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The U_L20 Gene of Herpes Simplex Virus 1 Encodes a Function Necessary for Viral Egress

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A recombinant virus from which the start codon and 53% of the U_L20 open reading frame had been deleted was constructed and characterized. We report the following: (i) The U_L20^- mutant formed small plaques in 143 tk^- cells but failed to form plaques in Vero cells. Virus yields were approximately 10- to 100-fold lower than those of wild-type virus in all cell lines tested. (ii) Electron microscopic examination of Vero cells infected with the U_L20^- mutant revealed that enveloped and unenveloped capsids accumulated in the cytoplasm, possibly in the space between the inner and outer lamellae of the nuclear membrane, and that virtually no virus was present in the extracellular space. (iii) Glycoproteins B, C, D, E, H, and I recovered from lysates of cells infected with the U_L20^- mutant could not be differentiated from those present in lysates of cells infected with the wild-type parent virus with respect to the electrophoretic mobility of mature and precursor forms. (iv) Repair of the deleted sequences restored the wild-type phenotype. (v) The gene product of the U_L20 gene was shown to be associated with cellular membranes and to possess characteristics of integral membrane proteins. We conclude that the U_L20 gene encodes an integral membrane protein with a hitherto unrecognized function in that it enables the transit of virions to the extracellular space. The function of the U_L20 gene product is complemented by some cell lines but not by Vero cells. The vesicles which serve to transport virions may have an origin different from those associated with transport of normal cellular proteins.

Current understanding of the rules governing egress from cells is that macromolecules present in the lumen of transport vesicles are destined for export unless they contain specific sequences which cause them to be retained in the cell (29). Consistent with this view, it has been assumed that herpes simplex viruses (HSV) become enveloped at the nuclear membrane and are transported by a default pathway past the Golgi into the extracellular space. The possibility that the egress is directed and does not occur by default emerged from studies on cells infected with a temperature-sensitive mutant in glycoprotein H (gH) and maintained at the nonpermissive temperature. Infectious virus containing gH was retained in these cells, whereas the virus which was released lacked gH and was not infectious (13). In this paper, we report evidence indicating that the transport of HSV from infected cells is ordered, that a viral protein is involved in the transport function, and that cellular functions may substitute for the viral function in some cells. Relevant to this report are the following considerations.

(i) The HSV-1 genome consists of two covalently linked components, L and S, each consisting of unique sequences (U_L and U_S) flanked by inverted repeats (16, 39, 46). Sequencing, mapping of mRNA, and analyses of mutants have shown that the HSV-1 genome encodes a minimum of 75 open reading frames, of which 69 map in U_L and U_S and 6 map in the inverted repeats flanking the unique sequences (11, 21, 26, 47).

(ii) The HSV-1 genome encodes seven well-characterized glycoproteins, designated gB, gC, gD, gE, gH, gG, and gI (1, 15, 22, 24, 28, 42, 43). In addition, McGeoch et al. (26) suggested that the products of the open reading frames U_L10, U_L20, U_L43, and U_L53 may be intrinsic membrane proteins on the basis of their predicted amino acid sequences. The U_L10 open reading frame has been deleted (2). In a recent report, this open reading frame was inactivated by insertion (25), but in the same studies attempts to inactivate the U_L20 gene were unsuccessful and the authors suggested that the U_L20 gene encoded an essential function. In this paper, we report on the construction and properties of a deletion mutant in the U_L20 gene.

MATERIALS AND METHODS

Cells and viruses. HSV-1 strain F [HSV-1(F)] is the prototype HSV-1 strain used in our laboratories (14). Recombinant viruses in this report were derived from HSV-1(F)Δ305, a thymidine kinase (*tk*) deletion mutant of HSV-1(F) described elsewhere (34). Titrations and preparation of HSV-1(F), HSV-1(F)Δ305, R7214, and R7230 stocks were done in Vero cells. For comparing the growth properties of viruses reported herein, viruses were titrated in 143 thymidine kinase-minus ($143TK^-$) cells originally obtained from Carlo Croce. Titrations and viral stocks of R7223 and R7225 were made from lysates of infected $143TK^-$ cells. Transfections were done in rabbit skin cells originally obtained from J. McClaren. Selection of tk^+ viruses was done in human $143TK^-$ cells. The cell lines were maintained in Dulbecco's modified Eagle medium supplemented with either 5% newborn calf serum (Vero and rabbit skin cells) or 5% fetal calf serum and 40 μg of bromodeoxyuridine per ml ($143TK^-$ cells).

Construction of plasmids. Restriction enzymes were obtained from New England Biolabs, Beverly, Mass. T4 DNA ligase and the Klenow fragment of DNA polymerase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. T4 DNA polymerase was obtained from U.S. Biochemical, Cleveland, Ohio.

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The *Bam*HI Q fragment of HSV-1(F) is contained within pRB1028, which has been described previously (2). pRB3367 contains the entire *tk* gene under the control of the α27 promoter (α27-*tk*) and has been described elsewhere (23).

A 5.7-kbp *Eco*RI/*Hin*dIII fragment which contains the right end of the *Eco*RI G fragment and left end of the *Hin*dIII A fragment of HSV-1(F) DNA (0.256 to 0.294 map units) was cloned into the *Eco*RI/*Hin*dIII sites of pGEM3Z (Promega), and the plasmid was designated pRB451. pRB452 contained a 3.4-kbp *Hin*dIII/*Nru*I fragment of pRB451 which spanned HSV-1(F) DNA from 0.256 to 0.277 map units. This fragment was cloned into the *Hin*dIII/*Sma*I sites of pGEM3Z. A 1.8-kbp *Hin*dIII/*Eco*RI fragment from pRB3367 which contained the α27-*tk* gene with ends which were blunted by T4 DNA polymerase was cloned into a similarly blunted unique *Bst*EII site of pRB452. This plasmid, designated pRB4127, contained the α27-*tk* gene between U_L20 and U_L21, with the direction of transcription of the *tk* gene the same as that of U_L20 (Fig. 1, lines 2 to 4). The 5′ end of the U_L20 gene was deleted by cutting pRB452 with *Bst*EII and *Hpa*I, blunting the ends with T4 polymerase, and ligating. This plasmid was designated pRB4128 (Fig. 1, line 5). A 2.1-kb *Hin*dIII/*Dra*III fragment of pRB452 was cloned into the *Hin*dIII/*Hin*cII sites of pGEM3Z and was designated pRB453. This plasmid was used to rescue the sequences deleted from the U_L20 gene (Fig. 1, line 6).

To tag the U_L20 open reading frame, a plasmid containing the *Bam*HI F′ fragment (pRB4082) of HSV-1(F) in pGEM3Z was cleaved with *Bgl*II and *Eco*RI, and a *Bgl*II/*Eco*RI fragment from pRB451 was inserted. The resulting plasmid, pRB4229, therefore contained DNA from the *Bam*HI F′ fragment to the *Eco*RI site in pRB451. pRB4229 was cleaved with *Bgl*II and was blunted with the Klenow fragment of DNA polymerase I. A double-stranded DNA oligomer (AAAAGGGACAGAAG CCCAACCTGCTAGACCGACTGCGACACCGCAAAAAC GGGTACCGACAC and its complement) encoding a human cytomegalovirus (CMV) epitope, N-KGQKPNLLDRLRHRK NGYRH-C, which is recognized by monoclonal antibody (MAb) CH28-2 (21), was cloned into the blunted *Bgl*II site of pRB4229, placing the epitope between amino acids 13 (Asp) and 14 (Leu) of U_L20. The plasmid, which was designated pRB4230 and is represented in Fig. 7, line 3, encodes N–Asp-13–Leu–epitope–Asp–Leu-14–C. Amino acids flanking the epitope (underlined) were duplicated to ensure that the open reading frame of U_L20 remained intact. The DNA encoding and flanking the CMV epitope of pRB4230 was sequenced to confirm that the epitope was in frame and intact.

Selection of recombinant viruses. tk^- progeny viruses were selected on 143TK$^-$ cells in the presence of bromodeoxyuridine. tk^+ progeny were selected on 143TK$^-$ cells overlayed with HAT medium, which is a mixture of Dulbecco's modified Eagle medium supplemented with hypoxanthine, aminopterin, thymidine, and 5% fetal calf serum.

Purification and analysis of viral DNA. Viral DNA intended for transfections was prepared from NaI gradients as previously described (48). Otherwise, viral DNA was purified by phenol-chloroform extraction of cytoplasmic extracts of infected Vero cells (44). Nick translations were done according to the manufacturer of a kit designed for this purpose (Du Pont, Wilmington, Del.). The separation of DNA fragments and transfer to nitrocellulose were done according to the method of Southern (41) as modified by Longnecker and Roizman (23). Hybridization conditions were as previously described (27, 33). Some fragments for nick translation and cloning were prepared by using glass powder in saturated sodium iodide (45). All DNA fragments were purified after separation in low-gelling-temperature agarose (FMC Bioproducts, Rockland, Maine).

Electron microscopy. Electron microscopic examinations were done in a Siemens 102 microscope. The procedures for staining and fixation were the same as previously described (7).

Glycoprotein biosynthesis. Vero cells infected with 5 PFU of HSV-1(F) or R7225 per cell were labeled between 7 and 16 h after infection with [^{35}S]methionine (1,300 Ci/mmol; The Radiochemical Centre, Amersham, England) at a concentration of 50 μCi/ml of medium containing 1/100 the usual concentration of unlabeled methionine. The glycoproteins were immunoprecipitated from cell lysates with the indicated MAbs as detailed elsewhere (6) and separated by electrophoresis in sodium dodecyl sulfate (SDS)–8.5% polyacrylamide gels cross-linked with *N*,*N*′-diallyltartardiamide (DATD). The gels were soaked in Amplify and exposed to Kodak X-Omat film for fluorography. The MAbs were as follows: H233 anti-gB, HCl anti-gC, HD1 anti-gD, H600.1 anti-gE (30), 52S anti-gH (40), and Fd69 anti-gI (22).

Preparation and analysis of membrane vesicles. HEp-2 cells were infected with at least 5.0 PFU per cell and were labeled at 16 to 20 h after infection with [^{35}S]methionine (>1,000 Ci/mmol; Amersham) at a concentration of 15 μCi/ml of medium 199 supplemented with 1% newborn calf serum. Infected cells were disrupted by 20 strokes of a Dounce homogenizer, and the lysate was clarified by low-speed centrifugation to remove the nuclei as previously described (43). The cytoplasmic fraction was made to 50% sucrose by weight and was placed in 38-ml ultracentrifuge tubes. A continuous 10 to 45% (wt/wt) sucrose gradient in phosphate-buffered saline (PBS) lacking Ca^{2+} and Mg^{2+} ions was layered over the cytoplasmic extract, and the gradients were spun at 20,000 rpm in an SW28 rotor for 20 h. Membrane vesicles floated approximately one-third from the bottom of the tube. Two-milliliter fractions were collected from the top of the gradients, diluted at least 10-fold with PBS, and pelleted at 30,000 rpm for 90 min in a Vti30 rotor. Fraction pellets were resuspended in 100 μl of PBSA*, which contains 1.0% Nonidet P-40, 1.0% sodium deoxycholate, 10 μM tolylsulfonyl phenylalanyl chloromethyl ketone, and 10 μM α-tosyl-L-lysine chloromethyl ketone in PBS. Disruption buffer (5% β-mercaptoethanol–2% SDS in 50 mM Tris-HCl [pH 7.0]) was added to each fraction, and the samples were sonicated for 1 min at room temperature. Samples were separated on 12.5% SDS-polyacrylamide gels cross-linked with DATD, transferred electrically to nitrocellulose, and allowed to react as previously described (4) with either MAb CH28-2, which recognizes the CMV epitope, or MAb 170-1, which recognizes gD. Both MAbs were kindly provided by Lenore Pereira.

Alkaline treatment of membrane vesicles. Purified membrane vesicles were obtained by sucrose flotation and pelleted as described above. The pellets were treated with 0.1 M sodium carbonate (pH 11.5) for 30 min on ice and were centrifuged for 30 min in an Eppendorf microfuge. The pellet and supernatant were then separated on a 12.5% DATD-polyacrylamide gel and analyzed as detailed above.

RESULTS

Deletion of U_L20. To delete the U_L20 gene, the α27-*tk* gene was cloned between U_L20 and U_L21 (plasmid pRB4127) such that the direction of transcription of the *tk* gene was the same as that of U_L20 (lines 3 and 4 of Fig. 1). Cotransfection of pRB4127 with viral DNA from the tk^- virus HSV-1

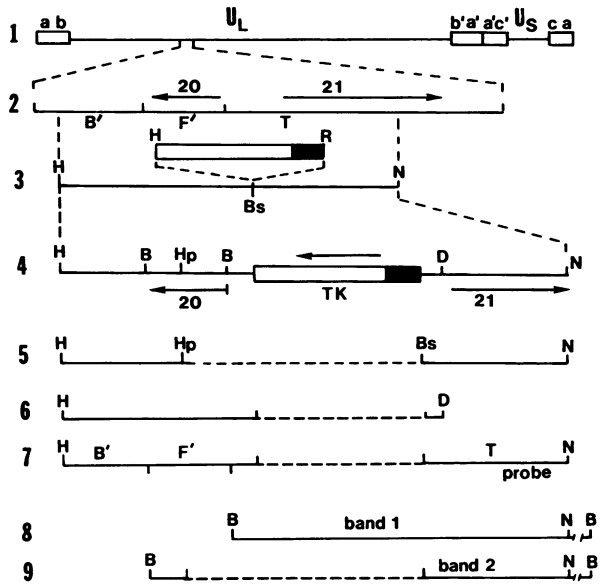

FIG. 1. Sequence arrangements of plasmids used for the deletion and repair of U_L20. Lines: 1 and 2, locations within the genome of HSV-1(F) of the *Bam*HI B', F', and T fragments and the U_L20 and U_L21 open reading frames; 3 and 4, sequence arrangements of insertion of the α27-*tk* gene between the U_L20 and U_L21 open reading frames as in pRB4127; 5, representation of pRB4128 in which 581 bp have been deleted, including the 5' end of the U_L20 open reading frame; 6, diagram of pRB453, which was used to repair the deletion of U_L20; 7, diagram of probe pRB451, which hybridizes with the depicted *Bam*HI B', F', and T fragments of HSV-1 DNA; 8, sequence arrangement of the DNA contained within band 1 in Fig. 2. This R7214-specific band, which hybridizes with the probe depicted in line 7, resulted from the insertion of the α27-*tk* gene within the *Bam*HI T fragment of HSV-1(F)Δ305; 9, sequence arrangement of the DNA within the R7225-specific band 2, which hybridizes with the pRB452 probe. Band 2 arose as a consequence of the deletion of U_L20 which caused the fusion of the truncated BamHI F' and T fragments. Restriction sites: B, *Bam*HI; Bs, *Bst*EII; Hp, *Hpa*I; D, *Dra*III; H, *Hin*dIII; N, *Nru*I; R, *Eco*RI. The filled box represents the α27 promoter. The open box represents the coding sequences of the *tk* gene. Arrows indicate the direction and approximate length of each indicated open reading frame.

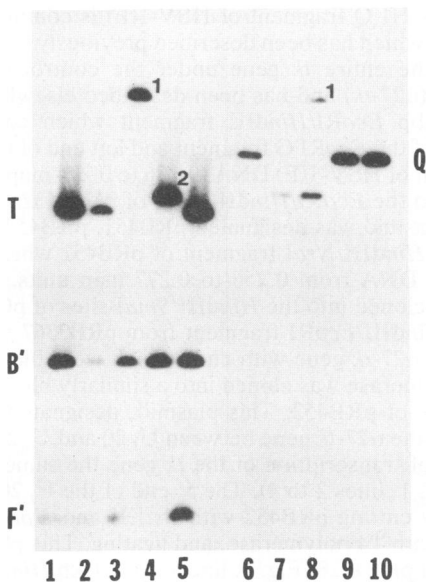

FIG. 2. Autoradiographic images of the electrophoretically separated restriction digests of the DNAs of viruses generated for and a result of the deletion of the U_L20 gene. The DNAs of HSV-1(F) (lanes 1 and 6), HSV-1(F)Δ305 (lanes 2 and 7), R7214 (lanes 3 and 8), deletion mutant R7225 (lanes 4 and 9), and the U_L20-repaired virus R7230 (lanes 5 and 10) were cleaved with *Bam*HI, separated on a 1.0% agarose gel, and transferred to two nitrocellulose sheets. The duplicate blots were then probed with pRB451 (lanes 1 through 5), which contains the U_L20 gene (see Fig. 1), or *Bam*HI-Q (lanes 6 through 10), which contains the *tk* gene. Positions of the *Bam*HI F', B', T, and Q fragments are indicated.

(F)Δ305 and subsequent selection of the tk^+ progeny in 143TK$^-$ cells overlaid with HAT medium yielded the recombinant virus R7214. Viral DNAs from HSV-1(F), HSV-1(F)Δ305, and R7214 were cleaved with *Bam*HI, electrophoretically separated on a 1.0% agarose gel, and transferred to two nitrocellulose sheets. One of the nitrocellulose sheets was then probed with nick-translated pRB452 (Fig. 2, lanes 1 to 5). The pRB452 probe hybridized with the *Bam*HI T fragment (2.8 kbp), *Bam*HI B' (1.5 kbp), and *Bam*HI F' (0.77 kbp) fragments in HSV-1(F) (Fig. 2, lane 1) and HSV-1(F)Δ305 (Fig. 2, lane 2) DNAs. Because of the insertion of the α27-*tk* gene into the *Bam*HI T fragment of R7214, this fragment increased in size to approximately 4.6 kbp (band 1, Fig. 2, lane 3) in R7214 DNA. The duplicate nitrocellulose blot was probed with *Bam*HI-Q, which contains the native *tk* gene. Band 1 in Fig. 2 (lane 8) hybridized with the *Bam*HI-Q probe because of the inserted *tk* gene within the *Bam*HI T fragment of R7214 DNA. The *Bam*HI-Q probe also hybridized with the 3.6-kbp *Bam*HI Q fragment of HSV-1(F) (Fig. 2, lane 6) and the truncated 3.1-kbp *Bam*HI Q fragments of HSV-1(F)Δ305 (Fig. 2, lane 7) and R7214 (Fig. 2, lane 8).

Viral DNA of R7214 was cotransfected with pRB4128 DNA into rabbit skin cells, and *tk*$^-$ progeny were selected under a bromodeoxyuridine overlay. pRB4128 contains, along with appropriate flanking sequences, a total deletion of 581 bp and a truncated U_L20 gene in which the start codon and 355 bp were removed from the 5' end of the 666-bp U_L20 open reading frame. The resulting virus, designated R7223, had deletions in both the *tk* gene and U_L20 open reading frame. Cotransfection of R7223 viral DNA with the *Bam*HI Q fragment containing an intact *tk* gene and cloned as pRB1028 followed by selection in 143TK$^-$ cells overlaid with HAT medium yielded R7225. The viral DNA of R7225 was digested with *Bam*HI, electrophoretically separated in a 1.0% agarose gel, transferred to two sheets of nitrocellulose, and then probed with pRB451. R7225 DNA differed from HSV-1(F) and HSV-1(F)Δ305 DNA in that it lacked the 0.77-kbp *Bam*HI F' and 2.8-kbp *Bam*HI T fragments (compare lanes 1 and 2 with lane 4 in Fig. 2). Removal of 666 bp from the 5' end of U_L20 also removed the *Bam*HI site that defined the junction of the F' and T fragments, and these fragments are fused in a single band of approximately 2.9 kbp (designated band 2 in lane 4 of Fig. 2). The duplicate nitrocellulose sheet that was probed with the *Bam*HI Q fragment revealed that the deletion of *Bam*HI-Q which is present in HSV-1(F)Δ305 DNA was repaired in R7225 DNA (compare lanes 7 and 10). We conclude that the deletion in R7225 is solely within the U_L20 open reading frame.

Rescue of U_L20. To repair the deletion in U_L20, rabbit skin cells were transfected with linearized pRB453 that contained approximately 2.1 kbp of HSV-1(F) DNA. This DNA contained the coding domain of U_L20, 440 bp upstream of the

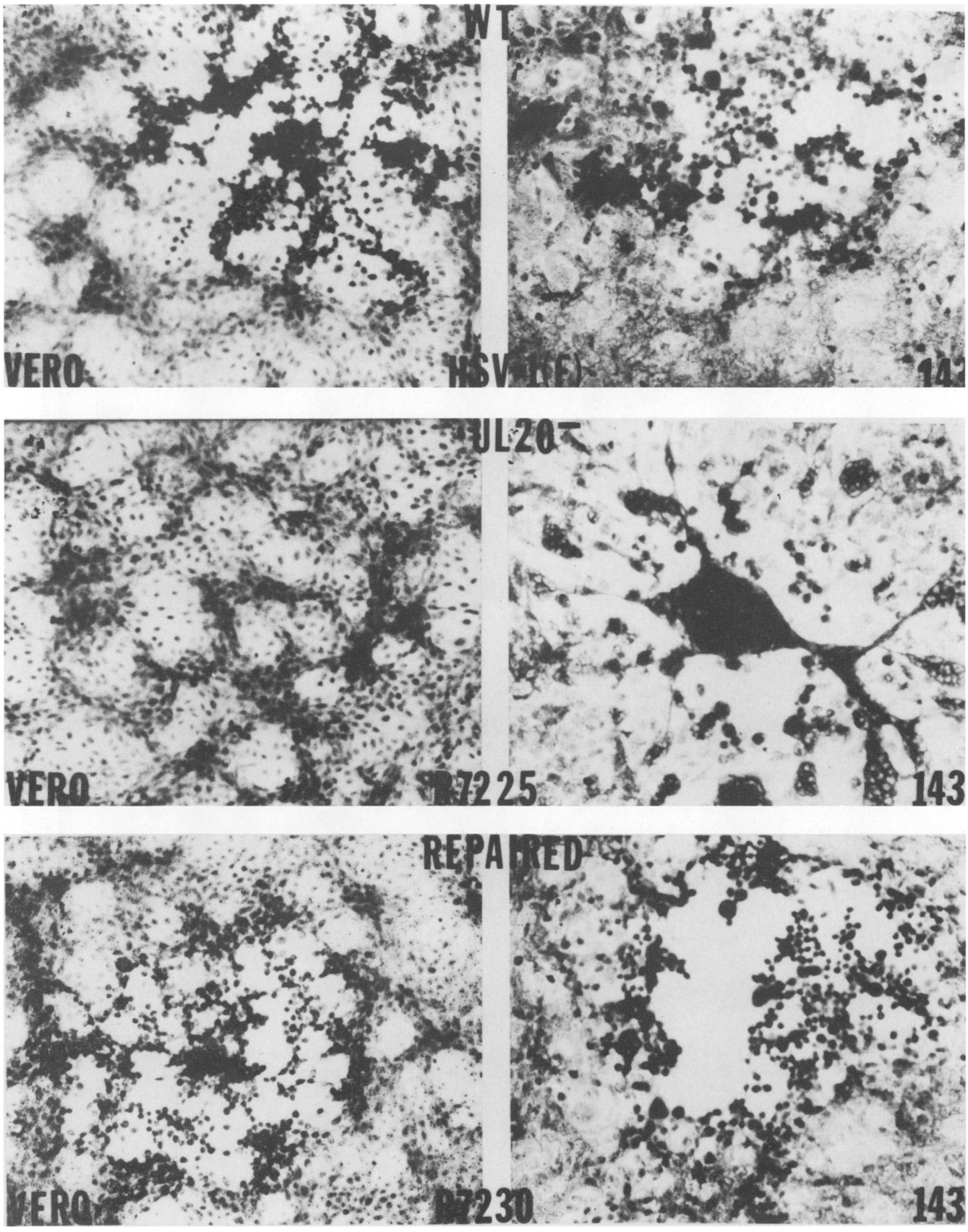

FIG. 3. Plaque morphology of R7225 in Vero and 143TK$^-$ cells. Monolayers of Vero or 143TK$^-$ cells were infected with HSV-1(F) (wild type [WT]), R7225, or R7230, as indicated, and were held at 37°C. Cells were fixed and stained 72 h after infection.

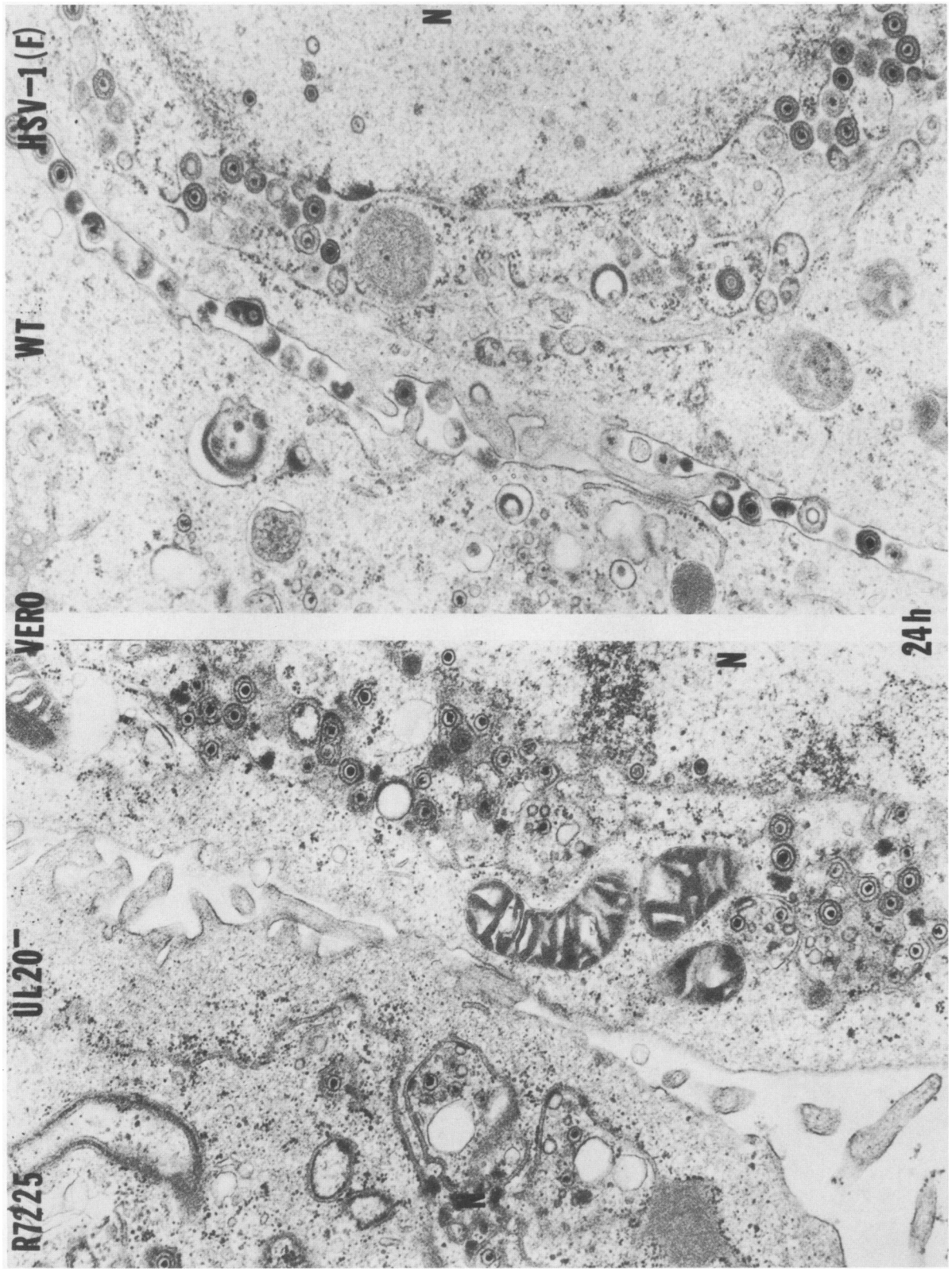

FIG. 4. Electron micrographs of Vero cells infected with R7225 or HSV-1(F). Monolayers of Vero cells were infected at 2.0 PFU per cell with either HSV-1(F) (wild type [WT]) or R7225 as indicated, incubated at 37°C for 24 h, and then fixed and prepared for electron microscopy. N, nucleus. The magnification may be calculated from the diameter of the capsid (105 nm). Note the absence of virions in the space between cells infected with R7225.

TABLE 1. Viral yield from replicate cultures 24 h postinfection with approximately 5 PFU of either wild-type, U_L20^- (R7225), or repaired (R7230) virus per cell[a]

Cell line	Yield (PFU/ml of cell extract)		
	HSV-1(F)	R7225	R7230
Vero	1.2×10^8	1.3×10^6	5.8×10^7
Rabbit skin	5.0×10^7	1.2×10^6	2.3×10^7
HEp-2	3.2×10^8	2.8×10^6	1.0×10^8
143TK$^-$	1.7×10^8	1.0×10^7	1.4×10^8

[a] Titrations were done in 143TK$^-$ cells.

U_L20 start codon, and 705 bp of the U_L19 open reading frame. Approximately 6 h after the transfection of rabbit skin cells with either pRB453 DNA or pGEM3Z, the cells were infected with R7225. Progeny virus was selected for the ability to form plaques in Vero cells. Whereas the progeny of transfection with pGEM3Z formed less than 10 PFU/ml in Vero cells, the progeny of pRB453 transfection followed by R7225 infection yielded approximately 10^3 PFU/ml in Vero cells. Four independent plaque isolates were examined by Southern analysis, and all contained a restored U_L20 gene (not shown). One of the plaque isolates was plaque purified two additional times in Vero cells and was designated R7230.

R7230 DNA was digested with *Bam*HI, separated on a 1.0% agarose gel, and transferred to two nitrocellulose sheets. The electrophoretically separated fragments in one of the blots were then hybridized with radiolabeled pRB451. As expected, the *Bam*HI T and F′ fragments of the rescued virus could not be differentiated from the corresponding fragments of the wild-type HSV-1(F) parent (compare lane 1 with lane 5 in Fig. 2). The duplicate nitrocellulose blot which was probed with *Bam*HI-Q revealed that the *Bam*HI Q fragment that was of a native size in R7225 DNA was also present in R7230 DNA (Fig. 2, lanes 9 and 10). We conclude that the capacity to form plaques in Vero cells was related to the deletion of U_L20 in R7225 inasmuch as viruses selected for growth in Vero cells exhibited a restored U_L20 open reading frame whereas viruses generated by transfection or infection with nonrelevant DNA failed to form plaques in these cells.

Plaque phenotype of the U_L20 deletion virus R7225. Viral stocks of HSV-1(F), R7225, and R7230 were plated on rabbit skin cells, Vero cells, and 143TK$^-$ cells and incubated for 48- and 72-h intervals. Plaques formed by R7225 were present and visible in 143TK$^-$ cell cultures at 48 h postinfection. These usually consisted of one large polykaryocyte (syncytium-negative [*syn*$^-$] morphology). At 72 h postinfection, the plaques in 143 cells corresponded to a titer of 10^7 PFU/ml and consisted of several polykaryocytes (Fig. 3). Small plaques in rabbit skin cells were detected at 72 h postinfection (not shown), but less than 10 PFU/ml were detected in Vero cells (Fig. 3). The repair of the U_L20 deletion in R7230 restored the ability to form plaques in Vero cells (Fig. 3, bottom panels) and conferred a *syn*$^+$ phenotype to the plaques formed in 143TK$^-$ cells.

Replication of U_L20^- virus in various cell lines. In this series of experiments, replicate cultures of Vero, rabbit skin, HEp-2, or 143TK$^-$ cells were infected, respectively, with wild-type [HSV-1(F)], R7225, or R7230 virus at 5.0 PFU per cell. The cells were harvested at 24 h postinfection and stored at −96°C until assayed on 143TK$^-$ cells. As shown in Table 1, the yield of R7225 was substantially lower in all cell lines tested. The 143TK$^-$ cell line yielded R7225 titers approximately 10-fold lower than those of the wild-type parent. In Vero and HEp-2 cell lines, R7225 titers were approximately 100-fold lower than those of the wild-type viruses. In rabbit skin cells, the titers were reduced by approximately 30-fold. The titers of R7230 were similar to those obtained from cells infected with HSV-1(F). We conclude that the deletion in the U_L20 open reading frame decreased viral yields, decreased viral plaque size in 143TK$^-$ and rabbit skin cells, and abolished plaque formation in Vero cells.

Egress of U_L20^- virus (R7225) from Vero cells is impaired. Since R7225 replicated in Vero cells but plaques did not form, it was of interest to determine whether the U_L20^- mutant virus was released from infected cells and available for infection of adjacent, uninfected cells. Vero cells were infected with 2.0 PFU of R7225 or HSV-1(F) per cell and were incubated at 37°C. At 14, 24, and 48 h postinfection, the cells were fixed and prepared for electron microscopy. The significant features of the infected cells were as follows.

(i) Incubation of cells at 37°C generally promotes egress of virus from cells as compared with lower temperatures (e.g., 34 to 35°C) of incubation. A remarkable feature was the near absence of R7225 virions in the space between Vero cells late in infection in contrast to those seen in cultures of cells infected with HSV-1(F) (Fig. 4). A particularly prominent feature was the presence of virus particles packed within membranes around the nucleus late in infection (Fig. 5).

(ii) A striking feature of the R7225-infected cells was the accumulation of large numbers of virions in the cytoplasm, possibly in the space between the inner and outer lamellae of the nucleus. Coincident with the accumulation of enveloped particles between membrane sheets readily seen in Fig. 5, there were numerous unenveloped capsids in the adjacent cytoplasmic space.

Fully processed glycoproteins of U_L20^- virus are present in lysates of infected Vero cells. Several studies have indicated that a block in the processing of the HSV glycoproteins performed by the Golgi enzymes leads to an intracytoplasmic accumulation of the virus and a reduction in virus egress from the cell (5, 9, 17, 38; for a review, see reference 9). Since the phenotype of R7225 in Vero cells was the virtual absence of extracellular virus, in the next series of experiments we investigated whether the block in R7225 egress from Vero cells was accounted for by defective processing of the glycoproteins. The autoradiographic images in Fig. 6 show that under the test conditions used, mature forms of all of the glycoproteins tested (gB, gC, gD, gE, gH, and gI) were present in Vero cells infected with either R7225 or wild-type HSV-1(F) in similar amounts. This was a surprising result that indicates that the block related to the U_L20 gene product relates specifically to transport and not to the processing of these HSV-1 glycoproteins.

The U_L20 gene product associates with cellular membranes. Given the phenotype of the U_L20 deletion, it was of interest to determine the intracellular location of the protein specified by the U_L20 gene. To track the protein, a DNA sequence encoding a CMV epitope and containing a *Kpn*I restriction site was inserted in frame within the 5′ end of the cloned U_L20 open reading frame (Fig. 7). As in a protocol described earlier in the text, rabbit skin cells were transfected with the resulting plasmid (pRB4230) and infected with R7225 virus. The progeny virus were plated on Vero cells, and a virus selected for its ability to form plaques on Vero cells was plaque purified and designated R7401.

To test for the presence of the inserted sequence encoding the epitope, the DNAs of HSV-1(F), R7225, R7230, and

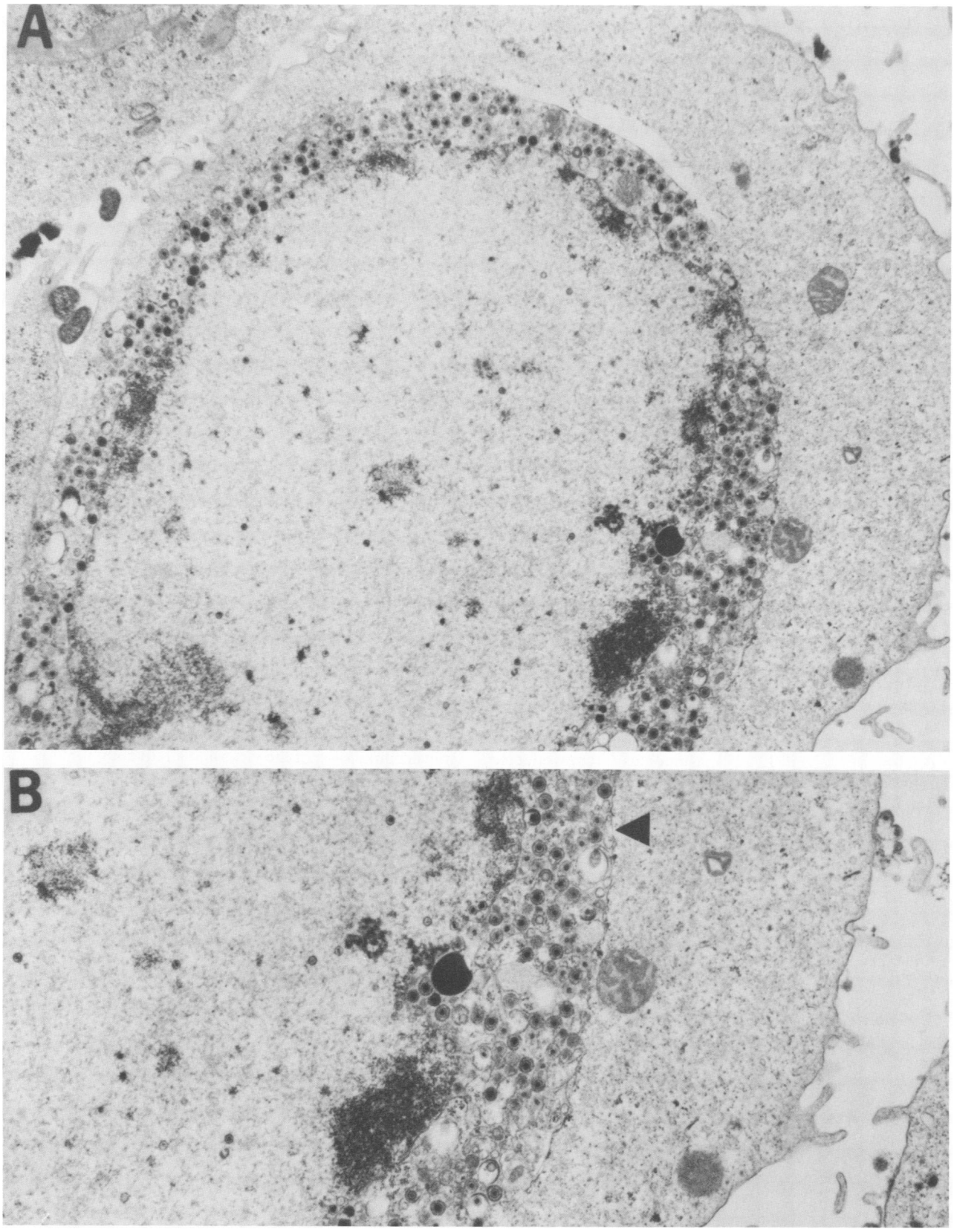

FIG. 5. Electron micrographs of Vero cells infected with R7225. The procedures were the same as those described in the legend to Fig. 4 except that cells were fixed 48 h after infection. (A) Low magnification of a cell infected with R7225; (B) higher magnification of a portion of the same cell. The arrowhead points to the membrane delimiting the cytoplasmic region containing extranuclear virions and unenveloped capsids.

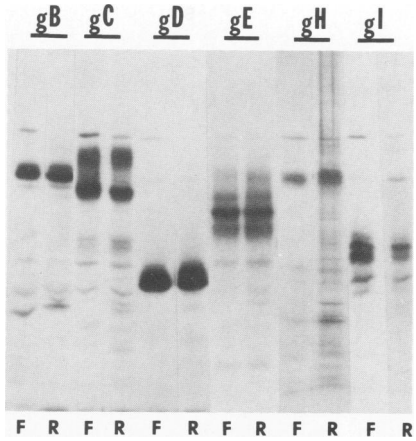

FIG. 6. Autoradiographic images of HSV-1 glycoproteins labeled with [^{35}S]methionine, immunoprecipitated with MAbs specific for gB, gD, gC, gE, gH, and gI, and electrophoretically separated in polyacrylamide gels. The Vero cells were labeled 7 to 16 h postinfection with either HSV-1(F) (lanes F) or R7225 (lanes R) as described in Materials and Methods.

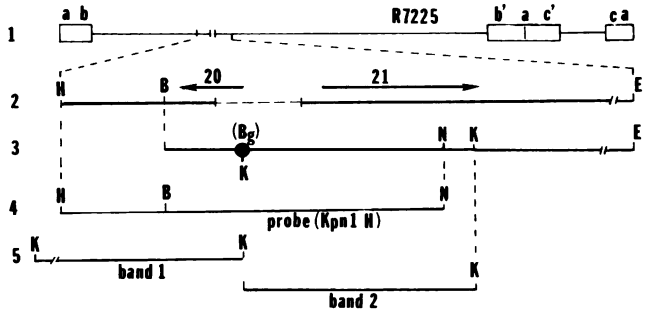

FIG. 7. Sequence arrangements of plasmids constructed in order to tag the U_L20 open reading frame with an epitope for a CMV antibody. Lines 1 and 2, sequence arrangement of the DNA contained within R7225 showing an internal deletion of the U_L20 gene. Line 3, sequence arrangement of the DNA within pRB4230 in which a CMV epitope (filled circle) containing a *Kpn*I site was cloned into the 5′ end of the U_L20 open reading frame. This plasmid was used to repair the deleted sequences in R7225 with the CMV epitope-containing U_L20 gene. Line 4, Probe pRB451, which hybridized with the *Kpn*I H fragment of the viral DNAs of HSV-1(F), R7225, R7230, and R7401 in Fig. 8. Line 5, diagram of CMV epitope insertion virus R7401-specific *Kpn*I fragments that hybridize with the pRB451 probe and appear in Fig. 8 as bands 1 and 2. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nru*I.

R7401 were digested with *Kpn*I, transferred to nitrocellulose sheets, and probed with pRB451 (Fig. 7, line 4). pRB451 hybridizes with the *Kpn* H fragment of HSV-1(F), which is approximately 9.4 kbp in length (Fig. 8, lane 1). A *Kpn*I fragment of R7230 DNA of a size indistinguishable from that of HSV-1(F) also hybridized with the *Kpn*I H probe (Fig. 8, lane 3). As expected, the corresponding fragment of R7225 DNA was smaller due to the deletion in the U_L20 gene (Fig. 8, lane 2). Placement of the CMV oligomer in the 5′ end of the U_L20 gene introduced a *Kpn*I site into the *Kpn*I H fragment of R7401 (Fig. 8, lane 4). As a result, two fragments of 7.3 and 2.1 kbp (Fig. 8, bands 1 and 2, respectively) hybridized with the *Kpn*I-H probe in R7401 DNA. We conclude that the epitope was inserted into the U_L20 open reading frame as designed.

McGeoch et al. indicated that on the basis of the predicted amino acid sequence, the U_L20 gene product should be capable of spanning cellular membranes two or three times (26). To determine whether the gene product was membrane associated, we separated infected cell membranes from denser cytoplasmic constituents by flotation through a continuous sucrose gradient. The infected cell materials in each gradient fraction were then centrifuged, solubilized and separated on a denaturing 12.5% DATD-polyacrylamide gel, transferred to nitrocellulose sheets, and reacted with MAb CH28-2. As a control, an antibody (H170-1) specific for gD was used to label membrane fractions of the cells. Under the conditions used, membrane-bound proteins migrate upward in the gradient and other structures (virions, mitochondria, etc.) remain in the lower part of the gradient. The results of a representative experiment (Fig. 9) indicate that the product of the U_L20 gene and gD were present in the same membrane fractions. It is clear from Fig. 9 that the U_L20 protein was present in membrane fractions of the sucrose gradient.

The U_L20 gene product shares properties of intrinsic membrane proteins. Unlike proteins that span the membrane once, membrane proteins that span the membrane more than once are resistant to extraction with alkali (reference 37 and references therein), and because of their hydrophobicity, they tend to aggregate on boiling in SDS. Purified membrane fractions were obtained by sucrose flotation and pelleted. The pellets were resuspended in sodium carbonate buffer (pH 11.5), allowed to incubate on ice, and then pelleted. Material from the supernatant fluid and pellet were then separated on a denaturing 12.5% DATD-polyacrylamide gel, transferred to nitrocellulose sheets, and reacted with MAb CH28-2 and MAb H170-1, respectively. The results (Fig. 10)

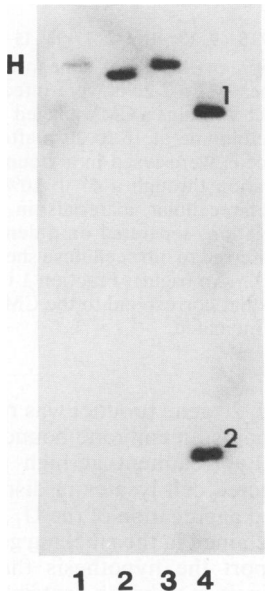

FIG. 8. Autoradiographs of *Kpn*I restriction digests of viral DNA of the U_L20-tagged R7401 recombinant virus. Viral DNAs of HSV-1(F) (lane 1), the deletion virus R7225 (lane 2), the repaired virus R7230 (lane 3), and the CMV-tagged virus R7401 (lane 4) were cut with *Kpn*I, separated on a 1.0% agarose gel, transferred to nitrocellulose, and hybridized with the *Kpn*I-H-specific probe pRB451.

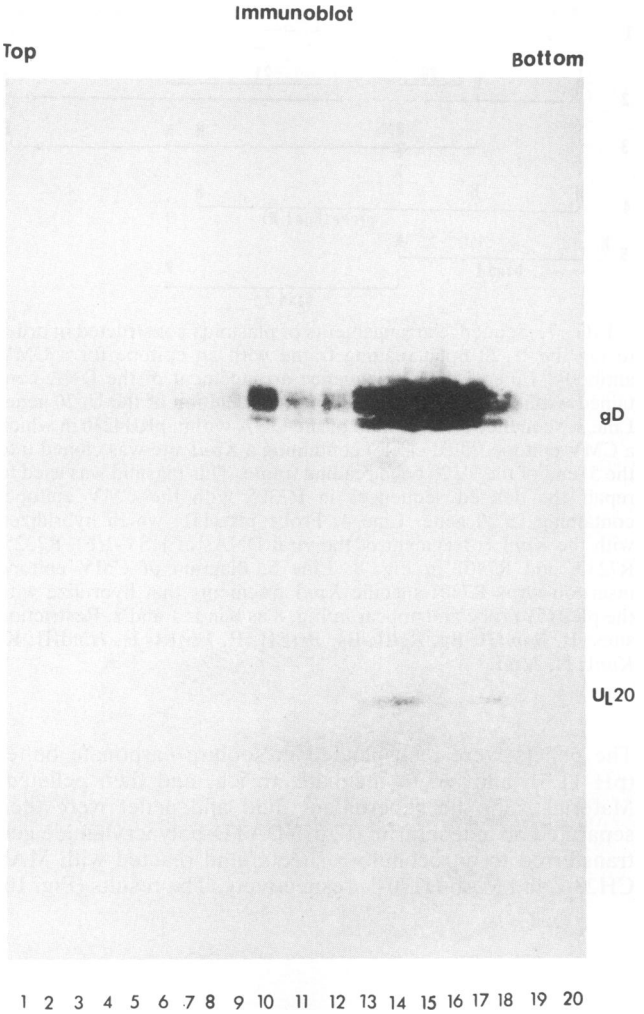

FIG. 9. Association of the U_L20 gene product with R7401-infected cell membranes. HEp-2 cells were infected at 10.0 PFU per cell with R7401, which contains a CMV-tagged U_L20 gene, and were labeled with [^{35}S]methionine at 16 to 20 h after infection. At 20 h after infection, the cells were lysed in a Dounce homogenizer and floated by centrifugation through a 45 to 10% (wt/wt) continuous sucrose gradient. The cellular materials in each fraction were pelleted by centrifugation, separated on a denaturing DATD-polyacrylamide gel, transferred to nitrocellulose sheets, and reacted with anti-CMV or anti-gD MAb (right). Fraction 1 is from the top of the gradient. The bands that correspond to the CMV-tagged U_L20 gene product and gD are indicated.

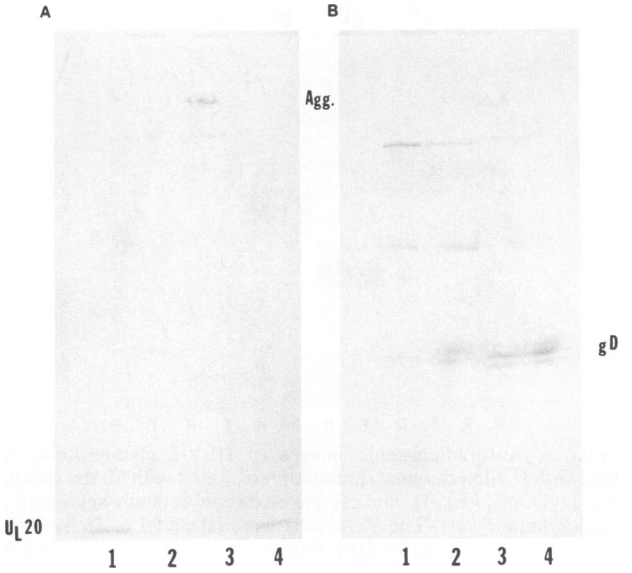

FIG. 10. Immunoblots of boiled or alkaline-treated R7401-infected cells. Lysates of HEp-2 cells which were infected with R7401 at 5.0 PFU per cell were either boiled (lanes 3) or incubated at room temperature in a disruption buffer that contained 2.0% SDS (lanes 4). The samples were separated on a denaturing DATD-polyacrylamide gel, transferred to nitrocellulose, and probed with anti-CMV antibody (A) or anti-gD antibody (B). For alkaline treatment of membrane fractions, purified membrane fractions of R7401-infected HEp-2 cells were incubated in sodium carbonate buffer (pH 11.5) and pelleted by centrifugation. Samples from the pellet (lanes 1) and supernatant fluid (lanes 2) were separated on a DATD-polyacrylamide gel, transferred to nitrocellulose, and probed with anti-CMV antibody (A) or anti-gD antibody (B). Note that upon reblotting of the nitrocellulose sheet with the anti-gD antibody, the band corresponding to U_L20 faded.

indicate that the U_L20 gene product was resistant to alkaline treatment and remained membrane bound, whereas gD was largely removed by treatment at high pH. Furthermore, boiling R7401-infected cell lysates in disruption buffer containing SDS caused aggregation of the U_L20 protein, and the aggregates were retained in the stacking gel. Together, these observations support the hypothesis that the U_L20 gene product is an intrinsic membrane protein.

DISCUSSION

The salient features of the results presented in this report are as follows: (i) the product of the U_L20 gene is a membrane protein with at least some of the properties of intrinsic membrane proteins; (ii) the U_L20 gene product is not required for the assembly, envelopment, or maturation of virions necessary for the acquisition of infectivity, and in this sense the gene is dispensable for viral growth in the cell lines tested; and (iii) the gene product is required for the efficient transport of virions from the space between the inner and outer lamellae of the nuclear membrane to the perinuclear space. Relevant to our findings are the following considerations.

(i) This is the first evidence that an HSV gene product is required for transport from the space between the inner and outer lamellae of the nuclear membrane to the extracellular space. The observation that this transport occurs in 143TK$^-$ cells and in rabbit skin cells but not in Vero cells indicates that there exist cellular gene products which perform such a function, that in some cell lines the cellular gene products can substitute for and complement U_L20^- mutants, and that this complementation cannot occur in Vero cells.

(ii) The association of the U_L20 gene product with membranes is supported by several lines of evidence. In addition to the observations that the U_L20 protein partitions with membranes containing gD and that it has some of the properties associated with intrinsic membrane proteins, the U_L20^- mutant has a *syn*$^-$ phenotype. The *syn*$^-$ phenotype is particularly significant. In general, wild-type viruses do not cause fusion of infected cells: fusion is associated with mutations in several genes. For the most part, these genes have been shown to encode membrane proteins. Among the

mutant genes with *syn*$^-$ phenotype are gB, U_L24, U_L53, and one which has been reported to map in U_L1 (3, 12, 20, 31, 32, 36). Since it is unlikely that several genes independently cause fusion of cells, it has been suggested (35) that the products of the *syn* genes form a complex and that the *syn*$^-$ phenotype is a consequence of mutations which destabilize or alter the tertiary structure of the complex.

(iii) The mechanism by which the product of the U_L20 gene enables the transport of virions through the extracellular space is unclear, and several issues relevant to a solution of this riddle remain unresolved. For example, our results show that total viral glycoproteins precipitated from cells infected with the deletion mutant cannot be differentiated from viral glycoproteins of wild-type virus-infected cells. However, the virion glycoproteins constitute a minority of the total viral glycoproteins: the majority are associated with cellular membranes. It is conceivable that the membrane-associated glycoproteins are transported through the Golgi and therefore are fully glycosylated whereas virions accumulate in a pre-Golgi compartment. If this were the case, it would indicate that the vesicles for the transport of virus to the extracellular space are distinct, virus specific, and different from the vesicles from the endoplasmic reticulum which normally proceed towards the Golgi.

What is clear is that the function of U_L20 is different from that observed in a number of experimental conditions in which processing of glycoproteins was affected. Inhibition of late glycosylation as observed in mutant cell lines defective in specific Golgi glycosyl transferases or induced by a number of glycosylation inhibitors (e.g., monensin, ammonium chloride, and brefeldin A) leads to a reduction in virus egress and concomitantly to an accumulation of virions in the cytoplasm (5, 10, 17, 19, 38). The steps in viral maturation that are blocked under these conditions are different from that blocked by the deletion of U_L20.

The U_L20 gene product of HSV-1 bears a slight similarity to the *vpu* gene product of HIV-1 in that both are small hydrophobic proteins that enable efficient virus maturation and release (18). As in the case of U_L20, the mechanism by which *vpu* renders release of virus more efficient is unknown.

(iv) Although we have not specifically quantitated the number of cytoplasmic unenveloped capsids in cells infected with the U_L20^- mutant, our impression is that the number of unenveloped capsids in the cytoplasm of R7225-infected cells was greater than that observed in cells infected with the wild-type parent. In principle, the fusion of the viral envelope with cytoplasmic membranes is likely to occur between the envelope containing viral glycoproteins and a region of the cellular membrane lacking gD, since the presence of wild-type gD precludes entry of virus into cells (8). The presence of large amounts of unenveloped capsids in the cytoplasm of Vero cells infected with the R7225 deletion mutant could be a consequence of accumulation of virions in the cytoplasm, since delay in the transit through the cytoplasm would increase the probability of membrane juxtapositions which lead to fusion. The accumulation of unenveloped capsids in the cytoplasm may also explain the lower titers of virus recovered from infected Vero cells.

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