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The Herpes Simplex Virus 1 U_L17 Gene Is Required for Localization of Capsids and Major and Minor Capsid Proteins to Intranuclear Sites Where Viral DNA Is Cleaved and Packaged

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In nuclei of cells infected with herpes simplex virus (HSV), synthesized viral DNA accumulates as concatamers that are cleaved into genomic lengths and inserted into preformed capsids. Whereas newly replicated DNA and enzymes required for DNA synthesis accumulate in sites of infected cell nuclei termed replication compartments, the intranuclear site of DNA cleavage and packaging is currently controversial. DNA packaging requires the U_L6, U_L15, U_L17, U_L25, U_L28, U_L32, and U_L33 genes in addition to the major capsid proteins. Using confocal immunofluorescence microscopy, it was observed that in >95% of HEp-2 cells fixed at late times after infection with wild-type HSV-1, capsids, major capsid proteins ICP5 and ICP35, and the U_L6-encoded minor capsid protein localized in DNA replication compartments. These data support the hypothesis that capsid assembly and DNA cleavage/packaging normally occur in HEp-2 cell replication compartments. In contrast, cells infected with a viral mutant lacking functional U_L17 contained antigenically dense nuclear aggregates that stained with ICP35, ICP5, and capsid specific antibodies. Cells infected with the U_L17 mutant virus also displayed U_L6-specific fluorescence in a diffuse pattern at the nuclear periphery in regions not containing ICP35 and ICP5. Displacement of ICP35 from replication compartments was not observed in cells infected with cleavage/packaging mutants lacking U_L28 and U_L33. We conclude that the U_L17 gene is required for correct targeting of capsids and major and minor capsid proteins to the DNA replication compartment of HEp-2 cells and deduce that this targeting reflects one functional role of U_L17 in viral DNA cleavage and packaging.

INTRODUCTION

Three types of capsids, designated A, B, and C, accumulate in nuclei of cells infected with herpes simplex virus (HSV) (Roizman and Furlong, 1974). B capsids consist of an internal core composed of VP22a (or ICP35) surrounded by an external shell composed largely of capsomeres of VP5 (or ICP5) linked by triplexes composed of one molecule of VP19C bound to two molecules of VP23 (Gibson and Roizman, 1972; Cohen *et al.*, 1980; Newcomb and Brown, 1989; Newcomb *et al.,* 1993, 1996). A protease (VP24) is also packaged within the core of the capsid (Gibson and Roizman, 1972; Davison *et al.,* 1992). The external shells of A, B, and C capsids are identical, but A and C capsids lack the internal core of B capsids (Gibson and Roizman, 1972; Schrag *et al.,* 1989; Booy *et al.,* 1991; Zhou *et al.,* 1994). Only C capsids contain genomic DNA (Gibson and Roizman, 1972).

Studies of HSV capsid assembly have been reviewed (Steven and Spear, 1997; Homa and Brown, 1997). In summary, (i) subunits consisting of VP22a, triplexes, and VP5 initially assemble in the cytoplasm (Nicholson *et al.,* 1994; Rixon *et al.,* 1996). (ii) Following nuclear importation of protein complexes, binding of triplexes to neighboring VP5 molecules and self-association of the scaffold protein may serve to drive simultaneous assembly of the inner and outer capsid shells (Newcomb *et al.,* 1996). (iii) Completion of the outer shell produces a roughly spherical structure termed the procapsid (Trus *et al.,* 1996). It has been proposed that the procapsid serves as the precursor of type A, B, and C capsids (Newcomb *et al.,* 1996). (iv) Maturation of the procapsid to other capsid types is closely linked to activation of the packaged protease that cleaves the C-terminal 25 amino acids from VP22a, freeing the scaffold from the outer shell (Preston *et al.,* 1983; Liu and Roizman, 1991; DiIanni *et al.,* 1993; Gao *et al.,* 1994; Matusick-Kumar *et al.,* 1995; Tatman *et al.,* 1994; Thomsen *et al.,* 1995). Possibly concurrent with extensive conformational changes resulting in an icosahedral, less porous outer shell, the freed scaffold is either lost (in A or C capsids) or collapses inwardly to produce a small internal core (in small-cored B capsids). Type A capsids result when scaffold expulsion/degradation is uncoupled from DNA insertion, whereas small-cored B capsids arise when the scaffold is not expelled but retained within the icosahedral outer shell. Alternatively, insertion of viral DNA into the procapsid and loss of the scaffold produces type C capsids which are preferentially enveloped at the inner nuclear membrane to form virions (Roizman and Furlong, 1974).

The U_L6, U_L15, U_L17, U_L28, U_L32, and U_L33 genes are

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required for cleavage of concatameric DNA into unit-length genomes but are dispensable for production of small-cored B-like capsids. Thus deletion or insertion mutations in any one of these genes precludes generation of C capsids presumably because cleaved DNA is not available for the packaging reaction (Al-Kobaisi *et al.*, 1991; Tengelsen *et al.,* 1993; Patel *et al.,* 1996; Baines *et al.,* 1997; Yu *et al.,* 1997; Lamberti and Weller, 1998; Salmon *et al.,* 1998). The U_L25 gene is dispensable for cleavage of viral DNA but is essential for production of C capsids (McNab *et al.*, 1998). At least the U_L6, U_L15, and U_L25 gene products are minor components of B and C capsids, suggesting that they might serve to link DNA to the capsid, or mediate DNA insertion (Patel and Maclean, 1995; McNab *et al.*, 1998; Salmon and Baines, 1998). Neither the U_L17 nor U_L32 gene products have been detected in B capsids (Lamberti and Weller, 1998; Salmon *et al.*, 1998). U_L17-encoded proteins are incorporated into virions and are likely associated with the tegument, a region of the virion between the outer capsid surface and inner surface of the virion envelope (Salmon *et al.*, 1998).

Sites of viral DNA synthesis, viral DNA, and enzymes required for HSV DNA replication localize in a central region of infected cell nuclei termed the DNA replication compartment (Quinlan *et al.,* 1984; de Bruyn Kops and Knipe, 1994; Phelan *et al.,* 1997). The HSV single-stranded DNA binding protein ICP8 serves as a marker for this compartment (Quinlan *et al.,* 1984). In Vero cells early in infection, major capsid proteins and at least some U_L32 gene product localize in the DNA replication compartment (Lamberti and Weller, 1998). In cells infected with a virus lacking U_L32 however, capsids are displaced from the DNA replication compartment indicating that viral proteins are required for localization of capsids within this compartment and suggesting that this localization is important for DNA cleavage and packaging (Lamberti and Weller, 1998). Later in infection, major capsid proteins and some tegument proteins often accumulate in intranuclear regions of Vero cells termed assemblons, which arise in regions peripheral to the DNA replication compartment but not abutting the inner nuclear membrane (Ward *et al.*, 1996). In contrast, U_L15-specific immunofluorescence remains largely within the DNA replication compartment of Vero cells even late in infection, indicating that some steps in viral DNA cleavage/packaging (e.g., processing of viral DNA) may also occur in the DNA replication compartment of Vero cells late in infection (Ward *et al.*, 1996).

The possibility that the U_L17 gene products act to properly target capsids and major and minor capsid proteins to specific intranuclear sites is addressed in this study. We determined that unlike in Vero cells, >95% of HEp-2 cells contain the major capsid proteins ICP5 and ICP35, as well as the U_L6-encoded minor capsid protein, in the DNA replication compartment late in infection. We also show that these capsid proteins no longer colocalize in the replication compartment in cells infected with a DNA cleavage/packaging mutant lacking functional U_L17. This suggests that one role of U_L17 proteins is to correctly target capsids and proteins required for capsid assembly to replication compartments, the likely sites in HEp-2 cells where DNA-containing capsids are assembled.

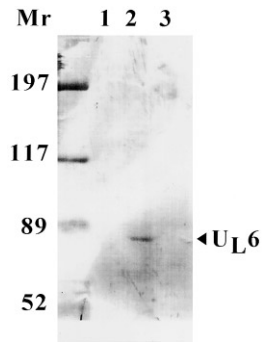


FIG. 1. Immunoblot of mock, HSV-1(F)- and U_L6^--infected HEp-2 cell lysates prepared 18 h p.i. Electrophoretically separated proteins from lysates of mock-infected or infected cells were transferred to nitrocellulose and probed with antiserum raised against the MBP-U_L6 fusion protein. Molecular weights are indicated in thousands to the left of the figure. Lane 1, U_L6^--infected cell lysate; lane 2, HSV-1(F)-infected cell lysate; lane 3, mock-infected cell lysate. The arrow head indicates the position of the U_L6 protein.

RESULTS

Identification of the U_L6 protein

As described under Materials and Methods, rabbits were immunized with an affinity-purified bacterial fusion protein containing the maltose binding protein fused to U_L6 sequences (MBP-U_L6). To determine the specificity of the antiserum, proteins in lysates of mock-, HSV-1(F)-, and U_L6^--infected HEp-2 cells were separated on a denaturing polyacrylamide gel, transferred to a nitrocellulose membrane, and reacted with the antiserum directed against MBP-U_L6. Bound antibody was identified by reaction of the nitrocellulose sheet with alkaline-phosphatase-conjugated anti-rabbit immunoglobulin followed by the fixation of colored substrate as described (Baines and Roizman, 1993). The anti-MBP-U_L6 antibody recognized a band of 79,000 apparent M_r in HSV-1(F)-infected lysates (Fig. 1, lane 2). This band was not present in lysates of cells infected with a virus that lacked the capacity to produce the last 298 amino acids of U_L6 protein because of a frameshift mutation at codon 378 (Patel *et al.*, 1996) (Fig. 1, lane 3) or lysates of mock-infected cells (Fig. 1, lane 1). Immunoblotting of mock-, HSV-1(F)-, and U_L6^- virus-infected cell lysates with the U_L21 polyclonal rabbit antiserum demonstrated the presence of U_L21-specific immunoreactive bands in lysates of HSV-1(F)- and U_L6^- virus-infected cells (data

not shown). We therefore conclude that (i) the U_L6^- mutant virus expresses viral proteins and (ii) the antiserum directed against MBP-U_L6 recognizes the product of the U_L6 open reading frame in infected cell lysates.

To assess the specificity of the indirect immunofluorescent staining with the MBP-U_L6 antiserum, cells were infected with wild-type virus [HSV-1(F)] or the U_L6 insertion virus (U_L6^- mutant virus), permeabilized in methanol at 18 h p.i., and reacted with ICP35-specific monoclonal antibody and MBP-U_L6-specific antiserum. To reduce possible reactions with host proteins in indirect immunofluorescence assays, the MBP-U_L6-specific antiserum was extensively adsorbed against uninfected HEp-2 cell lysates in the presence of 1% Tween 20. HSV glycoproteins E and I encode an Fc receptor that binds rabbit immunoglobulin (Johnson *et al.*, 1988). To reduce high background levels of fluorescence resulting from binding of antibody to the HSV-encoded Fc receptor, permeabilized cells were reacted extensively with 10–20% human serum as described under Materials and Methods. Bound rabbit immunoglobulin was visualized by reaction with either rhodamine- or Texas Red-conjugated anti-rabbit immunoglobulin and fluorescein-conjugated anti-mouse immunoglobulins that displayed minimal reactivity with human serum (Jackson Immunoresearch), and cells were viewed in light filtered for excitation of the appropriate conjugates. The results indicated that although HSV-1(F)-infected cells stained with the MBP-U_L6 antiserum demonstrated extensive intranuclear fluorescence (described in more detail below), levels of indirect immunofluorescent staining in cells infected with the U_L6 mutant virus (U_L6^-; Fig. 2) did not demonstrate extensive fluorescence in the nucleus or cytoplasm. Although some fluorescence was present in U_L6^- virus-infected cells stained with the MBP-U_L6 antiserum (Fig. 2), the intensity of indirect immunofluorescence in cells infected with the U_L6^- virus was considerably less than that seen in cells infected with viruses expressing U_L6 protein (Fig. 2) and was similar to levels seen in uninfected cells stained with MBP-U_L6 antiserum (not shown). Thus notwithstanding some background staining attributable to cross-reactivity with uninfected cell proteins, these data support the conclusion that the more intense intranuclear fluorescence produced upon staining with the preadsorbed MBP-U_L6 antiserum in permeabilized cells infected with wild-type virus reflects the presence of the U_L6 gene product.

To characterize the distribution of U_L6 protein in wild-type virus infected cells, HEp-2 cells were infected with the wild-type strain HSV-1(F) and were fixed and permeabilized at 18 h after infection in cold methanol. The permeabilized cells were incubated with MBP-U_L6-specific antiserum and monoclonal antibody directed against ICP8. Bound antibodies were detected by reaction with Texas Red-conjugated anti-rabbit antibody and FITC-conjugated anti-mouse antibody. As expected, ICP8-specific fluorescence accumulated in the nuclei of infected cells (Fig. 2). The U_L6-specific immunofluorescence largely colocalized with ICP8-specific fluorescence; minor amounts also accumulated in numerous small punctate regions located peripheral to the ICP8 containing DNA replication compartment. In ~5% of cells infected with wild-type virus, U_L6-specific staining localized within punctate regions peripheral to the DNA replication compartment that resembled previously described assemblons (Ward *et al.*, 1996). A cell that displays some assemblons can be seen in Fig. 2, third row, right-most cell. Taken together, these data indicate that U_L6 protein mostly accumulates in the DNA replication compartment of HEp-2 cells late after infection with wild-type HSV-1(F) (Fig. 2).

Colocalization of U_L6 protein with major capsid proteins

The next set of experiments was designed to test the possibility that the U_L6-encoded minor capsid protein colocalized with major capsid proteins late in infection. At 18 h p.i., HSV-1(F)-infected cells were incubated with MBP-U_L6 antiserum and monoclonal antibody against ICP5 or ICP35. Rhodamine- or Texas Red-conjugated anti-rabbit antibody was used to detect bound MBP-U_L6, and FITC-conjugated anti-mouse antibody was used to detect bound anti-ICP5 or anti-ICP35 antibodies. The appearance of cells viewed in light appropriate for excitation of the various conjugates revealed that U_L6-specific fluorescence colocalized with both ICP5- and ICP35-specific fluorescence in an even distribution throughout infected nuclei (Fig. 2). We conclude that the major capsid proteins ICP5 and ICP35 colocalize with U_L6 protein late in infection of HEp-2 cells and deduce that because U_L6 protein largely localized in the DNA replication compartment late in infection, that ICP5 and ICP35 also accumulate primarily in the DNA replication compartment late in infection. These data strongly suggest that the replication compartment represents the site of capsid assembly in HEp-2 cells.

U_L17 is required for proper intranuclear localization of major and minor capsid proteins

To test the possibility that proteins known to be required for cleavage and packaging might play a role in localization of capsid proteins in regions of the nucleus in which DNA cleavage/packaging takes place, we examined the intranuclear distribution of U_L6 protein, ICP35, and ICP5 in cells infected with available mutant viruses defective in viral DNA cleavage and packaging. The patterns of fluorescence in cells infected with the U_L17 mutant [HSV-1 $\Delta(U_L17)$] showed a striking departure from those of cells infected with wild-type virus and other cleavage packaging mutants. Specifically, ICP5 and ICP35 fluorescence no longer localized with ICP8 in

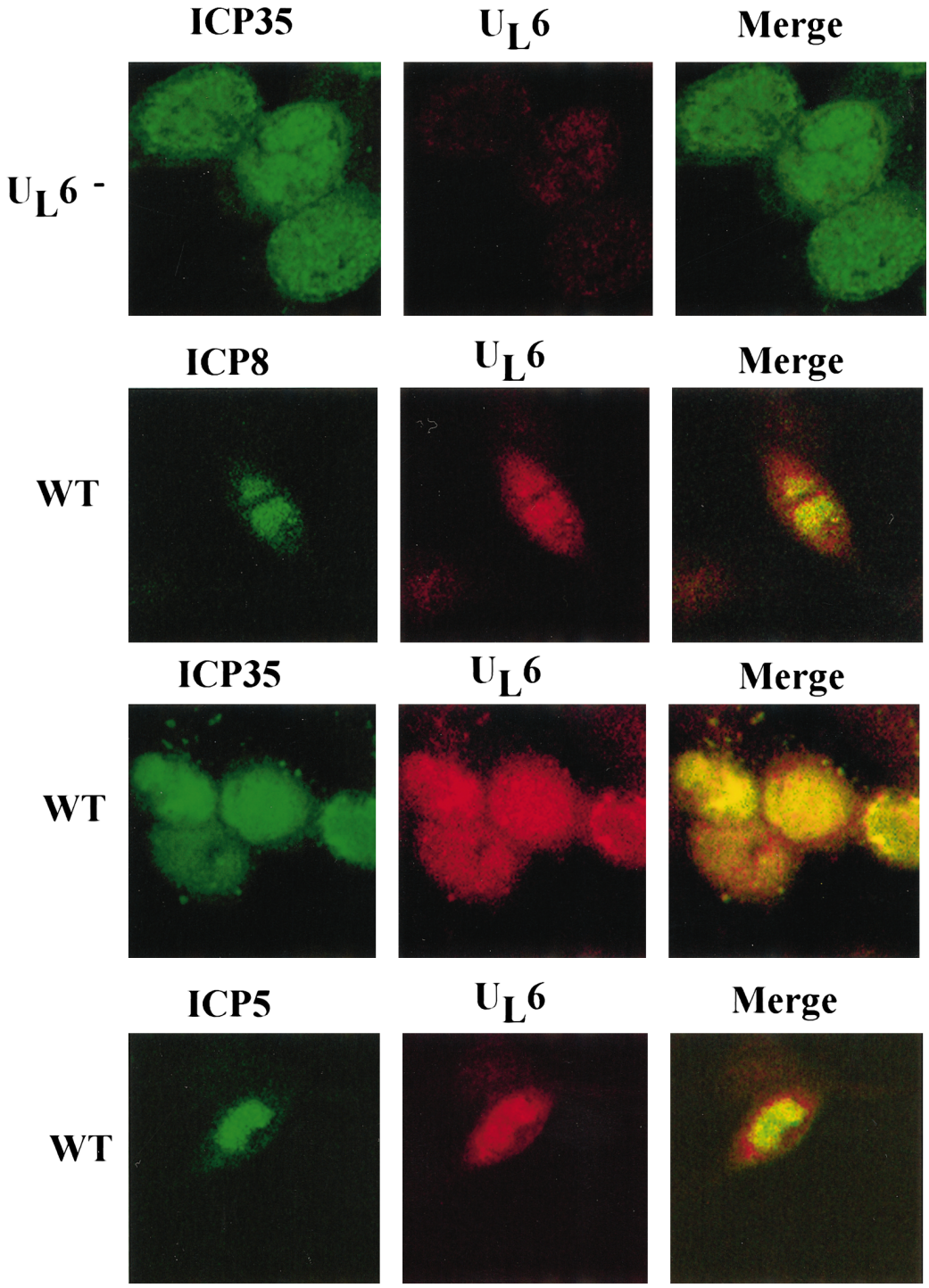


FIG. 2. Digital confocal images of infected HEp-2 cells fixed and stained at 18 h p.i. Cells were infected with a virus lacking an intact U_L6 gene (U_L6^-) or wild-type virus HSV-1(F) (WT) and were reacted with mouse monoclonal antibodies directed against the indicated HSV-1 proteins and rabbit polyclonal antiserum against U_L6-MBP. Bound monoclonal antibodies were visualized with fluorescein isothiocyanate (FITC)- conjugated donkey antimouse antiserum (green) and polyclonal antibody was visualized with lissamine rhodamine- or Texas Red-conjugated donkey anti-rabbit antibody (red). Single color images were recorded simultaneously and are shown in the left and middle columns. The right column represents the merged images of the left and middle panels in each row. Images were recorded with Bio-Rad software and printed on a Codonics NP1600 dye sublimation printer.

the replication compartment (Fig. 3). Rather, both ICP5- and ICP35-specific fluorescence accumulated in antigenically dense aggregates at the edges of infected cell nuclei (Fig. 3). U_L6-specific fluorescence appeared somewhat diffuse and no longer colocalized with ICP8-specific fluorescence (Fig. 3). Additionally, U_L6-specific

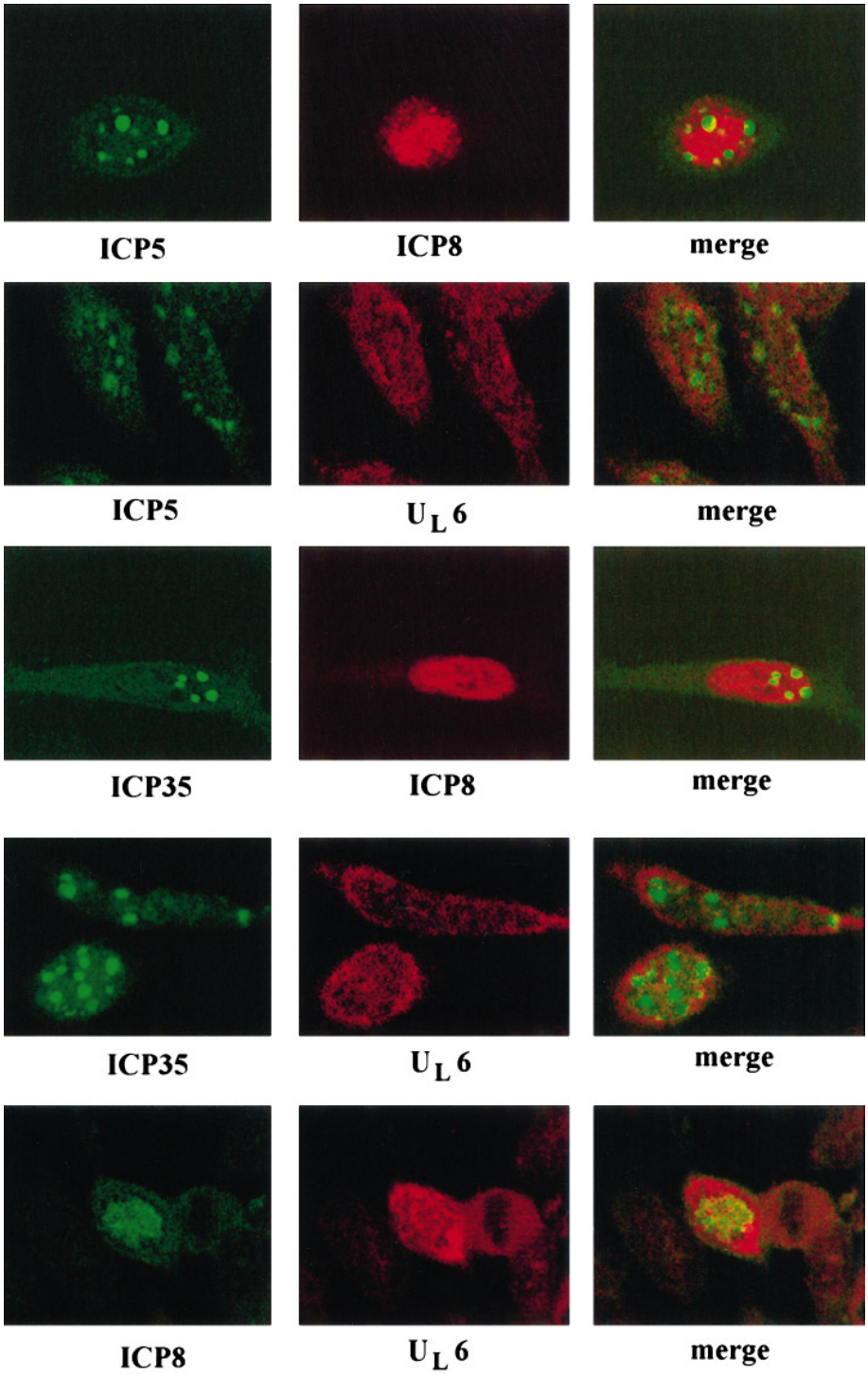


FIG. 3. Digital confocal images of infected HEp-2 cells fixed and stained 18 h after infection. Cells were infected with HSV-1(ΔU_L17), a virus lacking an intact U_L17 gene, and were reacted with monoclonal antibodies against ICP5, ICP35, or ICP8 and polyclonal antiserum against ICP8 or MBP-U_L6. Bound mouse immunoglobulin was visualized with FITC-conjugated donkey anti-mouse immunoglobulin (green) and bound rabbit immunoglobulin was visualized with Texas Red- or lissamine rhodamine-conjugated donkey anti-rabbit immunoglobulin (red). Single color images in the left and middle columns were recorded simultaneously or separately. The right column represents the merged images of the left and middle panels in each row. Images were recorded and printed as described in the legend to Fig. 2.

fluorescence was dissociated from regions exhibiting ICP5- and ICP35-specific fluorescence (Fig. 3). To confirm that the altered staining patterns observed in the U_L17 mutant virus [HSV-1 $\Delta(U_L17)$]-infected cells were due solely to the defect in the U_L17 gene, we examined the distribution of ICP5 and the U_L6 protein in cells

infected with a virus bearing a stop codon within the U_L17 gene [HSV-1(U_L17-stop)] and a virus derived from HSV-1(U_L17-stop) bearing a restored U_L17 gene [HSV-1(U_L17-restored)] (Fig. 4). These viruses have been described previously (Salmon *et al.,* 1998). The patterns observed in cells infected with U_L17-stop and U_L17-restored (Fig. 4) were similar to those seen in the U_L17 deletion virus [HSV-1 Δ(U_L17)] (Fig. 3) and HSV-1(F)-infected cells (Fig. 2), respectively. Thus reversal of the mutation in U_L17 allowed capsid proteins to distribute evenly throughout the DNA replication compartment, as in wild-type virus infected cells. We therefore conclude that the U_L17 gene is required for (i) proper distribution of ICP5 and ICP35 in the DNA replication compartment and (ii) colocalization of the minor capsid protein encoded by U_L6 with major capsid proteins ICP5 and ICP35.

In the above experiments, ICP35-specific staining of intact permeabilized cells is likely due to noncapsid associated ICP35 inasmuch as capsid associated ICP35 is sequestered in the capsid core (Newcomb and Brown, 1989). Similarly, at least some of the ICP5-specific immunofluorescence represents noncapsid-associated ICP5. To examine the role of U_L17 in distribution of capsids, cells were infected with HSV-1(F) or the U_L17 deletion mutant [HSV-1 Δ(U_L17)], permeabilized at 18 h after infection, and stained with a monoclonal antibody, 8F, which specifically recognizes an ICP5 epitope present only after ICP5 has undergone conformational changes during maturation of the outer capsid shell (Trus *et al.*, 1992). The same cells were also stained with an ICP8-specific polyclonal rabbit antiserum directed against ICP8 (Shelton *et al.,* 1994). At 18 h p.i., cells stained with the capsid-specific antibody demonstrated both cytoplasmic and nuclear staining. This observation was likely due to staining of intranuclear capsids and capsids present within cytoplasmic virions that accumulate in large numbers at late times after infection (Fig. 5). Intranuclear capsid-specific fluorescence colocalized with ICP8-specific fluorescence, indicating that capsids are normally evenly distributed in the DNA replication compartment (Fig. 5). In contrast to these observations, but consistent with the appearance of electron micrographs of U_L17 deletion virus-infected cells (Salmon *et al.*, 1998), capsid-specific fluorescence appeared predominantly in the nuclei of HEp-2 cells within brightly staining punctate regions that accumulated at the nuclear periphery in regions not containing ICP8 (Fig. 5). These data indicate that (i) intranuclear capsids accumulate primarily in the DNA replication compartment of HEp-2 cells late in infection with wild-type virus and (ii) the U_L17 gene is required for the normal distribution of capsids within this compartment.

One possibility to explain these observations is that aberrant targeting of capsid assembly components reflects a consequence of defective DNA cleavage and packaging rather than a specific role of U_L17 gene products in targeting of assembly products to DNA replication compartments. To address this possibility, cells were infected with cleavage/packaging mutant viruses lacking intact U_L15 [HSV-1(ΔU_L15ExII)] (Baines *et al.,* 1997) (data not shown), U_L28 (mutant virus gCB) (Fig. 6, U_L28^-), and U_L33 (mutant virus U_L33^-) (Fig. 6, U_L33^-) and were stained with ICP35-specific mouse monoclonal antibody and rabbit antiserum directed against MBP-U_L6 protein. The results indicated that fluorescence attributable to ICP35 and U_L6 protein coincided in an even distribution throughout infected cell nuclei (Fig. 6). Thus colocalization of major and minor capsid proteins is dependent on the U_L17 gene but not DNA cleavage and packaging. Parenthetically, these data also indicate that the colocalization of major and minor capsid proteins occurs in cells infected with viruses derived from a variety of viral strains including HSV-1(F) [HSV-1(ΔU_L15ExII)], HSV-1(17) (U_L17-restored), and HSV-1(KOS) (U_L28 mutant virus, gCB).

DISCUSSION

Using confocal immunofluorescence microscopy, we have determined that the minor capsid protein encoded by U_L6 and major capsid proteins ICP5 and ICP35 primarily localize within the DNA replication compartment of HEp-2 cells late after infection with wild-type HSV-1(F). As revealed by staining with a monoclonal antibody directed against a conformationally sensitive epitope in capsid-associated ICP5, we have also demonstrated that capsids localize in replication compartments late in infection of HEp-2 cells. These data, together with the observation that U_L6 protein and major capsid proteins ICP5 and ICP35 accumulate primarily in the replication compartment of HEp-2 cells early in infection (not shown), support the hypothesis that replication compartments are sites of capsid assembly and DNA cleavage and packaging in HEp-2 cells.

It has been shown that, at least early in infection of Vero cells, capsids and components required for capsid assembly accumulate in replication compartments (Lamberti and Weller, 1998), whereas later in infection, many viral assembly components accumulate within regions of infected Vero cell nuclei, termed assemblons, that accumulate at the periphery of the nucleus outside DNA replication compartments (Ward et al.,1996). Studies conducted in our laboratory indicated that in Vero cells infected with HSV-1(F) and stained with capsid-specific antibodies, assemblons were readily detectable late in infection (not shown). In contrast to observations in Vero cells, only 5% of HSV-1(F)-infected HEp-2 cells, fixed and stained identically as in the studies of Ward *et al.* (1996), contained assemblon-like foci as revealed by staining with antibodies against major capsid proteins and U_L6 protein (Fig. 2). The cellular factors that contribute to the differences in frequency of assemblon formation in

HEp-2 and Vero cells are unknown. Reasonable explanations include the possibilities that (i) HEp-2 cells produce fewer capsids or capsid proteins, leading to decreased frequency of aggregation of assembly byproducts, or (ii) HEp-2 cells are more resistant to HSV-mediated redistribution of intranuclear proteins.

In the absence of the U_L17 gene, the distribution of ICP5 and ICP35 is largely restricted to brightly staining punctate regions, whereas these proteins are more evenly distributed throughout the DNA replication compartment in HEp-2 cells infected with wild-type virus. The brightly staining foci in all cells infected with the U_L17 mutant were distinguishable from assemblons because these structures differed in appearance from assemblons; the structures occurred more frequently and occasionally were within the DNA replication compartment (Ward *et al.*, 1996). The minor capsid protein encoded by U_L6 was also displaced in cells infected with the U_L17 mutant. Thus in contrast to the appearance of U_L6-specific immunofluorescence that normally colocalizes with major capsid proteins, U_L6 protein accumulated at the nuclear periphery of cells infected with the U_L17 mutant in sites distinct from replication compartments and regions containing major capsid proteins (Fig. 3). This mistargeting of U_L6 protein does not preclude incorporation of U_L6 protein into capsids inasmuch as the protein is readily detected in capsids purified from cells infected with the U_L17 mutant (data not shown).

As seen in these indirect immunofluorescence studies, the absence of the U_L17 gene results in the redistribution of capsids from the DNA replication compartment to aggregates at the nuclear periphery of infected HEp-2 cells. Such aggregates were not present in cells infected with a virus bearing a restored U_L17 gene, indicating that aberrant distribution of capsid proteins was a consequence of the mutation in U_L17. This result is in agreement with previously reported studies in which electron microscopic examination of U_L17 mutant virus-infected Vero cells revealed intranuclear aggregates of capsids that accumulated near the inner nuclear membrane late in infection (Salmon *et al.*, 1998). Electron microscopic examination of cells infected with a virus derived from a U_L17 insertion mutant and bearing a restored U_L17 gene and of complementing cells infected with U_L17 mutants did not contain capsid aggregates to any appreciable extent (Salmon *et al.*, 1998). We have also shown in studies reported herein that capsid proteins remain in replication compartments in cells infected with DNA cleavage/packaging mutants lacking functional U_L28, U_L33 (Fig. 6), and U_L15 (not shown). These observations indicate that the unusual intranuclear distribution of capsids in cells infected with the U_L17 mutant is a result of the absence of the U_L17 gene products rather than a generic consequence of defective cleavage and packaging.

Paracrystalline arrays of capsids have been observed in thin sections of nuclei late in infection with wild-type virus, and these superficially resemble capsid aggregates seen in cells infected with the U_L17 mutant (Roizman and Sears, 1995). However, whereas capsid aggregates in wild-type virus-infected cells are relatively uncommon, all capsids in thin sections of cells infected with the U_L17 mutant virus were present in capsid aggregates (Salmon *et al.*, 1998). Data supporting the differences in distribution of capsids in wild-type and U_L17 mutant virus includes the even distribution of staining with hexon-specific antibody in wild-type virus-infected cells, as opposed to the brightly staining punctate regions detected in cells infected with the U_L17 mutant virus (Fig. 5).

The appearance of capsid distribution in cells infected with the U_L17 mutant superficially resembles that of cells infected with a virus bearing a temperature-sensitive mutation in the HSV protease and held at nonpermissive temperatures (Preston *et al.,* 1983; Gao *et al.*, 1994; Wilson and Church, 1997). Such cells contain aggregated procapsids that disperse after induction of capsid maturation upon shift to the permissive temperature (Wilson and Church, 1997). Although these considerations suggest that U_L17 may act to couple capsid maturation to capsid transport, it is not yet clear that the procapsid aggregates in cells infected with protease mutants reflects the distribution of procapsids in cells infected with U_L17 mutant viruses. In any case, the role that U_L17 plays in capsid trafficking is apparently different from that of the U_L32 gene product given the observation that capsid aggregates are not readily detected in cells infected with a viral mutant lacking U_L32 (Lamberti and Weller, 1998).

Taken together, the data reported herein suggest that one role of U_L17 proteins is to mediate distribution of capsid precursors and capsids throughout the DNA replication compartment where capsids are assembled and DNA is packaged. Defining the precise role of U_L17 in these processes and how such targeting is mediated and regulated will require additional studies.

MATERIALS AND METHODS

Cells and viruses

Vero cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% newborn calf serum, penicillin and streptomycin. HEp-2 cells and all other cell lines used in these studies were maintained in DMEM supplemented with antibiotics and 10% newborn calf serum. The following viruses were used in this study: HSV-1(F) (wild-type virus) (Ejercito *et al.,* 1968), U_L6^- (U_L6 mutant virus derived from HSV-1 strain 17) (Patel *et al.,* 1996), HSV-1(ΔU_L17) [U_L17 mutant virus derived from HSV-1(F)] (Salmon *et al.,* 1998), HSV-1(U_L17-stop) (U_L17 mutant virus derived from HSV-1 strain 17) (Salmon *et al.,* 1998), HSV-1(U_L17-restored) [U_L17-stop mutant virus derived from HSV-1(U_L17-stop) bearing a restored U_L17

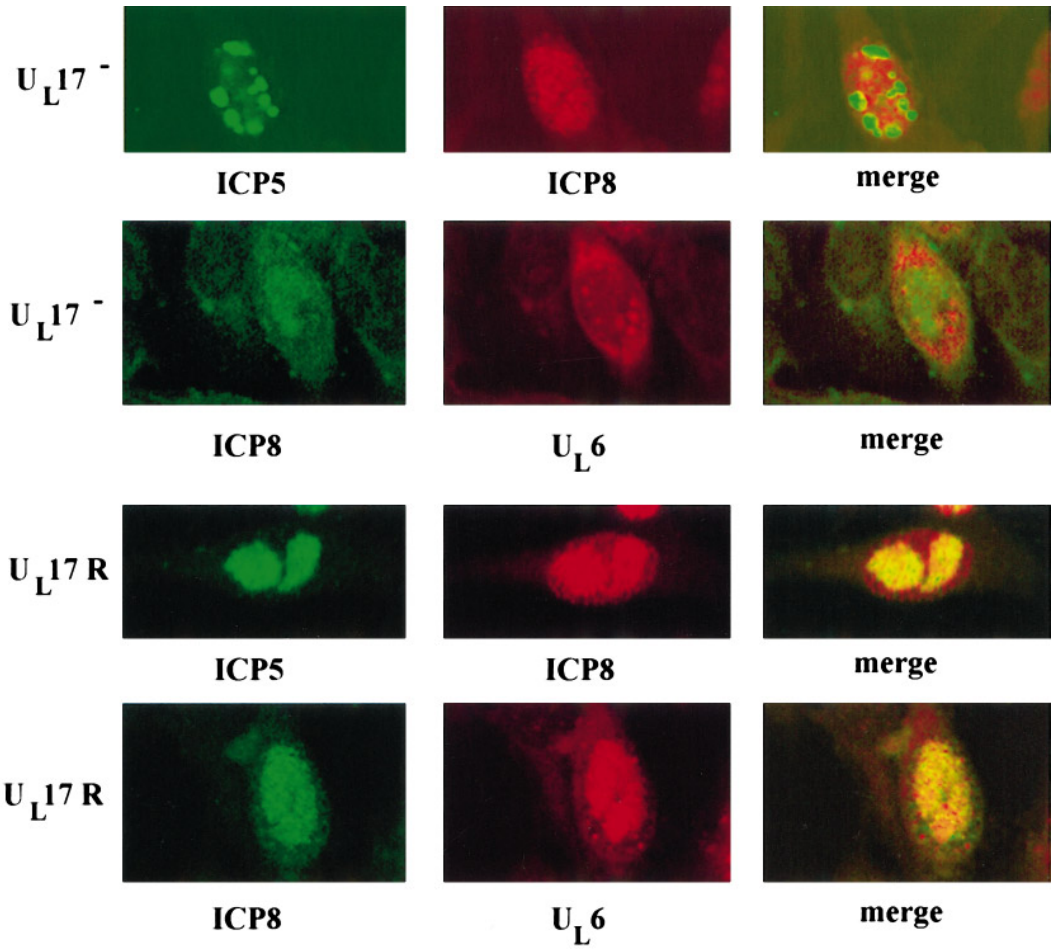


FIG. 4. Digital confocal images of infected HEp-2 cells fixed and stained 16 h p.i. Cells were infected with a virus bearing a stop codon in the U_L17 gene HSV-1(U_L17-stop) (designated U_L17^-) and a virus derived from HSV-1(U_L17-stop) but containing a restored U_L17 gene [HSV-1(U_L17-restored)] (designated U_L17R in the figure). Cells were permeabilized in cold methanol and reacted with monoclonal antibodies against ICP5 and ICP8 and polyclonal antibodies against ICP8 and MBP-UL6. Monoclonal antibodies were visualized with FITC-conjugated donkey antimouse immunoglobulin (green). Polyclonal antibodies were detected with Texas Red-conjugated donkey anti-rabbit immunoglobulin (red). Single color images in the left and middle columns were recorded simultaneously. The right column represents the merged images of the left and middle panels of each row. Images were recorded and printed as described in the legend to Fig. 2.

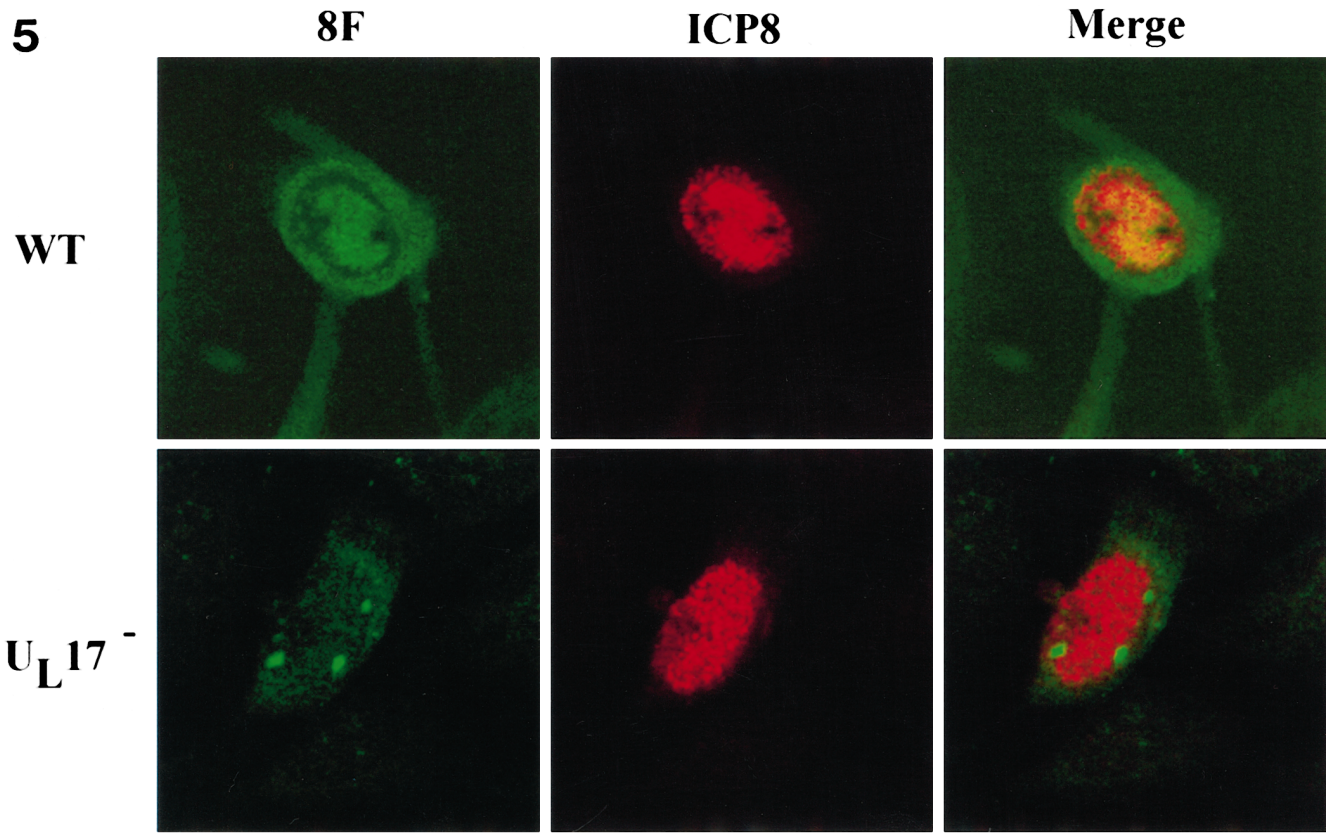
gene] (Salmon *et al.,* 1998), gCB (U_L28 mutant virus derived from HSV-1 strain KOS) (Tengelsen *et al.,* 1993), U_L33^- (U_L33 mutant virus derived from HSV-1 strain 17) (Cunningham and Davison, 1993), and HSV-1(ΔU_L15ExII) [U_L15 mutant virus derived from HSV-1(F)] (Baines *et al.,* 1997). The transformed cell lines G33, G5 (kindly provided by Dr. Arvind Patel, MRC Virology Unit, Glasgow), and C1 (kindly provided by Dr. Fred Homa, Pharmacia-Upjohn) were used for propagation of the U_L6, U_L33, and U_L28 mutants, respectively. These cell lines have been described previously (Cunningham and Davison, 1993; Tengelsen *et al.,* 1993; Patel *et al.*, 1996).

Antibodies

Monoclonal antibodies against HSV-1 ICP8 (GIRC No. 1115), ICP5 (GIRC No. 1117), and ICP35 (GIRC No. 1102) were purchased from Goodwin Institute for Cancer Research (Plantation, FL). The hexon specific monoclonal antibody, 8F, was a kind gift from Dr. Min Gao, Bristol-Myers Squibb, and has been described previously (Trus *et al.,* 1992). The rabbit polyclonal antibody against ICP8 was a kind gift of Dr. William Ruyechan, State University of New York at Buffalo, Buffalo, NY, and has also been described (Shelton *et al.,* 1994). The rabbit polyclonal antibody against U_L21 has been described (Baines *et al.,* 1994).

Antiserum production and Immunoblotting

To produce an antiserum directed against U_L6-encoded protein, a *Sal*I fragment containing bases 16,264–17,324 of HSV-1 (McGeoch *et al.*, 1988) was ligated into the *Sal*I site of pMal-c (New England Biolabs, Inc., Beverly, MA). This plasmid was predicted to encode the C-terminal 298 amino acids of U_L6 fused to the gene encoding maltose-binding protein (MBP). The junction of the two genes was sequenced to ensure that the open



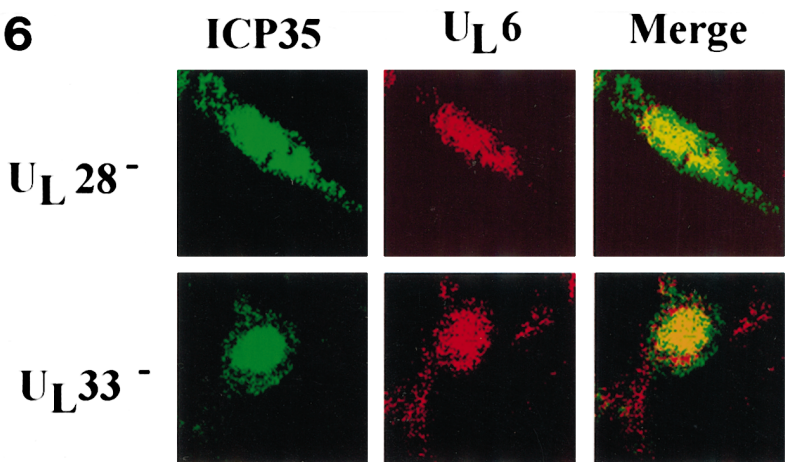


FIG. 5. Digital confocal images of infected HEp-2 cells fixed and stained 18 h p.i. Cells were infected with wild-type HSV-1(F) virus (WT) or HSV-1(ΔU_L17) (U_L17^-) and fixed cells were reacted with a hexon-specific monoclonal antibody (8F) and a polyclonal antibody directed against ICP8. Monoclonal antibody was visualized with FITC-conjugated donkey antimouse immunoglobulin (green). Bound anti-ICP8 antibody was identified with Texas Red-conjugated donkey anti-rabbit immunoglobulin (red). Single color images in the left and middle columns were recorded simultaneously. The right column represents the merged images of the left and middle panels of each row. Images were recorded and printed as described in the legend to Fig. 2.

FIG. 6. Digital confocal images of HEp-2 cells infected with U_L28 mutant (gCB) (U_L28^-) or U_L33 mutant (U_L33^-) viruses and fixed and stained 18 h after infection. Cells were reacted with monoclonal antibody against ICP35 and polyclonal antiserum against MBP-U_L6. ICP35-specific antibody was visualized with FITC-conjugated donkey anti-mouse antibody (green) and MBP-U_L6-specific antibody was visualized with Texas Red-conjugated donkey anti-rabbit immunoglobulin (red). Single color images in the left and middle columns were recorded simultaneously. The right column represents the merged image of the left and middle panels in each row. Images were recorded and printed as described in Fig. 2.

reading frame encoding the fusion protein was maintained (not shown). Expression of the soluble fusion protein (MBP-U_L6) was induced by the addition of IPTG to a final concentration of 0.1 mM, and MBP-U_L6 was purified by affinity chromatography on amylose beads (New England Biolabs, Inc.). The purified protein was eluted in 10 mM maltose and was used to immunize New Zealand White rabbits as previously described (Baines and Roiz-

man, 1993). Mock-, HSV-1(F)-, and U_L6^--infected HEp-2 cell lysates were prepared 18 h p.i. in phosphate-buffered saline (PBS), pH 7.4, supplemented with 1% Triton X-100, 1% sodium deoxycholate, 10 μM TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone) and 10 μM TLCK (α-tosyl-L-lysine chloromethyl ketone) and separated on a 7.5% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose, and immunoblotting was performed as described (Baines and Roizman, 1993) except that the MBP-U_L6 antiserum was diluted 1:1000 in PBS plus 1% bovine serum albumin (BSA) and 1% Tween 20. Immunoblotting with the U_L21 antibody was performed as previously described (Baines *et al.,* 1994).

Confocal immunofluorescence microscopy

Lab-Tek chamber slides (Nunc) were seeded with 30,000 HEp-2 cells per 1.0 cm^2 well, or six-well dishes containing sterile glass cover slips were seeded with 50,000 HEp-2 cells per 10 cm^2 well. Cells were allowed to grow overnight and were infected with various viruses as indicated in the text. At 18 h p.i., cells were permeabilized in ice-cold methanol for 10 min and air dried. Permeabilized cells were incubated with PBS containing 10–20% human serum (Sigma, S 2145) and 1% BSA to block nonspecific binding of rabbit immunoglobulin by the HSV-encoded Fc receptor containing gE/gI (Johnson *et al.*, 1988). Cells were reacted for 1–2 h at 37°C with diluted ascites fluid containing antibodies directed against ICP8 (1:500), ICP5 (1:1000), ICP35 (1:100), and the anti-MBP-U_L6 antiserum, which was extensively adsorbed against HEp-2 cells and diluted 1:50 in PBS plus 1% BSA. Alternatively, cells were incubated with polyclonal rabbit antiserum directed against ICP8 (1:1000) and monoclonal antibodies directed against ICP5, capsid-associated ICP5 (8F, 1:30), or ICP35. After extensive washing in PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse immunoglobulin and lissamine rhodamine- or Texas Red-conjugated donkey anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at 37°C. Samples were washed in PBS and mounted in Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA). Confocal microscopy was done as previously described (Nalwanga *et al.*, 1996). Digital images were printed on a Codonics NP1600 dye sublimation printer.

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