JOURNAL OF VIROLOGY, Feb. 1986, p. 647–655
0022-538X/86/020647-09$02.00/0

Vol. 57, No. 2

Identification of a New Glycoprotein of Herpes Simplex Virus Type 1 and Genetic Mapping of the Gene That Codes for It

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Received 6 August 1985/Accepted 29 October 1985

A type-specific monoclonal antibody, LP10, precipitated a glycoprotein with a molecular weight of approximately 59,000 from purified herpes simplex virus type 1. Although this glycoprotein was similar in size to glycoprotein D (gD), it was shown to be less abundant in both virions and infected cells, to migrate more rapidly in its precursor form, to incorporate glucosamine but not mannose, and to have a more stable precursor in tunicamycin-treated cells than the gD precursor (pgD). Immunoassays of cells infected with insertion recombinants and intertypic recombinants localized the gene coding for the target antigen of LP10 to the unique short (Us) region at map units 0.892 to 0.924 excluding gD. The target antigen of LP10 was then definitively mapped to the Us4 open reading frame by immunoprecipitation of a polypeptide synthesized by in vitro translation of a Us4-specific transcript prepared by using an SP6 cloning vector and RNA polymerase. This newly identified glycoprotein product of the Us4 gene of herpes simplex virus type 1 is distinct from the previously identified gB1, gC1, gD1, gE1, and gH1.

It is certain that the surface glycoproteins of herpes simplex virus (HSV) play a major role in defining the biological and pathogenic properties of the virus, and it is assumed that the glycoproteins are the primary targets of the immune response after HSV infection (26). Nevertheless, the glycoproteins of HSV remain poorly understood; we know little of their function, and the total number of glycoproteins specified by HSV is unknown. The existence of four HSV glycoproteins designated gB, gC, gD, and gE has been established for some time, and the nucleotide sequence of their corresponding genes has been determined (14; for a review, see reference 26). Marsden et al. (12, 13) identified a glycoprotein of HSV type 2 (HSV-2) which was distinguishable from gD and gE and which mapped in the unique short (Us) region of the HSV-2 genome. This probably corresponds to the polypeptide designated gG2 by Roizman et al. (22). Showalter et al. (23) prepared monoclonal antibodies that reacted with a 110K glycoprotein of HSV-1. Further characterization of this glycoprotein and genetic mapping of its coding sequence in the unique long (Ul) region established that it is distinct from previously described HSV-1 glycoproteins, and it was designated gH1 (3).

The complete nucleotide sequence of the Us region of HSV-1 predicts the existence of yet further virus-specified glycoproteins (14). In addition to the open reading frames representing sequences coding for gD and gE (Us6 and Us8, respectively), three other open reading frames (Us4, Us5, and Us7) probably code for transmembrane glycoproteins because their deduced translation products have characteristic signal peptides, membrane anchor sequences, and N glycosylation sites (14). In this report we describe a type 1-specific monoclonal antibody, LP10, that precipitates a target antigen which is distinct from previously described HSV-1 glycoproteins on the basis of electrophoretic mobility and glycosylation and which maps to the Us4 open reading frame.

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MATERIALS AND METHODS

Cells. BHK21 clone 13 cells and HEp-2 cells were grown in Glasgow modified Eagle medium containing 10% tryptose phosphate broth and 10% newborn bovine serum in an atmosphere of 5% CO_2 in air at 37°C.

Viruses. HSV-1 strain F (5) and HSV-2 strain 25766 (K. R. Dumbell, Wright-Fleming Institute, London) were used as control antigens in immunoassays. HSV-1 strain HFEM (31) was used as the inoculum for radioimmunoprecipitations. The insertion recombinant viruses were the generous gift of P. G. Spear (7). The intertypic recombinant viruses were gifts from H. Marsden, and the determination of their genome structures has been previously reported (8, 12, 13). For further fine mapping of intertypic recombinants, HSV-1 strain 17 (2), and HSV-2 strains HG52 (27) and 333 (W. Rawls, McMaster University, Ontario, Canada) were used.

Radiolabeled antigens. Radiolabeled purified virus was prepared by replacing the medium from two confluent roller bottle cultures of HEp-2 cells with 55 ml of 5% Glasgow modified Eagle medium–95% methionine-free Dulbecco modified Eagle medium, $10^{7.3}$ PFU of HSV-1 (multiplicity of infection of approximately 0.1), and 40 μCi of [^{35}S]methionine per ml (1,315 Ci/mmol; Amersham Corp., Amersham, England). After 48 h of incubation on a roller at 37°C, the medium was harvested and clarified by centrifugation at 3,200 rpm for 15 min at 4°C in an International-3 centrifuge (International Equipment Co., Div. Damon Corp., Needham Heights, Mass.). The virus in the clarified supernatant was pelleted at 21,000 rpm in an SW25 rotor of a Damon centrifuge at 4°C for 60 min. The pellet was suspended in 1 ml of phosphate-buffered saline (pH 7.4) containing 5% fetal bovine serum that had been heat inactivated at 56°C for 30

min, clarified at 34,000 rpm for 60 min, and filtered through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.). This was then layered on a 30-ml 10 to 40% potassium tartrate gradient in phosphate-buffered saline–fetal bovine serum. The gradient was centrifuged in an SW25 rotor at 25,000 rpm at 4°C for 60 min. The band was harvested with a needle and syringe, diluted in phosphate-buffered saline–fetal bovine serum, and pelleted in an SW40 rotor at 34,000 rpm at 4°C for 60 min. The pellet, after suspension in 1 ml of phosphate-buffered saline–fetal bovine serum, contained $10^{8.3}$ cpm and $10^{8.9}$ PFU.

Radiolabeled infected cell lysates were prepared by infecting confluent BHK cell monolayer cultures in 6-cm dishes at a multiplicity of infection of 10 PFU per cell. For pulse-labeling 2, 4, or 6 h after infection, the monolayers were washed twice with prewarmed phosphate-buffered saline and covered with 2 ml of prewarmed methionine-free Dulbecco modified Eagle medium containing 100 μCi of [^{35}S]methionine. The cells were harvested after 20 min of incubation at 37°C. For the prolonged labeling, cells were incubated in the radiolabel for the period of 2 to 8 h after infection. For the sugar labeling, reduced-glucose medium (0.9 g/ml) was used with 60 μCi of either D-[U-^{14}C]mannose (300 mCi/mmol; Amersham) or D-[1-^{14}C]glucosamine (300 mCi/mmol; Amersham) per ml; 2.5 μg of tunicamycin (Sigma Chemical Co., St. Louis, Mo.) per ml was included in the labeled medium where indicated. The cells were lysed and harvested in RIPA buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100) plus 2 mM phenylmethylsulfonyl fluoride and 50 U of micrococcal DNase (Sigma) per ml.

Monoclonal antibodies. LP10 is an HSV-1-specific monoclonal antibody prepared as described by McLean et al. (15). Preliminary immunoprecipitation experiments indicated that the target antigen was a glycoprotein of about 59,000 molecular weight, and reaction of the antibody with intertypic recombinants mapped the target gene to the Us region of the HSV-1 genome. On this basis the target antigen was tentatively identified as gD (3). The antibody has no neutralizing activity in the absence or presence of complement. The antibody is of the immunoglobulin G3 (IgG3) class. The monoclonal antibodies to gG2 (AP1) and gH1 (LP11) have been previously described (3, 12). Monoclonal antibodies to the NP (5/1) and M (174/4) proteins of influenza A virus were the generous gift of K. van Wyke Coelingh, Bethesda, Md. (29). The type 1-specific gD monoclonal antibody (II436) was a generous gift from P. Spear, Chicago, Ill. (7). The specificities of AP10 (gE2) and LP6 (gC2) are based upon radioimmunoprecipitation and map position determined by using intertypic recombinants (A. Buckmaster, unpublished results).

Radioimmunoprecipitation. Radiolabeled infected cell lysates were clarified by centrifugation at 34,000 rpm for 1 h at 4°C. Reticulocyte lysate translation reactions were diluted in RIPA buffer to yield 15,000 trichloroacetic acid-precipitable cpm per 10 μl. Precipitations were then performed in 500-μl Eppendorf tubes by mixing 10 μl of antigen, 10 μl of ascites fluid (1 μl for the influenza virus monoclonal antibodies), 230 μl of RIPA buffer, and 7 mg of staphylococcal protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N.J.) swollen in 50 μl of 25 mM Tris (pH 7.4). After 2 h of rotation at 4°C, the beads were pelleted in a microfuge and washed three times in 500 μl of RIPA buffer. The washed pellet was then incubated in 40 μl of Laemmli sample buffer (24 mM Tris [pH 6.8], 15.4 mg of dithiothreitol per ml, 2% SDS, 20% glycerol, 0.02% bromophenol blue) for 15 min at room temperature. After centrifugation the supernatant was harvested, and the pelleted beads were suspended in 20 μl of Laemmli sample buffer. After centrifugation the supernatant was pooled with the first harvest before scintillation counting and electrophoresis.

Polyacrylamide gel electrophoresis. Samples were immersed in boiling water for 3 min and electrophoresed for 4 h at 35 mA on 12.5% acrylamide–0.1% *N,N'*-methylenebisacrylamide resolving gels with 3% acrylamide–0.13% *N,N'*-methylenebisacrylamide stacking gels by using the SDS-buffer system of Laemmli (9). The gels were then fixed, dried, and fluorographed (1) with Kodak X-Omat X51 film (Eastman Kodak Co., Rochester, N.Y.).

Immunoassays. The radioimmunoassay measured antibody bound to antigen fixed to microtiter plates, using for detection ^{125}I-labeled staphylococcal protein A (Amersham) (12). The enzyme immunofiltration assay measured antibody bound to antigen captured with a microtiter filtration device, using for detection a staphylococcal protein A-horseradish peroxidase conjugate (19). The bound peroxidase was quantitated by the conversion of orthophenylenediamine to a colored product measured by absorbance (19).

Restriction enzyme analysis of DNA. Limited regions of the Us region of intertypic recombinants were analyzed in detail by hybridization of restriction enzyme digests with cloned fragments of HSV DNA. Confluent monolayers of BHK cells were infected at a multiplicity of 1 PFU per cell. When confluent cytopathic effect was visible, DNA was extracted by the method of Varmus et al. (30). Restriction enzyme digests of 2 μg of DNA were electrophoresed in 1% agarose gels and then transferred to nitrocellulose sheets (24). Blots were then hybridized with purified fragments from cloned subgenomic fragments of HSV-1 or HSV-2 DNA that were labeled with ^{32}P by nick translation (20), washed, and autoradiographed.

Molecular cloning of Us4 and Us7. The coding regions of Us4 and Us7 were subcloned from the *Bam*HI J fragment of HSV-1 (see Fig. 6) into the plasmid vector pSP64 (Promega-Biotec, Madison, Wis.), which contains the SP6 RNA polymerase promoter (16). Us4 was subcloned from the *Bam*HI J fragment of strain SC16 cloned in pAT153. The 1.4-kilobase *Bam*HI-*Nru*I fragment from the 5′ end of the *Bam*HI J fragment was obtained after double digestion and then partially digested with *Sau*3A. The 1-kilobase *Sau*3A-*Nru*I fragment (nucleotides 4111 through 5121) was harvested and inserted into the *Bam*HI-*Sma*I site in the polylinker region of pSP64. The open reading frame of this fragment consists of nucleotides 4140 through 4854 of Us (14).

Us7 was subcloned from the *Bam*HI J fragment of strain Patton (generously provided by R. Watson [32]) because this strain contains an *Nru*I restriction enzyme site (starting at nucleotide 7157) which is not present in strain SC16 (32). Double digestion with *Hin*dIII and *Hpa*I yielded a 3.2-kilobase fragment which primarily consists of the coding regions for Us6 (gD) and a portion of Us7 (gE). This fragment was then partially digested with *Nru*I, and the 1.8-kilobase *Nru*I-*Hpa*I fragment (nucleotides 7157 through 9005) containing the complete open reading frame of Us7 (nucleotides 7181 through 8351) was harvested and inserted into the *Sma*I site of pSP64.

The appropriate orientation of clones containing each insert in pSP64 was confirmed with double digestion with *Sph*I, which recognizes a single site in the 3′ end of each insert, and with *Sal*I, which has no site in either insert and a single site in the pSP64 polylinker, distal to the insert with

regard to the pSP64 promoter. The nucleotide sequence and number designations are those of McGeoch et al. (14).

The restriction enzymes used in these studies were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

In vitro transcription and translation. Purified DNA from cloned plasmids consisting of Us4 or Us7 in pSP64 was linearized with *Eco*RI. Capped transcripts were produced in 100-μl reactions containing 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 100 U of RNasin (Anglian Biotechnology, Colchester, Essex), 500 μM each ATP, CTP, and UTP, 25 μM GTP, 500 μM 7-methylguanosine triposphate [m^7G(5')pppG] (P-L Biochemicals, Milwaukee, Wis.), 2 μg of linearized DNA, and 20 U of SP6 RNA polymerase (Boehringer Mannheim). The reaction was incubated at 37°C for 2 h before one phenol extraction, one chloroform extraction, precipitation with ethanol, purification through a spin column of G-50 course Sephadex (11), and reprecipitation in ethanol. The precipitate was dissolved in 6 μl of diethylpyrocarbonate-treated water. Translation reactions (10 μl) were performed as described in detail by Pelham and Jackson (17) with 7.5 μl of methionine-deficient reticulocyte lysate mix, 0.5 μl of [^{35}S]methionine (5.9 μCi/μl, >800 Ci/mmol; Amersham), and 2 μl of transcription product. The reticulocyte lysate was prepared by T. Hunt, Department of Biochemistry, University of Cambridge. Oligo(dT) column-purified polyadenylated influenza A virus mRNA, used as a positive control, was the generous gift of S. C. Inglis, Department of Pathology, University of Cambridge.

RESULTS

Immunoprecipitation with monoclonal antibodies. Cell-free, [^{35}S]methionine-labeled HSV-1 was purified from the culture medium of infected HEp-2 cells. Three monoclonal antibodies each precipitated a single species of protein from lysates prepared from cell-free virus (Fig. 1). LP2 (lane 1) is known to react with gD, a glycoprotein of approximately 59,000 daltons (4, 25). LP11 (lane 2) is known to react with gH, a glycoprotein of approximately 115,000 daltons (3). LP10 (lane 3) precipitated a relatively small amount of a protein that migrated similarly to gD with regard to gel location and width of band.

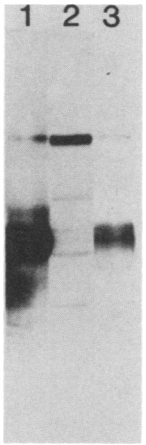

FIG. 1. Immunoprecipitation with monoclonal antibodies of proteins of purified HSV-1 radiolabeled with [^{35}S]methionine. Cell-free HSV-1, strain HFEM, was grown in HEp-2 cells in medium containing [^{35}S]methionine. After virus purification on a 10 to 40% potassium tartrate gradient, the ^{35}S-labeled virion lysate was used in immunoprecipitations with monoclonal antibodies. The precipitates were electrophoresed in an SDS–12.5% polyacrylamide gel. Lanes: 1, LP2 (to gD); 2, LP11 (to gH1); 3, LP10.

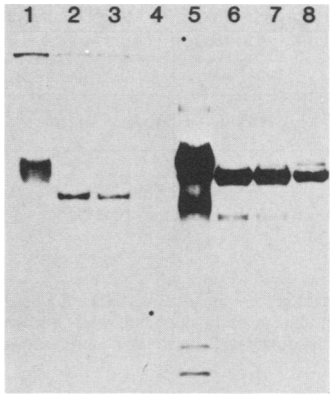

FIG. 2. Immunoprecipitation with monoclonal antibodies of lysates of HSV-1-infected cells radiolabeled for short or prolonged periods with [^{35}S]methionine. BHK cells were infected at a multiplicity of infection of 10 PFU per cell and incubated in the presence of [^{35}S]methionine for 20-min pulses at 2, 4, or 6 h postinfection or for the period 2 to 8 h postinfection. The cell lysates were immunoprecipitated with LP2 (to gD) or LP10, and the precipitates were resolved on SDS–12.5% polyacrylamide gels. Lanes: 1, prolonged radiolabeling, LP10; 2, 20-min pulse at 6 h, LP10; 3, 20-min pulse at 4 h, LP10; 4, 20-min pulse at 2 h, LP10; 5, prolonged radiolabeling, LP2; 6, 20-min pulse at 6 h, LP2; 7, 20-min pulse at 4 h, LP2; 8, 20-min pulse at 2 h, LP2.

Pulse-labeling of HSV-1-infected BHK cells was performed to determine whether the virion protein recognized by monoclonal antibody LP10 was different from the elec-

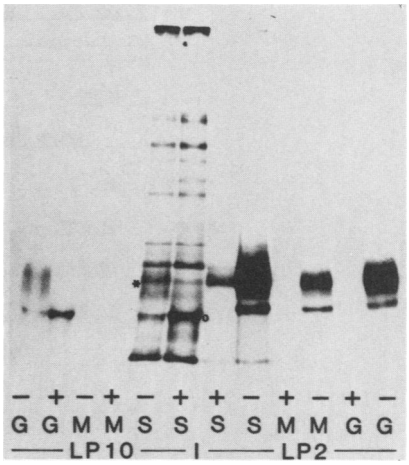

FIG. 3. Immunoprecipitation with monoclonal antibodies of lysates of HSV-1-infected cells radiolabeled with [^{14}C]glucosamine, [^{14}C]mannose, or [^{35}S]methionine in the presence or absence of tunicamycin. BHK cells were infected and radiolabeled for the period 2 to 8 h postinfection as in Fig. 2 in the presence (+) or absence (–) of 2.5 μg of tunicamycin per ml. The lysates were immunoprecipitated with LP2 (to gD) or LP10 and resolved on SDS–12.5% polyacrylamide gels. The radiolabel utilized is indicated by the following abbreviations: G, [^{14}C]glucosamine; M, [^{14}C]mannose: S, [^{35}S]methionine. *, Location of the fully glycosylated form of the protein precipitated by LP10; ○, location of the faster-migrating form of the protein present in tunicamycin-treated cells.

TABLE 1. Expression of LP10 target antigen and other glycoproteins by insertion-recombinant (IR) viruses

Monoclonal antibody	Antigen detected	Absorbance[a]					
		HSV-1	HSV-2	Control	IR 7-13	IR 4-1	IR 3-3
LP10		1.40	–	–	–	–	–
AP10	gE2	–	0.93	–	0.40	0.58	0.53
II436	gD1	1.18	–	–	1.45	1.55	1.23
AP1	gG2	–	1.56	–	1.16	1.22	1.06
LP6	gC2	–	0.71	–	–	–	–

[a] The values represent the mean absorbance values as determined by enzyme immunofiltration assay of duplicate wells corrected by zeroing the spectrophotometer on control wells in which no antibody was used. –, <0.02.

trophoretically similar gD precipitated by LP2 (Fig. 2). Cell lysates prepared from HSV-1-infected cells that were labeled for 20-min pulses with [^{35}S]methionine and then immunoprecipitated with LP2 (lanes 6 through 8) revealed the glycoprotein precursor (pgD), which has been estimated to have a molecular weight of approximately 52,000 (4, 25). When the same lysates were immunoprecipitated with LP10, approximately 20-fold longer time was required for fluorography of the gel to reveal bands of slightly lower intensity (data not shown). The pulse-labeled bands precipitated by LP10 migrated more rapidly through the 12.5% polyacrylamide gel than did pgD (lanes 2 through 4). The estimated molecular weight of these bands was 48,000.

Cell lysates prepared from HSV-1-infected cells that were labeled with [^{35}S]methionine for a prolonged period (2 to 8 h postinfection) were immunoprecipitated with each of these two monoclonal antibodies. LP2 precipitated an additional heavier wide band (gD), which has an approximate molecular weight of 59,000 (Fig. 2, lane 5). The additional band precipitated by LP10 was also wider and more slowly migrating than the pulse-labeled antigen. This heavier band migrated similarly to gD, but was much less intense (lane 1).

To determine whether the protein recognized by LP10 was a glycoprotein and to confirm further its nonidentity with gD, HSV-1-infected BHK cells were labeled for a prolonged period (2 to 8 h) with [^{35}S]methionine, [^{14}C]mannose, or [^{14}C]glucosamine either in the presence or in the absence of tunicamycin. The immunoprecipitates with LP2 or LP10 from these lysates were then characterized on 12.5% polyacrylamide gels and examined by fluorography (Fig. 3). LP2 precipitated both a pgD and gD protein from cells labeled with any of the three precursors in the absence of tunicamycin (lanes 8, 10, and 12). Lysates from tunicamycin-treated cells contained very little antigen recognized by LP2, confirming the observations of Pizer et al. (18) that gD precursors appear to be extremely labile in tunicamycin-treated cells (lanes 7, 9, and 11). The labeled antigen precipitated by LP10 differed in several characteristics from

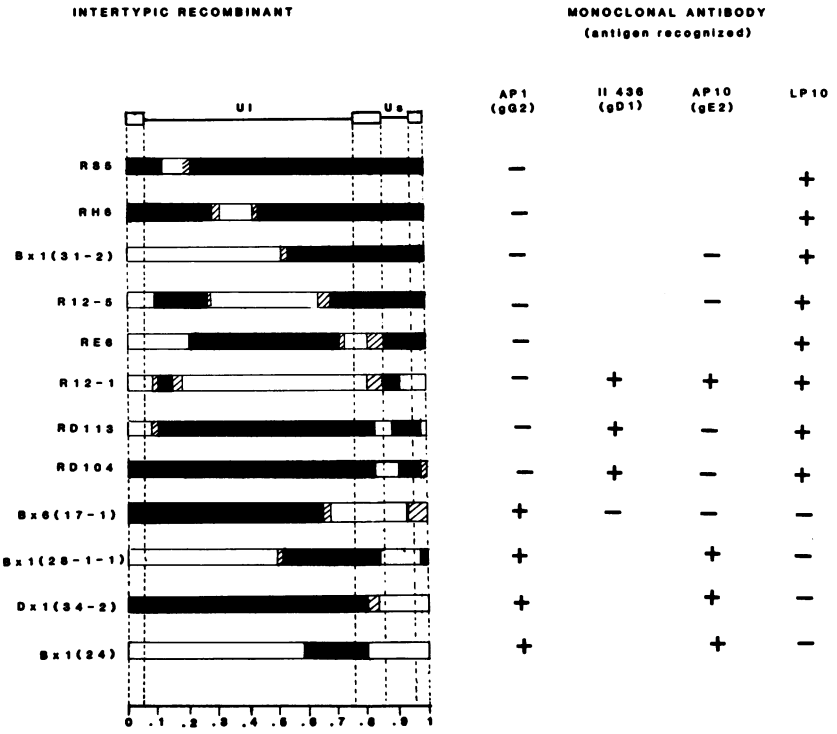

FIG. 4. Expression of glycoprotein antigens by intertypic recombinants as detected with type-specific monoclonal antibodies. The designations and genome structures of the 12 recombinants used in the study are shown at the left. The genome arrangement of HSV DNA is illustrated at the top of the figure, showing the U1 and Us regions indicated as lines bounded by repeat sequences indicated as boxes. Those sequences of the recombinant derived from the type 1 parent are represented by a solid bar, and those from the type 2 parent are indicated by an open bar. Large segments of uncertain parentage are represented by a cross-hatched bar. The units on the bottom represent the fraction of the genome length. The right side of the figure shows for each recombinant whether it induces (+) or does not induce (–) the indicated type-specific glycoprotein antigen. A blank space indicates that the assay was not performed. All recombinants were assayed by radioimmunoassay. Recombinants R12-1, RD113, RD104, and B6 (17-1) were also assayed by enzyme immunofiltration assay with complete concordance.

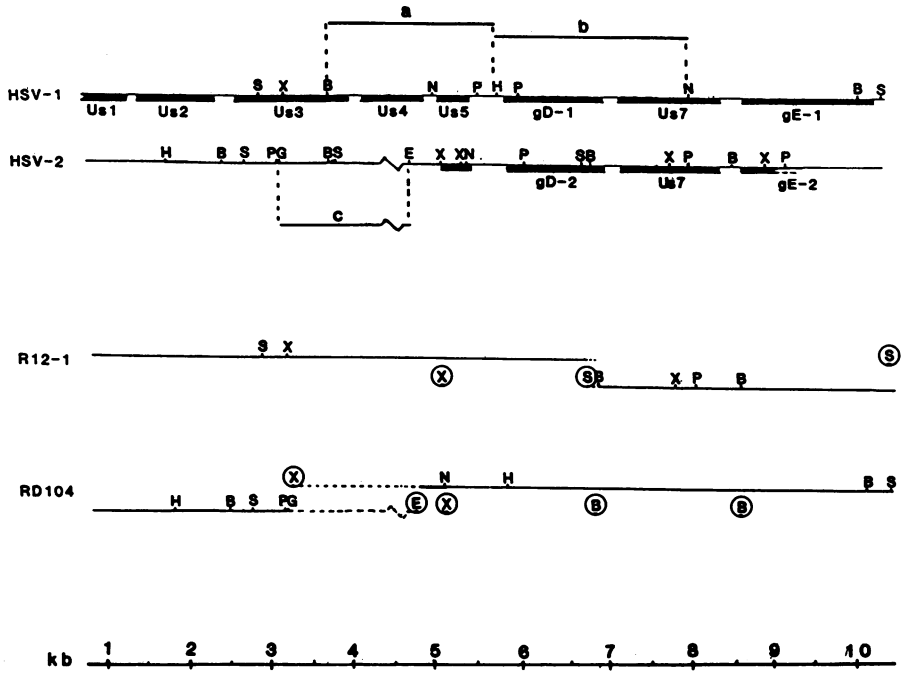

FIG. 5. Restriction maps of part of the Us region of the genomes of HSV-1 strain 17, HSV-2 strain 333, and intertypic recombinants R12-1 and RD104. The alignment of HSV-1 and HSV-2 restriction sites to the right of Us4 is based on sequence data (14; T. C. Hodgman, Ph.D. thesis, University of Cambridge, 1985). The alignment to the left is less certain. The *Bam*HI sites in Us3 are common sites in HSV-1 and HSV-2, and alignment of these sites requires compression of the HSV-2 map rightward of this *Bam*HI site and leftward at the *Eco*RI site. This is indicated by an interruption in the HSV-2 map. Sequencing studies have indicated the presence of additional nucleotide sequences in the HSV-2 genome in this region by comparison with HSV-1 (D. McGeoch, personal communication). The composition of recombinants R12-1 and RD104 is illustrated by an upper line for the HSV-1 sequence and a lower line for the HSV-2 sequence. Regions of uncertainty are shown as a dotted line. Restriction sites present are shown on the solid line. Absent sites are circled. The scale on the bottom line indicates the nucleotide designation of the Us region of HSV-1 genome in kilobases as described by McGeoch et al. (14). Restriction enzyme abbreviations: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; N, *Nru*I; P, *Pvu*II; S, *Sal*I; X, *Xho*I. ^{32}P-labeled probed fragments used to determine genome composition: a, HSV-1 *Bam*HI-*Hin*dIII; b, HSV-1 *Hin*dIII-*Nru*I; c, HSV-2 *Bgl*II-*Eco*RI.

gD. A wide [^{14}C]glucosamine-labeled band was present in the non-tunicamycin-treated cells (lane 1), whereas a narrower, faster-running band was seen in the tunicamycin-treated cells (lane 2). Little if any [^{14}C]mannose was incorporated into the antigen recognized by LP10 (lanes 3 and 4), although this label was well incorporated into gD (lane 10). In this experiment, [^{35}S]methionine gave considerable background labeling, but in the presence or absence of tunicamycin, the fully glycosylated and faster-migrating forms were observed, as in the [^{14}C]glucosamine-labeled lysates.

Genetic mapping the target antigen of LP10. The nonidentity of the LP10 target antigen with gD was further confirmed by using insertion recombinants that were prepared from an intertypic recombinant that basically consisted of an HSV-1 genome replaced in its Us region with HSV-2 genes (7). A segment of the Us region of HSV-1 consisting of the gD gene and part of the open reading frame contiguous to the 3′ terminus of gD was then inserted into the thymidine kinase gene of this recombinant. In either insertional orientation, these insertion recombinants expressed both gD1 and gD2 (Table 1) (7). As expected, they also expressed type 2 glycoproteins located in Us (gG and gE), but not a type 2 glycoprotein located in U1 (gC). None of the three recombinants expressed the type 1-specific target antigen of LP10, indicating a gene located in Us that was not gD (Table 1).

Intertypic recombinants were used to define more precisely the genetic location in Us of the target antigen of LP10. Figure 4 compares the known type-specific composition of intertypic recombinant viruses and the type-specific antigens that they express. The LP10 target antigen segregated with gD1 and away from gG2 in all recombinants tested, but could be distinguished from gE [recombinants R12-1 and Bx6(17-1)]. On the basis of these data the target antigen of LP10 was mapped between 0.892 and 0.924 map units excluding the gD gene.

Since the recombinant R12-1 specifies gD1 and gE2, a recombination must occur between these two genes (Us6 and Us8). Since the type 1-specific LP10 reacts with R12-1, it was of interest to determine whether the Us7 coding region of this recombinant was derived from type 1 or 2. To map the crossover point more precisely, digests of DNA from R12-1 and the parental type 1 and 2 strains were made with various restriction enzymes. Southern blots were then hybridized with a ^{32}P-labeled *Hin*dIII-*Nru*I fragment derived from the *Bam*HI J fragment of HSV-1 (Patton strain). This probe contains the coding region for gD and most of Us7 (Fig. 5). This analysis revealed that R12-1 contained the type 2 *Bam*HI restriction site, but not the *Sal*I site. The crossover therefore occurs near the C terminus within the gD coding region. These results support the hypothesis that Us7 in R12-1 is derived from type 2 and therefore is unlikely to be the target for LP10.

To define the 5′ limit of the gene for the LP10 target antigen, the intertypic crossover from type 2 to type 1 in the

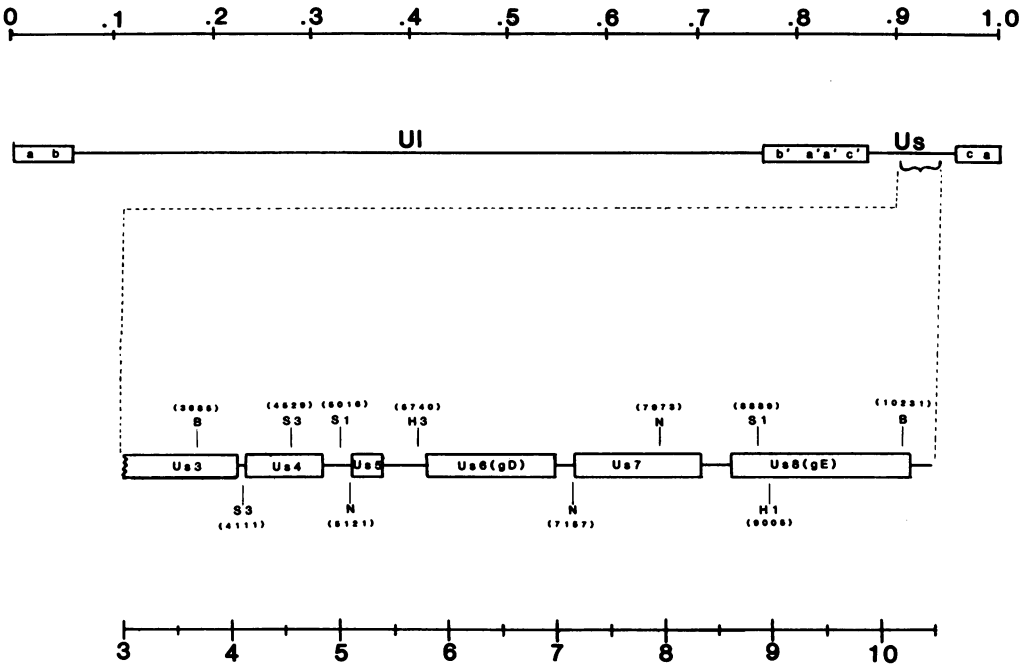

FIG. 6. Map of coding sequences and restriction enzyme sites utilized for in vitro transcription and translation of Us4 and Us7. The upper portion of the figure depicts the organization of the HSV-1 genome with a scale measuring the fraction of the genome length. The blowup from the Us region of the HSV-1 genome depicts the deduced coding regions in the *Bam*HI J fragment. The relative size and locations of these coding regions are defined in kilobases on the scale at the bottom of the figure. The arrows below the coding region indicate the four restriction enzyme sites used to subclone Us4 and Us7. Other relevant sites are indicated above. The numbers in parentheses indicate the nucleotide defining the 5' end of the restriction enzyme site. The deduced coding regions and nucleotide designation are from the complete published sequence of Us by McGeoch et al. (14). The *Nru*I site at nucleotide 7157 is specific to strain Patton (32). Restriction enzyme abbreviations: B, *Bam*HI; S3, *Sau*3A; S1, *Sph*I; N, *Nru*I; H3, *Hin*dIII; H1, *Hpa*I.

recombinant RD104 was examined. The region of uncertainty for the crossover is between the HSV-2 *Bgl*II site and the HSV-2 *Hin*dIII site (12). This area of uncertainty covers most of the Us3 and all of the Us4 and Us5 coding regions. LP10 reacts with RD104. It was therefore of interest to establish whether RD104 contains type 1 or type 2 DNA in the Us4 coding region.

The ^{32}P-labeled probes used to analyze restriction enzyme digests were the HSV-1 *Bam*HI-*Hin*dIII fragment and the HSV-2 *Bgl*II-*Eco*RI fragment (Fig. 5). After aligning the genomes of HSV-1 and HSV-2, the region of uncertainty for the crossover in RD104 could be narrowed slightly. The crossover was leftward of the *Nru*I site between Us4 and Us5. RD104 therefore contains the type 1 coding region for Us5, but the type composition of Us4 was not ascertained.

Immunoprecipitation by LP10 of polypeptides synthesized in vitro from Us4 and Us7. Reactions of LP10 with type-specific antigen expressed by intertypic recombinants implied that the gene coding for the target antigen lay very close and probably 5' to the gD gene. Definitive genetic mapping, however, was not possible with the recombinants available, and mapping with this method is in any case prone to error due to the possible presence of undetected crossovers in intertypic recombinants. From the complete nucleotide sequence of the Us region of the HSV-1 genome, McGeoch et al. (14) identified two open reading frames, designated Us4 and Us7, which lie on either side of the gD gene (Us6) and whose deduced translation products have the properties of transmembrane glycoproteins (Fig. 6). These two coding regions were therefore each subcloned from the *Bam*HI J fragment into the plasmid pSP64, which contains the promoter permitting the in vitro transcription of an inserted sequence by SP6 RNA polymerase. The resulting single-stranded RNA transcripts were then translated in vitro by using a rabbit reticulocyte lysate system (17). LP10 specifically immunoprecipitated the [^{35}S]methionine-labeled translation in vitro product from the Us4 coding region (Table 2).

LP10 immunoprecipitated solely the polypeptide synthesized from Us4 when the translation products of Us4 and Us7 were mixed (Fig. 7). The translation products of each open reading frame migrated as homogeneous bands. The estimated molecular weights of these products, with the migration of in vitro-synthesized influenza A virus polypeptides as standards, were 44,000 for Us4 and 52,000 for Us7.

DISCUSSION

LP10 is a nonneutralizing IgG3 mouse monoclonal antibody that immunoprecipitates a glycoprotein from purified

TABLE 2. Immunoprecipitation of [^{3}S]methionine-labeled in vitro translation products of Us4 and Us7

Monoclonal antibody	Antigen detected	cpm from mRNA[a]		
		Us4	Us7	poly(A) Flu A[b]
LP10		8,882	134	76
AP1	gG2 (HSV-2)	89	109	59
5/1	NP (Flu A)	ND	ND	1,011
174/4	M (Flu A)	56	ND	1,612

[a] The values represent the counts per minute immunoprecipitated from 15,000 cpm of trichloroacetic acid-precipitable translation product put into the reaction with each monoclonal antibody. ND, Not done.

[b] Flu A, Influenza A virus.

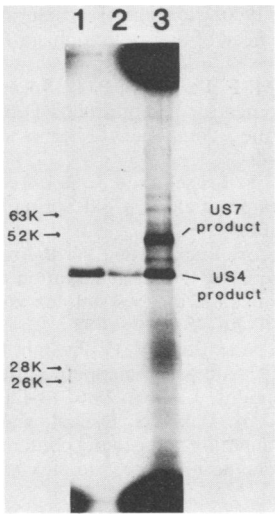

FIG. 7. Immunoprecipitation with LP10 of the in vitro translation products of the Us4 and Us7 open reading frames. Translations were carried out in a reticulocyte lysate system using as mRNA transcripts of Us4 or Us7 produced with SP6 RNA polymerase. The [^{35}S]methionine-labeled translation products or LP10 immunoprecipitates prepared from them were then electrophoresed on SDS–12.5% polyacrylamide gels. Lanes: 1, LP10 immunoprecipitate of 15,000 trichloroacetic acid-precipitable cpm of the Us4 translation product alone; 2, LP10 immunoprecipitate of the same material as lane 3; 3, mixture of 7,500 trichloroacetic acid-precipitable cpm each of Us4 and Us7 translation products. Molecular weight markers, indicated by arrows, were obtained by in vitro translation of poly(A)$^+$ RNA prepared from cells infected with influenza A virus (strain PR8).

HSV-1 virions and HSV-1-infected cells. Preliminary studies suggested that the target antigen of LP10 was gD1, based upon electrophoretic mobility and mapping the gene coding for the target antigen to the Us region by using intertypic recombinants. The autoradiographic signals produced by radioimmunoprecipitates with LP10 were weaker in 4 weeks than those produced with numerous monoclonal antibodies to gD in 2 days, thus prompting further investigation of the LP10 target antigen. As with gG2 and gH1, monoclonal antibodies permitted the identification of a glycoprotein whose recognition had been obscured by its comigration in gel electrophoresis with one of the previously identified and more abundant glycoproteins (3, 12, 22, 23).

Both pulse-labeling and prolonged labeling of the LP10 target antigen with [^{35}S]methionine indicated similar kinetics, but much less abundant product than with gD. Although the larger, more slowly labeled form of gD and the LP10 target antigen found in virions and infected cells migrated similarly (approximately 59,000 daltons), the precursor form of target antigen migrated more rapidly (48,000 daltons) than pgD (52,000 daltons) in 12.5% polyacrylamide gels.

The use of insertion and intertypic recombinants permitted the mapping of the gene coding for the LP10 target antigen to a part of the Us region of the HSV-1 genome (map units 0.892 through 0.924 excluding the gD gene). McGeoch et al. (14) have deduced from their nucleotide sequencing of the Us region that there are five consecutive coding regions (Us4 through Us8), which have been confirmed by mRNA mapping (21), that may code for glycoproteins. The products of two of these regions have been confirmed, gD (Us6) and gE (Us8). Consequently, to define precisely the gene coding for the LP10 target antigen we subcloned, transcribed, and translated in vitro Us4 and Us7, the sizes of which were consistent with the antigen in question. The polypeptide expressed in vitro from Us4 precipitated well with LP10, thus definitively mapping the LP10 target glycoprotein to Us4. Cytoplasmic vesicles were not included in the translation reaction, so that the polypeptide reacting with LP10 presumably underwent neither cleavage of a signal peptide nor glycosylation. It was fortunate for this study that such polypeptides expressed in vitro precipitated well with LP10 and with the monoclonal antibodies to M and NP proteins of influenza A virus. Only 1 of 7 monoclonal antibodies to gD precipitated the corresponding unprocessed polypeptide synthesized with the reticulocyte lysate system (10).

The immunoprecipitated LP10 target antigen from HSV-1-infected cells and the product expressed in the reticulocyte lysate system from Us4 both migrated significantly more slowly than would have been predicted from the sequence of the Us4 open reading frame. The molecular weight of the unprocessed polypeptide deduced from the nucleotide sequence is 25,236 (14). Using as molecular weight markers both influenza A virus polypeptides expressed in vitro and the published estimated molecular weights of pgD and gD (4, 24), the following molecular weights were estimated for LP10 target antigens: polypeptide synthesized in vitro from Us4, 44,000; partially glycosylated precursor antigen from infected cells, 48,000; and final glycoprotein in virions and infected cells, 59,000. Similarly, the predicted molecular weight of the unprocessed polypeptide synthesized in vitro from Us7 is 41,366 (14), although it migrated as a 52,000-molecular-weight protein in polyacrylamide gels. Comparable data were obtained by Lee et al. (10), who selected mRNAs from HSV-1-infected cells with cloned viral DNA fragments and then translated these mRNAs with a reticulocyte lysate system. They mapped polypeptides of 42,000 and 55,000 daltons to what in retrospect were probably Us4 and Us7, respectively. The basis for this discrepancy between estimated molecular weights in polyacrylamide gels and deduced molecular weight is not understood. It should be noted, however, that for some proteins, migration distance may not be a simple function of molecular weight. It has already been observed that the estimated molecular weight of gG2 is 92,000 or 124,000 depending upon whether the polyacrylamide gel is cross-linked with *N*,*N*′-methylenebisacrylamide or *N*,*N*′-diallyltartardiamide, respectively (12).

Similar discrepancies between the molecular weight deduced from nucleotide sequence and estimated by gel electrophoresis have been observed for gD1 and gC1 (6, 32). This discrepancy was attributed to proline-rich polypeptides (43 of 394 for gD1, 63 of 523 for gC1), which would be expected to disrupt α-helical structures (6, 32). This explanation is consistent with the observed slow migration of the Us4 and Us7 polypeptides, the deduced amino acids of which are proline rich (34 of 238 for Us4 and 51 of 390 for Us7) (14).

The LP10 target antigen incorporated [^{14}C]glucosamine readily but little, if any, [^{14}C]mannose. Tunicamycin, which inhibits N glycosylation (28), blocked a significant amount of sugar incorporation. Either [^{35}S]methionine or [^{14}C]glucosamine was incorporated into a relatively homogenously migrating glycoprotein precursor, which was the sole or predominant form that incorporated radiolabel during 20-min pulses. With prolonged labeling (hours), an increase in apparent molecular weight to 59,000 was observed. This two-step glycosylation, the second step of which was inhibited by tunicamycin, has been observed with the previously described HSV glycoproteins (25). The effect of tunicamycin

also discriminates the LP10 target antigen from gD. We confirmed the observation by Pizer et al. (18) that gD precursors are extremely labile in tunicamycin-treated cells. Three potential N glycosylation sites (Asn-X-Thr or Asn-X-Ser) can be identified from the published nucleotide sequence of Us4. The partial inhibition of glucosamine incorporation by tunicamycin and the relatively poor incorporation of mannose suggest O glycosylation of this glycoprotein. The sequence also contains a potential N-terminal signal peptide and a C-terminal hydrophobic transmembrane region, which are characteristic of transmembrane glycoproteins (14).

The newly identified glycoprotein is clearly distinct from the previously described glycoproteins of HSV-1 (gB1, gC1, gD1, gE1, and gH1) (3, 26). We cannot yet give this glycoprotein an alphabetical designation. It is probable, but not certain, that this protein is the HSV-1 equivalent of gG2 which maps similarly in the Us region of the HSV-2 genome with the same intertypic recombinants used in this study (12). LP10 and all of the gG2 monoclonal antibodies recognized at present are type specific. Homology or the lack of it between this newly identified HSV-1 glycoprotein and gG2 must await a type-cross-reactive monoclonal antibody or mapping and sequencing of the gene coding for gG2.

ACKNOWLEDGMENTS

We thank Howard Marsden and Duncan McGeoch for helpful discussion and Vivian Blok and Stephen Inglis for their valuable advice concerning in vitro transcription and translation. Duncan McGeoch kindly provided us with HSV-1 sequence data before publication. Therese McKendry gave us valuable technical assistance, and we thank Mary Wright for typing the manuscript.

This work was supported by the Medical Research Council, United Kingdom. While on sabbatical in Cambridge, D.D.R. was supported by the Veterans Administration, the University of California, San Diego, and the John Simon Guggenheim Memorial Foundation.

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