Transcription, Translation, and Cellular Localization of PDV-E66: A Structural Protein of the PDV Envelope of Autographa californica Nuclear Polyhedrosis Virus

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A late gene encoding a 66-kDa structural protein of Autographa californica nuclear polyhedrosis virus was mapped and sequenced (26.9–29.7 MU). Transcription initiates from two conserved TAAG motifs (−15 and −37) with transcripts detected from 12 to 72 hr pi. The protein is detected in infected Spodoptera frugiperda (Sf9) cells from 24 to 72 hr pi. Western blot and immunoelectron microscopic data identify this protein as specific for the polyhedra-derived virus (PDV) envelope. This protein has been named PDV-E66 to identify its viral origin, envelope location, and apparent molecular weight. In addition to being a structural protein of the PDV envelope, PDV-E66 is enriched in foci of microvesicles in the nuclei of infected Sf9 cells. © 1994 Academic Press, Inc.

INTRODUCTION

Autographa californica nuclear polyhedrosis virus (AcMNPV), the prototype of occluded baculoviruses, is an enveloped, double-stranded DNA virus (130 kb) which is distinguished by a unique biphasic life cycle in its lepidopteran host insect (Blissard and Rohrmann, 1990). Infection produces high titers of two forms of progeny virus, extracellular virus (ECV) and polyhedra-derived virus (PDV), and both forms are essential for natural propagation. Infection initiates when the insect feeds on a food source contaminated by viral occlusions containing PDV. The alkaline midgut fluids dissolve the protein occlusions, releasing PDV which infects the midgut epithelium. Early in infection nucleocapsids assemble in the nucleus, migrate to the cell surface, and obtain an envelope by budding through the plasma membrane into the insect hemolymph. This form of virus is named ECV and is responsible for secondary or systemic infection of a variety of tissues in the insect (Summers, 1971; Granados and Williams, 1986; Keddie et al., 1989). Previous studies (Granados and Williams, 1986; Keddie et al., 1989; Volkman and Keddie, 1990) indicate that ECV infects other tissues in a temporal sequence and the hemocytos may be a primary source for spreading infection within the insect, while Engelhard et al. (1994) give strong evidence that the host insect’s tracheal system may be an important conduit for virus dissemination and secondary infection of susceptible tissues. Late in infection the production of ECV decreases. Instead of budding from the cell surface, the nucleocapsids remain within the nucleoplasm where they are enveloped and occluded within the growing polyhedron crystal. This form of virus is named PDV.

PDV and ECV contain the same genome, yet exhibit significant differences in morphology, timing and cellular site of maturation, structural proteins, source of viral envelopes, antigenicity, and infectivity (Braunagel and Summers, 1994; reviews: Blissard and Rohrmann, 1990; Rohrmann, 1992). The differences between the envelopes of PDV and ECV may function to regulate important aspects of the role of these two viral forms in infectivity, host range, and tissue specificity.

The maturation of PDV and ECV occurs at different cellular membranes. Viral proteins of the ECV envelope are transported to the cell surface and incorporated into the viral envelope as the nucleocapsid buds through the plasma membrane (Kawamoto et al., 1977a,b; Volkman and Goldsmith, 1985; Fraser, 1986; Granados and Williams, 1986; Adams and McClintock, 1991). This strategy of ECV envelope maturation at the cell surface is similar to that observed for several RNA viruses (Wiley and Skehel, 1990) and poxviruses (Moss, 1990). However, the strategy utilized by PDV to obtain its envelope is unique. Not only does PDV become enveloped within the nucleoplasm, but it also remains in that location throughout infection. During AcMNPV infection, foci of unit membrane-like fragments and vesicle-like structures appear within the nucleus; these membranes are not observed in infected cells. Because of the small size and discrete vesicle-like appearance, these structures are called "microvesicles." These nuclear microvesi-
icles have been implicated to play a role in PDV maturation. Fraser (1986) observed that the capped ends of nucleocapsids associate with the microvesicles during PDV maturation, and other investigators have observed that nucleocapsids appear to “bud through” either microvesicles or unit-membrane-like structures in the process of envelopment (Stoltz et al., 1973; Tanada and Hess, 1976; Kawamoto et al., 1977a,b; Adams and McClintock, 1991).

The origin of the nuclear microvesicles, unit-membrane structures, and the PDV envelope is unknown. De novo membrane morphogenesis has been proposed to occur in the nucleoplasm of infected cells. This hypothesis is based upon the lack of apparent continuity of the membrane structures with the inner nuclear membrane and the differences in morphology between these two intranuclear membrane structures (Hughes, 1972; Stoltz et al., 1973; Maokinnon et al., 1974). However, other investigators have observed that upon viral infection, the inner nuclear membrane invaginates into the nucleoplasm (Summers and Arnott, 1986; Tanada and Hess, 1976). This observation raises the possibility that the inner nuclear membrane serves as a source for the microvesicles and/or the PDV envelope.

Acquisition of viral envelope at the inner nuclear membrane has been observed for herpes simplex viruses and subgroup B plant rhabdoviruses. Infection by herpes simplex viruses induces an inward invagination of the inner nuclear membrane and budding of nucleocapsids into the perinuclear space occurs in these regions (Roizman and Sears, 1990). The subgroup B plant rhabdoviruses, after obtaining their envelopes at the inner nuclear membrane, accumulate in the perinuclear space (Jackson et al., 1987). Maturation of both of these viruses results in enveloped nucleocapsids transported to the exterior of the cell and is clearly different from PDV maturation within the nucleus.

Since AcMNPV produces two functionally different progeny viruses which correlate with unique features of viral envelope maturation, it provides a novel system to comparatively study the maturation of viral envelope in both the nucleoplasm and cell surface. It is our goal to understand the molecular process of PDV envelope maturation. Proteins specific to the PDV envelope have been identified (Braunagel and Summers, 1994). In this study, we describe a late gene which encodes a PDV envelope-specific protein of 66 kDa. In infected cells, PDV-E66 not only localizes to the envelopes of newly assembled and occluded PDV, but also to the nuclear foci of microvesicles. Previous electron microscopic observations suggest that the nuclear microvesicles play a role in PDV envelopment. The identification of a PDV envelope-specific protein which also localizes to the microvesicles provides the first direct evidence that the microvesicles function as an intermediate or source of the PDV envelope.

MATERIALS AND METHODS

Cells and virus

AcMNPV (E2 strain) was used to infect Spodoptera frugiperda (Sf9) cells at a multiplicity of infection (m.o.i.) of 10 as described by Summers and Smith (1987). Time zero was defined as the time when cells were inoculated with the virus. The virus inoculum was removed after 1 hr absorption.

Virus purification, PDV-E66 band isolation, and N-terminal amino acid sequencing

Viral occlusions, PDV, ECV, viral envelope, and nucleocapsid fractions were prepared as described by Braunagel and Summers (1994). Purified PDV and ECV envelopes were denatured in disruption buffer [2% SDS, 1% β-mercaptoethanol, 25 mM Tris, 7% glycerol, and 0.1% bromophenol blue, pH 6.8] and proteins were separated on a 3% stacking/12.5% separating gel that was prerun for 30 min with 200 mM thiglycolic acid added to the upper buffer. The separated proteins were electrophoretically transferred to PVDF membrane (Pro-Blott; Applied Biosystems, Foster City, CA). Following transfer, the membrane was stained [45% methanol, 5% acetic acid, 0.1% Coomassie blue R250], destained [45% methanol, 5% acetic acid], and washed with water. A 60-kDa band unique to the PDV envelope was excised and the N-terminal amino acid sequence was determined to be QNNIQELQNF (Biotechnology Instrumentation Facility, University of California, Riverside, CA). The first six amino acids were used to synthesize a degenerate oligonucleotide (5' -CA'G'AC'G'GAT' C'AT'G'G'G'GA -3'). A PstI digestion of the AcMNPV genomic DNA was used in a Southern analysis to map the location of the gene.

Southern analysis

After agarose gel electrophoresis, DNA restriction fragments were transferred to Zeta-Probe GT membrane (Bio-rad, Richmond, CA) and hybridized with 32P end-labeled probes as recommended by the manufacturer.

Plasmid constructs and DNA sequencing

PstI-F (23.5–30.1 MU), EcoRI–KpnI (25.0–28.1 MU), KpnI–EcoRI (28.2–29.2 MU), and EcoRI–PstI (29.2–30.1 MU) genomic fragments were cloned into pUC18 (Stratagene, La Jolla, CA). Double-stranded DNA sequencing was done according to Sequenase 2.0 protocols (U.S.B., Cleveland, OH) using [α-32P]dATP (All radiochemicals are from DuPont NEN Research Products, Boston, MA). Oligonucleotides were synthesized on a Model 391 PCR-MATE DNA synthesizer (Applied Biosystems Inc.). DNA was sequenced on both strands and each nucleotide was sequenced an average of six times. The nucleotide and predicted amino acid sequences were analyzed using the program of genetics computer group (Devereux
et al., 1984; UWCG, version 7.3; Madison, WI). DNA databases, GenBank (release 81.0) and EMBL (release 37.0), and a protein database, Swisprot (release 27.0), were searched for genes and proteins demonstrating sequence homology with PDV-E66.

RNA isolation

Cell infection was done in 500-ml spinner flasks (m.o.i. = 10) and aliquots of cells were harvested at different time points postinfection. Total cellular RNA was isolated as described by Chirgwin et al. (1979). Poly(A) RNA was selected from total RNA, using Bioteck mRNA isolation protocols (Bioteck Laboratories, Inc., Houston, TX).

Northern analysis

Twenty micrograms of total RNA was separated on a 1.2% agarose–formaldehyde gel (Sambrook et al., 1989) and transferred to Zeta-Probe GT membrane using manufacturer’s protocols. The Drai–Drai (399 bp) and Drai–KpnI (463 bp) probes were 5’ end-labeled with T4 poly-nucleotide kinase (U.S.B.) and [γ-32P]ATP.

Primer extension analysis

Transcription initiation was mapped using primer extension techniques (Sambrook et al., 1989). The oligonucleotide 5’-CGGCAAGGATGACATAAATCAGC-3’ (nt 1341–1367 in Fig. 2) was 5’ end-labeled with T4 poly-nucleotide kinase and probed against 25 μg of total cellular RNA. Extension products were analyzed by denaturing gel electrophoresis (7.0 M Urea, 7% polyacrylamide, 100 mM Tris–borate, 20 mM EDTA, pH 8.3). The primer extension oligonucleotide was modified for sequencing as described below: 1.5 μg oligonucleotide was 5’ end-phosphorylated at 37°C for 60 min in a 20-μl reaction [50 mM Tris–HCl (pH 7.6), 10 mM MgCl2, 10 mM 2-mercapto-ethanol, 8 units T4 polymerase, and 5 μM ATP], precipitated with ethanol, and dissolved in 10 μl ddH2O; one microliter was used in each sequencing reaction. The sequencing ladder was obtained with the PstI-F plasmid DNA using standard Sequenase 2.0 protocols.

S1 nuclease analysis

Both 5’ and 3’ S1 nuclease analyses were conducted as described by Favolaro et al. (1980). The 5’ S1 probe was prepared by digesting the EcoRI–KpnI clone (25.0–28.1 MU) with KpnI, dephosphorylating with calf intestinal phosphatase (Boehringer-Mannheim, Corp., Indianapolis, IN), digesting with Drai, purifying the DNA through low-melting agarose, and 5’ end-labelling the fragment with T4 polymerase kinase. Fifty-thousand cpm of 5’ S1 probe was hybridized to 25 μg total RNA in a 30-μl reaction [80% formaldehyde, 40 mM PIPES, 0.4 M NaCl, and 1 mM EDTA, pH 6.4] at 42°C for 16 hr before S1 nuclease digestion (BRL, Gaithersburg, MD). The 3’ S1 probe was prepared by digesting the EcoRI–PstI clone (29.2–30.1 MU) with EcoRI and PstI, purifying the EcoRI–PstI fragment through low-melting agarose, and 3’ end-labelling the fragment at EcoRI site with Klenow (U.S.B.) and [α-32P]dATP (Sambrook et al., 1989). Ten-thousand cpm of 3’ S1 probe was hybridized to 30 μg total RNA in a 30-μl reaction [80% formaldehyde, 40 mM PIPES, 0.4 M NaCl, and 1 mM EDTA, pH 6.4] at 47°C for 16 hr before S1 nuclease digestion. Protected fragments were analyzed on a 5% polyacrylamide–urea gel.

SDS–PAGE and Western-blot analysis

Vertical slab SDS–PAGE was performed according to Laemmli (1970). A 3% stacking gel was used above a 10% separating gel. Samples were denatured in disruption buffer for 15 min at 65°C. Following electrophoresis, gels were stained with Coomassie blue, dried and exposed to X-ray film, or transferred to PVDF membrane using the wet transfer method described by Sambrook et al. (1989). For Western-blot analysis, the blot was treated with 1× BLOTTO (1% nonfat dry milk, 50 mM Tris–HCl, 150 mM NaCl, and 0.05% (v/v) Tween 20, pH 7.4) for 1 hr and then reacted with primary antibody for 1 hr. The blot was washed (3× 5 min) with TTBS [50 mM Tris–HCl, 150 mM NaCl, and 0.05% (v/v) Tween 20, pH 7.4], reacted with anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO) for 1 hr, washed with TBS, and color was developed.

In vitro transcription and translation

The region from Drai to PstI (27.7–30.1 MU, Fig. 1) was cloned into the HindIII and PstI sites of the pBS minus vector (Stratagene) to give rise to pBS-E66. The 3.3-kb cloned fragment contained 100 bp 5’ noncoding sequence of the PDV-E66 open reading frame (ORF). Five micrograms of pBS-E66 plasmid was linearized with HindIII and transcribed using T7 RNA polymerase (U.S.B.). The RNA transcript was purified through low-melting agarose and translated in rabbit reticulocyte lysate (Promega, Madison, WI) containing [35S]methionine. The translation mixture was separated by 10% SDS–PAGE and proteins visualized by exposure to X-ray film.

Heterologous protein expression and antibody production

The region from amino acid 24 to 705 of the PDV-E66 ORF in the plasmid clone was amplified by polymerase chain reaction (PCR) using two primers (forward: 5’-AAA-AAAAAGCTTGTAAACTATAAAAATGATGCC-3’ and reverse: 5’-AAAAAGGAGTTCAACAATTTTTAACAATTICACA-3’). Taq polymerase (Promega). The DNA was amplified using 25 cycles of 1 min at 94°C, 1 min at 37°C, and 3 min at 72°C with 5 sec extension after each cycle and the resultant PCR fragment was extracted with phenol/chloroform. The precipitated DNA was digested with HindIII and BamHI, sep-
arated on a 1% agarose gel, purified (GeneClean II, BIOL101, Inc., La Jolla, CA), and ligated into the HindIII and BamHI sites of pUC18 to give rise to PCR-pUC18. The FLAG epitope (IBI FLAG Biosystem, International Biotechnologies, Inc., New Haven, CT) was cloned in frame at the N-terminus of the amplified region (amino acids 24–708) to allow affinity purification of the fusion protein. A linker containing HindIII sites on both ends (underlined below) was cloned into the HindIII site of the PCR-pUC18 clone. The forward sequence of the linker is shown below: 5’-AAGCTTCAATGACTACAAAGCACGACGCAAGCCT-3’. Immediately following the HindIII site on the left, there is an NdeI site (italic) containing an ATG (bold) in frame with the PDV-E66 ORF. DNA sequencing confirmed that the final construct encodes the following N-terminal amino acid sequence: MDYDKDDDKLKN (amino acid 24 of PDV-E66 ORF is underlined). The N-terminal eight amino acids (italic) encode the FLAG epitope. Finally, the FLAG-E66 ORF was cloned into the NdeI and BamHI sites of PET-11a (Novagen, Inc., Madison, WI 53711) and expressed. The recombinant protein was found in the inclusion fraction of Escherichia coli.

Three New Zealand White female rabbits were injected intramuscularly with approximately 600 µg of the inclusion fraction in Freund's complete adjuvant (Pierce, Rockford, IL). The rabbits were then given two additional injections (Freund's incomplete) at monthly intervals. One week after the final injection, sera were collected by exsanguination of the rabbits. To eliminate the background reaction, the polyclonal antiserum was preadsorbed with uninfected Sf9 cells. Uninfected Sf9 cells were pelleted, resuspended in 1× BLOTTO, and sonicated until all cells were lysed. PDV-E66 antiserum or preimmune serum was added (1:500) and allowed to bind overnight at 4°C. The antibody was then clarified by centrifugation for 20 min at 15,000 rpm in a JA-21 rotor (Beckman, Palo Alto, CA). Preadsorbed polyclonal antiserum or preimmune serum was used throughout this study.

**Immunelectron microscopy (IEM)**

Infected cells and freshly purified virus were pelleted and sequentially fixed (1% paraformaldehyde, 0.5% glutaraldehyde, 0.05 M sodium cacodylate, pH 7.1, 10 min, 4°C; 2% paraformaldehyde, 2.5% glutaraldehyde, 0.05 M sodium cacodylate, pH 7.1, 30 min, 4°C). The fixed pellets were washed (0.05 M sodium cacodylate, pH 7.1; 3× 10 min, 4°C) and postfixed (1% osmium tetroxide, 0.05 M sodium cacodylate, pH 7.1; 30 min, room temperature). The pellets were washed again and dehydrated at −20°C with the following graded ethanol series: 30%, 50%, 70%, 90%, 100% (30 min/step; 30% ethanol dehydration was at 4°C). Samples were infiltrated with 50, 75, and 100% LR White/ethanol series −20°C, 1 hr per step (Electron Microscopy Science, Fort Washington, PA). Samples were then infiltrated with 100% LR White at −20°C overnight, followed by 8 hr infiltration. The resin was then polymerized in gelatin capsules heated to 50°C for approximately 44 hr.

Ultrathin sections were placed on nickel grids (Electron Microscopy Science), etched (0.56 M sodium periocarbide, 1 hr), washed (H2O, 2 min), incubated in 0.1 N HCl (10 min), and washed (H2O, 4× 2 min). The sections were blocked with TTBS–BSA (1% BSA, 50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 15 min, reacted with primary antibody (1:500; 16 hr at 4°C), and washed (TTBS: 4× 2 min). The bound primary antibody was detected using anti-rabbit IgG gold-conjugated antibody (16 nm; Amersham, Arlington Heights, IL). The sections were stained with uranyl acetate (Bozzola and Russell, 1992) and lead citrate (Venable and Coggeshall, 1965) and were visualized with a Zeiss 10 C transmission electron microscope.

**RESULTS**

**Identification, sequence, and analysis of the gene encoding PDV-E66**

To identify proteins unique to the PDV envelope, PDV and ECV were purified and fractionated into envelope and nucleocapsid fractions. A comparison of the structural proteins of PDV, ECV, and envelopes revealed a protein of 60 kDa unique to the PDV envelope (Braunagel and Summers, 1994). N-terminal amino acid sequencing of this protein predicted a sequence of QNNIQELQNF. Using a degenerate oligonucleotide predicted from the first six amino acids, the gene was mapped to the EcoRI–PstI region (6.6 kb; 25.0–30.1 MU; Fig. 1) of the AcMNPV genome.

The DNA sequence of the EcoRI–PstI region revealed five putative open reading frames (Figs. 1 and 2): ORF1, nt 679 to 1257; ORF2, nt 486 to 631; ORF3, nt 282 to 777;
Fig. 2. Nucleotide sequence and predicted open reading frames (ORFs) of the 26.9–23.7 MU region of AcMNPV genome. Consensus transcription initiation motifs and potential polyadenylation motif AATAAA for ORFs 1-4 (in 1314 and 1329) are underlined. Transcription from the PDV-E66 ORF initiates from two TAAG motifs (forward arrows). The N-terminus of the 60-kDa protein determined by amino acid sequencing begins at amino acid 70 (solid box). The putative nuclear targeting signal is indicated by the dotted box. Putative polyadenylation sites are shown (asterisk).
ORF4, nt 68 to 301; and the PDV-E66 ORF, nt 1242 to 3356. The N-terminus of the 60-kDa protein was 69 amino acids interior to the N-terminus predicted by the PDV-E66 ORF (Fig. 2). A reevaluation of the PDV envelope preparations revealed that multiple PDV envelope preparations routinely contained a 66-kDa protein, a 60-kDa
The gene encoding PDV-E66 is transcribed as a late 2.5-kb mRNA

Northern analysis of total-infected cell RNA and the 463-bp DraI–KpnI probe revealed four major RNA species with sizes of 1.5, 1.8, 2.5, and 3.2 kb, respectively (Fig. 3A). Some minor RNA species larger than 3.2 kb were also observed. These results were confirmed using poly(A) selected RNA (data not shown). Because of the complex patterns of the observed transcripts, a probe (399-bp DraI–Dral, Fig. 3C) was designed that would hybridize to all transcripts but PDV-E66. Using this probe, all transcripts except the 2.5-kb transcript were detected (Fig. 3B). Since the 463-bp DraI–KpnI probe was labeled on KpnI site (Fig. 3C), it should only detect mRNA transcribed in the genomic sense. Furthermore, Southern analysis of the PstI-digested AcMNPV genome with the DraI–Dral probe detected hybridization only to the PstI-F fragment (data not shown), indicating that all transcripts detected by the Northern analysis are from the PstI-F region. Taken together, these data indicate that the early 1.5- and late 1.8-kb transcripts correspond to transcripts which initiate from the upstream ORFs (Figs. 1 and 3C), while the 2.5-kb transcript is specific for PDV-E66. In addition, the 3.2-kb transcript is specific for PDV-E66.

PDV-E66 primer extension using total infected cell RNA and an oligonucleotide complementary to the PDV-E66 coding strand revealed five extension products which corresponded to transcripts initiating from two late transcription initiation motifs ATAAAG (−16) and TTAAG (−38) (Fig. 4). The transcripts were detected from 12 to 72 hr pi with maximal steady-state transcript levels between 18 and 24 hr pi. Those data correlate with temporal expression of the PDV-E66 2.5-kb transcript as determined by the Northern analysis (Fig. 3).

To confirm the results obtained by the primer extension analysis, 5' S1 nuclease mapping was performed. This experiment revealed two protected fragments of ap-
approximately 405 and 382 nt (Fig. 5A). These transcripts correspond to expected transcripts initiating proximal to TTAAG (−38) and ATAAG (−16) (Figs. 2 and 4). Three prime S1 mapping detected four protected fragments of approximately 216, 220, 228, and 233 nt (Fig. 5B). These correspond to the consensus polyadenylation sites (AAATAAA) downstream of PDV-E66 at nt 3437 and 3449 (Fig. 2).

Taken together, the data from the Northern, primer extension, and S1 nuclease analyses indicate that the size of the PDV-E66 transcripts is approximately 2240 nt and that there is approximately 260 nucleotides of polyadenylation tail. Data from all transcript mapping experiments indicate that PDV-E66 transcription occurs between 12 and 72 hr pi with maximal steady-state levels between 18 and 24 hr pi. Therefore, this gene is classified as a baculovirus late gene.

To detect whether the PDV-E66-noncoding DNA strand encodes any transcript, an oligonucleotide (5′-GATTCTAAATTTAAACCTTCC-3′; nt 732–752; Fig. 2) specific for the noncoding strand was tested in primer extension experiments. No transcript from the opposite strand was detected from 0 to 72 hr pi (data not shown).

**PDV-E66 protein analyses**

To confirm that PDV-E66 is a structural component of the PDV envelope, Western-blot analyses of purified virus, envelope, and nucleocapsid were conducted using the PDV-E66 monospecific antiserum obtained after preadsorption of the serum with uninfected SF9 cells. To prevent contamination of ECV by nonocluded PDV, which was released into the media by disintegrated cells, ECV was purified from culture media at 48 hr pi. PDV was purified from viral occlusions at 5 days pi. Two proteins at 66 and 60 kDa were detected in PDV and PDV envelope, but not in PDV nucleocapsid or ECV (Fig. 6A). The antiserum detected only one 66-kDa protein in AcMNPV-infected SF9 cells from 24 to 72 hr pi (Fig. 6B).

*In vitro* translation using rabbit reticulocyte lysate produced a protein of 66 kDa (Fig. 6C). This size is smaller than predicted from the DNA sequence (79 kDa), but is consistent with the size detected in the PDV envelope and infected cells (Fig. 6). Since the lysate used is incapable of N-glycosylation and protein cleavage, the apparent size difference resolved by SDS–PAGE is unlikely to be due to these modifications, but may be due to anomalous migration of protein (Pelham and Jackson, 1976; Walter and Blobel, 1983). Membrane proteins commonly adsorb more SDS and the increased binding of SDS could explain the anomalously low molecular weight of PDV-E66 (Hames and Rickwood, 1981).

**Immunoelectron microscopy (IEM)**

PDV was purified and immediately prepared for IEM to optimize the percentage of PDV virions which are intact. PDV-E66 localized to the PDV envelope (Figs. 7b–f) and to the PDV envelopes released during purification (Fig. 7b). It was not unusual to observe the label to be more highly concentrated at one end of the virus (Figs. 7d–f). No label was associated with PDV nucleocapsids. The control using preimmune serum displayed no binding to purified PDV (Fig. 7a) and label was not observed on purified ECV (data not shown).

Results from IEM of infected SF9 cells at 48 hr pi were consistent with those obtained with purified virus. Envelopes of newly assembled PDV (Figs. 8b–d), PDV in the process of being occluded (Fig. 8c), and occluded PDV (Fig. 8c) were labeled. In addition, the baculovirus-induced nuclear microvesicles were densely labeled (Figs. 8b and 8d). At an early stage of infection (i.e., 24 hr pi; data not shown), the microvesicles were typically located between the virogenic stroma and the nuclear envelope. At a more advanced stage of infection, the microvesicles were in juxtaposition to the nucleocapsids acquiring envelopes, fibrillar structures, and viral occlusions (Fig. 8b).
The control preimmune serum did not cross-react with the microvesicles (Fig. 8a). No labeling was observed in the virologic stroma and cytoplasm (data not shown). Nucleocapsids in both the nucleus (Fig. 8d) and cytoplasm (data not shown) were not labeled. ECV was also not labeled (data not shown). These experiments were repeated for cells at 24 hr pi and the results (data not shown) were consistent with the data described above.

**DISCUSSION**

This study describes a baculovirus late gene and the temporal patterns of its transcription, translation, and cellular localization. In multiple preparations of purified PDV and PDV envelopes, three patterns of PDV-E66 were observed: a 66-kDa protein, a 60-kDa protein, or both. N-terminal amino acid sequencing of both proteins revealed that the 66-kDa protein contains the N-terminus predicted by the PDV-E66 ORF, whereas the 60-kDa protein initiated 69 amino acids interior to the predicted N-terminus. The variable amount of the 60-kDa protein in different virus preparations coupled with detection of only the 66-kDa protein in infected cells suggested two possibilities: the 60-kDa protein may be a degradation product which occurs during purification, or it is the result of a specific cleavage. The 60-kDa protein was cleaved at a trypsin consensus site after a single arginine at amino acid 69 (Fig. 2). This cleavage site resembles the site utilized by rotavirus when VP4 is cleaved into VP8 and VP6 (Espejo et al., 1981; Estes, 1990). The activation of viral envelope protein precursors by trypsin cleavage after a single arginine has been shown for the F, protein of Sendai virus (Scheid and Choppin, 1974; Tashiro et al., 1993) and the HA, protein of influenza virus (Klenk et al., 1974; Huang et al., 1980; White et al., 1981). Experiments are under way to address which form of the PDV-E66 protein is active and/or if the apparent cleavage event has functional significance. A similar processing event was observed with another PDV envelope-specific protein, p25 (Russell and Rohrmann, 1993). The p25 protein is present as a doublet of approximately 25 kDa in Western-blot analyses of both OpMNPV-infected L. dispar and S. frugiperda cells. No evidence of either N- or O-linked glycosylation was found, suggesting that another form of processing may be responsible for the two observed sizes of the p25 protein.

To date, several structural proteins have been identified which are specific for PDV. Roberts (1989) identified a fatty acylated protein of 26.4 kDa (p26.4) which was expressed late in infection and was associated with AcMNPV PDV. P26.4 was solubilized by extraction with nonionic detergents, suggesting that it was associated
with the PDV envelope. The p74 protein is known to play a role in PDV infectivity (Kuzio et al., 1989) and is believed to be a PDV structural protein; however, its location within the virus has not been determined. A PDV structural O-linked glycoprotein, gp41, is predicted to reside between the envelope and nucleocapsid (Whitford and Faulkner, 1992a,b). Two PDV envelope proteins have been identified in other baculoviruses: vp17, an envelope protein of *Plodia interpunctella* granulosis virus (Funk and Consigli, 1993); p25, which was shown to be PDV envelope specific in OpMNPV-infected cells (Russell and Rohrmann, 1993). P25 was found to be enriched surrounding the virogenic stroma as well as in the envelopes of PDV in the process of being occluded and fully occluded. PDV-E66 is the only protein identified thus far which is specific for the PDV envelope and is also enriched in the nuclear microvesicles.

The source of the nuclear microvesicles induced by viral infection (Fraser, 1986) is unknown. Stoltz et al. (1973) and MacKinnon et al. (1974) observed unit mem-
brane-like structures within the nucleoplasm of infected cells; however, they were unable to determine any association of these membrane structures with the inner nuclear membrane. As a result, de novo membrane morphogenesis was postulated as the mechanism to produce the membrane structures within the nucleoplasm. However, baculovirus infection does induce an inward invagination of the inner nuclear membrane (Summers and Arnott, 1969; Tanada and Hess, 1976). These observations raise the possibility that the inner nuclear membrane could serve as the source of the nuclear microvesicles by pinching of small membrane vesicles into the nucleoplasm. Consistent with this possibility is our observation that the intranuclear location of the microves-
icles is altered as infection progresses. At 24 hr pi the microvesicles are frequently observed in close proximity to the inner nuclear membrane and/or viregenic stroma, while at 48 hr pi, they are concentrated at a more interior location where they are often observed to be adjacent to the nucleocapsids, fibrillar structures, noncocluded PDV, and viral occlusions (Fig. 8b).

A number of studies indicate that the nuclear unit membrane structures, or microvesicles, play a role in PDV envelopment. PDV nucleocapsids have been observed to bud through these membrane structures (Stoltz et al., 1973; Maekinin et al., 1974; Tanada and Hess, 1976; Kawamoto et al., 1977a,b; Adams and MacClintock, 1991). Fraser (1986) observed that the capped ends of nucleocapsids associated with the microvesicles. The identification of a PDV envelope-specific protein, which selectively localizes to the microvesicles, provides the first direct evidence that the nuclear microvesicles function as an intermediate in PDV envelope assembly.

Herpesviruses obtain their envelopes from the inner nuclear membrane which also invaginates inwardly during infection. Studies with herpes simplex virus and Epstein Barr virus show a pattern of viral envelope protein labeling over these areas of the inner nuclear membrane, indicating that viral envelope proteins are clustered in this region (Bibor-Hardy et al., 1982; Torrisi et al., 1989; Rolzman and Sears, 1990). If baculovirus is utilizing the inner nuclear membrane as the source of microvesicles, one may expect localization of PDV-E66 to the inner nuclear membrane, IEM using anti-PDV-E66 antisera did not reveal distinct labeling over the inner nuclear membrane significantly above the level of background. However, these data do not mean that PDV-E66 is not transiently associated with the inner nuclear membrane. It is also possible that it is transported into the nucleus through the nuclear pores to be incorporated into membranes. Analysis of the amino acid sequence of PDV-E66 reveals a putative nuclear targeting signal (amino acids 411–419, Fig. 2). Future experiments will address whether this signal is functional or not.

Baculovirus-induced membrane structures have not only been observed within the nucleoplasm, but also in the cytoplasm (Summers, 1971). The shrimp baculoviruses, Baculovirus panaei and Penaeus monodon, induce the development of an extensive membrane labyrinth which is derived from the outer nuclear and cytoplasmic membranes (Couch, 1991). It is not clear if the cytoplasmic membrane labyrinth plays a role in the nuclear envelopment of PDV; however, it is clear that baculoviruses have the ability to induce massive membrane morphogenesis in infected cells.

The identification of PDV envelope-specific proteins provides tools to study the biochemistry of viral envelopment in the nucleoplasm: a phenomenon which is not observed in uninfected insect cells or other viral systems. Knowledge of the types of protein processing, extent and types of glycosylation, potential acylation, or other post-translational events will provide information on the transport pathways leading to the nuclear localization of PDV envelope proteins. PDV-E66 and other PDV envelope proteins which are being identified in this laboratory will be used to determine the temporal course and location of protein incorporation into the membrane structures. In addition, the identification of proteins localizing to the microvesicles may allow these structures to be isolated and their lipid and protein compositions to be compared with those of the PDV envelope (Braunagel and Summers, 1994). These studies should begin to reveal the source of the nuclear microvesicles, the mechanism of the incorporation of PDV envelope proteins into membranes, and ultimately the mechanism of the nuclear envelopment of PDV.

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