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Characterization of the Gene Encoding Herpes Simplex Virus Type 2 Glycoprotein C and Comparison with the Type 1 Counterpart

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The gene encoding the glycoprotein C (gC) of herpes simplex virus type 1 maps to the region of the viral genome from 0.62 to 0.64. Recently, a herpes simplex virus type 2 glycoprotein previously designated gF and now designated gC was mapped to a homologous location. Analysis of the herpes simplex virus type 2 mRNA species encoded in this region revealed a major transcript of 2.5 kilobases, a 0.73-kilobase transcript (the 5′ ends of which were mapped by primer extension), and several minor species, all nearly identical to the herpes simplex virus type 1 pattern. A polypeptide of ca. 60,000 daltons was identified by in vitro translation of hybrid-selected mRNA. A smaller protein of ca. 20,000 daltons was also mapped to this region. The nucleotide sequence of a 3.4-kilobase segment of DNA encompassing gC was determined, and an open reading frame of 1,440 nucleotides specifying a 480-amino acid protein with properties consistent with that of a glycoprotein was identified. Comparative DNA sequence analysis showed regions of limited homology within the coding sequences for gC and a deletion which results in 31 fewer amino acids in the gC-2 near the amino terminus of the protein. The carboxy termini of gC-1 and gC-2 are very similar, as are the 20,000-dalton proteins.

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) specify a number of virally encoded glycoproteins designated gB, gC, gD, gE, gF, gG, and gX and occasionally by other designations. In the case of gB, gD, and gE, related counterparts of similar electrophoretic mobility have been identified (9, 25, 28), and mapping of the genetic loci encoding these proteins has shown their locations on the HSV-1 and HSV-2 genomes to be colinear (20, 25, 34). Ruyechan et al. (34) mapped the location of the glycoprotein genes with HSV-1 × HSV-2 intertypic recombinants. These studies placed the HSV-1 gC gene between 0.58 and 0.70 map units (m.u.) and the HSV-2 gC gene between 0.70 m.u. and the junction of the L and S components. The results were derived by associating glycoproteins of similar but not identical electrophoretic mobilities of around 130,000 daltons with particular restriction enzyme fragments of either HSV-1 or HSV-2 DNA. In another study with intertypic recombinant viruses (20), an HSV-2 glycoprotein of 63,000 daltons with no apparent HSV-1 counterpart was mapped between 0.55 and 0.65 m.u. Monoclonal antibodies (MABs) to HSV-2 glycoproteins also detected a small glycoprotein with a molecular weight of 60,000 which could be chased into higher-molecular-weight forms of 66,000 and 79,000 (3, 4) and was designated gF. Results from two studies with monoclonal antibodies (44, 45) showed that HSV-2 gF shared antigenic determinants with HSV-1 gC. To map the gene encoding HSV-2 gF, Para et al. (25) used monoclonal antibodies to two 75,000-dalton glycoproteins to immunoprecipitate extracts from intertypic recombinant viruses with defined crossover points. These studies indicated that HSV-2 gF was likely to be colinear with HSV-1 gC. Confirmatory evidence that gF maps between 0.62 and 0.64 m.u. came from experiments in which fragments of HSV-2 DNA were inserted into the thymidine kinase gene of HSV-1 and the recombinant progeny were examined for the ability to express gF (44). Based on the antigenic relatedness and the apparent colinearity of gF, it was proposed that gF be renamed HSV-2 gC, or gC-2. The protein previously designated gC-2 has recently been mapped to the short region of the HSV-2 genome and was renamed gG (31).

Many of the biological and biochemical characteristics of gC-2 have been reported. The newly synthesized and processed forms of gC-2 have been identified. In one report in which Vero cells were infected with HSV-2 strain 333, molecular weights of 60,000 for the precursor and 66,000 and 79,000 for the processed forms were reported (3). In another report, HEp-2 cells were infected with the same strain of virus and the precursor appeared as a doublet of 67,000 and 69,000 which could be chased into a 75,000-molecular-weight (75K) mature form (44). Both the nonglycosylated and processed forms of gC-1 are larger than gC-2, having molecular weights of 85,000, 110,000, and 130,000, respectively (26). In vitro translation of the gC-1 mRNA revealed a 69K protein, and the molecular weight predicted by the DNA sequence is ca. 55,000. It seems likely that the polypeptide moieties of gC-1 and gC-2 are similar in size but that gC-1 becomes more extensively glycosylated. The oligosaccharides of gC-2 were also characterized. Newly synthesized gC-2 was treated with endo-β-*N*-acetylglucosaminidase H to cleave the *N*-linked oligosaccharides of the high mannose type but not the complex type. A single band of 54,000 was found (44). The mature 75K form was insensitive to endo-β-*N*-acetylglucosaminidase H, suggesting that the processed form contained complex *N*-linked oligosaccharides. Treatment of the newly synthesized and processed forms of gC-2 with *N*-acetylgalactosamine oligosaccharidase demonstrated the presence of O-linked oligosaccharides in the mature form (44). Both forms of oligosaccharides have also been described for gC-1 (17, 24, 43). In the case of the gC-1 precursor, endo-β-*N*-acetylglucosaminidase H generates a 75K species (17, 43).

The kinetics of appearance of both gC-1 and gC2 in infected cells showed that the proteins could only be clearly detected in cells labeled 3 to 5 h postinfection and not in the presence of DNA synthesis inhibitors, whereas other glycoproteins could be detected as early as 1 to 3 h postinfection (3, 26). gC is present on the membrane of HSV-infected cells (3, 23, 44) as well as on the virion surface (44).

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The isolation of a *syn* mutant of HSV-2 which does not express gC-2 (44) indicates that gC-2 is not required for viral growth in tissue culture and suggests that gC-2 may be functionally analogous to its HSV-1 counterpart.

The gene encoding HSV-1 gC has been characterized in detail (12). The transcript which encodes gC-1 is a 2.5-kilobase (kb) unspliced message and is the major species of a family of mRNAs related by splicing. A 730-base-pair (bp) species which has a common 3′ end uses its own promoter and encodes an 18K polypeptide. The DNA sequence of gC-1 encoded by the KOS strain of HSV-1 was reported (12) and recently amended (7). These data predict a primary protein product of 511 amino acids with a predicted molecular weight of 55,000.

Preliminary information about the HSV-2 gC gene was obtained by studying the intertypic homology between 0.58 and 0.68 m.u. (8). At the 5′ end of gC and within the 3′ sequences encoding the 18K protein, there is moderate intertypic homology. Within the body of the gC-coding sequences there is strong homology, and only rightward of the 3′ end of the transcript is there no detectable homology. Additionally, we investigated whether the transcription pattern in HSV-2-infected cells is similar to that in HSV-1-infected cells. By Northern blot analysis with the *Eco*RI O fragment (0.64 to 0.644 m.u.) as a probe, a transcript the same size or slightly smaller than that encoding gC-1 (2.5 kb) was detected. A 730-bp mRNA corresponding to the HSV-1 transcript which encodes the 18K polypeptide was present, as well as a few very minor species which might be equivalent to the HSV-1-spliced RNAs.

In this study, we characterize the transcriptional starts of the HSV-2 gC and the 18K mRNAs, analyze the products of hybrid-selected in vitro translation, determine the nucleotide sequence of a 3.4-kb segment of HSV-2 DNA which encodes gC-2 and the 18K protein as well as the upstream regulatory sequences, and compare the sequence data with those of HSV-1.

MATERIALS AND METHODS

Construction of recombinant plasmids and M13 phage. Four recombinant plasmid subclones of the *Bam*HI-A region of HSV-2 DNA were constructed for use in the hybrid selection of mRNAs for in vitro translation and to aid in the preparation of M13 recombinant phages for DNA sequence analysis. A pBR322 recombinant plasmid pBamA containing the *Bam*HI A fragment (0.595 to 0.67 m.u.) of HSV-2 strain 333 was cleaved with *Eco*RI, and the resultant fragments were purified by electroelution from a 1% agarose gel. The 0.6-kb *Eco*RI fragment O (0.631 to 0.637 m.u.) was ligated to *Eco*RI-cleaved pBR322 to generate the recombinant plasmid pRI-O. The larger two *Eco*RI fragments were purified as a doublet and digested with *Bam*HI, and the products of this digestion were ligated together. Recombinant plasmids were selected for tetracycline sensitivity and were size selected on agarose gels to obtain a plasmid pRI/Bam, containing the 7.3-kb *Eco*RI-*Bam*HI fragment (0.637 to 0.67 m.u.). The subclone of pBamA, pBgl/RI, which contains the 0.9-kb *Bgl*II-*Eco*RI fragment (0.621 to 0.631 m.u.) was obtained by ligating the products of pBamA cleaved with *Eco*RI and *Bgl*II to a pBR322 *Eco*RI-*Bam*HI vector. Finally, a recombinant plasmid pBgl/Sal containing the 1.2-kb region between the *Sal*I and *Bgl*II sites (0.61 to 0.621 m.u.) was obtained by purifying this fragment from a plasmid containing the *Bgl*II fragment N of HSV-2 and ligating it to the plasmid vector pKC7, which had been cleaved with *Sal*I and *Bgl*II. The identities of all recombinant plasmids were confirmed by restriction analysis on agarose gels.

The M13 recombinant phages used for DNA sequencing and hybrid selection of mRNAs were obtained by one of two methods. In the first, restriction endonuclease digestions of the recombinant plasmids pBamA, pRI-O, pRI/Bam, pBgl/RI, or pBgl/Sal or digestions of DNA fragments purified from these plasmids were ligated to appropriately cleaved replicative forms of the vectors M13mp10, mp11, mp18, and mp19. Vectors were made by digesting the phages with *Eco*RI, *Bam*HI, *Acc*I, *Xma*I, and *Sal*I either alone or in pairs. Fragments to be inserted were generated with the enzymes *Eco*RI, *Bgl*II, *Xma*I, *Sal*I, *Sau*3A, and *Hpa*II. The second method (15) involved constructing deletions of M13 recombinant phages which contained HSV-2 DNA. By this method, the replicative forms of M13mp11, mp18, or mp19 recombinant phages were cleaved with two restriction endonucleases between the location of the inserted DNA and the hybridization site of the DNA sequencing primer. The restriction enzymes used were chosen so that a four-base 5′-protruding end was left adjacent to the inserted DNA and a four-base 3′ protrusion was left near the site of primer binding. Pairs of enzymes used in this way were *Bam*HI and *Sst*I and *Sal*I and *Sph*I. The linearized phage DNA was then sequentially treated with exonuclease III, S1 nuclease, Klenow DNA polymerase, and T4 DNA ligase and introduced into competent JM101 host cells. The extent of exonuclease III digestion was controlled by stopping the reaction at various times. The recombinant phages used as starting points for constructing a deletion series contained: the *Sal*I-*Bgl*II fragment (0.61 to 0.621 m.u.); the entire *Sal*I fragment (0.61 to 0.639 m.u.); or the *Eco*RI-*Xma*I fragment (0.637 to 0.644 m.u.). Selection and growth of recombinant phage and preparation of single-stranded phage DNA were done by standard techniques (37). Recombinants were characterized by T-track analysis (2) in which each template is used in a dideoxy-T sequencing reaction, allowing banding patterns to be compared before complete sequencing.

Isolation of viral RNA and proteins. BHK-21 cells were infected with HSV-2 (strain 333) at a multiplicity of 20 PFU per cell. The cells were exposed to virus to 60 min in Dulbecco modified Eagle medium without serum and subsequently maintained in medium containing 2% fetal calf serum. The cells were harvested by scraping into ice-cold phosphate-buffered saline at 10 to 16 h postinfection. Total cytoplasmic RNA was prepared as described by Anderson et al. (1). Radiolabeled extracts of viral proteins were prepared as follows. Cells were infected as described above. After adsorption, the inoculum was replaced with Dulbecco modified Eagle medium containing 2% fetal calf serum and [^{3}H]glucosamine (15 μ Ci/ml). Cultures were harvested 24 h after infection. Cell extracts were prepared for immunoprecipitation by suspending cell pellets in extraction buffer as described previously (13).

Selection hybridization and in vitro translation. Either plasmid DNA linearized by cleavage with a restriction enzyme and then denatured or single-stranded DNA prepared from M13 phage was applied to nitrocellulose and used to select complementary mRNA essentially as described by Ricciardi et al. (30). In a typical experiment, two 4-mmm squares of nitrocellulose, to which 20 μg of DNA had been applied were used, and these were hybridized to 100 to 250 μg of cytoplasmic RNA in a total mixture of 100 μl. The eluted RNA was used to program cell-free transla-

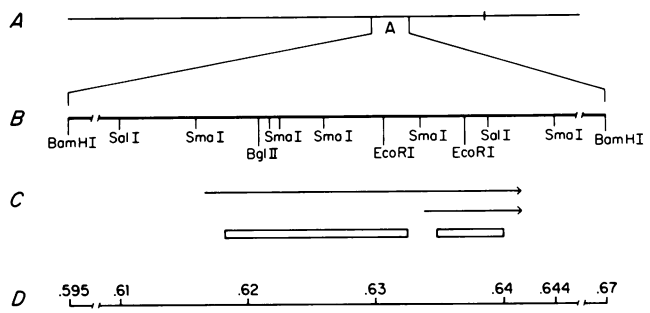

FIG. 1. Organization of the region of the HSV-2 genome encoding glycoprotein C. (A) HSV-2 genome and the location of the *Bam*HI fragment A. (B) Restriction map of the 3.4-kb *Sal*I-*Sma*I fragment, indicating sites that were used in the preparation of fragments for selection hybridization or DNA sequencing. (C) Summary of the location of the RNAs (arrows) and protein coding regions (open blocks) as determined by primer extension and DNA sequencing. (D) Map coordinates on the HSV-2 genome.

tion (total volume, 12.5 μ l) in a micrococcal nuclease-treated reticulocyte lysate, as described by Pelham and Jackson (27). The products of cell-free translation were labeled with [^{35}S]methionine and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography.

Primer extension analysis. The DNA primers used to probe the gC and 18K mRNAs were obtained by isolating Klenow polymerase ^{32}P-end-labeled *Hpa*II fragments from 8% acrylamide gels. For gC, a 600-bp *Bgl*II-*Sma*I fragment from pBgl/Sal was purified from low-melting agarose and was cleaved with *Hpa*II to yield a 102-bp fragment. The 100-bp fragment which served as the primer for the 18K message was isolated from an *Hpa*II digest of the *Eco*RI fragment O as above. Both primers were denatured with 0.3 M NaOH and loaded onto 8% acrylamide strand-separation gels from which single-stranded fragments were recovered. Primer annealing and extension were carried out by a modification of a procedure described by McKnight et al. (21). Probe DNA was coprecipitated with 10 μg of total late HSV-2 RNA and annealed at 68°C in 250 mM KCl–10 mM Tris (pH 8.3)–1 mM EDTA. Hybrids were then treated with AMV reverse transcriptase (100 U/ml) in a buffer containing 70 mM KCl, 10 mM Tris (pH8.3), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, and actinomycin D (100 μg/ml) at 37°C for 1 h. DNA products were ethanol precipitated, suspended in formamide-dye solution, heated at 100°C, and loaded onto 8 or 6% polyacrylamide–urea sequencing gels.

DNA sequencing methods. Sequence analysis by dideoxynucleoside triphosphate chain termination (36) was performed with the single-stranded M13 phage DNAs as a template. The DNA synthesis reactions were primed with a 17-residue fragment (New England Biolabs) or a 15-residue fragment (Bethesda Research Laboratories), either of which hybridizes adjacent to the inserted DNA. Reactions were extended with Klenow DNA polymerase (Bethesda Research Laboratories) in the presence of [^{32}P]dCTP, [^{32}P]dTTP, and unlabeled dATP and dGTP, one dideoxynucleotide in each of four reactions, for 15 min at room temperature as previously described (40). Thin sequencing gels (35) containing from 5 to 8% polyacrylamide were used to resolve the products. In cases in which anomalous mobilities of products in regions of high G-C content occurred, gels containing 15% acrylamide and 70% formamide were used to resolve the compressions. The dried or acid-fixed gels were exposed for 2 to 16 h on XAR-5 X-Omat film. Complete DNA sequence data were obtained from both strands of a 3.4-kb region and were entered, compiled, and analyzed on a Vax/VMS computer (Digital Corp.) with previously established programs (38, 39; J. Wallace, unpublished data).

RESULTS

Characterization of the RNA and protein products. By hybridization analysis, the region of the HSV-1 genome delineated by map coordinates 0.58 to 0.674 showed regions of high homology interspersed with regions of considerably lower homology and one region with no detectable homology (8). We have determined the nucleotide sequence of a 3.4-kb segment of HSV-2 DNA from map coordinates 0.61 to 0.644 which encodes glycoprotein C and an 18K protein. A map of this region of the HSV-2 genome is shown in Fig. 1.

We have previously shown (8) that the HSV-2 transcripts from a region homologous to that encoding gC-1 are very similar. A 5-μg sample of polyadenylic acid-containing mRNA from HSV-2-infected cells was fractionated through

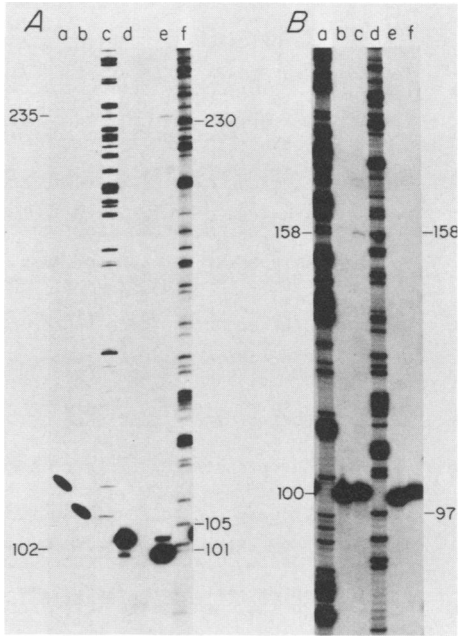

FIG. 2. Mapping the 5′ termini of the mRNAs by primer extension analysis. RNA from HSV-2-infected cells (10 μg) was hybridized to a 102-bp *Hpa*II fragment from the *Sma*I-*Bgl*II region (0.616 to 0.621 m.u.) (A) or to a 100-bp *Hpa*II fragment from the *Eco*RI O region (0.631 to 0.637 m.u.) (B). The individual strands of the primers were purified, and each one was tested. Lanes c and f in panel A and lanes a and d in panel B contain sequencing reactions as molecular weight markers. Lanes a and b in panel A and lanes e and f in Panel B contain no RNA. Lane e in Panel A shows a primer extension product of 235 bases which places the 5′ terminus of the message encoding gC at position 672 (see Fig. 3). Lane c in Panel B shows a primer extension product of 158 bases which places the 5′ terminus of the message encoding the 18,000-dalton protein at position 2390 (see Fig. 3). The numbers at the right of the panels indicate the length of bands in sequencing gels, and the numbers at the left of the panels indicate the sizes of the primers and the extension products.

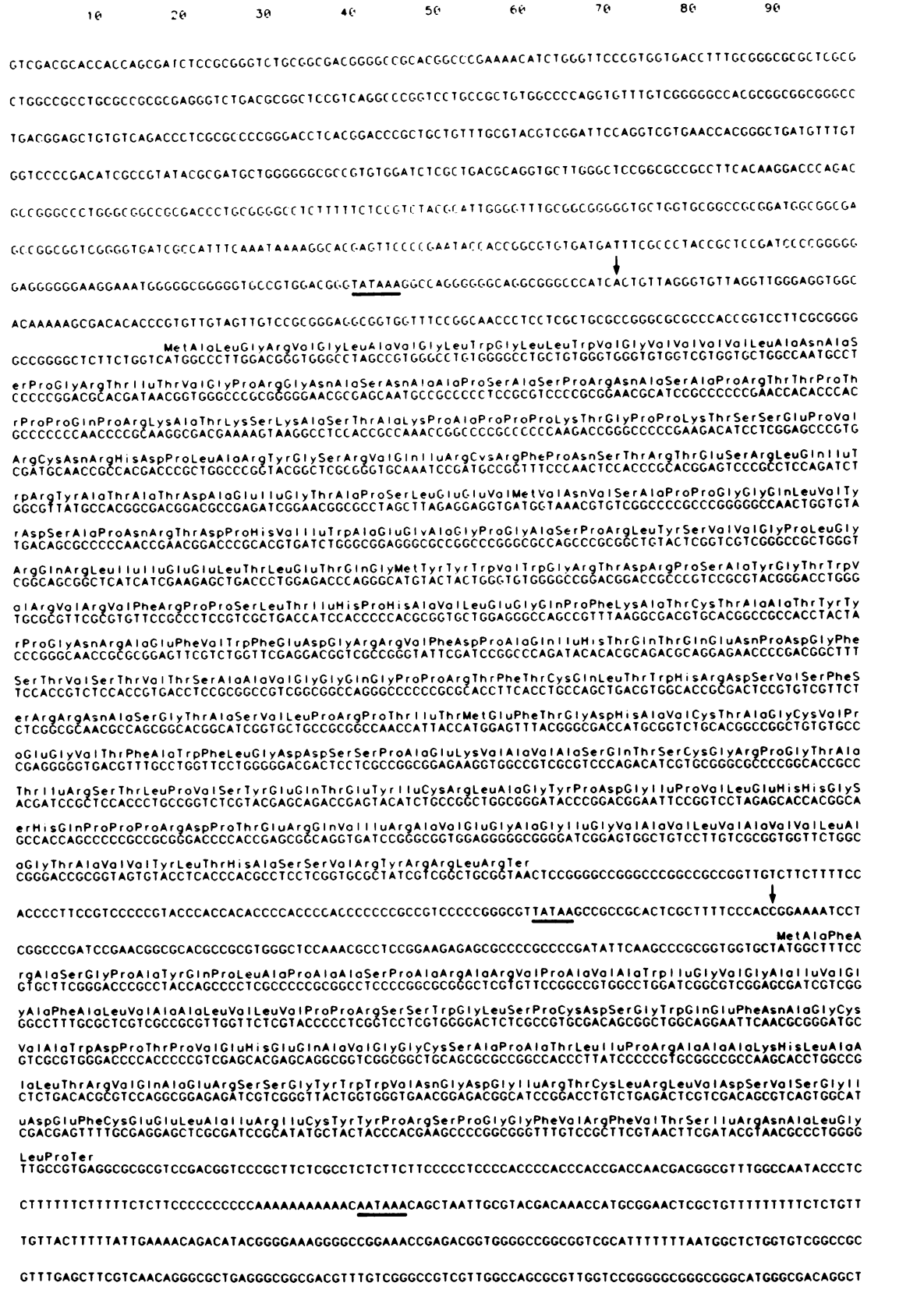

FIG. 3. Nucleotide sequence of a 3.4-kb segment of HSV-2 DNA encoding gC-2 and an 18K protein. The noncoding strand of the nucleotide sequence for gC-2 and the 18K protein is shown. The presumptive TATA boxes (nucleotides 641 through 645 and 2362 through 2366) are underlined for the two mRNAs, as is the polyadenylation signal (nucleotides 3141 through 3146). The 5′ termini (nucleotides 672 and 2390) of the two mRNAs are indicated by an arrow. The amino acid sequence predicted by the DNA sequence is shown for gC-2 and the 18K protein.

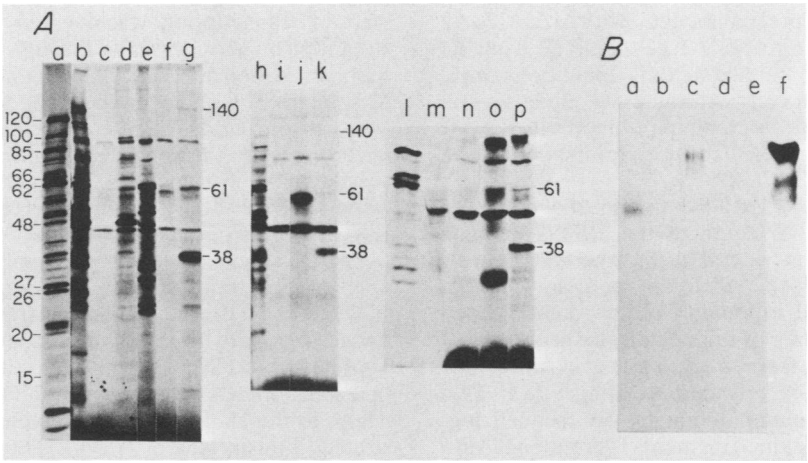

FIG. 4. Characterization of in vitro- and in vivo-synthesized proteins. (A) Cell-free protein synthesis was programmed by using RNA complementary to the following: pmtrIIa (0.59 to 0.60), lanes g, k, and p; pBglII J (0.32 to 0.40), lane d; pBam A (0.595 to 0.67), lane e; pBglII/EcoRI (0.621 to 0.631), lane f; M13mp10 BglII/EcoRI (0.621 to 0.631), lane j; and M13mp11, EcoRI/SalI (0.637 to 0.639), lane o. Total unselected HSV-2 RNA was translated in lanes b, h, and m, and no RNA was used in lanes c, i, and n. Size markers were obtained by using adenovirus 2 translation products (lane a). The products of cell-free translation in lanes a through k were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoreses with 15% acrylamide gels. In lanes l through o the products were fractionated through a gel containing 8 M urea and 17% polyacrylamide. (B) Immunoprecipitation of [^{3}H]glucosamine-labeled extracts of cells with specific MAB, infected by HSV-2 (lanes a, c, and e) or HSV-1 (lanes b, d, and f). Lanes a and b were reacted with 17αA2, a MAB specific for gC-2; lanes c and d were reacted with 13αC5, a MAB specific for gG; and lanes e and f were reacted with G11, a MAB specific for gC-1.

methyl mercury-containing agarose gels in parallel with HSV-1 mRNA and hybridized in situ with ^{32}P-labeled HSV-2 *Eco*RI fragment O (m.u. 0.631 to 0.637). The most abundant species was ca. 2.5 kb, the same size or slightly smaller than the message encoding gC-1 (8, 12). Also detectable was a 730-base message corresponding in size to the HSV-1 message encoding a 17.8K protein. To further analyze these RNAs, the 5′ termini were mapped by primer extension analysis. The choice of the primer was determined from the DNA sequence described below.

To determine whether the 5′ end of the 2.5-kb mRNA was in a homologous position to that of the HSV-1 message, we hybridized 10 μg of RNA from HSV-2-infected cells to an end-labeled 102-bp *Hpa*II fragment and extended it with reverse transcriptase. The elongation products were displayed on sequencing gels along with known sequencing reactions (Fig. 2). The major product extended 133 nucleotides past the *Hpa*II site placing the start of transcription within the sequences TCAC (nucleotides 670 through 673 in Fig. 3) with the most likely start on the A. This would place the 5′ terminus of the message encoding gC-2 exactly at or within one or two nucleotides of the start of the gC-1 mRNA. To map the 5′ end of the 730-bp message, RNA was hybridized to a 100-bp *Hpa*II fragment. The most prominent elongation product was 158 nucleotides (Fig. 2), placing the 5′ terminus of the 730-base message within the sequence CACCG (nucleotides 2387 through 2391 in Fig. 3) with the most likely start on the last C. Again, this placed the terminus in a nearly exact homologous position to its HSV-1 counterpart. A few faint bands extending past the major stop for the 730-base message could be seen which were interpreted to be due to hybridization to the 2.5-kb RNA which appears to be partially colinear (12).

The proteins encoded in this region were characterized by in vitro translation of hybrid-selected mRNAs (Fig. 4). Cell-free translation of RNA preparations from HSV-2-infected cells directs the incorporation of [^{35}S]methionine into numerous proteins (Fig. 4A, lane b). We had previously shown (13) that mRNA selected by hybridization to the left-hand 2.1 kb of the *Bgl*II fragment N, pmtrIIa, encodes three proteins of molecular weights 140,000, 61,000, and 38,000 which served as controls in these experiments (Fig. 4A, lanes g, k, and p). When DNA from the region homologous to HSV-1 gC was used to select mRNAs the following results were obtained: mRNAs selected by hybridization to the *Bam*HI fragment A (0.595 to 0.67 m.u.) encoded a number of polypeptides (Fig. 4A, lane e); with a *Bgl*II-*Eco*RI fragment (0.621 to 0.631 m.u.) which by DNA sequence analysis was shown to be within the coding sequences for gC, a single protein of ca. 60,000 daltons was seen (Fig. 4A, lane f). To visualize the 60,000-dalton protein more clearly, single-stranded DNA from an M13 phage containing the coding strand of the *Bgl*II-*Eco*RI fragment (0.621 to 0.631 m.u.) was used to select the mRNA (Fig. 4A, lane j) and a 60K protein could be clearly demonstrated. The 730-base message of HSV-1 has been reported to encode a 17,800-dalton protein (12), and HSV-2 sequence data (see below) has defined an open reading frame for this protein. mRNAs selected by hybridization to plasmid DNAs encoding this product have not detected this protein (Fig. 4A, lanes e and f), suggesting that the mRNA is not very abundant or that it is difficult to translate. However, when RNA was selected by hybridization to single-stranded DNA, an M13 phage containing the coding strand of the *Eco*RI-*Sal*I fragment (0.637 to 0.639 m.u.), a protein of 20,000 daltons was visualized when displayed on a urea-polyacrylamide gel (Fig. 4A, lane o). If plasmid pBR322 DNA was used for hybridization (data not shown), the products of cell-free translation were undistinguishable from those obtained when no RNA was added (Fig. 4A, lanes c, i, and n). When an unrelated region of the genome, the *Bgl*II fragment J (0.32 to 0.40 m.u.), was used to selected mRNAs, a totally different set of products was seen (Fig. 4A, lane d).

By using MABs obtained from S. Bacchetti that were previously shown to react with HSV-2 glycoproteins (3, 4), we could precipitate from extracts of [^{3}H]glucosamine-labeled HSV-2-infected cells a glycoprotein of 130,000 daltons, presumably gG, with MAB 13αC5 and a glycopro-

tein of ca. 70,000 daltons, presumably gC, with MAB 17αA2 (Fig. 4B). With MAB G11 to HSV-1 gC obtained from K. Shriver, a glycoprotein of 130,000 daltons could be precipitated from HSV-1-infected cells. Although we made several attempts, we were not able to precipitate the in vitro-translated 60,000-dalton protein selected by hybridization to the gC-2 region with the MAB 17αA2.

DNA Sequence of HSV-2 gC. To obtain the complete nucleotide sequence of the region of the HSV-2 genome encoding gC, the protein encoded by the smaller 3'-coterminal message, and the upstream and downstream regulatory sequences, and to identify other potential open reading frames on either side of this region, we sequenced a 3,410-bp segment of DNA from the *Sal*I site at position 0.61 to a *Sma*I site at approximately map position 0.644. Two strategies were used to obtain templates for sequencing. First, small restriction enzyme fragments were cloned into appropriately cleaved M13 vectors, and single-stranded phage were used directly as templates. The second approach was one recently developed by Henikoff (15). A series of deleted fragments were constructed by digestion of the replicative form of an M13 phage containing a large insert of viral DNA to produce a 5' end near the insert and a 3' end near the phage and digestion for various periods of time with exonuclease III, followed by S1 nuclease digestion and blunt-end ligation. The single-stranded phage DNAs were then prepared as templates. A minimum of a double-strand reading was obtained; generally three to four readings were obtained, and overlapping sequence through every restriction site was obtained to assure that small stretches of DNA between two closely spaced sites were not omitted.

The nucleotide sequence of the noncoding strand of the HSV-2 strain 333 gC gene is shown in Fig. 3. The start of transcription had been mapped to nucleotide 672. Upstream at nucleotides 641 through 646, the presumptive regulatory sequence TATAAA was found. The first ATG after the start of transcription was located 147 bases downstream at nucleotides 819 through 821. An open reading frame of 1,440 nucleotides was found which codes for a 480-amino acid protein. The protein structure predicted by the DNA sequence revealed many features characteristic of a membrane-bound glycoprotein. The polypeptide contains an amino-terminal hydrophobic membrane insertion or signal sequence (18, 26, 29) located between amino acids 6 (valine) and 25 (alanine) from nucleotides 834 through 893. A carboxy terminal transmembrane anchoring domain (33) was found between amino acid 443 (alanine) and amino acid 467 (leucine) from nucleotides 2148 through 2222. The transmembrane region was bounded by the basic amino acids arginine and threonine and was followed by an 11-amino acid charged cytoplasmic anchor. In addition, there are seven potential *N*-linked glycosylation sites (asparagine-X-serine or threonine [32]). The predicted weight of the gC protein is 51,600 as compared with the observed value of 60,000. No apparent polyadenylation signal could be found following the termination codon for gC-2 consistent with the apparent size of the mRNA as estimated on Northern blots (8) and with the mapping of the HSV-1 message which places the 3' terminus ca. 1 kb further downstream.

The start of transcription of the 730-bp message is at nucleotide 2390. Upstream from the cap site is the sequence TTATAA (2361 through 2366), suggesting that this mRNA is under the control of its own promoter and consistent with the observation that the 730-base mRNA could not be detected by Northern blot analysis with fragments of DNA upstream of this site as probes (8). The first ATG after the start of transcription was located 102 nucleotides downstream at nucleotides 2491 through 2493. An open reading frame of 516 nucleotides encoding a 172-amino acid protein was found. The polypeptide has no features of a membrane-associated protein. The calculated molecular weight of 18,200 is very close to the apparent weight of 20,000. The apparent polyadenylation signal AATAAA for both messages is located 132 bases downstream of the termination codon for the 18K protein (from nucleotide 3141 to 3146).

The DNA sequence was also analyzed for other potential open reading frames. Within the sequence which codes for gC-2, both of the other reading frames on that strand are closed, one with 14 termination codons, and the other with 4. Mapping of HSV-1 RNAs from this region has suggested that spliced RNAs may be present (11); however, additional RNAs or translation products have not been clearly demonstrated in this region of HSV-2. The mRNA which specifies the 18K protein uses a different reading frame from that of gC-2. Interestingly, on the complementary strand, there is an open reading frame which is longer than the one which encodes the 18K protein with a potential initiation codon at nucleotides 3271 through 3273 and a termination codon at nucleotides 2243 through 2245 which would encode a 344-amino acid polypeptide. No TATA homology was found 5' of the ATG; however, only 137 bases of sequence data were available, and similarly, no polyadenylation signal was found.

We have previously shown (13) that the right-hand end of the *Bgl*II N fragment (0.585 to 0.621 m.u.) encodes a 56K protein which is transcribed from left to right on the prototype arrangement of the genome. A termination codon (at nucleotides 515 through 517) is preceded by an open reading frame to the end of the available sequence encoding 172 amino acids and is followed by a polyadenylation signal at nucleotides 529 through 534. This sequence might encode the C terminus of the previously described 56K protein. Alternatively, Jenkins and Howett (16) have recently mapped a minor 1.5-kb message to the right of the message encoding the 56K protein and leftward of that encoding gC, which would be in the correct position for the signals and open reading frame identified by this sequence. Frink et al. (11) have described a set of two unspliced low-abundance RNAs transcribed from right to left which specify a 85K protein. The RNAs appear to overlap at the 5' end with the message that encodes gC-1. From the HSV-2 sequence data, no long open reading frame could be identified on this strand. If the coding sequences for a homologous HSV-2 protein map to the left of the available data the untranslated leader RNA would be at least 800 bases. It is possible that no homologous protein is encoded by HSV-2.

Comparative DNA sequence analysis between HSV-1 and HSV-2. The sequence of the gC gene of HSV-1 strain KOS has been published (12) and recently amended (7). Approximately 2.7 kb of DNA could be compared, beginning at residue 513 (Fig. 3) and extending to residue 3224. There are regions of both strong and weak homology, and as has been observed previously (14, 40), the intergenic spaces generally show the least homology.

The 5' regulatory sequences for the gC gene showed less intertypic homology than did the thymidine kinase gene (40) or the 38K-protein gene (14). In the gC-2 sequence, the TATA box is within the sequence CGGGTATAAA (nucleotides 637 through 646) and is identical in HSV-1. The start of transcription in HSV-1 is 21 bases downstream, and 25 bases downstream in HSV-2. Of the 21 bases, 9 are different, which is about twice the rate of mismatch observed in this region for either the 38K protein or thymidine kinase

genes. A second stretch of identity is the sequence GTGTATGATTTCGCC (nucleotides 563 through 578); however, in HSV-1 these two blocks of homology are separated by 26 bases, whereas in type 2 they are separated by 57 bases. The intervening sequences in HSV-2 contain guanosine-rich elements found upstream of other HSV genes (22, 42), GGGGGGAGGGGGG and GGGGGCGGGGG, separated by the AG-rich element AAGGAAA, also a common upstream feature of HSV genes (5, 22). These elements were not located within 120 bases upstream of the start of transcription of HSV-1 gC. No match of greater than 50% homology could be found for the sequences separating the two identical elements or for the sequences upstream of the distal element. The untranslated leader of the gC mRNA is 145 bases in HSV-1 and 147 bases in HSV-2 and shows no apparent homology. Interestingly, both HSV-1 and HSV-2 had the sequence AATAAA (nucleotides 529 through 534) preceded by TGA (nucleotides 515 through 517), the potential terminator and polyadenylation signal either for the 58K protein or for an unidentified protein specified by a minor 1.5-kb RNA.

The coding sequences for gC also showed areas of both strong and weak homology. For maximum DNA homology, three sites of insertion of HSV-1 DNA were made: after nucleotide 893, six bases were inserted; after nucleotide 1001, three bases were inserted; and after nucleotide 1070, 84 bases were inserted. All of the insertions were in phase and resulted in a gC-2 protein that maintained the same reading frame as gC-1. The 252 nucleotides which encode the amino-terminal 17.5% of the gC-2 protein differed in 81 sites from gC-1, a ca. 32% difference. After the large insertion of HSV-1 DNA, the remaining 1,188 nucleotides which code for the carboxy 82.5% of the protein, 242 nucleotide changes were observed for a rate of about 20% difference.

A comparison of the predicted amino acid sequences for gC-1 and gC-2 is shown in Fig. 5. The HSV-2 glycoprotein is 31 amino acids smaller than its HSV-1 counterpart. To maximize DNA and protein homology, deletions were placed at HSV-1 amino acids 26 through 27, 64, and 88 through 115. Of the first 84 amino acids in gC-2, 40 differed from those in gC-1 (48%). Of the remaining 396 amino acids of gC-2, 106 were not identical in gC-1 (27%). The similarity of the two proteins is even more striking if the substitution of an amino acid of related polarity is considered. In general, the functional arrangement seems to be conserved. Both glycoproteins possess a 20-amino acid hydrophobic membrane-signaling region. It should be noted that direct N-terminal sequencing of the glycoproteins will be required to confirm that these amino acids are absent from the mature protein. In gC-2, the hydrophobic membrane-anchoring region is 25 amino acids flanked at either side by a basic amino acid, whereas this domain in gC-1 is 26 residues flanked by a single basic residue. Each glycoprotein has a 10-amino acid highly charged cytoplasmic anchor at the carboxy terminus. There are eight potential *N*-linked glycosylation sites in gC-1 and seven in gC-2, five of which are at the same position on the glycoprotein. The predicted anhydrous molecular weights for gC-1 and gC-2 are 55,000 and 51,600, respectively.

In the intergenic region separating gC and the 18K protein, there is only patchy intertypic homology. The start of transcription of the two messages is the same with a few nucleotides. A TATA box is located 23 bases upstream in the gC-2 sequence and 27 bases upstream in gC-1. Farther upstream of the TATA box, only weak homology could be seen. The number of nucleotides separating the termination of gC translation and the TATA sequence was 99 nucleotides for gC-2 and 73 nucleotides for gC-1. No identical stretches of homology longer than seven bases were found, and even those appeared only to be runs of C. Within the untranslated leader mRNA little significant homology was detected, and the gC-2 leader was 102 bases, whereas the gC-1 leader was 79 bases.

The coding sequence for the 18K protein showed strong intertypic homology. Both proteins were encoded by a 516-nucleotide open reading frame. There were 110 differences at the nucleotide level (21%) which resulted in 43 nonidentical amino acids (25%), and the changes were scattered throughout the protein (Fig. 5). Both proteins had a predicted anhydrous molecular weight of 18,200.

In the intergenic region between the 18K protein and the next gene to the right, the homology is patchy for ca. 50 bases and is then lost completely. The polyadenylation signal AATAAA is located 112 nucleotides downstream of the HSV-1 termination signal and 131 nucleotides downstream of the HSV-2 terminator. The HSV-2 polyadenylation signal follows an unusual DNA sequence, e.g.,

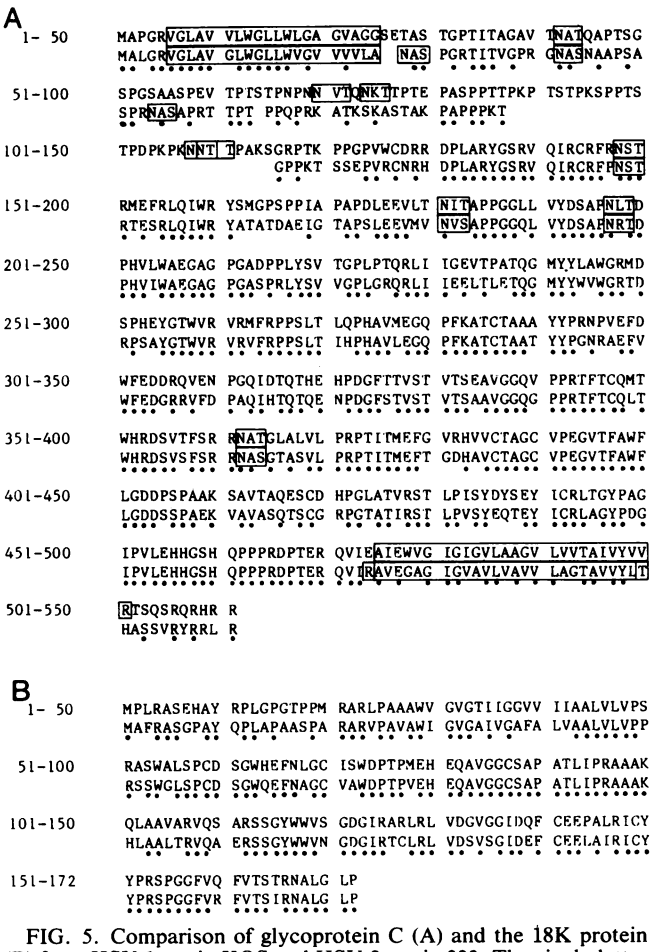

FIG. 5. Comparison of glycoprotein C (A) and the 18K protein (B) from HSV-1 strain KOS and HSV-2 strain 333. The single-letter amino acid code for HSV-1 is shown on the top line, and HSV-2 is shown on the bottom line. The sequences have been aligned to maximize DNA homology. The boxes indicate the hydrophobic membrane-signaling sequence near the amino terminus, the membrane-anchoring sequence at the carboxy terminus and the potential sites for *N*-linked glycosylation. Identical amino acids are indicated by a dot.

TTTTTTCTTTTTCTCTTCCCCCCCCCCAAAAAAAAAAA, which is not present in HSV-1.

DISCUSSION

This report has provided detailed proof that the HSV-2 genome encodes a glycoprotein in a homologous position to the HSV-1 glycoprotein C. HSV-2 is encoded by a transcriptional unit that is arranged identically to the HSV-1 unit in that the major unspliced 2.5-kb message encodes gC, and a smaller 0.73-kb message with a colinear 3′ terminus encodes a 18K protein. Cell-free translation of mRNAs selected by hybridization to DNA from m.u. 0.62 to 0.64 shows polypeptides with apparent molecular weights of 60,000 (gC) and 20,000 (18K protein). Nearly 80% of gC-2 shows a high degree of conservation with the HSV-1 counterpart. However, within the hydrophilic sequences near the amino terminus of the protein, there are significant differences. In total, DNA encoding 31 amino acids is missing from gC-2; the longest stretch is an 84-bp gap. In addition to the deleted sequences, there is only patchy homology, so that the amino acid sequence of the N terminus is quite dissimilar between HSV-1 and HSV-2. The 18K polypeptides appear to be identical in size and are well conserved.

The biological function of gC is not known precisely. It has recently been shown that gC-1 binds the third component of complement but that HSV-2 infected cells do not induce the formation of the C3b receptor (10). It is possible that the differences in the amino terminus, including the additional amino acids in gC-1, are specifically related to the ability to bind complement. Although most clinical isolates of HSV express gC, strains of both HSV-1 (19, 34) and HSV-2 (44) have been characterized which fail to express gC in tissue culture. Although gC is not essential for lytic growth, its presence appears to inhibit cell fusion, in that gC^- virus exhibits a syncytial plaque morphology (6). Neutralizing antibodies to appear to react with gC, and gC may be an antigen recognized by cell-mediated immunity.

The degree of variation between the genes encoding gC-1 and gC-2 and the 18K proteins is greater than has been observed in other intertypic comparisons. In general, the 5′ transcriptional regulatory sequences have been highly conserved in the case of thymidine kinase (40), a 38K protein mapping near position 0.60 (14), and gD (41). Only two identical stretches could be found upstream of the start of transcription of gC, and these were spaced differently. No other really good homology could be detected within 150 bases upstream. Since intertypic recombinants occur from crossover points throughout the genome, it is anticipated that the regulatory sequences would remain conserved. No detailed studies have measured the synthesis and turnover of gC-1 and gC-2 mRNAs, nor has it been shown that the regulation of the gene occurs in a similar fashion in an intertypic recombinant. A comparison of the coding sequences for gC has also shown a great deal of variation. In the case of thymidine kinase and gD, both HSV-1 and HSV-2 proteins had the same number of amino acids and were 73 and 80% homologous at the amino acid level, respectively. The 38K protein of HSV-1 had three more amino acids than its HSV-2 counterpart. These and other changes affected the 30 N-terminal residues; nevertheless, the overall homology was 89%. The HSV-2 gC has 31 fewer amino acids than gC-1 and several regions of low homology near the N terminus. On the other hand, the C-terminal 80% of the protein is highly conserved and shows about 80% amino acid homology. Although it might appear that a great deal of variation occurs because gC is not an essential protein for the viral life cycle, two observations argue against this possibility. First, the thymidine kinase gene is also not essential and yet it shows much less variation than gC. Secondly, intratypic comparison from a HSV-1 gC^+ strain (KOS) with a gC^- strain (MP) has revealed an intratypic variation of 1.5% in the 5′ and amino-terminal sequences (7), a rate comparable to that of the thymidine kinase gene (40). Less variation in the C-terminal sequences was seen, although there is no constraint imposed by the need to translate a functional protein.

One benefit of a detailed sequence comparison is that it becomes possible to identify nucleic acid sequences or polypeptides which can detect either type-common or type-specific regions of HSV. Zweig et al. (46) have recently synthesized a peptide that can elicit antibodies that recognize gC-1 and gC-2 in immunoblot assays. Given the high degree of homology in the C-terminal region and the ability to predict secondary and tertiary structure, it should be possible to synthesize peptides which elicit both sequence-dependent and conformation-dependent antibodies that would be useful in immunoprecipitation or other immunodiagnostic assays. An obvious example of a type-specific peptide would be the region of HSV-1 which is not present in gC-2. A possible gC-2-specific antibody is a peptide containing residues 162 through 172 (HSV-1 coordinants in Fig. 5) in which 9 of 10 amino acids are mismatched. Similar nucleic acid sequences can also be generated.

Comparative DNA sequence analysis will not only provide more information about the evolution of HSV-1 and HSV-2 but will also allow the generation of reagents which will facilitate studies on the function of specific proteins, the regulation of gene expression, and on the natural history of herpesvirus infections.

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ADDENDUM IN PROOF

Since the submission of this manuscript, Dowbenko and Laskey (J. Virol. **52**:154–163, 1984) have reported the sequence of the HSV-2 (strain G) equivalent of the gC gene presented here. The HSV-2(333) gC has one additional amino acid and several nucleotide differences.

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