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The Product of the UL12.5 Gene of Herpes Simplex Virus Type 1 Is a Capsid-Associated Nuclease

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The UL12 open reading frame of herpes simplex virus type 1 (HSV-1) encodes a deoxyribonuclease that is frequently referred to as alkaline nuclease (AN) because of its high pH optimum. Recently, an alternate open reading frame designated UL12.5 was identified within the UL12 gene. UL12.5 and UL12 have the same translational stop codon, but the former utilizes an internal methionine codon of the latter gene to initiate translation of a 60-kDa amino-terminal truncated form of AN. Since the role of the UL12.5 protein in the HSV-1 life cycle has not yet been determined, its properties were investigated in this study. Unlike AN, which can be readily solubilized from infected cell lysates, the UL12.5 protein was found to be a highly insoluble species, even when isolated by high-salt detergent lysis. Since many of the structural polypeptides which constitute the HSV-1 virion are similarly insoluble, a potential association of UL12.5 protein with virus particles was examined. By using Western blot analysis, the UL12.5 protein could be readily detected in preparations of intact virions, isolated capsid classes, and even capsids that had been extracted with 2 M guanidine-HCl. In contrast, AN was either missing or present at only low levels in each of these structures. Since the inherent insolubility of the UL12.5 protein prevented its potential deoxyribonuclease activity from being assayed in infected-cell lysates, partially purified fractions of soluble UL12.5 protein were generated by selectively solubilizing either insoluble infected-cell proteins or isolated capsid proteins with urea and renaturing them by stepwise dialysis. Initial analysis of these preparations revealed that they did contain an enzymatic activity that was not present in comparable fractions from cells infected with a UL12.5 null mutant of HSV-1. Additional biochemical characterization revealed that UL12.5 protein was similar to AN with respect to pH optimum, ionic strength, and divalent cation requirements and possessed both exonucleolytic and endonucleolytic functions. The finding that the UL12.5 protein represents a capsid-associated form of AN which exhibits nucleolytic activity suggests that it may play some role in the processing of genomic DNA during encapsidation.

Herpes simplex virus type 1 (HSV-1) encodes a diverse array of proteins involved in the propagation of its DNA genome. These include enzymes that are involved in general nucleotide metabolism, enzymes that direct the synthesis of viral DNA, and enzymes that promote the maturation of newly replicated DNA and its packaging into capsids. One viral protein which appears to play a critical role in the last two processes is a deoxyribonuclease encoded by the UL12 gene (4, 7, 13). The UL12 gene product is commonly referred to as alkaline nuclease (AN) since it requires an alkaline environment for optimal in vitro activity (1, 8, 18). It has an apparent molecular mass of 85 kDa and possesses both exonucleolytic and endonucleolytic activities (1, 3, 8, 9, 18). Although the precise function of AN in HSV-1 infections has yet to be defined, recent studies with a UL12 deletion mutant virus have indicated that this enzyme is required for efficient egress of capsids from the nucleus (17, 24) and for the processing of nonlinear or branched viral DNA intermediates that arise during replication (11).

Previous transcript-mapping studies identified five 3′-coterminal mRNAs which localized to the region of the HSV-1 genome containing the UL12 open reading frame (4, 7). AN was shown to be encoded by the 2.3-kb member of this family, whereas the 1.9-kb transcript in this group was predicted to encode an amino-terminally truncated form of AN. The latter mRNA initiated within the UL12 coding sequences and utilized an internal methionine codon as its start site for translation (Fig. 1). Translation of this 1.9-kb species in vitro yielded 60- and 54-kDa proteins; however, the identity of these polypeptides could not be confirmed due to their inability to react with immunological reagents specific for AN (4, 7). Nevertheless, more recent studies with a polyclonal antiserum raised against bacterially expressed HSV-1 AN demonstrated the existence of the 60-kDa protein in virus-infected cells (3, 12). It was therefore proposed that a previously unrecognized viral gene transcribed by the 1.9-kb mRNA did exist within the UL12 open reading frame, and this was designated UL12.5. This assignment was confirmed through the use of a mutant virus in which the UL12 but not the UL12.5 gene was disrupted; this virus failed to synthesize AN but still generated the 60-kDa protein (12). Characterization of this virus also revealed that the UL12.5 gene product could not substitute for the biochemical functions attributed to AN during an HSV-1 infection, since this mutant replicated as poorly as a virus in which both the UL12 and UL12.5 genes were deleted (12). Although all herpesvirus genomes for which sequence data is available contain obvious homologs of the UL12 gene (12), it is not known whether the ability to express alternate forms of AN is unique to HSV-1 or is common to other members of this virus family as well.

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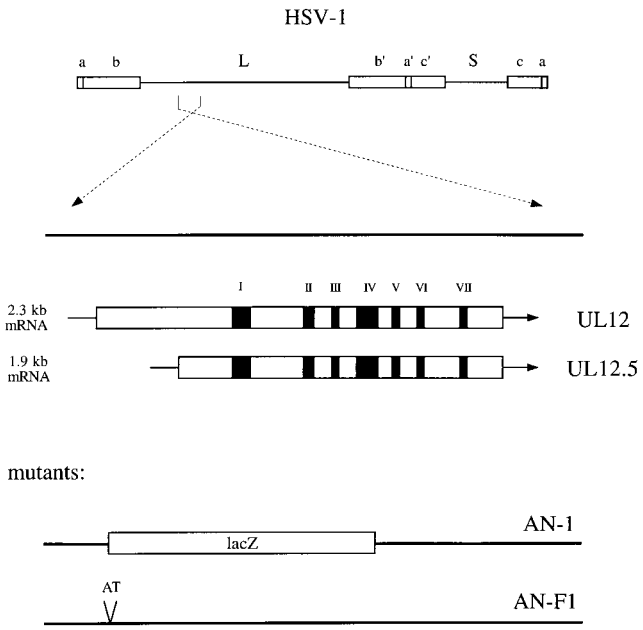

FIG. 1. Organization of the UL12 and UL12.5 genes of HSV-1. The HSV-1 genome is depicted at the top, including the long (L) and short (S) components and inverted repeat sequences *a*, *b*, and *c* (open boxes). The location of the UL12 and UL12.5 genes is indicated below. UL12 and UL12.5 encode the 626-amino-acid AN protein and a 500-amino-acid polypeptide, respectively; these represent the translation products of the 2.3- and 1.9-kb transcripts shown. UL12.5 is transcribed by a promoter within the UL12 gene and utilizes the Met-126 codon of UL12 as its initiation site for translation; otherwise, the transcripts and coding sequences of the two genes are identical. The locations of seven sequence motifs conserved in the AN homologs of all herpesviruses (12) are identified by solid boxes and Roman numerals. The mutations present in the two recombinant HSV-1 used in this study are identified at the bottom. AN-1 contains a large deletion that removes most of the UL12 and UL12.5 coding sequences and replaces them with a *lacZ* gene cassette; as a result, this virus expresses neither of the products of these two genes (17, 24). AN-F1 contains an AT dinucleotide insertion 14 bp downstream from the start codon of UL12. This frameshift mutation results in the production of a 15-amino-acid protein instead of AN but does not affect the expression of the UL12.5 gene (12).

It was determined in previous studies that the levels of UL12.5 protein that are synthesized in infected cells are significantly lower than those of AN (3, 12); however, the UL12.5 protein itself remained uncharacterized. Of particular interest was whether the UL12.5 protein was a functional nuclease, since the predicted gene product retained all seven of the sequence motifs conserved in the AN homologs of all herpesviruses (Fig. 1) (12). Previous analysis of crude extracts of cells infected with an HSV-1 mutant which expressed the UL12.5 protein but not AN revealed no detectable nucleolytic activity (12). However, this could have been due to the low levels of expression of this protein in HSV-1-infected cells or to its existence in a state that was refractory to the assay conditions used. The present study was therefore undertaken to address whether the UL12.5 protein possesses a nucleolytic function and to investigate its potential role in the HSV-1 life cycle. The UL12.5 protein was partially purified from virus-infected cells and found to be a capsid-associated polypeptide which possessed both exonucleolytic and endonucleolytic activities. Furthermore, the UL12.5 protein and AN were shown to be nearly indistinguishable with respect to their biochemical properties. These attributes of the UL12.5 protein suggest that it is involved in the maturation of replicated genomic DNA during infection and possibly in the processes of cleavage and encapsidation.

MATERIALS AND METHODS

Cells and viruses. African green monkey (Vero) cells and the UL12/UL12.5-expressing Vero-derived 6-5 cells (12) were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The wild-type virus used in this study was the *syn*17$^+$ strain of HSV-1. The HSV-1 recombinant AN-1 contained a large deletion within the UL12 and UL12.5 open reading frames accompanied by an insertion of a *lacZ* gene cassette (Fig. 1), so that it expressed neither AN nor the UL12.5 gene product. The HSV-1 recombinant AN-F1 contained a frameshift mutation at the start of the UL12 open reading frame (Fig. 1), so that it expressed the UL12.5 protein but not AN. Both AN-1 and AN-F1 were propagated in 6-5 cells and have been characterized previously (11, 12, 17, 24). Infections were carried out by infecting overnight cultures of Vero cells at a multiplicity of infection of 10 and harvesting the cells and/or media at 18 h postinfection unless noted otherwise.

Purification of extracellular virions. HSV-1 virions were purified from the extracellular media of cultures of infected cells by the procedure of Szilagyi and Cunningham (19). Briefly, cellular debris was removed by centrifugation at 1,500 × *g* for 20 min, and the extracellular virus in the supernatant was pelleted by centrifugation at 39,000 × *g* for 2 h; all centrifugations were done at 4°C. The virus pellet was then resuspended in medium lacking both phenol red and serum and layered onto a preformed continuous 5 to 15% (wt/vol) Ficoll 400 gradient in the same medium. Following centrifugation at 26,000 × *g* for 2 h in a swinging-bucket rotor, the virus band was collected by side puncture of the centrifuge tube, diluted in medium lacking both phenol red and serum, and pelleted at 85,000 × *g* for 1 h. The purified virus was resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for use in Western blot analysis.

Purification of intracellular capsids. Capsids were isolated from infected cells by a modification of the procedure described by Davison and Davison (5). Infected cells were pelleted by centrifugation at 1,500 × *g* for 20 min and resuspended in TNE buffer (20 mM Tris-HCl [pH 7.5], 0.5 M NaCl, 1 mM EDTA) containing 1% (vol/vol) Triton X-100 and a protease inhibitor cocktail of phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (10 μM), and pepstatin (10 μM). The cells were then lysed by three rapid freeze-thaw cycles followed by sonication. Cellular debris was pelleted by centrifugation at 1,500 × *g* for 20 min, and the capsid-containing supernatant was transferred to a fresh centrifuge tube and underlaid with 1 ml of a sucrose cushion (35% [wt/wt] in TNE buffer). Capsids were pelleted at 70,000 × *g* for 1 h, resuspended in TNE buffer by sonication, and layered onto a linear 20 to 50% (wt/wt) sucrose density gradient. Following centrifugation at 70,000 × *g* for 1 h in a swinging-bucket rotor, gradient fractions were collected by puncture of the bottom of the tube. Alternatively, individual capsid bands were collected by side puncture of the centrifuge tube, diluted in TNE buffer, and pelleted at 57,000 × *g* for 30 min. Treatment of purified capsids with guanidine-HCl to release DNA and penton proteins was carried out by the procedure of Newcomb and Brown (14). All capsid preparations were resuspended in SDS-PAGE loading buffer for use in Western blot analysis.

Western blot analysis. Protein samples were electrophoresed on either 8 or 12% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and subjected to Western blot analysis as described previously (3). The primary antibody used in all Western blot analyses was the BWp12 polyclonal antiserum specific for AN (3).

Solubilization and renaturation of UL12.5 protein. Cells infected with either AN-1 or AN-F1 were pelleted by centrifugation at 1,500 × *g* for 20 min and resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1% [vol/vol] Triton X-100) containing 150 mM NaCl. Following lysis on ice for 30 min, capsids were pelleted by centrifugation at 85,000 × *g* for 1 h and resuspended in lysis buffer containing 0.5 M NaCl. To maximize capsid recovery, this preparation was then treated with three rapid freeze/thaw cycles followed by sonication. Insoluble material was pelleted at 85,000 × *g* for 1 h and resuspended in solubilization buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM EGTA, 0.5 M NaCl, 50 mM dithiothreitol) containing 4 M urea. Following overnight incubation at room temperature with continuous mixing, the remaining insoluble material was pelleted and resuspended in solubilization buffer containing 8 M urea for an additional overnight incubation at room temperature. Alternatively, B capsids that had been purified by sucrose gradient centrifugation and pelleted as described above were resuspended in solubilization buffer containing 8 M urea for an overnight incubation at 37°C. The insoluble material remaining after this incubation was pelleted and discarded. The solubilized protein sample was renatured by stepwise dialysis as described previously (3) and stored at −70°C until further use. An insoluble form of bacterially expressed HSV-1 AN (3) was similarly solubilized in urea and renatured simultaneously with the preparations from the infected cells, so that the recovery and enzymatic activity of the UL12.5 protein could be directly compared to that of the functional nuclease AN.

Quantification of nuclease activity. Exonuclease activity was measured by quantification of acid-soluble nucleotides released during degradation of an ^{3}H-labelled DNA substrate as described previously (3). These reaction mixtures were incubated for 60 min at 37°C and contained either 20 μl of renatured solubilized protein from AN-1- or AN-F1-infected cells or 1.25 μl of renatured solubilized, bacterially expressed AN in a total volume of 200 μl. One unit (U)

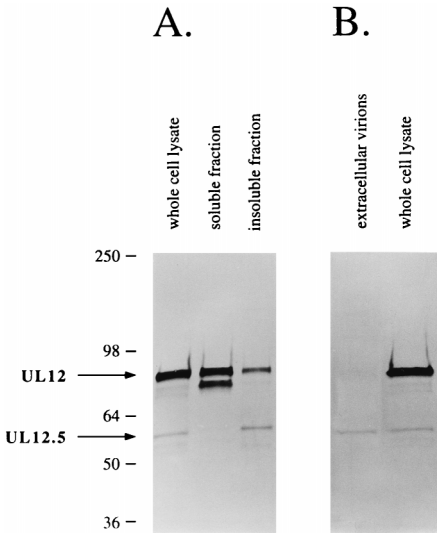

FIG. 2. Detection of the UL12.5 protein in HSV-1 virions. (A) Isolation of the UL12.5 protein in the insoluble fraction of HSV-1-infected cell lysates. Western blot analysis was carried out on whole-cell lysates that were either fractionated under high-salt detergent lysis into soluble and insoluble components or left untreated. The antibody used was BWp12, an affinity-purified rabbit antiserum raised against a recombinant AN protein (3, 12). (B) Detection of the UL12.5 protein in purified HSV-1 virions. Western blot analysis with the BWp12 antiserum was carried out on lysates of HSV-1 virions that had been purified from the media of infected cells. The mobilities of molecular mass markers (in kilodaltons) for both panels and the identities of the UL12 and UL12.5 proteins are shown on the left.

of exonuclease activity was defined as the amount (in micrograms) of acid-soluble nucleotides released after 1 min of incubation at 37°C.

Endonuclease and exonuclease activities were also measured by a plasmid DNA degradation assay as described previously (3); this used supercoiled covalently closed circular and *Hin*dIII-linearized pUC19 templates, respectively. These reaction mixtures were incubated for various times at 37°C and contained 50 ng of plasmid DNA and 5 μl of renatured solubilized protein from AN-1- or AN-F1-infected cells in a final volume of 25 μl. The reaction products were electrophoresed on agarose gels and quantified by densitometry as described previously (3). One unit (U) of endonuclease or exonuclease activity was defined as the amount (in micrograms) of plasmid DNA degraded, as determined by the reduction in undegraded DNA substrate, after 1 min of incubation at 37°C.

For specific activity determinations, total protein concentration was determined by the Bio-Rad protein assay. The concentrations of UL12.5 and AN protein present in renatured solubilized fractions were calculated by densitometric quantification of these proteins on Western blots with a Hoefer GS 300 transmittance/reflectance scanning densitometer; known amounts of purified recombinant AN (3) were used as standards in these quantitation experiments.

RESULTS

The UL12.5 protein is associated with viral capsids. In Western blot analyses with the AN-specific polyclonal antiserum BWp12, both the 85-kDa AN and the 60-kDa UL12.5 protein can be readily detected in whole-cell lysates of HSV-1-infected Vero cells (Fig. 2A). To perform an initial fractionation of the UL12.5 protein, cells infected with wild-type HSV-1 were separated into their soluble and insoluble components with 1% Triton X-100 plus 150 mM NaCl. Western blot analysis of these preparations revealed that the UL12.5 protein was completely absent from the soluble fraction and separated entirely within the insoluble fraction (Fig. 2A). In contrast, AN was almost entirely soluble under these lysis conditions (Fig. 2A). The UL12.5 protein also resisted solubilization in a native conformation when the lysis procedure was modified to include NaCl concentrations as high as 1 M or a variety of nonionic, anionic, cationic, and Zwitterionic detergents (2).

The insoluble nature of the UL12.5 protein suggested a possible association with the structural polypeptides which constitute the HSV-1 virion. To examine this possibility, virus particles were isolated from the extracellular media of infected cell cultures by Ficoll gradient centrifugation and subjected to Western blot analysis. The UL12.5 protein could be readily detected in extracellular virions isolated in this manner (Fig. 2B). In contrast, AN was not detected in these virion preparations, although it was synthesized at much higher levels during infection (Fig. 2B). These results indicated that the UL12.5 protein was not a fortuitous contaminant in these fractions but, rather, an actual component of the HSV-1 virion.

The UL12.5 protein was absent in soluble fractions of infected cell lysates that were prepared by high-salt detergent lysis (Fig. 2A), which represented conditions that should strip the virion of its envelope and tegument. This suggested that the UL12.5 protein might be a structural component of the capsid itself. To investigate this possibility, intracellular capsids were purified by sucrose gradient centrifugation and examined for the presence of the UL12.5 polypeptide. Distinct well-separated bands containing mature DNA-containing C capsids or empty B capsids could be readily generated in this manner, whereas a minor band corresponding to empty A capsids typically overlapped extensively with the B capsid band in these experiments. Continuous fractions derived from an entire sucrose gradient were collected and subjected to Western blot analysis (Fig. 3A).

Surprisingly, both AN and the UL12.5 protein were detected throughout the gradient. Moreover, a novel 54-kDa protein was also visible in these gradient fractions. This was determined to be a proteolytic degradation product derived specifically from the UL12.5 protein and not AN, since comparable levels of this 54-kDa protein were also detected in sucrose gradients prepared from cells infected with AN-F1, a recombinant HSV-1 strain which expresses the UL12.5 protein but not AN (Fig. 1) (2). The relative levels of the two UL12.5 polypeptides were significantly enhanced in four of the gradient fractions (fractions 7 through 10), whereas the levels of AN protein remained relatively uniform throughout the gradient. The four lanes which contained elevated concentrations of the UL12.5 protein corresponded to capsid-containing fractions, since the C capsid band had been collected in fractions 7 and 8 and the B capsid band had been collected in fractions 9 and 10 (Fig. 3A). The identity of these capsid-containing fractions was confirmed on Coomassie brilliant blue-stained SDS-PAGE gels, which revealed the presence of polypeptides indicative of C capsids (VP5, VP19, and VP23) and B capsids (VP5, VP19, VP22a, and VP23) in fractions 7 and 8 and fractions 9 and 10, respectively (Fig. 3B). Moreover, the secondary antibody used in the Western blot analysis was able to fortuitously react with the major capsid protein VP5 in these four fractions (Fig. 3A). The relative levels of AN, UL12.5, and VP5 proteins on duplicate Western blots were determined by densitometry and are summarized in Table 1. These results clearly show that significantly elevated levels of VP5 and the UL12.5 protein were detected in gradient fractions containing B and C capsids. This confirms and extends the analysis of extracellular virion particles presented above by demonstrating that the UL12.5 protein not only is a component of the HSV-1 virion but also represents a capsid-associated polypeptide.

Capsid preparations that were purified further by consecutive bandings in sucrose gradients retained both AN and the UL12.5 protein (2). Thus, it was of interest to determine the strength of the association between these polypeptides and the HSV-1 capsid. Purified capsids were therefore subjected to guanidine-HCl (GuHCl) extraction, since previous studies

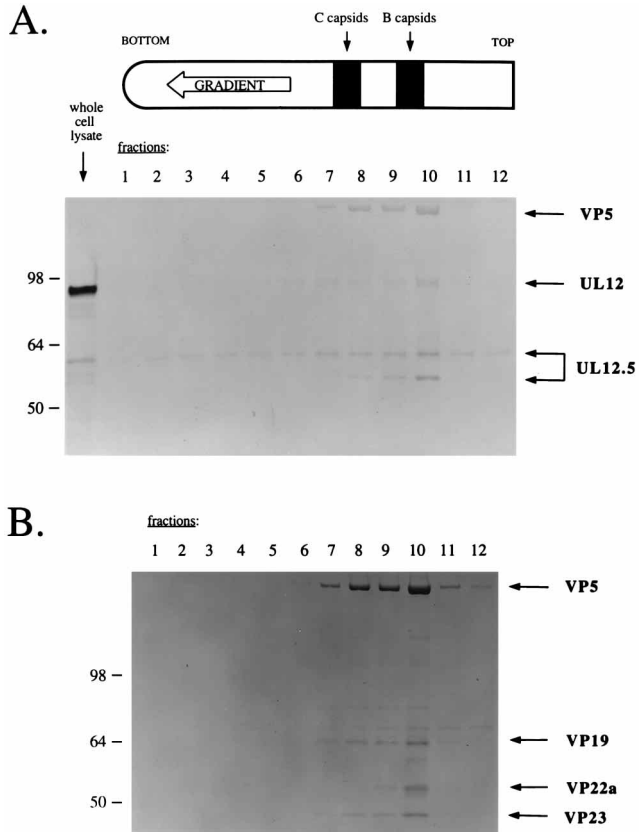

FIG. 3. Detection of the UL12.5 protein in HSV-1 capsids. (A) Western blot analysis of HSV-1 capsids isolated from sucrose gradients. Twelve fractions from a sucrose gradient containing capsids from HSV-1-infected cells were subjected to Western blot analysis with the BWp12 antiserum. A schematic diagram of the gradient is shown above the blot, indicating the positions of each of the 12 fractions as well as the locations of the C and B capsid bands (collected in fractions 7 to 8 and 9 to 10, respectively). Unfractionated infected cell lysate is included in the first lane to facilitate the identification of the UL12 and UL12.5 proteins. The mobilities of molecular mass markers (in kilodaltons) are shown on the left, and the identities of the VP5, UL12, and UL12.5 proteins are shown on the right. Note that preparations of sucrose gradient-purified capsids typically contain the full-length 60-kDa UL12.5 protein as well as a 54-kDa proteolytic degradation product. (B) Analysis of viral polypeptides in HSV-1 capsids isolated from sucrose gradients. The 12 gradient fractions in panel A were electrophoresed on an SDS-PAGE gel and stained with Coomassie brilliant blue. The mobilities of molecular mass markers (in kilodaltons) are shown on the left, and the identities of specific HSV-1 capsid proteins are shown on the right.

TABLE 1. Protein quantification in sucrose gradients of HSV-1 capsid preparations

Fraction no.	Relative protein concn[a]					
	UL12		UL12.5		VP5	
	Blot 1	Blot 2	Blot 1	Blot 2	Blot 1	Blot 2
3	1.1	1.0	1.5	2.1	0.0	0.0
4	1.1	2.1	2.4	2.2	0.0	0.0
5	1.2	1.7	2.0	3.2	0.0	0.0
6	1.0	1.7	2.0	3.7	0.0	0.0
7[b]	1.2	1.5	3.6	4.4	1.2	2.0
8[b]	1.2	1.8	3.1	8.6	3.0	5.8
9[c]	1.2	1.8	5.5	10.4	3.0	5.2
10[c]	1.9	4.4	9.5	17.5	6.4	11.9

[a] Fractions of sucrose gradients of HSV-1 capsid preparations were collected and subjected to duplicate Western blot analyses as shown in Fig. 3. The levels of UL12, UL12.5, and VP5 proteins present in each fraction were then quantified by densitometric analysis for both blots. In the case of the UL12.5 protein, the signals of both the 60-kDa protein and its 54-kDa degradation product (Fig. 3) were determined. The relative levels of the proteins were then calculated by using the ratio of the signal from each band to that of the UL12 band in fraction 3 of blot 2; the latter was used as a reference because it generated the lowest measurable signal for any of the bands and therefore gave a ratio of 1.0.

[b] C capsid-containing fraction.

[c] B capsid-containing fraction.

have shown that treatment of capsids with 0.5 M GuHCl results in the release of viral DNA while treatment with 2.0 M GuHCl results in the additional removal of the viral polypeptides that comprise the pentons of the capsid (14). Gradient-purified B capsids that had been extracted with GuHCl at concentrations as high as 2.0 M were found to retain essentially all of their UL12.5 protein (Fig. 4A). In contrast, this treatment completely stripped the VP22a, VP24, and VP26 proteins from the capsid (Fig. 4B), in agreement with previous studies (14), while treatment with GuHCl concentrations as low as 0.5 M eliminated most of the contaminating AN protein in the capsid preparation (Fig. 4A). Comparable results were obtained with purified C capsids (2). These data indicate that the UL12.5 protein represents a tightly associated component of the HSV-1 capsid which may be part of the hexon substructure. Moreover, these results do not rule out the possibility that trace amounts of AN are present in sucrose-gradient-purified capsids, although this would be inconsistent with the inability to detect this protein in extracellular virions (Fig. 2B).

The UL12.5 protein is a functional nuclease. Previous attempts at demonstrating a nuclease activity associated with the

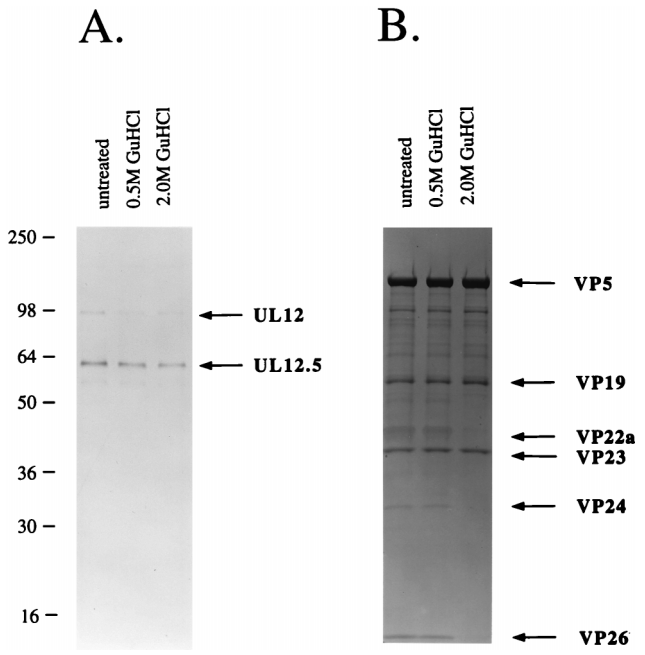

FIG. 4. Detection of the UL12.5 protein in GuHCl-extracted HSV-1 capsids. (A) Western blot analysis of GuHCl-extracted HSV-1 capsids. HSV-1 B capsids purified on sucrose gradients were extracted with either 0.5 or 2.0 M GuHCl or left untreated; these were then subjected to Western blot analysis with the BWp12 antiserum. (B) Analysis of viral polypeptides in GuHCl-extracted HSV-1 capsids. The capsid preparations in panel A were electrophoresed on an SDS-PAGE gel and stained with Coomassie brilliant blue. The mobilities of molecular mass markers (in kilodaltons) for both panels are shown on the far left. The identities of the UL12 and UL12.5 proteins or specific HSV-1 capsid proteins are shown on the right of panels A and B, respectively.

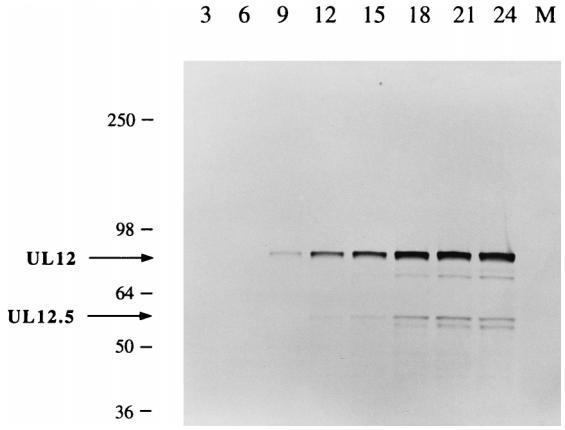

FIG. 5. Kinetics of accumulation of the UL12 and UL12.5 proteins. Cells infected with HSV-1 were used to prepare lysates at the indicated times (in hours) postinfection; lysates from mock-infected cells (M) were also prepared. These were subjected to Western blot analysis with the BWp12 antiserum. The mobilities of molecular mass markers (in kilodaltons) and the identities of the UL12 and UL12.5 proteins are shown on the left.

UL12.5 protein by using crude extracts of cells infected with the $UL12^{-}/UL12.5^{+}$ mutant AN-F1 (Fig. 1) were unsuccessful (12). This is perhaps not surprising, given the low levels of expression of this protein and its dramatic insolubility. It was therefore necessary to solubilize, renature, and partially purify this polypeptide from HSV-1-infected cells before its ability to function as a nuclease could be more stringently assessed.

The availability of the AN-F1 mutant conveniently allowed for the isolation of UL12.5 protein and the examination of its nucleolytic properties in the absence of contaminating AN. Moreover, identical preparations obtained from cells infected with a mutant which fails to express either AN or the UL12.5 protein, AN-1 (Fig. 1), provided appropriate controls for background nuclease activity. However, it was first necessary to assess the kinetics of UL12.5 gene expression in order to establish the optimal time for isolation of its gene product. A time course experiment was therefore performed in which lysates of wild-type-HSV-1-infected cells were prepared at 3-h intervals and then examined for the presence of AN and the UL12.5 protein by Western blot analysis (Fig. 5). The kinetics of expression of the UL12.5 protein and AN were found to be identical in this experiment. Both proteins were first detected at 9 h postinfection, continued to accumulate until 18 h postinfection, and maintained steady-state levels thereafter through 24 h postinfection. The UL12.5 protein was therefore routinely isolated from cells that had been infected with AN-F1 for a minimum of 18 h.

Cells infected with either AN-F1 or AN-1 were fractionated by high-salt detergent lysis as before; their insoluble protein fractions were then selectively solubilized with urea. This strategy had been successfully used in a previous study to purify insoluble AN that had been expressed in bacterial cells (3). Preliminary experiments indicated that high concentrations of urea (6 M or greater) were required to generate a soluble form of the UL12.5 protein. Nevertheless, a significant amount of the UL12.5 protein remained insoluble, even after overnight incubation in the presence of 8 M urea (2). Attempts were then made to refold the solubilized UL12.5 protein into an active form by gradual removal of the urea denaturant through stepwise dialysis. This procedure had been successfully applied in the renaturation of a bacterially expressed form of AN in a previous study (3). A preparation of this recombinant AN was therefore processed alongside the fractions from AN-F1- and AN-1-infected cells for use as a positive control.

The presence of the UL12.5 protein in renatured solubilized fractions from AN-F1-infected cells was verified by Western blot analysis (Fig. 6A, lane AN-F1); no UL12.5 protein was detected in comparable fractions from AN-1-infected cells (lane AN-1). The purity of the UL12.5 protein in this fraction was then assessed on Coomassie brilliant blue-stained SDS-PAGE gels. Although substantial levels of contaminating proteins were present in this preparation, a unique 60-kDa species could be detected in the renatured solubilized fraction from AN-F1-infected cells (Fig. 6B, lane AN-F1) which was not present in that from AN-1-infected cells (lane AN-1). Thus, the selective solubilization strategy that was used in this study was successful in partially purifying a soluble form of the UL12.5 protein.

The existence of a nuclease activity in this preparation of UL12.5 protein was then examined by an assay which measured the degradation of an ^{3}H-labelled DNA substrate. Release of acid-soluble nucleotides resulting from DNA template degradation was detected for the renatured solubilized fraction from AN-F1-infected cells but not for the comparable fraction from AN-1-infected cells. These results indicated that the UL12.5 protein present in the AN-F1-derived fraction was indeed a functional nuclease. The specific activity of this nuclease function was then estimated by using the actual amount of UL12.5 protein present in the renatured solubilized preparation; this was determined by quantitative Western blot analysis with known amounts of purified recombinant AN (3) as standards. The specific activity of the UL12.5 protein preparation was calculated to be 651 U/mg of protein, which was comparable to the 435 U/mg of protein obtained for the bacterially expressed AN that had been solubilized and renatured in an identical manner.

The absence of any nuclease function in the renatured sol-

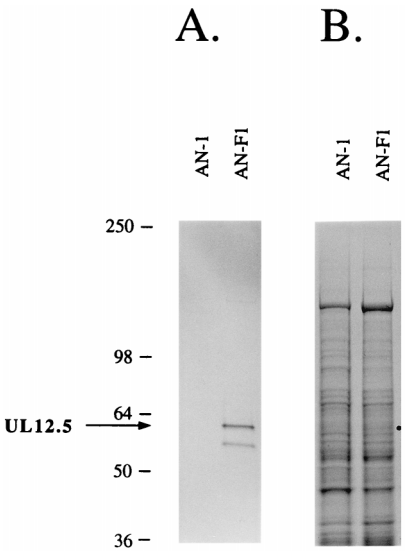

FIG. 6. Analysis of the insoluble fraction of infected-cell lysates following solubilization and renaturation. Insoluble fractions of lysates from AN-1- or AN-F1-infected cells were solubilized with urea and renatured by slow stepwise dialysis. These were then subjected to Western blot analysis with the BWp12 antiserum (A) or electrophoresis on Coomassie brilliant blue-stained SDS-PAGE gels (B). The mobilities of molecular mass markers (in kilodaltons) for both panels are shown on the far left. The identity of the UL12.5 protein is indicated by an arrow on the left of panel A and by a dot on the right of the AN-F1 extract-containing lane in panel B.

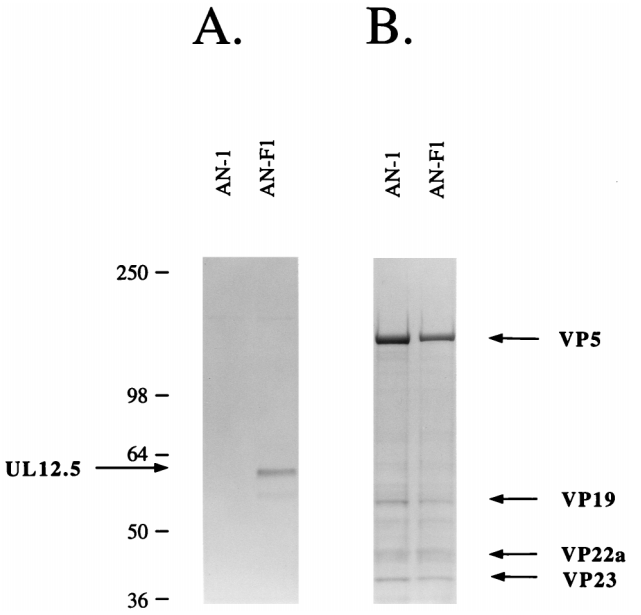

FIG. 7. Analysis of capsid proteins following solubilization and renaturation. Proteins of B capsids purified from AN-1- or AN-F1-infected cells were solubilized with urea and renatured by slow stepwise dialysis. These were then subjected to Western blot analysis with the BWp12 antiserum (A) or electrophoresis on Coomassie brilliant blue-stained SDS-PAGE gels (B). The mobilities of molecular mass markers (in kilodaltons) for both panels are shown at the far left. The identity of the UL12.5 protein is indicated by an arrow on the left of panel A. The identities of specific HSV-1 capsid proteins are shown on the right of panel B.

ubilized fraction from AN-1-infected cells indicated that the activity present in the comparable preparation from AN-F1-infected cells was mediated by the UL12.5 protein. However, since the latter fraction was significantly contaminated with other viral and cellular proteins (Fig. 6B, lane AN-F1), it was still formally possible that this nuclease activity was derived from another polypeptide. Thus, it was of interest to repeat these experiments with purified B capsids, which represented an alternate source of the UL12.5 protein that possessed far fewer contaminating polypeptides (Fig. 3 and 4). B capsids that had been isolated from either AN-1- or AN-F1-infected cells by sucrose gradient centrifugation were subjected to the same denaturation and renaturation procedure used previously with infected-cell lysates. Western blot analysis of the resulting fractions confirmed that UL12.5 protein was present in the renatured solubilized capsid preparations derived from AN-F1- but not AN-1-infected cells (Fig. 7A), and analysis on Coomassie brilliant blue-stained SDS-PAGE gels demonstrated that both preparations were comprised principally of the major capsid proteins of HSV-1 (Fig. 7B).

These renatured solubilized capsid proteins were then subjected to nuclease assays. As before, only the protein preparations derived from AN-F1-infected cells exhibited nucleolytic activity in these analyses. The specific activity of this nuclease function was found to be 126 U/mg of protein, as determined from the amount of UL12.5 protein present in the renatured solubilized preparation that had been estimated by quantitative Western blot analysis. This value was of the same order of magnitude as the specific activity of either the UL12.5 protein preparation derived from infected-cell lysates or the bacterially expressed AN that had been solubilized and renatured in an identical manner (651 and 435 units/mg of protein, respectively). These results confirmed that the nuclease function initially observed in the renatured solubilized fraction of infected cell lysates was likely to be due to the UL12.5 protein and not a contaminating nuclease. In addition to these experiments, other capsid preparations that contained the UL12.5 protein were examined for the presence of nuclease activity. Interestingly, neither intact native B capsids nor B capsids that had been opened by prior treatment with 2 M GuHCl had any detectable nucleolytic activity, even in assays involving significantly elevated protein concentrations (2). These results indicate that while the UL12.5 protein of the HSV-1 capsid may represent a functional nuclease, there is no evidence that its nucleolytic activity is manifest in assembled B capsids, since the only active preparations of the UL12.5 protein that have been obtained to date are those in which this protein has been dissociated from disrupted capsids.

Biochemical characterization of UL12.5 protein. Preliminary analysis of the two different preparations of partially purified UL12.5 protein described above had indicated that it was a functional enzyme which possessed an exonucleolytic activity that appeared to be comparable in strength to that of AN. Although less pure, those preparations derived from the insoluble fractions of infected-cell lysates generated much higher yields of UL12.5 protein than did those from purified capsids and were therefore used in all subsequent experiments. The UL12.5 protein and AN were further compared with respect to a variety of enzymatic parameters (Fig. 8). The effect of variations in pH revealed no significant difference between the UL12.5 protein and AN, in that they both required an alkaline environment (pH 9 to 10) for optimal exonucleolytic activity (Fig. 8A). The effect of increasing ionic strength on the activity of the two proteins was more distinct (Fig. 8B). NaCl had a stimulatory effect on the activity of UL12.5 protein at concentrations up to 50 mM; with increasing salt concentrations, enzyme activity was inhibited linearly. In contrast, AN activity was inhibited linearly from the lowest concentrations of NaCl tested (12.5 mM). Comparable results were obtained with KCl instead of NaCl (2). Finally, the effect of variations in the divalent cation cofactor concentration demonstrated that both the UL12.5 protein and AN had a strong preference for Mg^{2+} over Mn^{2+} (Fig. 8C). Peak enzyme activity was observed with Mg^{2+} concentrations of 5 mM for the UL12.5 protein and 3 mM for AN and an Mn^{2+} concentration of just 0.625 mM for both proteins. Thus, apart from a modest difference in sensitivity to salt concentration, the exonuclease activity of the UL12.5 protein was remarkably similar to that of AN.

It has been previously shown that in addition to its exonuclease activity, AN contains a weak endonuclease activity which may be of biological relevance (1, 3, 8, 9, 18). To determine whether an endonucleolytic function also resides in the UL12.5 protein, its enzyme activity was analyzed by a plasmid DNA degradation assay. This assay was performed with both supercoiled and linearized pUC19 DNA as templates, such that endonuclease and exonuclease activities, respectively, could be compared within the same protein preparation. These plasmid templates were incubated with the renatured solubilized fractions from either AN-F1- or AN-1-infected cells and then electrophoresed on agarose gels; representative results from these assays are shown in Fig. 9.

This analysis revealed that the UL12.5 protein did in fact possess an endonuclease activity, since the renatured solubilized fraction from AN-F1-infected cells was able to nick a supercoiled plasmid template and convert it into a linear form (Fig. 9B). Nevertheless, as with AN, this reaction was substantially slower than that mediated by the exonuclease activity of the protein. This is best illustrated at the later time points, when the endonuclease activity had still not nicked all of the

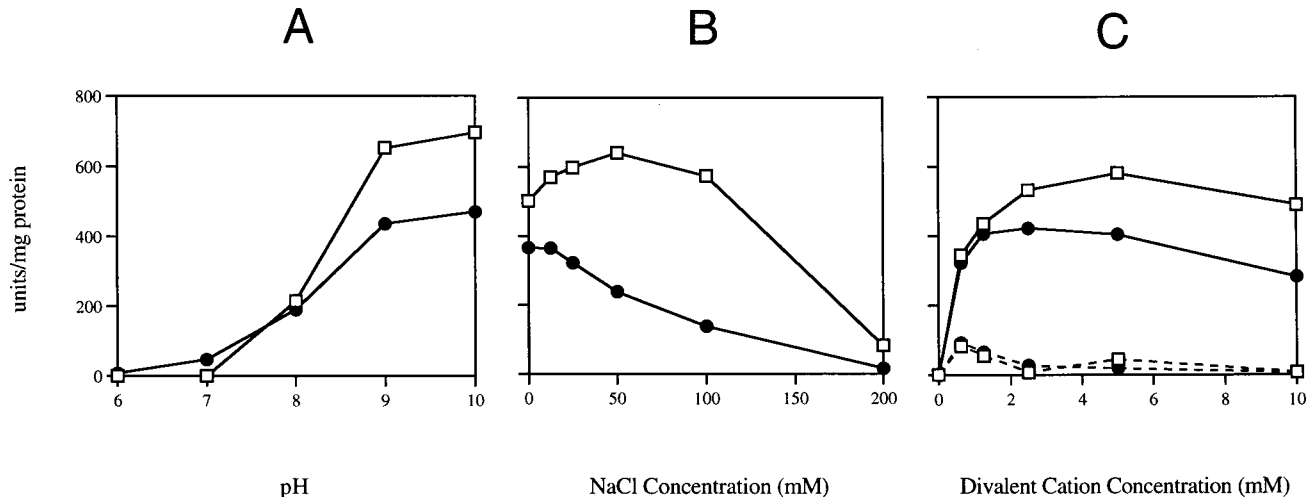

FIG. 8. Comparison of the nuclease activities in AN and the UL12.5 protein. (A) Effect of pH on nuclease activity. Specific activity in the presence of Tris-HCl at different pH values was determined by quantification of acid-soluble nucleotides released from degraded [^{3}H]DNA. (B) Effect of ionic strength on nuclease activity. Specific activity in the presence of various concentrations of NaCl was determined by quantification of acid-soluble nucleotides released from degraded [^{3}H]DNA. (C) Effect of variation of divalent-cation cofactor on nuclease activity. Specific activity in the presence of various concentrations of $MgCl_2$ (solid lines) or $MnCl_2$ (dashed lines) was determined by quantification of acid-soluble nucleotides released from degraded [^{3}H]DNA. In all three panels, datum points for AN and the UL12.5 protein are represented by solid circles and open boxes, respectively.

supercoiled plasmid template (Fig. 9B) but the exonuclease activity had completely degraded all of the linear plasmid template (Fig. 9A); this difference is much more dramatic when it is considered that the creation of a linear form by nicking requires a single cleavage event whereas the complete degradation of a linear plasmid involves many such events. Finally, a low level of nicking was unexpectedly mediated by the renatured solubilized fraction from AN-1-infected cells (Fig. 9B).

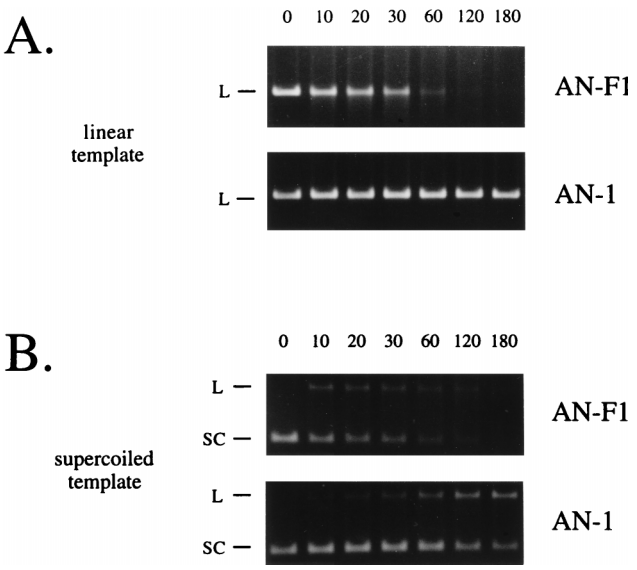

FIG. 9. Comparison of endonuclease and exonuclease activities associated with renatured solubilized protein from infected-cell lysates. Renatured solubilized protein fractions from AN-1- or AN-F1-infected cells were incubated with supercoiled and linearized pUC19 DNA for various times to assay their endonuclease and exonuclease activities, respectively. These digested samples were then electrophoresed on 1% agarose gels, stained with ethidium bromide, and quantified by densitometry to allow for calculation of reaction rates. Assay times (in minutes) are given at the top of each panel, the template used in the reactions (linear versus supercoiled) is indicated on the left, and the source of the protein present in the reactions is indicated on the right.

However, this background degradation was over an order of magnitude lower than the endonuclease activity of the UL12.5 protein and was devoid of any exonuclease activity, as confirmed by the absence of acid-soluble nucleotides generated in the ^{3}H-labelled DNA substrate assay described above and the lack of degradation in a linear plasmid template (Fig. 9A).

Reaction rates in these experiments that could be used for specific activity determinations were estimated by densitometric quantification of the undegraded DNA that was left in each gel lane; in the assays with a supercoiled template, the value for the background nicking activity was subtracted from that of the endonuclease activity of the UL12.5 protein. Specific activity was again calculated from the actual amount of UL12.5 protein present in an assay, as determined by quantitative Western blot analysis. By using this strategy, the specific activity of the endonuclease associated with the UL12.5 protein was determined to be 234 U/mg of protein, which was comparable to the 303 U/mg of protein previously reported for AN (3). In contrast, the specific activity for the exonuclease component of the UL12.5 protein, 571 U/mg of protein, was substantially lower than the 1,822 U/mg of protein previously reported for AN (3). However, the reaction rate used for the former value was calculated by measuring the complete degradation of linearized plasmid template, whereas that used for the latter value was calculated by measuring only the linearized plasmid template that had been degraded by 100 bp or more. The specific activity of the exonuclease function of AN was therefore recalculated by the same method used for the UL12.5 protein in this study; this value was determined to be 520 U/mg of protein, which was now comparable to the 571 U/mg of protein for the UL12.5 protein. Moreover, these values were similar to those obtained in the ^{3}H-labelled DNA substrate assay described above. Thus, the specific activities of the endonuclease and exonuclease components of the UL12.5 protein were nearly indistinguishable from those of AN. Together with the results of the biochemical characterization described above, this suggests that the enzymatic properties of the two proteins are very comparable.

DISCUSSION

The AN protein plays an important role in the life cycle of HSV-1 by facilitating both the resolution of nonlinear DNA replication intermediates and the egress of viral particles from the nucleus (11, 17, 24). Interestingly, HSV-1 encodes two forms of AN: an 85-kDa full-length polypeptide that is the product of the UL12 gene, and a 60-kDa amino-terminally truncated derivative of AN that is the product of the UL12.5 gene (3, 12). Although these two versions of AN differ only in the presence or absence of a 126-amino-acid segment at their amino-terminal ends, previous studies with the $UL12^{-}$/$UL12.5^{+}$ mutant AN-F1 demonstrated that the UL12.5 protein cannot functionally replace the full-length AN (12). There are three possible explanations for this complementation defect: (i) the UL12.5 protein lacks an essential functional domain encoded by the first 126 amino acids of AN; (ii) the low level of UL12.5 protein that accumulates during infection (12) or its marked insolubility prevents it from compensating for the complete loss of AN; or (iii) HSV-1 may possess independent requirements for both AN and the UL12.5 protein during productive infection, such that each mediates a function essential for replication. Each of these possibilities is currently being addressed experimentally through the construction of the appropriate recombinant viruses and through biochemical characterization of both proteins. This study represents an exploration of the first hypothesis, that the UL12.5 protein lacks one or more functional domains of AN, by effecting a comparison of the enzymatic properties of both proteins.

Previous analysis of the AN-F1 mutant had already failed to detect any enzymatic activity in crude extracts from cells infected with this virus (12). However, only low levels of the UL12.5 protein are expressed in AN-F1- and wild-type-HSV-1-infected cells compared to AN (12), so that its potential nucleolytic activity could have escaped detection in these experiments. Moreover, preliminary characterization of the UL12.5 polypeptide in this study revealed that it was highly insoluble (Fig. 2) and therefore unlikely to manifest any enzymatic activity in this state. This insolubility was probably due to the structural role of the UL12.5 protein during infection, since it was unexpectedly found to be associated with both extracellular virions and viral capsids (Fig. 2 to 4). As a result, the UL12.5 protein had to be first solubilized as a denatured polypeptide with urea and subsequently renatured by stepwise dialysis before its enzymatic properties could be compared with those of AN. Once the UL12.5 protein had been isolated and partially purified in this manner, it was characterized and found to possess properties that were essentially indistinguishable from AN; these included similar sensitivities to a variety of biochemical parameters, identical overall specific activities, and the presence of strong exonuclease and weak endonuclease components.

The simplest interpretation of these results is that the UL12.5 protein represents a capsid-associated form of AN that retains all of the enzymatic properties of the latter nuclease. However, since the UL12.5 protein cannot functionally replace the full-length AN during infection (12), its nuclease activity may not be sufficient for restoring normal replication in this virus. Thus, AN could perform an additional nonnucleolytic function during infection that is absent in the UL12.5 protein, and this putative activity would most probably be mediated by the 126-amino-acid domain that is unique to AN. The nature of this secondary function of AN is far from clear, but it could be involved in promoting the egress of viral particles from the nucleus (17), mediating interactions with the viral DNA binding protein ICP8 (20, 22), or fostering associations with host cell factors involved in debranching of replicated viral DNA (11).

Alternatively, the unique amino-terminal region of AN may not in itself constitute a separate functional domain but may instead alter some aspect of AN activity by its presence. For example, these sequences may actively prevent AN from being incorporated into the viral capsid, so that the bulk of virus-encoded nuclease synthesized during infection remains free in the nucleus. Additionally, the presence of the unique 5′ sequences of the AN mRNA or amino-terminal sequences of the AN polypeptide could impart an increase in transcript utilization or protein stability, respectively; this would account for the dramatically higher ratios of protein to RNA observed for AN compared to the UL12.5 protein (12). Finally, the presence of the 126-amino-acid region could affect the specificity of the nuclease activity of AN in a manner which is not apparent from the assays that were performed in this work.

The observations that the UL12.5 protein is sequestered into capsids and possesses a detectable endonuclease activity suggest a possible involvement in the cleavage of concatameric replicated viral DNA prior to its packaging into capsids. Although a number of viral polypeptides which play a role in this encapsidation process have been identified (reviewed in reference 23), the actual protein which performs the cleavage event itself has yet to be identified. However, a model for UL12.5 protein-mediated genomic cleavage can be proposed that is consistent with the results presented in this study. This involves the following steps: (i) initial incorporation of newly synthesized UL12.5 protein into immature B capsids; (ii) interactions with one or more components of the capsid to redirect the largely nonspecific endonuclease activity of the UL12.5 protein into precisely cleaving concatameric viral DNA at the DR1 repeats of the *a* sequence (6, 21); and (iii) retention of the UL12.5 protein following the encapsidation process as a component of fully mature C capsids. Although the properties of the UL12.5 protein seem to make it an attractive candidate for a terminase-like function, this assignment appears to be inconsistent with the high levels of genome cleavage observed in an HSV-1 UL12/UL12.5 null mutant (17, 24). It is formally possible that the UL12.5 protein does mediate cleavage of the viral DNA but that a cellular function may partially complement this function in its absence. Alternatively, the UL12.5 protein may be involved in DNA processing only after the cleavage event, which would be consistent with the failure of capsids of the UL12.5 mutant to egress from the nucleus despite the high levels of cleaved genomic ends generated during infection (17, 24).

In addition to its potential role in genome maturation, the presence of the UL12.5 protein in mature virion particles raises the possibility that this protein performs a crucial function at the onset of infection. For example, the UL12.5 protein could be involved in the circularization or processing of viral DNA that has just been released from the capsid into the nucleus (16). Alternatively, the release of the UL12.5 protein from the capsid could promote damage to host cell DNA in the same way that the vhs function of the virion degrades host cell RNA (10, 15). However, the observation that comparable yields of virus are generated in low-multiplicity infections with AN-F1 and AN-1 (12), which represents conditions that serve to compare the efficiency of reinfection in the presence or absence of capsid-associated UL12.5 protein, respectively, would seem to argue against a role for this polypeptide in the earliest stages of HSV-1 infection. The construction of a mutant virus which expresses AN but not the UL12.5 protein should help to clarify the individual functions these proteins carry out in HSV-1 infections; such studies are under way.

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