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Glycoprotein D of Herpes Simplex Virus Encodes a Domain Which Precludes Penetration of Cells Expressing the Glycoprotein by Superinfecting Herpes Simplex Virus

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Earlier studies have shown that herpes simplex viruses adsorb to but do not penetrate permissive baby hamster kidney clonal cell lines designated the BJ series and constitutively expressing the herpes simplex virus 1 (HSV-1) glycoprotein D (gD). To investigate the mechanism of the restriction, the following steps were done. First, wild-type HSV-1 strain F [HSV-1(F)] virus was passaged blindly serially on clonal line BJ-1 and mutant viruses [HSV-1(F)U] capable of penetration were selected. The DNA fragment capable of transferring the capacity to infect BJ cells by marker transfer contains the gD gene. The mutant gD, designated gDU, differed from wild-type gD only in the substitution of Leu-25 by proline. gDU reacted with monoclonal antibodies which neutralize virus and whose epitopes encompass known functional domains involved in virus entry into cells. It did not react with the monoclonal antibody AP7 previously shown to react with an epitope which includes Leu-25. Second, cell lines expressing gDU constitutively were constructed and cloned. Unlike the clonal cell lines constitutively expressing gD (e.g., the BJ cell line), those expressing gDU were infectable by both HSV-1(F) and HSV-1(F)U. Lastly, exposure of BJ cells to monoclonal antibody AP7 rendered the cells capable of being infected with HSV-1(F). The results indicate that (i) gD expresses a specific function, determined by sequences at or around Leu-25, which blocks entry of virus into cells synthesizing gD, (ii) the gD which blocks penetration by superinfecting virus is located in the plasma membrane, (iii) the target of the restriction to penetration is the identical domain of the gD molecule contained in the envelope of the superinfecting virus, and (iv) the molecular basis of the restriction does not involve competition for a host protein involved in entry, as was previously thought.

The series of events which culminate in the infection of susceptible cells by herpes simplex virus 1 (HSV-1) involve (i) attachment of components of the viral envelope to receptors in the plasma membrane, (ii) fusion of the viral envelope with the plasma membrane, (iii) release of the capsid into the cytoplasm and its transport to the nuclear pore, and (iv) release of the viral DNA into the nucleus (28). The observation that empty capsids accumulated at nuclear pores of cells late after infection with a mutant virus led to the hypothesis that a viral function precludes reinfection of cells by progeny of the infection (34). Our laboratories provided the first direct evidence for such a mechanism by showing that wild-type virus attaches to cells expressing a viral glycoprotein designated D (gD) but that penetration of cells leading to productive infection does not ensue (4). This report is a continuation of these studies; we selected spontaneous virus mutants which are capable of infecting cells expressing wild-type gD and report that (i) in cells restricting penetration, the functional gD molecule is on the surface of the plasma membrane, (ii) gD contains a functional domain which restricts infection independent of other functions of the molecule, and (iii) the target of the restriction, i.e., the virion component which is recognized by the restrictive domain, is an identical domain on the gD molecule situated in the virion envelope.

Relevant to this report are the following data.

(i) HSV encodes eight glycoproteins, designated gB, gC, gD, gE, gH, gG, gI (1, 9, 17, 19, 26, 32, 33), and gJ, the product of the US5 (21) open reading frame (N. Frenkel, personal communication). Of these, five, i.e., gC, gE, gG, gI, and gJ, are dispensable for replication in most cells in culture (11, 17–19, 36). By default, because these genes are not dispensable (3, 7, 16), all functions related to entry, maturation, egress, and restriction to superinfection can be performed by the glycoproteins gB, gD, and gH. The function of the dispensable glycoproteins is not known. Evidence presented elsewhere suggests that the dispensable components may, in some cells, perform functions similar to those of the three nondispensable glycoproteins with respect to entry into cells (5a).

(ii) The initial studies were done with baby hamster kidney cells transfected with a vector carrying a gene specifying a dihydrofolate reductase resistant to methotrexate and the HSV-1 *Bam*HI J fragment (2). This fragment encodes gG, gJ, gD, gI, and a portion of gE. The clonal lines of the methotrexate-resistant cells were analyzed for viral gene expression, and several clones expressing relatively large amounts of gD were then tested for the capacity to become infected with wild-type virus. Extensive protein and RNA analyses indicated that the cells expressed only gD and none of the other viral genes (2) and therefore that the restriction to penetration was due solely to the expression of the gD gene. This conclusion was confirmed by Johnson and Spear (13). Subsequently, Johnson et al. (12) reported that truncated gD molecules released from cells were able to bind to cells and to block infection with wild-type virus. The studies reported

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here were done with one of the clonal cell lines constitutively expressing gD and designated BJ-1 (4).

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney cells deficient in thymidine kinase (BHKtk$^-$); Vero, rabbit skin, and 293 (10) cells; and the BJ clone 1 of BHKtk$^-$ cells constitutively expressing gD (4), hereafter designated BJ cells, were grown in Dulbecco modified minimum essential medium containing 5% fetal calf serum. BHK-21 clone 13 cells were grown in the same medium containing 10% newborn calf serum. HSV-1 strain F [HSV-1(F)] (8), the wild-type virus used in these studies, and recombinant virus R7048 (17) were described previously. Recombinant R7037 is a derivative of R7036 (19) and differs from R7036 in that the deletion of the thymidine kinase gene was repaired by cotransfection with the HSV-1 *Bam*HI Q fragment.

Selection of HSV-1(F)U-10. BJ cells were exposed to 10 PFU of HSV-1(F) per cell. At 48 h postexposure, the cells were harvested and the lysates were used to reinfect BJ cells for three serial blind passages. Progeny virus from the fourth passage was grown in BHK cells and designated HSV-1(F)U. Sixteen plaque-purified viruses were obtained by plating HSV-1(F)U in Vero cell monolayers and were designated HSV-1(F)U-1 to HSV-1(F)U-16.

Plasmids. pGCF1068, pGCF1070, and pGCF1088 carry the *Bam*HI J and G fragments from HSV-1(F)U-10 DNA and the *Bam*HI J fragment from HSV-1(F) DNA, respectively, cloned in the *Bam*HI site of pUC19. The *Hin*dIII-*Tth*III subclones of pGCF1068 and pGCF1088 in pUC19 were named pGCF1068.7 and pGCF1088.11, respectively. pGCF1080 contains the *Bam*HI J fragment from HSV-1(F)U-10 DNA cloned in the *Sal*I site of pFR400 (31). pGCF1081 contains the 1,628-bp *Hin*dIII-*Tth*III fragment of pGCF1068.7 cloned in the *Bam*HI site of pRP-RSV vector (20).

Nucleotide sequence. *Rsa*I and *Taq*I subclones of pGCF1068.7 and pGCF1088.11 in pUC19 were sequenced on both strands with dideoxynucleotides, Sequenase 2 (ICN Laboratories), and [α-^{32}P]dCTP (3,000 Ci/mmol; Radiochemical Center, Amersham) as described previously (30).

Transfections. The *Bam*HI J and G DNA fragments were cleaved from pGCF1068 and pGCF1070, respectively, and electroeluted from agarose gels. Each fragment was cotransfected with HSV-1(F) DNA onto rabbit skin cells. Transfections were done as previously described (2).

Construction of BHK-BJU and 293-gDU cell lines. BHK-BJU cell lines were constructed by transfection of pGCF1080 DNA onto BHKtk$^-$ cells. The methotrexate-resistant cells were selected as previously described (2). 293-gDU cell lines were constructed by cotransfection of pGCF1081 DNA and pSV$_2$-neo DNA onto 293 cells. Cells acquiring resistance to neomycin G418 (GIBCO Laboratories) were selected. In both cases, clonal cell lines were prepared and tested for constitutive expression of gD as previously described (2).

Monoclonal antibodies. The monoclonal antibodies tested to differentiate between HSV-1(F) and the mutant HSV-1(F)U-10 were the gifts of L. Pereira (monoclonal antibodies HD1 [27], H106.1, H128.52, H170 [6], H606, H902, H919, H984, H997, H1101.4, H1034.1, H1270, H1296.1, H1378.3, H1380, H1423.2, H1522.3, H1526.1, H1557.3, H1589.2, H1591.1, and H1595.3), A. Minson (monoclonal antibody AP7 [22]), and P. G. Spear (monoclonal antibody III 114.4 [25]).

Labeling of cells and radioimmunoprecipitations. Cells were labeled at the indicated times postinfection with ^{35}S-methionine (1,300 Ci/mmol; Radiochemical Center, Amersham). gD was immunoprecipitated from cell lysates with the monoclonal antibodies specified in Results as detailed elsewhere (2). Samples were subjected to electrophoresis in sodium dodecyl sulfate–8.5% polyacrylamide gels cross-linked with *N,N'*-dialliltartardiamide. Fixed gels were soaked in Amplify and exposed to Kodak X-Omat films for fluorography.

Exposure of cells to monoclonal antibody AP7. To test the effect of AP7 monoclonal antibodies, replicate cultures in 24-well dishes were exposed to (i) indicated amounts of AP7 for 1 h at 37°C and then to HSV-1(F) or HSV-1(F)U-10 (10 PFU per cell) for 1 h in the same medium containing AP7, (ii) medium containing AP7 for 1 h at 37°C followed by a thorough rinse to remove the antibody and then infected as described above, or (iii) to an approximately 1,000-fold dilution of a mixture of virus and AP7 which had been preincubated for 1 h at 37°C. The cells were labeled with ^{35}S-methionine at the times indicated in Results.

RESULTS

Selection of spontaneous viral mutants capable of penetrating BJ cells. Monolayer cultures of BJ cells were exposed to 10 PFU of HSV-1(F) per cell, and lysates of the exposed cells were serially propagated on BJ cells three times. The appearance of cytopathic effects suggested the presence of mutants capable of penetrating and multiplying in BJ cells. Evidence of such mutants in the lysates of the fourth serial passage is shown in Fig. 1. In this experiment, BJ cells were exposed to either the parental HSV-1(F) or to the lysate of the fourth serial passage, designated F(U) virus, at multiplicities of 10 PFU per cell. The autoradiographic images of electrophoretically separated lysates of infected cells labeled with ^{35}S-methionine at different times after exposure to the viruses showed (Fig. 1) that the BJ cells infected with F(U) virus made the entire spectrum of viral proteins, whereas no viral proteins could be detected in cells infected with the parent virus.

Plaque purification of viruses unrestricted in BJ cells. To characterize the viruses unrestricted for growth in BJ cells, we selected and plaque purified 16 viral clones designated HSV-1(F)U-1 to HSV-1(F)U-16. The expression of viral genes of selected clones is shown in Fig. 2. All of the isolates expressed the entire spectrum of viral polypeptide bands in the parental BHKtk$^-$ cells. The HSV-1(F)U isolates, however, were less efficient in infecting the BJ cells than BHKtk$^-$ cells, probably because of a reduced efficiency in penetration. The clonal isolate HSV-1(F)U-10 was chosen for further studies.

The *Bam*HI J fragment of HSV-1(F)U-10 DNA transfers the capacity to penetrate BJ cells. Previous studies have shown that only three viral membrane proteins were essential for the entry of HSV-1 into cells (3, 4, 9, 16). As an initial approximation of the possible site of the mutation in HSV-1(F)U-10, we cloned the DNA fragments encoding gD (*Bam*HI-J) and gB (*Bam*HI-G) individually in pUC19 and cotransfected the fragments with HSV-1(F) DNA onto rabbit skin cells. The progeny of transfections with viral DNA alone or with *Bam*HI-J or *Bam*HI-G were then passaged once in BJ cells in order to enrich for recombinants. The progeny of this single passage were tested for the ability to form plaques and to specify viral protein synthesis in BJ and BHKtk$^-$ cells. The results (Table 1) show that the progeny

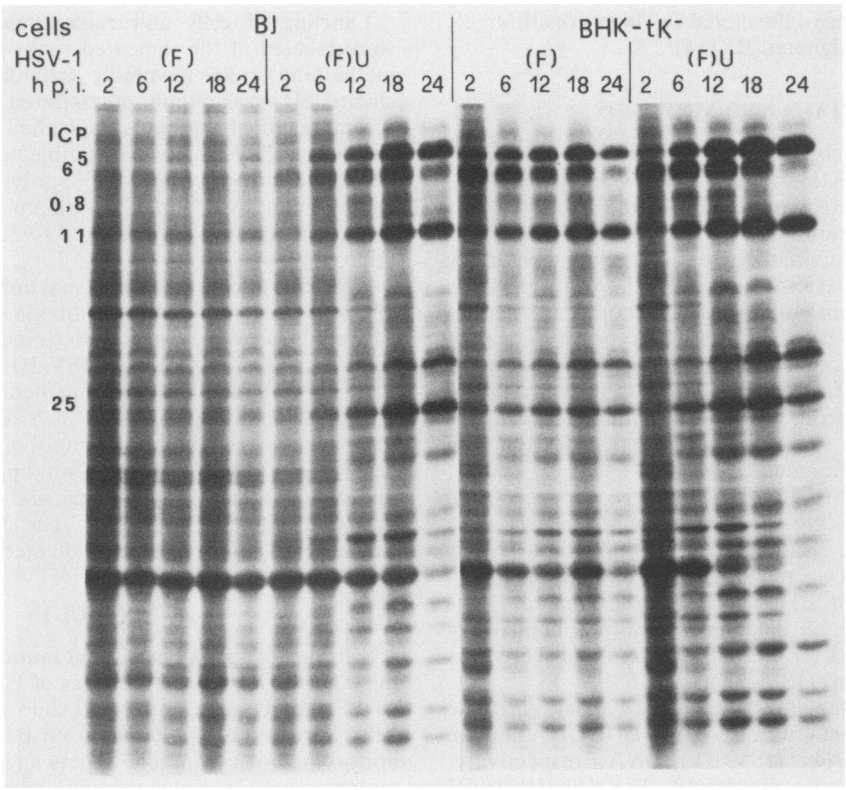

FIG. 1. Fluorographic image of proteins synthesized in BJ and BHKtk⁻ cells infected with HSV-1(F) [(F)] or with HSV-1(F)U [(F)U]. Cells were exposed to 10 PFU per cell, metabolically labeled for 1 h with ^{35}S-methionine at the indicated hour postinfection (h p.i.), harvested, solubilized in denaturing buffer, and electrophoretically separated in denaturing polyacrylamide gels. ICP, Infected-cell protein number.

of the transfection with *Bam*HI-J were enriched several hundredfold for the capacity to form plaques in BJ cells with respect to the progeny of transfection with parental DNA alone or in combination with *Bam*HI-G DNA. Furthermore, the progeny of transfection with *Bam*HI-J contained recombinant viruses in amounts sufficient to direct detectable amounts of viral protein synthesis in BJ cells (Fig. 3). We conclude therefore that the capacity to penetrate BJ cells was encoded in the *Bam*HI J fragment of HSV-1(F)U-10.

Nucleotide sequence of HSV-1(F)U-10 gD gene. The *Bam*HI J DNA fragment of HSV-1 encodes gG, gD, gI, and a portion of gE (15, 17, 19, 26, 29). Earlier studies have shown that only gD was expressed in BJ cells and that the restriction to penetration was due solely to gD (4, 13). In a preliminary series of experiments, BJ and, for comparison, BHKtk⁻ cells were each exposed to 10 PFU of either the parent HSV-1(F) or HSV-1(F) with deletion mutations in gG, gE, and gI-gE genes (designated R7037 [19], R7032 [18], and R7048 [17], respectively). The cells were labeled at 9 h postinfection with ^{35}S-methionine for 1 h. Analyses of electrophoretically separated proteins in denaturing gels (data not shown) did not reveal the presence of viral specific proteins in BJ cells. The results of this experiment excluded gG, gE, and gI as the targets of the restriction manifested by the BJ cells. The conclusion that the mutation which ablates the restriction to infection of BJ cells is located in the glycoprotein D gene rests on the nucleotide sequence described in this section and the evidence described below that the gD gene from HSV-1(F)U-10 virus has a novel biologic property in that cells expressing the mutated gD did not block infection by HSV-1(F). Nucleotide sequence analyses showed that in fact the coding sequence of the mutant gD differed from that of the wild-type gD. The results, reported in part in Fig. 4 and summarized in Table 2, indicate the following conclusions.

(i) A single base pair substitution was found within HSV-1(F)U-10 gD-coding sequence relative to that of the parental HSV-1(F) gene (Fig. 4A). Specifically, it was a T-149-to-C transition that predicts substitution of Leu-25 with proline in the mature form of gD after cleavage of the signal sequence. This is the only difference between the gD genes of the parent and mutant viruses.

(ii) Both HSV-1(F)U-10 gD and HSV-1(F) gD differ from the gD of HSV-1(17) (21) in 3 bp. The net effect of these substitutions is the change of the terminal amino acid of the signal sequence from glycine to serine and of amino acids 5 and 342 (post-signal-sequence cleavage) from alanine to valine and from arginine to histidine, respectively. The glycine, alanine, and arginine at positions −1, 5, and 342 are conserved in other sequences reported to date (14, 22, 35) (Table 2).

Immunoreactivity of mutant gD from HSV-1(F)U-10. The purpose of the experiment described in this section was to determine the effect of the mutation on the reactivity of monoclonal antibodies whose epitopes map in the vicinity of the mutation in HSV-1(F)U-10. The studies by Cohen and Eisenberg and others (reviewed in reference 23) have delineated several groups of monoclonal antibodies, each reactive with an epitope domain on HSV-1 gD. Of these, three are of interest. Thus, HD1 (27) was reported to react with the domain Ia of the discontinuous epitope I associated with virus entry (27) and cell fusion (5) located in part at the amino terminus and in part at or near amino acid 216 (24). Monoclonal antibody III 114.4 (25) belongs to the same

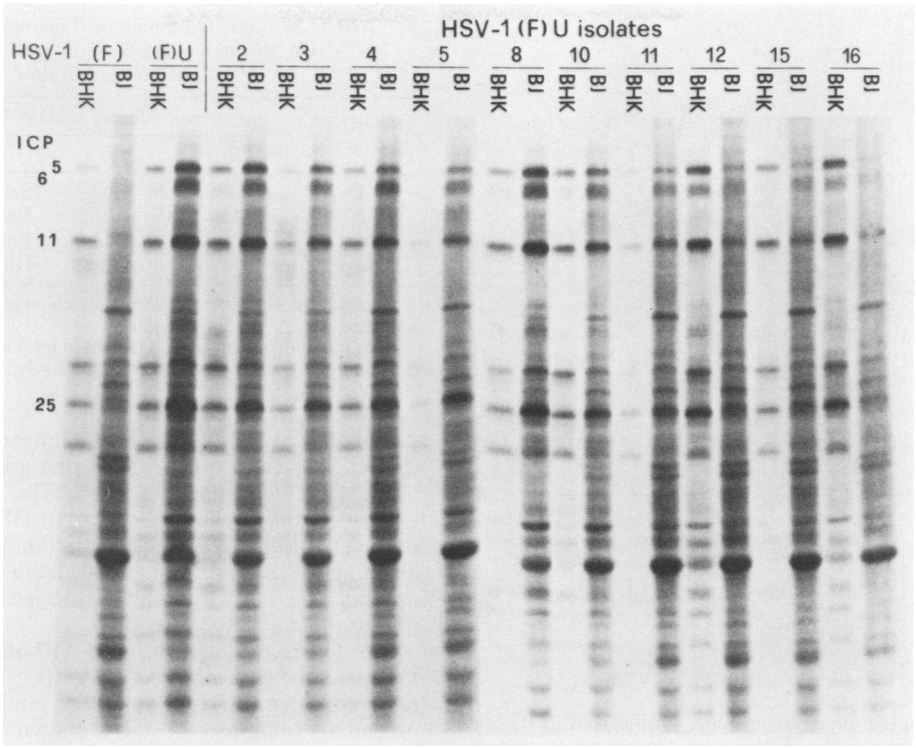

FIG. 2. Fluorographic image of proteins synthesized in BJ and BHKtk$^-$ cells infected with HSV-1(F), HSV-1(F)U, or representative plaque-purified HSV-1(F)U isolates identified by number. Cells were labeled for 1 h with ^{35}S-methionine at 16 h postinfection and processed as described in the legend to Fig. 1. ICP, Infected-cell protein number.

group but recognizes the domain Ib located between amino acids 134 and 140 (24). Monoclonal antibody 170 (6) reacts with the epitope VII mapped between amino acids 8 and 23. Of particular interest was also a monoclonal antibody (AP7) reported by Minson and associates (22) which does not fit into any of the groups identified by Cohen and Eisenberg (23). AP7 neutralized HSV-1 but only in the presence of a complement. An HSV-1 mutant resistant to AP7 was isolated and sequenced by Minson and associates (22). They reported that in the AP7 antibody-resistant virus, the only mutation involved the substitution of Leu-25 with proline, i.e., the genotype of the mutation is identical to that of HSV-1(F)U-10.

The results of the immune precipitations shown in Fig. 5 indicate that as expected, monoclonal antibody AP7 reacted with the parent but not with the mutant HSV-1(F)U-10 gD. All other monoclonal antibodies tested (as listed in Materials and Methods) reacted with both parent and mutant gD. The results with HD1 and III 114.4 indicate that the mutation in gDU does not modify the immunoreactivity of the epitope domains Ia and Ib previously related to the entry of virus into cells.

TABLE 1. Frequency of transfer of HSV-1(F)U-10 phenotype by *Bam*HI DNA fragments J and G

HSV-1(F)U-10 *Bam*HI fragment	No. of plaques in cells		Ratio (10^{-3})
	BJ	BHKtk$^-$	
None[a]	$<1.0 \times 10^1$	5.0×10^6	<0.002
J	4.0×10^4	2.7×10^6	14.8
G	4.2×10^2	1.8×10^7	0.02

[a] Parental HSV-1(F) DNA only.

Phenotype of cell lines expressing HSV-1(F)U-10 gD. In one series of experiments, we constructed cell lines expressing the gD gene cloned from HSV-1(F)U-10 designated gDU. Two constructs were made (Fig. 6). In the first, the *Bam*HI J fragment from HSV-1(F)U-10 DNA was cloned into pFR400 vector (31) and the resultant recombinant plasmid, pGCF1080, was transfected onto BHKtk$^-$ cells. Of the 24 individual methotrexate-resistant clones tested, those designated BHK-BJU-l, BHK-BJU-n, and BHK-BJU-z expressed the largest amounts of gD (Fig. 7A). In the second construction, the 1,628-bp *Hin*dIII-*Tth*III fragment from HSV-1(F)U-10 *Bam*HI fragment J DNA containing the coding sequences of gD was cloned under the control of the Rous sarcoma virus long terminal repeat in pRP-RSV vector (20). DNA from the resultant plasmid pGCF1081 and pSV_2-neo DNA were cotransfected onto 293 cells, a human kidney cell line that expresses adenovirus E1a and E1b (10). Of the 15 G418-resistant cell clones tested, those designated 293gDU-8 and 293gDU-3 produced the highest and intermediate amounts of gD, respectively (Fig. 7B). Comparisons showed that the amounts of gD available for precipitation by HD1 antibody in BJ, BHK-BJU-z, and 293gDU-3 were nearly equivalent but lower than those made in 293gDU-8 clonal cell lines (Fig. 7C).

In the next series of experiments, we exposed BHKtk$^-$, BJ, BHK-BJU-z, 293gDU-3, and 293gDU-8 clonal cell lines to 10 PFU of HSV-1(F) or HSV-1(F)U-10 per cell. The infected cells were labeled for 1 h with ^{35}S-methionine at 16 h postinfection and then harvested. The salient features of the results shown in Fig. 8 are as follows. All cell lines

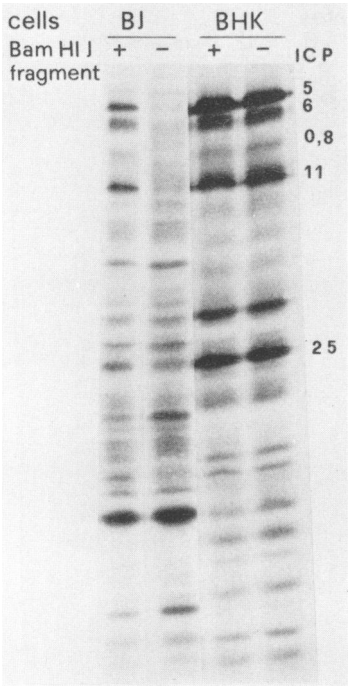

FIG. 3. Fluorographic image of proteins synthesized in BJ and BHKtk$^-$ cells exposed to the progeny of the cotransfection of HSV-1(F) DNA with (+) or without (−) the *Bam*HI J fragment from HSV-1(F)U-10 DNA cloned as pGCF1068. Cells were labeled with ^{35}S-methionine for 1 h at 16 h postinfection and processed as described in the legend to Fig. 1. ICP, Infected-cell protein number.

exposed to HSV-1(F)U-10 produced viral proteins at the time of harvest. Viral proteins were also made by all cell lines exposed to HSV-1(F) except the BJ cell line. We should note that although at 16 h the infected cells made predominantly HSV proteins, the accumulation of viral proteins in cells expressing gDU was slower than in the parental BHKtk$^-$ cells. The amount of gDU present at steady-state levels varied from one clone to another. Overall, BHK-BJU clonal cell lines were more restrictive than the 293-gDU

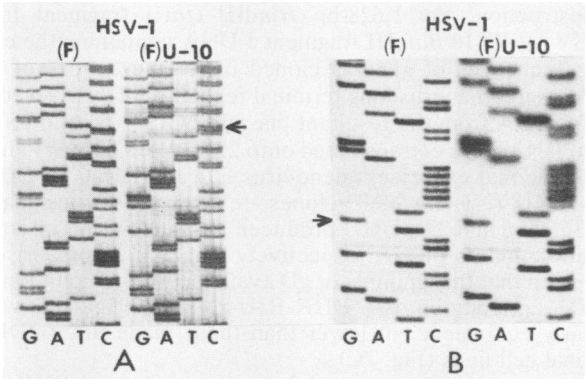

FIG. 4. Autoradiographic image of sequencing gels. (A). The arrow points to the T-149-to-C transition in the gD gene of HSV-1(F)U-10 relative to that of the parental HSV-1(F). (B) The arrow points to the G residue at position 685 in both HSV-1(F) and HSV-1(F)U-10. The substitution of C with G does not alter the serine at position 203.

TABLE 2. Predicted amino acid changes in gD sequence of HSV-1(F)U-10, parental HSV-1(F), and other HSV-1 strains sequenced to date

Amino acid no.[a]	Change in gD of HSV-1 strain[b]:				
	(F)U-10	(F)	17	Hzt	Patton
−1	Gly	Gly	Ser	Gly	Gly
5	Ala	Ala	Val	Ala	Ala
25	Pro	Leu	Leu	Leu	Leu
342	Arg	Arg	His	Arg	Arg

[a] Amino acid 1 is the first amino acid remaining after cleavage of the signal sequence.

[b] The nucleotide sequences were derived from references 21, 14, and 35 for HSV-1 strains 17, Hzt, and Patton, respectively.

clonal lines. The difference may reflect the amount of gD at the cell surface or other factors that are presently unknown.

We conclude that the phenotype of the mutated gD exhibits two key characteristics: (i) HSV-1 carrying gDU is relatively insensitive to the restriction imposed by wild-type gD, and (ii) cells expressing gDU have a reduced capacity to restrict infection with either wild-type or mutant virus relative to the capacity of BJ cells.

Effect of monoclonal antibody AP7 on the ability of BJ cells to restrict the penetration of HSV-1(F). Two observations are of significance. First, in an earlier publication (4) and in the preceding sections, we have shown that gD can restrict penetration of wild-type virus into cells expressing gD and that the mutation in gD which leads to a loss of restriction resulted in the substitution of Leu-25 with proline. These results suggest that the functional site in gD which restricts penetration is at or near Leu-25. Second, we have shown that monoclonal antibody AP7 reacted with the wild-type gD but not with the mutant gD, gDU. This antibody does not appear to play a role in the evolution of HSV-1 infection in the absence of complement (22). Substitution of Leu-25 with proline had no effect on the reactivity of gDU with HD1 and other monoclonal antibodies mapped to gD domains which include determinants of virus entry and cell fusion. If the restriction to penetration maps specifically to Leu-25 and is destroyed by the proline substitution, as these results suggest, it could be expected that exposure of the cells producing gD to AP7 antibody should result in removal of the restriction. To test this hypothesis, three series of experiments were done as follows.

In a preliminary experiment, the BJ and BHKtk$^-$ cell lines were preincubated for 1 h at 37°C with AP7 monoclonal

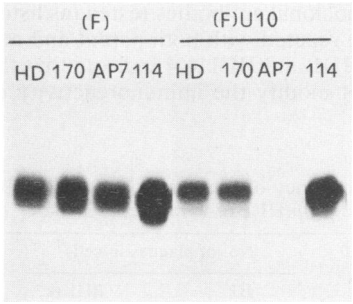

FIG. 5. Fluorographic image of gD immunoprecipitated from lysates of BHK cells infected with HSV-1(F) or HSV-1(F)U-10 and electrophoretically separated in denaturing polyacrylamide gels. The monoclonal antibodies tested were HD1 (HD), 170, AP7, and III 114.4 (114).

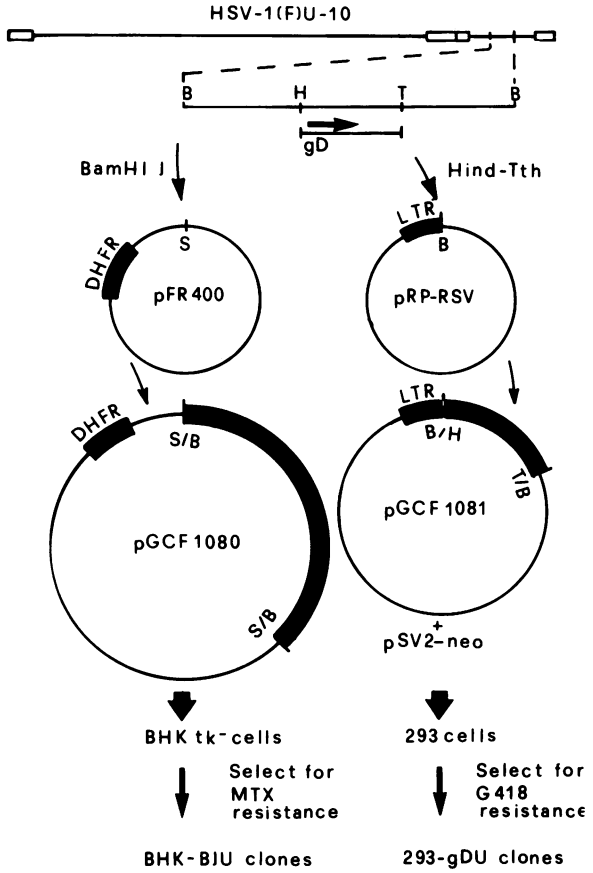

FIG. 6. Schematic representation of the construction of BHK-BJU and 293-dGU cell lines. The BHK-BJU cell line contains the *Bam*HI J DNA fragment and was selected for methotrexate (MTX) resistance. The 293-gDU cell lines contains the *Hin*dII-*Tth*III subfragment of *Bam*HI-J and was selected for resistance to G418. LTR, Long terminal repeat; DHFR, dihydrofolate reductase; B, *Bam*HI; H, *Hin*dIII; T, *Tth*III; S, *Sal*I.

antibody as ascitic fluid in the range of 0.1 to 2 μl/150 μl of medium. The cells were then exposed to HSV-1(F) at 10 PFU per cell for 1 h at 37°C in the presence of AP7, since the antibody does not neutralize the virus. Cells were labeled with ^{35}S-methionine at 16 h after infection. The results indicated that exposure of BJ cells to as little as 0.1 μl of AP7 ascitic fluid per 150 μl of medium resulted in a dramatic increase in viral protein synthesis (data not shown).

The second series of experiments was done to establish that the effect of AP7 was on the gD expressed by the uninfected cells rather than on the virus inoculum. The BJ cells were exposed to AP7 for 1 h prior to exposure to the virus (Fig. 9A, lane B), simultaneously with the virus (lane C), or both before and during exposure to the virus (lane D). Lane E shows the effect of incubation of the virus inoculum with AP7 prior to the exposure of the inoculum to the BJ cells. The results (Fig. 9A) show that exposure of BJ cells to AP7 enabled the expression of viral proteins and that pretreatment of BJ cells with AP7 was as efficient as exposure of the cells to antibody and virus concurrently. Conversely, exposure of the virus rather than the cells to AP7 did not enable the expression of viral proteins in these cells (Fig. 9A, lane E).

In the third series of experiments, BHKtk$^-$ and BJ cells were exposed to HD1 for 90 min to concentrations of monoclonal antibody ranging from 0 to 2 μl/150 μl of medium. We should note that the batches of ascitic fluids of HD1 and AP7 immunoprecipitated approximately equivalent amounts of gD from lysates of HSV-1-infected cells (Fig. 5). The cells were washed and exposed to 10 PFU of HSV-1(F) per cell. Viral protein synthesis was unaffected in BHKtk$^-$ cells exposed to the antibody (Fig. 9B, lanes A and B). In BJ cells exposed to the HD1 antibody, viral protein synthesis was barely detectable. Similar results were also obtained with H170 monoclonal antibody (data not shown).

DISCUSSION

In an earlier publication (4), our laboratories reported that cells expressing HSV-1 gD allowed attachment but restricted the penetration of wild-type HSV-1 and HSV-2. We suggested as one hypothesis that could explain our findings that for entry into cells, HSV-1 gD must interact with a host protein ligand, and therefore gD made in the uninfected cells could sequester the ligand and make it unavailable to the gD carried by the infecting virus. Subsequently, Johnson and colleagues (12) extended our studies and calculated that an uninfected cell must have as many as 4×10^5 such molecules per cell. In this paper, we report an extension of our initial studies. The rationale on which our studies were based is that if gD of HSV-1, a human herpesvirus, sequesters a nonhuman (BHK) ligand, we should be able to select spontaneous mutants which have a higher affinity for the ligand and by mapping the mutation define the nature of the binding sites. We have indeed selected mutants capable of overcoming the restriction to penetration. The properties of the mutated gD and of the HSV-1(F)U mutants investigated to date are as follows. (i) The mutant virus is capable of attaching to and penetrating cells expressing wild-type gD (the BJ cells). (ii) The mutation in gD is not extremely rare inasmuch as the viral mutants were selected in only four serial passages of the parent virus on BJ cells. (iii) Cells expressing the mutated gD (BHK-BJU and 293-dGU cell lines) are unrestricted, qualitatively allowing the attachment and penetration of both wild-type and mutant viruses (Table 3). We should note, however, that the BHK-BJU and the 293-DGU cell lines are not as susceptible to infection as the parental cell lines. The significant finding to emerge from these studies is that gD has a specialized domain which precludes penetration of cells making gD by HSV and that this domain maps at or near Leu-25. Moreover, the target of the restriction is the identical domain in gD. The central issues are (i) the phenotypes of the mutants, (ii) the significance of the mutation in gD which correlates with the altered phenotype, and (iii) the physiologic function of the restriction during productive infection.

Significance of the HSV-1(F)U phenotype. The results obtained in this study suggest that gD present in surface membranes restricts penetration by a mechanism other than by sequestering a ligand essential for penetration. The hypothesis to be considered here does not exclude the possibility that gD binds one or more host proteins which may act as receptors in cellular membranes for virus entry. The evidence favoring this hypothesis is twofold. First, if gDU had a lower affinity for the putative host ligand than wild-type gD, HSV-1(F)U viruses should not be able to infect BJ cells, since the ligand would be sequestered by gD. By contrast, if gDU had a higher affinity than wild-type gD for the host ligand, then the BJU and 293-gDU cell lines should be at the very least refractory to superinfection by the

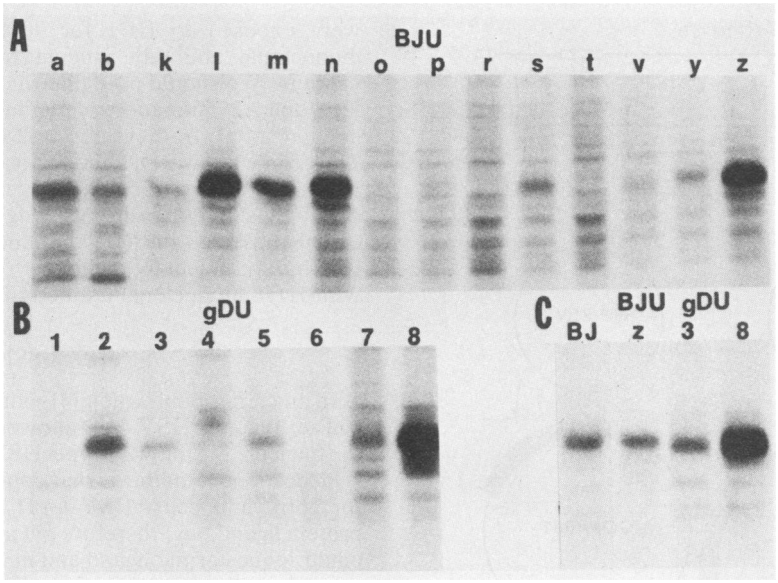

FIG. 7. Fluorographic image of gDU immunoprecipitated with monoclonal antibody HD1 from lysates of BHK-BJU (designated by letter) (A) and 293-gDU (designated by number) (B) clonal cell lines. (C) A comparison of the amounts of gD produced and immunoprecipitated from equivalent amounts of lysates of BJ, BHK-BJU-z, 293-gDU-3, and 293-gDU-8. The clonal cell lines were labeled with ^{35}S-methionine (30 μCi/ml of medium) between 12 and 24 h after seeding.

wild-type HSV-1(F) virus. The observation that HSV-1(F)U viruses are capable of infecting BJ cells and that both the BHK-BJU and the 293-gDU clonal cell lines can be infected by both viruses argues that a ligand required for penetration is not the limiting factor in the infection of BJ cells by HSV-1(F). Parenthetically, this line of evidence also argues against the hypothesis that the mutation in gDU enables the protein to bind to an entirely different host protein than that bound by wild-type gD.

The second line of evidence which argues against sequestration of a ligand essential for entry stems from two considerations. As noted in Results, exposure of the BJ cells to the monoclonal antibody AP7 removes the restriction to penetration of these cells by the HSV-1(F), the wild-type parent, whereas exposure to monoclonal antibody HD1 does not. The hypothesis that the AP7 antibody competes with the ligand for gD in the BJ cells is inconsistent with the available data on the functional domains of the gD molecule. Specifically, the interaction of AP7 antibody with gD does not lead to virus neutralization except in the presence of complement. The mutation in gD which leads to escape from both AP7 antibody binding and restriction to infection of BJ cells does not affect the interaction of gD with monoclonal antibodies (e.g., HD1 and III 114.4) which are capable of neutralizing the virus. Moreover, the known functional sites involved in virus entry and fusion of the envelope and the plasma membrane do not comap with the epitope defined by the AP7 monoclonal antibodies. It is most likely that a cellular receptor does interact with gD, but no evidence exists that still another host ligand binds to gD at or near Leu-25 or the AP7 epitope.

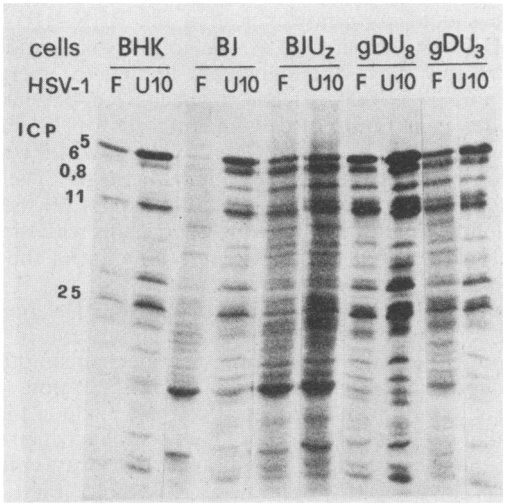

FIG. 8. Fluorographic image of electrophoretically separated proteins from lysates of BJ, BHK-BJU-z, 293-gDU-8, or 293-gDU-3 cells exposed to HSV-1(F) or HSV-1(F)U-10. The multiplicity of exposure was 10 PFU per cell. The cells were labeled for 1 h with ^{35}S-methionine at 16 h postinfection and processed as described in the legend to Fig. 1. ICP, Infected-cell protein numbers.

Significance of the HSV-1(F)U mutation which overcomes the restriction to penetration. As noted in Results, only a single mutation accounts for the ability of the mutant virus carrying gDU to infect BJ cells expressing wild-type gD. To date, no function related to the interaction of HSV-1 with cells has been mapped to that domain. The results presented in this report render untenable the hypothesis that the restriction to penetration by cells expressing gD is exercised through the same domain as the one which enables the virus to penetrate into cells. This is obviously not the case, inasmuch as viral mutants carrying gDU can infect cells, but penetration into cells expressing mutant gD is not restricted. Since gD appears to have distinct domains for entry and restriction of penetration, the question concerns how gD blocks penetration.

The model that best fits the available data is that the gD

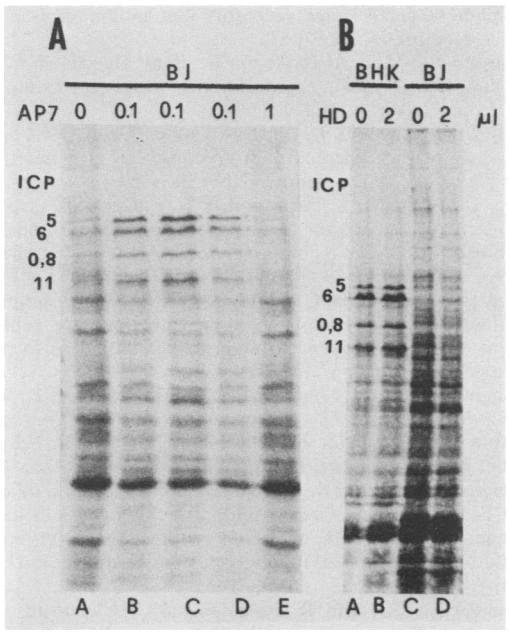

FIG. 9. Fluorographic image of electrophoretically separated proteins from lysates of BJ and BHKtk$^-$ cells exposed to HSV-1(F). (A) Lane A, Cells were exposed to HSV-1(F) with no prior treatment; lane B, cells were exposed to 0.1 µl of AP7 in 150 µl of medium for 1 h at 37°C, rinsed three times, and then exposed to HSV-1(F) for 1 h; lane C, cells were exposed at the same time to AP7 and HSV-1(F) for a 1-h interval; lane D, cells were exposed to 0.1 µl of AP7 in 150 µl of medium for 1 h at 37°C, and virus was added to the AP7-containing medium and allowed to adsorb to cells for an additional hour; lane E, concentrated HSV-1(F) was incubated with AP7 for 1 h at 37°C and then diluted approximately 1,000-fold prior to adsorption to cells. Cells were labeled with ^{35}S-methionine for 1 h starting at 9 h after exposure to virus, harvested, and processed as described in the legend to Fig. 1. (B) BHKtk$^-$ and BJ cells were exposed to 0 or 2 µl of HD1 ascitic fluid in 150 µl of medium for 90 min at 37°C. The cells were rinsed three times for 5 min each time and then were exposed to 10 PFU of HSV-1(F) for 1 h at 37°C. The cells were labeled with ^{35}S-methionine at 8 h postinfection and then harvested and processed as described in the legend to Fig. 1. ICP, Infected-cell protein numbers.

molecules accumulating in the plasma membrane of the uninfected or infected cell interact in a specific fashion with the gD molecules located in the envelope of the infecting virus and that the complex precludes the virus from penetrating into the cell. The model does not exclude the possibility that the gD molecule on the surface of the infected cell binds one or more cellular proteins. It is convenient to define the juxtaposition of cellular gD (CgD) and viral gD (VgD) as occurring in *trans*, whereas the juxtaposition which occurs between two molecules of gD in the same membrane would be in *cis*. According to the model, the interaction in *trans* between the juxtaposed gD would be analogous to that of functional and dysfunctional monomers forming a dimer, because the presence of gDU either as VgDU or as CgDU would enable penetration (Table 3).

The target of gD restriction is gD. The important features of the model are based on two findings described in this report. First, although we have previously shown that the restriction to penetration is exercised by gD, the specific target of gD, i.e., the molecule or structure on the infecting virus which is blocked by gD, was not known. Current studies indicate that

TABLE 3. Observed outcome of infection of cells expressing wild-type or mutated gD with viruses containing wild-type or mutated gD

gD expressed by cell line	Outcome with virus containing:	
	Wild-type VgD	Mutant VgdU
Wild-type CgD	CgD-VgD complex, no infection	CgD-VgDU complex, infection
Mutant CgDU	CgDU-VgD complex, infection	CgDU-VgDU complex, infection

the target is gD itself. Moreover, although prior evidence implicated gD, we had no evidence to conclude that it is the gD present in the plasma membrane rather than in other compartments of the cell which blocks penetration of HSV-1(F) into BJ cells. The studies of BJ cells treated with AP7 antibody indicate that the blocking molecule is accessible to antibody and therefore must be on the surface of the cell rather than internalized and inaccessible to the antibody. As indicated in the preceding section ("Significance of the HSV-1(F)U mutation"), none of the data generated in this study support the hypothesis that the block to penetration results from the interaction of gD with other molecules rather than in *trans* among themselves. The mutation in gD at Leu-25 may act to abolish this interaction.

Physiological significance of the restriction to penetration during productive infection. A mutation that appears to enable viral progeny to reinfect cells has been described elsewhere (34). In this report and a preceding paper (4), we have authenticated the existence of a specific mechanism for the exclusion of superinfecting virus. In view of the conclusions that gD contains a domain which functions to restrict infection of cells that express the glycoprotein and that the exclusion is not based on competition for a ligand essential for entry, it is appropriate to point out at least two reasons why exclusion of reinfection is desirable. Foremost, HSV-1 is enveloped at the nuclear membrane and transits through the cytoplasms inside membrane-delimited structures. During productive infection, the viral glycoproteins follow the exocytic pathway and are present in most of the intracellular membranes. In the course of the intracytoplasmic transport and in the absence of restriction, the envelope of the virion could fuse with the enclosing membrane, resulting in deenvelopment. Although juxtapositions of capsids to intracytoplasmic membranes consistent with deenvelopment have been seen, they are not very prominent. In the absence of this restriction, fusion of envelopes with intracytoplasmic membranes may significantly increase deenvelopment to a point of reducing the yield of infectious virus. Studies designed to test this hypothesis (G. Campadelli Fiume, F. Farabegoli, S. Di Gaeta, and B. Roizman, submitted for publication) indicate a significant increase in the number of unenveloped capsids in the cytoplasm of cells infected with HSV-1(F)U-10 virus compared with the number in cells infected with the wild-type parent. A second reason for the evolution of a mechanism for the prevention of reinfection is that in tissues the virus is released and accumulates between adjacent cells. In this instance, reinfection of previously infected cells would reduce the availability of the virus to spread in tissues and, by extension, in the host population.

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ADDENDUM IN PROOF

BJ cells infected with the AP7-resistant viruses isolated by Minson and coworkers (22) (derived from HSV-1 strains Sc-16 or HFEM) and labeled with [^{35}S]methionine at 16 h after infection synthesize the entire spectrum of viral proteins. This result confirms the above finding that the Leu-25 to proline substitution in mature gD confers upon HSV virions the ability to be unrestricted in cells expressing gD.

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