The U_L10 Gene of Herpes Simplex Virus 1 Encodes a Novel Viral Glycoprotein, gM, Which Is Present in the Virion and in the Plasma Membrane of Infected Cells

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The herpes simplex virus 1 U_L10 gene encodes a hydrophobic membrane protein dispensable for viral replication in cell culture (J. D. Baines and B. Roizman, J. Virol. 65:938–944, 1991). We report the following. (i) A fusion protein consisting of glutathione S-transferase fused to the C-terminal 93 amino acids of the U_L10 protein was used to produce a rabbit polyclonal antiserum. The antiserum reacted with infected-cell proteins which formed in denaturing polyacrylamide gels a sharp band (apparent M_r of 50,000) and a very broad band (M_r of 53,000 to 63,000). These bands were not formed by lysates of U_L10^- virus or by lysates of infected cells boiled in the presence of sodium dodecyl sulfate before electrophoresis. (ii) The proteins forming both bands were labeled by [^{3}H]glucosamine, indicating that they were glycosylated. (iii) The U_L10 protein in cells treated with tunicamycin formed a single band (apparent M_r of 47,000) reactive with the anti-U_L10 antibody, indicating that the 47,000-M_r protein was a precursor of N-glycosylated, more slowly migrating forms of U_L10. Treatment of the immunoprecipitate with endoglycosidase H increased the electrophoretic mobility of the 50,000-M_r species to that of the 47,000-M_r species, indicating that the 50,000-M_r species contained high-mannose polysaccharide chains, whereas the proteins forming the 53,000- to 63,000-M_r bands contained mature chains inasmuch as they were resistant to digestion by the enzyme. (iv) The U_L10 protein of R7221 carrying a 20-amino-acid epitope formed only one band with an M_r of 53,000. This band was sensitive to endoglycosidase H, suggesting that the epitope inserted in the R7221 U_L10 protein may have interfered with glycosylation. (v) The U_L10 protein does not contain a cleavable signal sequence inasmuch as the first U_L10 methionine codon was reflected in the 50,000-M_r protein. (vi) The U_L10 protein is present in virions and plasma membranes of unfixed cells that were reacted with the polyclonal rabbit antibody. In accordance with the current nomenclature, the U_L10 protein is designated glycoprotein M.

The 150-kbp genome of herpes simplex virus 1 (HSV-1) consists of two distinct covalently linked components designated long (L) and short (S). Each of these components is composed of unique sequences (U_L and U_S) which are flanked by inverted repeats (13, 32, 38). The genome of HSV-1 contains at least 76 genes (1, 5, 21, 25–27). Included among this number are 10 genes encoding glycoproteins designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, and gL (2, 7, 14–16, 22, 28, 34). In addition, the HSV open reading frames designated U_L10, U_L20, and U_L43 were predicted to encode membrane proteins on the basis of their deduced amino acid sequences (25). The product of the U_L43 gene has yet to be identified, although the gene is dispensable for growth in cells in culture (24). The product of the U_L20 gene has been identified recently and shares properties with other proteins containing multiple transmembrane domains (4, 24, 31). The gene is essential for virion egress from Vero cells but is dispensable for viral growth and spread in a number of other cell lines tested.

The protein encoded by U_L10 was predicted to contain six to eight transmembrane domains (see Fig. 1A) (25). Whereas initiation from the first methionine codon would produce a hydrophilic N terminus inconsistent with previously published signal sequences, initiation from the second in-frame methionine would produce an N terminus that could conceivably be cleaved by a signal peptidase after entry into

membranes. Earlier, we reported the genetic engineering of a viral mutant lacking the U_L10 gene and showed that the deletion mutant yielded titers 10-fold lower than the wild-type parent in various cell lines (3). With the aid of an antiserum directed against a peptide predicted to be present in the C terminus of the U_L10 gene product, MacLean et al. reported that the gene product existed as a single 47,000-M_r species in infected cells (24). Although a homolog of U_L10 in the human cytomegalovirus (CMV) genome has been shown to be associated with virions (20), the location in the infected cell and the function of the protein encoded by the HSV U_L10 open reading frame were unknown. In this report, we demonstrate that the protein encoded by U_L10 is N glycosylated, exists in multiple forms because of the extensive processing of its polysaccharide chains, becomes associated with infected-cell membranes without cleavage of a signal sequence, and is present in the virion.

MATERIALS AND METHODS

Reagents and plasmids. Enzymes used for cloning were obtained from commercial manufacturers as previously described (3). Endoglycosidase H (endo H) was obtained from Boehringer Mannheim (Indianapolis, Ind.). Tunicamycin was obtained from Sigma Chemical Co. (St. Louis, Mo.). [^{3}H]glucosamine (33 Ci/mmol) was obtained from Amersham (Downers Grove, Ill.).

pRB446 contains the right portion of the HSV-1(F) BamHI M DNA fragment (0.19 to 0.217 map units) in the form of a

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*Bam*HI-*Bgl*II fragment cloned into the *Bam*HI site of pGEM3Z (Promega, Madison, Wis.). This fragment contains a unique *Mlu*I site that lies between the U_L18 and U_L19 open reading frames. pRB446 was cleaved with *Mlu*I, the ends were blunted with T4 DNA polymerase, and a fragment from pRB3367 (23) which contains the thymidine kinase (*tk*) gene driven by the HSV-1 α27 promoter (α27-*tk*) was inserted. This plasmid, designated pRB4032, therefore contained the chimeric α27-*tk* gene inserted upstream of U_L18 and downstream of the proposed polyadenylation signals of U_L19 (9, 10) and therefore did not disrupt any known open reading frame of HSV-1.

pRB4113 contains a *Bam*HI-*Bgl*II fragment of HSV-1 strain F [HSV-1(F)] DNA (0.150 to 0.165 map units) cloned into the *Bam*HI site of pGEM3Z. pRB4113 was cut with *Nru*I, and a double-stranded DNA oligomer (AAG GGC CAG AAG CCC AAC CTG CTG GAC CGC CTG CGC CAC CGC AAG AAC GGG TAC CGC CAC and its complement) encoding the epitope N-KGQKPNLLDRLRHRK NGYRNH-C, which is recognized by monoclonal antibody CH28-2 (6), was inserted to yield pRB4122. Sequence analyses verified that the CMV epitope was inserted in frame between amino acids 380 and 381 of the U_L10 open reading frame. The HSV sequences in pRB4122 were cloned into the pGEM3Zf(+) vector (Promega), cleaved with *Bam*HI, and the *Bam*HI A' fragment of HSV-1(F) (0.140 to 0.150 map units) was inserted. Single-stranded DNA derived from this plasmid, designated pRB4204, served as the template for mutagenesis of the potential initiation codons of the U_L10 open reading frame.

Cells and viruses. HSV-1(F) is the prototype HSV-1 strain used in our laboratory (12). Recombinant viruses described in this report were derived from HSV-1(F)Δ305, which contains a 500-bp deletion in the *tk* gene and was described previously (29). Titrations and preparations of viral stocks and viral DNA were done in Vero cells. Transfections were done in rabbit skin cells originally obtained from J. Mc-Claren. *tk*$^+$ and *tk*$^-$ viruses were selected in human 143TK$^-$ cells originally obtained from Carlo Croce. HEp-2 cells were used for analyses of proteins and purification of virions. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum. For selection of *tk*$^-$ viruses, this medium was further supplemented with 40 μg of bromodeoxyuridine per ml.

The R7030 virus contains a *tk* gene driven by the HSV-1 α27 promoter in an *Hpa*I site within the coding sequences of the gene encoding glycoprotein E (22). The R7202 virus was obtained by cotransfection of R7030 viral DNA with plasmid pRB3992 and subsequent selection for *tk*$^-$ virus. pRB3992 contains a β-galactosidase gene driven by the mouse metallothionein promoter within a deletion defined by a *Hinc*II site at the 5' noncoding sequences of gE and a *Bam*HI site near the C terminus of gE. Thus, the R7202 virus lacks the majority of the gE codons, including the start codon. Unlike wild-type infected-cell lysates, lysates from cells infected with this virus do not exhibit Fc receptor activity (30). The R7216 virus, which has been described previously (3), lacks the C-terminal 972 bp of the 1,119-bp U_L10 open reading frame.

The recombinant R7209 was constructed by cotransfection of pRB4032 with intact HSV-1(F)Δ305 DNA and selection of *tk*$^+$ virus. The *tk* gene which was inserted into the space between the U_L18 and U_L19 open reading frames of the R7209 viral DNA served as a convenient marker for the selection of viruses containing tagged copies of U_L10 bearing mutations in either the first (R7231) or second (R7232)

TABLE 1. Genotypic characteristics of viruses used in these studies

Virus	Plasmid used for construction	Genotype or characteristics
Reported previously		
HSV-1(F)		Wild type
HSV-1(F)Δ305	pRB305	Δβ*tk*,[a] ΔU_L24
R7030	pRB3633	α27*tk* in gE, Δβ*tk*, ΔU_L24
R7216	pRB4036	ΔU_L10
R7212	pRB4035	α27*tk* between U_L10 and U_L11, Δβ*tk*, ΔU_L24
Reported in this article		
R7202	pRB3992	pMt[b] *lacZ* in ΔgE
R7221	pRB4122	Tagged U_L10 open reading frame
R7209	pRB4032	α27*tk* between U_L18 and U_L19
R7231	pRB4313	Two copies of U_L10 (one tagged, with first methionine mutated), Δβ*tk*, ΔU_L24
R7232	pRB4314	Two copies of U_L10 (one tagged, with second in-frame methionine codon mutated), Δβ*tk*, ΔU_L24

[a] β*tk* is the designation given to the thymidine kinase gene controlled by its own promoter and located at its natural position.

[b] pMT, mouse metallothionein promoter.

methionine codon in addition to the original copy of the gene. The selection was done by cotransfection of plasmid DNA bearing mutated U_L10 genes inserted between U_L18 and U_L19 coding sequences with R7209 viral DNA and subsequent selection for *tk*$^-$ virus in 143TK$^-$ cells overlaid with medium containing bromodeoxyuridine. *tk*$^-$ viruses were plaque purified serially three times on Vero cells, and the expected genomic structure was confirmed by hybridization of electrophoretically separated restriction enzyme digests of viral DNA with appropriate probes (data not shown).

A summary of viruses pertinent to this report and the plasmids used for their construction appears in Table 1.

Purification and analysis of viral DNA. Viral DNA intended for transfections was prepared from NaI gradients as described previously (39). Otherwise, viral DNA was purified by phenol-chloroform extraction of cytoplasmic extracts of infected Vero cells (36). DNA probes were prepared by nick translation as described previously (3). Restriction fragments of viral DNA were separated and transferred to nitrocellulose as described previously (23, 33). DNA was hybridized with denatured nick-translated probes overnight at 65°C in a mixture of 0.6 M NaCl, 50 mM sodium citrate (pH 7.0), 0.1% sodium dodecyl sulfate (SDS), 100 μg of denatured herring sperm DNA (Boehringer Mannheim) per ml, 0.02% Ficoll (type 400-DL; Sigma), 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin (fraction V). The blots were washed four times in an excess volume of 60 mM NaCl-20 mM sodium citrate (pH 7.0)–0.1% SDS for 15 min at 40 to 50°C.

Mutagenesis of the methionine codons and cloning of the U_L10 open reading frame for insertion into the viral genome as a second copy. Single-stranded DNA was generated from

pRB4204 with the R408 helper phage by standard procedures (37). To mutate the first methionine of the U$_L$10 open reading frame, the oligomer 5' GGG CCG GGC GTC A̲A̲C̲ TTG GGG ACG ACG AGT 3' (altered bases underlined) was phosphorylated in vitro and was used to prime second-strand DNA synthesis with T4 polymerase as specified by the manufacturer of a kit designed for this purpose (Bio-Rad, Rockville Centre, N.Y.). This introduced a *Hinc*II site for screening purposes and changed the proposed first initiation codon of U$_L$10 from methionine to isoleucine. In addition, the second amino acid was changed from GGA (glycine) to TGA (stop). The plasmid containing this mutation was designated pRB8084. Similarly, the oligomer 5' CGC GCC CCG GTC A̲C̲C̲ CCT TTC GTG GGG GGC TG 3' introduced into U$_L$10 a *Bst*EII site, changed the 18th codon from GGC to GGG (both encode glycine), and substituted GTG, which encodes valine, for the methionine codon at position 19. The plasmid containing this mutation was designated pRB8085. Both pRB8084 and pRB8085 were cleaved at the *Mlu*I site 119 bp downstream of the proposed polyadenylation signal of U$_L$10 and an additional *Mlu*I site 1,286 bp upstream of the first methionine codon of U$_L$10. The resultant 2.8-kbp *Mlu*I fragments of pRB8084 and pRB8085 were cloned into the unique *Mlu*I site of pRB446 to generate pRB4313 and pRB4314, respectively. Thus, the U$_L$10 open reading frame was placed upstream of U$_L$18 coding sequences and downstream of the polyadenylation signal that terminates U$_L$19 transcription. The direction of transcription of the inserted U$_L$10 open reading frame was the same as that of the native U$_L$10 gene (i.e., opposite that of the U$_L$18 and U$_L$19 genes).

Production of rabbit polyclonal antiserum. A female New Zealand White rabbit was inoculated subcutaneously four times at 15-day intervals with approximately 250 μg of purified fusion protein in a suspension of Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (other injections). For immunofluorescence, the antiserum was diluted 1:200 in phosphate-buffered saline (PBS). For probing nitrocellulose blots containing electrophoretically separated lysates of infected cells or virions, the antiserum was diluted 1:500,000 in PBS supplemented with 5% skim milk (Carnation).

Immunoprecipitation and treatment with tunicamycin and endoglycosidase H (endo H). HEp-2 cells were grown in 25-cm^2 cell culture flasks and were infected with 5.0 PFU of the various viruses per cell. Four to five hours after infection, the medium was replaced with 50 μCi of [^{35}S]methionine in 1 ml of DMEM containing 1% newborn calf serum and only 1/10 the normal amount of methionine. Infected cells were labeled with 50 μCi of [^{3}H]glucosamine in a similar manner, except that the 1 ml of labeling medium contained the normal amount of methionine. At 16 h after infection, the infected-cell monolayers were washed with PBS and solubilized in 0.35 ml of PBSA* (1.0% Nonidet P-40, 1.0% deoxycholate, 10 μM TPCK [tolylsulfonyl phenylalanyl chloromethyl ketone], and 10 μM TLCK [α-tosyl-L-lysine chloromethyl ketone] in PBS). The lysates were sonicated briefly, and 40 μl of a 50% slurry of staphylococcal protein A-Sepharose (Sigma) in PBS was added. After 30 min on ice with occasional mixing, the mixtures were centrifuged at maximum speed in an Eppendorf microcentrifuge for 5 min, and the supernatant fluids were removed. Three microliters of either anti-gD monoclonal antibody HD-1 or rabbit serum obtained either before or after immunization with a U$_L$10–glutathione S-transferase (GST) fusion protein was added to the supernatants, and the mixture was

allowed to incubate for 1 h on ice. Approximately 20 μl of a 50% slurry of staphylococcal protein A-Sepharose in PBS was added and, with occasional vortexing, was incubated for 1 h on ice. The Sepharose pellets were washed four times with 1-ml volumes of PBSA* and then resuspended in disruption buffer which contained 5% 2-β-mercaptoethanol (βME) and 2% SDS in 50 mM Tris-HCl (pH 7.6). The samples were either heated at 56°C or boiled for 2 min and were separated on denaturing polyacrylamide gels. The gels were soaked in 20% (wt/vol) sodium salicylate (Baker), dried, and fluorographed at −80°C.

In some cases, the immunoprecipitate from a single 25-cm^2 flask was resuspended in 40 μl of a buffer containing 100 mM sodium citrate (pH 5.5) and 100 mM βME and was incubated in the presence or absence of 0.1 mU of endo H overnight at 37°C. The protein was then denatured, separated, and fluorographed as described above.

Polyacrylamide gel electrophoresis and immunoblotting. Cell lysates were separated in 7.5% denaturing acrylamide gels, transferred to nitrocellulose, treated with blocking solution consisting of 5% skim milk in PBS (blocking solution) (30), and exposed to rabbit polyclonal antibody diluted in blocking solution either overnight at 4°C or for 90 min at room temperature. The nitrocellulose sheets were then washed four times in an excess volume of blocking solution, incubated for 1 h at room temperature with a 1:3,000 dilution of an alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse serum (Bio-Rad), washed again with blocking solution for 15 min, and then washed four times for 2 min each time in PBS. The immunoblots were then rinsed in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM MgCl$_2$) and allowed to react with alkaline phosphatase substrate diluted according to the directions of the manufacturer (Bio-Rad). In the final step before photography, the immunoblots were thoroughly rinsed with water.

Virion purification. Virions were purified as previously described (35). Briefly, HEp-2 cells in three 690-cm^2 roller bottles were infected at 5.0 PFU of HSV-1(F) per cell and were incubated for 24 h at 33°C in DMEM supplemented with 1% newborn calf serum. The cells were lysed by three strokes of a Dounce homogenizer, and the lysate was clarified by low-speed centrifugation. The supernatant fluid was then subjected to centrifugation at 20,000 rpm in an SW28 rotor of a Beckman centrifuge for 90 min. The pellet was resuspended in 3 ml of 1.0 mM phosphate buffer (pH 7.0). Virions were then purified by centrifugation through a continuous dextran gradient in a single SW28 centrifuge tube. A diffuse band near the middle of the gradient was collected with a Pasteur pipette, diluted fivefold with PBS, and subjected to centrifugation in an SW28 rotor for 1 h at 20,000 rpm. The virions were resuspended in 300 μl of PBS, and 50-μl aliquots were stored at −80°C.

RESULTS

Synthesis of a GST-U$_L$10 fusion protein for immunization of rabbits. A plasmid designated pRB4508 contained the last 93 amino acids of the U$_L$10 open reading frame fused to the gene encoding GST. A diagram of the predicted sequence arrangement of the fusion protein encoded by this plasmid is shown in Fig. 1B. The junction of the sequences encoding GST and U$_L$10 was sequenced to ensure that the two coding domains were in frame (not shown).

The 48,000-M_r fusion protein was purified by affinity chromatography to glutathione cross-linked to agarose beads (Sigma) and was checked for purity by separation on dena-
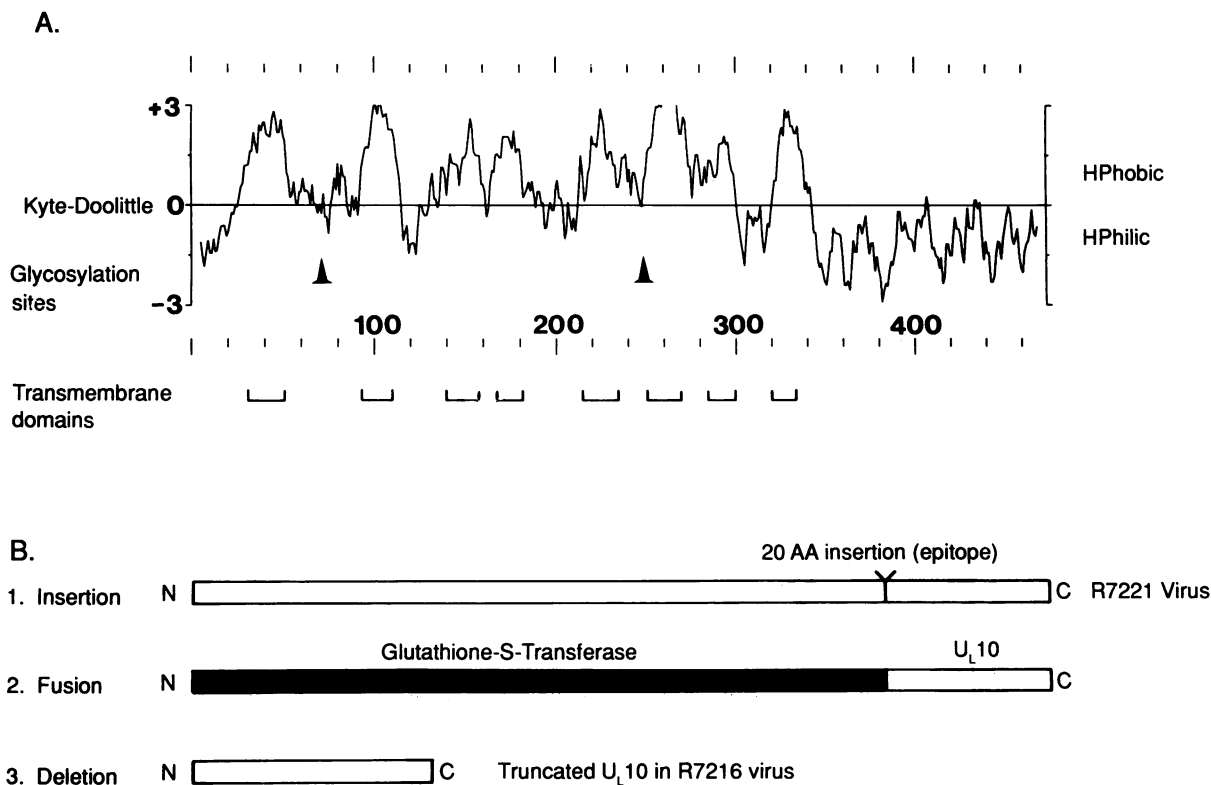
A.

B.

FIG. 1. (A) Kyte-Doolittle plot generated with the DNA sequence of the $U_L 10$ open reading frame by using a moving window of 9 amino acids. The sites of potential transmembrane domains (brackets) and N-glycosylation sites (filled triangles) are indicated. The amino acid number is indicated below the Kyte-Doolittle plot. (B) Schematic representations of the $U_L 10$ open reading frame. Line 1, position of the inserted CMV epitope in R7221. The open bar representing $U_L 10$ protein sequences is colinear with the Kyte-Doolittle plot shown above. Line 2, schematic representation of the 93-amino-acid carboxyl-terminal sequence of the $U_L 10$ protein (open bar) fused to GST (filled bar) for production of antibody. Line 3, $U_L 10$ amino-terminal sequence retained in deletion mutant R7216. AA, amino acid.

turing polyacrylamide gels followed by staining with Coomassie blue (Fig. 2). This affinity-purified preparation was used to immunize rabbits for the production of polyclonal antiserum as described in Materials and Methods.

Construction of a virus containing an epitope-tagged $U_L 10$. The recombinant virus R7212, which has been described elsewhere (3), contains a thymidine kinase marker between the $U_L 10$ and $U_L 11$ open reading frames (Figure 3, lines 1 and 2). We cotransfected intact R7212 viral DNA with pRB4122 plasmid DNA and selected for tk^- progeny. pRB4122 contains $U_L 10$ sequences in the C terminus of which a DNA oligomer that encodes a CMV epitope recognized by monoclonal antibody CH28-2 (6) was inserted (Fig. 3, line 3). The DNA encoding the epitope also contained a *Kpn*I site for screening purposes. Some of the tk^- progeny of the cotransfection contained an additional *Kpn*I cleavage site within their *Kpn*I E fragments, which is consistent with the insertion of the CMV epitope into the $U_L 10$ open reading frame (data not shown). One of these viruses was plaque purified three times and was designated R7221.

Purified viral DNAs obtained from cells infected with HSV-1(F), R7216 ($U_L 10^-$) (3), and R7221 (containing the inserted epitope) were each digested with *Kpn*I. The digests were electrophoretically separated on a 1.0% agarose gel, transferred to a nitrocellulose sheet, and hybridized with nick-translated pRB445, which contains HSV-1(F) DNA comprising the $U_L 10$ open reading frame and other sequences from the *Kpn*I E fragment (Fig. 3, line 6). The
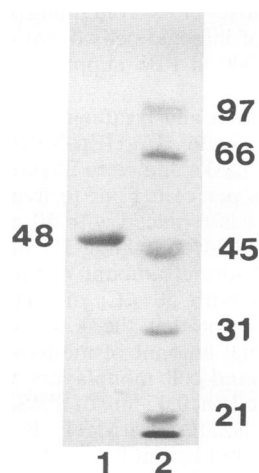

FIG. 2. Photograph of a Coomassie brilliant blue-stained, electrophoretically separated, affinity-purified $U_L 10$-GST fusion protein. The $U_L 10$-GST fusion protein from lysates of induced bacterial cultures was adsorbed to Sepharose beads cross-linked with glutathione and was eluted in 5 mM glutathione. The purified protein was separated on an SDS-polyacrylamide gel and stained with Coomassie brilliant blue. The apparent M_rs (in thousands) of the fusion protein and various molecular weight markers are indicated. Lane 1, affinity-purified $U_L 10$-GST fusion protein; lane 2, molecular weight standards (Bio-Rad).
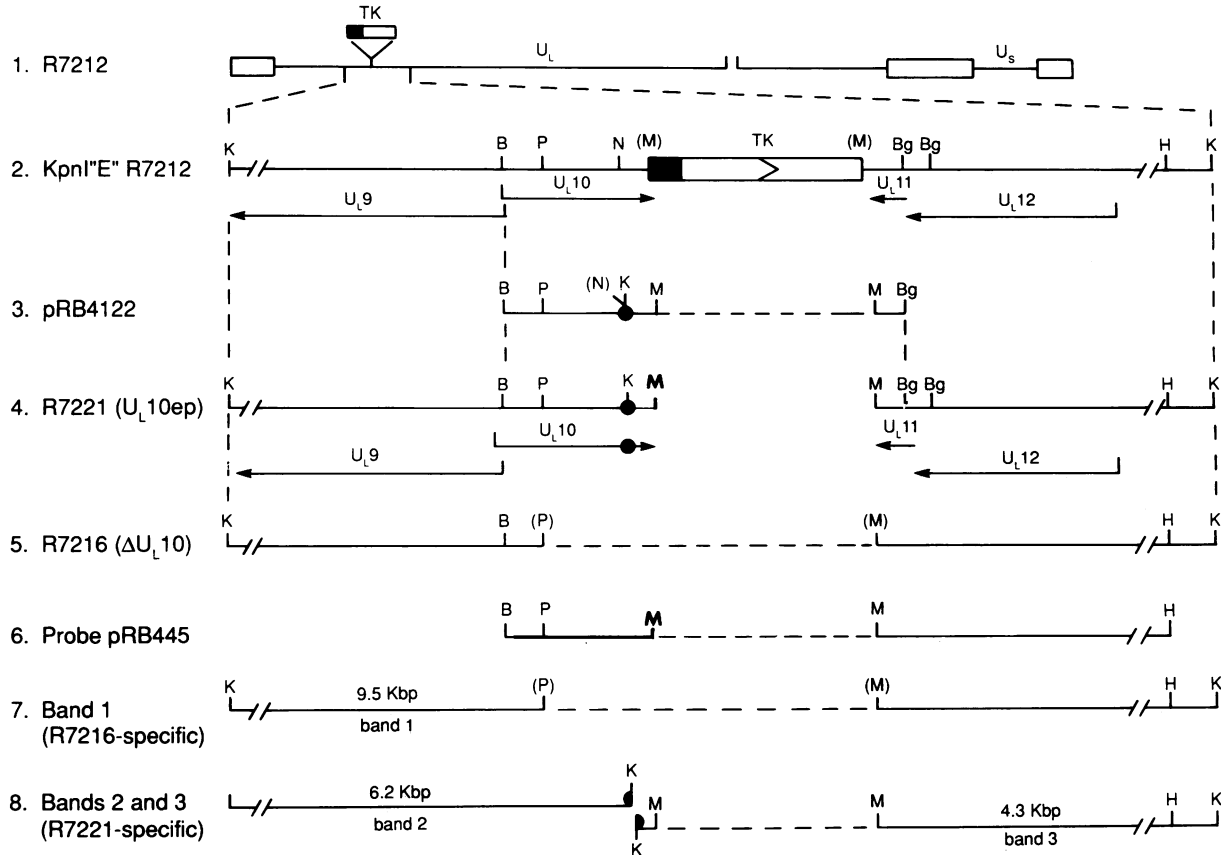
FIG. 3. Colinear schematic representations of the sequence arrangements of HSV-1 DNA and of the various plasmids used in these studies. Line 1, sequence arrangement of recombinant virus R7212 DNA. The genome of R7212 differs from that of the wild-type virus by virtue of a deletion within the *tk* gene (open space near the center of the U$_L$ segment) and the insertion of a *tk* gene (open rectangle above Y-shaped symbol) driven by an α27 promoter (filled rectangle) into the U$_L$ segment of the R7212 genome. Other open rectangles represent internal repeats flanking the unique sequences. Line 2, organization and direction of the inserted *tk* and U$_L$9 to U$_L$12 open reading frames within the KpnI E fragment of R7212 viral DNA. An HSV-1(F) *tk* gene (open rectangle) driven by the α27 promoter (filled rectangle) was inserted between the U$_L$10 and U$_L$11 open reading frames. Arrows delineate the lengths and directions of the indicated open reading frames. Line 3, sequence arrangement of HSV-1(F) DNA within plasmid pRB4122. DNA encoding a CMV epitope (filled circle) is present in the C terminus of the U$_L$10 open reading frame. Note that the chimeric *tk* gene is absent (dashed lines) from pRB4122 and that the DNA encoding the CMV epitope introduces a new KpnI site. Line 4, sequence arrangement of the U$_L$9 to U$_L$12 open reading frames located within the KpnI E fragment of R7221 viral DNA. The position of the inserted CMV epitope (ep) within the C terminus of the U$_L$10 open reading frame is indicated (filled circles). Note that the DNA encoding the epitope introduces a new KpnI site into the KpnI E fragment of R7221 viral DNA. Line 5, DNA within the KpnI E fragment of the U$_L$10 deletion virus R7216. Line 6, sequence arrangement of pRB445, which was nick translated and hybridized with viral DNA as shown in Fig. 4. Line 7, R7216 (ΔU$_L$10)-specific 9.5-kbp band 1 that hybridizes with probe pRB445 as shown in Fig. 4. As a result of the deletion of the majority of the U$_L$10 open reading frame, the KpnI E fragment of R7216 is approximately 1 kbp shorter than the wild-type KpnI E fragment. Line 8, sequence arrangements of R7221 (U$_L$10ep)-specific bands 2 and 3. The introduction of a KpnI site by virtue of the insertion of the epitopic tag within the U$_L$10 open reading frame divides the KpnI E fragment into 6.2- and 4.3-kbp subfragments (bands 2 and 3, respectively). B, BamHI; Bg, BglII; H, HindIII; K, KpnI; M, MluI; N, NruI; P, PstI. Restriction sites in parentheses were destroyed during cloning. DNA normally included in regions indicated by dashed lines was deleted.

results (Fig. 4) show that the 10.5-kbp KpnI E fragment of HSV-1(F) DNA was reduced in size by 1 kbp in the U$_L$10 deletion mutant R7216 (Fig. 4, lane 2, band 1), as predicted by the design of the deletion mutant presented in Fig. 3, line 7. The introduction of a KpnI site due to the insertion of the epitope in the U$_L$10 open reading frame caused the KpnI E fragment of R7221 to form two bands of 6.2 and 4.3 kbp (Fig. 4, lane 3, and Fig. 3, line 8). The results show, therefore, that the DNA oligomer encoding the epitope was inserted in the U$_L$10 open reading frame at the expected site.

Identification of the U$_L$10 gene product in lysates of infected cells. The results of four series of experiments are summarized in Fig. 5 to 8. In the first series, replicate cultures of

HEp-2 cells were each infected (5.0 PFU per cell) with HSV-1(F), the U$_L$10 deletion virus R7216, the R7221 virus carrying the 20-amino-acid CMV epitope in the U$_L$10 open reading frame, or the R7202 virus (gE⁻) and labeled with [^{35}S]methionine from 5 to 20 h postinfection. At 20 h postinfection, the cells were harvested and lysed, and the lysates were reacted with preimmune and immune rabbit anti-U$_L$10 serum induced by immunization with the U$_L$10 fusion protein described above or with anti-gD monoclonal antibody HD-1. The precipitates were then solubilized in disruption buffer and heated at 56°C before electrophoresis on a denaturing polyacrylamide gel. The preimmune antiserum precipitated bands in HSV-1(F) and R7216 lysates
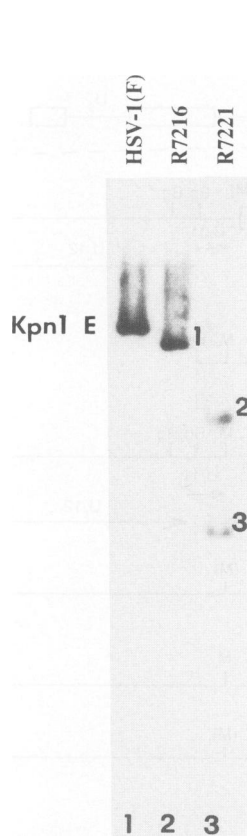
FIG. 4. Autoradiographic images of electrophoretically separated $KpnI$ restriction digests of the DNA of U_L10-tagged and U_L10 deletion viruses. Viral DNA was purified from HSV-1(F)-, U_L10 deletion virus R7216-, or U_L10-tagged virus R7221-infected cells, cleaved with $KpnI$, separated on a 1.0% agarose gel, transferred to nitrocellulose, and probed with radiolabeled pRB445. This plasmid contains sequences homologous to the $KpnI$ E fragment of HSV-1(F) DNA. The deletion in R7216 DNA reduces the size of $KpnI$ E by approximately 1.0 kbp (band 1). The insertion of the CMV tag in U_L10 introduces a new $KpnI$ site that cleaves $KpnI$ E into bands 2 and 3.

corresponding to the Fc receptor of HSV, which is composed of gE and gI (18). These bands were largely absent in immunoprecipitates of lysates obtained from R7202-infected cells, which lack the gE gene (Fig. 5, lanes 5 to 11). In contrast to the preimmune serum, the rabbit immune serum precipitated proteins which formed a prominent band with an apparent M_r of 50,000 and a broad, largely diffuse band with an apparent M_r ranging from 53,000 to 63,000 (Fig. 5, lane 9). The larger band comigrated with the glycosylated form of gD (compare lanes 11 [anti-gD] and 13 [anti-U_L10-GST] of Fig. 5). Neither the 50,000- nor the 53,000- to 63,000-M_r proteins were precipitated by the immune serum from lysates of cells infected with the U_L10 deletion mutant R7216 (Fig. 5, lanes 1 and 2). Because of the insertion of the epitope within the U_L10 open reading frame, the apparent molecular weight of the prominent band formed by the R7221 U_L10 protein increased from approximately 50,000 to approximately 53,000 in R7221 (compare lanes 4 [R7221] and 9 [R7202] of Fig. 5). These results indicate that the 50,000- and 53,000-M_r proteins were products of the U_L10 open reading frames of HSV-1(F) and R7221, respectively, inasmuch as their size was dependent on the size of the coding domain of the gene.

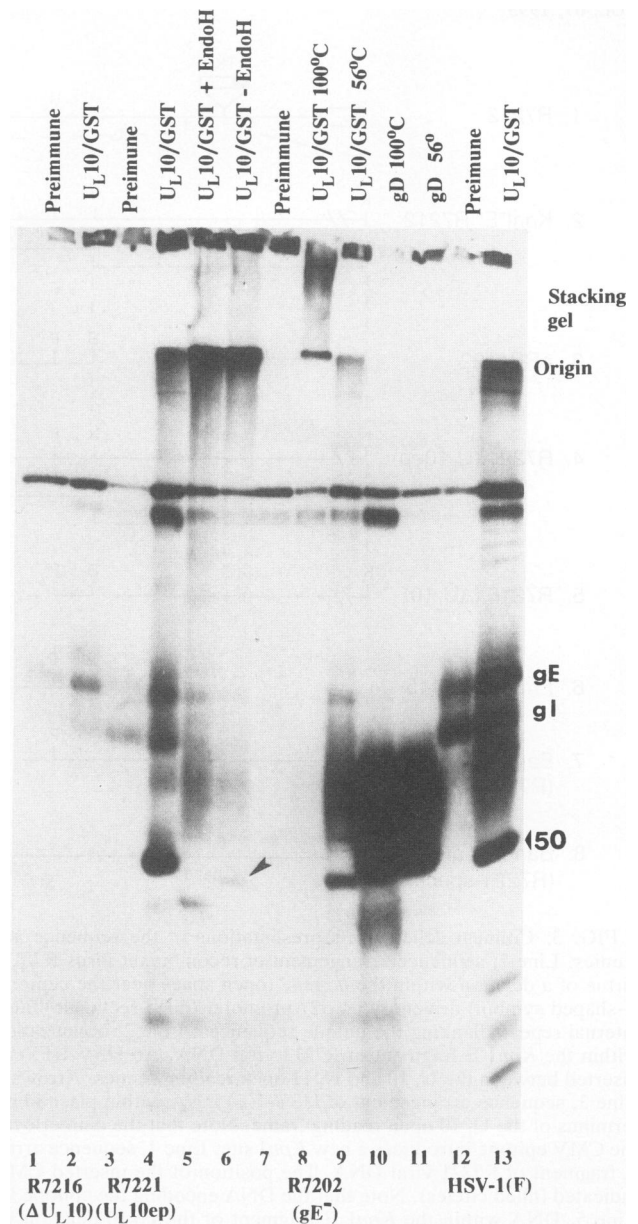The purpose of the second series of experiments was to

FIG. 5. Fluorographic image of electrophoretically separated proteins in denaturing gels. Infected cells were labeled 4 to 16 h after infection with [^{35}S]methionine. Cellular lysates were then immunoprecipitated with the antibodies indicated above the lanes. Immunoprecipitates were solubilized in a buffer containing 2% SDS and 5 mM βME before being separated on a 7.5% denaturing polyacrylamide gel and fluorographed at −80°C. The positions of the stacking gel and origin of the resolving gel are indicated on the right. The viruses that were used to produce the lysate are indicated below each lane as follows: lanes 1 and 2, R7216; lanes 3 and 4, R7221; lanes 5 to 11, R7202; lanes 12 and 13, HSV-1(F). The antibodies used in the immunoprecipitates are defined as follows: Preimmune, rabbit preimmune serum obtained before immunization with the U_L10-GST fusion protein; U_L10/GST, rabbit antiserum obtained after four immunizations with the U_L10-GST fusion protein; gD, anti-gD monoclonal antibody HD-1. Some immunoprecipitates were heated (56°C) or boiled (100°C) in sample buffer before being separated on the gel. Lanes marked EndoH contain immunoprecipitates that were treated in low-pH citrate buffer overnight at 37°C in the presence (+ [lane 5]) or absence (− [lane 6]) of 0.1 mU of endo H. The arrowhead indicates the position of the undigested U_L10-specific species that is indistinguishable in size from that seen in lysates of HSV-1(F)-infected cells. The positions corresponding to the migration of gE, gI, and a 50,000-M_r protein representing one of the U_L10 gene products are also indicated.
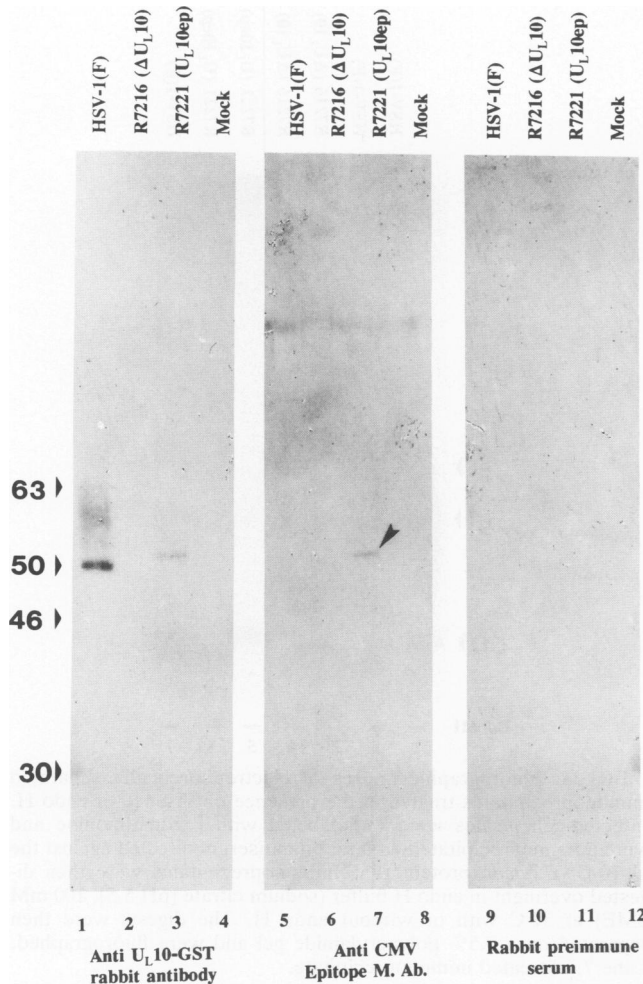
FIG. 6. Photograph of immunoblots of electrophoretically separated lysates probed with an anti-CMV mouse monoclonal antibody (M. Ab.), preimmune rabbit serum, or rabbit anti-U$_L$10-GST antiserum. Lysates obtained from infected or mock-infected cells were denatured at 56°C in a buffer containing 2% SDS and 5 mM βME and were electrophoretically separated on a 7.5% denaturing acrylamide gel. The separated proteins were then transferred electrically to a nitrocellulose sheet, which was divided and then probed with various antibodies. The presence of bound antibody was revealed by reaction with the appropriate alkaline phosphatase-conjugated anti-immunoglobulin followed by fixation of substrate. Left panel, immunoblot probed with U$_L$10-GST antiserum; middle panel, immunoblot probed with mouse monoclonal antibody directed against the CMV epitope inserted into the U$_L$10 open reading frame of virus R7221 (the tagged U$_L$10 gene product recognized by the mouse antibody is indicated with an arrowhead); right panel, immunoblot probed with preimmune rabbit serum.

address the identity of the slowly migrating, more diffuse band of proteins immunoprecipitated by the U$_L$10 rabbit immune serum. In this series of experiments, lysates of mock-infected and HSV-1(F)-, R7216-, or R7221-infected cells separated by electrophoresis on a denaturing polyacrylamide gel and electrically transferred to nitrocellulose were probed with the preimmune rabbit serum, the U$_L$10-GST-specific rabbit antiserum, or the monoclonal antibody CH28-2, which reacts with the epitope inserted into the U$_L$10 gene of R7221. The U$_L$10 antiserum reacted with both

the 50,000-M_r band and the broad band with the apparent M_r range of 53,000 to 63,000 (Fig. 6, lane 1). These bands were absent from the electrophoretically separated lysates of mock-infected cells and those of cells infected with the deletion mutant R7216. The insertion of the epitope within the U$_L$10 open reading frame caused the 50,000-M_r protein to migrate at a position corresponding to an M_r of 53,000 (Fig. 6, lane 3). The latter band reacted with both the rabbit immune serum (lane 3) and the monoclonal antibody to the CMV epitope (lane 7). In this instance, however, the slowly migrating broad band in lysates of HSV-1(F) largely disappeared.

The observations that the 53,000- to 63,000-M_r protein band reacted with the anti-U$_L$10 rabbit immune serum and that it was present in lysates of HSV-1(F) but not in lysates of the U$_L$10$^-$ virus indicates that the band contains a product of the U$_L$10 gene. Additional evidence concerning the nature of the product precipitated by the antibody was based on the observation that the U$_L$10 gene product, like many hydrophobic proteins (4, 31), aggregated when the samples were boiled in the presence of SDS and βME. An R7202-infected-cell lysate was immunoprecipitated with the rabbit antiserum and either heated at 56°C or boiled in the disruption buffer; samples boiled in disruption buffer failed to form the 50,000- and 53,000- to 63,000-M_r bands (Fig. 5, lane 8). As expected, the radiolabeled material was localized in the stacking gel. In contrast, the sample preparation described above had virtually no effect on the appearance or migration of gD (Fig. 5, lanes 10 and 11). The appearance and migration of gD and the 50,000- and 53,000-M_r bands were identical when lysates were solubilized in disruption buffer and heated at 25, 37, or 56°C in disruption buffer (data not shown). We conclude from this experiment that the 50,000- and 53,000- to 63,000-M_r proteins precipitated by the polyclonal antibody exhibited the highly hydrophobic properties predicted of the product of the U$_L$10 gene (25).

The purpose of the third series of experiments was to determine the nature of the modification of the U$_L$10 proteins which leads to the formation of multiple bands in denaturing polyacrylamide gels. As a general rule, HSV glycoproteins form several bands in denaturing polyacrylamide gels, depending on the extent of glycosylation (7, 34). To determine whether the proteins immunoprecipitated with the U$_L$10 antiserum were glycosylated, 50 μCi of [^{3}H]glucosamine per ml was added to the cell medium 4 to 14 h after infection with 5.0 PFU of HSV-1(F) per cell. As shown in Fig. 7, lane 3, [^{3}H]glucosamine was incorporated into both the slowly migrating and 50,000-M_r forms of U$_L$10 protein, indicating that these U$_L$10 gene products were modified by N glycosylation.

To further confirm that the protein encoded by U$_L$10 was glycosylated, cells were infected in the presence of 2 μg of tunicamycin per ml and labeled with either [^{3}H]glucosamine or [^{35}S]methionine 6 to 14 h after infection. Lysates made from the infected cells were then either (i) immunoprecipitated with the U$_L$10-GST antiserum and subsequently electrophoretically separated on a denaturing polyacrylamide gel and fluorographed or (ii) separated on a denaturing gel, transferred to nitrocellulose, and probed with the rabbit polyclonal antibody. In both cases, infection in the presence of tunicamycin caused a reduction of the molecular weight of the 50,000-M_r U$_L$10 gene product (Fig. 7, band 1) to approximately 47,000 (band 2). In addition, the more slowly migrating 53,000- to 63,000-M_r species virtually disappeared after treatment with tunicamycin (Fig. 7, lanes 2 and 6). As expected, treatment with tunicamycin reduced incorporation
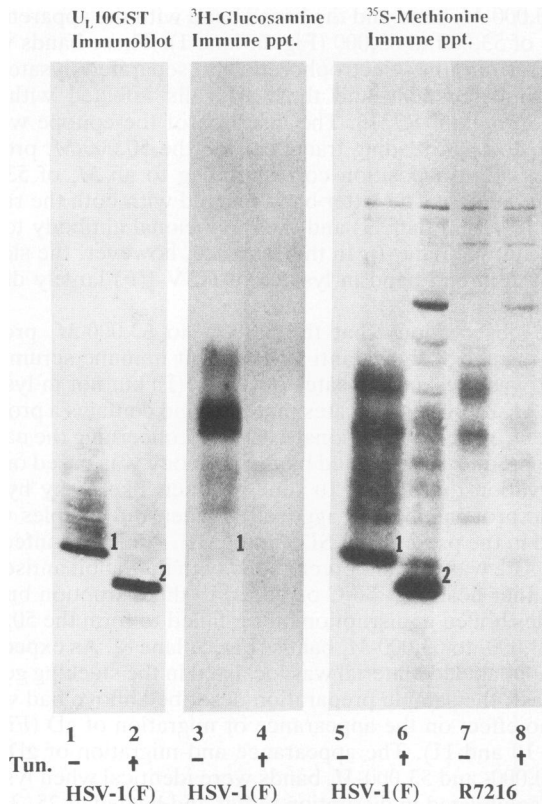
FIG. 7. Photographs of an immunoblot (lanes 1 and 2) and fluorographs of immunoprecipitates (lanes 3 to 8) reacted with U_L10-GST antiserum. Cells were infected and incubated in the presence or absence of 2 μg of tunicamycin (Tun.) per ml and were radiolabeled with [^{3}H]glucosamine or [^{35}S]methionine. Cell lysates either were separated on a denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with the U_L10-GST antiserum or were immunoprecipitated with the antiserum, separated on a denaturing polyacrylamide gel, and fluorographed. ppt., precipitate.
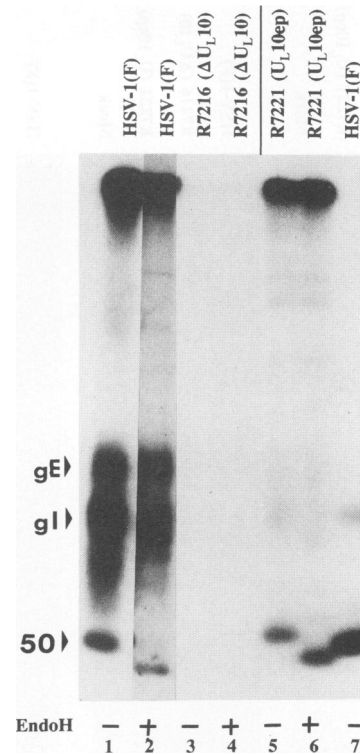

FIG. 8. Fluorographic images of electrophoretically separated immunoprecipitates treated in the presence or absence of endo H. Infected-cell lysates were radiolabeled with [^{35}S]methionine and were immunoprecipitated with rabbit antiserum directed against the U_L10-GST fusion protein. The immunoprecipitates were then digested overnight in endo H buffer (sodium citrate [pH 5.5], 100 mM βME) at 37°C with or without endo H. The digests were then separated on a 7.5% polyacrylamide gel and were fluorographed. Lane 7, untreated immunoprecipitate.

of glucosamine into the U_L10 gene products (Fig. 7, lane 4). These results indicate that the 50,000-M_r protein is N glycosylated and suggest that since it is virtually absent in tunicamycin-treated lysates, the more slowly migrating species is derived from further processing of the N-linked polysaccharide chains of the 50,000-M_r protein.

In the next series of experiments, immunoprecipitated proteins obtained by mixing the rabbit immune serum with lysates of HSV-1(F)-, R7221-, R7216-, and R7202-infected HEp-2 cells were reacted with endo H in a citrate buffer (pH 5.5) containing 100 mM βME. Endo H cleaves simple high-mannose N-linked glycoproteins. Resistance to endo H cleavage is attained by most glycoproteins, as they are processed in the medial Golgi compartment. In this series of experiments, the buffer caused some of the radiolabeled material to aggregate at the origin of the resolving gel (Fig. 5, lanes 5 and 6, and Fig. 8). Nevertheless, in the absence of endo H, HSV-1(F)- and R7202-infected-cell lysates reacted with the buffer alone formed the 50,000-M_r protein band, whereas in the presence of endo H, the 50,000-M_r protein band was replaced with a band with an apparent M_r of 47,000. Endo H had no effect on the 53,000- to 63,000-apparent-molecular-weight band. The treatment with endo H also reduced the size of the 53,000-M_r protein immunopre-

cipitated from R7221-infected-cell lysates to approximately 49,000 (Fig. 8, lane 6). We conclude from this series of experiments that the 50,000- and 53,000- to 63,000-M_r bands possess the properties of N-glycosylated proteins with high-mannose and more fully glycosylated polysaccharide chains, respectively, inasmuch as glycoprotein polysaccharide chains processed in a post-cis-Golgi compartment are not digested by this enzyme. The observation that the slowly migrating, diffuse band was absent from lysates of cells infected with a virus containing a U_L10 gene tagged with the CMV epitope suggests that this epitope disrupted the processing of the polysaccharide chains beyond the high-mannose stage.

Translation of U_L10 is initiated at the first methionine codon. The observation that the insertion of an epitope into the U_L10 open reading frame increased the size of the 50,000-M_r U_L10 gene product by 3,000, a size consistent with the predicted molecular weight of the epitope (2,958), suggested that both the tagged and untagged molecules were initiated at the same methionine codon. As noted by McGeoch et al. (25), initiation from the second in-frame methionine, which is 17 amino acids downstream from the first, would allow the use of a cleavable signal peptide for entry into cellular membranes. To determine which methionine was used for initiation of U_L10 translation, two viruses containing mutations in these methionine codons were con-
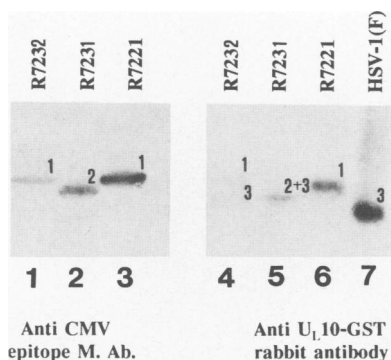
FIG. 9. Photograph of immunoblots of electrophoretically separated lysates of HSV-1(F)-, R7221-, R7231-, and R7232-infected cells probed with mouse anti-CMV monoclonal antibody (M. Ab.) or anti-U_L10-GST antiserum. R7221 contains a single tagged copy of the U_L10 gene. R7231 contains, in addition to a wild-type gene, a tagged gene with a mutation of the first methionine codon, while R7232 contains a wild-type gene and a tagged gene with a mutation of the second in-frame methionine codon. Infected-cell lysates were separated in various lanes of a denaturing polyacrylamide gel, electrically transferred to nitrocellulose, and probed with the indicated antibodies. Band 1 represents the tagged U_L10 gene product present in R7221 and R7232, band 2 represents the truncated tagged U_L10 gene product in R7231, and band 3 represents the wild-type gene product.

structed. In R7231, the first methionine codon of the tagged U_L10 open reading frame was replaced with isoleucine, whereas in R7232, the second in-frame methionine codon was replaced with valine. Because the first methionine codon of U_L10 overlaps U_L9, which encodes a product that is essential for replication of HSV-1 in tissue culture, we decided to construct recombinant viruses containing a second U_L10 gene at a site that did not overlap U_L9. In addition, we had at our disposal a recombinant virus, R7209, which contained a chimeric $\alpha27$-*tk* marker between the U_L18 and U_L19 open reading frames. We therefore cloned each of the mutated U_L10 genes into a plasmid containing HSV-1 DNA encoding U_L18 and U_L19 flanking sequences such that no known functional open reading frame was interrupted. The procedures by which the $\alpha27$-*tk* gene in R7209 was replaced with the mutant U_L10 gene are detailed in Materials and Methods. The viruses obtained by this procedure contained two U_L10 genes, i.e., a tagged U_L10 gene containing the mutated 1st or 19th codon and the wild-type, untagged gene.

Lysates of HSV-1(F)-, R7221-, R7231-, or R7232-infected cells were electrophoretically separated on polyacrylamide gels and were reacted with the rabbit polyclonal antiserum or with monoclonal antibody CH28-2, which recognizes the inserted epitope. The results (Fig. 9) were as follows.

(i) In lanes containing the R7231 lysate, monoclonal antibody CH28-2 reacted with a band (Fig. 9, band 2) with an electrophoretic mobility corresponding to a protein reduced in molecular weight by approximately 2,000 relative to the corresponding band in the R7221 lysate (Fig. 9, band 1). Thus, ablation of the first methionine codon truncated the U_L10 protein. The size of the truncation is consistent with the reduction in molecular weight of 1,939 that would be predicted were initiation to occur at the second in-frame methionine. The results therefore indicate that the ablated methionine codon is normally used to initiate translation and that the sequences at the amino terminus of the translated protein were retained in the partially glycosylated 50,000-M_r

protein. The insertion of the tag and truncation by virtue of the initiation codon mutation in R7231 caused the wild-type and mutant bands to migrate as a wide single band in lysates probed with the polyclonal rabbit antiserum (Fig. 9, lane 5).

(ii) Replacement of the second methionine (mutant R7232) had no effect on the electrophoretic mobility of the protein made by the U_L10 open reading frame. In this instance, the rabbit immune serum reacted with two bands (Fig. 9, bands 1 and 3), consistent with the conclusion that the wild-type and tagged U_L10 open reading frames specified 50,000- and 53,000-M_r proteins, respectively.

We conclude from these experiments that U_L10 does not specify an amino-terminal cleavable signal sequence and that the translation initiation site is at the first predicted methionine codon.

U_L10 protein is a virion component. Purified virions prepared as described in Materials and Methods were either boiled or heated at 56°C in disruption buffer and subjected to electrophoresis in a denaturing polyacrylamide gel. The gel was then either stained with Coomassie blue or electrically transferred to nitrocellulose and reacted with the U_L10-specific rabbit antiserum. The results (Fig. 10) were as follows.

(i) In lanes containing unboiled, electrophoretically separated virion polypeptides, the U_L10 protein migrated as a very broad band ranging in apparent molecular weight from 50,000 to 62,000. The 50,000-M_r protein was less prominent (relative to the 53,000- to 63,000-M_r protein) in immunoblots of virion preparations than in immunoblots of total infected-cell lysates.

(ii) In the boiled virion preparation, the anti-U_L10 rabbit polyclonal serum reacted with a band migrating slightly more slowly than VP1. This band, which is visible near the origin of the Coomassie blue-stained gel, was absent from the electrophoretic profile of unboiled virion polypeptides.

We conclude from these experiments that the U_L10 protein is a virion component and that, like its total infected-cell counterpart shown in Fig. 5, it aggregated when boiled.

U_L10 protein is associated with the plasma membrane of the infected cell. Vero cells were infected at a low multiplicity (approximately 200 PFU/25-cm² culture flask) with the gE⁻ recombinant R7202. After 48 h of incubation at 37°C, the unfixed monolayer culture was reacted with the rabbit immune serum, counterstained with biotinylated goat anti-rabbit immunoglobulin G and avidin-conjugated horseradish peroxidase, rinsed, and reacted with 4-chloro-1-naphthol substrate by the black-plaque technique previously described (19). The rabbit polyclonal antibody reacted with the U_L10 molecule at the surface of the HSV-1(F)-infected cells, as evidenced by the precipitate formed on the surface of the unfixed infected cells (Fig. 11). No such precipitate formed on plaques stained with the preimmune serum. These results are consistent with surface immunofluorescence detected in unfixed infected cell cultures stained with the anti-U_L10-GST antibody (data not shown) and indicate that at least a portion of the carboxyl-terminal 93 amino acids to which the rabbit polyclonal antibody was directed was exposed on the surface of the infected cells.

DISCUSSION

The salient features of the results presented in this report are as follows.

(i) The lowest apparent molecular weight of a protein whose synthesis is directed by the U_L10 open reading frames is 47,000, the size exhibited by the protein after treatment
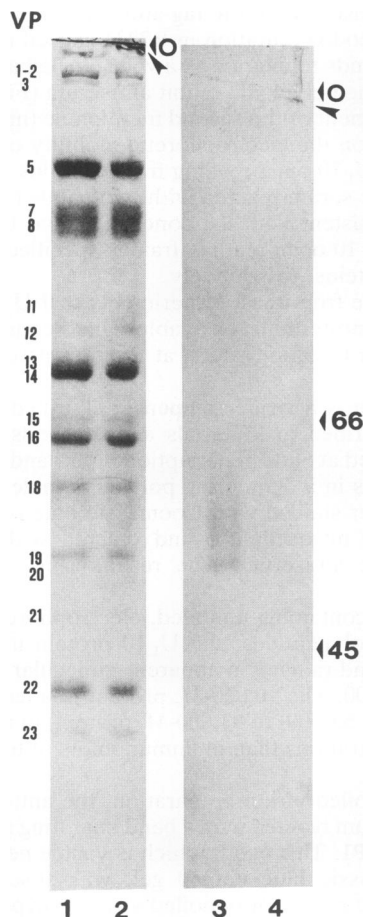
FIG. 10. Photographs of immunoblotted or Coomassie blue-stained electrophoretically separated virion polypeptides. Virions were purified from cells infected with HSV-1(F). Virion polypeptides (VP) were denatured in a buffer containing SDS and βME and were separated on a 7.5% denaturing polyacrylamide gel. The gel was divided, and the proteins were either stained with Coomassie brilliant blue (lanes 1 and 2) or transferred to nitrocellulose and probed with rabbit polyclonal antiserum directed against the U_L10-GST fusion protein (lanes 3 and 4). Lanes 1 and 3, virion proteins were denatured in buffer containing SDS and βME at 56°C; lanes 2 and 4, proteins were boiled in buffer containing SDS and βME. Lanes 3 and 4 contain 5% of the protein of lanes 1 and 2. The position of the origin (O) is indicated, as is the position of an aggregate (arrowhead), induced by boiling, that reacts with the rabbit antiserum. The positions corresponding to various virion polypeptides are indicated on the left. Molecular weight markers (in thousands) are indicated on the right.

with endo H and tunicamycin. The untreated proteins formed a sharp band consistent with a protein with an M_r of 50,000 and a more diffuse, more slowly migrating band. The 50,000-M_r protein most probably contains high-mannose polysaccharide chains inasmuch as the mobility of that protein was accelerated after endo H digestion. The more slowly migrating form was not affected by endo H, suggesting that it contained mature or more highly processed polysaccharide chains. These results are consistent with the hypothesis that the product of the U_L10 gene is N glycosylated and do not rule out the possibility that the protein is modified by other means.
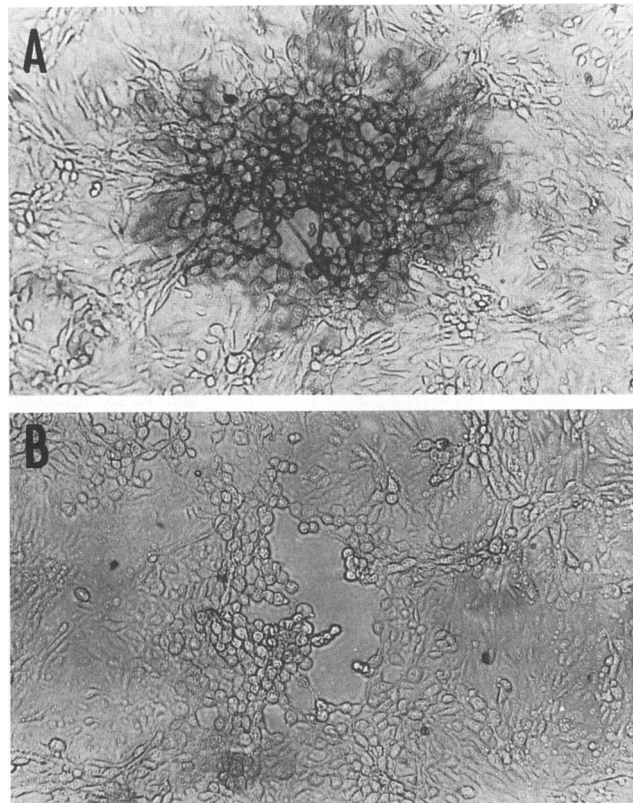
The DNA sequence of the U_L10 open reading frame

FIG. 11. Photomicrograph of unfixed gE⁻ R7202-infected cells probed with rabbit preimmune or rabbit anti-U_L10-GST serum. Forty-eight hours after infection, unfixed cells were probed with rabbit antisera. The bound antibody was detected with a biotinylated goat anti-rabbit antibody, followed by biotinylated horseradish peroxidase bound to avidin, followed by 4-chloro-1-naphthol substrate. (A) Viral plaque stained in the presence of a 1:100 dilution of rabbit preimmune serum; (B) viral plaque stained with a 1:500 dilution of rabbit antiserum directed against the U_L10-GST fusion protein.

predicts two potential N-glycosylation sites, one at amino acid 71 at a position within the first hydrophilic region following a hydrophobic domain and one at amino acid 247. Only the first glycosylation site is conserved within the U_L10 homolog of varicella-zoster virus and human CMV (8, 11).

In accordance with the current nomenclature, the U_L10-encoded glycoprotein is designated gM. As would be expected of N-glycosylated proteins, gM is present on the surface of infected cells and is also a virion constituent.

(ii) gM from infected cells differs from all other cell-associated HSV glycoproteins studied to date in that it is highly hydrophobic and aggregates when boiled in buffers containing SDS. In this respect, it resembles the multispanning membrane protein encoded by U_L20 more closely than it resembles the known glycoproteins (4).

Our results are consistent with and support the hypothesis that translation of U_L10 mRNA is initiated at the first predicted methionine codon and lead us to conclude that cleavage of an N-terminal signal sequence does not occur. The absence of a cleaved signal sequence predicts that the N terminus of gM expressed at the cell surface remains intracellular and the next hydrophilic domain, which contains the N-glycosylation site at 71 amino acids downstream, is extra-

cellular. Additional studies will be necessary to determine the topology of the remaining hydrophilic domains of gM and the sites of gM glycosylation. The topology of the U$_L$10 molecule is an important issue inasmuch as the structures of type III membrane proteins (proteins that span the membrane more than once) often give reliable clues regarding their functions (17). Studies of the conformation of the U$_L$10 protein in cells in culture are in progress.

It is of interest to note that gM carrying the 20-amino-acid hydrophilic CMV epitope does not form an endo H-resistant band, suggesting that its movement though the Golgi compartment or processing of the polysaccharide chains is restricted by the insert. Attempts to detect tagged gM on the surface of unfixed cells by using the CMV antibody or to immunoprecipitate tagged gM from lysates of infected cells with the CMV antibody were unsuccessful (not shown).

We also note that in contrast to our results, MacLean et al. reported that their antibody to U$_L$10 amino acids 458 to 467 reacted only with a 47,000-M_r protein (24). Hypotheses that could explain this result are (a) that boiling of the immunoprecipitate in buffers containing SDS diminished the amount of slowly migrating material that entered the gel and (b) that the antigenic site may be altered by fully processed polysaccharide chains.

(iii) We have previously reported that the U$_L$10 open reading frame is dispensable for replication in cells in culture, although the yield of the deletion mutant from infected cells is diminished relative to that of wild-type virus-infected cells. At this time, no clear phenotype attributable to the U$_L$10 gene product is available.

ACKNOWLEDGMENTS

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