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Antigenic Properties and Cellular Localization of Herpes Simplex Virus Glycoprotein H Synthesized in a Mammalian Cell Expression System

U. A. GOMPELS* AND A. C. MINSON

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom

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Herpes simplex virus type 1 glycoprotein H (HSV-1 gH) was synthesized in an inducible mammalian cell expression system, and its properties were examined. The gH coding sequence, together with the stable 5′ untranslated leader sequence from xenopus β-globin, was placed under control of the strong promoter from the human cytomegalovirus major immediate-early gene in an amplifiable plasmid which contains the simian virus 40 (SV40) virus origin for replication (ori). This expression vector was transfected into *ts* COS cells constitutively expressing a temperature-sensitive SV40 T antigen which allows utilization of the SV40 ori at permissive temperatures. The results of transient expression assays at the permissive temperature showed that HSV-1 gH could be synthesized in greater amounts than those produced by a high-multiplicity virus infection. The proteins produced were detected in Western blots (immunoblots) with a HSV-1 gH-specific polyclonal serum raised against a TrpE-gH fusion protein. The transfected gH had an apparent molecular weight of approximately 105,000, intermediate in size to those of the precursor (100,000) and fully processed forms (110,000) of HSV-1 gH from infections. Antigenicity was investigated by reactions with three virus-neutralizing monoclonal antibodies specific for conformational epitopes on gH. Only one of these monoclonal antibodies could immunoprecipitate the synthesized gH. However, equal recognition of the transfected gH was achieved by superinfection with virus. In addition, detectable amounts of gH were not expressed on the cell surface unless the cells were superinfected with virus. Studies with a temperature-sensitive mutant, *ts*1201, defective in encapsidation showed that the changes in antigenic structure and cell surface expression caused by superinfection with virus were not due simply to incorporation of gH into virions. These results suggest that gH requires additional virus gene products for cell surface localization and formation of an antigenic structure important for its function in mediating infectivity.

Glycoprotein H (gH) is one of seven glycoproteins, including gB, gC, gD, gE, gG, and gI, encoded by herpes simplex virus type 1 (HSV-1) which are located both on the virion envelope and the infected cell surface (1, 19, 38, 57, 64). Functions for these glycoproteins have been implicated in virus attachment and penetration (9, 16, 17, 25, 31, 37), as well as envelopment, egress, and membrane fusion (19, 45, 49). Because of their dual sites on the infected-cell surface and the virion envelope, HSV glycoproteins act as major antigenic determinants for the cellular and humoral immune responses of the host (50, 64, 65). gH may be an important target for this response. This glycoprotein is characterized by an apparent molecular weight of approximately 110,000, and the gene has been mapped (8, 19) and sequenced (19, 43). Although gH appears to be a relatively minor component of the infected-cell surface and virion envelope (8, 57), there is evidence which suggests that it plays a crucial role in the virus-replicative cycle. First, virus which lacks gH is not infectious. This was shown in studies with the temperature-sensitive mutant *ts*Q26 (70), which has a point mutation in the gH gene (15). At the nonpermissive temperature, although infectious intracellular virus is produced which retains gH, the extracellular virus lacks gH and is noninfectious (15). This is similar to the results of gD and gB deletion mutants which cannot initiate an infection (9, 37), whereas the other four virion glycoproteins appear to be nonessential for replication in vitro (23, 26, 38–40). Thus, like gD or gB, gH appears essential for infectivity. Second, coding sequences homologous to those of HSV-1 gH have been identified in representatives of all subgroups of herpesviruses: varicella-zoster virus, Epstein-Barr virus (14, 24, 33, 51), human cytomegalovirus (HCMV) (13, 53), and herpesvirus saimiri (20, 21). Thus, like gB but unlike gD, gH appears to be a member of a subset of genes essential among herpesviruses (12, 14, 20, 21, 42). Finally, like some antibodies specific for the nonconserved HSV-1 gD, the monoclonal antibodies (MAbs) to gH have pronounced biological effects on virus replication in vitro. Thus, some antibodies specific for gH and gD strongly neutralize virus infectivity in the absence of complement and can inhibit cell fusion by syncytial virus strains (19, 45, 49). Similarly, antibodies specific for the gH homologs in HCMV or Epstein-Barr virus also neutralize these viruses efficiently in the absence of complement (13, 21, 53, 56, 67), and one MAb specific for Epstein-Barr virus gH appears to prevent fusion rather than adsorption (44). MAbs to HSV-1 gH, unlike most other neutralizing antibodies, inhibit the transfer of virus from infected to uninfected cells during plaque formation by syncytial or nonsyncytial strains (19), and similar results have been shown using antibodies against the varicella-zoster homolog of gH (33). These observations imply functions for gH in virus entry and release or in formation of intercellular bridges.

The function of gH can be further studied in mammalian cell expression systems. Such systems allow correct processing of virus glycoproteins, formation of discontinuous

* Corresponding author.

antigenic sites, and analyses without interference from other virus gene products. If the glycoprotein produced is antigenically normal, then it can be used for measurement of immune response to infection, protection studies in animal models, immunization to produce neutralizing antibody, and functional assays. Mammalian cell expression systems have been used to express HSV glycoproteins which appear antigenically identical to the HSV-infected cell products (3, 4, 9, 11, 27–30, 32, 37, 41, 46, 60, 68, 72), and some studies have additionally shown identical glycosyl modifications and cellular localization (11, 46, 60, 68). Transient cell expression systems have been used to define both antigenic and functional sites on gB, gC, and gD through the use of deletion mutant genes and both monoclonal and polyclonal sera (9, 11, 27, 46, 60, 72). gD has been most extensively studied in this manner, and the gD thus produced has been recognized by a panel of MAbs, including neutralizing antibodies, which recognize both discontinuous and continuous epitopes (11, 27, 46, 72). We have constructed a transient cell expression system which overproduces gH. To test the antigenicity of this synthesized gH, we reacted cell lysates with three neutralizing MAbs which recognize conformational epitopes on gH (8, 19, 62). We show that only one of these three recognizes the gH produced, whereas all three antibodies recognize equally well the gH modified after superinfection with virus. Our results suggest that unlike the other HSV glycoproteins, gH appears to require virus gene expression for the formation of an antigenic structure which is important for infectivity.

MATERIALS AND METHODS

Antibodies. Hybridomas producing MAbs 52s and 53s (62) were supplied by the American Type Culture Collection (Bethesda, Md.). The hybridoma-producing MAb LP11 is from this laboratory as described previously (8, 19). Cell supernatants supplemented with 0.02% azide were prepared from 53s and 52s hybridoma cultures, and LP11 was prepared as an ascites fluid. Both 52s and LP11 are HSV-1 specific; 53s is types 1 and 2 common. The HSV-1 gH-specific polyclonal serum was raised in rabbits immunized with an *Escherichia coli* fusion protein containing gH sequences as described previously (15). The protein was a fusion between the TrpE gene product to amino acids 270 to 690 of HSV-1 gH.

Viruses and cells. BHK cells were grown in Glasgow modified Eagle medium supplemented with 10% tryptose phosphate broth and 10% newborn calf serum (NCS). The *ts* COS cells (58) were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal calf serum and maintained at 40°C. The virus strains used were HSV-1 HFEM, SC16, 65, *ts*1201, and *ts*Q26 and HSV-2 25766. Virus stocks were prepared by low-multiplicity passage in HEp-2 cells, and titers were determined by assay on BHK and Vero cells. HSV-1 65 is a recent clinical isolate (7) which is resistant to the effects of the HSV-1 gH-specific MAb LP11. This virus has a gH gene which differs by 10 nucleotides from the gH gene of HFEM, only one of these gives rise to a nonconservative amino acid substitution. This change is identical to one of three amino acid substitutions found in gH genes from virus mutants selected for resistance to neutralization by MAb LP11 (U. A. Gompels and A. C. Minson, manuscript in preparation). HSV-1 *ts*Q26 is a temperature-sensitive mutant which has a point mutation in the gH coding sequence (15). HSV-1 *ts*1201 is a temperature-sensitive mutant derived from HSV-1 17 which has a defect in the UL26 gene encoding the p40 capsid protein (1, 55). At the nonpermissive temperature, this mutant fails to encapsidate DNA (2). HSV-1 R *ts*1201 is a revertant of *ts*1201; both virus strains were kindly supplied by V. Preston (Medical Research Council Institute of Virology, Glasgow, United Kingdom).

Plasmid construction. The coding sequence for HSV-1 gH was taken from a plasmid, pUG102, which contains the 6,432-base-pair (bp) *Bgl*II m fragment of HSV-1 HFEM DNA inserted into the *Bgl*II site of the plasmid vector PKC7 (19). A partial *Nco*I digestion followed by *Xho*I digestion yielded a 3.4 kbp fragment containing the gH coding sequences. This DNA fragment was filled in with the Klenow fragment of DNA polymerase and inserted into the *Sma*1 site of plasmid pTZ (Bio-Rad Laboratories). The sequence 5′ to the initiating methionine codon was mutated by site-directed mutagenesis (35) from GGATCCCCCATGG to AGATCT TAAATGG, and this construct was designated pTZ gH. The gH coding sequence was released by *Bgl*II and *Xho*I digestion and ligated with *Bgl*II-digested plasmid vector SP64T (34) to give the gH coding sequence the transcription initiation site and stable 5′ untranslated leader sequence from xenopus β-globin. This plasmid was linearized by digestion with *Hin*dIII and ligated with *Hin*dIII-digested CDM8 (59). The plasmid CDM8 contains the strong transcriptional promoter from the HCMV major immediate-early gene, together with the origins of replications from simian virus 40 virus and polyomavirus which allow amplification of plasmid sequences in cells expressing simian virus 40 or polyomavirus T antigens such as *ts* COS, COS7, or MOP8 cells (18, 47, 58). The plasmid is selected via expression of its copy of SUPF, the amber suppressor tRNA gene, which allows for replication in the presence of ampicillin and tetracycline within *E. coli* M1061/P3 which contains the plasmid P3 carrying amber-mutated ampicillin and tetracycline resistance genes. The final 11.4-kilobase construct pUG-H1 (Fig. 1) contains the HSV-1 gH coding sequences flanked on the 5′ side by the HCMV major immediate-early promoter, the β-globin site for initiation of transcription, and the 5′ untranslated leader sequence of β-globin and on the 3′ side by the gH polyadenylation sequence and the β-globin polyadenylation sequence. The plasmid pUG-H2 is a deleted form of pUG-H1 derived by *Xho*I digestion, purification of the resulting 7.8-kilobase fragment containing the gH sequence and flanking control signals, followed by religation (Fig. 1).

Transfections and superinfections. Transfections were done by the modified calcium phosphate precipitation procedure described by Chen and Okayama (10). Plasmid DNA was prepared by scaled-down versions of the alkaline-sodium dodecyl sulfate method (6). The *ts* COS cells were plated 48 h before transfection at densities which gave monolayers 80 to 90% confluent before transfection. Calcium phosphate precipitates of plasmid DNA (25 μg) were added to monolayers in 25-cm^2 tissue culture flasks containing 10 ml of Dulbecco modified Eagle medium with 10% fetal calf serum. After incubation at various temperatures for 16 to 20 h with 3% CO_2, the cells were washed twice with 5 ml of medium and replaced with Dulbecco modified Eagle medium-10% fetal calf serum. The dishes were then harvested up to 96 h posttransfection directly into Laemmli buffer or immunoprecipitation buffer (see below). For experiments with superinfecting virus, the cells were first incubated 16 to 20 h in media with DNA, followed by a 24-h incubation in media without DNA. The cells were then infected with virus at a multiplicity of infection equal to 3 and harvested after an additional 24 h of incubation.

Immunoprecipitations and Western blot (immunoblot) analysis. The cells were washed twice with phosphate-buffered saline (PBS), cooled on ice for 5 min, and then lysed in 1 ml of immunoprecipitation buffer containing 0.05 M Tris hydrochloride, pH 7.2, 0.15 M NaCl, 1.0% deoxycholate, 0.1% SDS, 1.0% Triton X-100, 2.5 mM phenylmethylsulfonyl fluoride, and 50 μg of DNase I per ml. The lysate was collected, and 300-μl aliquots were reacted for 30 min at room temperature with MAb 52s or 53s at a final dilution of 1:2 or with LP11 at a dilution of 1:30. Protein A-sepharose swelled in Tris hydrochloride, pH 7.2, was added, and the samples were rotated at 4°C for 2 h. The sepharose was pelleted by spinning in a microfuge for 1 min followed by three washes in immunoprecipitation buffer. The sepharose pellet was resuspended in Laemmli sample buffer (36), incubated at room temperature for 20 min, and then spun in a Microfuge (Beckman Instruments, Inc.), and the supernatants were collected. Samples were boiled for 3 min and then loaded onto an SDS-7.0% polyacrylamide gel cross-linked with N,N'-methylene bis acrylamide (Bio-Rad Laboratories). At the completion of the separation, the proteins were transferred to nitrocellulose (BA 85; Schleicher & Schuell, Inc.) in a Tris-glycine buffer containing 20% methanol and 0.1% SDS as described previously (15). Transfer took place in an electroblot apparatus (Bio-Rad Laboratories) at 250 mA for 16 h. The nitrocellulose was blocked with PBS-3% bovine serum albumin for 16 h at room temperature and then incubated with polyclonal anti-gH-TrpE fusion protein serum diluted 1:200 in PBS-1% bovine serum albumin for 1 h. The blots were washed with PBS-1% Nonidet P-40 (NP40). Bound antibody was detected with [^{125}I]protein A (Amersham Corp.). Blots were wrapped in clingfilm and placed against Kodak X-Omat S film with an intensifying screen at −70°C for 1 to 7 days.

Immunoprecipitation with radiolabeled lysate was prepared as above, except cells were labeled for 16 h with [^{35}S]methionine (800 Ci/mmol; Amersham) at 25 μCi/ml in methionine-free medium containing 1% fetal calf serum. After separation of lysate products on a 10% polyacrylamide gel with SDS, the gel was dried down and exposed to film.

Immunofluorescence. Cells were seeded on glass cover slips and transfected or infected or both as described above. For surface staining, cells were fixed with 2% formaldehyde in PBS for 3 min and then washed three times with PBS. For internal staining, cells were additionally permeabilized with 1% NP40 in PBS for 5 min and then washed three times with PBS. Cells were blocked for nonspecific or Fc binding with a 1:1 mixture of NCS and preimmune rabbit serum (RS) and then incubated for 1 h at room temperature with MAb 52s diluted 1:2 in PBS-1% NCS-1% RS. The cells were washed with PBS-1% NCS-1% RS and then incubated for 1 h with fluorescein isothiocyanate conjugated to rabbit anti-mouse immunoglobulin G (DAKO Immunochemicals) diluted 1:50 in PBS-1% NCS-1% RS. The cells were washed with PBS-1% NCS-1% RS and then examined at 400× magnification through a microscope with a Leitz I2 filter allowing illumination at 450/410-nm wavelength.

RESULTS

Expression of HSV-1 gH in *ts* COS cells. The plasmid pUG-H1 was constructed to allow efficient expression of HSV-1 gH in a transient expression system (Fig. 1). This plasmid carried the gH coding sequence from HSV-1 HFEM DNA under control of the strong promoter for transcription from the major immediate-early gene from HCMV. This

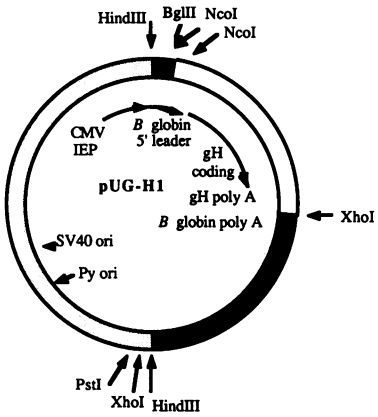

FIG. 1. Features of the gH expression plasmid pUG-H1. A 3.4-kbp partial *Nco*I-*Xho*I fragment containing the entire coding region of HSV-1 gH but lacking the promoter was ligated into plasmid SP64T (35), linearized with *Hin*dIII, and then cloned into vector CDM8 (60). Plasmid pUG-H2 has 3.0 kbp of SP64T and 400 bp of CDM8 sequences deleted by *Xho*I digestion followed by religation. ■, Plasmid SP64T sequences; ▩, CDM8 sequences; □, gH coding sequences. IEP, Immediate-early promoter.

contains the upstream enhancer region but not the cell-type-specific far upstream regions (48, 61). Transcription is initiated from the start site of β-globin from xenopus and transcribes the stable 5′ untranslated leader sequence of β-globin (34) where it is joined to the coding sequence of HSV-1 gH. The nucleotide sequence of the gH gene in this plasmid construct was shown to be identical to those from the parent virus DNA clone (19) and an independently isolated clone (data not shown). A *Xho*I deletion derivative of pUG-H1, pUG-H2, was also constructed which had 3.0 kbp of the SP64T sequence plus 400 bp of the CDM8 plasmid sequence deleted from the *Xho*I site in the gH sequence to the *Xho*I site in the CDM8 sequence (Fig. 1).

The plasmid pUG-H1 was transfected into *ts* COS cells and assayed for expression of gH by detection in Western blots by anti-TrpE-gH fusion protein antiserum (Fig. 2). The cells were transfected and then incubated for 3 days at various temperatures or combinations of temperatures: at 33°C, permissive for expression of the temperature-sensitive T antigen in *ts* COS cells; at 37°C, semipermissive; or at 40°C, nonpermissive. The expression of stable T antigen at the permissive temperature, 33°C, allows utilization of the plasmid simian virus 40 ori sequence and amplification of adjacent sequences. The apparent molecular weight of the gH produced was approximately 105,000, which is intermediate to those of the glycosylated precursor form (100,000) and the mature glycosylated form (110,000) of gH from infected cells. The greatest expression was achieved by incubating the transfected cells at 40°C overnight and then shifting down to 33°C for 48 h (Fig. 2), and similar results have been found with transfection of pUG-H2 (data not shown). The amount of gH thus produced exceeded the level of gH synthesized if the cells were shifted to the permissive temperature immediately after transfection (Fig. 2). This could be due to the toxic effect gH may have on cells to inhibit growth or metabolism. Thus, if the cells are maintained at 40°C overnight, cells may continue to divide, resulting in the expansion of the number of cells receiving plasmid, whereas continuous maintenance at the permissive temperature, 33°C, could result in levels of gH which are refractory to cell growth. Consistent with the idea that

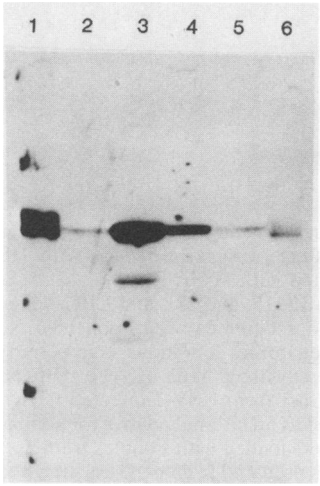

FIG. 2. Western blot analysis of HSV-1 gH (pUG-H1) produced at different incubation temperatures. Cell extracts were prepared from pUG-H1-transfected *ts* COS cells (lanes 2, 3, 4, and 5), HSV-1 SC16-infected BHK cells (lane 1), or HSV-1 SC16-infected cell DNA-transfected *ts* COS cells (lane 6). Cell extracts were then electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal TrpE-HSV-1 gH fusion protein antiserum. All lanes were loaded with extracts prepared from approximately 5×10^5 cells. Lane 1 is from cells infected with HSV-1 SC16 at a multiplicity of infection equal to five and then harvested 20 h postinfection, this was included as a reference for the largest amount of gH produced from an infection. Lanes 2 through 6 are extracts of cells harvested 72 h posttransfection at various incubation temperatures. Lanes: 2, 72 h at 40°C; 3, 24 h at 40°C and then 48 h at 33°C; 4, 72 h at 37°C; 5, 72 h at 33°C; 6, 72 h at 37°C.

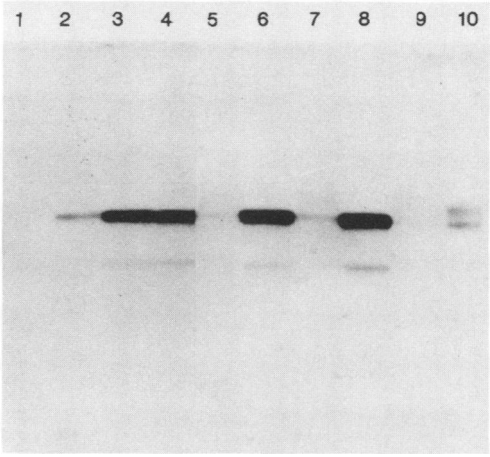

FIG. 3. Western blot analysis of HSV-1 gH (pUG-H1) produced at different times posttransfection. Cell extracts were prepared from pUG-H1-transfected *ts* COS cells harvested at various times posttransfection and then electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal TrpE-HSV-1 gH fusion protein antiserum. Lanes 5, 6, 7, 9, and 10 are samples from the experiment shown in Fig. 2 as lanes 2, 3, 4, 6, and 1, respectively. All lanes were loaded with extracts from approximately 5×10^5 cells. Lane 1 is the result of lysate harvested 20 h after transfection at 40°C. Lanes 2, 3, 4, and 8 show the results for extracts of cells which were treated for 20 h after transfection at 40°C and then shifted down to 33°C for 24 h (lane 2), 48 h (lane 3), 72 h (lane 8), and 96 h (lane 4).

constitutive expression of gH is toxic are the results from attempts we have made to establish cell lines expressing gH constitutively. In duplicate trials, all lines cotransfected with SV2 neo and pUG-H1 and then selected with G418 grew more slowly than those receiving SV2 neo and the CDM8 vector, resulting in eventual loss of gH expression with passage (data not shown). An alternative explanation for the high levels produced by treatment at 40°C for 24 h and then 33°C for 48 h may be that incubation for 72 h at the permissive temperature simply results in degradation of gH in cells dying from the known toxic effects of overproduction of T antigen (58).

To test the accumulation of gH expression in this system, a time course experiment was performed. After incubation overnight at 40°C, the transfected cells were incubated at 33°C, harvested at 24-h intervals until 96 h, and then analyzed for expression of gH. High levels of gH were expressed constantly between 48 and 96 h after the downshift in temperature (Fig. 3). More gH was synthesized 24 h after downshift to 33°C than if the cells were left at the permissive temperature for 72 h immediately after transfection. Expression of gH in pUG-H1-transfected MOP8 cells was equal to the level produced in transfected *ts* COS cells at 33°C for 72 h (not shown). MOP8 cells constitutively expressed at 37°C the polyomavirus T antigens, thus allowing utilization of the polyomavirus ori sequence in pUG-H1. The differences in gH expression with different temperature treatments were not due to differences in transfection efficiencies. The transfection efficiency in *ts* COS cells at 40 and 33°C or MOP8 at 37°C appeared similar as monitored by plaque formation after transfection of HSV-1 DNA, although transfection in *ts* COS cells at 37°C was only approximately twofold higher, as noted previously (58). Thus, incubation overnight at the nonpermissive temperature of 40°C in transfected *ts* COS before downshift to the permissive 33°C appears to be compatible with plasmid and/or cell survival resulting in overproduction of HSV-1 gH.

Antigenicity of HSV-1 gH in *ts* COS cells. Cells transfected with pUG-H1 were lysed in immunoprecipitation buffer and reacted with MAbs 53s, 52s, and LP11. MAbs 52s and LP11 are type 1 specific, neutralize HSV-1 infectivity efficiently in the absence of complement, prevent virus-induced cell fusion, and inhibit cell-to-cell spread (8, 19, 62). These antibodies appear to be directed at different sites, since mutants selected for resistance to one MAb were still sensitive to the other two (data not shown). MAb 53s is type common and immunoprecipitates both HSV-1 gH and HSV-2 gH. All three MAbs recognize conformational epitopes and will not react with denatured forms of gH in Western blots. Although all three MAbs recognized gH produced by infection of *ts* COS cells, only 52s immunoprecipitated equally well gH produced by transfection of the gene in *ts* COS cells (Fig. 4A, lanes 1 to 3 and 10 to 12). However, on longer exposure (Fig. 4B), it is apparent that a very small amount of gH was immunoprecipitated by MAbs 53s and LP11. This could be due either to a small proportion of gH molecules achieving the conformation recognized by these MAbs or to an altered conformation such that 53s and LP11 bind to gH with reduced affinity.

Ability of HSV to rescue antigenicity of gH in *ts* COS cells. The antigenicity of gH synthesized in transfected *ts* COS cells was examined after superinfection with HSV. To distinguish expression from the transfected gH gene from the virus product, both a mutant HSV-1 strain and HSV-2 were used as superinfecting viruses. The first experiment utilized a recent clinical isolate of HSV-1, strain 65, which is naturally resistant to LP11 (7) because of point mutations in

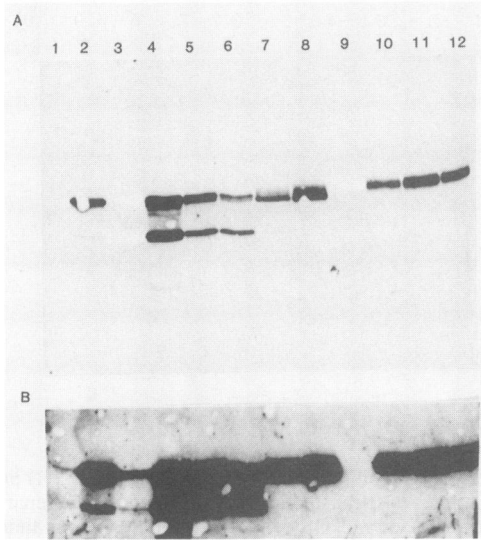

FIG. 4. Western blot analysis of immunoprecipitations of HSV-1 gH (pUG-H1): effect of superinfections with HSV-1 65. Cell lysates were reacted with MAbs 53s (lanes 1, 4, 7, and 10), 52s (lanes 2, 5, 8, and 11), or LP11 (lanes 3, 6, 9, and 12). Then immunoprecipitates were collected, electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal TrpE-HSV-1 gH fusion protein antiserum. All lanes were loaded with immunoprecipitates from approximately 2×10^6 cells. Lanes: 1, 2, and 3 lysates prepared from pUG-H1-transfected *ts* COS cells; 4, 5, and 6, lysates from pUG-H1-transfected *ts* COS cells which were then superinfected with HSV-1 65; 7, 8, and 9, *ts* COS cells infected with HSV-1 65; 10, 11, and 12, *ts* COS cells infected with HSV-1 HFEM. (A) 20-h exposure; (B) 7-day exposure.

the gH gene (Gompels and Minson, in preparation). When HSV-1 65 was used to superinfect the transfected cells, all three MAbs immunoprecipitated the synthesized gH equally well (Fig. 4A, lanes 4 to 6), whereas, infection of *ts* COS cells with HSV-1 65 alone resulted in production of a gH which was not immunoprecipitated by LP11. There was no detection even after prolonged exposure of the Western blot (Fig. 4A and B, lane 9). There were differences in apparent molecular weight between the forms of gH produced from transfection and those produced from transfection plus superinfection. Transfection with pUG-H1 produced gH of 105,000 in apparent molecular weight, with a minor species of 85,000, while transfection plus superinfection resulted in HSV-1 gH forms of 100,000, 105,000, and 85,000 (Fig. 4).

The antigenic rescue was repeated using HSV-2, while the superinfection with HSV-1 65 was repeated as an internal control. Figure 5 (lanes 2 to 10) shows a result identical to that in Fig. 4, while Fig. 5 (lanes 11 to 16) shows that superinfection with HSV-2 also allowed antigenic rescue. Since the Western blot of the immunoprecipitations was reacted with a HSV-1 gH-specific polyclonal serum, the HSV-2 gH produced by infection with HSV-2 was not detected. Thus, if the transfected *ts* COS cells were superinfected with HSV-2, the HSV-1 gH produced from the transfected gene was shown to be immunoprecipitated equally well by all three MAbs. The antigenic rescue caused by HSV-2 appears qualitatively different from that of the HSV-1 mutant since the 85,000 species remained a minor form (Fig. 5). This is not due to differences in affinity for the MAbs, since analysis of the total-cell lysates shows similar molar distinctions (Fig. 6).

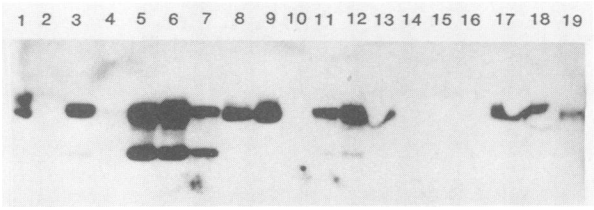

FIG. 5. Western blot analysis of immunoprecipitations of HSV-1 gH (pUG-H1): effect of superinfections with HSV-2 25766. Cell lysates were reacted with MAbs 53s (lanes 2, 5, 8, 11, 14, and 17), 52s (lanes 3, 6, 9, 12, 15, and 18), and LP11 (lanes 4, 7, 10, 13, 16, and 19). Immunoprecipitates were collected and then electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal TrpE-HSV-1 gH fusion protein antiserum. The cell extract from HSV-1-infected BHK cells is shown in lane 1 and included as an internal control for comparison to the other blots. All lanes were loaded with samples from 2×10^6 cells. Lanes: 2, 3, and 4, lysates prepared from pUG-H1-transfected *ts* COS cells; 5, 6, and 7, lysates from pUG-H1-transfected *ts* COS cells superinfected with HSV-1 65; 8, 9, and 10, lysates from HSV-1 65-infected *ts* COS cells; 11, 12, and 13, lysates from pUG-H1-transfected *ts* COS cells superinfected with HSV-2 25766; 14, 15, and 16, lysates from HSV-2 25766-infected *ts* COS cells; 17, 18, and 19, lysates from HSV-1 HFEM-infected *ts* COS cells.

Cell localization of HSV-1 gH. The cellular localization of gH under different conditions of transfection and superinfection was examined. The HSV-2 superinfection experiment was repeated, and the cells were also examined for cell surface expression by reacting unfixed cells with the HSV-1-specific MAb 52s, which can immunoprecipitate gH from transfected and infected cells. This was first examined by an enzyme-linked immunosorbent assay which showed surface expression of gH only on the superinfected transfected cells or the HSV-1-infected positive control (data not shown).

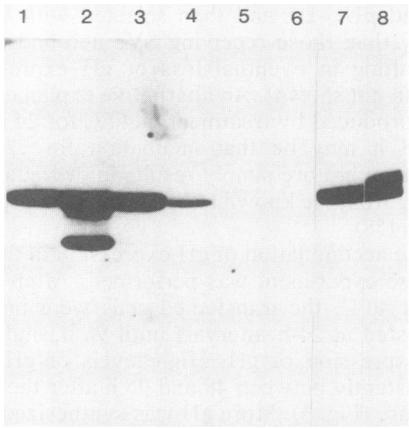

FIG. 6. Western blot analysis of HSV-1 gH (pUG-H1): effect of superinfections with HSV-1 65 and HSV-2 25766 on the total amount of HSV-1 gH produced. Cell extracts from the experiment shown in Fig. 5 were prepared without any reaction with antibody and directly electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal TrpE-HSV-1 gH fusion protein antiserum. All lanes are loaded with approximately 5×10^5 cells. Lanes: 1, *ts* COS cells transfected with pUG-H1; 2, *ts* COS cells transfected with pUG-H1 and then superinfected with HSV-1 65; 3, *ts* COS cells transfected with pUG-H1 and then superinfected with HSV-2 25766; 4, *ts* COS cells infected with HSV-1 65; 5, *ts* COS cells infected with HSV-1 HFEM; 6, *ts* COS cells infected with HSV-2 25766; 7, *ts* COS cells transfected with pUG-H1; 8, BHK cells infected with HSV-1 SC16.

Immunofluorescence was then used to investigate this in more detail, and the results are shown in Fig. 7. The gH produced in transfected *ts* COS showed a granular distribution in the cytoplasm along with marked perinuclear staining; little or no fluorescence was observed on the surface. However, gH produced from transfected *ts* COS cells superinfected with HSV-2 showed punctilate staining on the cell surface, whereas the cells infected with HSV-2 alone showed no specific staining (Fig. 7). Interestingly, in the pUG-H1-transfected *ts* COS cells without superinfection, approximately 30% of the cells expressing gH showed staining in multinucleate giant cells (Fig. 8). However, in those cells transfected with the vector CDM8 and stained for T antigen, similar multinucleate cells were observed, but only at a frequency of 1 to 5% (data not shown). These results suggest that gH contributes to cell fusion and will be described elsewhere.

Antigenicity and cell localization of gH in *ts* COS cells infected with an HSV-1 mutant deficient in production of virions. The cell surface localization and antigenic changes of gH in transfected and then superinfected cells could be due to insertion into the viral envelope and nonspecific association with the plasma membrane following virion egress. To rule out this interpretation, *ts* COS cells were infected with a temperature-sensitive mutant HSV-1 *ts*1201, which at the nonpermissive temperature fails to package DNA and does not form enveloped virions (2, 55). Immunoprecipitation of cell lysates prepared from infected cells at the nonpermissive and permissive temperatures shows that the gH produced in an infection where no virions are produced was still recognized by all three MAbs (Fig. 9). Cell localization by immunofluorescence using 52s shows that at permissive and nonpermissive temperatures, gH was expressed at the cell surface (Fig. 10). Thus, insertion into virions does not appear to be necessary for expression of the antigenic sites recognized by the three gH-specific MAbs or for transport to the cell surface.

DISCUSSION

Our results show that HSV-1 gH can be produced in a transient mammalian expression system. The gH was overproduced relative to that made during a high-multiplicity virus infection. Unlike other HSV virion glycoproteins expressed (3, 4, 9, 11, 27–30, 32, 37, 41, 46, 60, 68, 72), the characteristics of the gH produced during a transfection differed from those of gH produced during an infection. The gH was slightly smaller and antigenically distinct and was not transported to the cell surface. Similar results have been found with HSV-1 gH expressed by recombinant vaccinia virus (A. Forrester, V. Sullivan, G. Smith, and A. Minson, manuscript in preparation). In addition, HCMV gH expressed by recombinant vaccinia virus does not appear to be localized to the cell surface (13). Superinfection of HSV-1 gH-transfected *ts* COS cells with virus resulted in (i) changes in the apparent molecular weight, (ii) forms which appear antigenically identical to gH produced from an infection, and (iii) transport of gH to the infected-cell surface. The transfected gH could be distinguished from the gH produced from the superinfecting virus by molecular weight and antigenicity in reactions with three gH-specific MAbs and one gH-specific polyclonal serum. Antigenic rescue by the superinfecting virus was achieved by using either an HSV-1 mutant strain which produces a gH not recognized by MAb LP11 or with HSV-2 which produces a gH not recognized by MAb 52s or LP11 or the type 1-specific polyclonal TrpE-gH fusion protein antiserum used to detect the immunoprecipitation products in Western blots. In addition, the forms of gH produced by superinfection by the HSV-1 mutant were distinct from those produced by infection alone. Thus, it was possible to show that although MAb 52s recognized the transfected gH product, modification caused by other virus products was necessary to form antigenic structures of gH recognized efficiently by MAbs 53s and LP11.

The antigenic structures on HSV-1 gH recognized by MAbs 53s, 52s, and LP11 appear to be important for the function of gH in mediating infectivity. These three antibodies neutralized virus infectivity by binding to their respective conformationally dependent sites in gH. Although gH antibodies are as efficient as gD antibodies in neutralizing virus infectivity, gH does not appear equally immunogenic, at least during infections of the mouse, because MAbs to gH have been produced much less frequently than MAbs to gD (19, 21, 54); antibodies 53s, 52s, and LP11 are the only HSV gH-specific MAbs currently available (8, 19, 62). The gH-specific MAbs appear to be directed against distinct sites on gH, since mutants resistant to one MAb are still recognized by the other MAbs (data not shown). However, the biological properties of 52s and LP11 appear to be similar. They both neutralize virus infection efficiently and act to inhibit spread of virus from infected to uninfected cell; they also prevent virus-induced cell fusion of syncytial strains (8, 19, 62). However, while 52s recognized the gH produced by transfections, LP11 and 53s recognized this gH only after modifications resulting from virus superinfection. As determined by analyses of antibody-resistant mutants, we have shown that LP11 recognized a conformationally complex structure in the amino-terminal half of the cytoplasmic domain of HSV-1 gH and that changes in this domain affect virus viability (Gompels and Minson, in preparation). Although both LP11 and 53s recognized a very small amount of the transfected gH (Fig. 4B), normal recognition was achieved only after virus superinfection. Therefore, either the correct antigenic structures are formed in only a small proportion of the molecules or the structure is altered such that the affinity of binding is decreased. The expression of other virus genes appears to modulate this effect.

It could be argued that the effect of superinfection on immune recognition and cell localization of the transfected gH is due to insertion of gH into the viral membrane, with subsequent processing and cell surface transport achieved by egress of virus through the Golgi network and association of virus with the surface of the plasma membrane during exit. However, in the infected cell, both the precursor and fully mature products were recognized by all three antibodies, although only the fully mature product is found on excreted virions (15). Furthermore, in studies on the temperature-sensitive mutant *ts*Q26 at the nonpermissive temperature, mostly the precursor form of gH is synthesized and incorporated into intracellular virions, yet this virus can be neutralized by the gH-specific antibodies (15). Finally, for more direct evidence of the effect of gH processing and transport by insertion into the virus membrane, we studied the temperature-sensitive mutant virus *ts*1201 which at the nonpermissive temperature does not produce virions. This virus has a mutation in the UL26 gene encoding a capsid protein (p40), such that at the nonpermissive temperature, it fails to package DNA (2, 55). We showed, by infection of *ts* COS cells with *ts*1201 at the nonpermissive temperature, that in the absence of virions, a form of gH was produced which was still recognized by all three MAbs and, furthermore, this gH was transported to the cell surface. This

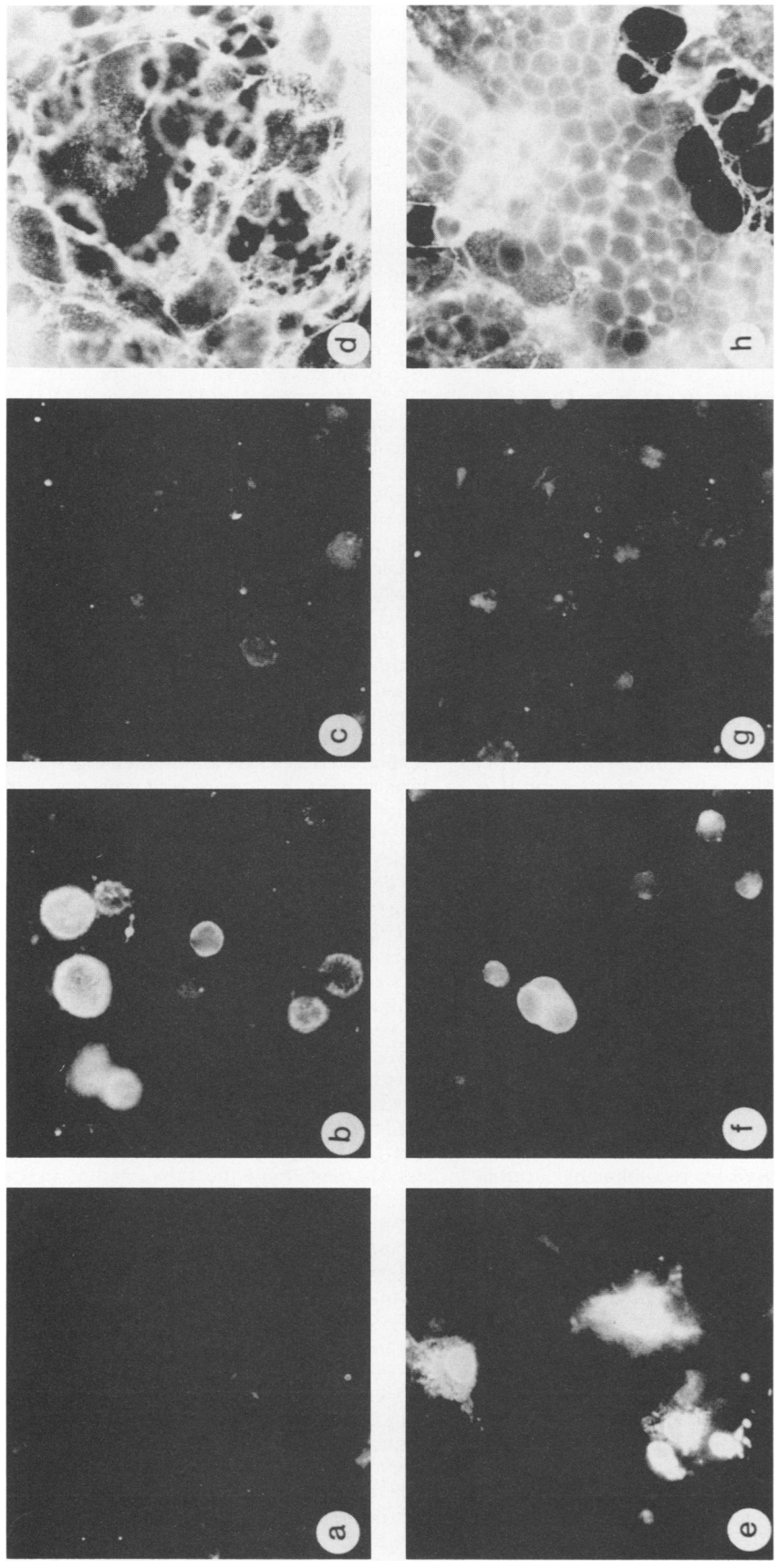

FIG. 7. Immunofluorescence analysis of HSV-1 gH (pUG-H1): effect of superinfection. Cells were prepared for surface fluorescence by fixation with 2% formaldehyde (a, b, c, and d) or for internal fluorescence by fixation with 2% formaldehyde followed by permeabilization with 1% NP40 (e, f, g, and h). Fixed cells were then reacted with the type 1-specific MAb 52s, bound antibody was detected by reaction with fluorescein isothiocyanate conjugated to rabbit anti-mouse immunoglobulins, and the cells were then viewed by fluorescence microscopy. Photographs a and e show the results for pUG-H1-transfected *ts* COS cells, b and f for pUG-H1-transfected *ts* COS cells superinfected with HSV-2 25766, c and g for HSV-2 25766-infected *ts* COS cells, and d and h for HSV-1 HFEM-infected *ts* COS cells.

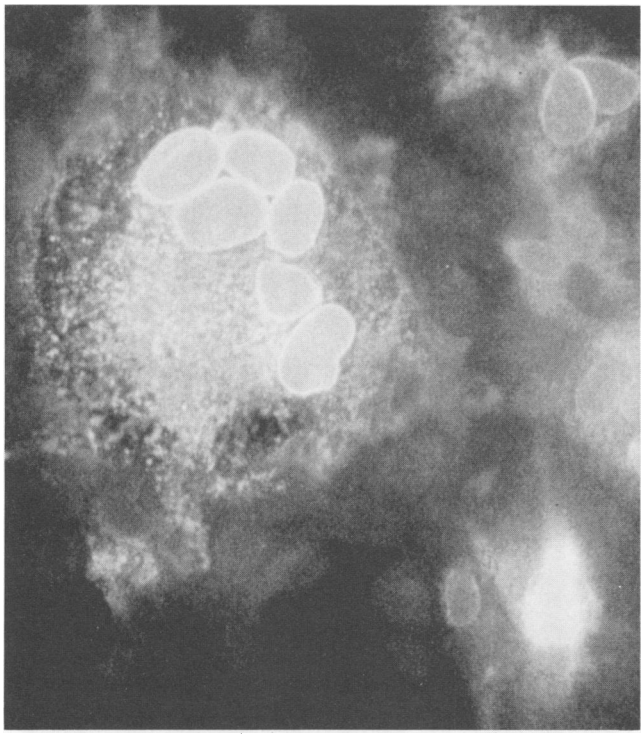

FIG. 8. Cell fusion in *ts* COS cells transfected with pUG-H1. Cells were fixed in 2% formaldehyde and permeabilized with 1% NP40. The *ts* COS cells were transfected with pUG-H1 and reacted with MAb 52s. Approximately 60% showed single-cell immunofluorescence (Fig. 7e), and 30% showed cytoplasmic fusion as above.

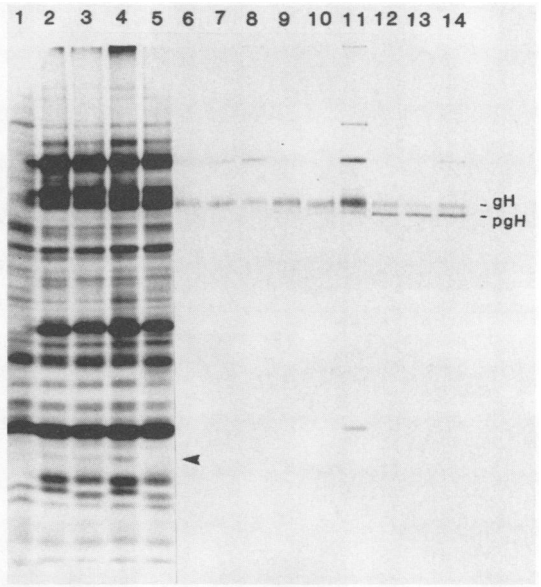

FIG. 9. Immunoprecipitation analysis of HSV-1 *ts*1201-infected *ts* COS cells. Cells were infected at a multiplicity of infection equal to 5 and then labeled with [^{35}S]methionine from 2 to 20 h postinfection. Infected-cell lysates were then reacted with MAb 53s (lanes 6, 9, and 12), 52s (lanes 7, 10, and 13), or LP11 (lanes 8, 11, and 14). The HSV-1 *ts*1201-infected cells were incubated at the permissive (32°C; lanes 3, 9, 10, and 11) or nonpermissive (40°C; lanes 5, 12, 13, and 14) temperature. The total antigen from HSV-1 HFEM-infected cells incubated at 33 or 40°C is shown in lanes 2 and 4; total mock-infected antigen is shown in lane 1; total antigen from HSV-1 *ts*1201-infected cells at 33 and 40°C is in lanes 3 and 5, respectively. The arrow shows the lack of the 39,000-molecular-weight form of p40 at the nonpermissive temperature in cells infected with HSV-1 *ts*1201 (56). Other lanes: 6, 7, and 8, immunoprecipitates of HSV-1 HFEM-infected cells; 9, 10, and 11, immunoprecipitates of HSV-1 *ts*1201-infected cells incubated at 32°C; 12, 13, and 14, immunoprecipitates of HSV-1 1201-infected cells incubated at 40°C.

implies that the incorporation of gH into virions does not explain the results of transfection-superinfection experiments and that additional virus gene expression is necessary for achieving the correct antigenic structure and cell localization of gH.

Several factors have been described which may affect the conformation and transport of gH. Although limited, the current analyses of HSV virion glycoproteins gB, gC, gD, gE, gG, and gI synthesized in mammalian cell expression systems show no differences in antigenic structure, glycoprotein modification, or cell surface localization (3, 4, 9, 11, 27–30, 32, 37, 41, 46, 60, 68, 72), unlike our results for gH expression. The most extensive studies of antigenic sites have been in transient expression assays in COS cells of gD, the other major target for neutralizing antibodies against HSV (11, 27, 46, 72). These studies investigated the antigenic structure of gD with 13 MAbs representing seven groups of antibodies which recognize both continuous and discontinuous sites. Six of the MAbs used also neutralize virus infectivity (11, 46, 72). It was shown that the discontinuous sites in the cytoplasmic domain, including the major neutralization site, have their structures maintained by six conserved cysteine residues (46, 70). In gH, the major conserved features are residues which surround four conserved cysteines in the carboxy-terminal half of the cytoplasmic domain (13, 19, 21, 43). It has been shown for Sendai virus that high-mannose oligosaccharide addition must precede disulfide bonding for formation of proper folding of virus glycoproteins (69). It is possible that HSV infection modifies the endoplasmic reticulum-Golgi network such that cotranslational processing and subsequent transport through the Golgi of membrane glycoproteins may be altered. The transport of gD in HSV-infected cells has been shown to be slower than that in uninfected cells, suggesting that virus infection may affect glycoprotein interactions with the endoplasmic reticulum (28). In this respect, it is notable that the human immunodeficiency virus envelope glycoprotein gp160 when expressed in a HSV vector is transported to the cell surface but not incorporated into the viral envelope (71). It is possible that gH has internal signals which allow for retention in the endoplasmic reticulum which can only be overcome after specific interactions with a virus-modified endoplasmic reticulum-Golgi network. Two observations are useful in this regard. First, the VP7 glycoprotein of rotavirus sets a precedent for such signals, since both the signal sequence and the first 110 amino acids are necessary for the specific retention of VP7 in the endoplasmic reticulum (66). Second, different sialyl and galactosyl transferases have been observed in HSV-infected cells (52). This could affect transport of gH through the Golgi or result in inappropriate glycosylation which may mask antigenic sites as has been shown for the influenza virus hemagglutinin (63). It remains to be studied further whether there is a link between the antigenic changes and transport to the cell surface of gH provided by superinfection of the transfected cells. Finally, a more specific effect on gH folding may be mediated by interaction with another virus protein. It has been shown, for example, that alterations in the M2 integral membrane protein of influenza virus may affect the conformation of the

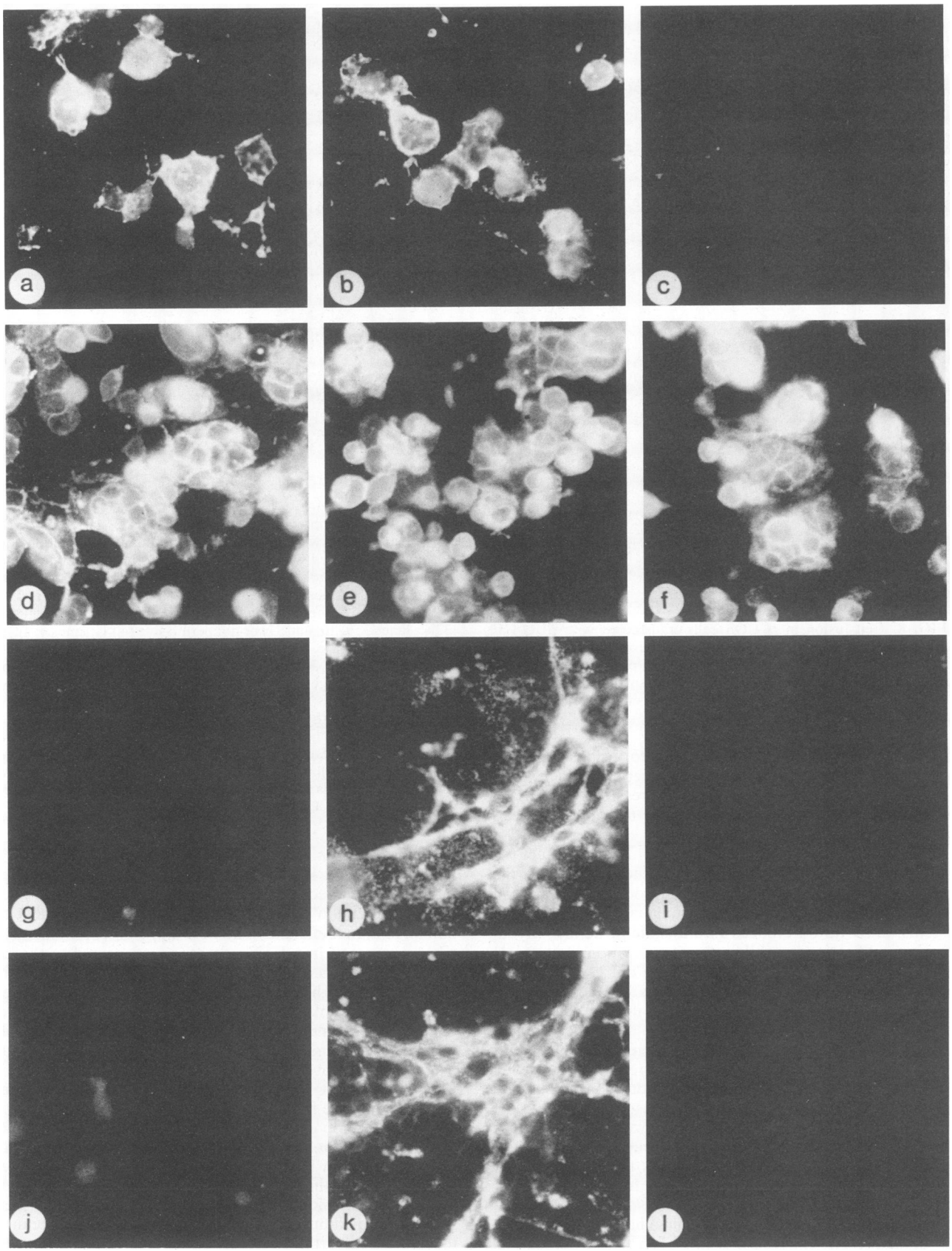

FIG. 10. Immunofluorescence analysis of HSV-1 *ts*1201-infected *ts* COS cells. Cells were infected at a multiplicity of infection equal to 1, incubated at 40°C for 20 h, fixed, and then reacted with antibodies as described in the legend to Fig. 8. In panels a, b, c, g, h, and i, surface fluorescence is shown, while in panels d, e, f, j, k, and l, internal staining is shown. Cells infected with HSV-1 *ts*1201 are shown in panels a and d, and HSV-1 *ts*R1201, a revertant of mutant strain *ts*1201, is shown in panels b and e. HSV-1 *ts*Q26, a temperature-sensitive mutant which does not express gH at the cell surface at 40°C (16), is shown in panels c and f. HSV-2 25766-infected cells are shown in panels g and j. HSV-1 HFEM-infected cells are shown in panels h and k, and uninfected cells are in panels i and l.

hemagglutinin glycoprotein (5, 22). It is not certain whether there is a more general effect of herpesvirus infection on cellular mechanisms of intracellular transport affecting glycoprotein modification and folding or whether a specific virus product is responsible for the differences shown here in gH antigenic structure and cell localization. What is clear is that gH expressed by itself is not transported to the cell surface and does not form an antigenic structure which is important for the function of gH in mediating virus infection, while expression of other virus products allow both cell surface localization and an antigenically normal form of gH to be produced.

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