing low, moderate, and extensive amounts of cell-cell fusion, respectively. Infected Vero cell monolayers were fixed at 30 h postinfection, stained with X-Gal to detect β -Gal activity, and subsequently counterstained with eosin. Monolayers were examined and photomicrographs were taken with an Olympus CK-2 microscope.

Cell-free, Cre-mediated recombination reactions were used to introduce shuttle plasmid DNA at a *loxP* site within the *tk* locus of the *KΔT::lox1* recipient virus (28), which is gB^- because of a large *Bst*EII deletion within the viral *gB* locus. Mutant plasmids identified while determining the mutational frequency were introduced individually. In order to expedite the introduction and screening of additional potential mutants, small shuttle plasmid DNA stocks were prepared from randomly chosen transformants and mixed together in batches of four at equimolar ratios prior to use in recombination reactions. Since 36% of the plasmids should contain one or more missense mutations, one or two plasmids in each batch of four would encode a mutant *gB*, and the rest would encode the wild-type molecule. Reaction mixtures were subsequently transfected into the *gB*-complementing D6 cell line to produce infectious virus, including recombinant viruses containing shuttle plasmids encoding either functional or lethal *gB* mutations. After transfection lysates were replated on D6 cells, recombinant viruses producing β -Gal⁺ plaques in the presence of Blueo-gal were isolated, and small stocks of each recombinant virus isolate were prepared on D6 cells. Sufficient β -Gal⁺ viruses were isolated from experiments including batches of four plasmids in the Cre reaction to ensure that each shuttle plasmid present in the original recombination reaction would be represented in the β -Gal⁺ virus population sampled. A panel of 450 recombinant viruses, representing approximately 100 shuttle plasmids, were isolated by this method.

Identification of two discrete *syn* regions within the *gB* cytoplasmic domain. To identify viruses expressing *gB* mutants affecting membrane fusion, each β -Gal⁺ recombinant virus isolate was tested for the mutant *syn* phenotype. Since it is well established that functional *gB* is required for cell-cell fusion, plaque assays were performed on Vero cells. A total of eight independent *syn* mutant viruses, A2.1, A5.6,

B1.4, B1.8, B2.8, B10.1, B11.6, and 108.38, were identified and purified. These viruses varied in the amount of cell-cell fusion induced (Fig. 2). A2.1 produced plaques having from a few to several polykaryocytes each, A5.6 induced extensive cell-cell fusion, and the other six viruses induced intermediate levels of cell-cell fusion.

To demonstrate that the observed *syn* mutant phenotypes could be directly attributed to expression of mutant *gB* polypeptides encoded on the inserted shuttle plasmids, Cre-mediated recombination was used to recover the shuttle plasmid from the genomic DNA of each *syn* mutant virus and reintroduce the shuttle plasmids de novo into the *KΔT::lox1* genome. Plaque assays on Vero cells of the resulting transfection lysates were assayed with X-Gal, and the plaque phenotype of the β -Gal⁺ recombinants was again determined. In each case, reinsertion of the recovered shuttle plasmid produced a second-generation β -Gal⁺ recombinant virus with a *syn* mutant plaque phenotype (data not shown). *syn* mutant plaques were never observed after Cre-mediated insertion of the parental pJG108 shuttle plasmid into *KΔT::lox1*. Therefore, the observed *syn* mutant phenotypes were directly attributable to expression of a mutant *gB* polypeptide from the inserted shuttle plasmid.

The eight shuttle plasmids, named for the viruses from which they were recovered, were sequenced throughout the mutagenesis target region to identify base changes (Table 1). Plasmids pA2.1, pB1.4, pB2.8, and p108.38 encoded *gB* polypeptides having the single amino acid substitutions T813I, R796C, R800W, and R858C, respectively (Fig. 3). Together, they identified two new residues within the *gB* cytoplasmic domain which affect cell-cell fusion. The R858C mutation occurs at the same position as the *tsB5* R858H *syn* mutation, implying that it is loss of the arginine which leads to the mutant phenotype rather than the specific amino acid substitutions. The remaining four shuttle plasmids encoded

TABLE 1. Nucleotide and amino acid substitutions in *syn* mutant gB shuttle plasmids^a

Plasmid ^b	Nucleotide ^c	Codon change	Residue no. ^d	Amino acid change
pA2.1	3108	AAC to AAT	773	— ^e
	3227	ACC to ATC	813	Thr to Ile
pA5.6	3095	TCC to TTC	769	Ser to Phe
	3307	CTA to TTA	836	—
	3331	CGG to TGG	848	Arg to Trp
	3419	ACC to ATC	877	Thr to Ile
pB1.4	3111	CCC to CCT	774	—
	3175	CGT to TGT	796	Arg to Cys
	3246	AAC to AAT	819	—
	3303	GCC to GCT	838	—
	3372	CAC to CAT	861	—
	3400	CTG to TGT	871	—
pB1.8	3133	CTG to TTG	782	—
	3361	CGC to TGC	858	Arg to Cys
	3403	CTC to TTC	872	Leu to Phe
pB2.8	2985	TTC to TTT	732	—
	3111	CCC to CCT	774	—
	3187	CGG to TGG	800	Arg to Trp
	3214	CTG to TTG	809	—
pB10.1	3175	CTG to TGT	796	Arg to Cys
	3249	CCC to CCT	820	—
	3285	GGC to GGT	832	—
	3397	CGG to TGG	870	Arg to Trp
pB11.6	3096	TCC to TCT	769	—
	3175	CGT to TGT	796	Arg to Cys
	3212	GCC to GTC	808	Ala to Val
	3223	CTA to TTA	812	—
	3317	GCC to GTC	843	Ala to Val

^a Each plasmid was sequenced throughout the mutagenesis target region.^b Plasmids were named for the virus from which they were recovered.^c The first base of the *Xho*I site at 0.372 map units in the viral genome is numbered 1.^d The initiation methionine is numbered 1.^e —, no change.

gB polypeptides containing multiple amino acid substitutions. pB1.8 (R858C and L872F) and pB10.1 (R796C and R870W) each encoded two amino acid substitutions, while pA5.6 (S769F, R848W, and T877I) and pB11.6 (R796C, A808V, and A843V) each encoded three amino acid substitutions (Table 1 and Fig. 3). The R858C mutation in B1.8 and the R796C mutation in B10.1 and B11.6 were identified as *syn* mutations by comparison with the single-mutation viruses 108.38 and B1.4, respectively. Therefore, the additional mutations in these viruses may be irrelevant to the *syn* mutant phenotype. The three mutations in A5.6 occurred at residues not previously associated with the *syn* mutant phenotype and therefore define a new *syn*-affected residue.

To determine whether the additional mutations in B1.8, B10.1, and B11.6 contribute to their *syn* mutant phenotypes and to identify the A5.6 *syn* mutation, these mutations were segregated either by exchanging homologous restriction fragments between the mutant plasmid and pJG108 or by introducing the mutation in question into pJG108 by a PCR-based site-directed mutagenesis approach as described above. Each new plasmid was individually introduced into KAT::lox1 by Cre-mediated recombination. The β -Gal⁺

plaques produced by the resulting transfection lysates on Vero cells were analyzed directly, without purification. The A808V, A843V, and R870W mutations alone did not lead to the *Syn*[−] phenotype (Table 2), demonstrating that the R858C and R796C mutations are the *syn* mutations in B1.8 and in B10.1 and B11.6, respectively. None of the individual mutations derived from A5.6 induced the *syn* mutant phenotype, despite the previous demonstration that reinsertion of the pA5.6 shuttle plasmid into the KAT::lox1 genome again induced the *syn* mutant phenotype. Apparently, two or more of the three amino acid substitutions in A5.6 collaborate to induce the *syn* mutant phenotype.

The newly identified single *syn* mutations, together with those described previously, allow resolution of sequences within the gB cytoplasmic domain which function in membrane fusion into two discrete regions (Fig. 4). *syn* region I lies immediately proximal to the predicted transmembrane domain and includes residues R796, R800, and T813 and the insertion mutations between residues E816 and L817. *syn* region II is distal to the transmembrane domain, towards the C terminus, and includes residues A855 and R858. Identification of the A5.6 *syn* mutation will modify the limits of one or both of the *syn* regions. Notably, no *syn* mutations within the gB transmembrane domain were isolated, even though this region of the molecule was mutagenized as well.

Identification of an *roe* mutation within the gB cytoplasmic domain. To determine whether gB expressed from the inserted shuttle plasmids could complement the defective gB expressed from the native viral gB locus, the plating efficiency of each virus in the panel was compared following infection of the D6 cell line and noncomplementing Vero cells. Although no significant differences in plating efficiencies were observed (data not shown), isolate 108.15 produced very small plaques on Vero cells. In contrast, 108.15 produced normal-sized plaques on the D6 cell line, demonstrating that the small-plaque phenotype was due to a defect in the gB gene and was likely related to virus penetration or spread. Virus 108.15 was thus purified for further analysis.

An IC assay was used to determine whether 108.15 was defective in its ability to spread from cell to cell. In this assay, the combined rate of spread by a constant amount of input virus via both extracellular and direct cell-to-cell routes is measured by counting the number of IC at various time points after infection. To account for variations in input PFU, the IC titer for each time point was standardized to the IC titer at 4 h postinfection, at which time no spread of the input virus would have occurred. The rate of increase in IC per input PFU over time is a measure of the rate of spread for a given virus. The wild-type gB virus KAT::108 produced $10^{1.5}$ and $10^{3.1}$ IC per input PFU by 24 and 48 h postinfection, respectively. In contrast, 108.15 produced only 10^1 and $10^{2.5}$ IC per input PFU at the equivalent times postinfection, a difference by 48 h of $10^{0.6}$ IC per input PFU compared with KAT::108. This reduction in the rate of virus spread for 108.15 correlated well with the small-plaque phenotype observed on Vero cells.

Previously, both *ts* and rate-of-entry (*roe*) mutations have been described for gB (7, 54), either of which might explain the phenotype of 108.15. Plating efficiency and virus yield assays were used to establish that 108.15 has no *ts* defect (data not shown). Isolate 108.15 was tested for an *roe* defect by using a low-pH buffer-restricted entry assay (40), in which unpenetrated virus exposed on the surface of cells are inactivated with low-pH citrate buffer at various time points after infection. The rate at which preabsorbed virus becomes resistant to inactivation is a measure of the entry kinetics for

FIG. 4. Summary of *syn* and *roe* mutations. The positions of the *roe* and *syn* mutations ($\blacktriangle$, insertion) are shown in the context of the relevant region of the gB polypeptide. Both the new and previously reported *syn* mutations are included. The approximate limits of *syn* regions I and II are also shown. Amino acid numbering begins from the initiation methionine.

TABLE 3. Rate-of-entry kinetics for KΔT::108.15

Expt no.	Virus	% Survival ^a at time postinfection (min):						
		0	20	40	60	90	120	180
1	KOS321	0	1	7	42	85	56	77
	KΔT::108.15	0	0	0	12	32	30	34
2	KOS321	0	0	10	65	67	82	84
	KΔT::108.15	0	0	0	26	34	45	59

^a Reported as percent survival at each time point relative to the untreated control sample. Values based on triplicate samples for each time point.

function in both processes (11) had been the strongest evidence for this connection. However, this mutation also resulted in a significant reduction in posttranslational modification and intracellular transport of gB (10), both of which have previously been shown to affect gB function indirectly (50, 53, 70). Therefore, it was not clear to what extent these additional phenotypes might have been responsible for lack of function by the residue 851 truncation mutant. The 108.15 mutant gB protein must be incorporated into the virion envelope, since it functions in virus penetration, albeit at a reduced efficiency, and gB is processed normally, inasmuch as 108.15 gB is similar to wild-type gB in molecular size (27a). The observation that the deletion of *syn* region II is precisely the difference between the nonfunctional 851-residue gB mutant and an 863-residue truncation mutant which is fully functional (11, 41) further strengthens the conclusion that this region provides a common function in both membrane fusion processes.

Several lines of evidence support the conclusion that the two general regions identified represent the critical functional gB cytoplasmic sequences relative to membrane fusion. Clustering of the *syn* mutations within two discrete regions of the extended sequences mutagenized presents a strong genetic argument. Furthermore, the regions identified coincide with the highly conserved sequences within the gB cytoplasmic domain. In contrast, the intervening sequences and those C-terminal to region II are poorly conserved, and the sequences C-terminal to residue 863 are dispensable for gB function. Finally, computer-assisted analysis of the wild-type and mutant sequences predicts that the individual amino acid substitutions have minimal effects on hydrophobicity or secondary structure. However, it should be noted that from the present data, we cannot formally rule out the possibility that any of the *syn* mutations exerts its effects from a distance. In addition, identification of the A5.6 *syn* mutations will require modification of the limits of at least one of the two regions.

Although these data provide a more detailed genetic map of the gB cytoplasmic domain regions which function in HSV-induced membrane fusion events, their precise function(s) remains unknown. HSV-induced membrane fusion is a regulated process (64), and an active inhibitor of fusion is apparently induced late in infection (9, 64). Previously, a regulatory role during membrane fusion has been postulated for the gB cytoplasmic domain, likely via interaction with other HSV genes known to carry *syn* mutations (10). Loss of three individual arginine residues leads to the *syn* mutant phenotype, suggesting that either arginines specifically or charged molecules in general are important for the extent or timing of membrane fusion. Arginines have previously been identified as critical residues in the binding sites for a wide variety of ligand molecules, including proteins, and enzy-

matic substrates (3, 20, 44–46, 63). The identification of arginines as critical residues may imply that the gB cytoplasmic domain interacts with an unknown ligand in a process which is important for the extent or timing of membrane fusion.

The A2.1 *syn* mutation may also provide clues to gB function. Recently, evidence for threonine/serine phosphorylation of the gB molecule has been reported (22). The T813 residue mutated in A2.1 is one of a doublet of threonine residues which are highly conserved among gB homologs, particularly among alphaherpesviruses. Such conservation implies an important function. Although not previously associated with virally induced membrane fusion, protein phosphorylation has been shown to regulate other membrane fusion events, including exo- and endocytosis and myoblast fusion during myogenesis (48, 51, 75, 79). In light of these data, phosphorylation of gB within the cytoplasmic domain might play a role in regulating HSV-induced membrane fusion.

The identification of an *roe* mutation in the cytoplasmic domain together with those identified previously in the extracellular domain has implications for the overall function of gB. The observation that mutations in these two remote regions of the molecule have the same effect strongly suggests that gB functions as an integrated molecule during membrane fusion. Although only *syn* region II has been molecularly associated with both virus penetration and cell-cell fusion, it seems likely that the two processes share other functional domains on gB as well. This hypothesis is supported by recent reports demonstrating that certain anti-gB MAbs can block virus penetration or cell-cell fusion or both (33, 59). The *roe* mutations further demonstrate that events in the cytoplasmic domain can affect the function of the extracellular domain and vice versa, suggesting that intramolecular communication between these two remote regions of the molecule is an essential mechanism by which gB functions. Such signal transduction, via allosteric changes transmitted through the transmembrane domain, is a common communication mechanism employed by cell surface receptors for a wide variety of ligands and growth factors (4, 19, 32, 73).

Given the high degree of conservation and predicted unusual structure, it was rather surprising that no individual mutations affecting either cell-cell fusion or viral penetration were identified within the gB transmembrane domain. It may be that the assays used in this study were not appropriate for or capable of detecting mutations affecting the function of this region of the molecule. For example, the panel of viruses isolated were not generally assayed for a *ts* phenotype. Alternatively, it could be that critical functional residues within this region of the molecule are so sensitive to alteration that any perturbations abrogate function or appropriate insertion into the membrane. Although it was anticipated that initial isolation of recombinant viruses on the gB-complementing D6 cell line would allow the recovery of such mutations, it could be that severely crippled molecules inhibited in *trans* the function of the wild-type gB expressed by the D6 cells. Such negative complementation has been reported previously for gB (11). If such a phenomenon occurred, it may have caused lethal mutations within the transmembrane to be missed. Finally, the transmembrane domain may be conserved for functions unrelated to those studied here.

The results presented in this article extend previous knowledge of gB function by providing direct genetic evidence for a common gB function during virion-cell and

cell-cell fusion. In addition, the mutations identified suggest potential functional mechanisms for the gB molecule, particularly the cytoplasmic domain, in membrane fusion. Further genetic and biochemical analysis will be required to test these hypotheses. For example, it would be of interest to determine whether additional charged residues within *syn* regions I and II are essential for the membrane fusion activity and whether gB is in fact phosphorylated on threonine and on which residues.

ACKNOWLEDGMENTS

We thank Neal DeLuca, Prashant Desai, William Goins, Stan Person, and Ramesh Ramakrishnan for suggestions, discussions of the data, and critical reading of the manuscript.

This work was supported by Public Health Service grant AI26937-04.

REFERENCES

- Abremski, K., and R. Hoess. 1984. Bacteriophage P1 site-specific recombination: purification and properties of the Cre recombinase protein. *J. Biol. Chem.* **259**:1509-1514.
- Addison, C., F. J. Rixon, J. W. Palfreyman, M. O'Hara, and V. G. Preston. 1984. Characterization of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration and assembly of capsids. *Virology* **138**:246-259.
- Borders, C. L., and J. F. Riordan. 1975. An essential arginyl residue at the nucleotide binding site of creatine kinase. *Biochemistry* **14**:4699-4704.
- Bourne, H. R., D. A. Sanders, and F. McCormick. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature (London)* **348**:125-132.
- Buckmaster, E. A., U. Gompels, and A. C. Minson. 1984. Characterization and physical mapping of an HSV-1 glycoprotein of approximately 115×10^3 molecular weight. *Virology* **139**:408-413.
- Bzik, D. J., C. Debroy, B. A. Fox, N. E. Pederson, and S. Person. 1986. The nucleotide sequence of the gB glycoprotein of HSV-2 and comparison with the corresponding gene of HSV-1. *Virology* **155**:322-333.
- 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.
- Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person. 1984. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. *Virology* **137**:185-190.
- Bzik, D. J., S. Person, and G. S. Read. 1982. The active inhibition of herpes simplex virus type 1-induced cell fusion. *Virology* **117**:504-509.
- Cai, W., B. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J. Virol.* **62**:2596-2604.
- Cai, W., S. Person, C. Debroy, and B. Gu. 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1. *J. Mol. Biol.* **201**:676-688.
- Cai, W., S. Person, S. Warner, J. Zhou, and N. A. DeLuca. 1987. Linker insertion nonsense and restriction-site deletion mutations of the gB glycoprotein of herpes simplex virus type 1. *J. Virol.* **61**:714-721.
- Campadelli-Fiume, G., M. Arsenakis, F. Farbegoli, and B. Roizman. 1988. Entry of herpes simplex virus type 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. *J. Virol.* **62**:159-167.
- Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vector: coexpression of β -galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell. Biol.* **5**:3403-3409.
- Cranage, M. P., T. Kouzarides, A. T. Bankier, S. Satchwell, K. Weston, P. Tomlinson, B. Barrell, S. E. Bell, A. C. Minson, and G. L. Smith. 1986. Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. *EMBO J.* **5**:3057-3063.
- DeLuca, N. A., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes. 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein B (VP7). *Virology* **122**:411-432.
- DeLuca, N. A., D. J. Bzik, S. Person, and W. Snipes. 1981. Early events in herpes simplex type I infection: photosensitivity of fluorescein isothiocyanate-treated virions. *Proc. Natl. Acad. Sci. USA* **78**:912-916.
- Desai, P. J., P. A. Schaffer, and A. C. Minson. 1988. Excretion of noninfectious virus particles lacking gH by a temperature-sensitive mutant of herpes simplex virus type I: evidence that gH is essential for virion infectivity. *J. Gen. Virol.* **69**:1147-1156.
- Dinarello, C. A., and N. Savage. 1989. Interleukin-1 and its receptor. *Crit. Rev. Immunol.* **9**:1-20.
- Duerksen-Hughes, P. J., X. X. Xu, and K. D. Wilkinson. 1987. Structure and function of ubiquitin: evidence for differential interactions of arginine-74 with the activating enzyme and the protease of ATP-dependent proteolysis. *Biochemistry* **26**:6980-6987.
- Eberle, R., D. Black, and J. K. Hilliard. 1989. Relatedness of glycoproteins expressed on the surface of simian herpes-virus virions and infected cells specific to HSV glycoproteins. *Arch. Virol.* **109**:233-252.
- Edson, C. M., B. A. Hosler, and D. J. Waters. 1987. Varicella-zoster virus gpI and herpes simplex virus gE: phosphorylation and Fc binding. *Virology* **161**:599-602.
- Ejercito, P. M., E. D. Kieff, and B. Roizman. 1968. Characterization of herpes simplex virus strains differing in their effects on cell social behavior. *J. Gen. Virol.* **2**:357-364.
- Engel, J. P., E. P. Boyer, and J. L. Goodman. 1990. A single nucleotide change in the glycoprotein gB of herpes simplex virus type 1 (HSV-1) is responsible for a syncytial phenotype and unique mouse pathogenic properties, workshop 21, p. 32. 9th Am. Soc. Virol. Meet.
- Forrester, A., H. Farrel, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson. 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J. Virol.* **66**:341-348.
- Fuller, A. O., R. E. Santos, and P. G. Spear. 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment but prevent penetration. *J. Virol.* **63**:3435-3443.
- Fuller, A. O., and P. G. Spear. 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection of herpes simplex virus 1 prevent virion-cell fusion at the cell surface. *Proc. Natl. Acad. Sci. USA* **84**:5454-5458.
- Gage, P. J. Unpublished data.
- Gage, P. J., B. Sauer, M. Levine, and J. C. Glorioso. 1992. A cell-free recombinant system for site-specific integration of multicistronic shuttle plasmids into the herpes simplex virus type 1 genome. *J. Virol.* **66**:5509-5515.
- Gompels, U., and A. C. Minson. 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. *Virology* **153**:230-247.
- Graham, F. L., and A. J. Van der Eb. 1973. A new technique for the assay of infectivity of human retrovirus. *Virology* **52**:456-467.
- Hayatsu, H. 1976. Bisulfite modification of nucleic acids and their constituents. *Prog. Nucleic Acid Res. Mol. Biol.* **16**:75-124.
- Hershkowitz, I. 1989. A regulatory hierarchy for cell specialization in yeast. *Nature (London)* **342**:749-757.
- Highlander, S. L., W. Cai, S. Person, M. Levine, and J. C. Glorioso. 1988. Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. *J. Virol.* **62**:1881-1888.
- Highlander, S. L., D. J. Dorney, P. J. Gage, T. 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.
35. Highlander, S. L., W. F. Goins, S. Person, T. C. Holland, M. Levine, and J. C. Glorioso. 1991. Oligomer formation of the gB glycoprotein of herpes simplex virus type 1. *J. Virol.* **65**:4275–4283.
 36. Higuchi, R. 1989. Using PCR to engineer DNA, p. 61–70. *In* H. A. Erlich (ed.), *PCR technology: principles and applications for DNA amplification*. Stockton Press, New York.
 37. Holland, T. C., and S. Person. 1977. Ammonium chloride inhibits cell fusion induced by *syn* mutants of herpes simplex virus type 1. *J. Virol.* **23**:213–215.
 38. Holmes, I. H., and D. H. Watson. 1961. An electron microscope study of the attachment and penetration of herpes simplex virus in BHK21 cells. *Virology* **21**:112–123.
 39. Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine. 1986. Transcriptional control signals of a herpes simplex virus type 1 late (γ_2) gene lie within bases –34 to +124 relative to the 5' terminus of the mRNA. *Mol. Cell. Biol.* **6**:3652–3666.
 40. Huang, A. S., and R. R. Wagner. 1964. Penetration of herpes simplex virus into human epidermoid cells. *Proc. Soc. Exp. Biol. Med.* **116**:863–869.
 41. Huff, V., W. Cai, J. C. Glorioso, and M. Levine. 1988. The carboxy-terminal 41 amino acids of herpes simplex virus type 1 glycoprotein B are not essential for production of infectious virus particles. *J. Virol.* **62**:4403–4406.
 42. Hutchinson, L., H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A. C. Minson, and D. C. Johnson. 1992. A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. *J. Virol.* **66**:2240–2250.
 43. Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F. L. Graham, and D. C. Johnson. 1992. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. *J. Virol.* **66**:5603–5609.
 44. Jaikaria, N. S., L. Rosenfeld, M. Y. Khan, I. Danishevsky, and S. A. Newman. 1991. Interaction of fibronectin with heparin in model extracellular matrices: role of arginine residues and sulfate groups. *Biochemistry* **30**:1538–1544.
 45. Jorgensen, A. M., C. L. Borders, and W. W. Fish. 1985. Arginine residues are critical for the heparin-cofactor activity of antithrombin III. *Biochem. J.* **132**:59–63.
 46. Kasher, J. S., K. E. Allen, K. Kasamo, and C. W. Slayman. 1986. Characterization of an essential arginine residue in the plasma membrane H^+ -ATPase of *Neurospora crassa*. *J. Biol. Chem.* **261**:10808–10813.
 47. Keller, P. M., A. J. Davidson, R. S. Low, C. D. Bennett, and R. W. Ellis. 1987. Identification and structure of the gene encoding gP11, a major glycoprotein of varicella-zoster virus. *Virology* **152**:181–191.
 48. Kim, H. S., I. H. Lee, C. H. Chung, M. S. Kang, and D. B. Ha. 1992. Ca^{2+} /calmodulin-dependent phosphorylation of the 100 kDa protein in chick embryonic muscle cells in culture. *Dev. Biol.* **150**:223–230.
 49. Ligas, M. W., and D. C. Johnson. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. *J. Virol.* **62**:1486–1494.
 50. Little, S. P., and P. A. Schaffer. 1981. Expression of the syncytial (*syn*) phenotype in HSV-1, strain KOS: genetic and phenotypic studies of mutants in two *syn* loci. *Virology* **112**:686–702.
 51. Lognonne, J. L., and J. P. Wahrmann. 1988. A 48kDa cell surface phosphoprotein specific for myoblast fusion. *Cell Differ.* **22**:245–258.
 52. Manservigi, R., P. G. Spear, and A. Buchan. 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. *Proc. Natl. Acad. Sci. USA* **74**:3913–3917.
 53. Marlin, S. D., S. L. Highlander, T. C. Holland, M. Levine, and J. C. Glorioso. 1986. Antigenic variations (*mar* mutations) in herpes simplex virus glycoprotein B can induce temperature-dependent alterations in gB processing and virus production. *J. Virol.* **59**:142–153.
 54. Marsden, H. S. 1987. Herpes simplex virus glycoproteins and pathogenesis. *Molecular basis of virus disease*. Cambridge University Press, Cambridge.
 55. McGeoch, D. J., M. A. Dalrymple, A. J. Davidson, A. Dolan, M. C. Frame, D. M. McNab, L. Perry, J. E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region of the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69**:1531–1574.
 56. Mettenleiter, T. C., I. Rauh, and A. Koop. 1991. Abstr. 6B-02, p. 126. Abstr. 16th Int. Herpesvirus Workshop, Pacific Grove, Calif.
 57. Misra, V., and E. L. Blewett. 1991. Construction of herpes simplex viruses that are pseudodiploid for the glycoprotein B gene: a strategy for studying the function of an essential herpes virus gene. *J. Gen. Virol.* **72**:385–392.
 58. Misra, V., R. Nelson, and M. Smith. 1988. Sequence of a bovine herpesvirus type-1 glycoprotein gene that is homologous to the herpes simplex gene for the glycoprotein gB. *Virology* **166**:542–549.
 59. Navarro, D., P. Paz, and L. Pereira. 1992. Domains of herpes simplex virus I glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion. *Virology* **186**:99–112.
 60. O'Brian, S. S., C. A. Greenamoyer, E. D. Anton, and T. R. Dambaugh. 1990. Abstr. 15th Int. Herpesvirus Workshop, Washington, D.C., p. 230.
 61. Pellett, 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 1. *J. Virol.* **56**:807–813.
 62. 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.
 63. Pullan, L. M., and E. A. Noltman. 1985. Specific arginine modification at the phosphatase site of muscle carbonic anhydrase. *Biochemistry* **24**:635–640.
 64. Read, G. S., S. Person, and P. M. Keller. 1980. Genetic studies of cell fusion induced by herpes simplex virus type a. *J. Virol.* **35**:105–113.
 65. Riggio, M. P., A. A. Cullinane, and D. E. Onions. 1989. Identification and nucleotide sequence of the glycoprotein B gene of equine herpesvirus 4. *J. Virol.* **63**:1123–1133.
 66. Robbins, A. K., D. J. Dorney, M. W. Wathen, M. E. Whealy, C. Gold, R. J. Watson, L. E. Holland, S. D. Weed, M. Levine, J. C. Glorioso, and L. W. Enquist. 1987. The pseudorabies virus g11 gene is closely related to the gB glycoprotein gene of herpes simplex virus. *J. Virol.* **61**:2691–2701.
 67. Roizman, B., and A. Sears. 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsh, J. L. Melnick, and T. P. Monath (ed.), *Virology*, 2nd ed. Raven Press, New York.
 68. Ross, L. J. N., M. Sanderson, S. D. Scott, M. M. Binns, T. Doel, and B. Milne. 1989. Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of HSV-1. *J. Gen. Virol.* **70**:1789–1804.
 69. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 70. 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.
 71. Shortle, D., and D. Nathans. 1978. Local mutagenesis: a method for generating viral mutants with base substitutions in preselected regions of the viral genome. *Proc. Natl. Acad. Sci. USA* **75**:2170–2174.
 72. Spatz, S. J., R. K. Maes, and C. E. Beisel. 1990. Abstr. 15th Int. Herpesvirus Workshop, Washington, D.C., p. 233.
 73. Storms, R. W., and H. R. Bose, Jr. 1989. Oncogenes, protooncogenes, and signal transduction: toward a unified theory? *Adv. Virus Res.* **37**:1–34.
 74. Stuve, L. L., S. Brown-Shimer, C. Pachl, R. Najarian, D. Dina,

- and R. L. Burke. 1987. Structure and expression of the herpes simplex virus type 2 glycoprotein gB gene. *J. Virol.* **61**:326-335.
75. Walsh, D. A., P. Newsholme, K. C. Cawley, S. M. van Patten, and K. L. Angelos. 1991. Motifs of protein phosphorylation and mechanisms of reversible covalent regulation. *Physiol. Rev.* **71**:285-304.
76. Whalley, J. M., G. R. Robertson, N. A. Scott, G. C. Hudson, C. W. Bell, and L. M. Woodworth. 1989. Identification and complete nucleotide sequence of a gene in equine herpesvirus 1 analogous to the herpes simplex virus gene encoding the major envelope glycoprotein gB. *J. Gen. Virol.* **70**:383-394.
77. Whitbeck, J. C., L. J. Bello, and W. C. Lawrence. 1988. Comparison of the bovine herpesvirus 1 gI gene and the herpes simplex virus type 1 gB gene. *J. Virol.* **62**:3319-3327.
78. White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped animal viruses. *Q. Rev. Biophys.* **16**:151-195.
79. Woodman, P. G., D. I. Mundy, P. Cohen, and E. Warren. 1992. Cell-free fusion of endocytic vesicles is regulated by phosphorylation. *J. Cell Biol.* **116**:331-338.