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Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensable for growth in cell culture

(gene deletion/selectable marker/viral glycoprotein/dispensable gene)

RICHARD LONGNECKER*, SUBHENDRA CHATTERJEE†, RICHARD J. WHITLEY†, AND BERNARD ROIZMAN*‡

*Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, IL 60637; and †The Department of Pediatrics and Microbiology, University of Alabama, Birmingham, AL 35294

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ABSTRACT **The genome of herpes simplex virus 1 consists of two components, L and S, each containing unique sequences flanked by inverted repeats. Current and earlier studies have shown that 11 of the 12 open reading frames contained in the unique sequences of the S component can be deleted and are dispensable for growth in cell culture. Analyses of one recombinant virus containing a deletion in the open reading frame US7 permitted the identification of a monoclonal antibody specific for the product of this gene. The protein encoded by this gene has a predicted translated molecular weight of 41,366 and an apparent molecular weight of approximately 65,000 in denaturing polyacrylamide gels. The electrophoretic mobility of the protein synthesized by cells in the presence of inhibitory concentrations of tunicamycin is faster than that of the protein accumulating in lysates of untreated infected cells. We conclude that the product of US7 is a glycoprotein subject to N-linked glycosylation, and we have designated it glycoprotein I. These studies indicate that the unique sequences of the S component encode four glycoproteins (G, D, I, and E) of which at least three (G, I, and E) are dispensable for growth in continuous lines of primate cells.**

The DNA genome of herpes simplex virus 1 (HSV-1) consists of two components, L and S, that invert relative to each other (refs. 1 and 2; see also Fig. 1 legend). Each component consists of unique sequences flanked by inverted repeats (3, 4). Analyses of the nucleotide sequence of the unique sequences of the S component have shown that it consists of 12 open reading frames designated US1 through US12 (5). Analyses of the amino acid sequences of the predicted proteins encoded by these open reading frames permitted the assignment of the genes specifying the previously identified and mapped glycoproteins G (gG, refs. 6 and 7), D (gD, ref. 8), and E (gE, ref. 8) to the open reading frames US4, US6, and US8, respectively. These studies also predicted that the US7 product may be a glycosylated membrane protein and might correspond to the 55,000 apparent molecular weight protein identified by Lee *et al.* (8) by *in vitro* translation of hybrid-selected RNA from the US7 region. We report that US7 does encode a glycoprotein, which we have designated glycoprotein I (gI). The circumstances that led to the authentication of the product of the US7 open reading frame as gI were as follows.

Comparison of the genome arrangements of the various herpesviruses that infect humans and animals suggested that the S component sequences diverged in their evolution (2, 9). One hypothesis to explain this conclusion is that these genes evolved from DNA sequences acquired by a progenitor virus. In the course of selective adaptation of the virus to its host, the sequences evolved into genes whose functions are relevant to the survival of the individual herpesviruses in their specific ecological niches, the hosts in which these viruses multiply, rather than functions necessary for the selective replication of their genomes or maturation of the virus. To test this hypothesis, the genes mapping in the S component have been systematically probed by a site-specific deletion mutagenesis technique (10–12). All the US open reading frames, except US6 (encoding gD), have been systematically deleted and shown not to be essential for growth of the viruses in primate cell lines (refs. 10–12 and this report). Among the genes deleted was US7. By screening a panel of monoclonal antibodies directed against HSV-1 proteins with molecular weights in the range predicted for a glycosylated product of the US7 open reading frame, we identified one monoclonal antibody that reacted only with HSV-1 viruses with an intact US7 coding region. This monoclonal antibody was then used to characterize the product of the gene.

MATERIALS AND METHODS

Viruses and Cells. The properties of HSV-1(F), HSV-1(F)Δ305, a virus carrying a deletion in the thymidine kinase (TK) gene, and R7023, from which several genes have been deleted, were reported elsewhere (11, 13, 14). Vero and BS-C-1 are both African green monkey kidney cell lines, and HEp-2 is a human epidermoid carcinoma cell line.

DNAs. The cloning and purification of plasmid and viral DNA were as described (15, 16). DNA probes were labeled with [α-^{32}P]dCTP by nick-translation with a kit from New England Nuclear.

Immunoprecipitations, Polyacrylamide Gel Electrophoresis, and Immunoblots. Procedures for immunoprecipitations, electrophoresis in denaturing polyacrylamide gels, solubilization of proteins, electrical transfer of electrophoretically separated proteins to nitrocellulose, and reaction with monoclonal antibodies in an immunoperoxidase-coupled reaction or with ^{125}I-labeled protein A were as described (7, 17–19).

RESULTS

Construction of gE$^-$ and US7$^-$ Recombinant Viruses. The strategy for the construction of the viruses with deletions in US8 (the gE gene) and US7 were detailed elsewhere (10, 12) and consisted of two steps. In the first, consistent with previous results showing that the gE gene was dispensable for growth in cell culture (11), a selectable marker—consisting of a chimeric HSV-1 TK gene constructed by fusion of the promoter-regulatory domain of the α27 gene to the sequences

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Abbreviations: HSV-1 and HSV-2, herpes simplex virus 1 and 2; TK, thymidine kinase; α27–TK, coding sequences of the TK gene fused with promoter-regulatory domain from the α27 gene; prefix g, glycoprotein (e.g., gB is glycoprotein B).

‡To whom reprint requests should be addressed at: Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 East 58th Street, Chicago, IL 60637.

encoding the TK enzyme—was inserted into the unique *Hpa* I site of a cloned copy of the gE gene. The recombinant α27–TK DNA fragment, cloned as plasmid pRB3633, was cotransfected with intact DNA of the TK^- mutant HSV-1(F)Δ305 DNA, and a TK^+ virus designated R7030 (Fig. 1, line 8) was selected by plating the progeny of transfection on human $143TK^-$ cells in medium containing methotrexate (200 μg/ml). The DNA of this virus was shown to contain the chimeric TK gene inserted into the gE gene as follows. *Bam*HI digests of R7030 DNA (Fig. 2) revealed the loss of the wild-type *Bam*HI J fragment and the appearance of a new band designated no. 1. As expected, the new band hybridized with labeled DNA sequences containing the TK gene (Fig. 2, band 1, probe pRB103) and with *Bam*HI J-specific sequences (Fig. 2, band 1, probe pRB123). The smaller *Bam*HI Q fragment designated ΔQ in Fig. 2 contained the 700-base-pair deletion within the TK gene present in the parental HSV-1(F)Δ305 virus. *Hin*dIII digests of R7030 DNA (Fig. 2) revealed the replacement of the wild-type *Hin*dIII G fragment with a new DNA band designated no. 2. This new band hybridized with labeled DNA specific for the TK gene (Fig. 2, band 2, probe pRB103). Moreover, as expected, band 2 hybridized with labeled DNA of *Bam*HI J (Fig. 2, probe pRB123), which contains sequences from both the *Hin*dIII fragments N and G.

In the second step, the sequences encoding the domains of the US7 and gE genes were deleted from the *Bam*HI J fragment cloned as pRB123 by digesting the plasmid DNA to completion with *Bst*EII restriction endonuclease and religating the plasmid DNA to generate a plasmid designated pRB3703 (Fig. 1, line 9). The plasmid DNA was then cotransfected with intact R7030 viral DNA, and a TK^- virus (R7048) was selected by plating the progeny of the transfection on $143TK^-$ cells in medium containing 5-bromo-2′-deoxyuridine (40 μg per ml of medium). The recombinant R7048 was then isolated and analyzed by restriction endonuclease cleavage and hybridization. Analyses of the DNA of R7048 indicated that the deletion in the viral DNA extended beyond the right terminus of the sequences deleted in pRB3703 (Fig. 1, line 9) and included *Bam*HI X, *Bam*HI Z, and ≈1 kilobase pair of *Bam*HI Y (Fig. 1, line 12). This conclusion is supported by (*i*) the appearance in the *Bam*HI digests of R7048 DNA of a novel DNA band that hybridized to sequences specific for *Bam*HI J (Fig. 3, probe pRB123) and

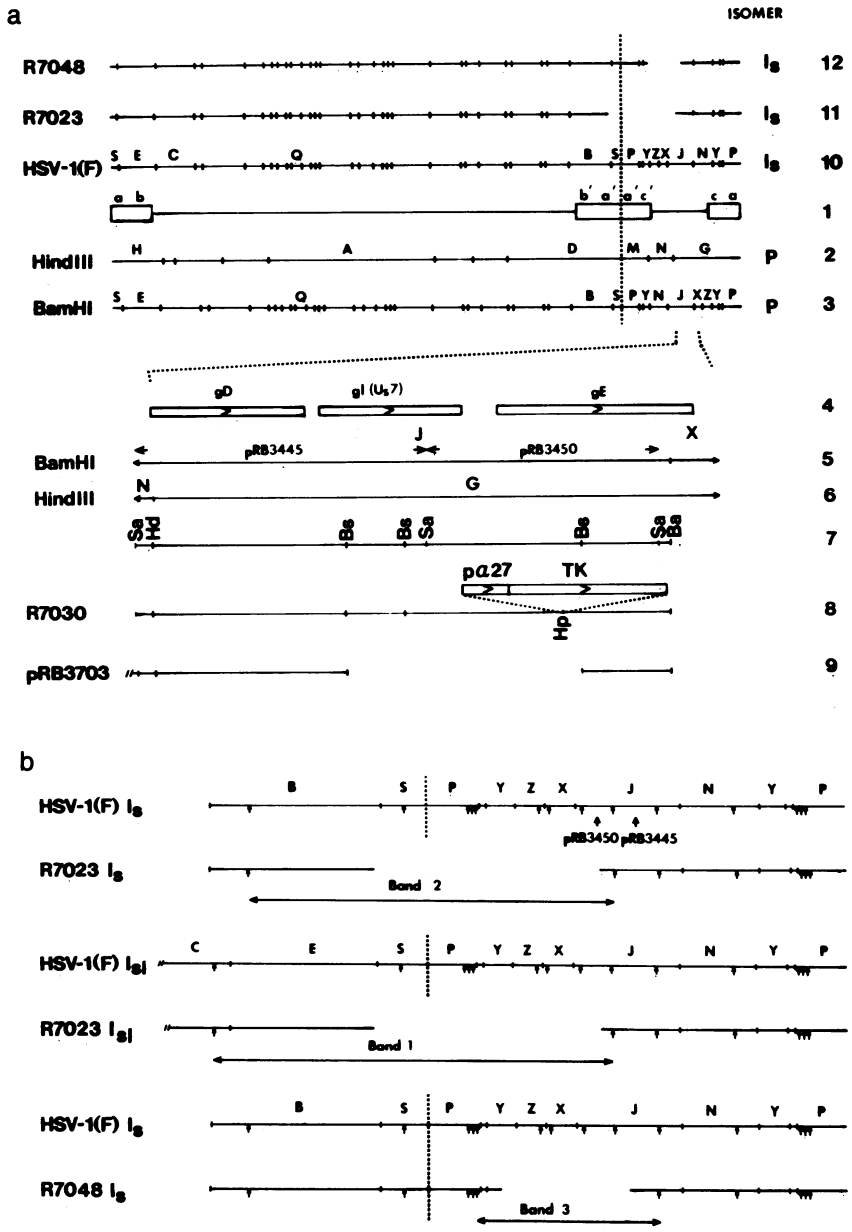

FIG. 1. Schematic representations of the DNA sequence arrangements in HSV-1(F) and of the various viral recombinants and plasmids used in these studies. (*a*) Line 1: sequence arrangement of the HSV-1(F) genome. Boxes represent the terminal sequences repeated internally in inverted orientation and dividing the genome into the long (L) and the short (S) components. Because the L and S components invert, the HSV-1 genome forms four isomers designated P (prototype), I_s (inversion of the S component) I_l (inversion of the L component), and I_{sl} (inversion of both components). Lines 2 and 3: *Hin*dIII and *Bam*HI restriction endonuclease maps of HSV-1(F) DNA in the prototype (P) arrangement. The DNA fragments pertinent to this report are shown with their letter designation. Line 4: coding domains of gD, US7, and gE genes. Lines 6 and 7: restriction endonuclease maps for the *Bam*HI J and X DNA fragments shown in line 5 (Sa, *Sac* I; Hd, *Hin*dIII; Bs, *Bst*EII; Ba, *Bam*HI; Hp, *Hpa* I). Line 8: unique *Hpa* I site into which the α27–TK gene fusion was inserted, interrupting the coding domain of the gE gene. Line 9: sequence arrangement of plasmid pRB3703, from which the *Bst*EII subfragments have been deleted. Lines 10, 11, and 12: *Bam*HI maps of HSV-1(F), R7023, and R7048, respectively, shown in the I_s orientation. To visualize R7048 in the P arrangement, invert the S component of the R7048 genome structure shown in line 12. (*b*) Expanded portion of restriction endonuclease maps of HSV-1(F) and R7048 DNAs in the I_s arrangement and of R7023 DNA in the I_s and the I_{sl} arrangements. Vertical dotted line represents the junction between the L and S components. Letters indicate the *Bam*HI fragments of HSV-1(F) DNA. Vertical arrows immediately below the maps indicate the location of *Sac* I cleavage sites. The two *Sac* I subfragments of *Bam*HI J were cloned as pRB3450 and pRB3445 and their locations are shown in the HSV-1(F) I_s map. Bands 1 and 2 are the two *Sac* I fragments generated by the sequence arrangement of the two isomers of R7023 DNA. Band 3 corresponds to the *Sac* I fragment generated by the fusion of *Bam*HI J and *Bam*HI Y in the sequence arrangement of R7048 DNA.

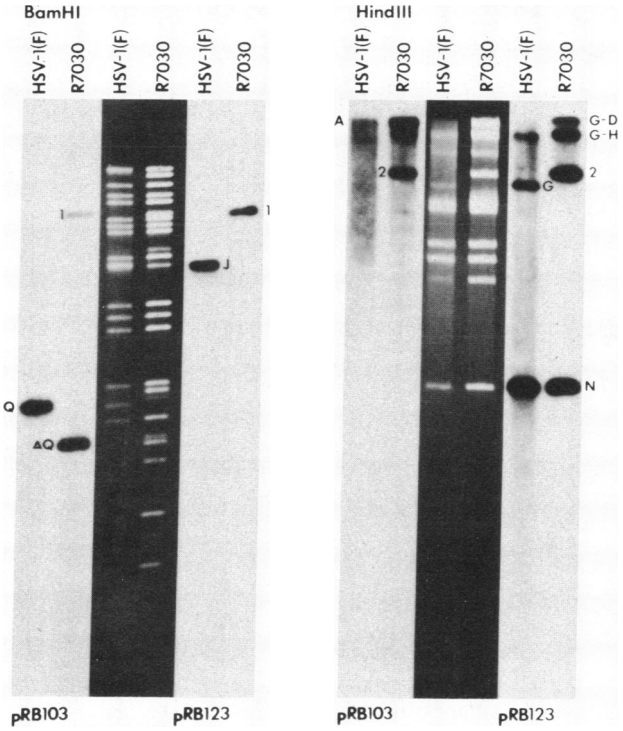

FIG. 2. Photographic and autoradiographic images of electrophoretically separated *Bam*HI or *Hin*dIII restriction endonuclease digests of HSV-1(F) and R7030 viral DNAs. The ^{32}P-labeled DNA of plasmid pRB103, carrying the *Bam*HI Q fragment (20), or of pRB123, carrying the *Bam*HI J fragment (20), was hybridized to the electrophoretically separated viral DNA fragments. Letters refer to fragment designations given to wild-type HSV-1(F) DNA. Bands designated by two letters represent the products of fusion of terminal fragments spanning the junction between the L and S component. Bands designated 1 and 2 are the bands generated by the insertion of the chimeric α27–TK gene into the parental R7030 viral genome. Band ΔQ identifies the *Bam*HI Q fragment of HSV-1(F)Δ305 genome from which 700 base pairs have been deleted. The procedures for restriction endonuclease digestion, electrophoretic separation of DNA digests in agarose gels, visualization with ethidium bromide, transfer to nitrocellulose sheets, hybridization with ^{32}P-labeled DNA, and autoradiography were as described elsewhere (16, 20, 21).

*Bam*HI Y (Fig. 3, probe pRB113) and (*ii*) the disappearance of *Bam*HI fragments of J, X, and Z and one of the two copies of *Bam*HI Y fragment.

In subsequent investigations we studied the immunoreactivity of polypeptides in lysates of cells infected with the recombinant R7048 (described here) or the recombinant R7023 [described previously (11)]; for this reason it was of interest to compare the structures of the two recombinants. Recombinant R7023 is arrested in the I_s and I_{sl} arrangements of HSV-1 DNA (Fig. 1). It lacks the gE gene and all of the DNA sequences to the terminus of the S component of the viral genome and portions of adjacent inverted repeat sequences of the L component. The remaining sequences of the S component are fused to the remaining portions of the inverted repeats of the L component (Fig. 1*a*, line 11, and Fig. 1*b*). To illustrate the structure of the two recombinants, *Sac* I digests of R7048 and R7023 DNAs were hybridized with each of the two *Sac* I subfragments (Fig. 1*a*, line 5, and Fig. 1*b*), cloned as pRB3445 and pRB3450, respectively, of the *Bam*HI J fragment. The *Sac* I subfragment containing gE (pRB3450) hybridized to two novel bands in digests of R7023 DNA (Fig. 3, bands 1 and 2) that correspond to the two bands indicated in Fig. 1*b*. This probe failed to hybridize with the digests of R7048 DNA, consistent with the loss of this DNA sequence from the R7048 genome. The *Sac* I subfragment containing the gI and gD genes (pRB3445) hybridized to a wild-type fragment in the electrophoretically separated digests of R7023 DNA (Fig. 3, probe pRB3445) indicating that these genes are intact. Hybridization of this probe with digests of R7048 DNA revealed the presence of a new band (Fig. 3, band 3) corresponding to the fragment labeled band 3 in Fig. 1*b*. These analyses indicate that the only DNA sequences absent from R7048 DNA that were not absent from R7023 DNA were those that corresponded to the open reading frame US7.

Absence of the US7 Gene Product from the Lysates of R7048-Infected Cells. The nucleotide sequence of US7 predicts its polypeptide product would have a translated molecular weight of 41,366. This compares with translated molecular weights of 59,090 and 25,237 for gE and gG, respectively. On the assumption that monoclonal antibodies to US7 may already exist but may not have been specifically identified, we analyzed a panel of monoclonal antibodies that reacted with proteins of apparent molecular weight in the range between those of gG and gE. Lysates of virus-infected cells were electrophoretically fractionated in denaturing polyacrylamide gels, and the separated proteins were electrically transferred to nitrocellulose and tested for reactivity, by procedures previously described (7), with the monoclonal antibodies. These analyses yielded a monoclonal antibody designated Fd69, which reacted with lysates of cells infected with R7023 or with the parent HSV-1(F) virus but not with those of cells infected with R7048. The proteins reactive with Fd69 formed at least two bands, corresponding to approximate molecular weights of 65,000 and 55,000 (Fig. 4*b*). All lysates of infected cells reacted in these tests with a monoclonal antibody (H1163) specific for the HSV-1 glycoprotein gB (Fig. 4*b*). Immunoprecipitations with monoclonal antibodies to gE (H600) and to gD (H1380) indicated, as expected, that the two recombinant viruses R7023 and R7048 expressed gD but not gE (Fig. 4*c*). Also as expected (data not shown), the recombinant viruses R7023 and R7048 reacted with monoclonal antibody H1379 (7) specific for HSV-1 gG, whereas Fd69 reacted with R7036 (12), a previously described recombinant virus from which the gene specifying gG had been deleted.

Tunicamycin Inhibits the Posttranslational Processing of Proteins Reactive with Monoclonal Antibody Fd69 to a Higher Apparent Molecular Weight. To verify the prediction that the product of US7 is a glycoprotein, we tested the effect of tunicamycin, an inhibitor of N-linked glycosylation, on the processing of the proteins reactive with the monoclonal antibody Fd69. In these experiments, BS-C-1 cells were pretreated with tunicamycin (1 or 3 μg per ml of medium) for 1 hr. The cells were then infected with HSV-1(F) and maintained in the presence of tunicamycin for 14 hr. The lysates of the cells harvested at that time were electrophoresed in denaturing polyacrylamide gels, and the separated proteins were electrically transferred to nitrocellulose and tested for reaction with monoclonal antibody Fd69. As shown in Fig. 4*d*, Fd69 reacted with two protein bands from lysates of untreated infected cells, but the antibody reacted with a faster-migrating band from the lysates of infected cells treated with tunicamycin. These results are consistent with the expectation that tunicamycin inhibited the N-linked glycosylation of the HSV-1(F) protein reactive with the monoclonal antibody Fd69.

The observation that Fd69 reacted with surfaces of cells infected with wild-type virus (data not shown) is consistent with the conclusion that the product of US7 is a membrane glycoprotein. In accordance with convention, it has been designated with the next available letter (I) as glycoprotein I (gI).

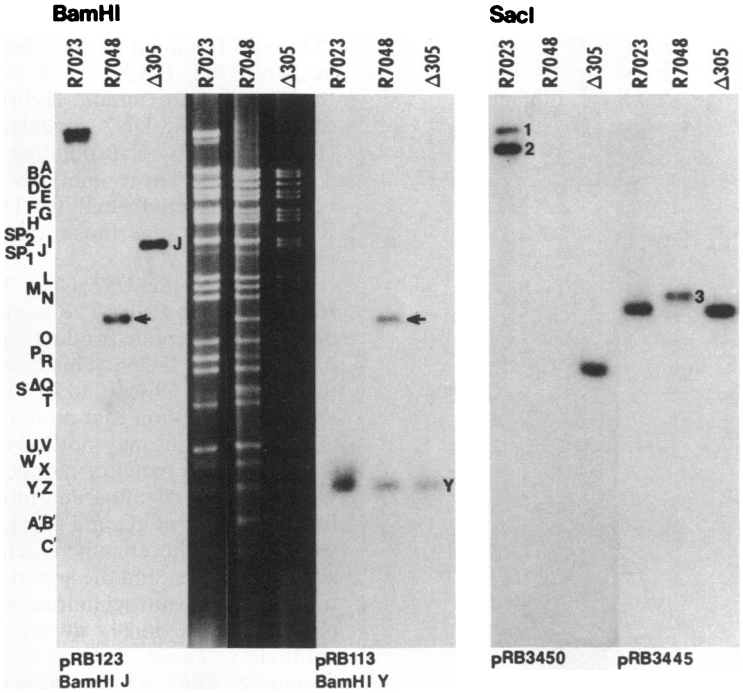

FIG. 3. Photographic and autoradiographic images of electrophoretically separated *Bam*HI or *Sac* I restriction endonuclease digests of HSV-1(F), R7023, and R7048 viral DNAs. The ^{32}P-labeled hybridization probes were plasmid DNAs pRB103 and pRB123 (described in the legend to Fig. 2), pRB3445, carrying the *Sac* I subfragment from *Bam*HI J containing the coding regions for gD and gI (11), and pRB3450, carrying the *Sac* I subfragment from *Bam*HI J containing the coding region for gE (11). Letters identify the *Bam*HI fragments of HSV-1(F). Bands indicated by arrows correspond to the fusion product of *Bam*HI J and *Bam*HI Y that is present in the R7048 genome. The two bands in the *Bam*HI digests of R7023 that hybridize with *Bam*HI J arose from the fusion of *Bam*HI J with either *Bam*HI E (I_{sl} arrangement) or *Bam*HI B (I_s arrangement) in the R7023 viral genome. The fusion site is contained in the *Sac* I fragments designated bands 1 and 2 and shown in Fig. 1*b*. Band 3 corresponds to the *Sac* I band generated by the fusion of *Bam*HI J with *Bam*HI Y in the R7048 viral genome and is shown in Fig. 1*b*.

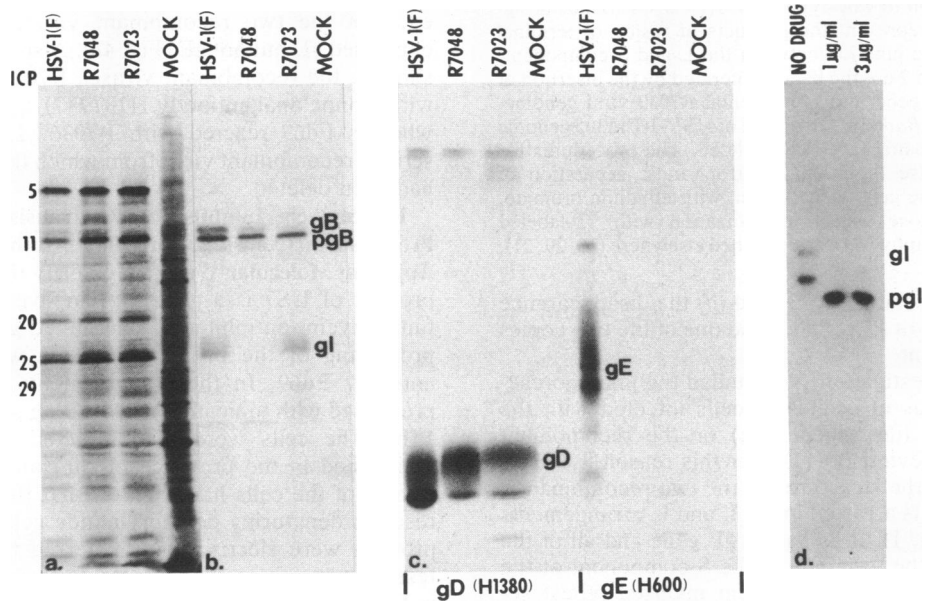

FIG. 4. Autoradiographic images and immunoreactivity of infected-cell polypeptides electrophoretically separated in a denaturing polyacrylamide gel, transferred to nitrocellulose, and reacted with monoclonal antibodies. (*a*) Autoradiographic images of [^{35}S]methionine-labeled proteins from HEp-2 cells infected with HSV-1(F), R7023, or R7048. Monolayer cultures of HEp-2 cells (4×10^6 cells) were exposed to 10 plaque-forming units of virus per cell. (Mock-infected cells were handled similarly but not exposed to virus.) After 1 hr of adsorption, the inoculum was replaced with maintenance medium consisting of mixture 199 supplemented with 1% calf serum. During the period 10–18 hr after infection, the cells were incubated with [^{35}S]methionine (25 μCi/ml; 1 Ci = 37 GBq) in maintenance medium containing only 10% of the usual amount of methionine. Infected-cell polypeptides (ICPs) are numbered at left according to ref. 17. (*b*) Immunoreactivity of the electrophoretically separated, immobilized proteins shown in *a* with monoclonal antibody to gI (Fd69) and to gB (H1163) (7, 12) in an immunoperoxidase-coupled reaction. pgB designates the precursor form of the mature gB. (*c*) Extracts of cells infected with HSV-1(F), R7023, or R7048 were subjected to immunoprecipitation with monoclonal antibodies to gD (H1380, ref. 7) and gE (H600, ref. 7). (*d*) Electrophoretic mobility of protein bands reactive with Fd69 in lysates of cells infected and mock-treated or treated with tunicamycin. BS-C-1 cells were pretreated for 1 hr with tunicamycin (0, 1, or 3 μg/ml), infected with HSV-1(F) at 5 plaque-forming units per cell, and then maintained in the presence of tunicamycin. Cells were harvested 14 hr after infection. Infected-cell proteins were electrophoretically separated, transferred to nitrocellulose, and reacted with monoclonal antibody Fd69 and ^{125}I-labeled protein A as described (18, 19). pgI designates the precursor form of the mature gI.

DISCUSSION

The significant features of our results are as follows. (*i*) The deletion of the open reading frame US7 brings the number of open reading frames or known genes deleted from the unique sequences of the S component to 11 of a total of 12. This cluster of dispensable genes contrasts sharply with the rather small number of largely scattered dispensable genes in the L component of the viral DNA. As noted in the Introduction, the clustering of genes dispensable for growth in cell culture suggests that the proteins encoded by these genes do not uniquely recognize cis-acting sites in viral nucleic acids or enable the function of viral proteins required for replication or maturation of the virion. The clustering of these genes in the S component of the HSV genome is consistent with the hypothesis that these sequences evolved independently and were not conserved among the various herpesviruses. As suggested earlier, they may have evolved from DNA sequences acquired by a progenitor of the modern herpesviruses and used for generation of genes necessary for the maintenance of the virus in the human population rather than for the functions related to replication of its genome or maturation of the virion (2, 9).

(*ii*) The glycoprotein (gI) described in this report is the seventh glycoprotein shown to be encoded by HSV-1. Consistent with the colinearity of the HSV-1 and HSV-2 genomes, an open reading frame corresponding to that of HSV-1 US7 was mapped in the HSV-2 genome (22). Of the seven glycoproteins (gB, gD, gC, gE, gH, gG, and gI), four (gG, gD, gI, and gE) map in the unique sequences of the S component. Conditional mutants have been reported in gB (23–25) and deletion mutagenesis studies suggest that gH and gD may also be required for infection and replication in cells (A. E. Sears, R.L., and B.R., unpublished data). All other known glycoproteins have been shown to be dispensable for growth in cell culture (11, 12, 26). The presence of so many glycoprotein genes and especially of four that encode dispensable glycoproteins is puzzling. One plausible explanation is that the host-cell ranges of HSV-1 and HSV-2 include a wide variety of cells differing in the composition of their membrane and that these glycoproteins are required for the infection of these cells and, by extension, for the maintenance of the virus in the human host. Consistent with this hypothesis is the observation that the pathogenicity in mice of the viruses carrying deletions in the S component is significantly reduced relative to the wild-type parent virus (B. Meignier, R.L., and B.R., unpublished data).

(*iii*) The construction of the recombinant virus R7048 greatly facilitated the identification of a monoclonal antibody that recognized the product of the deleted gene. Studies of the properties and function of gI will be facilitated by the use of monoclonal antibody directed against it.

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