

## Localization and Putative Function of the U<sub>L</sub>20 Membrane Protein in Cells Infected with Herpes Simplex Virus 1

PATRICIA L. WARD,<sup>1</sup> GABRIELLA CAMPADELLI-FIUME,<sup>2</sup> ELISA AVITABILE,<sup>2</sup>  
AND BERNARD ROIZMAN<sup>1\*</sup>

*The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637,<sup>1</sup>  
and Section of Microbiology and Virology, Department of Experimental Pathology,  
University of Bologna, Bologna, Italy<sup>2</sup>*

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**The U<sub>L</sub>20 protein of herpes simplex virus 1, an intrinsic membrane protein, is required in infected Vero cells in which the Golgi apparatus is fragmented for the transport of virions from the space between the inner and outer nuclear membranes and for the transport of fully processed cell membrane-associated glycoproteins from the *trans*-Golgi to the plasma membrane. It is not required in the human 143TK<sup>-</sup> cell line, in which the Golgi apparatus remains intact. We report the following. (i) The U<sub>L</sub>20 protein was detected in infected cells beginning at 6 h postinfection and was regulated as a  $\gamma_1$  gene. (ii) Pulse-chase experiments revealed no detectable alteration in the mobility of the U<sub>L</sub>20 protein in polyacrylamide gels. (iii) In both infected Vero and infected 143TK<sup>-</sup> cells, the U<sub>L</sub>20 protein was detected by immunofluorescence in association with nuclear membranes and in the cytoplasm. Some of the cytoplasmic fluorescence colocalized with  $\beta$ -COP, a protein associated with Golgi-derived transport vesicles. U<sub>L</sub>20 protein was present in virions purified from the extracellular space but could not be detected in the plasma membrane. These results are consistent with the hypothesis that U<sub>L</sub>20 is a component of virion envelopes and membranes of virion transport vesicles and is selectively retained from the latter in a Golgi compartment.**

The 152-kbp herpes simplex virus 1 (HSV-1) genome encodes at least 15 membrane proteins. These include the glycoproteins gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM, the gene products specified by the U<sub>L</sub>20, U<sub>L</sub>24, and U<sub>L</sub>34 open reading frames, and the predicted gene product of open reading frame U<sub>L</sub>43 (1, 5, 6, 7, 14, 15, 17, 19, 20, 30, 33, 37, 45). Of these proteins, more than half, i.e., gC, gE, gG, gI, gJ, gM, U<sub>L</sub>20, U<sub>L</sub>24, and U<sub>L</sub>43, are dispensable for viral replication in at least some cells in culture (1, 4, 6, 16, 21, 26-29, 50). The function of these dispensable membrane proteins is of considerable interest inasmuch as their existence could suggest that they function only in cells not cultured in vitro or that cultured cells express functions similar to those of the dispensable viral proteins. Recent studies have shown that at least some of the dispensable viral glycoproteins may be required for apical but not basolateral entry into polarized cells (43). Little is known about the functions of the U<sub>L</sub>24 and U<sub>L</sub>43 proteins. The function of the U<sub>L</sub>20 protein, however, has been studied in some detail and is of considerable interest. Thus, viral mutants from which the U<sub>L</sub>20 gene has been deleted multiply and form small, at times syncytial plaques in some cell lines (e.g., 143TK<sup>-</sup>); they multiply but do not form plaques in other cell lines (e.g., Vero) (6). A differential feature of the two cell lines is that the Golgi apparatus is fragmented and dispersed in infected Vero cells but not in infected 143TK<sup>-</sup> cells (8). Detailed studies of cells infected with the U<sub>L</sub>20<sup>-</sup> virus revealed the following.

(i) In Vero cells infected with the U<sub>L</sub>20<sup>-</sup> virus, virions accumulated between the inner and outer nuclear membranes, and virus particles were absent from the space between

infected cells (6). In cells infected with wild-type virus, this space is filled with virions. The absence of extracellular virions explained, at least in part, the absence of plaque formation in Vero cell monolayer cultures.

(ii) The glycoprotein oligosaccharides of virions purified from infected Vero cells were unprocessed. The oligosaccharides of the bulk of the viral glycoproteins extracted from Vero cells infected with U<sub>L</sub>20<sup>-</sup> virus were fully processed in that they contained terminal sialic acid residues. Nevertheless, the glycoproteins were not transported to the plasma membranes. In contrast, the glycoproteins associated with the membranes of 143TK<sup>-</sup> cells infected with the U<sub>L</sub>20<sup>-</sup> virus were fully processed and transported to the plasma membrane (3). In cells in which the Golgi apparatus is fragmented and dispersed, U<sub>L</sub>20 protein appears to function at the point of entry of virions into the exocytic pathway from the outer nuclear membrane and in the egress of viral glycoproteins from the *trans*-Golgi.

In this report we describe studies designed to elucidate the distribution of the U<sub>L</sub>20 protein in the infected cells as a key step toward an understanding of its function. As a first step in the characterization of the protein, we constructed a fusion protein to raise antiserum against U<sub>L</sub>20 protein. With the aid of this antiserum and an antibody to a constituent of the Golgi apparatus, we determined the following: (i) the U<sub>L</sub>20 gene is regulated as a  $\gamma_1$  gene, and the protein can be detected in infected-cell lysates by 6 h after infection; (ii) pulse-chase analysis of U<sub>L</sub>20 revealed no discernible alterations in mobility in polyacrylamide gels; (iii) the protein was not expressed at the cell surface but was present in virions purified from extracellular fluid and from the cytoplasm; and (iv) U<sub>L</sub>20 protein was found to be present in the nuclear membranes, in the Golgi apparatus, and dispersed in the cytoplasm but was not detected in the plasma membranes of infected cells.

\* Corresponding author. Mailing address: The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 E. 58th St., Chicago, IL 60637. Phone: (312) 702-1898. Fax: (312) 702-1631.

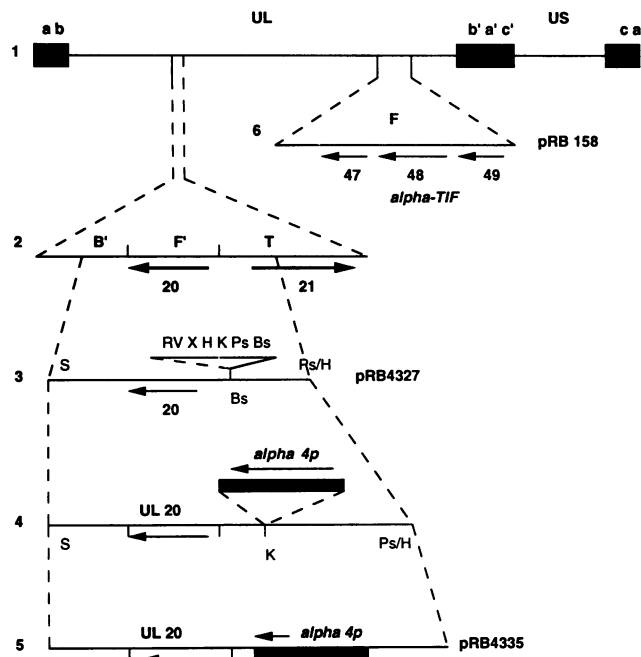


FIG. 1. Schematic representations of the sequence arrangements of HSV-1 DNA and of the various plasmids used in these studies. Lines 1 and 2, locations within the genome of HSV-1(F) of the *Bam*HI B', F', and T fragments and the U<sub>L</sub>20 and U<sub>L</sub>21 open reading frames. Line 3, sequence arrangement of plasmid pRB4327; a double-stranded oligonucleotide containing multiple cloning sites and *Bst*EII cohesive ends was inserted into the unique *Bst*EII site between the U<sub>L</sub>20 and U<sub>L</sub>21 open reading frames. Lines 4 and 5, sequence arrangement of pRB4335; a *Kpn*I fragment containing 1.9 kb of the HSV-1 *Bam*HI Z fragment (containing the  $\alpha$ 4 promoter [ $\alpha$ 4p] sequences) was inserted into the *Kpn*I site of pRB4327 in the proper transcriptional orientation to the U<sub>L</sub>20 gene. Line 6, sequence arrangement of pRB158, the *Bam*HI F fragment of HSV-1 containing the  $\alpha$ -TIF gene and its regulatory sequences. S, *Sac*I; RV, *Eco*RV; X, *Xba*I; H, *Hind*III; K, *Kpn*I; Ps, *Pst*I; Bs, *Bst*EII.

## MATERIALS AND METHODS

**Cells and viruses.** The isolation and properties of HSV-1(F) and HSV-2(G), the prototype HSV-1 and HSV-2 strains used in our laboratories, have been described elsewhere (13). Viral stocks of wild-type viruses were made in HEp-2 cells, and titers were determined in Vero cells. The genetically engineered mutants used in these studies were as follows. R7032 lacks the entire U<sub>S</sub>8 gene, which encodes glycoprotein E (32); it was used in experiments in which it was desirable to avoid reactivity of immunoglobulin G (IgG) with the viral Fc receptor (e.g., black plaque assays and pulse-chase experiments). R7405 (49) contains an epitope derived from glycoprotein B of human cytomegalovirus (CMV) (6) inserted into the U<sub>L</sub>20 gene and a portion of the U<sub>L</sub>26.5 gene promoter sequences (25) oriented such that this promoter is in the proper transcriptional orientation to the U<sub>L</sub>20 gene. R7225 lacks 355 bp from the 5' end of the coding domain of the U<sub>L</sub>20 gene. (6). All recombinants except R7225 were grown, and titers were determined, in Vero cells. Inasmuch as recombinant R7225 does not form plaques in Vero cells, it was grown, and titers were determined, in 143 thymidine kinase-minus (143TK<sup>-</sup>) cells originally obtained from Carlo Croce. Purified virion preparations were prepared from infected BHK cells. The cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with ei-

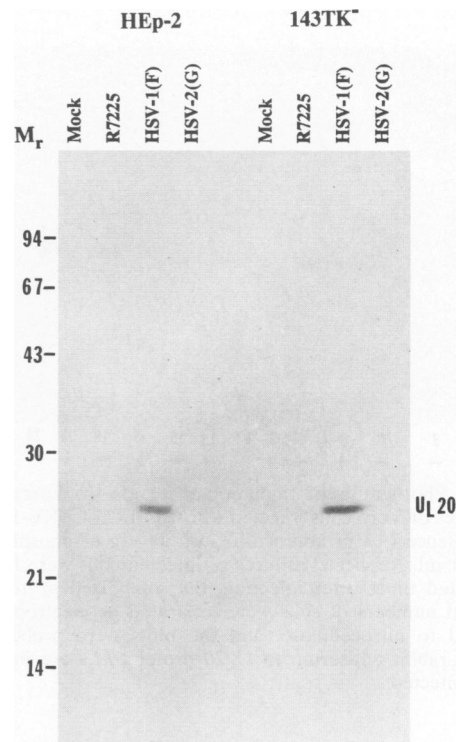


FIG. 2. Photograph of immunoblot of electrophoretically separated lysates of HEp-2 and 143TK<sup>-</sup> cells infected with the indicated viruses. The blots were probed with rabbit antiserum raised against the U<sub>L</sub>20<sup>-</sup> $\beta$ -galactosidase fusion protein. *M<sub>s</sub>* are in thousands.

ther 5% newborn calf serum (Vero or HEp-2 cells) or 5% fetal calf serum (143TK<sup>-</sup> cells).

**Plasmids.** Figure 1 illustrates the construction of pRB4335 and pRB158; line 1 shows the sequence arrangement of the HSV-1 genome, and line 2 shows the sequence arrangement of the HSV-1 *Bam*HI B', F', and T fragments and the orientations of the U<sub>L</sub>20 and U<sub>L</sub>21 genes. A 1.99-kb *Pst*I-*Sac*I fragment of pRB451 containing DNA sequences spanning map units 0.256 to 0.294 (6) was cloned into the *Pst*I and *Sac*I sites of pGEM3Z (Promega). This plasmid was designated pRB4324. The *Pst*I and *Hind*III sites in the polylinker of this plasmid were destroyed by cleavage with *Pst*I and *Hind*III restriction enzymes (New England Biolabs, Inc., Beverly, Mass.) and treatment with T4 polymerase (U.S. Biochemical, Cleveland, Ohio) and the Klenow fragment of DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). This cleaved plasmid was religated by using T4 ligase (New England Biolabs), and a double-stranded DNA oligonucleotide containing multiple cloning sites and *Bst*EII-compatible cohesive ends (5'GTAACGATATCTAGAAGCTTGGTACCTGCAG3' and its complement 5'GTTACCTGCAGGGTACCAAGCTTCTAGATATC3') was ligated into the unique *Bst*EII site (Fig. 1, line 3). Restriction enzyme cleavage sites in this polylinker were *Eco*RV, *Xba*I, *Hind*III, *Kpn*I, *Pst*I, and *Bst*EII. Insertion of this oligonucleotide destroyed the *Bst*EII site at the 5' end, creating an *Eco*RV site, but preserved the *Bst*EII site at the 3' end. This plasmid was designated pRB4327 (Fig. 1, line 3). The 1.9-kb *Kpn*I fragment from pRB178 (HSV-1 *Bam*HI Z fragment cloned into pUC18) containing the  $\alpha$ 4 promoter sequences and portions of the 5' transcribed noncoding sequences of the  $\alpha$ 4 gene starting with nucleotide

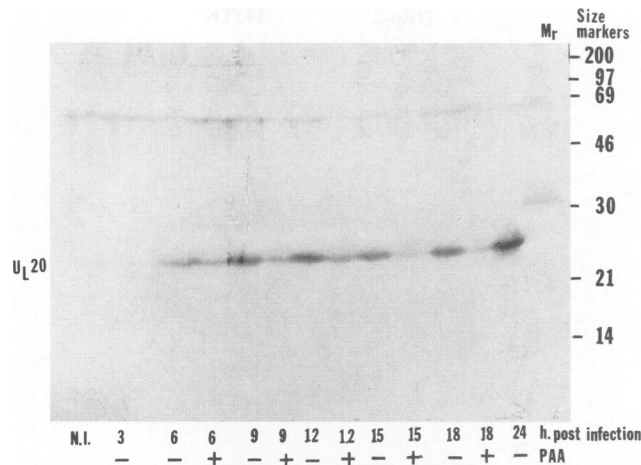


FIG. 3. Photograph of immunoblot of electrophoretically separated lysates of Vero cells infected with 10 PFU of HSV-1(F) per cell in the presence (+) or absence (-) of 300 µg of phosphonoacetate (PAA) per ml. Replicate cultures of infected cells were harvested at the indicated times after infection, the solubilized proteins derived from equal numbers of cells were separated by electrophoresis and transferred to nitrocellulose, and the blots were probed with the polyclonal rabbit antiserum to U<sub>L</sub>20 protein. *M<sub>r</sub>*s are in thousands. N.I., not infected.

+33 was inserted into the *Kpn*I site of pRB4327 such that the  $\alpha$ 4 promoter would be in the proper transcriptional orientation to the U<sub>L</sub>20 gene. This plasmid was designated pRB4335 (Fig. 1, line 5). In other experiments we used a promoter consisting of the 5' nontranscribed domain of the  $\alpha$ 4 gene fused to the transcribed noncoding domain of the transcription initiation site and the 5' transcribed noncoding domain of the  $\gamma$ <sub>1</sub>U<sub>L</sub>19 gene encoding the major capsid protein. This promoter is expressed throughout the reproductive cycle of the virus and allows the accumulation of large amounts of protein (23).

**Production of the U<sub>L</sub>20- $\beta$ -galactosidase fusion protein and polyclonal rabbit antiserum to the U<sub>L</sub>20 protein.** The *Bam*HI F' fragment of HSV-1(F) DNA was cloned into pUC18 as pGCF1053. The 670-bp *Bgl*II-*Bam*HI fragment from pGCF1053 was filled in with the Klenow fragment of DNA polymerase, ligated with 12-mer *Eco*RI linkers, and cloned into the *Eco*RI site of pGEM to yield plasmid pGCF1057. The *Eco*RI fragment from pGCF1057 containing 95% of the U<sub>L</sub>20 coding sequence was cloned into the *Eco*RI site of the pEX-2 vector (Biochemia, Mannheim, Germany), which contains a *cro-Escherichia coli lacZ* gene fusion. The recombinant plasmid pGCF1136 was introduced into POP2136 cells. To prepare the U<sub>L</sub>20- $\beta$ -galactosidase fusion protein, an overnight culture was diluted 1:100 and allowed to grow at 30°C to an optical density at 600 nm of 0.1. The culture was then shifted to 42°C for 3 h. Pelleted cells from a 2-liter culture were sonicated, and the inclusion bodies were pelleted, washed two times in phosphate-buffered saline (PBS) containing 1% deoxycholate and 1% Nonidet P-40, and subjected to preparative denaturing polyacrylamide gel electrophoresis. The protein corresponding to the U<sub>L</sub>20- $\beta$ -galactosidase fusion protein was electroeluted in electrophoresis buffer containing 0.01% sodium dodecyl sulfate (SDS). New Zealand White rabbits were inoculated subcutaneously with 200 µg of fusion protein emulsified with complete Freund's adjuvant. The rabbits were boosted five times at 2-week intervals with the same amount of fusion protein in incomplete Freund's adjuvant. Serum samples were

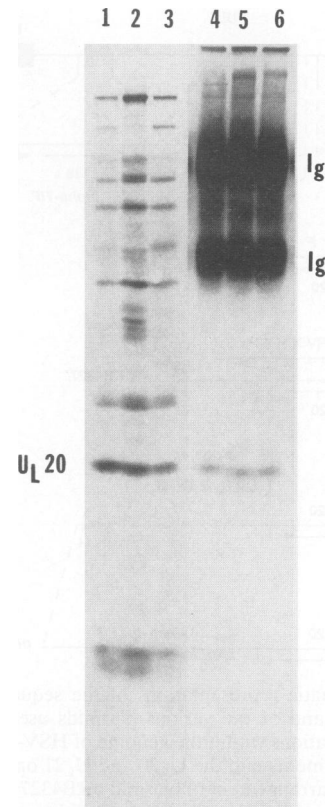


FIG. 4. Photographs of [<sup>35</sup>S]methionine-labeled proteins immunoprecipitated with the U<sub>L</sub>20 rabbit serum (lanes 1 to 3) and of an immunoblot of the identical immunoprecipitates reacted with U<sub>L</sub>20- $\beta$ -galactosidase antiserum (lanes 4 to 6). HEp-2 cells were mock infected or exposed to 10 PFU of R7032 (gE<sup>-</sup>) virus per cell. At 8 h after infection, the medium was replaced with 1 ml of medium lacking methionine and serum. The cells were labeled for 1 h with 50 µCi of [<sup>35</sup>S]methionine at 9 or 16 h after infection. In the cultures labeled at 9 h after infection, the radiolabeled methionine was chased with cold methionine at 37°C for an additional 12 h. The cells labeled at 16 h after infection were harvested immediately after the labeling period. Lanes 1 and 4 cells pulse-labeled at 9 h; lanes 2 and 5, cells pulsed at 9 h and chased for 12 h; lanes 3 and 6, cells pulsed at 16 h. Cell lysates were immunoprecipitated with the U<sub>L</sub>20 antiserum, heated at 37°C for 30 min, separated by electrophoresis on a denaturing polyacrylamide gel, transferred to nitrocellulose, and exposed to X-ray film (lanes 1 to 3). The identical nitrocellulose blot was then probed with the U<sub>L</sub>20 antiserum (lanes 4 to 6). The secondary antibody used in the immunoblot reacts with the rabbit U<sub>L</sub>20 antiserum used in the immunoprecipitation (bands labeled Ig). Since U<sub>L</sub>20 protein aggregates on boiling in SDS and does not enter gels (6), the immunoprecipitated samples were not boiled. As a consequence, IgG present in the samples was not completely solubilized and appears as multiple species in the immunoblot.

collected and analyzed for reactivity with the U<sub>L</sub>20 protein in lysates of cells electrophoretically separated in denaturing gels and transferred to a nitrocellulose sheet.

**Antibodies.** The polyclonal rabbit antiserum to U<sub>L</sub>20 was used at dilutions of 1:1,000 for immunofluorescence, 1:100,000 for immunoblotting, and 1:200 for immunoprecipitations. The mouse monoclonal antibody to  $\beta$ -COP, the Golgi coat protein of non-clathrin-coated vesicles (2), was provided by T. Kreis. The CH-28 monoclonal antibody to glycoprotein B of CMV and the monoclonal antibodies specific for HSV-1 glycoprotein D (H170) and for ICP4 (H640) were provided by L. Pereira.

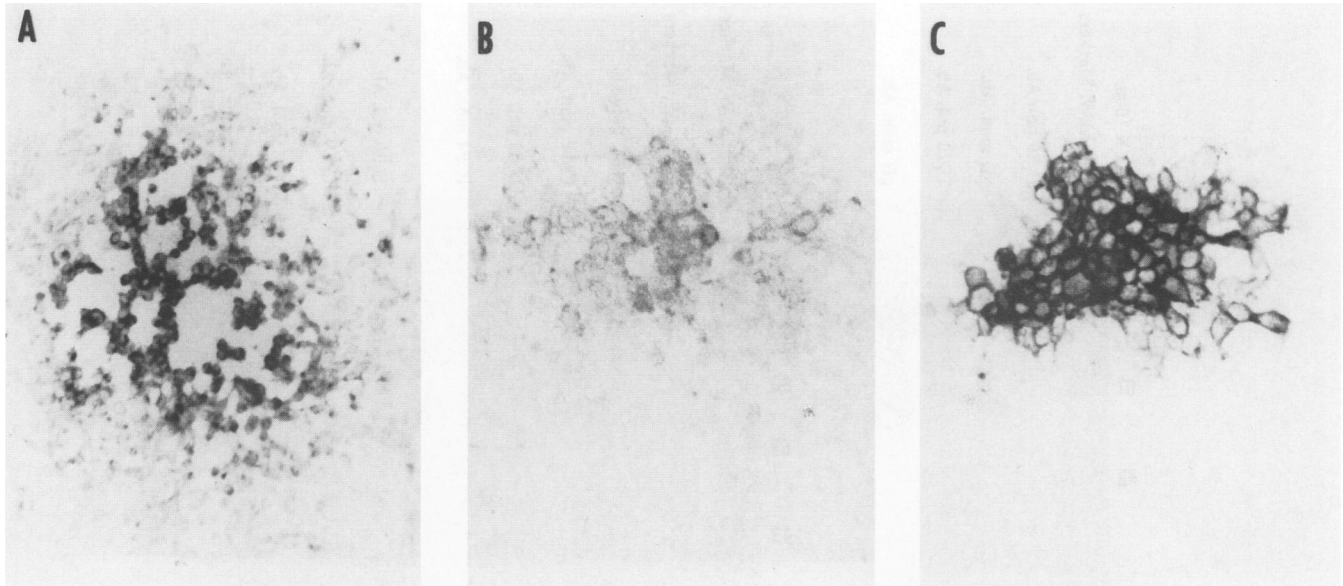


FIG. 5. Photomicrograph of methanol-fixed and unfixed gE<sup>-</sup> virus (R7032)-infected Vero cells probed with rabbit anti-U<sub>L</sub>20- $\beta$ -galactosidase serum or rabbit anti-gM serum. Forty-eight hours after infection, the cells were probed with rabbit antisera. The bound antibody was detected with a biotinylated goat anti-rabbit antibody followed by biotinylated horseradish peroxidase bound to avidin and by 4-chloro-1-naphthol substrate. (A) Fixed cells; a viral plaque reacted with a 1:200 dilution of rabbit anti-U<sub>L</sub>20 serum. (B) Unfixed cells; a viral plaque reacted with a 1:200 dilution of rabbit anti-U<sub>L</sub>20 serum. (C) Unfixed cells; a viral plaque reacted with a 1:200 dilution of rabbit anti-gM serum.

The rabbit anti-U<sub>L</sub>53 antibody was obtained from David Johnson. The goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated antibody was purchased from Sigma Chemical Co., (St. Louis, Mo.). The goat anti-mouse IgG Texas red-conjugated antibody was purchased from Molecular Probes, Inc. (Eugene, Oreg.). Commercial antibodies were used as recommended by the manufacturers.

**Transfections.** Vero or 143TK<sup>-</sup> cells were seeded onto glass coverslips and allowed to adhere. Transfections were done essentially as previously described (31) except that 20  $\mu$ g of each plasmid was used and cells were fixed for immunofluorescence analysis 30 h after transfection.

**Polyacrylamide gel electrophoresis and immunoblotting.** Infected-cell lysates were separated in denaturing gels consisting of 12.5 or 15% polyacrylamide and 0.1% SDS, and the proteins were electrically transferred to nitrocellulose sheets. The sheets were soaked at room temperature for 10 to 30 min in PBS containing 5% skim milk (Carnation) and then were reacted at room temperature for 90 min with the U<sub>L</sub>20 polyclonal rabbit antiserum (1:100,000 dilution) in PBS containing 1% bovine serum albumin (BSA). The blots were washed once in PBS-5% milk for 15 min, rinsed four times in PBS, and reacted for 1 h in a 1:3,000 dilution of goat anti-rabbit or goat anti-mouse antiserum conjugated to alkaline phosphatase. The blots were washed as described above and developed by using reagents and protocols supplied in a Bio-Rad Laboratories (Richmond, Calif.) kit.

**Black plaque assay.** The procedures for the black plaque assay were essentially as previously described (24). Vero cell monolayer cultures were exposed to 10 to 50 PFU of R7032 (gE<sup>-</sup>) virus per 25-cm<sup>2</sup> cell culture flask and incubated at 34°C for 48 h. The monolayers were either fixed in methanol to permeabilize the cells or left intact. The monolayers were incubated at room temperature in blocking medium consisting of medium 199 supplemented with 1% heat-inactivated horse serum (199v) for 1 h. Cells were then incubated in a solution

containing the appropriate primary antibody at room temperature for 90 min. For analysis of U<sub>L</sub>20 expression at the plasma membrane, infected cells were reacted with either U<sub>L</sub>20 polyclonal rabbit antiserum or anti-gM (U<sub>L</sub>10 protein) polyclonal rabbit antiserum (1:200 dilution). The monolayers were washed three times with medium 199 supplemented with 1% fetal calf serum (199v), and incubated in a 1:500 dilution of biotinylated goat anti-mouse or goat anti-rabbit antibody at room temperature for 1 h. Monolayers were again washed three times and reacted with avidin bound to biotin-conjugated horseradish peroxidase (Vector Laboratories), and the color reaction was developed by using 1-chloro-4 naphthol substrate as previously described (24).

**Immunoprecipitations.** For pulse-chase experiments, radio-labeled, infected HEp-2 cell lysates were solubilized in 200  $\mu$ l of immunoprecipitation buffer (consisting of a mixture of 1% Nonidet P-40, 1% sodium deoxycholate, 10  $\mu$ M TPCK [tolyl-sulfonyl phenylalanyl choromethyl ketone], 10  $\mu$ M TLCK [ $\alpha$ -tosyl L-lysine chloromethyl ketone] in PBS), sonicated briefly, and clarified by centrifugation for 20 min at 4°C in an Eppendorf microcentrifuge. Fifty microliters of a 50% slurry of protein A-conjugated Sepharose beads (Sigma) was added to the clarified lysates on ice and left for 1 h to remove materials which might react nonspecifically. The beads were removed by centrifugation, and the supernatant fluids were reacted with 1  $\mu$ l of U<sub>L</sub>20 antiserum overnight at 4°C. The immune complexes were recovered by addition of 50  $\mu$ l of a 50% slurry of protein A-Sepharose beads and incubation on ice for 1 h. The beads were washed four times with immunoprecipitation buffer and once with 100 mM NaCl-10 mM Tris-HCl, pH 7.4. The immunoprecipitated proteins were eluted from the beads by addition of 50  $\mu$ l of sample buffer containing 2% SDS and 5%  $\beta$ -mercaptoethanol and heating of the samples for 30 min at 37°C, electrophoretically separated on a denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, reacted with the U<sub>L</sub>20 antiserum, and exposed to X-ray film.

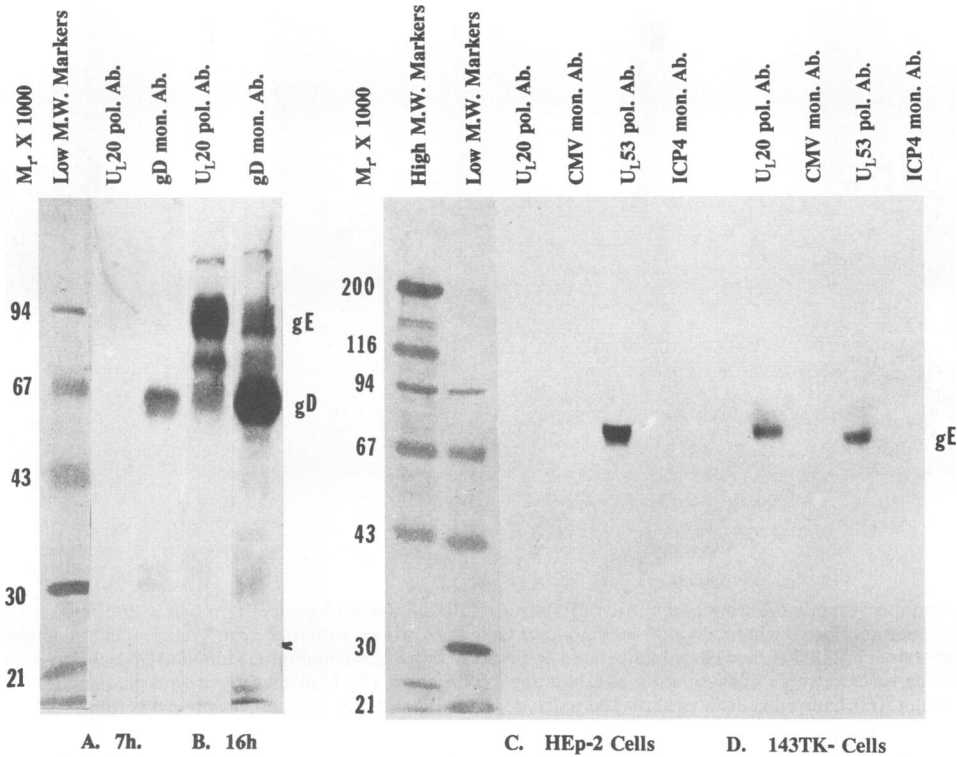


FIG. 6. Photographs of biotinylated, immunoprecipitated proteins. (A and B) Vero cell monolayers were infected with HSV-1(F) virus (10 PFU per cell), and the monolayers were exposed to NHS-LC-biotin at 7 or 16 h postinfection. (C and D) HEP-2 or 143TK<sup>-</sup> cells were infected with HSV-1(F) or R7405 (U<sub>L</sub>20-CMV tagged, used with CMV antibody) virus (10 PFU per cell), and the monolayers were exposed to NHS-LC-biotin at 16 h postinfection. All cells were maintained at 34°C. Surface-biotinylated cells were washed, harvested, and lysed in immunoprecipitation buffer. The cell lysates were reacted with either U<sub>L</sub>20 rabbit antibody (lanes U<sub>L</sub>20 pol. Ab.), monoclonal antibody to the CMV epitope (lanes CMV mon. Ab.), monoclonal antibody to gD (lanes gD mon. Ab.), rabbit antibody to U<sub>L</sub>53 (lanes U<sub>L</sub>53 pol. Ab.), or monoclonal antibody to ICP4 (lanes ICP4 mon. Ab.). All immunoprecipitated samples other than U<sub>L</sub>20 or U<sub>L</sub>53 were boiled before electrophoresis. The precipitates containing U<sub>L</sub>20 or U<sub>L</sub>53 rabbit antiserum were heated at 37°C for 30 min prior to electrophoresis. The immunoprecipitated proteins were separated on denaturing polyacrylamide gels (panels A and B, 12.5% gel; panels C and D, 11% gel), transferred to nitrocellulose sheets, and reacted with streptavidin-horseradish peroxidase conjugate. The precipitated proteins were visualized by using the ECL chemiluminescence detection system from Amersham. The arrowhead in panel B indicates the position of U<sub>L</sub>20 migration as detected by immunoblots (not shown). M.W., molecular weight.

For immunoprecipitation of cell surface proteins, infected-cell monolayers were washed with PBS and incubated at room temperature in a solution of 75 µg of NHS-LC-biotin (Pierce Chemical Co., Rockford, Ill.) per ml of PBS for 30 min. The cells were rinsed with PBS, harvested, and lysed in immunoprecipitation buffer. Immunoprecipitations, electrophoresis, and transfer of the precipitated proteins were done as described above except that goat anti-mouse IgG-coated agarose beads (Sigma Chemical Co.) were used to immunoprecipitate samples incubated with monoclonal antibodies and all samples except those precipitated with antibodies to U<sub>L</sub>20 or to U<sub>L</sub>53 were boiled before being loaded on SDS-polyacrylamide gels. The nitrocellulose sheets were then blocked overnight at 4°C in a solution of 5% milk in PBS, rinsed in PBS containing 0.1% Tween 20, and reacted with streptavidin-horseradish peroxidase conjugate (1:1,500 dilution in PBS-0.1% Tween 20) (Amersham Life Sciences) for 1 h. The membranes were washed extensively in PBS-0.1% Tween 20 and developed by using reagents and protocols for chemiluminescence obtained from Amersham Life Sciences. The developed blots were exposed to X-ray film to visualize the precipitated proteins.

**Immunofluorescence.** Approximately 10<sup>5</sup> Vero or 143TK<sup>-</sup> cells were seeded onto sterilized glass coverslips in 24-well tissue culture plates (Costar) and allowed to attach overnight.

The cells were exposed to 5 PFU of R7032 (gE<sup>-</sup>) virus per cell and fixed in ice-cold methanol at 14 to 16 h after infection. The coverslips were first blocked for 30 min at room temperature in PBS containing 1% BSA and then reacted with primary antibody for 1 h at room temperature, washed in PBS, and reacted with the appropriate fluorescein- or Texas red-conjugated secondary antibody for 1 h at room temperature. The coverslip cultures were rinsed again in PBS, mounted on glass microscope slides on a drop of 90% glycerol in PBS containing 1 mg of *p*-phenylenediamine per ml. and examined in a Zeiss confocal fluorescence microscope. Digital images of the fluorescent profiles were acquired by using software provided with the Zeiss confocal microscope and printed by a CP210 Codonics digital printer.

**Virion preparation.** Virions were purified essentially as described by Szilagyi and Cunningham (47). Briefly, medium containing virions was centrifuged at 1,000 × *g* for 30 min. Virions were then pelleted by centrifugation for 2 h at 80,000 × *g*, resuspended in Dulbecco's modified Eagle's medium lacking phenol red, and layered onto a 5 to 15% Ficoll gradient made in the same medium. Bands containing the virion particles were collected by side puncture, diluted with medium lacking phenol red, pelleted by centrifugation (80,000 × *g* for 2 h at 4°C), gently resuspended in 200 µl of PBS, and stored at

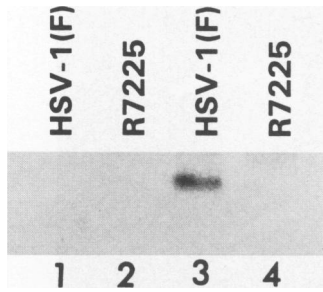


FIG. 7. Photographs of biotinylated, immunoprecipitated proteins. HEp-2 cells were infected with HSV-1(F) (lanes 1 and 3) or with U<sub>L</sub>20<sup>-</sup> virus R7225 (lanes 2 and 4) and reacted with biotin at 16 h postinfection. Lanes 1 and 2, infected cell monolayers were washed and exposed to NHS-LC-biotin as described in the legend to Fig. 6. The surface-biotinylated cells were washed, harvested, and lysed in immunoprecipitation buffer. The cell lysates were reacted with anti-U<sub>L</sub>20 rabbit serum. Lanes 3 and 4, infected cells were harvested, washed, and disrupted in 0.4% Nonidet P-40. The cytoplasmic extracts were then exposed to 300 μg of NHS-LC-biotin per ml for 15 min at room temperature. The reaction was quenched by the addition of 100 mM Tris-HCl, pH 7.5, and the cell lysates were reacted with anti-U<sub>L</sub>20 antiserum. Immunoprecipitated, biotinylated proteins were separated by electrophoresis and transferred to nitrocellulose blots, and the proteins were visualized as described in the legend to Fig. 6.

-80°C. A fraction of the virion preparation was processed for electron microscopic observation in order to monitor the extent of purification.

**RESULTS**

**Production of U<sub>L</sub>20 antiserum.** New Zealand White rabbits were inoculated subcutaneously with U<sub>L</sub>20-β-galactosidase fusion protein that had been electroeluted from denaturing polyacrylamide gels as described in Materials and Methods. The resulting antiserum was reacted with electrophoretically separated infected-cell proteins that had been transferred to nitrocellulose sheets. The results are shown in Fig. 2. The antiserum reacted with a single protein band with an apparent *M<sub>r</sub>* of approximately 24,000 in lysates of HEp-2 or 143TK<sup>-</sup> cells infected with HSV-1(F). An identical pattern was observed for lysates of infected Vero cells (data not shown). The specificity of the antiserum was demonstrated by its reactivity with a single protein band with lysates of HSV-1(F)-infected cells but not with lysates of mock- or R7225 (U<sub>L</sub>20<sup>-</sup>)-infected cells (Fig. 2). The antiserum did not react with infected-cell polypeptides from lysates of cells infected with HSV-2(G) (Fig. 2).

**Requirements and timing of expression of the U<sub>L</sub>20 gene.** Vero cells were exposed to 10 PFU of HSV-1(F) per cell and incubated at 37°C in medium 199v in the presence or absence of phosphonoacetate (300 μg/ml; Sigma). At this concentration, phosphonoacetate totally abolishes viral DNA synthesis. At intervals the cells were harvested, solubilized in disruption buffer containing 2% SDS and 1% β-mercaptoethanol, incubated at 37°C for 20 min, subjected to electrophoresis in a denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted with the anti-U<sub>L</sub>20 rabbit serum. The results (Fig. 3) indicate that the U<sub>L</sub>20 protein accumulated in readily detectable amounts by 6 h postinfection, and no appreciable change in the amounts of protein was detected beyond 9 h postinfection. The observation that the synthesis of U<sub>L</sub>20 protein was diminished but not abolished by phosphonoacetate suggests that the U<sub>L</sub>20 gene was regulated as a γ<sub>1</sub> gene.

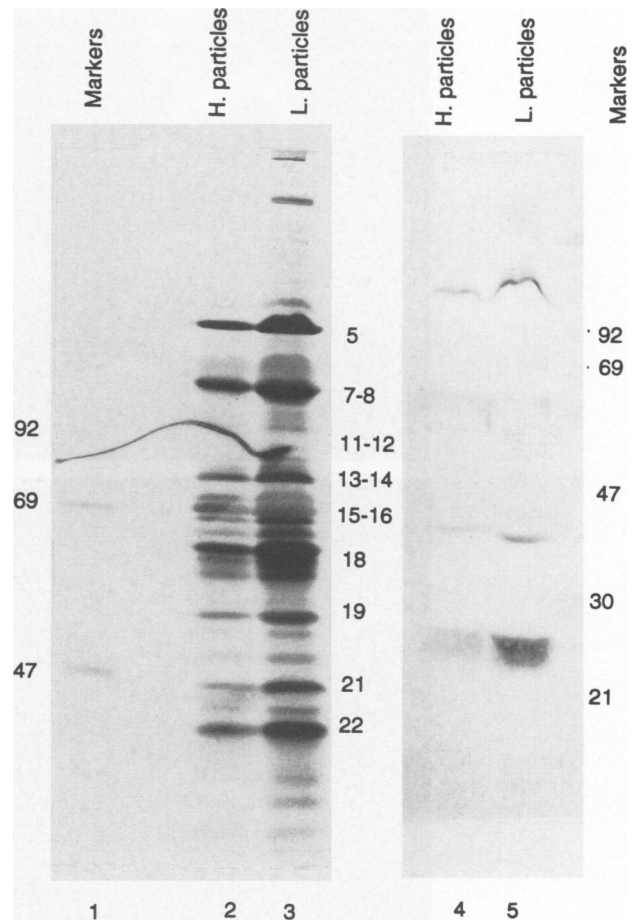


FIG. 8. Photographs of silver-stained or immunoblotted electrophoretically separated virion polypeptides. Virions were prepared from the extracellular fluid of HSV-1(F)-infected BHK cells. Virion polypeptides were denatured in a buffer containing SDS and β-mercaptoethanol and were separated on a denaturing polyacrylamide gel. The proteins were either silver stained (lanes 1, 2, and 3) or transferred to nitrocellulose sheets and reacted with the anti-U<sub>L</sub>20 rabbit antiserum (lanes 4 and 5). Virion protein designations (center) and molecular weight size markers (in thousands) (sides) are indicated. H., heavy; L., light.

**The electrophoretic mobility of pulse-labeled U<sub>L</sub>20 protein is not altered after a chase.** The majority of membrane-associated proteins encoded in the HSV-1 genome have been shown to be modified by glycosylation. Although the predicted amino acid sequence of the U<sub>L</sub>20 open reading frame present in HSV-1 strain 17 does not contain consensus sites for N-linked glycosylation (30), we were interested to see whether there was any change in the electrophoretic mobility of the U<sub>L</sub>20 protein in a pulse-chase experiment. In this series of experiments we used HEp-2 cells inasmuch as earlier studies have shown that Vero cells contain an endogenous protease which may alter the electrophoretic mobility of viral proteins during a chase (35). HEp-2 cells grown in 25-cm<sup>2</sup> tissue culture flasks were exposed to 10 PFU of R7032 (gE<sup>-</sup>) per cell. At 8 or 15 h after infection, the medium was replaced with 1 ml of the same mixture but without methionine or the serum supplement. The cells were pulse-labeled at either 9 or 16 h after infection with 50 μCi of [<sup>35</sup>S]methionine for 1 h at 37°C. In the cultures labeled at 9 h, the labeling medium was then replaced

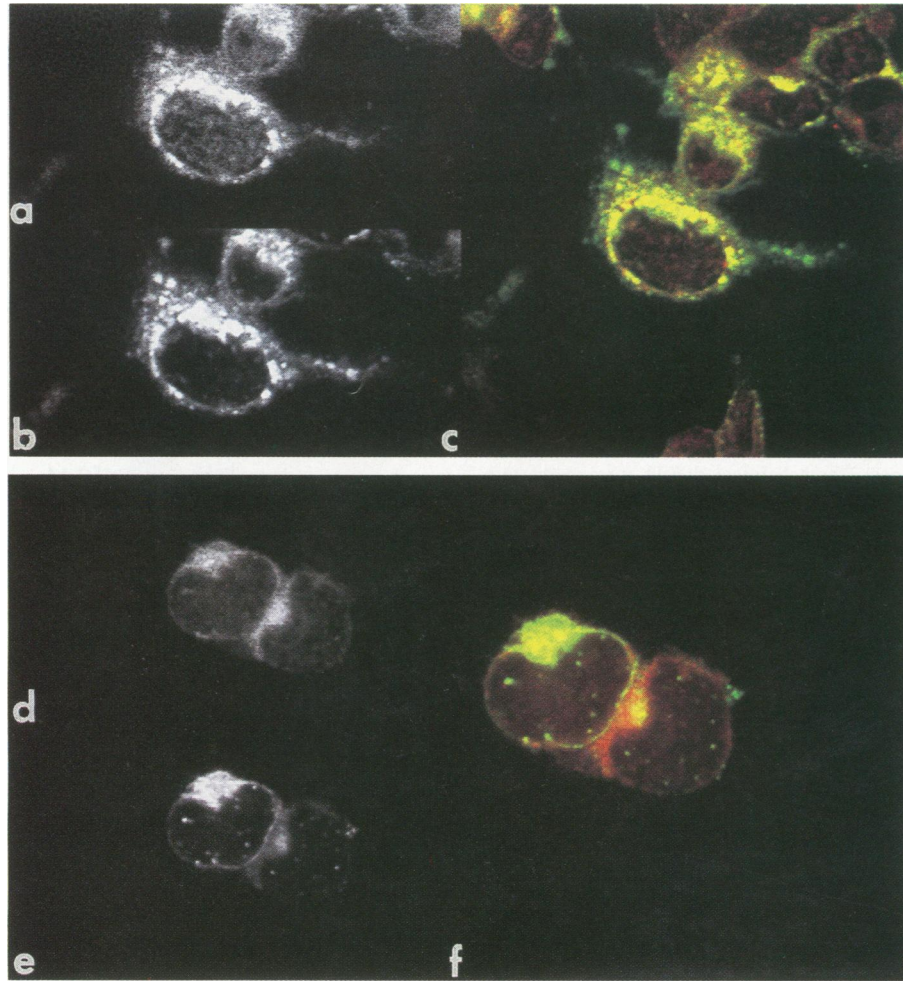


FIG. 9. Confocal, digital images of infected Vero (a, b, and c) or 143TK<sup>-</sup> (d, e, and f) cells reacted with antibodies to U<sub>L</sub>20 (green fluorescence) and  $\beta$ -COP (red fluorescence). Cells were grown on glass coverslips, infected with 3 to 5 PFU of R7032 (gE<sup>-</sup>) virus per cell, and fixed in ice-cold methanol at 14 to 16 h after infection. The coverslips were reacted with the primary antibodies for 1.5 h at room temperature, washed in PBS, and subsequently reacted with the appropriate fluorescein or Texas red-conjugated secondary antibody for 1 h at room temperature. (a and b) Split images of Vero cells double stained with antibodies to  $\beta$ -COP (a; Texas red fluorescence) and to U<sub>L</sub>20 (b; FITC fluorescence). (c) Overlay of images in panels a and b. (d and e) Split image of 143TK<sup>-</sup> cells double stained with antibodies to  $\beta$ -COP (d; Texas red fluorescence) and to U<sub>L</sub>20 (e; FITC fluorescence). (f) Overlay of images in panels d and e. The images were captured with software provided by Zeiss with the instrument and printed by a Codonics CP210 printer. The image in panel c was attenuated to reduce the intensity of fluorescence.

with medium containing normal levels of unlabeled methionine, and the cells were reincubated at 37°C for an additional 12 h. The cells labeled at 16 h after infection were harvested immediately after the labeling interval. The [<sup>35</sup>S]methionine-labeled immunoprecipitated proteins are shown in Fig. 4, lanes 1 to 3. The relative migration of U<sub>L</sub>20 protein was not altered at 12 h after the [<sup>35</sup>S]methionine pulse (lane 2), nor was there evidence of any alteration when cells were pulse-labeled at 16 h after infection (lane 3). Furthermore, the migration of U<sub>L</sub>20 protein was also unaltered in pulse-chase experiments done with cells treated with monensin from the time of infection (data not shown), suggesting that U<sub>L</sub>20 is not modified by O-linked glycosylation (22). The identification of the [<sup>35</sup>S]methionine-labeled species as U<sub>L</sub>20 was verified by reacting the nitrocellulose sheet with the U<sub>L</sub>20 polyclonal serum (Fig. 4, lanes 4 to 6). The two more slowly migrating species in lanes 4 to 6 represent reactivity of the U<sub>L</sub>20 antiserum present in the immunoprecipitation reaction with the alkaline phosphatase-

conjugated anti-rabbit antibody used in the immunoblot. We should note that (i) the duration of the pulse was not excessive given the slow rate of glycosylation of viral proteins in infected cells and (ii) the anti-rabbit alkaline phosphatase-conjugated secondary antibody used in the immunoblot does not detect the light chain of IgG present in the immunoprecipitation reactions (data not shown).

**U<sub>L</sub>20 is not detected at the plasma membrane.** Earlier studies have shown that the U<sub>L</sub>20 protein is associated with cellular membranes. To determine whether U<sub>L</sub>20 was present in the plasma membrane, two series of experiments were done. In the first, unfixed Vero cells infected with gE<sup>-</sup> (R7032) virus were exposed to rabbit anti-U<sub>L</sub>20 polyclonal antibody and then to biotinylated goat anti-rabbit immunoglobulin followed by avidin bound to biotinylated peroxidase (ABC complex). In a parallel experiment, a replicate infected-cell culture was exposed to rabbit anti-gM polyclonal antibody (5). The procedure, designated the black plaque technique (24), detected the

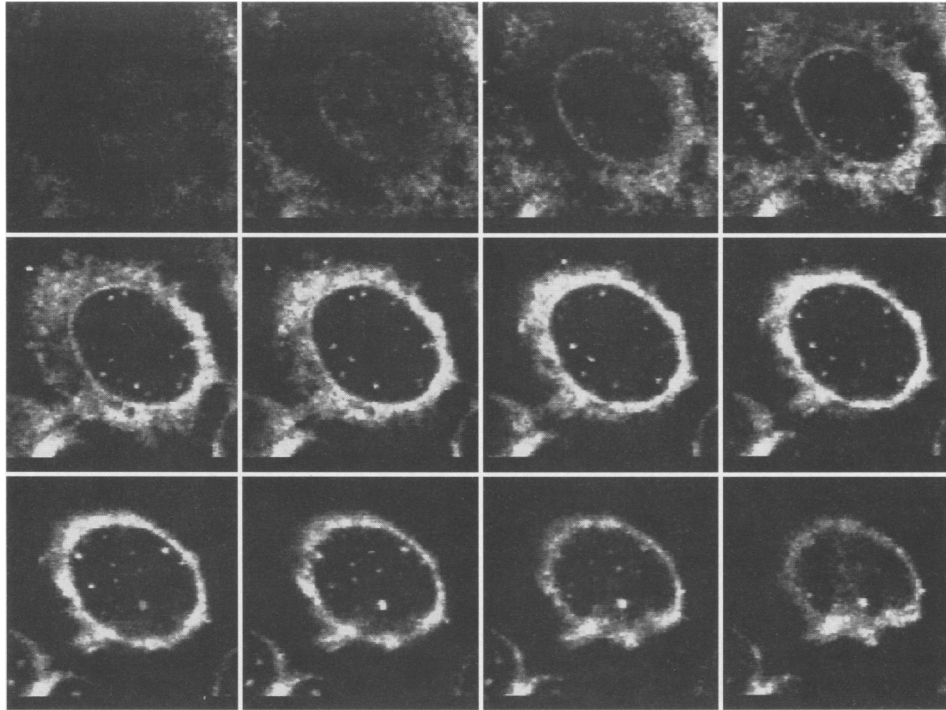


FIG. 10. Digital, unprocessed images of an R7032-infected Vero cell reacted with antibody to U<sub>L</sub>20 and anti-rabbit IgG conjugated to FITC. Cells were infected, fixed, and stained as described in the legend to Fig. 8. Twelve 0.5- $\mu$ m sections through the z axis were captured and printed by a Codonics CP210 printer. The section in the top left panel is from the basolateral side of the cell.

presence of abundant amounts of gM (Fig. 5C) but not of U<sub>L</sub>20 proteins on the surface of infected cells (Fig. 5B). The U<sub>L</sub>20 protein does react with the antibody in the same system but only after fixation of the cells with methanol, which renders the cells permeable to the antibody (Fig. 5A).

In the second series of experiments, surface proteins from cells infected with wild-type virus were biotinylated at 16 h after infection as described in Materials and Methods. The cells were harvested and lysed, and the cell lysates were reacted with the rabbit anti-U<sub>L</sub>20 polyclonal antibody or antibodies to CMV epitope for detection of CMV-tagged U<sub>L</sub>20. Antibodies to the viral glycoprotein D were included as a positive control for surface protein expression. The anti-U<sub>L</sub>53 rabbit polyclonal serum or anti-ICP4 monoclonal antibody was included as a negative control, since these proteins are not expressed at the cell surface. The immunoprecipitated proteins were separated on denaturing polyacrylamide gels and transferred to nitrocellulose sheets. The sheets were reacted with streptavidin-horseradish peroxidase conjugate and developed by chemiluminescence (ECL). Trace amounts of U<sub>L</sub>20 were detected by chemiluminescence in immunoprecipitates from the surfaces of infected 143TK<sup>-</sup> and HEP-2, but not Vero, cells maintained at 37°C (data not shown). A repeat of this experiment at 34°C failed to yield detectable amounts of U<sub>L</sub>20 protein on the surface of infected Vero, 143TK<sup>-</sup>, or HEP-2 cells (Fig. 6, lanes U<sub>L</sub>20 pol. Ab.), whereas large amounts of gD were readily detectable at both 7 and 16 h postinfection (Fig. 6A and B, lanes gD mon. Ab.). Furthermore, U<sub>L</sub>20 could be biotinylated if infected cells were first disrupted with Nonidet P-40 (Fig. 7, lane 3), while U<sub>L</sub>20 was not detected on the surface of wild-type or U<sub>L</sub>20 virus-infected cells or on U<sub>L</sub>20 virus-infected cells that had been disrupted (Fig. 7, lanes 1, 2, and 4).

Two comments regarding these experiments are appropri-

ate. First, we used a gE<sup>-</sup> virus in the first series of experiments to avoid nonspecific interaction of gE with the Fc portion of the IgG molecule. Since the absence of U<sub>L</sub>20 protein on the surface of infected cells could be due to the absence of gE, wild-type virus (gE<sup>+</sup>) was used in the second series. As expected, gE was immunoprecipitated nonspecifically to various degrees, depending on cell type and rabbit serum (Fig. 6). In this instance, discrimination between specific and nonspecific binding was feasible and in fact reinforced our concerns regarding the interaction of gE with the Fc domain of IgG. Second, we suspect that the trace amounts of U<sub>L</sub>20 detected on the surface of 143TK<sup>-</sup> or HEP-2 cells incubated at 37°C (data not shown) was due to the presence of virions at the cell surface, since we could not detect any U<sub>L</sub>20 protein at the cell surface of cells maintained at 34°C. The egress of virions from cells is delayed at 34°C (18). We cannot exclude leakage of the infected cells incubated at 37°C, however, neither ICP4 nor U<sub>L</sub>53 (both internal proteins) was labeled with biotin and precipitated from the surface of infected HEP-2 or 143TK<sup>-</sup> cells maintained at 34°C (Fig. 6C and D). We conclude from these studies that the plasma membrane of infected cells contains at most small or negligible amounts of U<sub>L</sub>20 protein.

**U<sub>L</sub>20 is present in extracellular virions.** Inasmuch as U<sub>L</sub>20 is required to facilitate transport of virions from the space between the inner and outer nuclear membranes to the extracellular space, it was of interest to determine whether U<sub>L</sub>20 is physically associated with virions. In these experiments virions were collected from extracellular fluid and banded in Ficoll gradients as described by Szilagyi and Cunningham (47). This procedure allowed the differentiation between noninfectious (light) particles and infectious (heavy) particles. The electrophoretically separated virion proteins obtained as described in Materials and Methods were either silver stained or



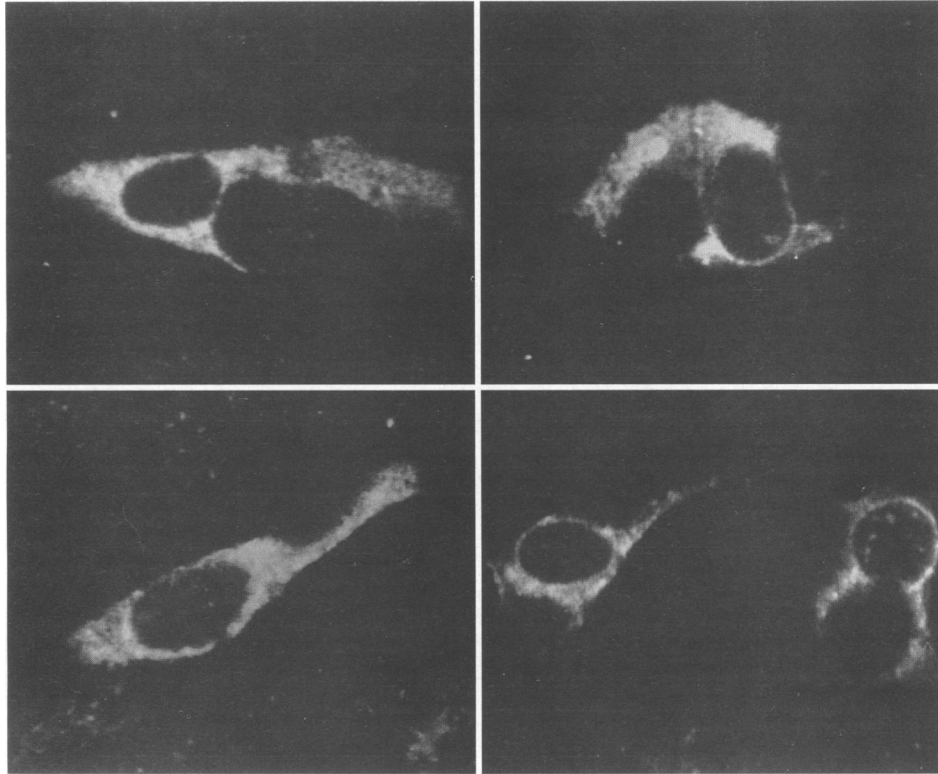


FIG. 11. Digital, unprocessed images of Vero cells transfected with plasmids containing the  $U_L20$  open reading frame as described in Materials and Methods. The cells were reacted with antibody to  $U_L20$  and anti-rabbit IgG conjugated to FITC. Cells were fixed in methanol and stained 30 h after transfection.

transferred to nitrocellulose sheets and stained with the anti- $U_L20$  polyclonal rabbit antiserum. The results shown in Fig. 8 indicate that  $U_L20$  was present in extracellular virions. We should note that the electrophoretic profile of the virions presented in Fig. 8 (lanes 2 and 3) differs from that published previously by this laboratory. The problem stems from the observation that while boiling in SDS is essential for the solubilization of many virion proteins, it causes the aggregation of integral membrane proteins with multiple transmembrane domains (see reference 44 and references therein; 46). As a consequence,  $U_L20$  protein in preparations boiled in SDS does not enter the gel. Conversely, failure to boil results in electrophoretic profiles of virion proteins in which some proteins are underrepresented.

**Localization of  $U_L20$  protein in infected cells.** The objective of these experiments was to determine the sites of localization of  $U_L20$  in the infected cells by immunofluorescence with selected reagents. Two series of experiments were done.

In the first series, the cells were infected with  $gE^-$  virus (R7032) to preclude nonspecific fluorescence caused by interaction of IgG with Fc receptors. The infected cells were stained with the rabbit anti- $U_L20$  serum alone or double stained with the  $U_L20$  antiserum and a monoclonal antibody to the Golgi-derived vesicle coat protein  $\beta$ -COP. Since in the course of these studies it became apparent that  $U_L20$  protein colocalizes in part with the Golgi- and intermediate compartment-associated protein  $\beta$ -COP, the studies focused primarily on infected Vero and human 143TK<sup>-</sup> cells. The selection of these cells for our studies was based on the observation that the Golgi apparatus is fragmented and dispersed in infected Vero cells but not in infected 143TK<sup>-</sup> cells (8).

The results are shown in Fig. 9 and 10. In Fig. 9 the photographs show individual Vero (a) and 143TK<sup>-</sup> (d) cells reacted with the anti- $\beta$ -COP monoclonal antibody and anti-mouse IgG conjugated to Texas red. Figure 9b and e show the same cells reacted with antibody to  $U_L20$  and anti-rabbit IgG conjugated to FITC. Figure 9c and f show the two images of each cell superimposed. These results indicate that  $U_L20$  is present in nuclear membranes and that the strongest signals in the cytoplasmic domains of both Vero and 143TK<sup>-</sup> cells were colocalized with  $\beta$ -COP. Moreover, the distribution of  $\beta$ -COP was consistent with the fragmentation of the Golgi in Vero cells but not in 143TK<sup>-</sup> cells. To make the coincidence of the  $\beta$ -COP and  $U_L20$  colocalization more visible, the  $U_L20$  antibody fluorescence in Vero cells was diminished (Fig. 9b and c). Figure 10 shows sections of another infected Vero cell reacted with  $U_L20$  antibody and anti-rabbit IgG conjugated to FITC. The results show a strongly fluorescent ring around the nucleus which extends into but does not fill the cytoplasm.

On the basis of the association of  $U_L20$  with membranes (6) and its localization in infected cells, we conclude that this protein is present in nuclear membranes and in various cytoplasmic structures, including all or portions of the Golgi apparatus. As noted above,  $U_L20$  is not present in detectable amounts on the infected-cell surface.

In a second series of experiments, Vero cells were cotransfected with a plasmid containing the entire coding sequence of  $U_L20$  driven by either the  $\alpha 4$  or the  $\alpha$ - $\gamma$  promoter (see Materials and Methods) and with a second plasmid containing the  $\alpha$ -TIF gene and its regulatory sequences. Figure 11 shows that in the absence of viral infection, the fluorescence due to the  $U_L20$  protein was diffused throughout the cytoplasm and

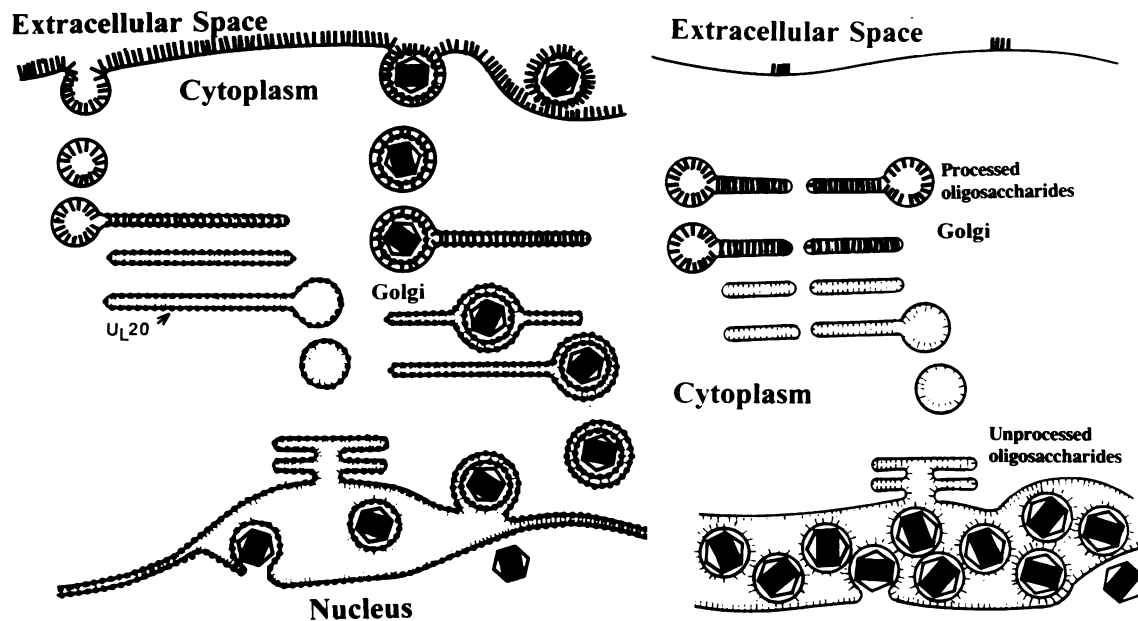


FIG. 12. Schematic representation of the distribution of U<sub>L</sub>20, virions, and cellular membrane-associated glycoproteins in Vero and 143TK<sup>-</sup> cells infected with wild-type and U<sub>L</sub>20<sup>-</sup> virus. The right panel shows the events occurring in U<sub>L</sub>20<sup>-</sup> virus-infected Vero cells. From the bottom of the diagram up, capsids become enveloped at the outer nuclear membrane and accumulate in the space between the nuclear membranes. The oligosaccharides on the glycoproteins in virions and those contained in the nuclear membranes are not processed (3). However, the glycoproteins associated with membranes do reach the Golgi apparatus and are fully processed, but they are retained in the *trans*-Golgi compartment (i.e., in a compartment after addition of the terminal sialic acid) and are not transported to the plasma membrane. The thin dashed lines represent unprocessed oligosaccharides, the Golgi stacks are shorter to emphasize that they are fragmented and dispersed, and the thicker dashed lines represent processed oligosaccharides. The left panel represents 143TK<sup>-</sup> cells infected with wild-type virus. In these instances the glycoproteins associated with cellular membranes transit from the *trans*-Golgi to the plasma membrane, and the virions enter the exocytic pathway through transport vesicles formed at the outer nuclear membranes. The dots lining all membranes except the plasma membrane represent U<sub>L</sub>20 protein, which does not reach the plasma membrane. The Golgi here is shown to be intact, as would be expected in infected 143TK<sup>-</sup> cells. Note that the same general pattern of protein and virion distribution would occur in 143TK<sup>-</sup> cells infected with U<sub>L</sub>20<sup>-</sup> virus, except that U<sub>L</sub>20 protein would be absent, or in Vero cells infected with wild-type virus, except that the Golgi apparatus would be fragmented and dispersed.

included the perinuclear space. It is noteworthy that the amount of U<sub>L</sub>20 fluorescence detected in the transfected cells was as high or higher than that seen in infected cells, possibly because the promoters fused to the U<sub>L</sub>20 coding sequences were stronger than the natural promoter of the gene.

## DISCUSSION

The studies presented in this report show the following.

(i) Antiserum raised against a chimeric protein consisting of the β-galactosidase gene fused to 95% of the coding sequences of the U<sub>L</sub>20 protein reacted with a polypeptide with an *M<sub>r</sub>* of approximately 24,000. This is consistent with the predicted molecular weight of the protein and with the results of previous studies with epitopically tagged protein as well as with antipeptide antisera used to immunoprecipitate U<sub>L</sub>20 (*M<sub>r</sub>* ~ 21,000) from infected cells (6, 29).

(ii) Addition of phosphonoacetate to cells infected with HSV-1(F) did not block synthesis of U<sub>L</sub>20. The protein accumulated and was detected by immunoblotting beginning at 6 h after infection. We conclude that U<sub>L</sub>20, a protein dispensable for viral replication in cells in culture, is regulated as a γ<sub>1</sub> gene. Our studies are at variance with a previous report which suggested that the U<sub>L</sub>20 protein is encoded by an essential gene and is expressed as early as 2 to 4 h postinfection (29).

(iii) The predicted amino acid sequence of the U<sub>L</sub>20 protein encoded by HSV-1 strain 17 (30) does not contain consensus

sites associated with N-linked glycosylation, and pulse-chase analysis of U<sub>L</sub>20 did not reveal any significant alterations in the relative electrophoretic mobility of the protein in either the presence (Fig. 4) or absence (data not shown) of monensin.

(iv) U<sub>L</sub>20 could not be detected at the cell surface as measured either by antibody staining of unfixed cells by the black plaque assay (Fig. 5) or by immunoprecipitation of cell surface proteins (Fig. 6 and 7). These results were surprising since all of the membrane proteins that have been shown to be virion associated have also been detected in the plasma membrane (5, 17, 19, 37, 39). Whether U<sub>L</sub>20 contains specific retention signals to prevent it from reaching the cell surface is unknown.

(v) U<sub>L</sub>20 was present in virions isolated from extracellular medium.

(vi) Immunofluorescence analyses of infected cells double stained with antibodies to U<sub>L</sub>20 and β-COP protein revealed that U<sub>L</sub>20 is present in a cytoplasmic compartment involved in the exocytic pathway. In addition, U<sub>L</sub>20 was detected in a perinuclear ring, possibly the nuclear membrane. Of significance is the finding that in certain cell types (Vero and HEp-2) infected with HSV-1, the Golgi becomes fragmented and dispersed, and there is a concomitant redistribution of Golgi-resident enzymes and of a Golgi coat protein (8). This redistribution is a late event in the viral replicative cycle and possibly reflects a disequilibrium between anterograde and

retrograde transport caused by an overwhelming influx of viral glycoproteins and virions into the exocytic pathway (8). Our results showed that regardless of cell type, the distribution of U<sub>L</sub>20 closely approximated that of the Golgi-associated protein.

The results reported here raise several key issues with respect to viral egress and the role of U<sub>L</sub>20 in this process. While it is generally agreed that capsids assemble in the nucleus and are enveloped at the inner nuclear membrane (38, 39), the precise events which govern virion transport between the site of envelopment and the extracellular space have been the subject of much debate (reviewed in reference 40). The results obtained from several studies support the model, proposed on the basis of electron microscopic studies by Schwartz and Roizman (42), that virions are transported through the cytoplasm in membrane-bound vesicles (9, 22, 38, 40, 48) as would be expected for macromolecules that are destined for export from the cell. Vesicularly transported proteins follow a default pathway from the endoplasmic reticulum through the Golgi to the plasma membrane unless the proteins contain specific retention signals (reviewed in references 34, 36, and 41). That virions and viral glycoproteins utilize the normal cellular exocytic pathway has been suggested by the observations that viral glycoprotein processing and maturation as well as virion exocytosis are impaired in mutant cells defective in Golgi enzymatic activities or in competent cells exposed to agents which disrupt this pathway (10, 11, 22). Although in cells infected with the U<sub>L</sub>20<sup>-</sup> virus, virion exocytosis was blocked in a pre-Golgi compartment, viral glycoproteins were transported at least to the *trans*-Golgi inasmuch as they contained terminal sialic acid residues (3). These observations suggest that the vesicles which transport virions may have an origin different from that of vesicles which transport viral glycoproteins (3, 6). The apparent presence of U<sub>L</sub>20 in the nuclear membrane is consistent with the requirement for U<sub>L</sub>20<sup>-</sup> to facilitate the exocytosis of virions from the space between the inner and outer nuclear membranes. The phenotype of the U<sub>L</sub>20<sup>-</sup> virus, as well as that of a virus carrying a temperature-sensitive mutation in the gH gene and characterized by retention of infectious virus containing gH in infected cells maintained at the nonpermissive temperature (12), suggests that viral egress may not occur by default, as would normally be expected for vesicularly transported macromolecules, but rather as a directed series of events.

The results of our studies and the considerations cited above lead us to propose the model illustrated in Fig. 12. According to this model, U<sub>L</sub>20 is present in the nuclear membranes, possibly in the endoplasmic reticulum (although this remains to be proven), in the Golgi apparatus, and, as a consequence of envelopment, also in virion envelopes. The U<sub>L</sub>20 present in transport vesicles is retained in some Golgi compartment and does not reach the plasma membrane. Finally, the U<sub>L</sub>20 protein present in virions remains associated with the virion envelope throughout its transit through the cytoplasm. The U<sub>L</sub>20 protein is required for exocytosis of virions in infected Vero and HEP-2 cells and, most probably, in infected cells in which the Golgi apparatus is fragmented and dispersed throughout the cytoplasm. Conceivably the gene encoding the U<sub>L</sub>20 proteins may have evolved to compensate for the fragmentation of the Golgi apparatus.

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