Molecular Basis of the Glycoprotein C-Negative Phenotypes of Herpes Simplex Virus Type 1 Mutants Selected with a Virus-Neutralizing Monoclonal Antibody

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Previously (Holland et al., *J. Virol.* 52:566-574, 1984; Kikuchi et al., *J. Virol.* 52:806-815, 1984) we described the isolation and partial characterization of over 100 herpes simplex virus type 1 mutants which were resistant to neutralization by a pool of glycoprotein C- (gC) specific monoclonal antibodies. The genetic basis for the inability of several of these gC⁻ mutants to express an immunoreactive envelope form of gC is reported here. Comparative nucleotide sequence analysis of the gC gene of the six mutants gC⁻3, gC⁻8, gC⁻49, gC⁻53, gC⁻85, and *synLD70*, which secrete truncated gC polypeptides, with that of the wild-type KOS 321 gC gene revealed that these mutant phenotypes were caused by frameshift or nonsense mutations, resulting in premature termination of gC translation. Secretion of the gC polypeptide from cells infected with these mutants was due to the lack of a functional transmembrane anchor sequence. The six secretor mutants were tested for suppression of amber mutations in mixed infection with a simian virus 40 amber suppressor vector. Mutant gC⁻85 was suppressed and produced a wild-type-sized membrane-bound gC. Nucleotide sequence analysis of the six gC deletion mutants gC⁻5, gC⁻13, gC⁻21, gC⁻39, gC⁻46, and gC⁻98 revealed that they carried identical deletions which removed 1,702 base pairs of the gC gene. The deletion, which was internal to the gC gene, removed the entire gC coding sequence and accounted for the novel 1.1-kilobase mRNA previously seen in infections with these mutants. The mutant gC⁻44 was previously shown to produce a membrane-bound gC protein indistinguishable in molecular weight from wild-type gC. This mutant differed from wild-type virus in that it had reduced reactivity with virus-neutralizing monoclonal antibodies. Nucleotide sequence analysis of the gC gene of mutant gC⁻44 demonstrated a point mutation which changed amino acid 329 of gC from a serine to a phenylalanine.

Cells infected with herpes simplex virus type 1 (HSV-1) synthesize a number of virus-specified glycoproteins which are incorporated into the virion envelope and plasma membranes (30). The glycoproteins induce both humoral (11, 22) and cell-mediated (21) immune responses. The viral glycoprotein designated gC has been shown to function as a major HSV-1 antigen (3-5, 9, 10, 23, 26). Some mutants of HSV-1 do not express gC, yet they grow as well as wild-type virus in cell culture. Thus, gC is considered to be nonessential for viral replication in vitro (2, 13, 14, 29). However, this glycoprotein may play an important role in natural infection because nearly all clinical isolates of HSV-1 express gC (25, 32). Indeed, a gC⁻ mutant of HSV-1 (strain KOS) is less pathogenic than wild-type virus following intracranial inoculation of mice (17).

We have been investigating the antigenic properties of gC, using gC-specific virus-neutralizing monoclonal antibodies to select mutants of HSV-1 (strain KOS), which are altered in antigenic structure. Two distinct mutant classes have been detected: monoclonal antibody-resistant (*mar*) mutants and gC⁻ mutants. *mar* mutants are resistant to neutralization by the selecting antibody but are sensitive to at least one other gC-specific monoclonal antibody. The gC *mar* mutants induce the synthesis of antigenically altered proteins which are wild type in size and processed normally. The gC⁻ mutant virions lack an immunoreactive envelope form of gC and are

resistant to neutralization not only by the selecting antibody but by a pool of gC-specific monoclonal antibodies.

Because the gC gene product is not essential for virus replication in cell culture, diverse genetic changes would be expected to give rise to the gC⁻ phenotype. In a previous study (13) we characterized 103 gC⁻ mutants and showed that the gC⁻ phenotype can be caused by a variety of molecular mechanisms. These include deletions of portions of the gC gene and mutations which result in secretion of truncated gC gene products. In this report, nucleotide sequencing data is described which define the genetic basis for the gC⁻ phenotype of several of these mutants.

MATERIALS AND METHODS

Cells and viruses. Vero cells or CV-1 cells were grown and maintained in Eagle minimum essential medium containing Hank salts (GIBCO Laboratories, Grand Island, N.Y.) supplemented with tryptose phosphate broth-nonessential amino acids-10% heat inactivated fetal calf serum (GIBCO)-100 µg of streptomycin per ml-100 U of penicillin per ml. A plaque-purified isolate of the wild-type KOS strain, designated KOS-321 (14), and gC⁻ mutant viruses derived from KOS 321 were grown by infection of Vero cells at low multiplicity and titrated by plaque assay on Vero cells.

Enzymes. Restriction enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, Md.). Digestions were performed by following specifications of the manufacturer. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals (Indianap-

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TABLE 1. Differences in the DNA sequence of the gC gene of KOS 321 with that of the published sequence of Frink et al. (7)

Base no. ^a	Nucleotide Difference	Predicted amino acid
128	A to G	None
154	T to G	Pro to Pro, gC amino acid 3
185	G to A	Gly to Ser, gC amino acid 14
713	C to T	Leu to Leu, gC amino acid 190
1044	A to T	Asp to Val, gC amino acid 300
1201	T to C	His to His, gC amino acid 352
1215	C to T	Thr to Met, gC amino acid 357
1689-1694	6 Cs to 7 Cs	None
1725-1730	6 Cs to 5 Cs	None
1807	C to G	None
1809	G to C	None
1861	C to A	None
2459	T to C	None

^a Numbering is that described Fig. 2 of Frink et al. (7), as modified previously (2).

olis, Ind.). Phage T4 polynucleotide kinase (P-L Biochemicals, Inc., Milwaukee, Wis.) was used for 5' end-labeling of DNA restriction fragments.

Isolation of viral DNA and cloning of the HSV-1 *Sa*II T fragment. Viral DNA was purified from KOS and gC⁻ mutant-infected Vero cell lysates by CsCl density gradient centrifugation (12). Viral DNA was digested with *Sa*II and electrophoresed on 0.8% low-melting-point agarose gels. *Sa*II T fragments (map units 0.620 to 0.645) were isolated from the gels and ligated into the *Sa*II site of pBR322.

Nucleotide sequencing. Nucleotide sequence analysis was carried out by the procedure of Maxam and Gilbert (20). Sequencing reactions were performed on 5'-end labeled restriction fragments gel purified from digests of the *Sa*II-T recombinant plasmids of KOS or the appropriate gC⁻ mutant.

Simian virus 40 amber suppression. The amber suppressor system developed by Laski et al. (18) was used to test suppression of chain termination. Simian virus 40 (SV40), containing a genetically engineered *Xenopus laevis* *su*⁺ tRNA tyrosine gene cloned into the late region of the virus, was prepared originally by mixed transfection with helper virus DNA in CV-1 cells by the method of Laski et al. (18). Second-passage stock virus was prepared by the method of Summers et al. (31) in CV-1 cells by infecting cells with progeny of the original mixed transfection, and a fraction of second-passage stock was kindly provided to us. A third-passage stock (P3) was prepared by infection of CV-1 cells with second-passage virus. Approximately 4×10^6 cells were infected with 0.2 ml of second-passage virus (2,500 particles per cell), incubated at 37°C, and harvested when the cytopathic effect was evident at 7 to 10 days postinfection. Virus was released into the medium by sonication, and samples were stored at -70°C.

Enzyme-linked immunosorbent assay for viral cell surface antigens. CV-1 cells were plated in 96-well trays at 4×10^4 cells per well. One-day-old monolayers were infected with the P3 stock of SV40 *su*⁺ tRNA vector (50 µl) diluted 1:5 or mock infected. Virus was absorbed for 1 h before the medium was replenished, and the infection was allowed to proceed for 48 h at 37°C. SV40-infected cell monolayers were then superinfected with one of the gC⁻ mutants or wild-type strain KOS at an input multiplicity of 10 PFU per cell. After 18 h at 37°C the cell monolayers were washed once with phosphate-buffered saline (PBS), fixed for 10 min

with 0.25% glutaraldehyde in PBS, and washed twice with PBS. A previously described pool (pool 2) of gC-specific monoclonal antibodies (13) was added at dilutions of 1:500, 1:1,000, or 1:2,000 (50 µl), incubated at 37°C for 1 h, and washed twice with PBS. Each well then received 50 µl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) diluted 1:500 in PBS. One hour later the wells were washed twice with PBS, and 50 µl of substrate [2 mM 2,2-azino-di(3-ethylbenzthiazoline sulfonic acid) in 0.1 M sodium acetate-0.05 M NaH₂PO₄-250 mM H₂O₂] was added. The plates were photographed, and the absorbance at 405 nm was determined for each well.

Radiolabeling, immunoprecipitation, and electrophoresis. Confluent monolayers of CV-1 cells (3×10^6 cells) were either mock infected or infected with SV40 *su*⁺ tRNA vector, and 48 h later they were superinfected with HSV-1 at an input multiplicity of 10 to 20 PFU (0.2 ml) per cell. Three hours after infection with HSV-1, the cells were radiolabeled with [¹⁴C]glucosamine at 1 µCi/ml. Cells were harvested at 18 h post infection with HSV-1, and immunoprecipitation was done as described previously with a pool of gC-specific monoclonal antibodies (14). Immunoprecipitates were analyzed by polyacrylamide gel electrophoresis as described previously (27).

RESULTS

Nucleotide sequence analysis of the KOS 321 gC gene. The nucleotide sequence of the glycoprotein C gene of the KOS strain of HSV-1 has been published by Frink et al. (7), with modifications to the sequence described in a later report (2). To determine the genetic basis for the mutations in the gC genes reported previously (13), it was necessary to determine the exact sequence of the gC gene for the plaque-purified isolate KOS 321 that was used to generate these mutants. We sequenced the 2,697-base-pair region described by Frink et al. (7) and found 13 differences. The differences in the nucleotide sequence and the predicted changes in the amino acids of the proteins encoded in this portion of the HSV-1 genome are shown in Table 1. Of the 13 base differences, 6 occurred within the gC-coding sequence, but there were only 3 changes in the amino acid composition. The three amino acids of the gC protein which differ from those of the sequence of Frink et al. (7) are located at amino acids 14, 300, and 357. Only two of these amino acid changes are in the mature protein, however, because amino acid 14 is in the signal sequence which is cleaved during maturation of the protein (16). The methionine instead of threonine at amino acid 357 has been confirmed by the amino acid sequencing data of Kikuchi et al. (16). They also reported that amino acids 34 and 48 were prolines. Our DNA sequence predicts that these amino acids are theonines, as was originally reported by Frink et al. (7).

The 3' end of the 2,700-base gC mRNA is coterminal with a 730-base mRNA which Frink et al. (7) have shown by in vitro translation encodes a 17,800-molecular-weight protein. None of the 13 base changes occurred within the proposed coding sequence of this protein. The two base changes which either deleted or added a nucleotide to the sequence were located between the termination codon for gC and the start of transcription for the 730-base message.

Nucleotide sequence analysis of mutants which secrete truncated gC gene products. Previously, we described the isolation of over 100 HSV-1 mutants that were resistant to neutralization by a pool of gC-specific antibodies (13). The majority (ca. 90%) of the mutants made no detectable gC

FIG. 1. Nucleotide sequencing of the *synLD70* and KOS gC gene. DNA sequence ladders comparing the DNA segment containing the *synLD70* frameshift mutation and the effect that this mutation has on the amino acid sequence of the gC polypeptide. The altered base in *synLD70* relative to that in KOS is indicated with an asterisk. The sequence presented is that of the gC-coding strand for nucleotides 959 to 976. The nucleotide sequence of cloned DNA fragments of *synLD70* and KOS were determined as described in the text.

protein, despite the synthesis of an apparently normal gC mRNA. Six mutants, gC⁻³, gC⁻⁸, gC⁻⁴⁹, gC⁻⁵³, gC⁻⁸⁵, and *synLD70*, produced truncated gC proteins which were secreted into the medium in which the cells were growing. The mutations for four of these mutants were physically mapped by marker rescue. The mutations in *synLD70* and gC⁻⁸ were mapped to a 600-base-pair *EcoRI*-*SstII* fragment (map units 0.633 to 0.637), placing their mutations in the middle third of the gC-coding sequence. The mutations for mutants gC⁻³ and gC⁻⁴⁹, which secrete truncated proteins larger than those of *synLD70* and gC⁻⁸, were mapped to a 1,500-base-pair *EcoRI*-*BamHI* fragment (map units 0.633 to 0.643), placing their respective mutations in the 3' two-thirds of the gC-coding sequence.

TABLE 2. Mutations in the gC secretion and deletion mutants

Mutant	Mutation	Base no. ^a	gC mutant termination codon and its base no. ^b
<i>synLD70</i>	Deletion of a C	965	TGA, 972-974
gC ⁻⁵³	C to T	1064	TAG, 1064-1066
gC ⁻⁸⁵	C to T	1064	TAG, 1064-1066
gC ⁻⁸	Deletion of a G	1039 ^c	TGA, 1137-1139
gC ⁻⁴⁹	C to T	1223	TGA, 1223-1225
gC ⁻³	Deletion of a G	1585 ^c	TAA, 1623-1625
gC ⁻⁵ , gC ⁻¹³ , gC ⁻²¹ , gC ⁻³⁹ , gC ⁻⁴⁶ , gC ⁻⁹⁸	Deletion of 1,702 bases	121-1822 or 122-1823	

^a Numbering is that described in Fig. 2 of Frink et al. (7), as modified previously (2).

^b Wild-type gC translation initiation ATG, base numbers 146 to 148; wild-type gC translation termination TAA, base numbers 1678 to 1680.

^c Because the deleted base was located in a repeat of four cytidines for *synLD70* and a repeat of four guanidines for gC⁻³, we randomly chose the last base of the repeat as the deleted base.

To locate the precise sites of the mutations, the *SalI* T fragment (map units 0.621 to 0.646) from *synLD70*, gC⁻⁸, gC⁻³, and gC⁻⁴⁹ was cloned into pBR322, and the region to which these mutants were physically mapped was sequenced. A summary of the sequencing data is presented in Table 2. Mutants *synLD70*, gC⁻⁸, and gC⁻³ each underwent deletion of a single nucleotide. The frameshifts generated by these nucleotide deletions caused premature termination of gC translation because the new reading frame for each mutant contained a number of stop codons. An example of the sequencing data for one of these mutants is given in Fig. 1. A comparison of the nucleotide sequence between bases 959 and 976 for *synLD70* and KOS DNA shows that the deletion of a C:G base pair, nucleotide 965, in *synLD70* causes gC translation to terminate at an opal stop codon six nucleotides from the site of the deletion. In the case of mutants gC⁻⁸ and gC⁻³, the deletion of a G:C base pair causes glycoprotein C translation to terminate at stop codons 97 (opal codon) and 37 (ochre codon) nucleotides from the site of the deletion, respectively (Table 2). The fourth mutant, gC⁻⁴⁹, had a point mutation that changed base 1223 from a cytidine to a thymidine. This mutation changed the codon of amino acid 360 of gC from a CGA coding for arginine to TGA, which is the opal termination codon (Table 2).

The mutations in the two remaining gC secretor mutants, gC⁻⁵³ and gC⁻⁸⁵, were not physically mapped by marker rescue. Instead, an estimate of the physical location of the mutations was made by comparing the size of the gC polypeptides secreted by these two mutants with those secreted by gC⁻⁸ and *synLD70*. In a previous report (13) we determined the molecular weights of the nonglycosylated gC polypeptide secreted by each of the six secretor mutants. The apparent size of the gC polypeptides secreted by gC⁻⁵³ and gC⁻⁸⁵ (molecular weights, 50,000 and 51,000, respec-

FIG. 2. Nucleotide sequencing of the gC⁻85 and KOS gC gene. DNA sequence ladders comparing the DNA segment containing the gC⁻85 point mutation and the effect that this mutation has on the amino acid sequence of the gC polypeptide. The altered base in gC⁻85 relative to that in KOS is indicated with an asterisk. The sequence presented is that of the gC coding strand for nucleotides 1055 to 1069.

tively) fell between the apparent sizes of the proteins secreted by *synLD70* and gC⁻8 (molecular weights, 48,000 and 54,000, respectively). Because termination of gC translation for *synLD70* and gC⁻8 is at base numbers 972 to 974 and 1137 to 1139, respectively (Table 2), we reasoned that nonsense codons for gC⁻53 and gC⁻85 must be within the 163 bases defined by these two mutants. Nucleotide sequencing of this region showed that both gC⁻53 and gC⁻85 have a point mutation at base 1064 (Table 2). Comparison of the nucleotide sequence between base numbers 1055 and 1069 for gC⁻85 and KOS DNA (Fig. 2) showed that the change of base 1064 from a C to a T generates an amber termination codon in the gC-coding sequence.

A comparison of the truncated gC proteins produced by each of the six secretor mutants, as deduced from the nucleotide sequencing data, is shown in Fig. 3. It is immediately apparent that five of the six mutants, gC⁻8, gC⁻49, gC⁻53, gC⁻85, and *synLD70*, produce proteins that lack the putative transmembrane region (amino acids 478 to 500) of gC (7). The sixth mutant, gC⁻3, makes a protein that is only 19 amino acids smaller than that of KOS gC. It can be predicted that the gC polypeptide made by gC⁻3 would have 14 of the 22 amino acids of the transmembrane sequence. However, the frameshift mutation within the codon of amino acid 480 changes the 12 remaining amino acids of gC⁻3 from exclusively hydrophobic amino acids to a sequence that contains some charged and uncharged residues. The loss or alteration of the putative transmembrane sequence would account for the secretion of gC from cells infected with these mutants.

Nucleotide sequence analysis of gC deletion mutants. Six of the 103 gC⁻ mutants studied by Holland et al. (13), gC⁻5, gC⁻13, gC⁻21, gC⁻39, gC⁻46, and gC⁻98, were shown to have deletions of a major portion of the gC gene. From restriction enzyme analysis, it was estimated that the dele-

tion in each of these mutants was approximately 1,600 base pairs in size and that it was within the region defined by the *Sst*II sites at map units 0.631 and 0.642. Nucleotide sequencing of these mutants showed that the endpoints of the deletion were identical for all six mutants. The deletion removed 1,702 base pairs starting at nucleotide 121 or 122 and ending at nucleotide 1822 or 1823. The ambiguity in the initial and final bases of the deletion is due to the presence of two cytidine residues at the deletion junction, one or both of which could come from the 5' end of the deletion. The deletion removed the entire coding sequence of gC but retained 120 bases of the 5'-noncoding and nearly all of the 3'-noncoding region of the gC mRNA, based on the transcription mapping data of Frink et al. (6, 7). The size and position of the deletion would account for the abundant 1.1-kilobase (kb) message which was seen in infections with the six deletion mutants (13). Because the 1,702 base pairs deleted do not affect the transcription initiation or polyadenylation signals, a truncated gC mRNA of approximately 1,000 bases would be predicted.

Nucleotide sequence change in mutant gC⁻44. The mutant gC⁻44 differs from the other gC⁻ mutants in that it produces a normal-sized gC protein that remains associated with infected cells. In virus neutralization assays, however, gC⁻44 is only partially susceptible to a pool of gC-specific monoclonal antibodies, resulting in its classification as a gC⁻ mutant (13). Quantitative enzyme immunoassays showed that the amount of gC present on the surface of gC⁻44-infected cells is only about half that found in wild-type infected cells. Detergent-disrupted gC⁻44 virions give a gC band identical in size to that of wild-type gC on sodium dodecyl sulfate-polyacrylamide gels, and this protein can be immunoprecipitated from such preparations by gC-specific monoclonal antibodies (data not shown). These findings indicate that gC is present on the envelope of gC⁻44 virions

but that the protein has either reduced reactivity with neutralizing antibodies or that the amount of gC present on the virion is reduced. Physical mapping of the gC⁻44 mutation by marker rescue is not possible because the level of expression of gC in the plasma membrane of infected cells makes gC⁻44 plaques indistinguishable from wild-type plaques by immunoperoxidase staining (15).

In an attempt to locate the mutation responsible for the gC⁻44 phenotype, we sequenced the DNA between the 360 bases 5' of the cap site for gC mRNA down to base 1770, which is 30 nucleotides 3' of the stop codon for gC translation. Except for the change of a cytidine to a thymidine at nucleotide 1131, the remaining sequence of gC⁻44 was identical to that of KOS. The point mutation at nucleotide 1131 changed amino acid 329 of gC from serine to phenylalanine.

Amber suppression of the gC⁻85 mutation. Before the DNA sequencing data were available, suppression of the truncated gC phenotype was tested by coinfecting CV-1 cells with the SV40 *su*⁺ tRNA vector and each of the six gC secretor mutants. An enzyme-linked immunosorbent assay (ELISA) with a pool of gC-specific monoclonal antibodies should be positive, if these mutants inserted gC into the membrane of infected cells when the amber suppressor gene was present. CV-1 cells were infected with the SV40 *su*⁺ tRNA vector, and 48 h later they were superinfected with each of the gC⁻ mutants. KOS infections were used as positive controls, and gC⁻39 deletion mutant infections were used as negative controls. The infections were terminated 18 h later by fixing the cells with glutaraldehyde, and the ELISA was carried out as described above. CV-1 cells that were not preinfected with the SV40 vector showed a positive ELISA reaction only for wild-type virus KOS and gC⁻44

FIG. 3. Schematic diagram comparing the wild-type gC protein with the truncated gC polypeptides produced by the six secretor mutants. The numbers below the line denote the C-terminal amino acid (a.a.) of the gC protein. The numbers above the line for *synLD70*, gC⁻8, and gC⁻3 represent the last amino acid of wild-type gC before a frameshift mutation changed the reading frame for these three mutants. The signal sequence and the putative transmembrane sequence are shown as shaded areas.

FIG. 4. ELISA of HSV-1-infected cell monolayers with and without suppression. CV-1 cells were infected with SV40 *su*⁺ tRNA virus (Su⁺) or were mock infected (Su⁻), and then 48 h later they were superinfected with the indicated gC⁻ virus. Eighteen hours later the cells were fixed, and the ELISA was carried out as described in the text. The P3 stock of SV40 vector was used at a dilution of 1:5, and the pool of gC-specific monoclonal antibodies was used at dilutions of 1:2,000 (A), 1:1,000 (B), and 1:500 (C).

infections (Fig. 4). We have already described gC⁻44 as a mutant that puts gC in infected cell membranes. Cells which were first infected with the SV40 vector and then superinfected with HSV-1 were positive by ELISA for KOS, gC⁻44, as expected, and, in addition, gC⁻85. This demonstrates that the gC⁻85 mutation, which is responsible for altering the expression of gC on the surface of infected cells, can be suppressed by the SV40 *su*⁺ tRNA vector. To confirm that this represented translational suppression resulting in a gC product of wild-type size, SV40 *su*⁺ tRNA-infected CV-1 cells were infected with gC⁻85 or KOS and labeled with [¹⁴C]glucosamine from 2 to 18 h postinfection. Cells were harvested, and a pool of gC-specific monoclonal antibodies was used in immunoprecipitation from Nonidet P-40 extracts of the cells. In the presence of suppressor (Su⁺), wild-type-sized gC was readily detected in gC⁻85-infected cells, while in control cells (Su⁻) only the truncated form of gC⁻85 was found (Fig. 5). The presence of the amber termination codon in the reading frame of gC for this mutant was in agreement with the DNA sequence described above for gC⁻85. In contrast, mutant gC⁻53 was found to have an amber mutation by DNA sequence analysis, but the mutation was not suppressed in coinfections with the SV40 *su*⁺ tRNA vector (Fig. 4).

DISCUSSION

In a previous report (13) we described the isolation of over 100 HSV-1 mutants that were resistant to neutralization by a pool of gC-specific monoclonal antibodies. These mutants

FIG. 5. Immunoprecipitation of [^{14}C]glucosamine-labeled proteins from cell extracts of cultures infected with KOS or gC⁻85 with or without suppression. CV-1 cells were infected with SV40 *su*⁺ tRNA virus (*Su*⁺) or were mock infected (*Su*⁻), and then 48 h later they were superinfected with KOS or gC⁻85. Pool 2 of gC-specific monoclonal antibodies was used in immunoprecipitations from Nonidet P-40 extracts of the cells. The immunoprecipitated proteins were electrophoresed on a 8.5% sodium dodecyl sulfate-polyacrylamide gel and visualized by fluorography.

produced virus which did not contain an immunoreactive envelope form of gC and were thus designated gC⁻ mutants. We showed that the gC⁻ phenotype can be caused by several molecular mechanisms. These include deletions of portions of the gC gene and mutations which result in secretion of truncated gC gene products. In this report we have described nucleotide sequencing data which define the genetic basis for the gC⁻ phenotype for several of these mutants.

Nucleotide sequencing of the six gC secretor mutants revealed that each has a mutation which creates a chain-terminating nonsense codon in the gC-coding sequence. Three of the six mutations were the consequence of frameshift deletions, and three were due to base substitutions resulting in the formation of nonsense codons. The predicted termination codon of mutants *synLD70*, gC⁻53, gC⁻85, gC⁻8, and gC⁻49 are located to the amino-terminal side of the putative transmembrane anchor sequence of gC. These mutants therefore would be expected to secrete a truncated form of gC from infected cells. The mutant gC⁻3 is particularly interesting because it has a frameshift mutation located within the sequence coding for the transmembrane anchor which changes both the number and sequence of amino acids in this region of the gC protein. These changes resulted in secretion of a nearly wild-type-sized gC protein from cells infected with this mutant. The truncated proteins produced by these mutants have provided valuable material for analysis of both the biochemical and immunological properties of this HSV-1 glycoprotein.

Kikuchi et al. (16) have reported on the isolation and

characterization of peptides generated by cyanogen bromide cleavage of purified glycoprotein C. CNBr cleavage of gC yields five fragments (CN-A, CN-B1, CN-B2, CN-C, CN-D) that can be resolved on a Sephacryl S-200 column and five fragments (CN-E) that are too small to be resolved on this column. The alignment of these fragments on the gC molecule, as deduced from the predicted amino acid sequence of gC, are from N terminal to C terminal: CN-A, CN-E, CN-B1, CN-E, CN-E, CN-E, CN-C, CN-E, CN-D, CN-B2. Comparison of the CNBr peptides generated from the three gC secretor mutants *synLD70*, gC⁻8, and gC⁻49 with that of the wild-type CNBr peptides indicates that these mutants lack the three C-terminal CNBr fragments of gC and, in addition, that *synLD70* and gC⁻8 are missing all or portions of the CN-C fragment. CNBr fragment B2 contains the putative transmembrane sequence of the gC protein. The DNA sequence analysis of *synLD70*, gC⁻8, and gC⁻49 agree with the CNBr data.

The CNBr data predicted that *synLD70* is missing the four C-terminal CNBr fragments CN-C, CN-E, CN-D, and CN-B2. The deletion of a single base pair at nucleotide 965 in mutant *synLD70* causes gC translation to terminate at a TGA codon six nucleotides from the point of the deletion. Because the CN-C peptide begins with amino acid 278, which corresponds to nucleotides 974 to 976, the truncated gC protein produced from a *synLD70* infection should lack the four C-terminal CNBr fragments.

The CNBr data predicted that mutant gC⁻8 should have a mutation located within the CN-C fragment, such that C-terminal fragments CN-E, CN-D, and CN-B2 should be missing and a smaller form of the CN-C fragment should be present. The CN-C fragment consists of amino acids 278 to 349 of gC. Mutant gC⁻8 has a frameshift mutation at nucleotide 1039 (amino acid 297) that should cause termination of translation after a protein of 330 amino acids is made. According to the DNA sequence data, CNBr cleavage of gC isolated from cells infected with gC⁻8 would be expected to yield a CN-C fragment 19 amino acids smaller than the wild type.

The chromatographic profile of the CNBr fragments of gC isolated from cells infected with gC⁻49 indicates that it has a mutation located near the C terminus of the CN-C fragment. This should result in the loss of C-terminal fragments CN-E, CN-D, and CN-B2 but not fragment CN-C. In fact, gC⁻49 has a point mutation at nucleotide 1223 which causes gC translation to terminate after a polypeptide of 351 amino acids is produced. Because the last amino acid of the CN-C fragment is residue 349, the DNA sequence data would predict that a wild-type CN-C fragment should be present in CNBr digests of this protein.

The CNBr fragment profile and the DNA sequencing data of mutants *synLD70*, gC⁻8, and gC⁻49 indicate that the C-terminal 30% (amino acids 350 to 511) of the gC protein contains an essential sequence needed for anchoring gC in plasma membranes. The secretor mutant gC⁻3 further defines the amino acids which are important for anchoring gC. This mutant secretes a protein similar in molecular weight to that of wild-type gC. Nucleotide sequencing suggests that this protein should be 19 amino acids smaller than wild-type gC because of the deletion of a single nucleotide. This deletion occurs in the codon of amino acid 480 which is the third residue of the proposed transmembrane sequence of gC (7). The 23 amino acids which comprise the putative transmembrane sequence (amino acids 478 to 500) contain 17 hydrophobic and 5 uncharged amino acids. The frameshift in gC⁻3 changes these residues such that the 15 C-terminal

amino acids (amino acids 478 to 492) of this mutant polypeptide now consist of 3 charged, 7 uncharged, and 5 hydrophobic amino acids. The gC⁻³ polypeptide is quantitatively secreted from cells infected with this virus, indicating that the integrity of the hydrophobic core of the transmembrane domain is necessary for anchoring gC in cell membranes. Similar results have been obtained for other integral membrane proteins. It has been demonstrated for the hemagglutinin of influenza virus (8) and for the G protein of vesicular stomatitis virus (28) that the hydrophobic core is an essential element of the functional transmembrane anchor domain because deletion of this sequence from cloned copies of these genes results in secretion of the mutant protein. Davis et al. (1) have studied the membrane anchor sequence of the pIII protein from bacteriophage ϕ 1 by constructing a series of overlapping deletions extending into the 3' (carboxy-terminal) end of the pIII gene. A gradual loss of anchor function was found for deletions extending into the hydrophobic domain, but no sharp boundary was encountered at which the protein was either membrane bound or secreted. They concluded that the gradual loss of anchor function was probably due to a gradual loss of thermodynamic stability with the membrane as hydrophobic residues were removed.

The truncated gC proteins produced by these secretor mutants have also been used to define the physical location of the two known antigenic sites on gC. By examining the ability of individual monoclonal antibodies to immunoprecipitate the truncated gC polypeptides, Marlin et al. (19) showed that antigenic sites I and II are located on different parts of the gC molecule. Group II antibodies were distinguished from group I antibodies by their ability to immunoprecipitate the proteins produced by all of the secretor mutants, thus localizing antigenic site II to the *synLD70* polypeptide, the smallest truncated polypeptide. DNA sequence analysis of *synLD70* indicates that antigenic site II would therefore be located within the N-terminal 273 amino acids of gC. Antibodies defining antigenic site I were found not to react with the gC⁻⁸ or *synLD70* polypeptides, but they did immunoprecipitate the gC⁻⁴⁹ polypeptide. This would indicate that sequences unique to the gC⁻⁴⁹ polypeptide, which are not contained in the gC⁻⁸ polypeptide (amino acids 297 to 359), are important in maintaining a functional antigenic site I. Evidence supporting this comes from nucleotide sequence analysis of two monoclonal antibody-resistant (*mar*) mutants isolated with group I monoclonal antibodies C11 and C14 (19). The *mar* mutants C11.1 and C14.1 both carry a point mutation which changes amino acid 307 from a glutamine to an arginine (F. Homa, unpublished data).

The remaining 90 gC⁻ mutants described by Holland et al. (13) have been screened in an attempt to isolate gC polypeptides smaller than those described in this report. These mutants synthesize an apparent wild-type-sized gC mRNA, but we have been unable to detect the expression of any gC-related polypeptide (13). These mutants may make mRNA which is not translatable or they may make proteins which are unstable. Evidence for the latter comes from the DNA sequence of the macroplaque strain of HSV-1 (2). The gC⁻ phenotype of this virus is the result of a frameshift mutation. The truncated and secreted gC polypeptide which should be expressed from cells infected with this virus could not be detected. Because this protein would be smaller than any of the secreted gC proteins reported here, it is reasonable to assume that there may be a minimum size to the truncated polypeptide that is necessary for stability and secretion.

Conditional lethal mutants of the nonsense type have not been of practical use in mammalian cell genetic studies, as they have been in procaryotic and lower eucaryotic organisms, because naturally occurring mammalian cell systems capable of nonsense suppression have not been detected. Recent development of such a system capable of translational suppression of nonsense mutations stems from the elegant engineering by in vitro mutagenesis of a *X. laevis* tryptophan gene to create an amber (UAG) suppressor tRNA (18). This tRNA gene functions as a suppressor gene when cloned into a SV40 genome for use as a vector in mixed infections with amber mutants of hybrid adenovirus type 2 (18) and influenza virus (33). Summers et al. (31) have shown that the SV40 *su*⁺ tRNA vector can be used to suppress a nonsense mutation in the thymidine kinase gene of HSV-1. In this report, we show that the SV40 *su*⁺ tRNA vector can also be used to suppress nonsense mutations in an HSV-1 structural gene. Mixed infection of the SV40 *su*⁺ tRNA vector with the gC secretor mutant gC⁻⁸⁵ resulted in restoration of membrane-bound gC. In the presence of the suppressor tRNA, approximately 35 to 50% of the gC produced by this amber mutant was found on the surface of infected cells. The suppressor was specific for the UAG nonsense mutant, gC⁻⁴⁹. The nucleotide sequencing data of the gC secretor mutant gC⁻⁵³ predicts that it should also be suppressed by the SV40 *su*⁺ tRNA vector, but it was not. This may be due to the presence of a second mutation in the gC gene of this mutant. Because suppression was measured by an ELISA with infected cell monolayers as antigens, a second nonsense mutation downstream from the amber mutation could affect gC anchorage to the infected cell membrane.

The mutant gC⁻⁴⁴ is similar to wild-type KOS in that it produces a normal-sized gC protein that can be found on the surface of infected cells and in the envelope of gC⁻⁴⁴ virions. But, unlike KOS, gC⁻⁴⁴ shows reduced sensitivity to gC-specific neutralizing monoclonal antibodies and is therefore classified as a gC⁻ virus (13). DNA sequencing of the gC gene of this mutant revealed a point mutation which changed amino acid 329 from serine to phenylalanine. Because the site of the gC⁻⁴⁴ mutation has not been physically mapped, it is not possible to say with certainty that this amino acid change is responsible for the mutant phenotype. Quantitative enzyme immunoassays indicate that gC⁻⁴⁴-infected cells contain only about 50% of the gC found on KOS-infected cells (13). If the reduced levels of gC apply to the virion envelope, then the decrease in reactivity with neutralizing antibodies may be due to a lack of sufficient antigen on the surface of the virus. It is also possible that the gC⁻⁴⁴ phenotype is due to a mutation located outside the gC gene. Pancake et al. (24) have reported on the isolation of a class of mutants selected for resistance to immune cytolysis. The majority of these mutants synthesize wild-type levels of the glycoproteins at nonpermissive temperatures but express the glycoproteins in reduced amounts on the surface of infected cells. These mutants have been mapped by marker rescue to several regions of the HSV-1 genome.

DNA sequencing of the six deletion mutants (gC⁻⁵, gC⁻¹³, gC⁻²¹, gC⁻³⁹, gC⁻⁴⁶, gC⁻⁹⁸) showed that each had an identical 1,702-base-pair deletion which removed all of the gC-coding sequence. Because these mutants were all isolated from a virus stock produced from a plaque-purified isolate of HSV-1, it is likely that they are sister clones. These mutant viruses produce a truncated (1.1-kb) form of the 2.7-kb gC mRNA. Examination of the DNA sequence of

these mutants shows that the truncated 1.1-kb message is actually encoded by a chimeric gene composed of the 5'-noncoding and upstream sequences of the gC gene and the 5'-noncoding and coding sequences of the 730-base mRNA which is 3' coterminal to the 2.7-kb gC message (6, 7). The fusion of the control sequences of the gC gene onto the coding sequences of the smaller message drastically alters the steady-state level of this 730-base message (13). In a wild-type infection, the 730-base mRNA is a low-abundant message, but in infections with the six deletion mutants it is expressed at levels comparable to that of the 2.7-kb gC mRNA. Frink et al. (6) have shown by in vitro translation that the 730-base mRNA encodes a 17,800-molecular-weight protein.

The variety of mutations in the gC gene reported here were detected because this glycoprotein is not required for replication of HSV-1 in cell culture. Peptide chain-terminating mutations and deletion mutations in the gC gene are not lethal to the virus. Truncated and deleted proteins have great utility for the study of structure-function relationships and would be important tools for the analysis of such relationships in essential proteins. We are attempting to use the SV40 amber suppressor system to induce, isolate, and perpetuate amber mutants in the essential glycoprotein B gene of HSV-1. We are also attempting to produce viruses that carry otherwise lethal mutations in the gB gene which can be complemented for growth in permissive cells transformed with that gene.

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