JOURNAL OF VIROLOGY, May 1994, p. 2830–2839
0022-538X/94/$04.00+0

Vol. 68, No. 5

A Herpes Simplex Virus 1 U_S11-Expressing Cell Line Is Resistant to Herpes Simplex Virus Infection at a Step in Viral Entry Mediated by Glycoprotein D

RICHARD J. ROLLER AND BERNARD ROIZMAN*

The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637

Received 24 August 1993/Accepted 24 January 1994

A baby hamster kidney [BHK(tk$^-$)] cell line ($U_S11$cl19) which stably expresses the U_S11 and α4 genes of herpes simplex virus 1 strain F [HSV-1(F)] was found to be resistant to infection with HSV-1. Although wild-type HSV-1(F) attached with normal kinetics to the surface of $U_S11$cl19 cells, most cells showed no evidence of infection and failed to accumulate detectable amounts of α mRNAs. The relationship between the expression of U_L11 and resistance to HSV infection in $U_S11$cl19 cells has not been defined, but the block to infection with wild-type HSV-1 was overcome by exposing cells with attached virus on their surface to the fusogen polyethylene glycol, suggesting that the block to infection preceded the fusion of viral and cellular membranes. An escape mutant of HSV-1(F), designated R5000, that forms plaques on $U_S11$cl19 cells was selected. This mutant was found to contain a mutation in the glycoprotein D (gD) coding sequence that results in the substitution of the serine at position 140 in the mature protein to asparagine. A recombinant virus, designated R5001, was constructed in which the wild-type gD gene was replaced with the R5000 gD gene. The recombinant formed plaques on $U_S11$cl19 cells with an efficiency comparable to that of the escape mutant R5000, suggesting that the mutation in gD determines the ability of the mutant R5000 to grow on $U_S11$cl19 cells. The observation that the $U_S11$cl19 cells were slightly more resistant to fusion by polyethylene glycol than parental BHK(tk$^-$) cells led to the selection and testing of clonal lines from unselected and polyethylene glycol-selected BHK(tk$^-$) cells. The results were that 16% of unselected to as much as 36% of the clones selected for relative resistance to polyethylene glycol fusion exhibited various degrees of resistance to infection. The exact step at which the infection was blocked is not known, but the results illustrate the ease of selection of cell clones with one or more sites at which infection could be blocked.

Earlier we reported the construction of a cell line, designated $U_S11$cl19, from a clonal cell line (α4cl13) which expresses the α4 gene and was derived from BHK(tk$^-$) cells (1). The $U_S11$cl19 cells express both U_S11 and α4 genes of herpes simplex virus 1 strain F [HSV-1(F)] (34). This cell line was made to test for various activities of the U_S11 protein in the absence of other virally encoded infected cell products. In the course of these studies, we observed that cultures exposed to HSV at high PFU/cell ratios showed no evidence of cytopathic effects, suggesting that they might be resistant to infection. Here we report that $U_S11$cl19 cells are in fact resistant to infection, and we demonstrate that the block to infection occurs at viral entry on a path mediated by the viral glycoprotein D (gD).

Relevant to this report are the following.

(i) The U_S11 gene of HSV (22, 30, 37) encodes a site- and conformation-specific RNA-binding protein (32). This protein regulates the abundance of at least one viral RNA, designated Δ34, such that this RNA is six- to eightfold less abundant in cells infected with wild-type HSV than in cells infected with a virus that does not express U_S11 (33). The U_S11 protein is the product of a γ_2 (true-late) gene (15) and is incorporated into the HSV-1 virion at 600 to 1,000 copies per virion (34). As such, it is present in both the α (immediate-early) and γ (late) stages of HSV infection. In both the α and γ stages of infection, cytoplasmic U_S11 protein is found associated specifically with the 60S subunit of ribosomes (34). In the late stage of infection, U_S11 protein is found associated with nucleoli (19, 29). These associations with ribosomes and nucleoli occur independently of other viral functions, since they also occur in a cell line that constitutively expresses a stably transfected copy of the U_S11 gene (34).

(ii) gD plays an essential role in viral entry at a step subsequent to attachment and at or prior to fusion of the viral and cellular membranes. Neutralizing antibodies directed against gD do not prevent attachment of the virus to the cell surface, and their inhibition may be overcome by exposure of cells and attached virus to the fusogen polyethylene glycol (PEG) (9, 12). HSV virions that lack gD attach to cells with normal kinetics but do not infect (9, 18). They may be induced to infect by exposure of cells and virus to PEG (18). gD was reported to mediate infection at least in part by binding to a cell surface receptor, as shown by the ability of UV-irradiated, gD-bearing virions to block superinfection in a saturable fashion and the failure of virions lacking gD to do so (14). Binding studies with soluble gD suggested that the gD receptor is present at about 4×10^5 to 5×10^5 copies per cell and binds to gD with a dissociation constant of about 0.23 to 0.25 μM (13).

(iii) Cell lines that express the HSV-1 gD gene have also been found to be resistant to HSV infection (3, 16). This resistance has been shown to be due to the cell surface expression of gD, since preincubation of cells with antibodies directed against gD results in loss of resistance (5). Incubation of HSV-1 with gD-expressing cells does not result in infection but rather results in endocytosis and degradation of the virus (3). Escape mutants of HSV-1 that can grow on gD-expressing cells have been isolated (2, 5), and marker transfer experi-

* Corresponding author. Mailing address: The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 E. 58th St., Chicago, IL 60637. Phone: (312) 702-1898. Fax: (312) 702-3791.

ments suggest that two mutations in gD (a Leu-25 to Pro substitution and an Ala-186 to Ser substitution), singly or in combination, confer on the virus that carries them the ability to infect cells that express wild-type gD. Cell lines that express these mutant gD molecules are not resistant to infection by either wild-type or mutant viruses (2, 5). In addition to its apparent role in binding to a cell surface receptor, gD may also be directly involved in the fusion of viral and cellular membranes, inasmuch as BJ cells, which constitutively express gD, show spontaneous cell-cell fusion (4), and antibodies to gD can block the cell-cell fusion induced by viral infection (25).

MATERIALS AND METHODS

Cells and viruses. Vero cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% newborn calf serum. BHK(tk$^-$) cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS). The construction and properties of the α4cl13 cell line were previously described (1). The α4cl13 cell line was maintained in DMEM–5% FBS supplemented with 400 μg of G418 per ml. The construction of the U_S11cl19 cell line was described previously (34). The U_S11cl19 cells were maintained in DMEM–5% FBS supplemented with 400 μg of G418 per ml and 440 mM methotrexate. Since neomycin has been shown to inhibit attachment of HSV-1 to BHK cells (17), cells maintained in medium supplemented with G418 were washed twice with medium lacking G418 before exposure to virus. The properties of HSV-1(F) and the methods for growth and preparation of high titer viral stocks have been described previously (7, 31). HSV-1(F) mutant viruses U10, U21, and U30 and the recombinant U21rec, carrying mutations in the gD coding sequence, were obtained from G. Campadelli-Fiume (2). The U10 virus carries a mutation resulting in the substitution of Leu-25 with Pro. The U30 virus carries a mutation resulting in the substitution of Ala-185 with Ser. The U21 virus carries both of the mutations found in U10 and U30.

Plasmid constructions and synthesis of RNA probes. Standard methods were used for all plasmid constructions described here (20). Plasmid pRB4716, which served to generate RNA probes to detect the α27 mRNA, was constructed by ligating the 1.1-kb *Bam*HI-*Sal*I fragment of the α27 gene of HSV-1(F) into *Bam*HI-*Sal*I-cut pGEM-3Z(f+). Plasmid pRB3952, for the generation of RNA probe to detect the α47 and α22 genes, was constructed in two steps. In the first step, the 0.7-kb *Nru*I-*Xho*I fragment from the α47 gene of HSV-1(F) was ligated into *Sal*I-*Sma*I-cut pGEM-3Z to generate pRB3951. In the second step, pRB3951 was digested with *Apa*I and *Hin*dIII, the ends were filled by treatment with Klenow enzyme in the presence of deoxynucleoside triphosphates, and the plasmid was religated to generate pRB3952. Plasmid pRB4352, for the generation of RNA probe to detect the α0 mRNA, was constructed by ligating the 1.35-kb *Nru*I-*Sal*I fragment containing exon III sequences of the α0 gene from HSV-1(F) into *Sal*I-*Sma*I cut pGEM-3Z(f+). RNA probes were synthesized from uncut plasmids with SP6 RNA polymerase (New England BioLabs) in reactions containing [α-^{32}P]CTP, using protocols provided by Promega Biotec.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels as described by Gibson and Roizman (10, 11), electrically blotted onto nitrocellulose, and probed with a 1:2,000 dilution of anti-gD monoclonal antibody H170 (26) as previously described (33).

DNA sequencing. The sequence of nucleotides between the *Nco*I and *Eco*NI restriction sites in the gD coding sequence from the mutant R5000 was determined on both strands by the dideoxy termination method, using a Sequenase version 2.0 kit (United States Biochemicals). Sequencing reactions were primed with 1 of a set of 14 synthetic DNA oligonucleotides complementary to the gD coding sequence and separated from each other by 150 bp.

Isolation and analysis of RNA. Total RNA was isolated as described by Puissant and Houdebine (28). Fractionation of RNAs on formaldehyde-agarose gels was done as described by Maniatis et al. (20). Gels were blotted to Zeta-Probe membranes and probed with strand-specific RNA probes, using protocols recommended by the manufacturer except that hybridization and washing of blots were carried out at 80°C.

Black plaque assay. Cultures were washed twice with phosphate-buffered saline (PBS), fixed with methanol at −20°C for 20 min, and dried under a stream of air. Cells were rehydrated by incubating 5 min in PBS and then reacted sequentially with a 1:2,000 dilution of anti-gD monoclonal antibody H170 (26) in PBS, a 1:1,000 dilution of biotinylated goat anti-mouse immunoglobulin G (Zymed Laboratories) in PBS, and a 1:500 dilution of streptavidin-alkaline phosphatase conjugate (Zymed Laboratories) in PBS, with two 5-min washes in PBS following each incubation. Plaques were visualized by exposure to color development solution (made by mixing 1 part 30-mg/ml nitroblue tetrazolium in 70% [vol/vol] *N,N*-dimethyl formamide, 1 part 15-mg/ml 5-bromo-4-chloro-3-indolyl phosphate in *N,N*-dimethylformamide, and 98 parts 0.1 M $NaHCO_3$–1 mM $MgCl_2$ [pH 9.8]) for 2 to 10 min.

Marker transfer and the construction and purification of recombinant virus. The procedures for cotransfection of cloned fragments of R5000 DNA and full-length HSV-1 DNA have been described previously (27). For marker transfer experiments, U_S11cl19 cells were infected with serial dilutions of virus progeny of the cotransfection. Infected cultures were maintained for 3 days in DMEM supplemented with 5% FBS and 165 μg of human immune gamma globulin (Armour Pharmaceutical Co.) per ml, providing fresh medium each day. Cultures were then assayed for plaque formation by black plaque assay. For construction of the recombinant virus R5001, HSV-1(F) DNA was cotransfected with the 846-bp *Nco*I-*Eco*NI fragment of the gD gene from R5000 into rabbit skin cells. After 4 days, when all cells in the culture showed cytopathic effect, the culture was frozen and a viral stock was prepared. Portions of a 10^{-6} dilution of this stock and dilutions of HSV-1(F) and the mutant R5000, each containing 1,000 PFU, were reacted on ice for 2 h with serial dilutions of anti-gD monoclonal antibody DL11 (6, 23) and then used to infect Vero cell cultures. After 2 days, plaques were counted on the cultures infected with HSV-1(F) and R5000 to determine the dilution of the antibody giving the greatest ratio of R5000 to HSV-1(F) plaques (1:412). Plaques were picked from the dish infected with transfection stock that had been exposed to the appropriate antibody dilution, and these isolates were carried through two additional cycles of plaque purification in the absence of selection.

Attachment assays. Ten cultures each of BHK(tk$^-$), α4cl13, and U_S11cl19 cells were each exposed to 1,000 PFU of HSV-1(F) in 1.5 ml at 4°C. At 15, 30, 45, 60, and 90 min after virus addition, inocula were removed from duplicate dishes of each cell type and transferred directly to cultures of Vero cells in 25-cm^2 dishes. After 1 h, inocula were removed from Vero cultures and replaced with medium 199 supplemented with 1% newborn calf serum and 165 μg of human immune gamma globulin per ml. After 2 days, plaques were counted in the Vero cell cultures.

PEG fusion. Cell monolayers in 25-cm^2 dishes were exposed

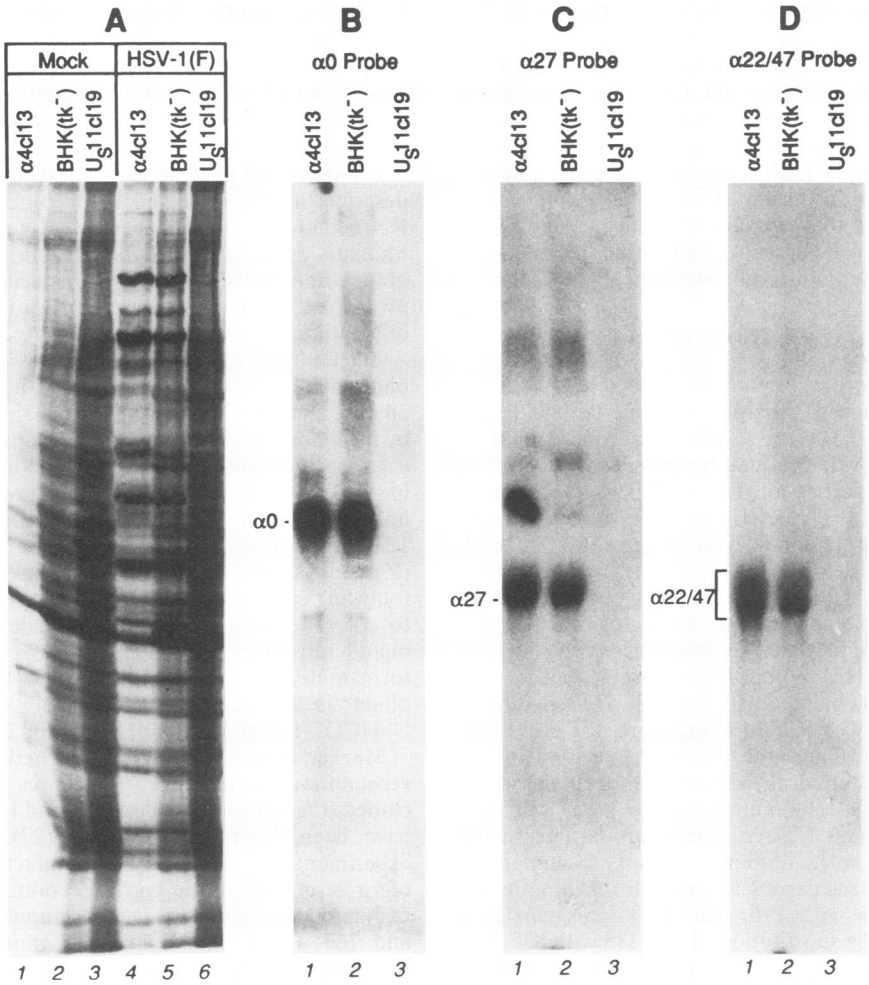

FIG. 1. Assay for HSV-1 gene products in U_S11cl19 cells. (A) Autoradiographic image of [^{35}S]methionine-labeled, electrophoretically separated polypeptides from uninfected (lanes 1 to 3) and HSV-1(F)-infected (lanes 4 to 6) BHK(tk$^-$), α4cl13, and U_S11cl19 cells. (B) Autoradiographic image of a Northern (RNA) blot of formaldehyde-agarose gel-separated RNAs purified from BHK(tk$^-$) (lane 1), α4cl13 (lane 2), and U_S11cl19 cells (lane 3) infected with HSV-1(F) and probed with labeled SP6 transcript of pRB4352. (C) Same as panel B but probed with labeled SP6 transcript of pRB4716. (D) Same as panel B but probed with labeled SP6 transcript of pRB3952. The positions of the α0 and α27 mRNAs are indicated with dashes. The positions of the α22 and α47 mRNAs (only partially resolved) are indicated with a bracket.

to virus in 1.5-ml inocula at 4°C for 90 min. The inocula were decanted, and monolayers were washed twice with cold PBS. Cultures were then rapidly treated with a 48% (wt/wt) solution of PEG 6000 in PBS as described previously (35). Cultures were incubated at 37°C for 6 h and then assayed for α27 protein by immunofluorescence as previously described (34), using a 1:500 dilution of anti-α27 monoclonal antibody H1113 as the primary antibody.

RESULTS

The U_S11cl19 cell line was made by transfecting the U_S11 coding sequence under the control of the promoter/regulatory domain of HSV-1 glycoprotein B (gB), linking the sequence to the *dhfr* gene in α4cl13 cells, and cloning the methotrexate-resistant progeny (34). In initial experiments to characterize the U_S11cl19 cell line, the cells were infected at high multiplicity with HSV-1(F) and found to show no cytopathic effect, suggesting that they might be resistant to infection. This possibility was examined by testing infected cultures of U_S11cl19 for the production of virally encoded gene products. Cultures of BHK(tk$^-$), α4cl13, and U_S11cl19 cells were either mock infected or infected with 10 PFU of HSV-1(F) per cell. After 18 h of infection, the cells were incubated in medium containing 50 μCi of [^{35}S]methionine per ml for an additional 3 h. Cellular extracts were prepared and subjected to denaturing gel electrophoresis (Fig. 1A). Infected BHK(tk$^-$) and α4cl13 cells (lanes 4 and 5) showed a normal late pattern of viral protein synthesis coupled with shutoff of host protein synthesis. Infected U_S11cl19 cells (lane 6) exhibited neither host shutoff nor detectable viral protein synthesis. This result suggested that U_S11cl19 cells exhibit a block to infection at a point in the viral life cycle prior to the synthesis of late proteins. Subsequently, we tested for synthesis of viral products from increasingly earlier stages of infection. These experiments culminated in a test for production of α mRNAs. Cultures of BHK(tk$^-$), α4cl13, and U_S11cl19 were infected with 10 PFU of HSV-1(F) per cell. At 6 h after infection, total RNA was prepared from each culture, separated on a formaldehyde-agarose gel, transferred to a Zeta-Probe membrane,

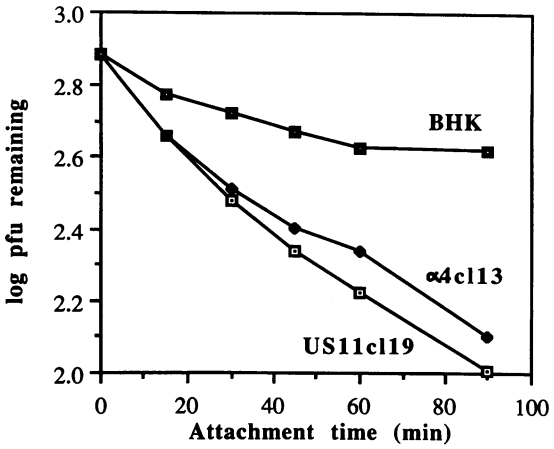

FIG. 2. Assay for attachment of HSV-1(F) to BHK(tk$^-$), α4cl13, and U_S11cl19 cells. Shown is a plot of the logarithm of PFU remaining in an inoculum after incubation with BHK(tk$^-$), α4cl13, or U_S11cl19 cells versus the time of incubation with cells. Each point represents the mean of duplicate measurements.

and probed with labeled, SP6-transcribed transcripts of sequences from the α0, α27, and α22/47 genes (Fig. 1B to D). Transcripts of all of these genes could be readily detected in RNA from BHK(tk$^-$) and α4cl13 cells (lanes 1 and 2) in equivalent amounts. In contrast, none of these transcripts were detectable in RNA from U_S11cl19 cells, suggesting that U_S11cl19 cells exhibit a block to infection at some point prior to accumulation of α mRNAs. It should be noted, however, that the apparent degree of resistance exhibited by U_S11cl19 cells was observed to be dependent on the passage history of the cells. Cultures of cells which had been passaged more times than those used for the assay shown in Fig. 1 were more susceptible to infection and synthesized detectable amounts of α mRNAs (not shown).

HSV-1 attaches normally to U_S11cl19 cells. The results shown in Fig. 1 prompted us to examine whether HSV-1(F) could attach to and penetrate into U_S11cl19 cells. To test for attachment, monolayers of U_S11cl19, α4cl13, and BHK(tk$^-$) cells in 25-cm^2 dishes were exposed to 1,000 PFU of HSV-1(F) at 4°C. At various times after infection, the viral inocula were removed from duplicate cultures of each cell type and tested for residual (i.e., unattached) virus on Vero cell monolayers (Fig. 2). Attachment of virus to cells is indicated by the depletion of PFU from the inoculum. HSV-1(F) virus attached to U_S11cl19 and α4cl13 cells with similar kinetics. In the experiment shown, virus attached about twice as efficiently to U_S11cl19 and α4cl13 cells as to BHK(tk$^-$) cells.

The fusogen PEG enhances infection of U_S11cl19 cells. The results shown in Fig. 1 and 2 above suggested that the block to infection in U_S11cl19 cells occurs after viral attachment and before α gene expression, possibly prior to fusion of the viral and cellular membranes. To test the latter possibility, we exposed U_S11cl19 cell monolayers to HSV-1(F) for 1 h at 4°C. Cultures were then either shifted to 37°C without treatment or treated with the fusogen PEG as described in Materials and Methods and then incubated at 37°C. After 6 h, cell monolayers were fixed and assayed for expression of the α27 protein by immunofluorescence (Fig. 3). Two observations should be noted. (i) In the absence of PEG treatment (Fig. 3A and C), some cells were permissive to infection, indicated by their expression of ICP27 protein. The number of these cells did not increase significantly with the amount of infecting virus when the multiplicity of infection exceeded 10 (compare Fig. 3A and C), suggesting that the susceptible cells comprise a subpopulation which is saturated at the multiplicities of infection used in this experiment. The proportion of such permissive cells in U_S11cl19 cultures varied depending on the passage history of the cells, such that cultures that had been passaged for a longer time had a greater proportion of permissive cells. Subcloning of U_S11cl19 cells gave rise to fully resistant lines with no permissive cells and lines in which only a small proportion (always less than 10%) of the cells were permissive. We recovered no subclones that contained only permissive cells. (ii) PEG treatment (Fig. 3B and D) clearly increased the number of permissive cells in the culture, allowing the initiation of infection in the resistant population of cells. PEG treatment did not increase the number of positive cells in cultures of BHK(tk$^-$) cells (not shown), suggesting that the increase in susceptibility to infection was specific for U_S11cl19 cells and that the block to infection in these cells occurs at or prior to fusion of the viral and cellular membranes. It should be noted that BHK(tk$^-$) and U_S11cl19 cells differed in their response to PEG treatment such that BHK(tk$^-$) cells were fused into giant syncytia at PEG concentrations tolerated by U_S11cl19 cells.

Isolation of the escape mutant R5000. U_S11cl19 cells in a 150-cm^2 flask were infected with 5×10^8 PFU of HSV-1(F). One day after infection, cells were propagated once at 1:5 dilution, and the resulting culture was incubated until the cells reached confluence, at which time viral plaques were evident. Cells were propagated again at a 1:5 dilution, and the resulting culture was incubated at 37°C until all cells showed cytopathic effect, at which time the culture was frozen and a viral stock was prepared. Serial dilutions of the viral stock were plated on Vero cell monolayer cultures, and six plaques were picked and brought through a second cycle of plaque purification. One of the isolates, designated R5000, was chosen for further studies.

The mutation in R5000 that permits growth on U_S11cl19 cells maps to the viral gene encoding gD. Cloned *Eco*RI fragments from R5000 were cotransfected with full-length HSV-1(F) DNA into rabbit skin cells, and viral stocks were prepared from the cultures when all cells showed cytopathic effect. These stocks were then plated onto U_S11cl19 cells, and cultures were examined for plaques by a black plaque assay 3 days after infection. The results of this series of experiments are summarized in Fig. 4. In the first experiment, only the *Eco*RI H fragment transferred the ability to plaque on U_S11cl19 cells. The *Eco*RI H fragment contains the coding regions for all of the genes in the U_S sequence of the genome and the promoter/regulatory sequences of the α4 and Ori_S transcriptional units. In the second experiment (Fig. 4B), eight subclones of the *Eco*RI H fragment were tested, and two of these, containing sequences between the *Sph*I restriction enzyme site located between the gG and gI coding sequences and the *Bgl*II site located within the gE coding sequence, were found to transfer the ability to plaque on U_S11cl19 cells. In the third experiment, subclones of the 6.6-kb *Ssp*I-*Bgl*II fragment were tested (Fig. 4C), and four of these transferred the ability to form plaques on U_S11cl19 cells. The only sequence common to all four of these subclones is an 846-bp region between *Nco*I and *Eco*NI sites in the gD coding sequence. These results suggested that a mutation in the gD gene could confer the ability to form plaques on U_S11cl19 cells.

The sequence from R5000 that transfers the ability to form plaques on U_S11 contains a single nucleotide change. The sequence between the *Nco*I and *Eco*NI sites in the gD coding sequence in the mutant R5000 was determined and found to contain a single nucleotide change from the sequence of the

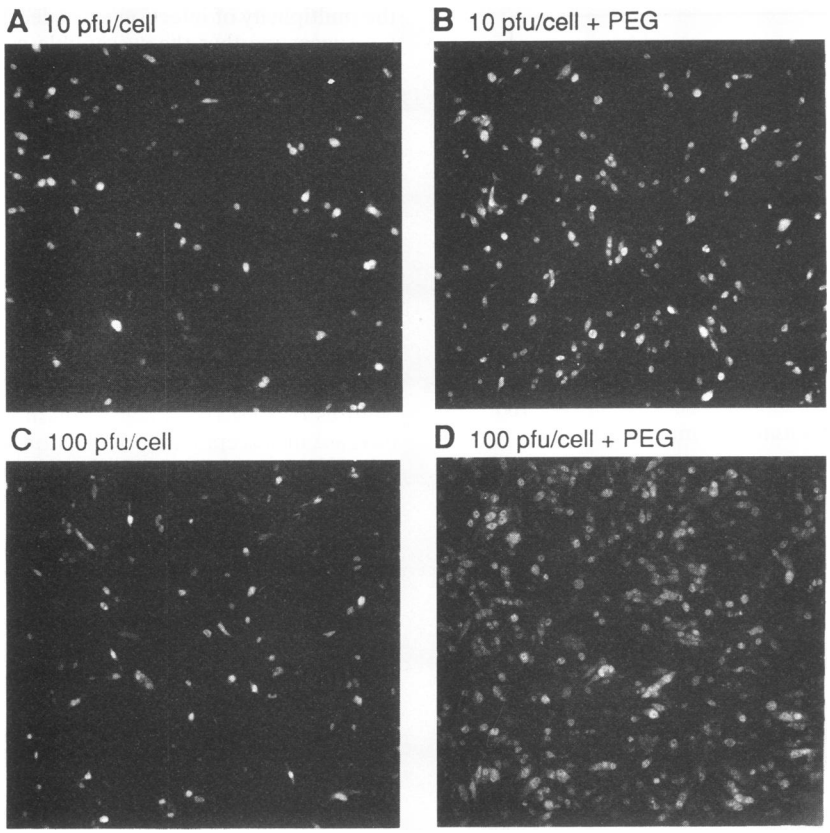

FIG. 3. (A) Photographic image of U_S11cl19 cells infected with 10 PFU of HSV-1(F) per cell without PEG treatment, reacted with anti-α27 monoclonal antibody and fluorescein isothiocyanate-conjugated secondary antibody, and viewed with UV illumination. Nuclei of cells expressing α27 protein show fluorescence. (B) Same as panel A except that after exposure to virus, cells were treated with PEG. (C) Same as panel A except that cells were infected with HSV-1(F) at 100 PFU per cell. (D) Same as panel B except that cells were infected with HSV-1(F) at 100 PFU per cell.

corresponding region of HSV-1(F) (Fig. 5) (5, 22). This change in the nucleotide sequence creates a single amino acid substitution in gD, changing the serine at position 140 to an asparagine and creating a potential N-linked glycosylation site. The same amino acid substitution had been previously identified by Muggeridge et al. (24) in a mutant selected for resistance to the anti-gD monoclonal antibody DL11. Muggeridge et al. determined that the potential N-linked glycosylation site created by the mutation was used in the infected cell, resulting in the generation of a hyperglycosylated gD. To determine whether this mutation in R5000 was completely responsible for the growth properties of the mutant on U_S11cl19 cells, the *Nco*I-*Eco*NI fragment of R5000 was cotransfected with HSV-1(F) DNA, and a recombinant virus carrying the mutation was isolated by infection of Vero cells in the presence of DL11 antibody and subsequent plaque purification. At no point was this recombinant virus selected by growth on U_S11cl19 cells. Virus isolates carrying the desired mutation were identified by probing blots of infected cell protein with anti-gD monoclonal antibody H170 and looking for a change in migration due to the use of the additional N-linked glycosylation site. The result for one such isolate, designated R5001, is shown in Fig. 6. Total proteins from BHK(tk$^-$) cells infected with 10 PFU of HSV-1(F), the mutant R5000, or the recombinant R5001 per cell and maintained for 18 h were separated on an SDS-polyacrylamide gel, electroblotted onto nitrocellulose, and probed with monoclonal antibody H170 directed against gD. As seen by Muggeridge et al., the electrophoretic mobility of the mutant gD molecule (lanes 3 and 4) was lower than that of the wild-type gD (lane 2) in SDS-polyacrylamide gels as a result of the additional asparagine-linked oligosaccharide. No wild-type gD was detectable in the blot of R5001 proteins (lane 4), suggesting that the virus was essentially pure. The plaquing efficiencies of HSV-1(F), R5000, and R5001 were tested on BHK(tk$^-$) and U_S11cl19 cells (Table 1). R5001 formed plaques at an efficiency comparable to that of the mutant R5000, suggesting that the ability of that mutant to form plaques on U_S11cl19 cells was completely determined by the mutation in the gD gene.

Behavior of gD U mutants on U_S11cl19 cells. Specific mutations in gD have been associated with the ability of viruses that carry them to overcome resistance to HSV infections displayed by BHK cell lines that express HSV-1 gD. We tested a set of these mutations along with HSV-1(F), R5000, and R5001 for their ability to form plaques on U_S11cl19 cells. Serial dilutions of each virus were plated on BHK(tk$^-$) and U_S11cl19 cells, and after 3 days of infection, plaques were visualized by immunostaining as described in Materials and Methods. The results of three such experiments are presented in Table 1. All of the mutant viruses tested formed plaques on U_S11cl19 cells at efficiencies much higher than that of wild-type HSV-1. The plaquing efficiencies of all of the mutant viruses were comparable. It should be noted that the plaquing efficiencies of all of these viruses on U_S11cl19 cells were

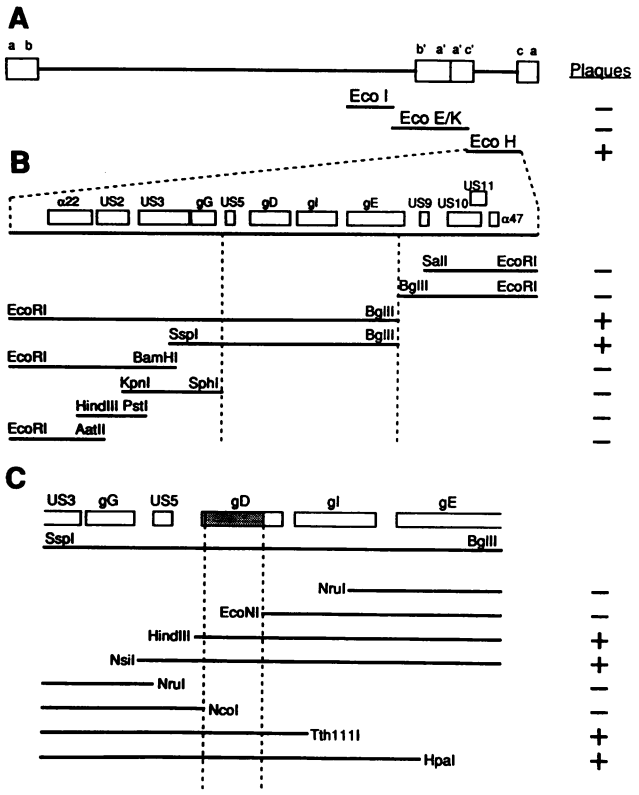

FIG. 4. Marker transfer between the mutant R5000 and HSV-1(F). (A) Schematic diagram of the HSV-1 genome in prototype arrangement, showing the unique sequences (thin lines) flanked by inverted repeats (open boxes). Also shown are the positions of the *Eco*RI I, E/K, and H fragments in the HSV-1 genome. Fragments that transferred the ability to forms plaques on U_S11cl19 cells are indicated with a plus sign; those that did not are indicated with a minus sign. (B) Schematic diagram of the sequence arrangement in the *Eco*RI H fragment of HSV-1 showing the positions of mapped protein coding regions (open boxes). Also shown beneath are the positions of subcloned sequences of the *Eco*RI H fragment tested for marker transfer. (C) Schematic diagram of the arrangement of sequences between the *Ssp*I and *Bgl*II restriction enzyme sites in the *Eco*RI H fragment of HSV-1. Open boxes indicate protein coding regions. The stippled area in the gD protein coding sequence indicates the position of sequences held in common by all fragments that transferred to HSV-1(F) the ability to form plaques on U_S11cl19 cells.

dependent on the passage history of the cells, such that all of the viruses formed plaques more efficiently on U_S11cl19 cells that had been passaged more extensively. Thus, in initial experiments on low-passage cells, HSV-1(F) formed no plaques even after infection with 10^9 PFU. In later experiments on cells that had been passaged more than 10 times after cloning, HSV-1(F) formed plaques after infection with $>10^7$ PFU. Similarly, the first measurements of the plating efficiency of mutants showed much lower efficiencies on U_S11cl19 cells relative to BHK(tk$^-$) cells than the last. The experiments shown in Table 1 are the last three such measurements.

Isolation of resistant BHK(tk$^-$) cell clones. The observation that resistance to infection in U_S11cl19 cells was accompanied by resistance to fusion with PEG prompted us to test whether we could isolate cell lines resistant to infection by selection with PEG. Monolayers of BHK(tk$^-$) cells were subjected to three selections by treatment with either 50.0% (wt/wt) or 52.6% (wt/wt) PEG as described in Materials and Methods.

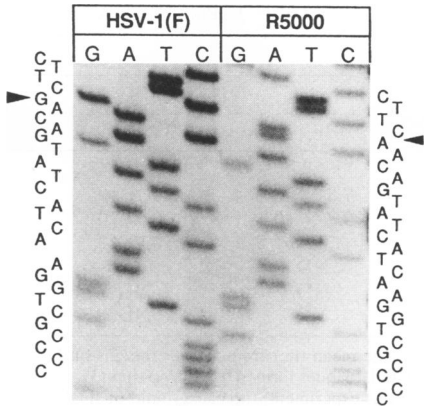

FIG. 5. Autoradiographic image of a sequencing gel showing part of the sequence from the gD genes of HSV-1(F) (lanes 1 to 4) and mutant R5000 (lanes 5 to 8). Nucleotides that differ between the two sequences are indicated with arrowheads. The substitution of A (in R5000) for G [in HSV-1(F)] changes a serine codon at position 140 in the mature gD protein to an asparagine codon.

After the final selection, cells were cloned by limiting dilution. Untreated BHK(tk$^-$) cells were cloned also in order to assess the frequency of spontaneously arising resistant cells. Initial screening of a collection of 79 clones was done by infecting monolayers of each clone with 2,000 PFU of HSV-1(F) and assaying plaque formation 3 days after infection. This input multiplicity yielded 200 to 400 plaques on unselected, uncloned BHK(tk$^-$) cells. Clones that showed no plaques (i.e., that

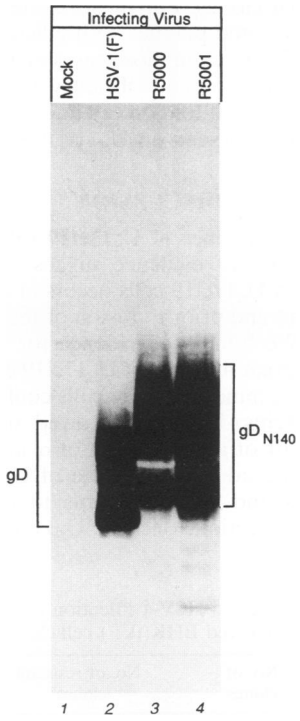

FIG. 6. Photographic image of a Western blot (immunoblot) of electrophoretically separated proteins from BHK(tk$^-$) cells infected with no virus (lane 1), HSV-1(F) (lane 2), mutant R5000 (lane 3), and recombinant R5001 (lane 4) probed with anti-gD monoclonal antibody H170.

TABLE 1. Plating efficiencies of mutant and recombinant viruses on BHK(tk$^-$) and U_S11cl19 cells

Virus	Titer						Efficiency on U_S11cl19 relative to BHK(tk$^-$)[a]
	Expt 1		Expt 2		Expt 3		
	BHK(tk$^-$)	U_S11cl19	BHK(tk$^-$)	U_S11cl19	BHK(tk$^-$)	U_S11cl19	
HSV-1(F)	3.5×10^8	3.0×10^1	2.6×10^8	3.0×10^2	1.2×10^8	NC[b]	6.8×10^{-7}
R5000	7.8×10^8	2.1×10^7	4.2×10^8	6.6×10^7	3.0×10^8	8.4×10^7	0.114
R5001	5.2×10^8	1.0×10^7	1.7×10^8	2.9×10^7	2.4×10^8	2.9×10^7	0.074
U10	ND[c]	ND	2.4×10^9	2.2×10^8	1.8×10^9	2.4×10^8	0.110
U21	ND	ND	7.1×10^8	5.0×10^7	6.0×10^8	8.2×10^7	0.100
U30	ND	ND	1.4×10^9	7.3×10^7	1.1×10^9	1.3×10^8	0.077
U21rec	ND	ND	9.8×10^9	3.9×10^8	5.2×10^9	4.2×10^8	0.055

[a] Calculated as the mean titer from all experiments on U_S11cl19 cells divided by the mean titer on BHK(tk$^-$) cells.
[b] NC, not counted. Plaques formed by HSV-1(F) on U_S11cl19 cells even after many passages were small, often composed of only a few cells on a background of singly infected cells. In this experiment, the background of singly infected cells was too high to permit a reliable count of plaques.
[c] ND, not determined.

depressed the plating efficiency by at least 100-fold) were classified as resistant. The results of the initial screen (Table 2) were quite surprising. Even in the absence of selection, 15.8% of cell clones were resistant to infection. PEG selection did, however, result in the isolation of a larger percentage of resistant clones. Some of the cell clones that were found to be resistant in the initial screen were further characterized by determining the plating efficiency of HSV-1(F), mutant U21, and mutant R5000. Monolayers of resistant cell clones and of unselected, uncloned BHK(tk$^-$) cells were infected with serial dilutions of HSV-1(F), U21, and R5000 and examined 3 days after infection for the formation of plaques. The results for each of the viruses on the resistant cell clones are presented in Table 3 as plating efficiencies relative to unselected, uncloned BHK cells. The degree of resistance to infection varied greatly between individual clones. For most of the cell lines, R5000 and U21 viruses formed plaques with efficiencies comparable to that of HSV-1(F). Even in those cases in which these viruses showed a significantly higher efficiency than HSV-1(F), the enhancement (at most 70-fold on cell lines U12 and 95-27) was much smaller than that seen on U_S11cl19 cells.

DISCUSSION

Mechanism of resistance of U_S11cl19 cells to infection by HSV-1. Several lines of evidence suggest that the block to HSV-1 infection in U_S11cl19 cells occurs at a step subsequent to viral attachment and prior to fusion of the viral and cellular membranes. (i) We found no evidence for the expression of viral genes in the great majority of U_S11cl19 cells that had been exposed to virus. A minority of the cells could be infected and expressed α proteins, but most showed no evidence of α protein synthesis or of accumulation of α mRNAs. (ii) Infectious particles attached to the surface of U_S11cl19 cells. (iii) Exposure of cells and adsorbed virus to the fusogen PEG greatly enhanced infection. Because U_S11cl19 cells consist of at least two subpopulations of differing resistances, the data of Fig. 3 do not allow quantitation of the stimulation of viral entry by PEG. That is, in a mixture of resistant and susceptible cells, it is not possible to see a stimulation greater than the reciprocal of the fraction of susceptible cells. The crucial point is that the highly resistant subpopulation of U_S11cl19 is rendered largely susceptible to infection by treatment with PEG. (iv) A mutation in the gD coding sequence allowed the mutant virus to at least partially overcome the block to infection. gD has been shown to be essential for viral entry at a step subsequent to infection and prior to the fusion of viral and cellular membranes (8, 9, 12, 14, 18). It has further been shown that gD mediates interaction with a cell surface molecule inasmuch as gD-bearing virions can saturate and inhibit binding of subsequently added virions, but virions that do not bear gD cannot (14). Furthermore, a soluble form of gD has been shown to have a saturable binding site on the cell surface (13). One hypothesis that could account for our results is that U_S11cl19 cells, or at least that fraction of the population that is resistant, do not express a recognizable gD receptor. Such a receptor might be absent altogether or present in an altered form. Absence or alteration of the receptor might take place as a result of U_S11-mediated regulation or mutation of the receptor itself or as a result of a change in its processing due to regulation or mutation of some component of the processing pathway. Alternatively, U_S11cl19 cells may express a functional receptor but be altered in some property of the cell surface required for membrane fusion. As noted in results, U_S11cl19 cells were more resistant to PEG-induced cell-cell fusion than BHK(tk$^-$) cells.

TABLE 2. Resistance to HSV-1 infection in PEG-selected and unselected BHK(tk$^-$) cell clones

Selection	No. of clones	No. of resistant clones[a]	% Resistant clones
None	19	3	15.8
50.0% PEG	41	15	36.6
52.6% PEG	19	6	31.6

[a] Resistant clones are defined as those that did not support plaque formation when infected with 2,000 PFU of HSV-1(F).

We note that it is formally possible that the block to viral entry evidenced by the lack of α mRNA accumulation and stimulation of α protein expression by PEG and the block to plaque formation that is overcome by the mutation in the gD gene are not the same block and that the mutation in gD may overcome a block to some process mediated by gD later in the infectious cycle. While we believe that U_S11cl19 cells may display more than one form of resistance (see below), the accumulated evidence in the literature overwhelmingly supports the conclusion that viral entry is the only process in which a mutation in the gD gene is likely to make as large a difference as the mutation in R5000 makes in infection of U_S11cl19 cells. Most compelling are the data of Ligas and Johnson (18), who showed that even a virus in which the gD gene has been deleted can complete the remainder of infection efficiently if its entry is effected.

TABLE 3. Susceptibility of PEG-selected and unselected BHK(tk$^-$) cell clones to infection with HSV-1(F), R5000, and U21

Selection	Cell line	Plating efficiency on cell line relative to BHK(tk$^-$) cells[a]			R5000/ HSV-1(F)	U21/ HSV-1(F)
		HSV-1(F)	R5000	U21		
None	BHK(tk$^-$)	1.0	1.0	1.0	1.0	1.0
	U1	1.5×10^{-2}	2.5×10^{-2}	7.1×10^{-2}	1.7	4.7
	U8	1.2×10^{-2}	3.9×10^{-2}	8.4×10^{-2}	3.3	7.0
	U12	7.8×10^{-4}	1.1×10^{-2}	5.5×10^{-2}	14.1	70.5
50.0% PEG	95-1	4.7×10^{-4}	6.8×10^{-4}	1.3×10^{-3}	1.5	2.8
	95-8	2.7×10^{-4}	1.2×10^{-4}	5.7×10^{-4}	0.4	2.1
	95-12	7.4×10^{-5}	1.0×10^{-4}	3.9×10^{-4}	1.4	5.3
	95-14	1.4×10^{-4}	2.3×10^{-4}	6.2×10^{-4}	1.6	4.4
	95-18	1.3×10^{-3}	6.4×10^{-3}	1.3×10^{-2}	4.9	10.0
	95-19	5.8×10^{-5}	1.9×10^{-4}	6.3×10^{-4}	3.3	10.9
	95-22	5.7×10^{-5}	2.4×10^{-4}	4.9×10^{-4}	4.2	8.6
	95-27	1.7×10^{-4}	1.2×10^{-3}	1.2×10^{-2}	7.1	70.6
	95-29	2.4×10^{-4}	3.1×10^{-4}	2.1×10^{-3}	1.3	8.8
	95-33	8.3×10^{-4}	2.4×10^{-2}	2.4×10^{-2}	28.9	28.9
	95-36	6.5×10^{-3}	1.5×10^{-2}	5.5×10^{-2}	2.3	8.5
	95-38	1.6×10^{-4}	1.2×10^{-3}	1.8×10^{-3}	7.5	11.3
52.6% PEG	100-5	2.1×10^{-3}	5.4×10^{-3}	1.2×10^{-2}	2.6	5.7
	100-6	1.9×10^{-5}	2.5×10^{-5}	5.6×10^{-5}	1.3	2.9
	100-7	1.6×10^{-4}	2.2×10^{-4}	7.2×10^{-4}	1.4	4.5
	100-12	1.2×10^{-3}	1.4×10^{-3}	1.3×10^{-2}	1.2	10.8
	100-14	2.3×10^{-3}	4.4×10^{-3}	2.2×10^{-2}	1.9	10.0

[a] Calculated as viral titer on cell line tested divided by the viral titer on BHK(tk$^-$) cells.

Relationship of resistance to infection in gD-expressing cells and in U_S11cl19 cells. Viral mutants selected for growth on gD-expressing cells were found to be able to grow on U_S11cl19 cells far more efficiently than wild-type HSV-1(F). The mutant R5000, selected for growth on U_S11cl19 cells, was able to grow efficiently on the gD-expressing BJ cell line (2). Taken at face value, the ability of the same set of mutants to overcome resistance in these two cell lines suggests that the mechanisms of resistance to infection in these two cell lines must be closely related. Two considerations suggest that the mechanisms of resistance in BJ and U_S11cl19 cells cannot be identical.

(i) The mutant R5000, as mentioned, multiplied efficiently on BJ cells, whereas the recombinant R5001, carrying only the mutation in the *Nco*I-*Bgl*II fragment of the gD coding sequence, did not grow efficiently on BJ cells. This observation suggests that there is some secondary mutation in R5000 that is responsible for its growth on gD-expressing BJ cells. Also, although the mutant U21 and the recombinant U21rec plated with comparable efficiencies on U_S11cl19 cells, the recombinant formed much smaller plaques than the mutant and at high multiplicity (10 PFU per cell) synthesized substantially less viral protein (not shown). It thus seems reasonable to conclude that any and all of the mutants may contain secondary mutations that strongly affect their growth properties on U_S11cl19 and BJ cells.

(ii) Resistance to infection in BJ cells has been shown to be caused by expression of gD, inasmuch as preincubation of these cells with anti-gD antibody results in loss of resistance. U_S11cl19 cells express no gD.

Two hypotheses present themselves to account for the behavior of wild-type viruses and viruses carrying mutations in gD on U_S11cl19 and BJ cells. (i) Resistance to HSV infection in both cell lines is caused by absence of functional gD receptors on the cell surface. Mutant gD molecules allow the viruses that carry them to use an alternate receptor and thereby infect the cells. This alternative receptor might be the product of an allele of the normal receptor gene or the product of a distinctly different gene. The incomplete reciprocity of infection shown by the recombinant U21 and R5001 viruses would suggest that there are differences in the alternate receptors available on U_S11cl19 and BJ cells.

(ii) U_S11cl19 and BJ cells are resistant by dissimilar mechanisms, one perhaps lacking functional receptor and the other resistant to some other gD-mediated process leading to fusion of viral and cellular membranes. In this case, the ability of viruses carrying identical mutations in gD to infect both cell types would suggest that some mutations in gD can alter multiple functions of the glycoprotein without inactivating it. There is at present no evidence that gD operates at multiple steps in the entry process. The first of these two hypotheses is directly tied to a demonstrated function of gD, is ultimately simpler, and is, therefore, preferable.

Relationship of resistance to infection and expression of U_S11 protein. Two general hypotheses may be framed to address the role of U_S11 in the resistance to infection shown by U_S11cl19 cells. (i) The resistance to infection displayed by U_S11cl19 cells may require the expression of U_S11 protein. U_S11 is a regulatory protein that can suppress the accumulation of an RNA to which it binds (33). It is possible that U_S11 protein can recognize cellular RNAs that encode membrane proteins or proteins that affect processing and/or transport of membrane proteins as regulatory targets and, consequently, alter the properties of the cell surface. (ii) The resistance to infection may be a peculiarity of this clone that is unrelated to the expression of U_S11 protein. We do not have evidence that differentiates between these hypotheses. U_S11cl19 was the only clone from the original transfection of α4cl13 that displayed high-level resistance to infection, but it expressed U_S11 protein at a much higher level than any of the other clones isolated and so is not directly comparable. As U_S11cl19 cells have been passaged, total U_S11 protein expression and resistance to infection have both declined. We have subcloned cell lines from U_S11cl19, and while it is true that the two lines that express the highest level of U_S11 protein expression and resistance to infection have both declined. We have subcloned

cell lines from U_S11cl19, and while it is true that the two lines that express the highest level of U_S11 protein are also completely resistant to infection, there is no correlation in the collection of other subclones between the level of U_S11 protein expression and the resistance to infection (data not shown). The design of the U_S11cl19 cell line should have permitted an easy test of the hypothesis that U_S11 expression is required for resistance. U_S11 coding sequence was placed under the control of the α4-responsive gB promoter in cells that express the HSV-1 strain F α4, which is temperature sensitive. It should thus have been possible to diminish expression of U_S11 protein by shifting cells to the nonpermissive temperature for α4 function. Unfortunately, growth at the nonpermissive temperature does not appreciably diminish U_S11 protein expression in U_S11cl19 cells, suggesting that U_S11 protein is expressed in an α4-independent manner.

The ease with which we were able to clone highly resistant cell lines from unselected BHK(tk^-) cells (Tables 2 and 3) demonstrates that spontaneous isolation of a resistant cell line is not a rare event. None of the cell lines so isolated, however, was very much more susceptible to the mutant R5000 than to HSV-1(F), suggesting that we did not spontaneously isolate cells showing the same mechanism of resistance as U_S11cl19 cells. The high frequency of isolation of BHK(tk^-) cell clones that are resistant to infection by a different mechanism(s) does, however, suggest that any cloned BHK(tk^-) cell line, including U_S11cl19, may be resistant to infection by multiple mechanisms. We are confident that we have characterized one such mechanism in U_S11cl19 cells but cannot presently rule out the possibility that there are others superimposed.

ACKNOWLEDGMENTS

We thank Lenore Pereira, Roselyn Eisenberg, and Gary Cohen for the gift of monoclonal antibodies.

These studies were aided by Public Health Service grants from the National Cancer Institute (CA47451) and the National Institute for Allergy and Infectious Diseases (AI124009) and by an unrestricted grant from the Infectious Diseases Program of Bristol Myers Squibb.

REFERENCES

1. **Arsenakis, M., J. Hubenthal-Voss, G. Campadelli-Fiume, L. Pereira, and B. Roizman.** 1986. Construction and properties of a cell line constitutively expressing the herpes simplex virus glycoprotein B dependent on functional α4 protein synthesis. J. Virol. **60:**674–682.
2. **Brandimarti, R., T. Huang, B. Roizman, and G. Campadelli-Fiume.** Mapping of herpes simplex virus 1 genes with mutations which overcome host restrictions to infection. Proc. Natl. Acad. Sci. USA, in press.
3. **Campadelli-Fiume, G., M. Arsenakis, F. Farabegoli, and B. Roizman.** 1988. Entry of herpes simplex virus 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.
4. **Campadelli-Fiume, G., A. Avitabile, S. Fini, D. Stirpe, M. Arsenakis, and B. Roizman.** 1988. Herpes simplex virus glycoprotein D is sufficient to induce spontaneous pH-independent fusion in a cell line that constitutively expresses the glycoprotein. Virology **166:**598–602.
5. **Campadelli-Fiume, G., S. Qi, E. Avitabile, L. Foa-Tomasi, R. Brandimarti, and B. Roizman.** 1990. Glycoprotein D of herpes simplex virus encodes a domain which precludes penetration of cells expressing the glycoprotein by superinfecting herpes simplex virus. J. Virol. **64:**6070–6079.
6. **Cohen, G. H., V. J. Isola, J. Kuhns, P. W. Berman, and R. J. Eisenberg.** 1986. Localization of discontinuous epitopes of herpes simplex virus glycoprotein D: use of a nondenaturing ("native" gel) system of polyacrylamide gel electrophoresis coupled with Western blotting. J. Virol. **60:**157–166.
7. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. J. Gen. Virol. **2:**357–364.
8. **Fuller, A. O., and W.-C. Lee.** 1992. Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. J. Virol. **66:**5002–5012.
9. **Fuller, A. O., and P. G. Spear.** 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. Proc. Natl. Acad. Sci. USA **84:**5454–5458.
10. **Gibson, W., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10:**1044–1052.
11. **Gibson, W., and B. Roizman.** 1974. Proteins specified by herpes simplex virus. X. Staining and radiolabeling properties of B capsid and virion proteins in polyacrylamide gels. J. Virol. **13:**155–165.
12. **Highlander, S., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso.** 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. **61:**3356–3364.
13. **Johnson, D. C., R. L. 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.
14. **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.
15. **Johnson, P. A., C. MacLean, H. S. Marsden, R. G. Dalziel, and R. D. Everett.** 1986. The product of gene U_S11 of herpes simplex virus type 1 is expressed as a true late gene. J. Gen. Virol. **67:**871–883.
16. **Johnson, R. M., and P. G. Spear.** 1989. Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. J. Virol. **63:**819–827.
17. **Langeland, N., H. Holmsen, G. R. Lillehaug, and L. Haarr.** 1987. Evidence that neomycin inhibits binding of herpes simplex virus type 1 to the cellular receptor. J. Virol. **61:**3388–3393.
18. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62:**1486–1494.
19. **MacLean, C. A., F. J. Rixon, and H. S. Marsden.** 1987. The products of gene U_S11 of herpes simplex virus type 1 are DNA-binding and localize to the nucleoli of infected cells. J. Gen. Virol. **68:**1921–1937.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Mavromara-Nazos, P., M. Ackermann, and B. Roizman.** 1986. Construction and properties of a viable herpes simplex virus 1 recombinant lacking the coding sequences of the α47 gene. J. Virol. **60:**807–812.
22. **McGeoch, D. J., A. Dolan, S. Donald, and F. J. Rixon.** 1986. 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.
23. **Muggeridge, M. I., V. J. Isola, R. A. Byrn, T. J. Tucker, A. C. Minson, J. C. Glorioso, G. H. Cohen, and R. J. Eisenberg.** 1988. Antigenic analysis of a major neutralization site of herpes simplex virus glycoprotein D, using deletion mutants and monoclonal antibody-resistant mutants. J. Virol. **62:**3274–3280.
24. **Muggeridge, M. I., T.-T. Wu, D. C. Johnson, J. C. Glorioso, R. J. Eisenberg, and G. H. Cohen.** 1990. Antigenic and functional analysis of a neutralization site of HSV-1 glycoprotein D. Virology **174:**375–387.
25. **Noble, A. G., G. Lee, R. Sprague, M. L. Parish, and P. G. Spear.** 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 1. Virology **129:**218–224.
26. **Pereira, L., D. V. Dondero, D. Gallo, V. Devlin, and J. D. Woodie.** 1982. Serological analysis of herpes simplex virus types 1 and 2 with monoclonal antibodies. Infect. Immun. **35:**363–367.
27. **Post, L. E., and B. Roizman.** 1981. A generalized technique for the deletion of specific genes in large genomes: α gene 22 of herpes

simplex virus 1 is not essential for growth. Cell **25:**227–232.
28. **Puissant, C., and L.-M. Houdebine.** 1990. An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. BioTechniques **8:**148–149.
29. **Puvion-Dutilleul, F.** 1987. Localization of viral-specific 21 kDa protein in nucleoli of herpes simplex infected cells. Eur. J. Cell. Biol. **43:**487–498.
30. **Rixon, F. J., and D. J. McGeoch.** 1984. A 3′ co-terminal family of mRNAs from the herpes simplex virus type 1 short region: two overlapping reading frames encode unrelated polypeptides one of which has a highly reiterated amino acid sequence. Nucleic Acids Res. **12:**2473–2587.
31. **Roizman, B., and P. G. Spear.** 1968. Preparation of herpes simplex virus of high titer. J. Virol. **2:**83–84.
32. **Roller, R. J., and B. Roizman.** 1990. The herpes simplex virus U_S11 open reading frame encodes a sequence-specific RNA-binding protein. J. Virol. **64:**3463–3470.
33. **Roller, R. J., and B. Roizman.** 1991. Herpes simplex virus 1 RNA binding protein U_S11 negatively regulates the accumulation of a truncated viral mRNA. J. Virol. **65:**5873–5879.
34. **Roller, R. J., and B. Roizman.** 1992. The herpes simplex virus 1 RNA binding protein U_S11 is a virion component and associates with ribosomal 60S subunits. J. Virol. **66:**3624–3632.
35. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B_2) in virion infectivity. J. Virol. **29:**1149–1158.
36. **Schwartz, J., and B. Roizman.** 1969. Similarities and differences in the development of laboratory strains and freshly isolated strains of herpes simplex virus in HEp-2 cells: electron microscopy. J. Virol. **4:**879–889.
37. **Watson, R. J., and G. F. Vande Woude.** 1982. DNA sequence of an immediate early gene (IE mRNA-5) of herpes simplex virus type 1. Nucleic Acids Res. **10:**979–991.