

# The Pseudorabies Virus UL28 Protein Enters the Nucleus after Coexpression with the Herpes Simplex Virus UL15 Protein

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**Herpesvirus DNA is packaged into capsids in the nuclei of infected cells in a process requiring at least six viral proteins. Of the proteins required for encapsidation of viral DNA, UL15 and UL28 are the most conserved among herpes simplex virus type 1 (HSV), varicella-zoster virus, and equine herpesvirus 1. The subcellular distribution of the pseudorabies virus (PRV) UL28 protein was examined by in situ immunofluorescence. UL28 was present in the nuclei of infected cells; however, UL28 was limited to the cytoplasm in the absence of other viral proteins. When cells expressing variant forms of UL28 were infected with a PRV UL28-null mutant, UL28 entered the nucleus, provided the carboxyl-terminal 155 amino acids were present. Additionally, PRV UL28 entered the nucleus in cells infected with HSV. Two HSV packaging proteins were tested for the ability to affect the subcellular distribution of UL28. Coexpression of HSV UL15 enabled PRV UL28 to enter the nucleus in a manner that required the carboxyl-terminal 155 amino acids of UL28. Coexpression of HSV UL25 did not affect the distribution of UL28. We propose that an interaction between UL15 and UL28 facilitates the transport of a UL15-UL28 complex to the infected-cell nucleus.**

Herpesvirus DNA is packaged into preassembled capsids in the nuclei of infected cells in a process that is not clearly understood. At least six genes that are required for the encapsidation of concatemeric viral DNA into preassembled nucleocapsids have been identified in herpes simplex virus type 1 (HSV) (2, 4, 6, 11, 18, 21–23, 26). Viruses with deletions or temperature-sensitive mutations in these genes possess similar phenotypes. Under nonpermissive conditions the mutants fail to cleave monomer-length genomes from the concatemeric form, resulting in the accumulation of concatemeric virus genomes and capsids lacking DNA.

Pseudorabies virus (PRV) is an alphaherpesvirus closely related to HSV and serves as a valuable tool for the study of herpesvirus DNA encapsidation. The UL28-null encapsidation mutant PRV(332-31) is characterized by the same phenotype as the UL28-null HSV gCB mutant (14, 23). In several cases, PRV and HSV proteins can functionally substitute for their homologs (15, 16).

Efforts to better characterize and understand the properties of the six proteins required for encapsidation have recently been made. PRV UL28 had been visualized within the nuclei of infected cells (19), and it was presumed that UL28 contained a nuclear localization signal (NLS) similar to that reported for the simian virus 40 (SV40) T antigen (9). In this work, experiments were performed to demonstrate that UL28 does not possess a classical NLS, that UL28 requires another viral protein in order to enter the nucleus, and that the HSV UL15 protein can fulfill that requirement.

## MATERIALS AND METHODS

**Cells and viruses.** African green monkey kidney cells (Vero; ATCC CRL-1586) were propagated in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (HyClone) plus 50 U of penicillin G per ml and 50 µg of streptomycin sulfate (Life Technologies) per ml. PRV(Becker) and HSV-(KOS) were used as the wild-type strains.

A cell line transformed with the PRV UL28 and gB genes was constructed as follows. Vero cells were cotransfected with pECP108 plus pSV2neo and selected for resistance to 500 µg of G418 (Life Technologies) per ml. Colonies were screened for functional PRV UL28 and PRV gB activities by their ability to support plaque formation of the HSV gB-null mutant HSV(KΔT) (5) and the PRV UL28-null mutant PRV(332-31) (14). A cell line that expressed both UL28 and gB upon infection was named V108. The ability of PRV gB to complement an HSV gB-null mutant was previously demonstrated (15). V108 cells were maintained in media containing 100 µg of G418/ml.

**Antisera.** Polyclonal rabbit antisera were raised against purified fusion proteins expressed in *Escherichia coli*. PRV UL28 was expressed as a Cro-UL28 fusion to produce D71 antiserum (19). HSV UL15 was expressed as a maltose-binding protein-UL15 (exon 2) fusion to generate AS9 antiserum (27), and HSV UL25 was expressed as a glutathione *S*-transferase-UL25 fusion to generate ID1 antiserum.

**Plasmids.** PRV UL28 was cloned into the expression vector pLuxF3 (24) as pECP93 to express UL28 under the control of the SV40 promoter. A series of UL28 deletion mutations was derived from pECP93. UL28Δ1 contained a deletion between the *StuI* and *MscI* sites to eliminate codons 93 through 218. UL28Δ2 contained a deletion between the *MscI* and *SphI* sites to eliminate codons 219 through 393. UL28Δ3 contained a deletion between the *SphI* and *HincII* sites to eliminate codons 393 through 569. UL28Δ4 contained a deletion between the *HincII* and *NheI* sites to eliminate codons 570 through 724. UL28Δ2, UL28Δ3, and UL28Δ4 had *EcoRI* oligonucleotide linkers inserted to maintain the reading frame. An NLS from the SV40 T antigen was inserted between codons 37 and 38 of UL28 by ligating the oligonucleotide 5' CGC GTA TGG CCA AAA AAG AAG AGA AAG GTC 3' and its complement with appropriate ends into the *MluI* site to generate UL28/NLS.

HSV proteins were expressed under the control of the cytomegalovirus major immediate-early promoter in plasmid pF1'-CMV (8). HSV UL15 cDNA was constructed by insertion of the exon 1 and exon 2 splice junction into the genomic DNA (27). HSV UL25 was from a genomic clone (pKEF-B5 [7]).

Plasmid pECP108, containing the PRV UL28 and gB genes, was used to construct the recombinant cell line V108. pECP108 contained a *NotI-NcoI* fragment spanning UL28 and gB.

**Transfection and immunofluorescence.** Vero cells were plated at 10 to 20% confluence on coverslips and transfected with plasmid DNAs. Plasmid DNAs in serum-free media (Opti-MEM I; Life Technologies) were mixed with a cationic lipid (pFx-3; Invitrogen) in serum-free medium and added to the cell monolayer for 24 h. The medium was replaced with Dulbecco's modified Eagle's medium

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plus fetal calf serum and antibiotics and incubated at 37°C for an additional 24 h. Cells were fixed with formaldehyde and permeabilized with Nonidet P-40 as previously described (19). The primary antisera (1:250 dilution) in phosphate-buffered saline (PBS) plus 5% nonfat dry milk were incubated with the cells for more than 90 min. The cells were rinsed in PBS and incubated with a secondary, tetramethyl rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antiserum (1:50 dilution; Sigma) for more than 90 min. The coverslips were rinsed in PBS and mounted onto slides. Cells were observed by epifluorescence on a Zeiss Axioplan microscope with a 100-W UV lamp and photographed.

## RESULTS

**Subcellular distribution of PRV UL28.** PRV UL28 has been shown to accumulate in the nuclei of infected cells (19). A series of experiments was performed to identify sequences required for localization of UL28 to the nucleus. Vero cells were infected with PRV(Be) and examined by in situ indirect immunofluorescence for the distribution of UL28 (Fig. 1A). UL28 was distributed throughout the cell but was most prominent in the nucleus. When a plasmid encoding PRV UL28 was transfected into Vero cells and the cells were examined by immunofluorescence, UL28 was limited to the cytoplasm (Fig. 1B). This demonstrated that UL28 did not possess a distinct functional NLS and suggested that an interaction with one or more other virus-induced proteins was responsible for the nuclear localization during infection. Previous evidence suggested that UL28 associated with another protein during infection (19). To demonstrate that UL28 was not actively retained in the cytoplasm, a decacodon NLS derived from the SV40 T antigen (9) was inserted within *UL28* (Fig. 2). UL28/NLS was expressed in Vero cells and found within the nucleus (Fig. 1C). The UL28/NLS protein was not absent from the cytoplasm but had acquired the ability to enter the nucleus.

The immunofluorescent signals in the photographs are significantly above the background level in this and subsequent experiments. Every cell is adjacent to cells that do not express UL28. These nonexpressing cells represent the background level and are too weak to be detected under the conditions that permit the visualization by immunofluorescence of the expressing cells. The possibility that the transient-expression plasmid pECP93 had acquired one or more mutations that may have caused the restriction to the cytoplasm was addressed. A stable pECP93-transformed cell line that permitted plaque formation by PRV(332-31) was isolated, indicating that pECP93 encodes and expresses functional UL28 (20).

**Identification of the sequences within UL28 required for nuclear localization.** Full-length UL28 did not possess a functional NLS and was likely to require another virus-induced protein for transport to the nucleus. A series of experiments were conducted to identify the domain within UL28 likely to be necessary to signal its transport to the nucleus. A series of deletion mutations which spanned *UL28* was made (Fig. 2). The *UL28* deletion plasmids were transfected singly into Vero cells, and the distribution of UL28 within these cells was examined by immunofluorescence (Fig. 3). All of the proteins were detected at significant levels in transfected cells, suggesting that they retained epitopes for detection by the D71 polyclonal antiserum and were stable under the conditions used. Protein stability was addressed by examining the strength of immunofluorescence signals at 24 and 48 h posttransfection. The signals were of greater intensity at 48 h than at 24 h, suggesting that the protein levels accumulated with time. Although the signal intensities increased with time, the individual UL28 variant proteins exhibited different distribution patterns. The UL28, UL28Δ1, UL28Δ3, and UL28Δ4 proteins exhibited signals limited to the cytoplasm (Fig. 3A, B, D, and E). However, the UL28Δ2 protein was evenly distributed throughout

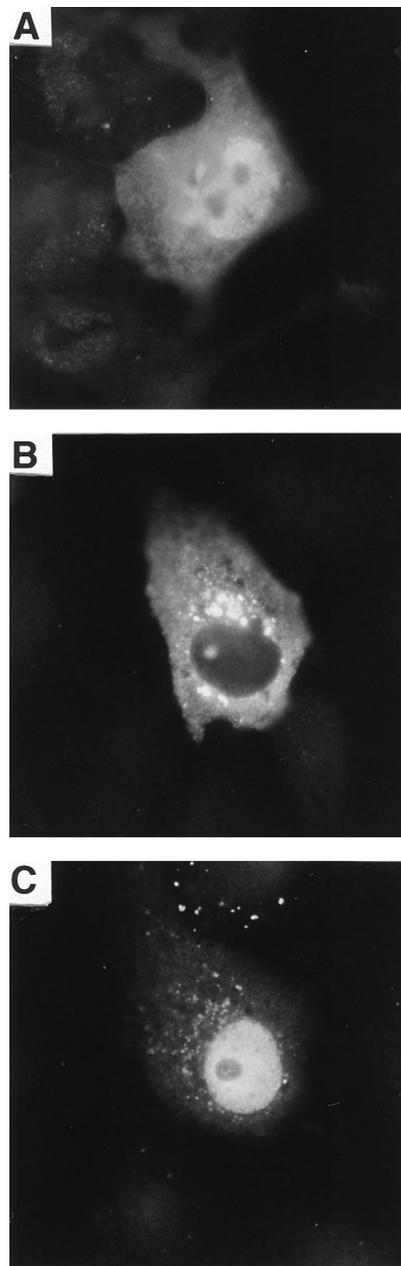


FIG. 1. In situ detection of PRV UL28. (A) Vero cells grown on coverslips were infected with PRV at an MOI of 0.1 and harvested 6 h postinfection for immunofluorescence. (B and C) Vero cells were grown on coverslips and transfected with plasmids to express either UL28 (B) or UL28/NLS (C). The proteins were detected by indirect in situ immunofluorescence with D71 as the primary antiserum and tetramethyl rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G as the secondary antiserum. Immunoreactive cells were visualized at a magnification of  $\times 790$  by epifluorescence and recorded on Kodak Tri-X film.

the cell (Fig. 3C). The means by which UL28Δ2 entered the nucleus is not understood. The amino acids joined as a result of the deletion did not fit consensus nuclear localization domain patterns. It is also unlikely that the protein's smaller size was responsible for its ability to enter the nucleus, since all of the shortened proteins were of comparable sizes. Another possibility is presented in the Discussion.

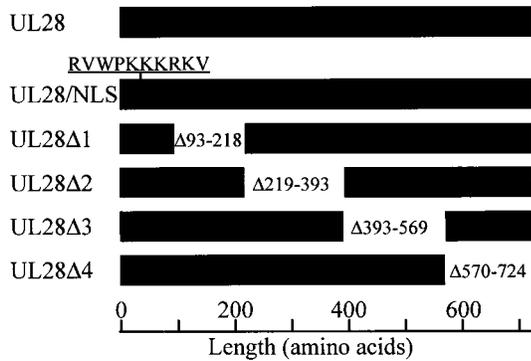


FIG. 2. Structure of PRV UL28 protein variants. The UL28 proteins expressed in cells are diagrammed to scale. The names of the proteins, their features, and the extent of their deletions are given.

The UL28 deletion proteins were then tested for the ability to be directed to the nucleus during infection with the UL28-null mutant PRV(332-31). This experiment tested the ability of the UL28 deletion proteins to enter the nucleus in the presence of all other virus-induced proteins. Vero cells were transfected and infected 24 h later with PRV(332-31) at a multiplicity of infection (MOI) of 10. The infection was terminated 6 h postinfection, and the cells were processed for examination by immunofluorescence detection of UL28. Under these conditions, the UL28, UL28Δ1, UL28Δ2, and UL28Δ3 proteins were transported to the nucleus (Fig. 4A to D). The UL28Δ4 protein remained in the cytoplasm; its distribution was unaffected by the infection. These data were consistent with the specific transport of UL28 to the nucleus facilitated by one or more other virus-induced proteins. Two additional observations were made: (i) there were no detectable UL28-positive revertants contaminating the PRV(332-31) stock, as determined by a >6-log difference in plaque formation on Vero and V108 cells as well as the inability to detect UL28 by immuno-

fluorescence in cells infected at an MOI of 10 with PRV(332-31); and (ii) there was no detectable UL28 in the PRV(332-31) virion preparation resulting from UL28 packaged in the virion, as determined by the inability to detect UL28 in PRV(332-31)-infected cells by immunofluorescence shortly after infection. Therefore, the UL28 proteins detected were due to expression from the transfected plasmids.

**HSV-induced proteins provide nuclear transport for PRV UL28.** There is considerable conservation at the amino acid level of several of the proteins involved in the packaging/cleavage reaction among herpesviruses. The possibility that an HSV-induced function could complement the PRV-induced entry of transiently expressed UL28 into the nucleus was tested. PRV UL28-transformed cells (V108 cells) were infected with HSV(KOS) and examined by immunofluorescence for the expression and distribution of PRV UL28 (Fig. 5A). HSV infection induced the expression of PRV UL28 from the V108 cell chromosome, and PRV UL28 was detected in both the cytoplasm and the nucleus. The D71 antisera against PRV UL28 did not cross-react with HSV UL28 as determined by immunofluorescence or Western blot analyses (20). These results indicated that one or more HSV-induced proteins effectively relocated UL28 to the nucleus. These results also suggested that the virus-induced protein that transports UL28 to the nucleus was conserved in HSV infection and that PRV UL28 interacts with an HSV protein.

**HSV UL15 directed PRV UL28 to the nucleus.** The finding that an HSV-induced function facilitated the transport of PRV UL28 to the nucleus was explored. The sequence of the HSV genome is known (12, 13), and the proteins required for packaging are relatively well characterized compared to the PRV proteins. Two HSV proteins required for packaging were tested for their potential to direct UL28 to the nucleus. HSV UL15 was chosen because (i) UL15 and UL28 are the two most conserved proteins involved in encapsidation among herpesviruses and (ii) of the predicted masses of the known packaging proteins, that of HSV UL15 was the closest to, and

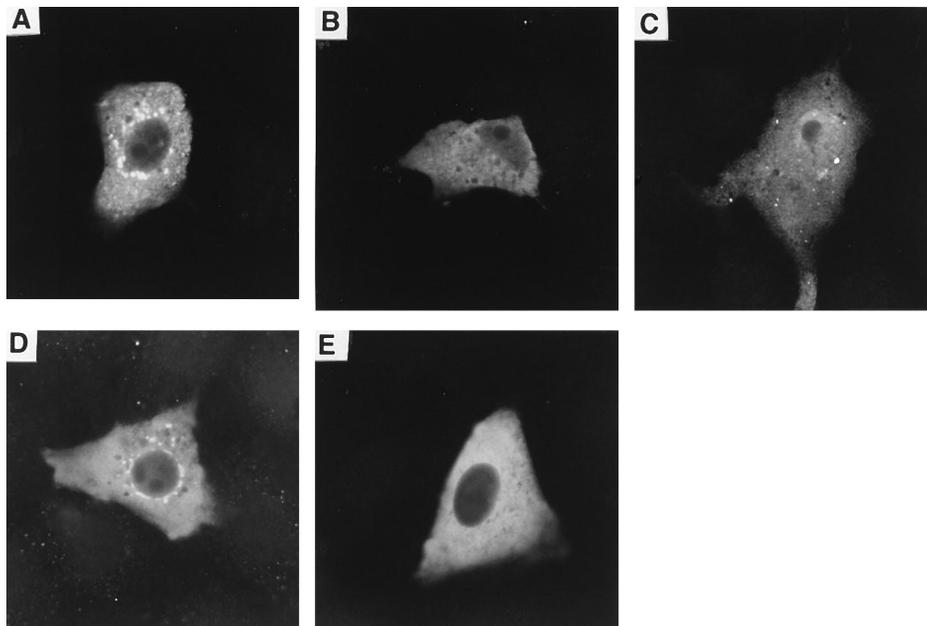


FIG. 3. UL28 variant protein expression in Vero cells. Vero cells grown on coverslips were transfected and processed for immunofluorescent detection of PRV UL28. (A) UL28; (B) UL28Δ1; (C) UL28Δ2; (D) UL28Δ3; (E) UL28Δ4. Magnification,  $\times 580$ .

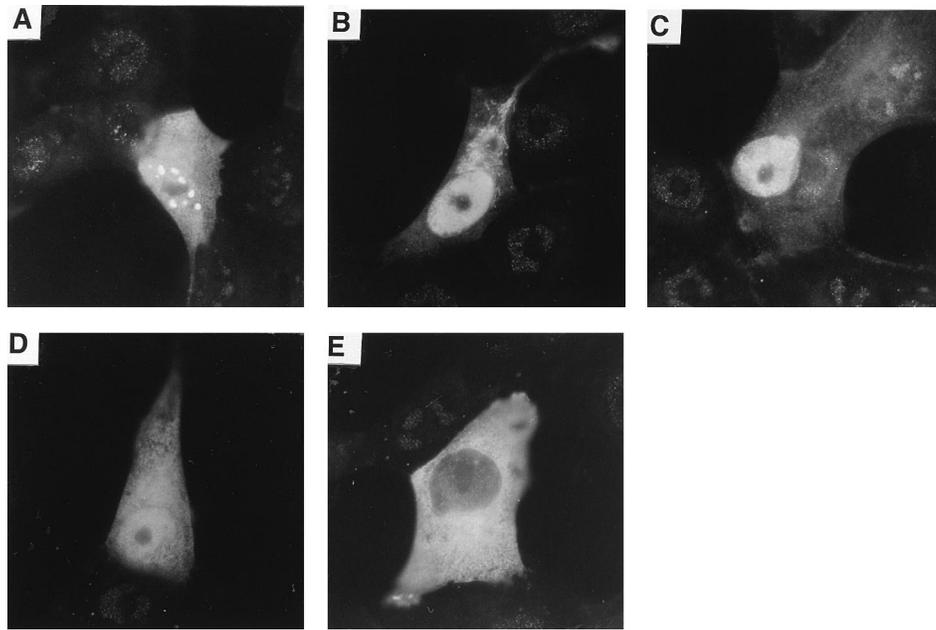


FIG. 4. UL28 variant protein expression in PRV(332-31)-infected cells. Vero cells grown on coverslips were transfected with plasmids to express the indicated proteins and infected with PRV(332-31) at an MOI of 10 24 h later. The cells were processed for immunofluorescent detection of PRV UL28 6 h postinfection. (A) UL28; (B) UL28 $\Delta$ 1; (C) UL28 $\Delta$ 2; (D) UL28 $\Delta$ 3; (E) UL28 $\Delta$ 4. Magnification,  $\times$ 580.

slightly less than that of HSV UL28, reminiscent of the slightly smaller protein which coimmunoprecipitated with PRV UL28 (19).

Plasmids encoding PRV UL28 and HSV UL15 were co-transfected into Vero cells and examined by immunofluorescence 48 h later. Full-length PRV UL28 was redistributed to the nucleus upon coexpression of HSV UL15 (Fig. 5B). The

effect of HSV UL15 on PRV UL28 localization was examined further by testing the PRV UL28 deletion proteins. Each of the plasmids encoding the PRV UL28 deletion proteins was co-transfected with the plasmid encoding HSV UL15 (Fig. 5C to F). Upon coexpression with HSV UL15, the UL28, UL28 $\Delta$ 1, UL28 $\Delta$ 2, and UL28 $\Delta$ 3 proteins accumulated in the nucleus whereas the UL28 $\Delta$ 4 protein did not. The proteins UL28,

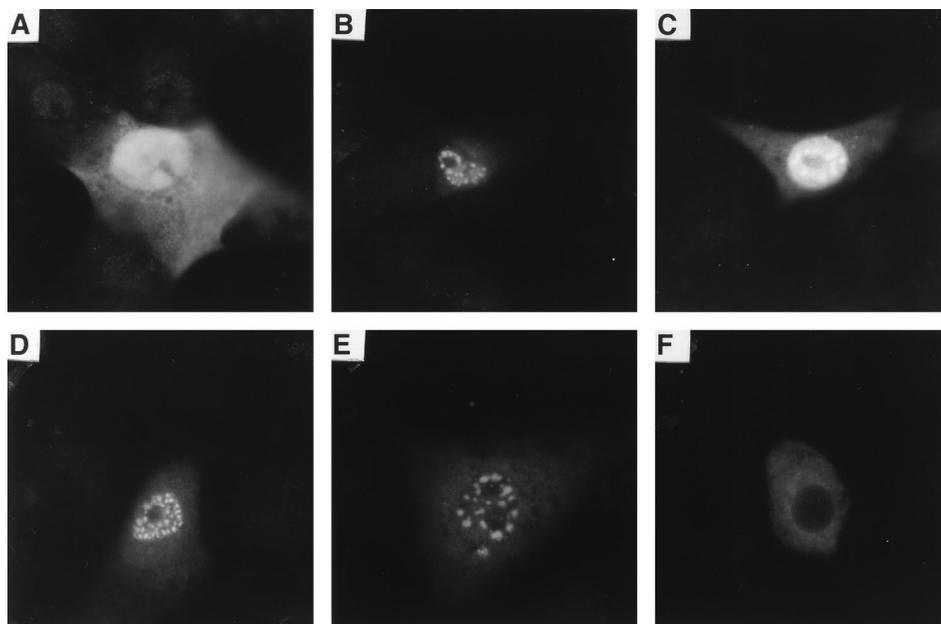


FIG. 5. UL28 variant proteins expressed in HSV-infected cells or coexpressed with HSV UL15. (A) V108 cells grown on coverslips were infected with HSV at an MOI of 0.1 and processed for immunofluorescent detection of PRV UL28 6 h later. Alternatively, Vero cells grown on coverslips were transfected with plasmids to express HSV UL15 plus UL28 (B), UL28 $\Delta$ 1 (C), UL28 $\Delta$ 2 (D), UL28 $\Delta$ 3 (E), or UL28 $\Delta$ 4 (F) and were processed for immunofluorescent detection of PRV UL28 48 h later. Magnification,  $\times$ 590.

UL28 $\Delta$ 1, UL28 $\Delta$ 2, and UL28 $\Delta$ 3 appeared in the nucleus in punctate patterns quite distinct from that seen in PRV(332-31)-infected cells transfected with the deletion plasmids (Fig. 4). However, this experiment demonstrated that HSV UL15 was sufficient for directing PRV UL28 to the nucleus in a process that required the carboxyl terminus of UL28.

HSV UL15 has been shown to enter the nuclei of infected cells (4, 25). However, the distribution of HSV UL15 within uninfected cells has not been described. Since the pattern of PRV UL28 was altered by the expression of HSV UL15, the subcellular distribution of UL15 was examined for possible differences in localization due to PRV UL28. HSV UL15 was examined by immunofluorescence in transfected Vero cells (Fig. 6A) and was found within the nucleus, with distinct punctate nuclear regions. When PRV UL28 was coexpressed with HSV UL15 (Fig. 6B), no appreciable differences in the localization of UL15 could be attributed to the expression of UL28.

The second protein examined for its ability to affect the localization of PRV UL28 was HSV UL25. HSV UL25 did not affect the distribution of full-length or deletion forms of PRV UL28 in transfected cells (20). Although no change in the distribution of UL28 could be attributed to HSV UL25, the expression of HSV UL25 was examined to confirm that UL25 was expressed from the plasmid and to determine whether UL25 was capable of entering the nucleus in the absence of other viral proteins (Fig. 6C). UL25 was distributed throughout the cell, and its distribution was not affected by the expression of UL28 (20). Therefore, although UL15, UL25, and UL28 are involved in viral DNA encapsidation, only UL15 facilitated the transport of UL28 to the nucleus, the site of encapsidation.

## DISCUSSION

The phenotype of all UL28-null and UL28-temperature-sensitive mutants is a cessation of viral replication at the point of DNA cleavage and encapsidation (1, 6, 14, 17, 23). Therefore, the presumed function of UL28 is to act in concert with other proteins in the complex process of packaging viral DNA into preformed capsids within the nuclei of infected cells. Consistent with that finding, PRV UL28 was shown previously to be in the nuclei of infected cells (19). Additionally, PRV UL28 was shown to be a component of an oligomer as detected by immunoprecipitation of radiolabeled fractions from a sucrose gradient (19). The experiments described above confirmed localization of UL28 to the nuclei of infected cells. Surprisingly, in the absence of other viral proteins, UL28 was limited to the cytoplasm. Further experiments revealed that the carboxyl-terminal 155 amino acids of UL28 were required for nuclear localization during PRV infection. These findings support the model that UL28 forms an oligomer with another viral protein for transport to the nucleus. These data also suggest that the required domain lies within the carboxyl-terminal 155 amino acids of UL28. This domain does not lie within the sequences eliminated in UL28 $\Delta$ 1, UL28 $\Delta$ 2, or UL28 $\Delta$ 3, since those proteins were able to be transported to the nucleus in the presence of other viral proteins. This model does not explain the whole-cell distribution of the UL28 $\Delta$ 2 protein in the absence of other viral proteins.

At least two other possibilities remain. The required domain may not lie in the C-terminal residues deleted in UL28 $\Delta$ 4 but in the amino-terminal 92 amino acids. The folding of the truncated UL28 $\Delta$ 4 protein may have affected access to, or the structure of, the amino-terminal region so that the effect of HSV UL15 was not realized. This model does not address the distribution of UL28 $\Delta$ 2 either. Another model must be considered to explain the

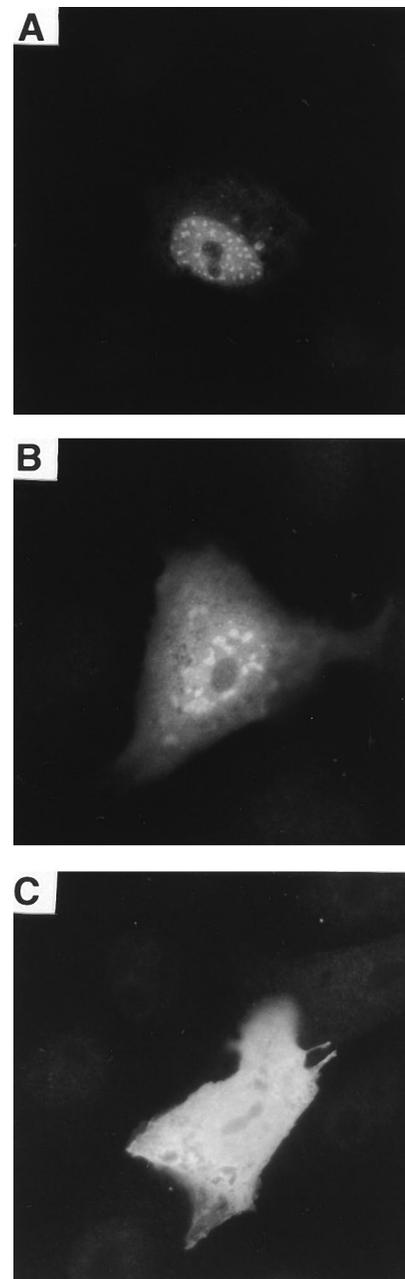


FIG. 6. Expression of HSV UL15 and HSV UL25. Vero cells grown on coverslips were transfected with plasmids to express HSV UL15 (A), HSV UL15 plus PRV UL28 (B), or HSV UL25 (C). The cells were processed for immunofluorescent detection of HSV UL15 (A and B) or HSV UL25 (C). Magnification,  $\times 790$ .

distribution of UL28 $\Delta$ 2 in the nucleus and cytoplasm in the absence of other viral proteins. UL28 may possess an NLS that is normally "masked" by a different domain. The masking effect may be reversed by the binding of another viral protein, such as UL15. If the NLS exists in the region deleted in UL28 $\Delta$ 4 and the masking domain exists in the region deleted in UL28 $\Delta$ 2, then UL28 $\Delta$ 2 would be expected to enter the nucleus in the absence of other viral proteins. Consistent with the data, each of the UL28 deletion proteins except for UL28 $\Delta$ 4 would be expected to be able to enter the nucleus in the presence of UL15. Although this

model accounts for UL28 distribution patterns in the absence of other viral proteins, it is inconsistent with the data for the distribution patterns when UL15 was coexpressed. If the effect of UL15 or another protein was to bind to the region deleted in UL28 $\Delta$ 2, then UL28 $\Delta$ 2 would not be expected to be affected in the presence of UL15 or other infected-cell proteins. A significant difference was seen, however (Fig. 3C, 4C, and 5D). Further work is needed to exclude one or more of these models.

The identification of a domain within UL28 that is required for UL28 to reach the nucleus is significant because the region acts unlike a typical NLS. HSV UL15 was found to be sufficient to enable PRV UL28 to enter the nucleus. Other viral proteins may also be capable of directing UL28 to the nucleus, but the inability of UL25 to direct UL28 to the nucleus suggests that the interaction is specific. It remains to be confirmed that PRV UL15 affects PRV UL28 in a similar manner. Currently, only the DNA sequence of the second exon of PRV UL15 has been reported (10). The sequences of the PRV UL15 splice junction and first exon remain to be determined. Similarly, the effect of HSV UL15 coexpression on PRV UL28 localization suggests, but does not confirm, that HSV UL15 affects HSV UL28 localization.

The degree of similarity of the required domains of the PRV UL28 and HSV UL15 proteins may contrast with that of other domains required for encapsidation of the respective virus genomes. The HSV(gCB) UL28-null mutant did not form plaques on the PRV UL28-complementing cell line V108, which suggests that there is at least one other virus-specific domain of UL28 (20). The possibility of additional independently functioning domains within UL28 is supported by the complementation of different temperature-sensitive HSV UL28 mutants (6).

The finding that HSV UL15 was capable of entering the nucleus in the absence of other proteins further substantiates the observation that UL15 enters the nucleus during infection with HSV (4, 25). The significance of the punctate pattern of UL15 (Fig. 6A) in the absence of other viral proteins is not known. The significance of the punctate pattern of UL28 in the presence of UL15 is not known, but the pattern has also been seen at early times in PRV infection (19). Another consideration is whether the effect on UL28 is due to UL15 or UL15.5 (3, 27) or both, since both proteins are presumably expressed in transfected cells but cannot be distinguished in immunofluorescence analyses by existing antisera.

An effect of one protein involved in the packaging/cleavage reaction on another has been identified in living cells. This represents the first demonstrated potential interaction between any of the proteins required for viral DNA encapsidation among herpesviruses. The significance is strengthened by the finding that the specificity of the interaction is maintained between different alphaherpesviruses.

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