JOURNAL OF VIROLOGY, Aug. 1992, p. 5168–5174
0022-538X/92/085168-07$02.00/0

Vol. 66, No. 8

The U_L11 Gene of Herpes Simplex Virus 1 Encodes a Function That Facilitates Nucleocapsid Envelopment and Egress from Cells

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Received 30 January 1992/Accepted 1 May 1992

The U_L11 gene of herpes simplex virus 1 was reported to encode a myristylated protein (C. A. MacLean, B. Clark, and D. J. McGeoch, J. Gen. Virol. 70:3147–3157, 1989). To determine the function of the gene product, a recombinant virus (R7219) lacking 61% of the codons (176 bp of the 288-bp coding domain) was genetically engineered. The deletion mutant replicated in all cell lines tested, albeit to titers 30- to 250-fold lower than those obtained from cells infected with wild-type virus. Electron microscopic analyses indicated that both full and empty capsids accumulated in the nuclei, juxtaposed with the inner lamellae of the nuclear membranes, and that increased numbers of naked particles were present in the cytoplasm of cells infected with the mutant virus. There was a >1,000-fold decrease in the amount of infectious extracellular virus released from Vero cells infected with the deletion mutant compared with that from cells infected with wild-type virus. Furthermore, the onset of release of infectious virus from cells infected with the U_L11^- mutant was significantly delayed: levels of extracellular U_L11^- virus increased 15-fold between 20 and 26 h after infection, while levels of wild-type extracellular virus increased 500-fold between 8 and 14 h after infection. A virus in which the U_L11 gene was restored produced wild-type levels of total and extracellular virus and was indistinguishable from wild-type virus upon analysis by electron microscopy. Taken together, the data indicate that the absence of the U_L11 gene causes a reduced capacity to envelope and transport virions into the extracellular space.

The herpes simplex virus 1 (HSV-1) genome can be viewed as containing two covalently linked components, L and S, consisting of unique sequences (U_L and U_S, respectively) flanked by inverted repeats (16, 18). At least 70 open reading frames map in U_L and U_S, and 6 map in the inverted repeats flanking the unique sequences (3, 5, 7, 8, 10, 11). The function of many of the genes is unknown. In an attempt to define the functions of HSV genes, this laboratory has systematically probed HSV-1 genes for their role in replication of the virus in cells in culture.

The 288-bp open reading frame designated the U_L11 gene of HSV-1 was predicted to encode a myristylated protein (10). MacLean et al. reported that this was, in fact, the case and that the product of the U_L11 gene was associated with purified virion preparations (9). As in the case of other cellular and viral proteins, the myristylated residue is likely to be a glycine located in a consensus myristyl acceptor site located at the N terminus of the protein (9, 17). To determine the function of the U_L11 gene, we constructed a recombinant virus lacking a large portion of the coding sequences of this gene. Descriptions of the genotypes of this virus and of other viruses relevant to this report are presented in Table 1.

The general procedure for construction of the recombinant virus was described elsewhere in detail (13). It involves first the insertion of a selectable marker, a thymidine kinase (*tk*) gene linked to the α27 gene promoter at or near the target gene, followed by the selection of recombinants which lack both the marker gene and the adjacent sequences targeted for deletion. In this instance, we took advantage of the recombinant virus R7212 described earlier (1). This virus was constructed by the insertion of the α27-*tk* gene between the U_L10 and U_L11 open reading frames and is derived from HSV-1(F)Δ305, a recombinant virus constructed by the deletion of approximately 500 bp of the *tk* gene from the parent, HSV-1 strain F [HSV-1(F)] (13). To generate the U_L11 deletion mutant, R7212 viral DNA was cotransfected into rabbit skin cells with the plasmid pRB4117, and the progeny of the transfection was plated at low multiplicities of infection on $143TK^-$ cells in the presence of bromodeoxyuridine (40 μg/ml of medium). pRB4117 contained a deletion that extended from an *Eco*RV site within the chimeric α27-*tk* gene to an *Eco*RV site within the U_L11 open reading frame. As illustrated in Fig. 1, lines 1 to 3, the deletion removed a total of 1,366 bp, of which 1,190 bp were derived from the α27-*tk* gene and 176 bp (61% of the codons) were derived from the 3′ end of the coding sequence of the U_L11 open reading frame. The deletion did not affect the U_L12 gene, the 3′ end of which overlaps 87 bp (30%) of the 5′ end of U_L11, and left the extreme 5′ end of the *tk* gene and the α27 gene promoter intact. The recombinant *tk*-minus virus selected by growth in medium containing bromodeoxyuridine was designated R7218 and should contain deletions in both the *tk* and the U_L11 genes.

In order to repair the natural *tk* gene of R7218, rabbit skin cells were transfected with the *Bam*HI Q fragment of HSV-1(F), which contains the *tk* gene (pRB1028). After 6 h, the transfected cells were infected with R7218. Selection for *tk*-plus virus among the viral progeny in hypoxanthine-aminopterin-thymidine medium produced R7219, which should contain a deletion solely within the U_L11 open reading frame.

To confirm the genetic structure of the recombinant viruses, the viral DNAs of HSV-1(F), HSV-1(F)Δ305, R7212, and the deletion virus R7219 were purified, digested with *Sal*I, and transferred to two nitrocellulose sheets by methods

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TABLE 1. Genotypes of viruses used in this study

Virus	Plasmid used for construction	Genotype[a]
Constructed previously		
HSV-1(F)	None	wt
HSV-1(F)Δ305	pRB305[b]	ΔU_L23, ΔU_L24
R7212	pRB4035[c]	α27-*tk* between U_L11 and U_L10; ΔU_L23, ΔU_L24
Constructed in these studies		
R7218	pRB4117	ΔU_L11, ΔU_L23, ΔU_L24
R7219	pRB1028	ΔU_L11
R7237	pRB455	U_L11R

[a] wt, wild type; Δ, deletion; R, deleted sequences were restored.
[b] The deletion in the *tk* gene (U_L23) also inactivated the adjacent, partially overlapping U_L24 open reading frame (13).
[c] The insertion mutant was described in reference 1.

described by Longnecker and Roizman (8). One sheet was hybridized with the ^{32}P-labeled pRB455 that contained the entire U_L11 gene (Fig. 2, lanes 1 to 5). This probe hybridized with the 7.0-kbp *Sal*I D fragment of HSV-1(F) and HSV-1(F)Δ305 (Fig. 2, lanes 1 and 2). As described previously (1), the insertion of the α27-*tk* gene between the open reading

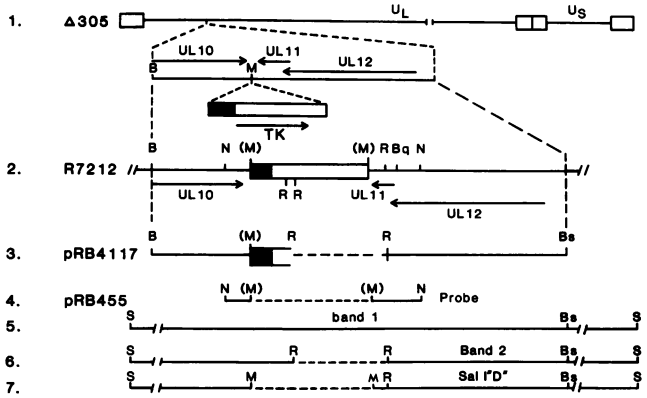


FIG. 1. Schematic representation of sequence arrangements of the DNA of the plasmids and viruses used for the deletion and repair of U_L11. (Lines 1 and 2) Sequence arrangements of the insertion of a chimeric *tk* gene between the U_L10 and U_L11 open reading frames as in R7212 DNA as previously described (1). (Line 3) Representation of the deletion of the majority of the chimeric *tk* gene and the U_L11 open reading frame as in pRB4117 and the deletion virus R7219. (Line 4) Diagram of pRB455. The HSV-1 sequences in pRB455 served as a probe for the studies whose results are shown in Fig. 2, lanes 1 to 5; they were also used in the transfections to restore the U_L11 deletion to generate R7237. (Line 5) Sequence arrangement of the R7212-specific band 1 of Fig. 2 (lanes 3 and 8). The band contained the chimeric *tk* gene and the U_L11 open reading frame and therefore hybridized with both probes in Fig. 2. (Line 6) Sequence arrangement of the R7219-specific band 2 of Fig. 2 (lanes 4 and 9). This band arose as a consequence of the deletion of the majority of the *tk* gene and U_L11 open reading frame. (Line 7) Sequence arrangement of the native *Sal*I D fragment of HSV-1(F) DNA. Filled boxes, α27 promoter used to drive the *tk* gene; arrows, indicated open reading frames; dashed lines, sequences either not present or deleted in that particular construct. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; M, *Mlu*I; N, *Nru*I; R, *Eco*RV; S, *Sal*I.

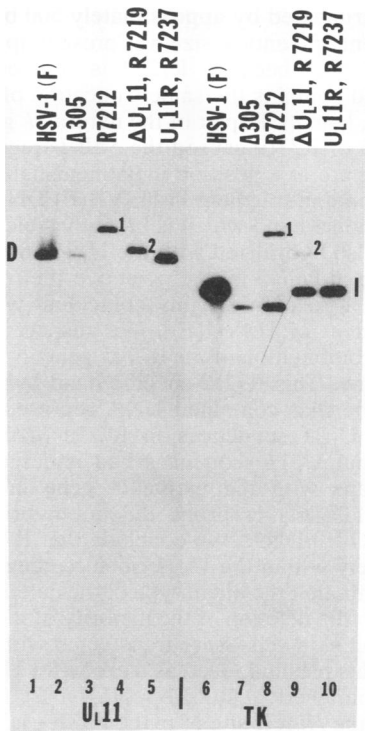


FIG. 2. Autoradiographic images of the electrophoretically separated *Sal*I restriction digests of the DNAs of viruses generated for and as a result of the deletion of the U_L11 gene. The DNAs of HSV-1(F) (lanes 1 and 6), HSV-1(F)Δ305 (lanes 2 and 7), R7212 (lanes 3 and 8), the deletion virus R7219 (lanes 4 and 9), and the U_L11-restored virus R7237 (lanes 5 and 10) were cleaved with *Sal*I, separated on a 1.0% agarose gel, and transferred to two nitrocellulose sheets. The duplicate blots were then probed with either pRB455 (lanes 1 through 5), which contained the entire U_L11 open reading frame, or *Bam*HI Q (lanes 6 through 10), which contained the native *tk* gene. The position of the migration of the *Sal*I D and I fragments are indicated.

frames of U_L10 and U_L11 of R7212 DNA caused the 7.0-kbp *Sal*I D fragment to migrate at a position that corresponded to 8.8 kbp. This fragment appears as band 1 in Fig. 2. To confirm the fact that the increase in size of the *Sal*I D fragment was due to the insertion of the *tk* marker, the duplicate nitrocellulose sheet was allowed to hybridize with a ^{32}P-labeled *Bam*HI Q fragment of HSV-1(F) DNA, which contains sequences that correspond to the *tk* gene (Fig. 2, lanes 6 to 10). As expected, band 1 also hybridized with the *tk* probe (Fig. 2, lane 8), indicating that the chimeric *tk* gene was inserted near the U_L11 open reading frame as designed.

The *Sal*I D fragment of the U_L11 deletion virus R7219 (lanes containing R7219 DNA are marked "ΔU_L11" in Fig. 2) migrated at a position that corresponded to 7.4 kbp (Fig. 2, band 2) as a consequence of the deletion of the majority of the *tk* gene and U_L11 open reading frame. As stated above, the plasmid that was used to generate the deletion of U_L11 in R7219 DNA contained a small portion of the 5′ end of the chimeric *tk* gene. The fact that band 2 of R7219 DNA hybridized with the *tk* probe (Fig. 2, lane 9) confirms this fact.

In HSV-1(F) DNA, the *Sal*I I fragment contains the natural *tk* gene. As a result of the deletion in the *tk* gene of HSV-1(F)Δ305, the *Sal*I I fragment that hybridized with the

tk probe is truncated by approximately 500 bp (Fig. 2, lane 7). A fragment of identical size was present upon digestion of R7212 with *Sal*I because R7212 is derived from HSV-1(F)Δ305 and contains the same truncation of the natural *tk* gene (Fig. 2, lane 8). Repair of the natural *tk* gene during the construction of R7219 caused the *Sal*I I fragment of R7219 DNA to migrate at a position indistinguishable from that of the corresponding fragment in HSV-1(F) DNA (Fig. 2, lane 9). An additional band which is barely visible in lanes 2 and 8 of Fig. 2 also hybridized with the U_L11 and *tk* probes [the band is seen in lane 8 migrating above the truncated *Sal*I I fragment at a position roughly coincident with that of the *Sal*I I fragment of HSV-1(F)]. We suspect that this band arose by recombination of the α27-*tk* gene in R7212 with the native *tk* gene. This R7212-specific band hybridized with a smaller probe that contained DNA representing the exact deletion of U_L11 sequences in R7219 (data not shown), indicating that U_L11 sequences had undergone rearrangement, perhaps with the native *tk* gene in R7212 DNA. However, this smaller probe did not hybridize with any portion of R7219 DNA. We conclude that R7219 contains a deletion solely within the U_L11 open reading frame.

To ascertain that the phenotype of the deletion mutant was due solely to the deletion of the majority of the U_L11 gene in R7219, rabbit skin cells were transfected with pRB455 (Fig. 1, line 4). This plasmid, used as a probe for U_L11 sequences (Fig. 2), contained a 1,067-bp HSV-1(F) *Nru*I fragment cloned into the *Hin*cII site of pGEM-3Zf(+). The sequences cloned into this fragment extended from 0.156 to 0.165 map units of HSV DNA and included the entire U_L11 open reading frame and portions of the 3′ ends of the U_L10 and U_L12 open reading frames. The transfected cells were infected with R7219. Approximately 40 h later, the progeny of the transfection-infection was plated on Vero cells. Five plaques that appeared larger than R7219 plaques were plaque purified two additional times. The DNA from these viruses was purified and analyzed by Southern analysis. The DNAs of the progeny of all five plaques contained a restored U_L11 gene (data not shown). One of these viruses was designated R7237. To verify that the deleted U_L11 sequences of R7219 were restored in R7237, the virus was plaque purified two additional times on Vero cells and the viral DNA was purified, cleaved with *Sal*I, separated on a 1.0% agarose gel, and hybridized with *tk* and pRB455 probes. As expected (Fig. 2), both the *Sal*I I fragment, which contained the native *tk* gene, and the *Sal*I D fragment, which contained U_L11, were restored to sizes indistinguishable from those of the corresponding fragments of HSV-1(F) (Fig. 2, compare lane 1 with lane 5 and lane 6 with lane 10). Moreover, neither band 1 nor band 2 was present in R7237 DNA. Transfection of the vector pGEM3Z with subsequent R7219 infection failed to produce any large plaques which contained the restored U_L11 gene (not shown). Since wild-type plaques containing a restored U_L11 gene were obtained from R7219 infection of cells in which pRB455 was transfected but not from cells transfected with vector DNA, the differences in plaque size between R7219 and R7237 (and other differences described hereafter) were due to the deletion of the U_L11 open reading frame and not to another spontaneous mutation.

TABLE 2. Viral yields from infected cultures

Cell line	Virus yield[a]		
	HSV-1(F)	R7219	R7237
Vero	5.1×10^8	3.1×10^6	3.0×10^8
Rabbit skin	7.5×10^8	7.2×10^6	5.0×10^8
HEp-2	4.8×10^8	1.8×10^6	1.5×10^8
143TK$^-$	8.1×10^7	2.6×10^6	9.3×10^7

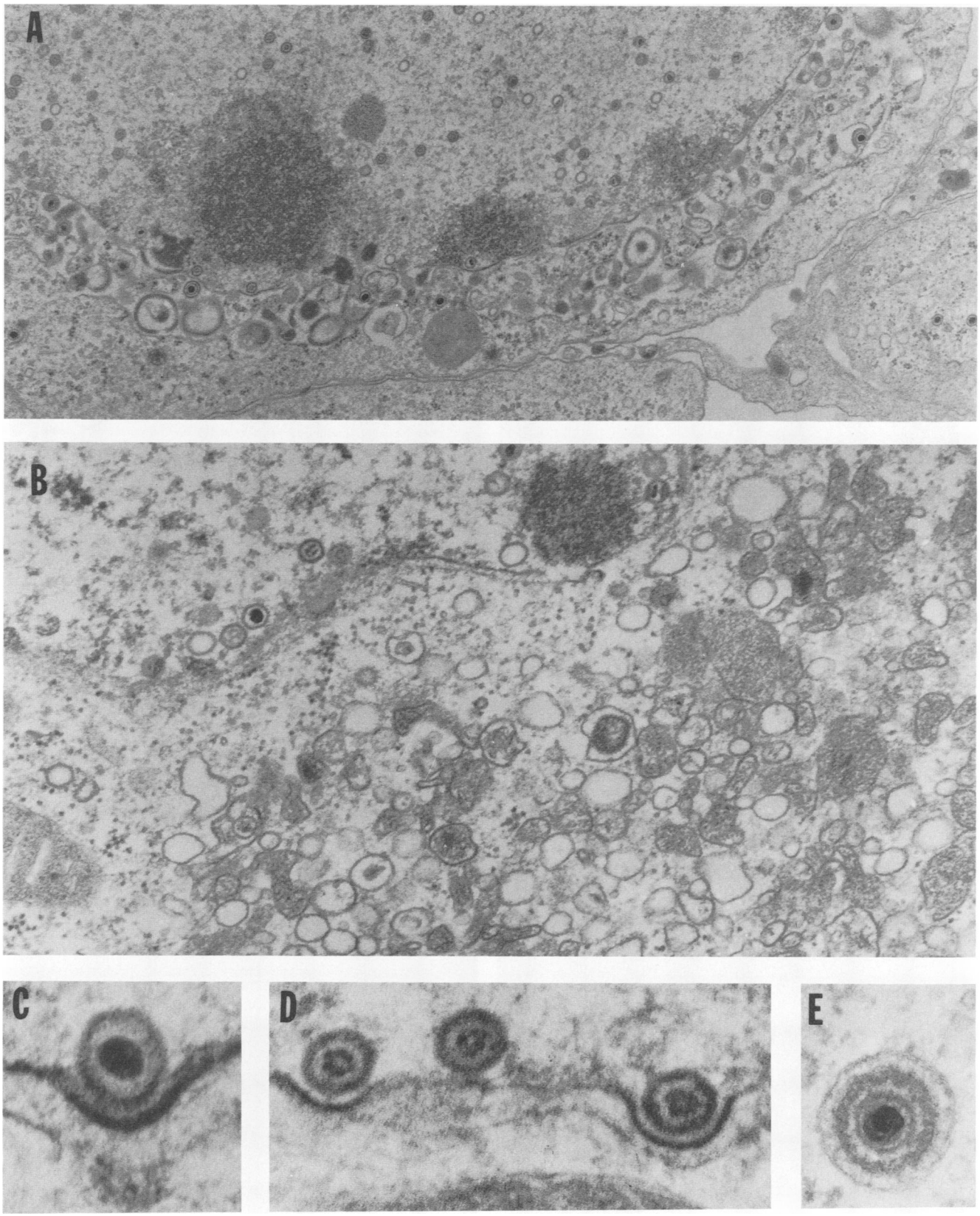
[a] PFU per milliliter of culture fluid. Titrations were done in replicate Vero cell monolayer cultures 24 h after infection with 5 PFU of virus per cell.

Replicate cultures of HEp-2, Vero, rabbit skin, and 143TK$^-$ cell cultures were each infected with 5 PFU of R7219, R7237, or HSV-1(F) virus per cell. The progeny virus harvested at 24 h after infection and incubation at 37°C was titrated on Vero cell cultures. As shown in Table 2, the U_L11 deletion virus R7219 replicated to titers approximately 30-, 100-, 250-, and 150-fold lower than those of wild-type HSV-1(F) in 143TK$^-$, rabbit skin, HEp-2, and Vero cells, respectively. Moreover, we conclude that the reduced amount of infectious virus was due to the deletion in the U_L11 gene, since the U_L11-restored virus R7237 replicated to titers approximately equal to those obtained upon infection with HSV-1(F).

In order to determine the reasons for decreased yields in cells infected with the U_L11 deletion virus, we infected Vero cells with 5.0 PFU of the wild-type, deletion (R7219), or restored (R7237) virus per cell. The cells were incubated at 37°C for 24 h, at which time they were fixed in glutaraldehyde, sectioned, and examined in a Siemens 102 electron microscope. To obtain a quantitative estimate of the distribution of capsids and virions in infected cells, intracellular and extracellular particles were counted in approximately 30 random sections of infected cells. The results (Fig. 3 and 4 and Table 3) were as follows.

(i) The incidence of U_L11^- capsids juxtaposed with the inner lamellae of the nuclear membranes was greater than that of the wild-type or restored virus. Approximately 3% of particles in the nuclei of cells infected with the wild-type and restored viruses were in contact with the inner lamellae, whereas 11% of nuclear capsids rested against the inner lamellae of cells infected with the mutant virus. Of further interest was the increased numbers of capsids that were observed to be partially enveloped by the inner lamellae of cells infected with the U_L11^- virus (Fig. 3C and D). Such partially enveloped capsids were only occasionally seen in thin sections of cells infected with wild-type virus (one is present in Fig. 4B), inasmuch as envelopment is a rapid process. These observations suggest that the deletion in the U_L11 gene affects the envelopment at the nuclear membrane by either slowing down or reducing the incidence of the process.

FIG. 3. Electron micrographs of Vero cells infected with the R7219 U_L11 deletion virus. Monolayers of Vero cells were infected with approximately 2.0 PFU of R7219 per cell. The cells were then incubated for 24 h at 37°C, at which time they were fixed in glutaraldehyde and prepared for electron microscopy. (A) Note the lack of extracellular particles between cells. (B) Note the accumulation of both empty and full capsids near the inner lamellae of the nuclear membrane. (C) Capsid containing DNA partially enveloped at the inner lamellae of the nuclear membrane. (D) Empty capsids attached to and partially enveloped by the inner lamellae of the nuclear membrane. (E) R7219 virion in a cytoplasmic vesicle.


A
B
C
D
E

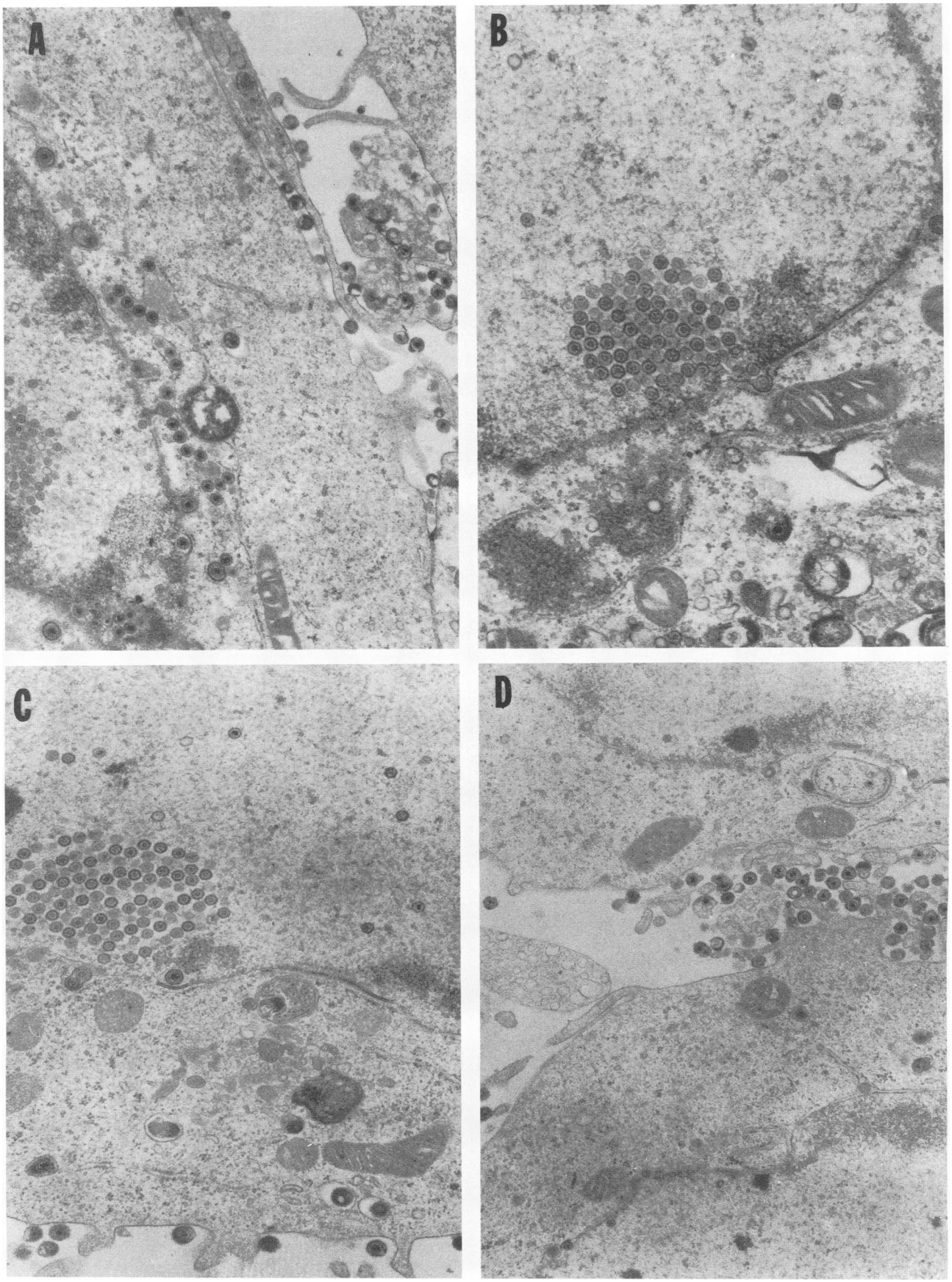

A
B
C
D

FIG. 4. Electron micrographs of Vero cells infected with wild-type virus [HSV-1(F)] (A and B) or rescued virus (R7237) (C and D) and maintained as described in the legend to Fig. 3. Note differences in the accumulation of extracellular virions in cells infected with wild-type and rescued viruses compared with the amounts seen in cells infected with the deletion mutant shown in Fig. 3.

(ii) In cells infected with the wild-type, deletion, and restored viruses, enveloped particles and, to a lesser extent, unenveloped particles accumulated in the space between the lamellae of the nuclear membrane at 24 h after infection (Fig. 3A and 4A). The numbers of particles accumulating in this space were similar in cells infected with each virus (Table 3). This suggests that the deletion in the U_L11 gene does not affect transit across the perinuclear space.

(iii) The ratio of unenveloped U_L11^- to enveloped capsids in the cytoplasm was greater than the corresponding ratios of wild-type and restored viruses. However, as in cells infected with the wild-type and restored viruses, normal enveloped particles were occasionally observed within cytoplasmic vesicles, presumably en route to the extracellular space (Fig. 3E).

(iv) The most striking difference between the appearance of cells infected with the U_L11^- virus and that of cells infected with the wild-type and restored viruses was a decrease in the number of extracellular virions in cells infected with the deletion mutant. The U_L11^- virions present in the extracellular environment could not be differentiated from those of wild-type and restored viruses.

The observation that the numbers of extracellular particles are decreased and those of naked cytoplasmic particles are increased in cells infected with the U_L11^- mutant suggests that the deletion in U_L11 decreases the frequency or prolongs the time of transit from the cytoplasm to the extracellular space.

In order to verify the observation that there was an overall decrease in extracellular U_L11^- virus relative to the amount of extracellular virus in wild-type-infected cells and to determine whether there was a detectable delay in virion release from cells infected with the U_L11 deletion mutant, we quantified the amount of infectious extracellular virus released from cells and total virus production in cells infected with the three viruses as a function of time. Vero cell monolayers were infected with 5.0 PFU per cell. To remove the bulk of the input extracellular virus, the infected monolayers were washed twice with medium containing anti-HSV antibody and 1% newborn calf serum and three times with medium containing 1% newborn calf serum. At various times after infection, 4 of the 5 ml of medium overlying the cells was removed and clarified at 1,500 rpm for 5 min at 4°C in a Beckman model TJ-6 tabletop centrifuge. The cell-free medium and cells were stored separately at −80°C until the titers were determined on Vero cells. The results (Fig. 5) show that while 9,000- and 6,000-fold increases in extracellular virus were detectable between 8 and 20 h after infection with HSV-1(F) and R7237, respectively, only a 2-fold increase in infectious extracellular virus was evident between 8 and 20 h after infection with the R7219 deletion virus. At 26 h after infection, the amount of infectious virus in the medium obtained from cells infected with the deletion virus was reduced at least 1,000-fold relative to the amount obtained from cells infected with wild-type virus. In addition, the onset of release of infectious virus from cells infected with the U_L11^- mutant was significantly delayed: levels of extracellular U_L11^- virus increased 15-fold between 20 and 26 h after infection, while wild-type extracellular virus increased more than 500-fold between 8 and 14 h after infection. The amount of infectious virus obtained from lysates of cells infected with the deletion mutant increased 35-fold between 8 and 26 h after infection. This was a 23-fold reduction relative to the 800-fold increase in infectious virus

TABLE 3. Distribution of capsids in different compartments of infected Vero cell cultures[a]

Location or condition	No. (%) of capsids		
	HSV-1(F)	R7219	R7237
Nucleus			
Total	1,241 (45)	887 (69)	915 (39)
At the inner lamellae	112 (3.0)	168 (11)	104 (3.2)
Between the lamellae	446 (12)	199 (13)	355 (11)
Cytoplasm			
Total	231 (6.2)	323 (21)	359 (11)
Enveloped	133 (3.6)	73 (4.6)	228 (7.1)
Unenveloped	98 (2.6)	250 (16)	131 (4.1)
Extracellular	1,818 (49)	164 (10)	1,597 (50)
Total (all locations)	3,736 (100)	1,573 (100)	3,226 (100)

[a] Tabulations are from approximately 30 randomly selected sections of Vero cells 24 h after infection with 2.0 PFU per cell. Electron micrographs of representative thin sections are shown in Fig. 3 and 4.

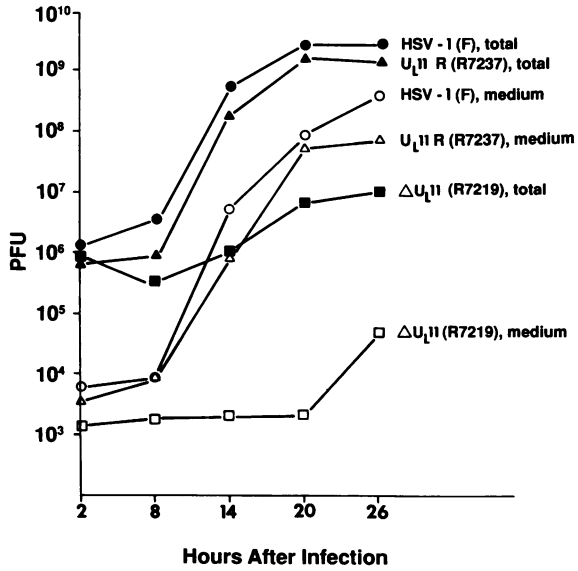


FIG. 5. Quantitation of infectious cell-free virus ("medium") and virus obtained from Vero cell lysates ("total") infected with HSV-1(F), the U_L11 deletion mutant R7219, and the restored virus R7237. Vero cell monolayers in 25-cm² dishes were infected with 5.0 PFU of virus per cell. After the virus was allowed to adsorb, the monolayers were washed to remove input extracellular virus. At various times after infection, culture medium was removed and clarified by low-speed centrifugation. The clarified cell-free medium and the adherent cells were then frozen separately at −80°C. The medium was thawed, the cells were thawed and sonicated to release intracellular virus, and titrations were performed on Vero cell monolayer cultures. For comparative purposes, a given titer was multiplied by the sample volume to reach the value presented.

produced in wild-type virus-infected cells over the same period. Thus, relative to results with wild-type virus, the amount of infectious U_L11^- virus that was released from Vero cells into the medium was substantially reduced, the release of infectious virus into the medium was significantly delayed, and the rate of production of infectious cell-associated virus was significantly reduced. Because the amounts of infectious virus obtained from the medium and cell lysates of cells infected with the wild-type and restored viruses were virtually identical, we conclude that the decreased production of infectious virus from cells infected with the U_L11 deletion mutant is due solely to the absence of the majority of the U_L11 gene.

There are several instances in which myristylated proteins have been shown to be important for the maturation or transport of both unenveloped and enveloped viruses. For example, mutation of the consensus myristylation site of the murine leukemia $Pr65^{gag}$ protein prevents normal cleavage of $Pr65^{gag}$, precludes its packaging into virions, promotes its secretion into the medium, and prevents viral assembly. These data indicate that interaction of the myristylated $Pr65^{gag}$ with the plasma membrane is an essential early step in retroviral particle assembly (15). An example of involvement of myristylated protein in transport is afforded by the pre-S1 envelope protein of hepatitis B virus. In this instance, the pre-S1 envelope protein is secreted in particles containing only nucleocapsids, even though empty particles containing nonmyristylated envelope proteins are secreted from infected cells. Mutation of the pre-S1 myristylation site causes some of the protein to be secreted in subviral particles (14). Myristylated proteins that are not associated with membranes may exploit the modification as a means to promote protein interaction. For example, mutation of the capsid protein VP1 of poliovirus so that it is no longer myristylated prevents capsid formation (12).

In recent years, mutations in a number of genes have been shown to affect the transit of herpes simplex virions across the cytoplasm to the extracellular space. These include mutations in glycoproteins D and H and a host range-specific effect of the deletion in U_L20 (2, 4, 6). In the case of the latter gene, Vero cells infected with the deletion mutant do not exhibit excessive accumulation of capsids abutting the inner lamellae of the nuclear membranes. However, these cells accumulated virions in the space between the inner and outer lamellae and failed to exhibit extracellular virions (2). In contrast, the U_L11 deletion mutant is at least partially defective in envelopment and exhibits a decrease in, but not total elimination of, extracellular virions. The molecular interactions responsible for the defects in egress and envelopment of the U_L11^- virus are not known. We cannot exclude the possibility that in the absence of fully functional U_L11 capsids lack the proper surface to become enveloped or the possibility that U_L11 associates with membranes and is involved in both envelopment of capsids and transport of virions.

We should note that 61% of the codons of the U_L11 gene were deleted from the genome of R7219. We are not aware of any viral gene product which, when truncated to this extent, is still capable of functioning as the wild-type counterpart. Because the N terminus of U_L11 overlaps with another gene, strategies designed to delete the remaining gene fragment are not entirely satisfactory. Nevertheless, experiments in progress should resolve the question of whether the residual peptide accumulates at the presumed sites of its activity.

We thank Suzanna Rudofsky and Shu-Fen Chow for expert technical assistance.

These studies were aided by Public Health Service grants from the National Cancer Institute (CA47451) and the National Institute for Allergy and Infectious Diseases (AI124009 and AI1588) and by an unsolicited grant from Bristol-Myers Squibb.

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