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Release of the Catalytic Domain N_o from the Herpes Simplex Virus Type 1 Protease Is Required for Viral Growth

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The herpes simplex virus type 1 (HSV-1) protease and its substrate, ICP35, are involved in the assembly of viral capsids and required for efficient viral growth. The full-length protease (Pra) consists of 635 amino acid (aa) residues and is autoproteolytically processed at the release (R) site and the maturation (M) site, releasing the catalytic domain N_o (VP24), Nb (VP21), and a 25-aa peptide. To understand the biological importance of cleavage at these sites, we constructed several mutations in the cloned protease gene. Transfection assays were performed to determine the functional properties of these mutant proteins by their abilities to complement the growth of the protease deletion mutant *m*100. Our results indicate that (i) expression of full-length protease is not required for viral replication, since a 514-aa protease molecule lacking the M site could support viral growth; and that (ii) elimination of the R site by changing the residue Ala-247 to Ser abolished viral replication. To better understand the functions that are mediated by proteolytic processing at the R site of the protease, we engineered an HSV-1 recombinant virus containing a mutation at this site. Analysis of the mutant A247S virus demonstrated that (i) the mutant protease retained the ability to cleave at the M site and to *trans* process ICP35 but failed to support viral growth on Vero cells, demonstrating that release of the catalytic domain N_o from Pra is required for viral replication; and that (ii) only empty capsid structures were observed by electron microscopy in thin sections of A247S-infected Vero cells, indicating that viral DNA was not encapsidated. Our results demonstrate that processing of ICP35 is not sufficient to support viral replication and provide genetic evidence that the HSV-1 protease has nuclear functions other than enzymatic activity.

Assembly of herpesvirus capsids is an essential step of virion maturation that occurs in the nuclei of infected cells (16, 18). Three types of capsids have been isolated from virus-infected cells (12). On the basis of their increasing sedimentation distance on sucrose gradients, these capsids are designated A, B, and C (12, 13, 33). A and C capsids have the same protein contents, but C capsids contain the viral genome and are thought to be able to mature into infectious virions (12, 31). B capsids, like A capsids, lack viral DNA but contain the abundant viral assembly protein ICP35 (VP22a) inside the icosahedral capsid shell (1, 3, 12, 28, 38).

It has been postulated that B capsids are intermediates in capsid assembly, because ICP35, which is present in B capsids but absent from mature virions, is analogous to the scaffold protein of double-stranded DNA bacteriophage (1, 4). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis has shown that B capsids are composed of at least seven proteins, i.e., VP5, VP19C, Na (VP21), ICP35 (VP22a), VP23, N_o (VP24), and VP26 (1, 13, 39, 43). The major capsid protein, VP5, accounts for about 70% of the mass of the surface shell and is the structural subunit of both hexons and pentons (28, 29, 39, 43, 47). It appears that VP5 adopts different conformations during capsid assembly because some monoclonal antibodies (MAbs) specific for VP5 can distinguish between hexons and pentons (42). Nuclear transport of VP5 is dependent on the presence of ICP35, indicating an interaction between these two proteins (10, 30). It has been recently reported that the UL6 gene product is also associated with capsids (32).

Three of these seven capsid proteins, N_o, Nb, and ICP35, are encoded by the overlapping genes UL26 and UL26.5 (5, 15, 19, 26, 34, 44). The herpes simplex virus type 1 (HSV-1) protease (Pra) encoded by the UL26 gene consists of 635 amino acids (aa) and undergoes autoproteolytic processing at two sites; between Ala and Ser at positions 247-248 (release [R] site) and positions 610-611 (maturation [M] site), releasing N_o, Nb, and a 25-aa peptide (Fig. 1A) (5, 8, 20–22, 34). Although genetic and functional analyses clearly demonstrate that N_o, consisting of the first 247 aa of Pra, contains the catalytic domain (18–21), the enzymatic activity of the protease can be independent of capsid assembly (5, 6, 20–22, 24, 43). Autoprocessing and *trans* cleavage of cytomegalovirus protease have been extensively studied elsewhere (2, 45, 46). An additional autoproteolytic site has been identified in the catalytic domain of the cytomegalovirus protease (2, 31, 45).

Synthesis of the more abundant protein, ICP35, encoded by the UL26.5 gene, is initiated at residue 307 (Met) of Pra. The entire amino acid sequence of ICP35, therefore, is identical to residues 307 to 635 of Pra. Consequently, ICP35 c,d can be *trans* cleaved at its C terminus by the protease to generate ICP35 e,f and a 25-aa peptide (5, 8, 20–22). Since only fully processed forms of the protease (N_o and Nb) and ICP35 (ICP35 e,f) are detected in B capsids, it is likely that cleavage at these sites is required for virion maturation. In support of this assumption is the fact that if ICP35 is not processed, assembly of VP5 into hexons of capsids is comformationally altered (10).

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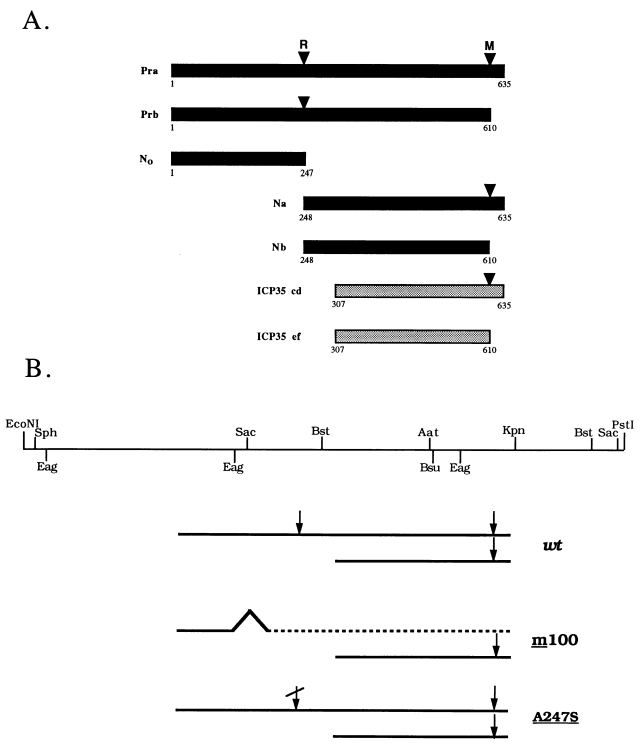

FIG. 1. (A) Polypeptide products of UL26 and UL26.5 open reading frames. The HSV-1 protease (Pra), substrate (ICP35 c,d), and cleavage products (Prb, N_o [VP24], Na, Nb [VP21], and ICP35 e,f [VP22a]) are described in the text. The sites of cleavage of Pra and ICP35 c,d are indicated by arrows. The numbers of amino acids for the N and C termini of each protein are indicated. (B) The UL26 and UL26.5 open reading frames of HSV-1 wt, mutant *m*100, and A247S genomes. Restriction enzyme sites for the plasmid pRB4057 are indicated in the top line. Aat, *Aat*II; Bst, *Bst*EII; Bsu, *Bsu*36I; Eag, *Eag*I; Kpn, *Kpn*I; Sac, *Sac*I; Sph, *Sph*I.

It has been demonstrated that the protease is essential for viral replication by using a temperature-sensitive mutant and deletion mutants of HSV-1 protease (7, 10, 35, 36). In contrast, full-length ICP35 is not absolutely required for viral growth, because of the fact that Na and ICP35 can probably functionally substitute for each other in ΔICP35 mutant-infected cells (23). Although autoproteolytic and *trans* cleavage products (N_o, Nb, and ICP35 e,f) are components of B capsids, the fate of the 25-aa peptide during capsid assembly is unknown. Our recent results have shown that these 25 aa are involved in the closure of capsids (24). Despite the fact that the precise positions of autoproteolytic and *trans* cleavage processing have been identified, the biological importance of these cleavages during virion maturation is unclear. In this paper, we report on the examination of HSV-1 protease cleavage site mutants and demonstrate that autoproteolytic processing at the R site, but not at the M site, is essential for viral replication. However, this conclusion is valid only when full-length wild-type (wt) ICP35 is provided. Our data provide evidence that the HSV-1 Pra is a multifunctional protein and that its enzymatic activity can be genetically separated from its other functions that are required for virion maturation.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained as described previously (17). The growth medium for the neomycin-resistant cell line BMS-MG22 included 250 μg of the antibiotic G418 per ml (10).

The HSV-1 wt strain KOS1.1 was propagated and assayed as described previously (17). The mutant viruses *m*100 and A247S were grown in BMS-MG22 cells, and ΔICP35 was grown in 35J cells (23).

Plasmids. The plasmid pRB4057 of HSV-1 strain F was kindly provided by B. Roizman (University of Chicago). Construction of plasmids pM307L, pSVPra, pSVPrbM307L, and $pSVN_o$ has been described previously (10, 23, 24). Plasmid pSV563M307L was constructed by three-way ligation of the 1,606-bp *Xba*I-*Bsu*36I fragment of pSVPrbM307L and the 141-bp *Bsu*36I-*Asp*718-digested PCR fragment (see below) into the 3.5-kbp *Xba*I-*Asp*718 fragment of the vector pJ3Ω (27). To engineer a stop codon (indicated by boldface) after aa 563 of the UL26 open reading frame, oligonucleotides containing the sequences 5′-AATTGGC CATGGGTCTTTCCCAGCATTACCCTCCCCACG and 5′-CAGCAATTGG TACC**TA**GGCAGCTGGGGCATACG were used to create the 613-bp fragment by PCR with pT7HSV (5) as a template and then by digesting with *Bsu*36I and *Kpn*I (indicated by underlining) for cloning. Thus, pSV563M307L encodes the first 563 aa of Pra. Plasmid pSV514M307L was constructed by three-way ligation of the 657-bp *Sac*I-*Eag*I fragment of pSVPrbM307L (24) and the 643-bp *Xba*I-*Sac*I fragment of Pra into the *Xba*I-*Eag*I fragment of the vector pSVc122 (23). Thus, pSV514M307L encodes the first 514 aa of Pra as well as an additional amino acid, His. Plasmid pSV419M307L was constructed as follows. A 286-bp *Bam*HI-*Eco*RI fragment of pT7419K (44) was ligated into pSVICP35 e,f (24) to generate pSVc216. Plasmid pSV419M307L was constructed by three-way ligation of the 657-bp *Sac*I-*Eag*I fragment of pSVPrbM307L (24) and the 643-bp fragment of *Xba*I-*Sac*I of Pra into the *Xba*I-*Eag*I fragment of the vector pSVc216. Thus, pSV419M307L encodes the first 419 aa of Pra. To ensure that the correct mutant proteins were made, protease mutant plasmids encoding the N-terminal 419 and 514 aa were recloned back to T7 promoters from pSV419M307L and pSV514M307L and the desired proteins were reexamined in the *Escherichia coli* expression system. Plasmid pSVA247SM307L was constructed as follows. A 1,344-bp *Kpn*I-*Sac*I fragment of pT7A247SA610SC (44) was cloned into the same sites of $pSVN_o$ to generate pSVA247SA610S. A 469-bp *Kpn*I-*Aat*II fragment of pSVA247SA610S was replaced by the same fragment derived from pM307L to generate pSVA247SM307L. Plasmid pRB4057A247S was constructed by replacement of a 94-bp *Apo*I-*Hpa*I fragment of pRB4057 with the same fragment derived from pT7A247SK (44). Plasmid pSVA610SA247SM307L was constructed by replacement of a 1.1-kb *Bst*EII-*Kpn*I fragment of pSVA 247SM307L with the same fragment of pSVA247SA610S. Plasmid pSVA610 SM307L was constructed by replacement of a 1.1-kbp *Bst*EII-*Kpn*I fragment of pSVPra with the same fragment of pSVA610SA247SM307L.

Isolation of mutant A247S virus. An HSV-1 protease release site mutant virus was constructed by cotransfection of BMS-MG22 cells with genomic ΔICP35 DNA and plasmid pRB4057A247S. The progeny viruses from the marker transfer were tested for their abilities to grow in BMS-MG22 and Vero cells.

Analysis of viral proteins. For Western blot (immunoblot) analysis of infected cell lysates, cell monolayer cultures were infected with KOS1.1 or A247S virus at a multiplicity of infection of 10 and were harvested as indicated in the text. Procedures for SDS-PAGE and Western blot analysis were described previously (9, 44). The anti-ICP35 MAb MCA406 (1:1,000 dilution; Serotec) was used to detect ICP35 and the protease-related products Pra, Prb, Na, and Nb.

Electron microscopy. Thin sections of infected cells to be examined by electron microscopy were prepared as previously described (10, 23).

Indirect immunofluorescence. Indirect immunofluorescence assays were performed as described previously (8, 37), with the following antibodies: anti-ICP35 MAb MCA406 (1:150 dilution), anti-N_o polyclonal serum (1:150 dilution) (10), anti-VP5 MAb 5C (1:40 dilution) (42), or MAb 3B (1:75 dilution) (42); fluorescein-conjugated goat anti-mouse antibody (1:150 dilution); and fluorescein-conjugated goat anti-rabbit antibody (1:150 dilution).

RESULTS

Construction and characterization of mutant proteases expressed from plasmids. In an attempt to define the functional domains of the HSV-1 protease, we constructed two types of mutations in the cloned UL26 gene (Fig. 2): (i) nonsense mutations and (ii) cleavage site (R- and/or M-site) mutations. Since ICP35 begins at residue 307 of Pra, it shares amino acid identity with the C-terminal 329 residues of the protease (5, 7, 20–22). Thus, any protease nonsense mutants would also affect ICP35 polypeptides in virus-infected cells. To ensure that the phenotype of the mutant reflects mutations in only the protease gene, we used the plasmid pM307L encoding the protease with a Met-to-Leu change at residue 307 as the parental plasmid for construction of all protease nonsense and cleavage site mutants in this study (Fig. 2). The M307L mutation in the protease gene eliminates the synthesis of full-length ICP35 (23) but does not affect any functions of the protease which support viral growth (10). Therefore, any mutations in pM307L used in this study should affect only the protease gene.

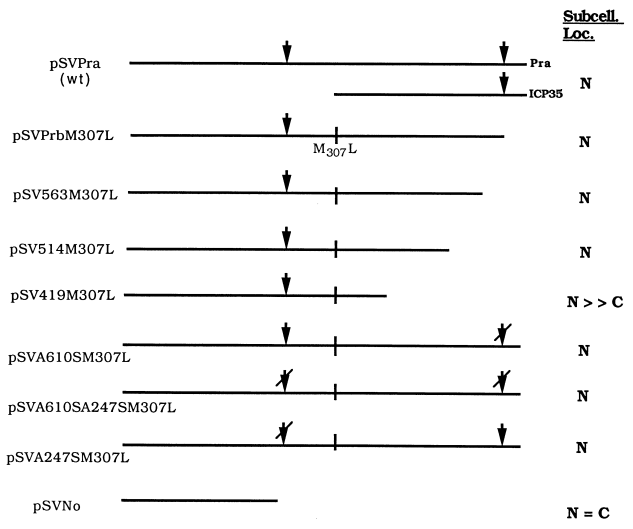

FIG. 2. Structures and subcellular locations (subcell. loc.) of mutant protease molecules. Protease gene plasmids containing the indicated mutations were transfected into Vero cells, and the subcellular locations of the mutant proteins were determined by indirect immunofluorescence staining for N_o and/or ICP35 after 20 h. Cleavage sites of proteins are indicated by arrows.

We first examined the expression and the subcellular distribution of mutant proteases by indirect immunofluorescence. These mutant genes were expressed under the control of the simian virus 40 early promoter in transfected Vero cells. Therefore, no other viral gene products would affect the subcellular distribution of mutant proteases. The observed subcellular localization of the proteins is summarized in Fig. 2. To ensure that the mutant protease rather than the proteolytic products due to autoproteolytic processing of wt or mutant proteases was examined, two antibodies (one specific for the catalytic domain [N_o] and another specific for the C-terminal portion of the protease [MAb MCA406]) were used.

Representative immunofluorescence data are shown in Fig. 3. Following transfection of Vero cells with nonsense mutant pSVPrbM307L or pSVA610SM307L (results not shown) or cleavage site mutant pSVA247SM307L (Fig. 3B and C), proteases were predominantly localized to the nucleus by using either polyclonal anti-N_o serum or MAb MCA406 (summarized in Fig. 2). Similar results were obtained following transfection of wt plasmid pSVPra (Fig. 3A) and mutant plasmid pSV563M307L (or pSV514M307L [Fig. 3D] when an antibody specific for N_o was used). The mutant protease consisting of only the N-terminal 419 aa expressed from pSV419M307L showed increasing cytoplasmic staining but was still predominantly localized in the nucleus (Fig. 3F), while the catalytic domain N_o consisting of the first 247 aa showed approximately equal cytoplasmic and nuclear localizations (Fig. 3E). With MCA406 MAb, the mutant proteases encoded by pSV563 M307L, pSV514M307L, and pSV419M307L were not recognized because the epitope is contained between residues 563 and 610 (11).

To determine whether any of these mutations would affect an essential function of the protease, we examined the abilities of the plasmid-encoded mutant proteins to complement the growth of the protease deletion mutant *m*100 virus (10). The mutation in the *m*100 virus eliminates synthesis of the functional protease but does not alter the expression of ICP35 (10). Therefore, in our *trans* complementation experiments, ICP35 was produced by *m*100 virus, while mutant protease molecules were provided from transfected plasmids. All nonsense and cleavage site mutant proteases were previously shown to be capable of *trans* cleaving ICP35, and the desired mutant proteins were made in the *E. coli* coexpression system (references 5, 25, and 44 and results not shown). The results of complementation experiments are shown in Table 1. The wt protease or the protease with a Met-to-Leu change at residue 307 expressed from the UL26 gene under its own promoter (pRB4057 or pM307L) or the simian virus 40 early gene promoter (pSVPra) complemented the growth of *m*100. It appears that neither cleavage at the M site nor the C-terminal 25 aa of the protease is essential for viral replication, since the cleavage site mutant protease A610SM307L and nonsense mutants encoding the N-terminal 610, 563, or 514 aa of the protease molecule could partially support the growth of *m*100. However, we do not know whether the cleavage occurs at residue 610 of A610SM307L and A247SA610SM307L in our complementation experiments. Nevertheless, our results demonstrate that the full-length protease is not required for viral growth when ICP35 is provided. That the N-terminal 610-aa molecule retains all functions of the protease necessary to support viral growth was further confirmed by our recent isolation and characterization of *Prb* virus (24). However, the N-terminal 419-aa protease encoded by pSV419M307L, like the catalytic domain N_o, was not sufficient to support the growth of *m*100, suggesting that an essential function of the protease between residues 419 and 514 was inactivated. In contrast to the mutation at the M site, the double-cleavage site mutant protease encoded by pSVA610SA247SM307L failed to support mutant growth, suggesting that processing at the R site of Pra is essential. This was further confirmed by the fact that elimination of just the R site by changing Ala-247 to Ser in pSVA247SM307L abolished viral replication. These results suggest that release of the catalytic domain N_o from Pra is required for viral growth.

Isolation of the R-site mutant A247S virus. The transient complementation experiments described above demonstrated that the mutation at the R site specifically affected an essential function of the protease. To better understand the functions mediated by proteolytic processing at the R site, we constructed a recombinant virus bearing this mutation.

Our strategy for isolation of the R site mutant A247S was similar to that used for isolation of *Prb* virus (24). The procedure involves transferring the A247S mutation to the viral genome accompanied by rescue of the ΔICP35 lesion. This procedure differs from the previous procedure (10) in that the infectious ΔICP35 genome was cotransfected with pA247S directly into BMS-MG22 cells which express only Pra. Since both Pra and ICP35 are essential for efficient viral growth, all surviving progeny virus after the marker transfer should be recombinants, i.e., either A247S or wt virus. The A247S virus can be distinguished from wt virus by its failure to replicate on Vero cells. Six plaques were picked from the marker transfer experiment, and five of them displayed mutant growth property. One isolate was further plaque purified twice and was designated A247S (Fig. 1B).

To verify that the mutation in A247S virus corresponds to the desired mutation, a 2,232-bp *Sph*I-*Pst*I fragment was cloned from A247S viral DNA, and the region spanning the R site was sequenced. Comparison of sequences revealed that nucleotides GCG encoding Ala at the residue 247 from wt DNA were replaced by AGC encoding Ser in A247S DNA (Fig. 4). Therefore, we conclude that the intended mutation was introduced into the A247S genome.

Growth property of the mutant A247S virus. A plaque assay was performed to determine whether the mutant A247S is growth defective on Vero cells. In keeping with the results of

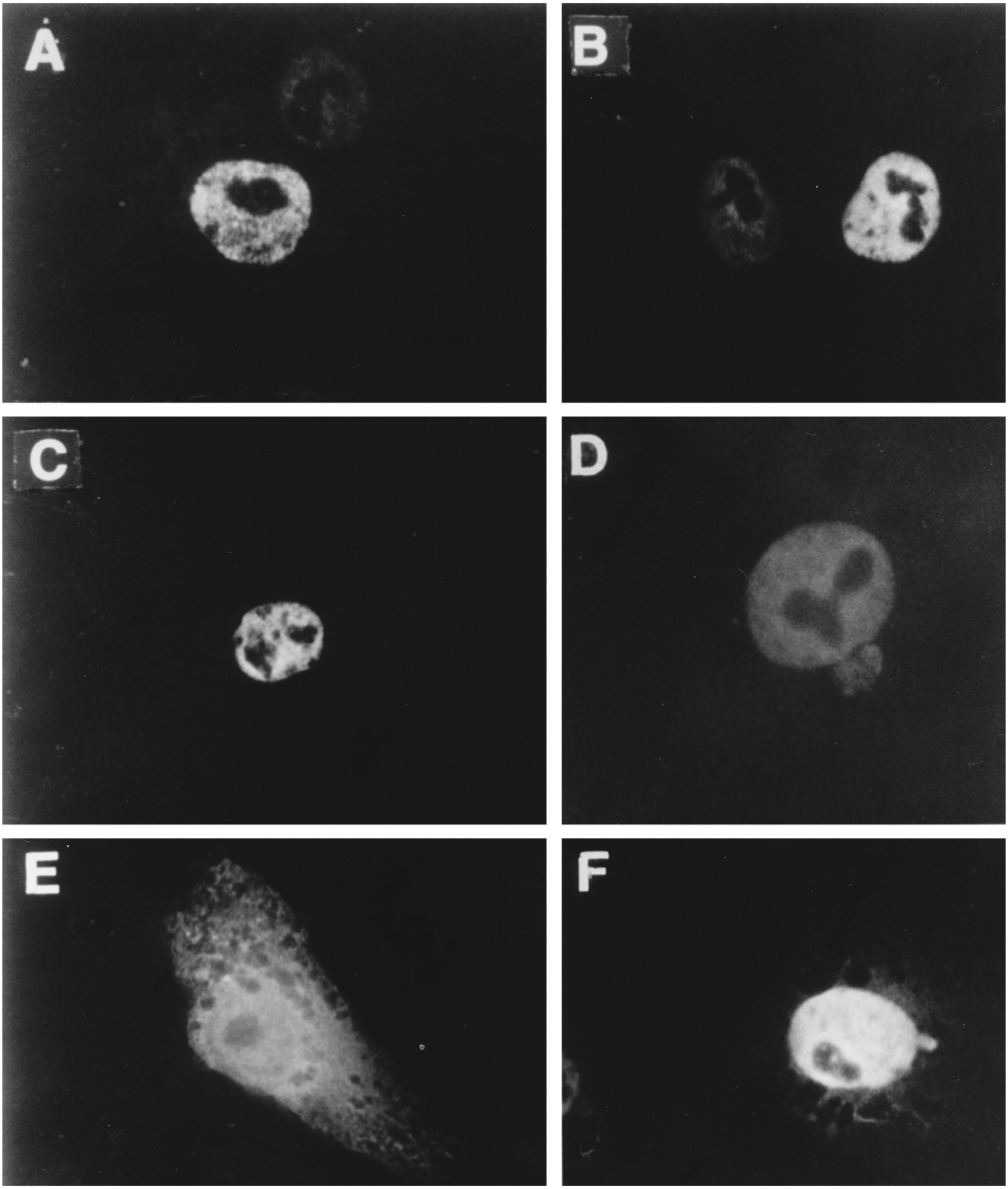

FIG. 3. Immunofluorescence staining for HSV-1 protease in Vero cells transfected with protease plasmids. Twenty hours after transfection, Vero cells were fixed, permeabilized, and incubated with either anti-N_o polyclonal antiserum (A, B, D, E, and F) or the anti-ICP35 MAb MAC406 (C). (A) Pra (wt)-transfected cells; (B and C) pSVA247SM307L-transfected cells; (D) pSV514M307L-transfected cells; (E) $pSVN_o$-transfected cells; (F) pSV419M307L-transfected cells.

TABLE 1. Ability of mutant protease plasmids to complement *m*100

Plasmid transfected[a]	Expt.	Virus yield (PFU/ml)[b]	Complementation index[c]
pUC18	1	1.2×10^3	1
	2	4.0×10^3	1
	3	1.6×10^3	1
pRB4057 (wt)	1	8.2×10^5	683
	3	6.7×10^5	419
pM307L	3	5.9×10^5	369
pSVPra (wt)	1	4.6×10^5	383
	2	5.0×10^5	125
pSVPrbM307L	1	2.4×10^5	200
	2	4.6×10^5	120
	3	2.4×10^5	150
pSV563M307L	2	1.6×10^5	40
pSV514M307L	1	1.4×10^5	117
	2	1.7×10^5	43
pSV419M307L	1	1.6×10^3	1.3
	2	4.2×10^3	1
pSVA610SM307L	1	3.4×10^5	283
pSVA610SA247SM307L	1	8.0×10^2	0.7
	3	9.0×10^2	0.6
pSVA247SM307L	1	1.4×10^3	1.2
	3	1.8×10^3	1.1
$pSVN_o$	1	6.2×10^3	5.2
	3	3.3×10^3	2

[a] Vero cells were transfected with the plasmids indicated. At 20 h posttransfection, the cells were infected with 3 PFU of *m*100 per cell and were incubated for a further 20 h before being harvested.
[b] Determined by plaque assays on BMS-MG22 cells.
[c] Expressed as virus yield relative to transfection with pUC18 DNA.

trans complementation experiments, the mutant grew on BMS-MG22 cells; however, it was unable to form plaques on Vero cells, demonstrating that the A247S mutation is lethal for the virus (Table 2). We also found that titers of A247S viral stocks are usually around 10^7 PFU/ml, which is significantly lower than those of our other protease mutant stocks (10, 24). These results suggest that the A247S mutation in the mutant virus may exhibit a slightly *trans*-dominant phenotype.

Protease activity of the mutant A247S virus. We then examined the expression and activity of the mutant A247S protease. Vero or BMS-MG22 cells were either mock infected or infected with virus, cell extracts were prepared at 10 h postinfection (p.i.), and proteins were analyzed by Western blotting (Fig. 5). Since the protease is produced in very low quantities (7, 10, 23), twice as much infected cell lysates was loaded in lanes 6 and 7 as was loaded in lanes 2 and 3 in order to examine autoproteolysis in wt-infected Vero and BMS-MG22 cells. wt Pra was autoproteolytically processed into Prb, Na, and Nb (Fig. 5, lanes 6 and 7). N_o was not detected, because the Western blot was probed with MCA406, a MAb specific for ICP35 (5, 14, 43). In contrast, in A247S-infected Vero cells, both Pra and Prb were detected, suggesting that the A247S protease is capable of autoprocessing at the M site. However, autoprocessing at the R site was eliminated, as indicated by lack of the release of Na and Nb (Fig. 5, lane 4). In A247S-infected BMS-MG22 cells, Na and Nb were provided by the cell line (Fig. 5, lane 5). The level of processing of ICP35 c,d to e,f in A247S-infected Vero cells appeared to be slightly reduced compared with that in wt-infected cells (compare lanes 2 and 4 of Fig. 5), but the mutant protease retains its ability to process its M site and to *trans* cleave ICP35. Therefore, we conclude that processing of ICP35 is not sufficient to support viral growth.

TABLE 2. Growth of the A247S mutant

Virus	Titer (PFU/ml) in[a]		Titer ratio (Vero cells/MG22 cells)
	Vero cells	MG22 cells	
wt	4×10^8	2.2×10^8	1.8
A247S	1.4×10^3	1.8×10^7	7.8×10^{-5}

[a] Titers on virus stocks were determined by plaque assays on the cell lines indicated, and plaques were counted 2 days p.i.

Electron microscopy studies. The A247S mutant virus was examined by electron microscopy to determine whether it forms mature capsids or only capsid precursors under nonpermissive conditions. Thin sections of wt- or A247S-infected Vero cells were prepared at 16 h p.i. Normal capsid structures were observed throughout wt-infected cells at low magnification (results not shown). At higher magnification, most of these intranuclear structures were the electron-translucent (B-capsid) type (Fig. 6A). However, in A247S-infected Vero cells, large aggregations of capsid structures were observed at low magnification (Fig. 6B). Higher magnification revealed that all

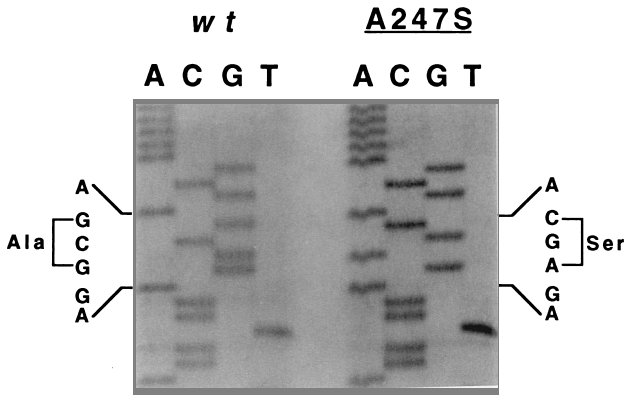

FIG. 4. DNA sequencing of the A247S mutation. The region spanning the R site of wt and A247S DNA was sequenced. Comparison of sequences revealed that nucleotides GCG encoding Ala at the residue 247 from wt DNA were replaced by AGC encoding Ser in A247S DNA.

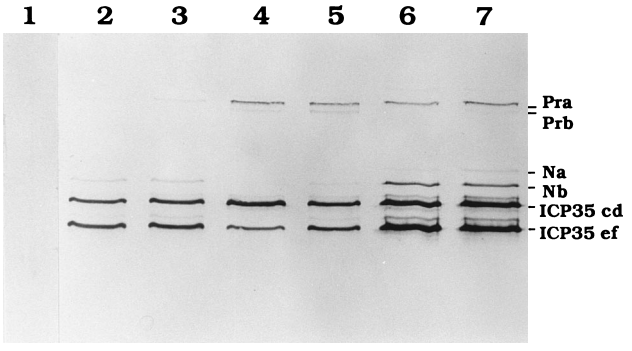

FIG. 5. Western blot analysis of HSV-1 protease-related polypeptides in wt- or A247S-infected cells. Vero (lanes 2, 4, and 6) and BMS-MG22 (lanes 1, 3, 5, and 7) cells were mock infected (lane 1) or were infected with wt (lanes 2, 3, 6, and 7) or A247S (lanes 4 and 5) virus. Total proteins were prepared at 10 h p.i., separated by SDS-PAGE, and transferred to a nitrocellulose filter. Lanes 6 and 7 were identical to lanes 2 and 3, except that twice the amounts of samples were loaded. The filter was probed with a MAb, MCA406, specific for ICP35.

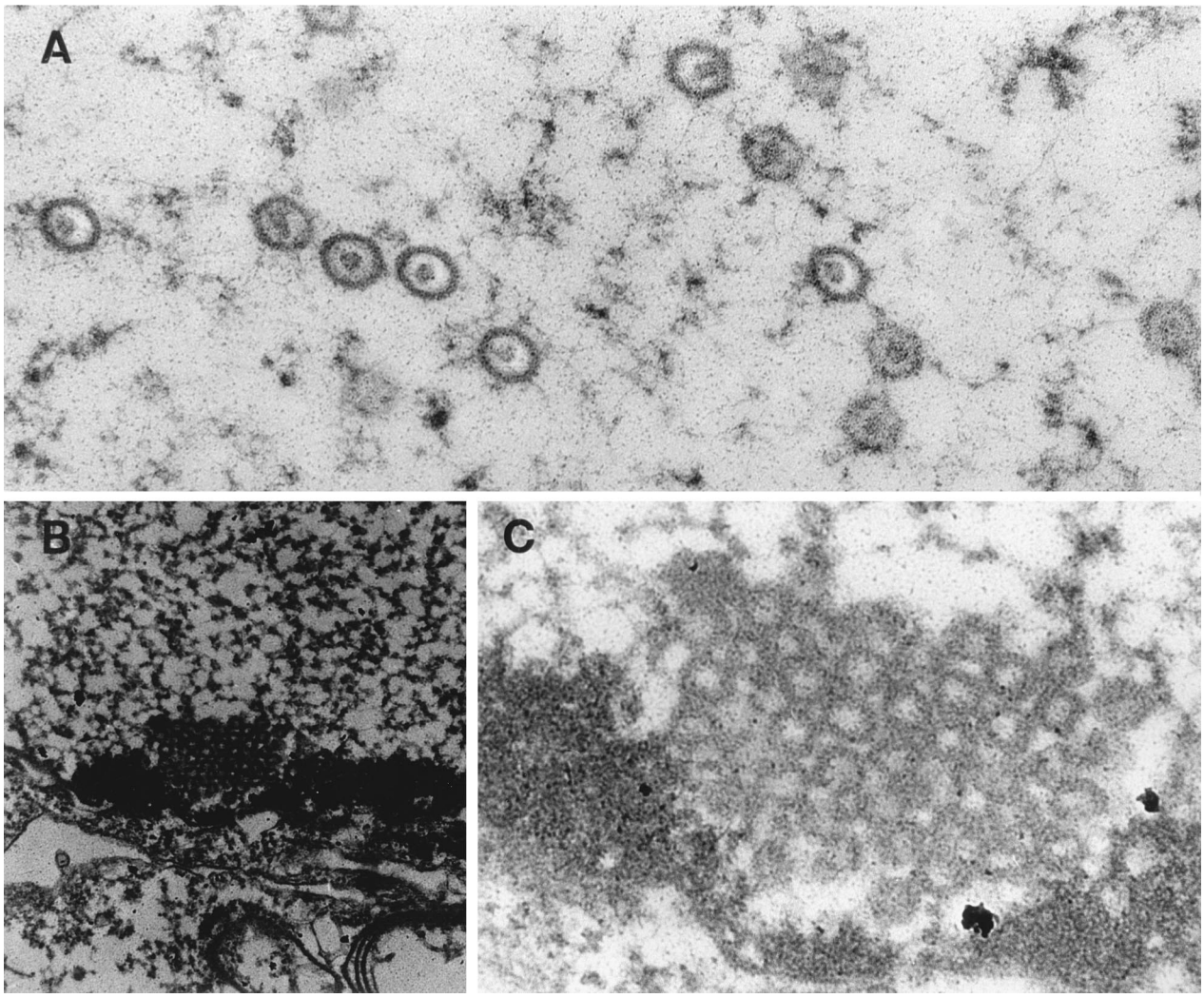

FIG. 6. Electron micrographs of thin sections of wt- and A247S-infected cells. Vero cells were infected with wt or A247S at a multiplicity of infection of 10 PFU per cell. The cells were fixed and prepared at 16 h p.i. (A) wt-infected Vero cells; (B and C) A247S-infected Vero cells. Magnification, 80,400 (A and C) or 24,400 (B).

of these structures were empty capsids (DNA^-) (Fig. 6C), indicating that viral DNA was not packaged.

Antibody recognition of VP5. We previously reported that in protease deletion mutant *m*100-infected Vero cells, in which ICP35 c,d is not processed, assembly of the major capsid protein VP5 into hexons, but not pentons, was conformationally altered (10). To determine whether hexons were altered in A247S-infected Vero cells, in which ICP35 c,d is processed, we performed indirect immunofluorescence.

Vero cells were infected with either wt or A247S virus and were stained at 6 h p.i. with a panel of MAbs specific for VP5. These MAbs recognized different conformation-specific epitopes of VP5 in B capsids (42). MAbs 8F5 and 5C recognize hexons, while 3B recognizes pentons of B capsids (42). VP5 accumulated in the wt-infected cell nucleus as determined by reactivity with all VP5 MAbs (Fig. 7B and E; results not shown). MAb 3B showed the wt nuclear staining pattern of VP5 in mutant-infected cells (Fig. 7C), indicating that assembly of VP5 into pentons of capsids was not affected. In contrast, reactivity with MAb 5C was dramatically reduced in mutant-infected cells (Fig. 7F), indicating that assembly of VP5 into hexons of capsids was conformationally altered. However, a staining pattern similar to that for the wt was observed in A247S-infected BMS-MG22 cells, suggesting that the assembly defect could be restored when the wt protease was provided in *trans* (results not shown). These results demonstrate that processing of ICP35 is not sufficient for capsids to assemble correctly and that release of N_o is required.

DISCUSSION

During the past few years, considerable evidence has indicated that the HSV-1 protease plays a crucial role in capsid assembly and virion maturation (10, 24, 35, 40, 41). In this study, we described construction of several different types of mutations in the HSV-1 protease gene and directly examined the biological importance of proteolytic processing by and of the protease in lytic infection.

Liu and Roizman reported that the N-terminal region of the protease appears to be critical for activity because deletion of the N-terminal 32 aa of Pra inactivates the ability of the protease to process ICP35 (21, 22). Therefore, we concentrated our mutagenic analyses on the C-terminal portion of the protease. Our complementation experiments indicate that the C-

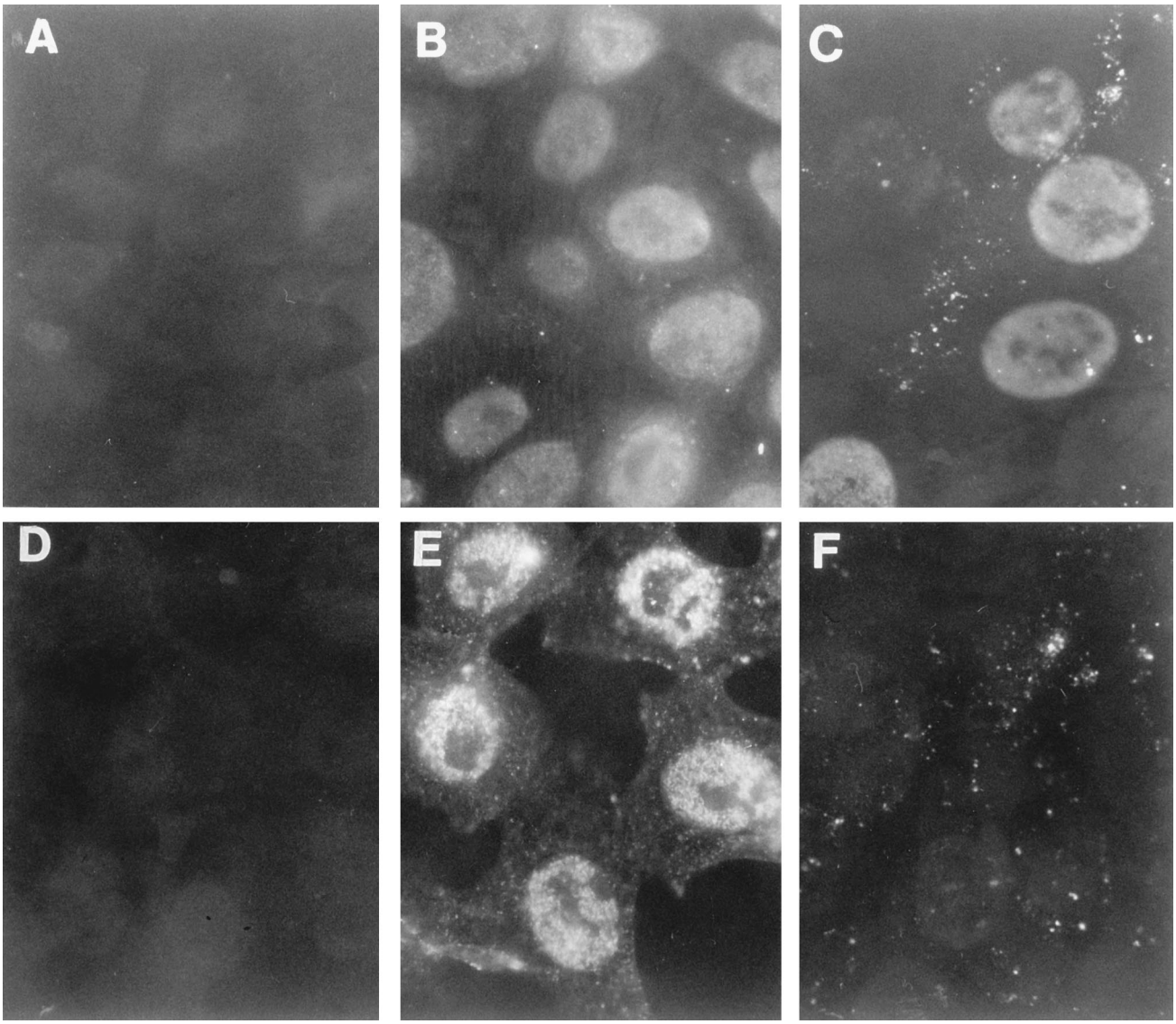

FIG. 7. Recognition of VP5 in A247S-infected Vero cells. Vero cells were either mock infected (A and D) or infected with wt (B and E) or A247S (C and F) virus and were processed for indirect immunofluorescence at 6 h p.i. The cells were fixed, permeabilized, and incubated with anti-VP5 MAbs 3B (A, B, and C) and 5C (D, E, and F).

terminal region of Pra, in contrast to the N-terminal portion of Pra, is dispensable. It appears that neither cleavage at the M site nor the C-terminal 25 aa of the protease are essential, because the first 514 aa of Pra could support viral growth, demonstrating that downstream of the residue 514 of Pra is dispensable. However, in order for the first 514 aa of Pra to support viral growth, full-length ICP35 must be supplied in *trans*. In agreement with our previous report (23), these results imply that Na and ICP35 can functionally substitute for each other.

The results that the first 419 aa of Pra predominantly localized in the nucleus but still failed to support viral replication suggest that the lack of the nuclear localization by N_o cannot account exclusively for its inability to support viral growth. Since complementation experiments revealed that the first 514 aa of Pra retain their ability to support viral growth, at least one essential function must be located between residues 248 and 514 of Pra. Nb (VP21), like ICP35 e,f, is present in B capsids and absent from mature virions (5, 44), but its function during capsid assembly is unknown. However, results from our experiments and those of others indicate that Nb and ICP35 can substitute for each other under certain conditions, suggesting that Nb may also function as a scaffold protein. In this case, the essential region of Nb required for viral growth could be further narrowed down to the region between residues 307 and 514, because this region is shared by both ICP35 and Pra.

Using marker transfer, we introduced the A247S mutation into the ΔICP35 viral genome and rescued the ΔICP35 lesion. However, in previous experiments, the marker transfer was performed with a cell line (X4) which could support the growth of both mutants, i.e., the parental mutant and the desired mutant (10). It is known that active viral DNA replication greatly facilitates the recombination frequency. Since viral DNA replication proceeds at the wt level in ΔICP35-infected nonpermissive cells (11), recombination between the transfected mutant plasmid and the viral gemone should not be

affected. Therefore, we decided to perform the marker transfer with the cell line BMS-MG22, which has some restrictions for the replication of the parental ΔICP35 virus and supports the growth only of the desired A247S virus. As expected, a high percentage of progeny viruses examined from the marker transfer displayed the phenotype of the desired mutant.

The growth of A247S was similar to that of the previously reported *m*100 virus, i.e., growth on the protease-complementing cell line BMS-MG22 but no growth on Vero cells (10). However, we previously could not distinguish whether the growth defect of *m*100 was due to the fact ICP35 is not processed or was due to the fact that the protease molecule was deleted. The data presented in this paper clearly demonstrate that processing of substrate ICP35 is not sufficient to support viral growth. Our current working model is that the catalytic domain N_o, like Nb and ICP35 e,f, which are present in B capsids (6, 11, 44), plays a structural role in addition to its enzymatic activity in the capsids, and this role can be achieved only after release from Pra. These results provide further evidence that the full-length protease Pra is a multifunctional protein required for capsid assembly and virion maturation. One growth difference among our protease mutants (10, 24) is that it is rather difficult to generate high-titer stocks of A247S on wt protease expressing BMS-MG22 cells, suggesting that the mutant bearing the A247S mutation may exhibit a *trans*-dominant phenotype. It is not surprising that some cleavage site mutants would exhibit a *trans*-dominant phenotype, because they not only could compete with the wt protease for interactions with other capsid proteins during capsid formation but could also directly affect the ability of wt protease to bind substrates.

The major capsid protein VP5 may adopt different conformations during capsid assembly (10, 23, 42). However, the nature of these conformational changes is unknown. Although ICP35 is processed in A247S-infected cells and unprocessed in *m*100-infected cells, they both show the same phenotype in our immunofluorescence experiments. In both cases, the VP5 conformation-dependent MAb 5C failed to recognize hexons in mutant-infected Vero cells. However, this evidence is not compelling enough to support the conclusion that processing of ICP35 is independent of antibody recognition, because we know only that ICP35 can be processed in A247S-infected cells, not whether processed or unprocessed ICP35 is present in mutant capsids. An attempt to address this question by examining mutant-infected cell lysates in sucrose gradients was not conclusive. It appears that A247S capsids, like those of *ts*1201 (35, 36) and *m*100, were not stable in the gradients, and no capsid band was observed. This may be due to the low amount of capsid proteins made in HSV-1-infected cells, because these aberrant capsid structures could be isolated from a baculovirus system (40, 41).

A key question which remains to be answered regarding the virion maturation pathway is where and when the autocleavage of Pra and the *trans* processing of ICP35 occur during capsid assembly. It has been demonstrated that the catalytic domain of Pra, N_o, can *trans* cleave ICP35 in transfected cells (19), in an *E. coli* coexpression system (5, 25), and in vitro (8). These results suggest that the enzymatic activity of the protease is not coupled to the assembly of capsids. This is further supported by the finding that wt levels of autocleavage and *trans* processing of ICP35 were observed in VP5 or VP23 mutant-infected Vero cells (6). In both cases, no capsid structures were observed either by sucrose gradient analysis or electron microscopy (6). However, these experiments may not reflect the precise role of the protease in capsid assembly, because our previous results indicated that release of N_o from Pra in the cytoplasm or prior to participating in the capsid assembly inactivates its ability to support virus growth (10). Further experiments will be required to expand these findings and elucidate the precise role of the protease during capsid assembly and virion maturation.

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