

# Herpes Simplex Virus Type 1 Glycoprotein C-Negative Mutants Exhibit Multiple Phenotypes, Including Secretion of Truncated Glycoproteins

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A virus-neutralizing monoclonal antibody specific for glycoprotein C (gC) of herpes simplex virus type 1 strain KOS was used to select a number of neutralization-resistant mutants. A total of 103 of these mutants also were resistant to neutralization by a pool of gC-specific antibodies and thus were operationally defined as gC<sup>-</sup>. Analysis of mutant-infected cell mRNA showed that a 2.7-kilobase mRNA, comparable in size to the wild-type gC mRNA, was produced by nearly all mutants. However, six mutants, gC<sup>-</sup>5, gC<sup>-</sup>13, gC<sup>-</sup>21, gC<sup>-</sup>39, gC<sup>-</sup>46, and gC<sup>-</sup>98, did not produce the normal-size gC mRNA but rather synthesized a novel 1.1-kilobase RNA species. These mutants had deletions of 1.6 kilobases in the coding sequence of the gC structural gene, which explains their gC<sup>-</sup> phenotype. Despite the production of an apparently normal mRNA by the remaining 97 mutants, only 7 mutants produced a detectable gC polypeptide. In contrast to wild-type gC, which is a membrane-bound glycoprotein with an apparent molecular weight of 130,000 (130K), five of these mutants quantitatively secreted proteins of lower molecular weight into the culture medium. These were *syn*LD70 (101K), gC<sup>-</sup>8 (109K), gC<sup>-</sup>49 (112K), gC<sup>-</sup>53 (108K), and gC<sup>-</sup>85 (106K). The mutant gC<sup>-</sup>3 secreted a protein that was indistinguishable in molecular weight from wild-type KOS gC. Another mutant, gC<sup>-</sup>44, produced a gC protein which also was indistinguishable from wild-type gC by molecular weight and which remained cell associated. Pulse-labeling of infected cells in the presence and absence of the glycosylation inhibitor tunicamycin demonstrated that these proteins were glycosylated and provided estimates of the molecular weights of the nonglycosylated primary translation products. The smallest of these proteins was produced by *syn*LD70 and was 48K, about two-thirds the size of the wild-type polypeptide precursor (73K). Physical mapping of the mutations in *syn*LD70 and gC<sup>-</sup>8 by marker rescue placed these mutations in the middle third of the gC coding sequence. Mapping of the mutations in other gC<sup>-</sup> mutants, including two in which no protein product was detected, also placed these mutations within or very close to the gC gene. The biochemical and genetic data available on mutants secreting gC gene products suggest that secretion is due to the lack of a functional transmembrane anchor sequence on these mutant glycoproteins.

Cells infected with herpes simplex virus type 1 (HSV-1) synthesize at least four virus-specified glycoproteins which are found in the plasma membrane and the virion envelope (1, 14, 15, 35). The viral glycoprotein designated gC is not expressed by some HSV-1 mutants (17, 20, 22, 32). Since many of the mutants which do not express gC grow as well as wild-type HSV-1 in cell culture, gC is considered to be nonessential for virus replication in vitro (20, 32). However, nearly all clinical isolates of HSV-1 express gC (28, 38), suggesting that this glycoprotein may have an important function in virus replication in humans. Although the function of gC remains uncertain, the role of gC as a major HSV-1 antigen has been established in numerous studies (4-6, 10, 11, 26, 29).

In our laboratories, the antigenic properties of HSV-1 glycoproteins are under investigation. In a previous report (20), we have described the production of virus-neutralizing monoclonal antibodies and their use in selecting mutants of HSV-1 (strain KOS) which were altered in antigenic struc-

ture. Selections with gC-specific monoclonal antibodies resulted in two distinct mutant classes: monoclonal antibody-resistant (*mar*) mutants and gC<sup>-</sup> mutants. *mar* mutations within the gC gene result in the expression of gC that is antigenically altered but otherwise similar to wild-type gC. These mutants are resistant to the selecting antibody but are sensitive to at least one other gC-specific neutralizing monoclonal antibody. Mutations giving rise to *mar* mutants upon selection with a gC-specific monoclonal antibody should be of the missense type. Mutants with the gC<sup>-</sup> phenotype produce virions which do not contain an immunoreactive envelope form of gC. Since the gC gene product is not essential for virus replication in vitro, it might be expected that a wide variety of genetic changes could give rise to this phenotype.

In this report, we extend our observations on gC<sup>-</sup> mutants selected with monoclonal antibodies. The characterization of 103 gC<sup>-</sup> mutants showed that this phenotype can be caused by several molecular mechanisms. These mechanisms include deletions of portions of the gC gene and mutations which result in secretion of truncated gC gene products. These findings demonstrate that antibodies can be used as powerful selective agents for the isolation of diverse sets of mutants altered in nonessential membrane proteins.

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**MATERIALS AND METHODS**

**Cells and viruses.** Vero cells, human embryonic lung (HEL) cells, and rabbit kidney cells were grown in modified Eagle minimal essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS) (GIBCO) as described previously (9). A plaque-purified isolate of the wild-type KOS strain, designated KOS 321, was used in all experiments (20). The mutant *synLD70* was isolated and characterized as gC<sup>-</sup> previously (20, 33). Virus stocks were prepared by infection of Vero cells at a low multiplicity of infection (MOI), and titers were determined on Vero cell monolayers.

**Monoclonal antibodies.** The isolation, characterization, and production of ascites fluids of the gC-specific monoclonal antibodies C2, C3, C4, and C7 have been described previously (20). Monoclonal antibodies C9, C10, C11, C13, C14, C15, C16, and C17, which are specific for gC, and B6, which is specific for gB, were isolated by similar procedures and will be fully characterized elsewhere (S. D. Marlin, T. C. Holland, S. Highlander, M. Levine, and J. Glorioso, manuscript in preparation). Two pools of gC-specific antibodies were used in these experiments. Pool 1 consisted of equal volumes of antibodies C2, C3, C4, C7, C9, C10, C11, C13, C14, C15, and C16. Pool 2 consisted of equal volumes of antibodies C3, C10, C13, C16, and C17.

**Mutagenesis and selection of mutants.** Mutants with the

gC<sup>-</sup> phenotype were selected on the basis of resistance to neutralization by monoclonal antibody C11 at a dilution of 1:100. As noted below, some mutants were selected from bromodeoxyuridine-mutagenized stocks of KOS 321, which were prepared as described previously (20). Neutralization reactions were carried out as described previously (20), except that 10<sup>6</sup> PFU was used. Surviving virus was plaqued on Vero cell monolayers, and isolated plaques were picked after 3 days, put into 0.2 ml of MEM containing 5% FCS and 10 mM N-2-hydroxylthylpiperazine-N'-2-ethanesulfonic acid, and freeze-thawed to release virus. Fractions of each isolate were tested for resistance to C11 and pool 1 of gC-specific monoclonal antibodies in plaque reduction assays, as described by Rawls et al. (30). Isolates resistant to both C11 and pool 1 were plaque purified twice more and then retested for resistance to these antibodies. Isolates resistant to C11 and pool 1 were designated gC<sup>-</sup> mutants. A mutant virus was considered to be resistant to neutralization by an antibody or antibody pool if the neutralization titer of that antibody in neutralization assays with the mutant was five or more twofold dilutions lower than the antibody titer obtained in assays with wild-type virus (20).

**Labeling of infected cells.** For [<sup>35</sup>S]methionine labeling of the mature forms of the viral glycoproteins, a 16-h labeling period was used. HEL cell monolayers (10<sup>6</sup> cells) in T25 flasks (Costar, Cambridge, Mass.) were infected with wild-type or mutant viruses at an MOI of 10. After adsorption for

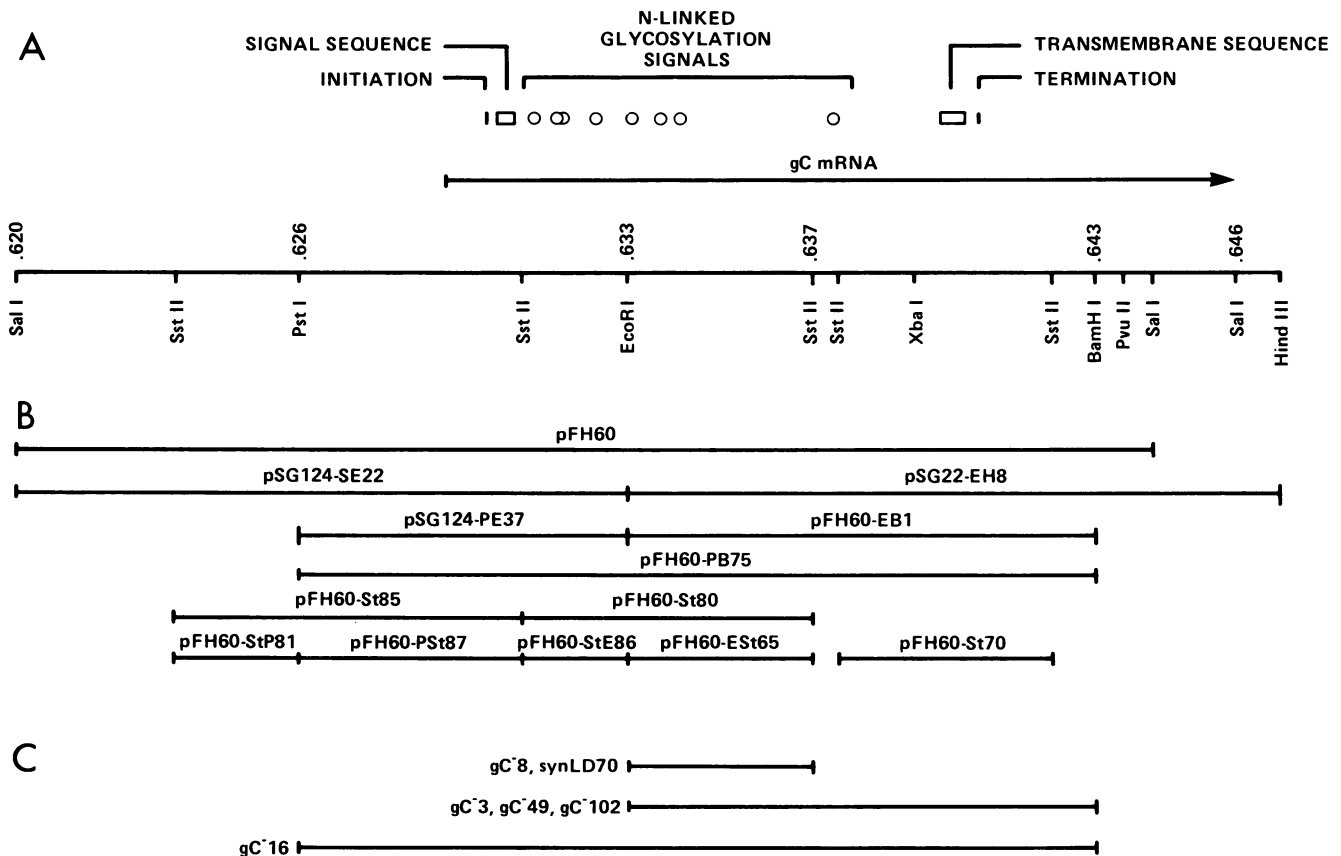


FIG. 1. Map of the region of the HSV-1 genome containing the gC gene. (A) Restriction enzyme sites, location of the gC mRNA, and location of the sequences encoding important domains of gC (7). (B) Cloned fragments of viral DNA. (C) Marker rescue of gC<sup>-</sup> mutants. The mutations conferring the gC<sup>-</sup> phenotype mapped within the limits shown for each of these viruses. The limits were determined by the overlap of the cloned wild-type restriction fragments which rescued the mutants.

1 h, the monolayer was overlaid with MEM-2% FCS and incubated at 37°C. After an additional 4 h, the medium was removed and replaced with methionine-free MEM-2% FCS-40  $\mu$ Ci of [<sup>35</sup>S]methionine (New England Nuclear Corp., Boston, Mass.) per ml. Approximately 20 h after infection, the labeling medium was removed and reserved. The infected cells were solubilized by the direct addition of 0.25 ml of lysis buffer (150 mM NaCl, 20 mM Tris, 1% Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride, 0.1 mM L-1-tosylamide 2-phenylethylchloromethyl ketone, 0.1 mM *N*-alpha-*p*-tosyl-L-lysine chloromethyl ketone [pH 8]) to the infected cell monolayer. Both the medium and cell extract were clarified by centrifugation at 15,000  $\times$  *g* for 15 min.

For determination of the molecular weights of the precursor forms of the viral glycoproteins, a 30-min pulse-labeling interval was used. Duplicate cultures of HEL cells were infected as described above. Seven hours after infection, the glycosylation inhibitor tunicamycin (Sigma Chemical Co., St. Louis, Mo., and Calbiochem-Behring, San Diego, Calif.) was added to the medium to a final concentration of 2  $\mu$ g/ml where indicated. After incubation at 37°C for an additional 2 h, the medium was decanted and replaced with Dulbecco phosphate-buffered saline, plus tunicamycin where indicated, plus 100  $\mu$ Ci of [<sup>35</sup>S]methionine per ml. The cultures were then incubated at 37°C for 30 min, the labeling medium was decanted, and the cells were extracted with lysis buffer as described above. The cell extracts were clarified by centrifugation at 15,000  $\times$  *g* for 15 min before immunoprecipitated.

**Immunoprecipitation.** Immunoprecipitation was done as described previously (20), except that pool 2 of gC-specific monoclonal antibodies was used instead of single monoclonal antibodies. Antigen-antibody complexes were collected with protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N.J.).

**Polyacrylamide gel electrophoresis.** Immunoprecipitates were solubilized in electrophoresis sample solution (20) and electrophoresed in 8.5% acrylamide gels cross-linked with *N,N'*-diallyltartardiamide (18:1 [wt/wt]) (9). Radioactive bands were visualized by fluorography (20). Molecular weights were estimated with <sup>14</sup>C-labeled protein molecular weight standards (New England Nuclear).

**Plasmid construction.** The regions of the HSV-1 genome contained in plasmids used in this study are shown in Fig. 1. The plasmid pFH60 was constructed by cloning the *Sall* T fragment of KOS 321 into the *Sall* site of pBR322 (12). The *EcoRI-BamHI* fragment contained within the *Sall* T fragment was subcloned by digesting pFH60 with *EcoRI* and *BamHI* and ligating into the *EcoRI-BamHI* site of pBR322. Plasmids pFH60-PB75, pFH60-St85, pFH60-St80, pFH60-St70, and pFH60-EST65 were constructed by insertion of the appropriate fragment from pFH60 into the *PstI* site of pBR322 by oligodeoxyguanosine-oligodeoxycytidine tailing, such that the *PstI* site of pBR322 remained intact. Plasmids pFH60-StP81 and pFH60-PSt87 were made by digesting pFH60-St85 with *PstI* and inserting the resultant fragments into the *PstI* site of pBR322. To construct pFH60-STE86, pFH60-St80 was digested with *PstI* and *EcoRI*, and the resultant fragment was cloned in the *PstI-EcoRI* site of pBR322. The plasmids pSG22-EH8 and pSG124-SE22 were obtained by religation of pSG22 and pSG124 (12) after digestion with *EcoRI* and *HindIII* and with *EcoRI* and *Sall*, respectively. Plasmid pSG124-PE37 was constructed by digestion of pSG124-SE22 with *PstI* and *EcoRI* and ligation into the *PstI-EcoRI* site of pBR322.

**Marker rescue analysis.** The physical map locations of gC<sup>-</sup>

mutations were determined by marker rescue transfections as described by Goldin et al. (12). Primary rabbit kidney cells were cotransfected with infectious mutant virus DNA and a chimeric plasmid containing an HSV-1 restriction fragment. Plasmids were digested with the appropriate restriction enzymes to excise the insert before use in transfections. Progeny virus from the transfections were examined for the presence of gC<sup>+</sup> recombinants by use of an immunoperoxidase staining technique, the "black plaque" assay (21). In this assay, gC<sup>+</sup> plaques stain black, whereas gC<sup>-</sup> plaques remain unstained. Pool 2 of gC-specific monoclonal antibodies was used in these assays as the primary antibody, since the antibodies in this pool bind only to plaques formed by viruses which express gC in the plasma membranes of infected cells. These plaques were then visualized by use of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) as the second antibody and 4-chloro-1-naphthol as the substrate. This substrate forms a black precipitate on cells which bound the peroxidase.

**Isolation of viral RNA.** Monolayer cultures of Vero cells (2  $\times$  10<sup>7</sup> cells per flask) were infected at an MOI of 10 for 60 min at 37°C in MEM containing 10% FCS. The cells were pulse-labeled with 5  $\mu$ Ci of [<sup>3</sup>H]uridine (New England Nuclear) per ml for 60 min just before extraction. Polyribosome-associated RNA was isolated from cells by the method of Palmiter (27), followed by digestion with proteinase K (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) and phenol-chloroform extraction (2). Polyadenylated RNA was purified by the use of oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc., Waltham, Mass.).

**Size fractionation and hybridization of RNA.** Polyadenylated RNA was denatured by glyoxylation and size-fractionated by electrophoresis in 1% agarose gels (3, 25). The RNA was transferred to nitrocellulose paper, prehybridized, and then hybridized to probes <sup>32</sup>P-labeled by nick translation as described by Holland et al. (19). The sizes of the hybridizing transcripts were determined by their migration relative to single-stranded DNA standards as described by Holland et al. (19).

**Isolation of viral DNA.** Vero cell monolayers in roller bottles were infected with wild-type or mutant virus at an MOI of 0.01 at 37°C. When the cytopathic effect was generalized, cells were scraped into the medium. Cytoplasmic virions and virions which were released into the medium were isolated as described by Holland et al. (18). The viral DNA released from the virions was further purified by cesium chloride gradient centrifugation (12).

**Transfer of restriction endonuclease-digested DNA to nitrocellulose and hybridization.** Viral DNA was digested for 6 h at 37°C with *Sall* and *EcoRI* (Bethesda Research Laboratories, Gaithersburg, Md.) with 5 U of enzyme per  $\mu$ g of DNA. The DNA was electrophoresed in 1% agarose slab gels, denatured by soaking the gel in a solution containing 0.5 M NaOH and 1.5 M NaCl for 2 h, and then neutralized in 0.5 M Tris-hydrochloride (pH 7.4) containing 3 M NaCl for 2 h and transferred to nitrocellulose (34).

The DNA blot was prehybridized for 12 to 16 h at 42°C in buffer containing 50% formamide; 5 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate); 50 mM sodium phosphate (pH 6.5); 100  $\mu$ g of sonicated salmon sperm DNA per ml; 0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone; and 0.1% sodium dodecyl sulfate (SDS) (37). Hybridization with nick-translated probes was done in buffer containing four parts of prehybridization buffer and one part of 50% dextran sulfate for 24 h at 42°C.

The hybridized blots were washed twice in  $2\times$  SSC containing 0.1% SDS at room temperature and twice in  $0.1\times$  SSC containing 0.1% SDS at  $65^\circ\text{C}$ . The nitrocellulose was then wrapped in Saran Wrap and exposed to Kodak AR-5 film.

### RESULTS

**Isolation of  $gC^-$  mutants.** The  $gC$ -specific monoclonal antibody C11 was used to select HSV-1 (KOS) mutants that were resistant to neutralization by this antibody. Two independent selection experiments were done. Isolates were plaque purified from among the survivors of the selections and tested for resistance to neutralization by the selecting antibody C11 and by pool 1 of  $gC$ -specific monoclonal antibodies. Those mutants resistant to C11 but not to pool 1 were tentatively designated *mar* mutants and were analyzed separately (Marlin et al., manuscript in preparation). Those

mutants resistant to both C11 and pool 1 were designated  $gC^-$  mutants. In the first selection experiment, mutants  $gC^-1$  through  $gC^-50$  were isolated from an unmutagenized stock. In the second selection experiment, mutants  $gC^-51$  through  $gC^-83$  were isolated from an unmutagenized stock, and mutants  $gC^-84$  through  $gC^-103$  were isolated from a stock which was mutagenized with  $2.5\ \mu\text{g}$  of bromodeoxyuridine per ml. These isolates were plaque purified twice more. With the exception of  $gC^-44$ , which is discussed below, the  $gC^-$  mutants were resistant to neutralization at antibody concentrations over 1,000-fold greater than those which neutralized wild-type HSV-1.

**Analysis of infected cell RNA.** The  $gC^-$  phenotype could be caused by a number of different genotypes, including those which alter transcription of the  $gC$  gene. Accordingly, cells infected with the mutants were screened for the production of  $gC$  mRNA. Infected cell RNA was analyzed by isolating polyribosome-associated RNA at 6 h postinfection from Vero cells infected with each of the  $gC^-$  mutants. The RNA was denatured by glyoxylation, size-fractionated by agarose gel electrophoresis, and transferred to nitrocellulose paper. The RNA was then hybridized with a  $^{32}\text{P}$ -labeled probe specific for the  $gC$  gene.

Cultures infected with all but six of the  $gC^-$  mutants produced an RNA similar in size to the wild-type 2.7-kilobase (kb)  $gC$  mRNA (data not shown). Polyadenylated RNA isolated from four of these six mutants, along with polyadenylated RNA isolated from KOS and  $gC^-16$ , which is representative of the 97 other  $gC^-$  mutants, was hybridized with the  $gC$ -specific probe pSG22-EH8 (Fig. 2). The 2.7-kb message present in KOS- and  $gC^-16$ -infected cells was not found in cells infected with the four mutants  $gC^-5$ ,  $gC^-13$ ,  $gC^-39$ , and  $gC^-46$ . Instead, these mutants produced a novel 1.1-kb mRNA. Mutants  $gC^-21$  and  $gC^-98$  also had similar novel RNA profiles (data not shown).

The limits of the 1.1-kb RNA were mapped by hybridizing the RNA blots with  $^{32}\text{P}$ -labeled probes specific for different regions of the  $gC$  gene. The viral sequences in plasmids pFH60-PSt87 and pFH60-St70, which are probes for the 5' and 3' ends, respectively, of the  $gC$  gene (Fig. 1), hybridized to both the wild-type 2.7-kb mRNA and the mutant 1.1-kb RNA. The viral sequences in pFH60-StE86 and pFH60-ESt65, which were used to probe for the central region of the  $gC$  gene (Fig. 1), hybridized to the wild-type mRNA but not to the mutant 1.1-kb RNA (data not shown). Thus, the RNAs transcribed from the mutant genomes lacked major portions of the  $gC$  coding sequence.

**Analysis of viral DNA.** To determine whether the mutants which produced the 1.1-kb mRNA had a deletion in the  $gC$  coding sequence, viral DNA was isolated from KOS and  $gC^-13$ ,  $gC^-39$ , and  $gC^-46$ . The DNA was digested with *SalI* or *SalI-EcoRI*, size-fractionated on agarose gels, transferred to nitrocellulose, and hybridized with  $^{32}\text{P}$ -labeled pFH60. This plasmid contains the *SalI* T fragment (map units 0.620 through 0.645) of HSV-1 strain KOS cloned into the *SalI* site of pBR322 (Fig. 1). This 3,700-base-pair fragment contains the entire coding sequence for glycoprotein C (7). By using pFH60 as a hybridization probe, a single band was detected in the *SalI* digest of both the wild-type and mutant viral DNA (Fig. 3). However, the *SalI* band from the mutant DNA was ca. 1,600 base pairs smaller than the wild-type band. When the DNA from these samples was digested with *SalI-EcoRI*, the hybridization pattern of these three mutants did not change, whereas two bands were detected in the wild-type digest. This indicated that a deletion of ca. 1,600 base pairs occurred within the *SalI* T fragment of these three mutants

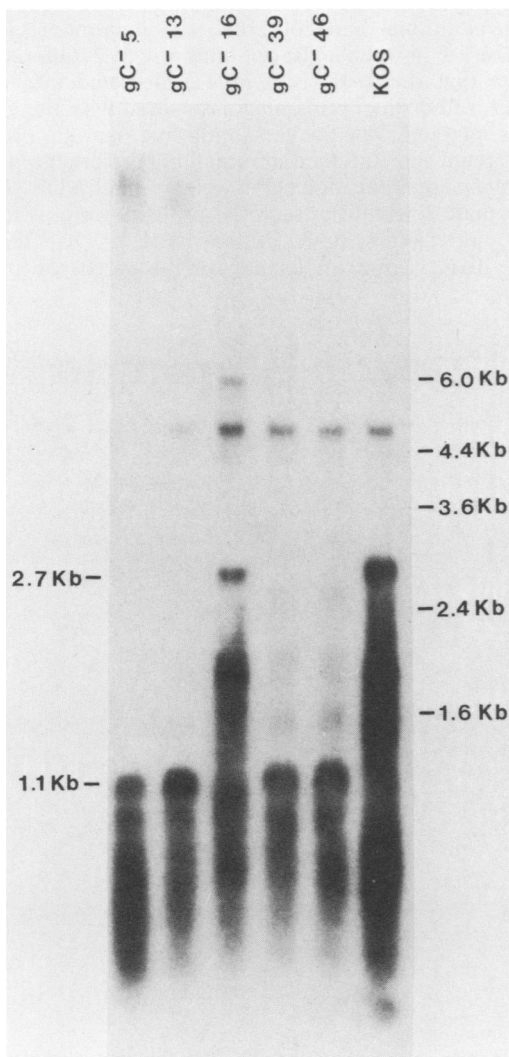


FIG. 2. Analysis of infected cell mRNA. Polyadenylated RNA extracted from KOS- and mutant-infected cells was isolated on an oligodeoxythymidylic acid-cellulose column. Glyoxalated RNAs were separated on agarose gels and blotted onto nitrocellulose paper. The blots were hybridized with pSG22-EH8 that had been labeled with  $^{32}\text{P}$  by nick-translation and then autoradiographed at  $-70^\circ\text{C}$ .

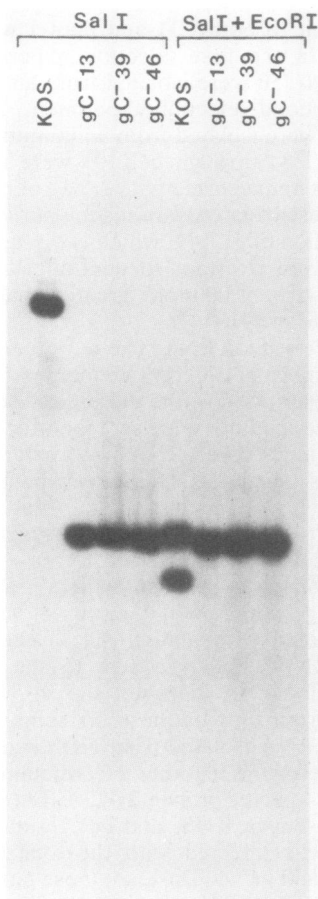


FIG. 3. Analysis of mutant viral genomes. Viral DNA was purified on CsCl gradients, cleaved with *SalI* or *SalI-EcoRI*, and separated on a 1% agarose gel. The separated fragments were blotted onto nitrocellulose paper and probed with the *SalI* T fragment which was gel purified from a *SalI* digest of pFH60.

and that this deletion spanned the single *EcoRI* site within this fragment. Fine mapping of the deletion was done by cloning the relevant *SalI* fragment from the three mutants shown in Fig. 3. Restriction mapping of these fragments determined that the 1,600-base-pair deletion lies within the region defined by the *SstII* sites at map units 0.631 and 0.642 (data not shown). The deletion of this region does not alter the transcription initiation or polyadenylation signals (7) and would account for the size of the truncated message seen in the mutant infection. The restriction maps of the three mutants were identical, suggesting that they were sister clones. The deletion of the majority of the gC coding sequence accounts for the gC<sup>-</sup> phenotype of these mutants. Analysis of DNA from mutants gC<sup>-</sup>5, gC<sup>-</sup>21, and gC<sup>-</sup>98 showed that these mutants had deletions similar to those in the mutants shown in Fig. 3 (data not shown).

**Immunoprecipitation of gC gene products.** Since nearly all of the gC<sup>-</sup> mutants produced a normal-sized gC mRNA, cells infected with these mutants were analyzed for the production of gC. HEL cells infected at an MOI of 10 were labeled with [<sup>35</sup>S]methionine from 5 to 20 h postinfection. Pool 2 of gC-specific monoclonal antibodies was used to make separate immunoprecipitations from both the medium and detergent extracts of the infected cells. Immunoprecipi-

tated viral proteins were analyzed by SDS-polyacrylamide gel electrophoresis.

The majority of the gC<sup>-</sup> mutants tested, including the deletion mutants, did not produce any [<sup>35</sup>S]methionine-labeled proteins which were immunoprecipitable with the gC-specific antibody pool. However, seven mutants, including *synLD70*, which had been isolated previously (33), produced proteins which were immunoprecipitated by the antibody pool. A fluorogram of the immunoprecipitated proteins is shown in Fig. 4. Wild-type KOS infections produced a gC protein with an apparent molecular weight of 130,000 (130K). This protein was predominantly found in the cell extract, although a small quantity of KOS gC was detected in the medium. Also detected in the extract of KOS-infected cells was a precursor form of gC of 102K. Two mutants, gC<sup>-</sup>44 and gC<sup>-</sup>3, also produced immunoprecipitable proteins of 130K and 102K. Mutant gC<sup>-</sup>44 was similar to KOS in that both the 130K and 102K proteins were found in the cell extract. However, this mutant differed from KOS in that an additional band of 110K was immunoprecipitated from the gC<sup>-</sup>44 cell extract. Mutant gC<sup>-</sup>3 differed from KOS in that the 130K protein was secreted into culture medium, rather than remaining associated with the cell.

Cells infected with the remaining five mutants produced and secreted into the medium immunoprecipitable proteins with lower apparent molecular weights than KOS gC. The approximate sizes of the secreted proteins were as follows: gC<sup>-</sup>49, 112K; gC<sup>-</sup>8, 109K; gC<sup>-</sup>53, 108K; gC<sup>-</sup>85, 106K; *synLD70*, 101K. Lower-molecular-weight precursor proteins

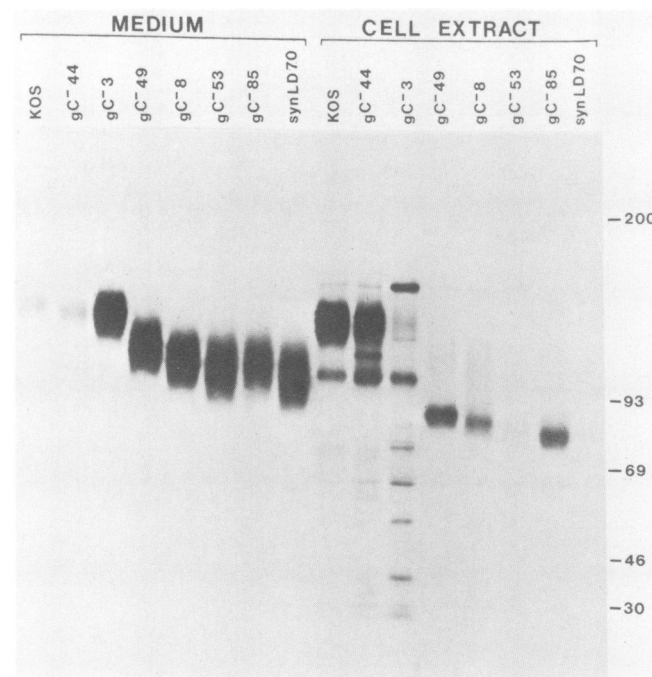


FIG. 4. Immunoprecipitation of [<sup>35</sup>S]methionine-labeled proteins from the medium and cell extracts of cultures infected with gC<sup>-</sup> mutants. HEL cells were infected at an MOI of 10 and labeled with [<sup>35</sup>S]methionine from 5 to 20 h after infection. Pool 2 of gC-specific monoclonal antibodies was used in immunoprecipitations from the culture medium and from the Nonidet P-40 extracts of the cells. The immunoprecipitated proteins were electrophoresed on a 8.5% SDS-polyacrylamide gel and visualized by fluorography. The locations of molecular weight markers are indicated on the right.

were clearly seen in extracts of cells infected with  $gC^-49$ ,  $gC^-8$ , and  $gC^-85$  (Fig. 4). Failure to detect the precursors in extracts of  $gC^-53$ - and  $synLD70$ -infected cells may be due to reduced synthesis of viral proteins by these mutants late in the infectious cycle.

Since  $gC$  is a glycoprotein, it was not possible to estimate the molecular weights of the polypeptide portion of the glycoproteins produced by the mutants from the data presented in Fig. 4. To obtain these data and to determine how much of the change in molecular weights could be attributed to alterations in the length of the polypeptide, infected cells were labeled with a 30-min pulse of [ $^{35}S$ ]methionine in the presence or absence of tunicamycin. The labeled gene products were extracted with Nonidet P-40, immunoprecipitated, and electrophoresed. These labeling conditions allowed the estimation of the molecular weights of both the nonglycosylated polypeptide (from tunicamycin-treated cells) and the polypeptide containing the N-linked core oligosaccharides (from control cells). A fluorogram of the gel obtained in this experiment is shown in Fig. 5. It is evident that in the absence of tunicamycin, two or more precursor bands were detected in each immunoprecipitation, differing

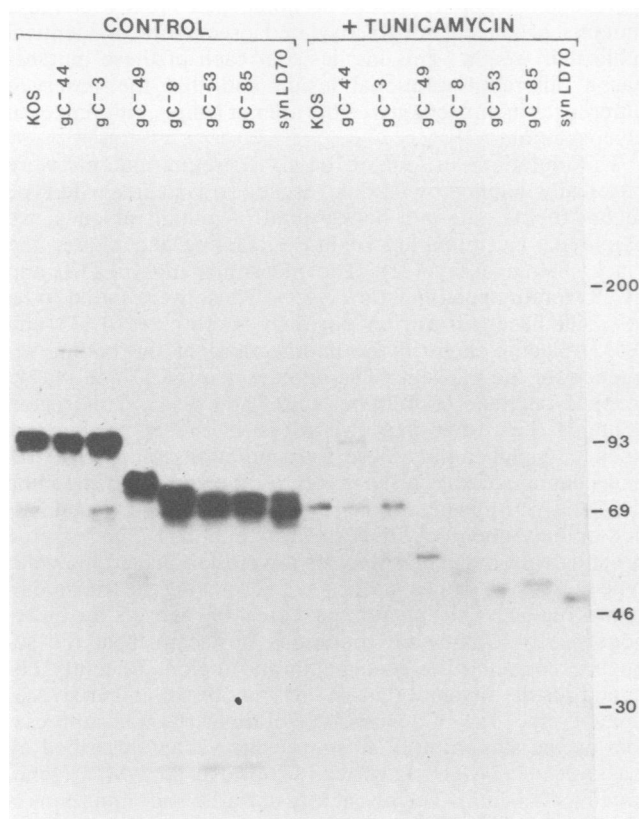


FIG. 5. Determination of the molecular weights of the mutant glycoprotein precursors. HEL cells were infected with KOS or  $gC^-$  mutants at an MOI of 10. Tunicamycin (2  $\mu$ g/ml) was added to the indicated cultures at 7 h after infection. All cultures were pulse-labeled with [ $^{35}S$ ]methionine for 30 min at 9 h after infection. Cells were disrupted with Nonidet P-40, and pool 2 of  $gC$ -specific monoclonal antibodies were used to immunoprecipitate the precursors from the clarified cell extracts. The immunoprecipitated proteins were separated on an 8.5% SDS-polyacrylamide gel and visualized by fluorography. The locations of molecular weight markers are indicated on the right.

by several thousand in apparent molecular weight. The double bands were more easily seen in a shorter exposure of this fluorogram (data not shown). This heterogeneity may be due to differences in glycosylation. It was also noted that incorporation of [ $^{35}S$ ]methionine into the precursors was reduced in the tunicamycin-treated cells. This was observed with tunicamycin obtained from two sources, Sigma Chemical Co. (this experiment) and Calbiochem-Behring (data not shown). Some tunicamycin preparations have been shown to cause some inhibition of protein synthesis (13, 16).

The precursors produced in tunicamycin-free cultures of  $gC^-44$  and  $gC^-3$  were similar in size to that produced by the wild-type virus (Fig. 5). The apparent size of the precursor band with the lowest relative mobility was 102K. The precursors produced by control cultures of  $gC^-49$  and  $gC^-8$  were 83K and 80K, respectively, whereas those produced by  $gC^-53$ ,  $gC^-85$ , and  $synLD70$  were very similar in size, ca. 78K. In the presence of tunicamycin, only single precursor bands were detected. These precursors were as follows: KOS, 73K;  $gC^-44$ , 73K;  $gC^-3$ , 73K;  $gC^-49$ , 59K;  $gC^-8$ , 54K;  $gC^-53$ , 50K;  $gC^-85$ , 51K;  $synLD70$ , 48K. Thus, the  $gC^-44$  and  $gC^-3$  precursors were not distinguishable from each other or KOS by molecular weight. The precursors produced by the other mutants in this group all differed from each other and KOS.

**Analysis of  $gC^-44$ .** The mutant  $gC^-44$  differed from the other  $gC^-$  mutants in that it produced a normal-sized  $gC$  protein that remained associated with the cell. To confirm and further quantitate the difference between this mutant and the wild-type virus, we conducted additional virus neutralization tests. In these experiments, the mutant  $gC^-101$ , which produced no detectable  $gC$ , was used as a  $gC^-$  control virus. Pool 2 of  $gC$ -specific monoclonal antibodies was used as neutralizing antibody. Wild-type and both mutant viruses were highly susceptible to neutralization by antibody B6, specific for  $gB$ , which was used as a control antibody (Table 1). Mutant  $gC^-44$  was partially susceptible to neutralization by the  $gC$ -specific antibody pool, whereas KOS was highly sensitive to this pool and  $gC^-101$  was highly resistant to it. The partial susceptibility of  $gC^-44$  to the  $gC$ -specific antibody pool indicated the presence of at least some  $gC$  in  $gC^-44$  virions. In addition, quantitative enzyme immunoassays, done with single  $gC$ -specific monoclonal antibodies, easily detected  $gC$  in the plasma membranes of infected  $gC^-44$  cells, although the amount expressed was only about 50% of that found in wild-type-infected cells (data not shown).

**Physical mapping of  $gC^-$  mutations.** The physical map locations of several  $gC^-$  mutations were determined in marker rescue experiments. Mutant viral DNA and cloned wild-type DNA fragments were cotransfected into rabbit kidney cells. The fraction of wild-type virus among the progeny was determined by immunoperoxidase staining of  $gC^+$  plaques. The results of several marker rescue experiments conducted on the  $gC^-$  mutants are presented in Table 2. The marker rescue data are summarized in Fig. 1.

The two mutants which were mapped most precisely were  $synLD70$  and  $gC^-8$ . These viruses, which produced secreted proteins smaller than wild-type  $gC$ , contained mutations which mapped between coordinates 0.633 and 0.637 (ca. 600 base pairs). This places the mutations of  $synLD70$  and  $gC^-8$  in the middle third of the  $gC$  coding sequence. Although  $synLD70$  was not rescued by pFH60-EST65 (Table 2), the limits within which this mutation maps have been confirmed by several rescue experiments with plasmids pFH60-St80 and pSG22-EH8 (data not shown). The mutants  $gC^-3$  and

TABLE 1. Neutralization of  $gC^{-44}$ 

Virus	% Virus survival with:	
	$gC$ pool 2	B6
KOS	0.1	0.0
$gC^{-101}$	100.9	0.0
$gC^{-44}$	52.7	0.0

$gC^{-49}$ , which also produced secreted proteins (Fig. 4), mapped within coordinates 0.633 and 0.643 (ca. 1,500 base pairs), which contains the 3' two-thirds of the  $gC$  coding sequence. Viruses  $gC^{-16}$  and  $gC^{-102}$  produced no proteins that could be immunoprecipitated by the pool of  $gC$ -specific antibodies (data not shown). The mutations in these viruses mapped to the vicinity of the  $gC$  gene and may be within the  $gC$  coding sequence. Unfortunately, it was not possible to map the mutation in  $gC^{-44}$ , since the level of expression of  $gC$  in the plasma membranes of infected cells made  $gC^{-44}$  plaques indistinguishable from wild-type plaques by immunoperoxidase staining.

### DISCUSSION

We used monoclonal antibodies to select over 100 HSV-1 mutants that were resistant to neutralization by a pool of  $gC$ -specific antibodies. By this criterion, we failed to detect  $gC$  in these mutant virions, and consequently, they were designated  $gC^{-}$  mutants. Our experiments were designed to determine the nature of the defects which caused the  $gC^{-}$  phenotype in these viruses. Perhaps the most interesting mutants were those which secreted the  $gC$  gene product from the infected cell. We also found mutants which had substantial deletions from the  $gC$  gene. One mutant,  $gC^{-44}$ , was identified which either had reduced expression of  $gC$  in virions and in the plasma membrane of infected cells or had reduced reactivity with our  $gC$ -specific monoclonal antibodies. However, ca. 90% of the mutants made no detectable  $gC$  protein, despite their expression of an apparently normal  $gC$  mRNA. Mutations of this type are presently under investigation to determine why they fail to make any detectable  $gC$  gene product.

Although wild-type  $gC$  is membrane bound, we found that several mutants ( $gC^{-3}$ ,  $gC^{-8}$ ,  $gC^{-49}$ ,  $gC^{-53}$ ,  $gC^{-85}$ , and *synLD70*) secreted immunoprecipitable glycoproteins into the culture medium. Only minimal amounts of  $gC$  were found in the medium of cultures infected with wild-type

virus. Of this group of mutants, one ( $gC^{-3}$ ) produced and secreted a glycoprotein similar in molecular weight to wild-type  $gC$ , whereas the glycoproteins produced by the remaining five mutants were lower in molecular weight than those of wild-type  $gC$ .

The processing of  $gC$  has been extensively studied (for a review, see P. G. Spear, in B. Roizman (ed.), *The Herpesviruses*, vol. 3, in press). It is known that N-linked oligosaccharide cores are added to  $gC$  in the rough endoplasmic reticulum either cotranslationally or immediately posttranslationally. The signal sequence is most probably cleaved at this time also. The partially glycosylated protein is transported to the Golgi-apparatus, in which the N-linked oligosaccharide cores are modified and the O-linked sugars are added, after which the now mature form of  $gC$  is transported to the plasma membrane.

To determine the molecular weights of the polypeptide backbones of the glycoproteins produced by the  $gC^{-}$  mutants, infected cells were pulse-labeled in the presence of tunicamycin, an inhibitor of N-linked glycosylation (36). The immunoprecipitable polypeptide found in tunicamycin-treated  $gC^{-3}$ -infected cells was similar in molecular weight to the wild-type precursor of  $gC$ , whereas the tunicamycin precursors produced by the remaining five mutants were lower in molecular weight. It should be noted that no two of these mutants produced nonglycosylated precursors of identical molecular weight. This implies that each of these mutants has a different mutational lesion and that the observed differences in molecular weights are not simply differences in glycosylation.

The mutations in four of the six secreting mutants were physically mapped by marker rescue. To visualize wild-type recombinants among a background of mutant plaques, we employed an immunoperoxidase staining technique, the black plaque assay (21). The mutations in *synLD70* and  $gC^{-8}$  were mapped most precisely. These were found to be in a 600-base-pair region between coordinates 0.633 and 0.637, placing them in the middle third of the coding sequence for the  $gC$  gene. The mutations in  $gC^{-3}$  and  $gC^{-49}$  mapped between coordinates 0.633 and 0.643. This region contains the 3'-terminal two-thirds of the  $gC$  coding sequence. Significantly, these four mutations mapped to regions encompassing, or upstream of, the sequence encoding the putative transmembrane region of  $gC$ , which was identified in the sequencing study of Frink et al. (7). The secreted proteins with molecular weights lower than that of the wild-type  $gC$  may be truncated forms of  $gC$  lacking the transmembrane region. This phenotype could be caused by either nonsense or frame-shift mutations upstream from the sequence encoding the transmembrane region. Recently obtained results indicate that  $gC^{-85}$  may be an amber (UAG) mutant, since the  $gC^{-}$  phenotype of this virus was suppressible by the simian virus 40 suppressor vector described by Laski et al. (24) (D. Purifoy, J. Glorioso, and M. Levine, unpublished data). The possibility of frame-shift mutations is supported by the finding that numerous stop codons exist in the unused reading frames of  $gC$  (7). Secretion of truncated glycoprotein gene products produced from genes engineered to delete the transmembrane region has been reported in several laboratories (8, 23, 31). At this time, however, we cannot rule out the possibility that missense mutations within the  $gC$  gene might lead to abnormal proteolytic cleavage of the mutant protein, cutting the transmembrane region away from the extracellular domain. However, this cleavage would have to proceed rapidly and quantitatively, as we found no evidence for wild-type-size  $gC$  precursors in

TABLE 2. Percentage of  $gC^{+}$  progeny in marker rescue transfections of  $gC$  mutants<sup>a</sup>

Mutant	% $gC^{+}$ progeny with the following rescuing plasmids <sup>b</sup> :				
	pFH60-ES165	pFH60-PB75	pFH60-St80	pSG22-EH8	pSG22
$gC^{-3}$	<0.5	6	<0.5	ND	60
$gC^{-8}$	18	ND	ND	ND	ND
$gC^{-49}$	<0.5	6	<0.5	ND	22
<i>synLD70</i>	<0.5	12	27	12	39
$gC^{-16}$	<0.5	12	<0.5	<0.5	ND
$gC^{-102}$	<0.5	7	<0.5	ND	33

<sup>a</sup> In all cases, at least 200 plaques were tested. If no  $gC^{-}$  plaques were observed, this was recorded as less than 0.5% rescue. ND, Not done.

<sup>b</sup> The positions of the cloned restriction fragments are shown in Fig. 1. The plasmid pSG22 contains *EcoRI* fragment I (map coordinates 0.633 through 0.721) (12).

cells infected by these mutants in either 30-min (Fig. 5) or 15-min (data not shown) pulse-labeling experiments.

Although the above discussion applies to mutants secreting truncated gC gene products, the mutation in gC<sup>-3</sup> may be of a different nature. The protein secreted by gC<sup>-3</sup>-infected cells and its partially glycosylated precursor are similar in molecular weight to the corresponding wild-type forms. This phenotype may be due to a missense mutation which substitutes a charged amino acid for one of the hydrophobic amino acids within the transmembrane region. Such a substitution could destroy the hydrophobic interactions required for stable integration of the transmembrane region in the membrane.

Several mutants (gC<sup>-5</sup>, gC<sup>-13</sup>, gC<sup>-21</sup>, gC<sup>-39</sup>, gC<sup>-46</sup>, and gC<sup>-98</sup>) had similar deletions in the gC gene. These mutants produced no detectable 2.7-kb gC mRNA but did produce a novel 1.1-kb RNA. This was due to a 1.6-kb deletion which included most of the gC coding sequence. The deletion occurred within the region bounded by the *Sst*II sites at map units 0.631 and 0.642. The transcription initiation (map unit 0.629) and polyadenylation (map unit 0.646) sites of gC were not affected by the deletion, which accounts for the presence of the 1.1-kb mRNA.

With the exception of the deletion mutants, all of the gC mutants produced a 2.7-kb gC mRNA. However, gC gene products were detected in only seven of these mutants (Fig. 4). We were unable to detect any gC-related protein produced by the remaining mutants by the techniques of virus neutralization, immunoperoxidase staining, immunoprecipitation from culture medium and cell extracts, and acetone precipitation of the proteins in the culture medium (data not shown). These mutants may make mRNAs which are not translatable, or they may make proteins which are unstable. Initially, we also expected to find mutations affecting transcriptional control of the gC gene. This could include mutations within the gC promoter and regulatory sequences and mutations in genes responsible for the regulation of gC, such as the *Cr* locus described by Ruyechan et al. (32). However, we found no evidence for mutations of this type.

Others have noted that mutants selected by their syncytial plaque morphology (*syn* mutants) often have the gC<sup>-</sup> phenotype as well (20, 32, 39). Of the mutants isolated in these experiments, only one, gC<sup>-103</sup>, caused syncytia formation. Mutant *syn*LD70, isolated previously (33), produces small syncytia. The relationship between the gC<sup>-</sup> and the *Syn* phenotypes remains to be elucidated.

We anticipate that the mutants secreting soluble forms of gC will be useful in the study of the biochemical and immunological properties of this viral glycoprotein. For example, we studied the reactions of individual monoclonal antibodies with the truncated forms of gC. Some of the antibodies failed to precipitate the protein produced by *syn*LD70, which produces the smallest truncated gC. Using this approach in combination with our analysis of *mar* mutants of gC (20; Marlin et al., manuscript in preparation), we located one of the major antigenic sites on gC to the amino-terminal two-thirds of the polypeptide, and a second major antigenic site is at least partly contained within the carboxyl-terminal one-third of gC. We are also exploring the possibility of making truncated variants of the other viral glycoproteins for similar purposes.

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