Autographa californica Nuclear Polyhedrosis Virus: Subcellular Localization and Protein Trafficking of BV/ODV-E26 to Intranuclear Membranes and Viral Envelopes

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INTRODUCTION

Baculoviruses infect larvae of susceptible insects and produce two functionally distinct virus progeny during the infection cycle: budded virus (BV) and occlusion-derived virus (ODV). BV acquires a viral envelope early in the infection process by budding through the plasma membrane, whereas ODV matures later in infection by obtaining a viral envelope from an intranuclear source of membrane microvesicles. The protein composition of BV and ODV envelopes are different (Smith and Summers, 1978, 1981; Braunagel and Summers, 1994), and while several ODV envelope-specific proteins are now identified, gp64 is the only protein known to be specific to the BV envelope (Volkman and Goldsmith, 1985; Whitford et al., 1989). The maturation of BV and ODV is temporally regulated. Optimal BV maturation occurs prior to 24 h postinfection (p.i.) and then decreases. Concomitant with the decrease of BV maturation, ODV maturation in the nucleus is increased and continues throughout infection. The factors that regulate the maturation of BV versus that of ODV are currently unknown.

Expression of the da26 gene has been shown to stimulate late gene transcription in cooperation with da41 (Guarino and Summers, 1988). Fusion constructs with β-galactosidase did not adversely affect infectivity indicating that this protein is not essential for infectivity (O’Reilly et al., 1990). When the da26 locus was disrupted, a few polyhedra phenotype were produced (O’Reilly et al., 1990). Unlike most genes which are expressed early and late in infection, da26 transcription does not initiate from the well-defined baculovirus early and late promoters, but rather from a cryptic sequence (Guarino and Summers, 1988). Da26 RNA transcripts are detected as early as 3 h p.i. and accumulate during infection (O’Reilly et al., 1990).

In this study, we demonstrate that the product of the da26 gene is the viral envelope protein, BV/ODV-E26 (ORF16; Ayres et al., 1994). Early in infection BV/ODV-E26 (-E26) locates to cytoplasmic vesicles, and later in infection -E26 is incorporated into membranes within the nucleus. The fact that -E26 is a structural protein of BV suggests that early in infection the protein is incorporated into the plasma membrane of the host cell. The identification of a protein that exhibits temporally regulated transport to the cell surface, and into membranes within the nucleus, provides a unique tool to study intracellular transport and regulatory factors required for the maturation of BV and ODV. This study also shows that the viral protein FP25K and cellular actin may form a complex with -E26 and potentially participate in the regulation of transport of -E26.
MATERIALS AND METHODS

Recombinant virus, protein expression, and antibody production. The EcoRI A fragment of AcMNPV (E2 strain) containing da26 was digested with EcoRII, filled with Klenow, and subsequently digested with BamHI and cloned into the Smal, BglII sites of pUC 18 (pUC-da26). This fragment (~800 nt) contained 21 nucleotides of upstream nontranslated leader, the da26 gene, and da26 polyadenylation sites. The insert containing the da26 gene was then excised using EcoRI and PstI (provided by the pUC vector) and further subcloned into pVL1393 (Webb and Summers, 1990) and pBS(−). Final constructs were confirmed by DNA sequencing.

Recombinant virus was generated using pVL1393-da26 and Bsu36I-digested BacPak6 viral DNA (CLONTech, Palo Alto, CA; Summers and Smith, 1987). Recombinant virus lacked the polyhedrin protein (occ−) and showed a marked increase production of a 26-kDa protein. This recombinant virus was used for abundant expression studies and as the source of protein used as injection antigen for production of antisera. Polyhedrin-expressed 26-kDa protein was cut from SDS–PAGE gels, eluted, and used as antigen for preparation of antibody. Rabbits were injected every 28 days, for a total of four injections with antigen (RIBI; Hamilton, MA). To generate high affinity antisera, the second and third injections contained approximately one-half the amount of the first injection, and the final injection contained approximately one-fourth the initial amount of antigen. Ten days after the last injection, sera were collected and titer was evaluated.

SDS–PAGE and Western blot analysis. Spodoptera frugiperda cells (Sf9) were infected with the E2 strain of AcMNPV at a multiplicity of infection (m.o.i.) of 20, virus was absorbed for 1 h (time 0 is the time of addition of virus) and then unabsorbed virus and media were removed and replaced with TMNFH, 10% FBS. Cells were harvested at designated times post infection, pelleted, and the protein concentration of the cell pellet was determined by the method of Bradford (1976). Purified BV, ODV, envelope and nucleocapsid fractions were determined by the method of Bradford (1976). Purified BV, ODV, envelope and nucleocapsid fractions were prepared as described in Braunagel and Summers (1994). Vertical slab SDS–PAGE was performed according to Laemmli (1970). A 4% stacking gel was used above a 12.5% separating gel. Before loading, samples were incubated in 15% SDS, 0.5% β-mercaptoethanol, 25 mM Tris±HCl (pH 6.8) and 7% glycerol for 15 min at 65°C.

Western blot analysis was performed using protein blotted onto PVDF membrane (Immobilon-P; Millipore, Bedford, MA). The membranes were blocked with TTBS±BLotto (150 mM NaCl, 10 mM Tris, 1% nonfat dry milk, 0.002% sodium azide, 0.1% Tween 20, pH 8.0). Antibody was bound overnight (4°C; No. 7554, 1:1000), blots were washed twice with TTBS±BLotto, and horseradish peroxidase-linked anti-rabbit IgG (1:10,000) was bound for 1 h at room temperature. Blots were washed three times with TTBS, reacted for 1 min with Renaissance (NEN, Boston, MA) chemiluminescence reagent and exposed to X-ray film.

Testing for immunorelatedness of the different molecular weight protein species was performed by using large scale SDS–PAGE which clearly separated the three molecular weight species close to 26 kDa. Western blot was performed on these gels and the location of each of the proteins was identified by immunostaining a small strip of the membrane. The remaining unstained bands were bound to polyclonal sera to immunoselect antibodies that were specific for each molecular weight protein species and the blots were washed to remove unbound antibody. A SDS–PAGE gel (15 μg of 48 h p.i. infected cell lysate) and Western blot was completed and each lane was incubated overnight (4°C) with the strip containing the immunoselected antibody to one of the protein species around 26 kDa. In this manner, the immunoselected antibody was exchanged from one blot to the other without exposing the antibody to denaturing conditions. Antibody binding against the blot containing the 48-h p.i. sample was detected using the chemiluminescence techniques described above.

Immunoelectron microscopy (IEM). At the designated time postinfection, the cell samples (m.o.i. = 20) were pelleted, fixed, and suspended in LR White (Hong et al., 1994). Ultrathin sections were prepared, blocked with TBS±BSA (150 mM NaCl, 10 mM Tris, 0.1% Tween 20, 1% BSA, pH 8.0) for 15 min, reacted with primary antibody (1:1000, 12 h, 4°C) and washed with TBS (4 × 2 min). The bound primary antibody was detected using gold-conjugated anti-rabbit IgG (30 nM, Amersham, Arlington Heights, IL). The sections were stained with uranyl acetate (Bozolla and Russell, 1992) and lead citrate (Venable and Coggeshall, 1965). Cells were observed using a Zeiss 10C transmission electron microscope (Texas A&M University Electron Microscopy Center).

Immunofluorescence microscopy. Cells were processed for light microscopy using a modification of previously described procedures (Charlton and Volkman, 1991). A suspension of 5 × 10⁶ Sf9 cells was applied to a coverslip, allowed to adhere for 1 h, and infected at a m.o.i. of 20 (27°C). At the appropriate time, cells were rinsed with Grace’s media and fixed with 3.7% paraformaldehyde in PBS (pH 7.2, 20 mM phosphate, 140 mM NaCl) for 10 min at room temperature. Subsequent steps were performed at room temperature. Fixative was removed and coverslips were rinsed twice with PBS±BLotto (PBS containing 1% nonfat milk). Cells were permeabilized by treatment with methanol (10 min) with subsequent treatment of 0.5% Triton X-100 (10 min), followed by two rinses with PBS. Nonspecific reactivity was blocked by treatment with normal goat serum (1:20 in PBS, 30 min), and cells were incubated in primary antibody (No. 7554; 1:1000 in PBS±BLotto) for 30 min. Cells...
were rinsed twice with PBS±BLOTTO and secondary antibody [fluorescein isothiocyanate-conjugated anti-rabbit IgG (FITC); Sigma, 1:100 in PBS±BLOTTO] was added and incubated for 30 min. Cells were washed two times with PBS±BLOTTO and nucleus was visualized by staining the cells with DAPI (0.1 µg/ml in PBS) for 5 sec and rinsing three times with PBS. Cells were mounted for viewing using nonbleach mountant (10 mg phenylenediamine/ml in 90% glycerol). Cells were viewed and photographed with a Zeiss Axiovert 135 Photomicroscope.

Membrane fractionation and triton X-114 assay. Cellular membranes were prepared as described by Wang and Haunerland (1994). Briefly, this technique includes lysing the cells (20 mM Tris, 140 mM NaCl, 1 mM CaCl$_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol, pH 8.0) separating the cell debris (10 min at 30,000 g. 800 g to ethanol, pH 8.0) separating the cell debris (10 min at 30,000 g). The insoluble material was separated from the membrane pellet by centrifugation (100,000 g, 1 h). A 100-µg fraction of the membrane pellet (see above; from 24 h p.i. SF9 cells) was solubilized in 200 µL of Triton X-114 TN buffer (% Triton X-114, 10 mM Tris, 0.15 M NaCl, pH 8.0) on ice for 20 min with occasional agitation. The solubilized pellet was centrifuged at 13,000 g for 20 min at 4°C and the soluble supernatant fraction overlaid onto a 300 g cushion. Phase separation was repeated as above. The final aqueous phase was removed without disturbing the sucrose cushion, mixed with Triton X-114 (1%), incubated on ice for 10 min, and overlaid onto the original sucrose cushion. Phase separation was repeated as above. The final aqueous phase was removed and extracted again by adding Triton X-114 (2%). The phases were separated as described above and the aqueous phase was collected. The detergent droplet at the bottom of the sucrose cushion was recovered as the final detergent phase. Both aqueous and detergent phases were acetone precipitated and analyzed using SDS±PAGE and Western blotting techniques.

Immunoprecipitation. SF9 cells were labeled between 18 and 24 h p.i. with [35S]translabel (ICN) using 100 µCi per 3 × 10^6 cells in 1 ml of methionine-free Grace's media. Cells were harvested at 24 h p.i. and lysed on ice for 30 min in RIFT buffer (3 × 10^6 cells/ml RIFT; 50 mM Tris, 100 mM NaCl, 0.2% Tween-20, and 10 µg/ml aprotinin, pH 8.0). Cells were disrupted by passage through a 25-gauge needle and debris and unbroken cells were removed by centrifugation (13,000 g for 10 min, 4°C). Cell extracts were mixed with antisera (1:1000) and incubated overnight at 4°C with gentle agitation. Protein-A agarose beads (Sigma, 20 µL of 50% slurry) were added, incubated for 1 h at 4°C and immunoprecipitates were collected by centrifugation (1000 g for 3 min, 4°C). Beads were washed two times with RIFT buffer and once with TBS. A sample of the beads were incubated at 65°C for 15 min in 20 µl of SDS±PAGE sample buffer and the equivalent of 3 × 10^6 cells/lane were analyzed by Western blotting. 35S-labeled proteins were visualized by autoradiography.

Yeath two-hybrid assay. Using oligonucleotides and PCR, an EcoRI site was incorporated immediately upstream of the da26 gene and a BamHI site was incorporated immediately after the stop codon. These restriction sites were used to clone da26 into the yeast vectors pACT2 and pAS2-1 in frame with gal4. Restriction sites were also incorporated upstream and downstream of the FP25K gene using oligonucleotides and PCR. Immediately upstream of the FP25K initiation codon, both EcoRI and NcoI restriction sites were added, while a BamHI site was incorporated immediately downstream of the stop codon. The gene was cloned into pUC 18 using the EcoRI and BamHI restriction sites. The FP25K clone was then transferred to the yeast plasmid, pAS2-1 using the NcoI and BamHI restriction sites (pAS2-1/FP25K). Using the pAS2-1/FP25K plasmid as source, the FP25K gene was transferred to the yeast plasmid pACT2 using NdeI (provided by pAS2-1) and BamHI sites. Yeast expression constructs were confirmed by DNA sequencing. Transfection and chromogenic reactions were performed according to manufacturer's protocol (CLONTECH, Palo Alto, CA).

RESULTS

BV/ODV-E26 (-E26) is a structural protein of BV and ODV. To determine the time course expression of the da26 gene product, cell extracts were collected at various times postinfection and analyzed by Western blotting techniques using polyclonal antibodies generated to -E26. The blot was overexposed in order to visualize the low amounts of protein associated with the cell lysate early in infection. Through 24 h p.i. one predominant immunoreactive band was detected at 26 kDa. This protein was detected as early as 4 h p.i. (Fig. 1, lane 5), accumulated to higher levels by 16 h p.i. and remained at high levels throughout infection (Fig. 1, lanes 5±13). It should also be noted that these experiments were performed using cell lysates, thus protein that was produced, incorporated into budded virus and released into the media, would not be detected. By 36 h p.i. several lower and higher molecular weight immunoreactive proteins were also detected (Fig. 1, lanes 10±13). To determine if the broad banding pattern of both the 26- and 18-kDa proteins represented multiple proteins, the blot was exposed for less time. The decreased exposure showed that in both molecular weight regions (26 and 18 kDa), the broad diffuse banding pattern was the result of multiple protein species (Fig. 1, lane 14, arrows). In vitro
translation results predicted one major protein product of 26 kDa; however, minor lower molecular weight products were also detected (Fig. 1, lane 21).

The banding pattern of the immunoreactive proteins species at time points later than 24 h.p.i. was complex. To address the nature of this banding pattern, a large format SDS–PAGE, Western blot experiment was performed which clearly separated the three protein bands clustered at 26 kDa. Each of the protein species was cut from the blot and used to immunoselect antibodies specific for that protein species (1 to 3 represents highest to lowest molecular weight, respectively). A second SDS–PAGE and Western blot was then prepared using 48-h.p.i. cell extract samples and reacted with each immunoselected antibody. As shown in Fig. 1B (lanes 1±3), each immunoselected antibody not only recognized the 26-kDa protein species but also the additional species around 34 and 18 kDa. The 26- and 18-kDa protein species were each highly reactive with all of the immunoselected antibodies (Fig. 1B, lanes 1±3), while the 34 kDa was highly reactive to the middle band (Fig. 1B, lane 2), but less so with the highest and lowest protein species-selected antibodies (Fig. 1B, lanes 1 and 3). These data indicate that the complex protein-banding pattern either represents the same protein that is oligomerized, or processed, or represents different proteins that are so immunologically related that the antibody cannot distinguish between them.

BV, ODV, viral envelope, and nucleocapsid fractions were analyzed to determine if the immunoreactive proteins were components of the mature virus. The purity of the virus envelope and nucleocapsid preparations were tested using antibodies to other known ODV and BV structural proteins. The banding patterns of p39, ODV-E56, gp67, ODV-E18, ODV-EC27 were consistent with previous results (data not shown). In total BV, the major immunoreactive protein was 26 kDa; however, the purified envelope contained both 26- and 18-kDa proteins, although the 26 kDa was the major reactive band (Fig. 1; lanes 15 and 16). Total ODV contained both 18- and 26-kDa proteins, and these were also present in the purified envelope (Fig. 1, lanes 18 and 19). Unlike BV, ODV contained much more of the 18-kDa protein species, in both total virus and purified envelope. Both BV and ODV
The protein was named BV/ODV-E26 to identify it as a structural protein of BV and ODV, was localized in the envelope, and has an apparent molecular weight of 26 kDa for the predominant form.

Because of the multiple forms of immunoreactive proteins that are detected after 24 h p.i in infected cell lysates, studies designed to identify the fine structure...
localization of -E26 were performed at early time points when the 26-kDa protein was the predominant form (i.e., up to and including 24 h p.i.). IEM studies revealed that at 6 h p.i., -E26 was observed in cytoplasmic vesicles (Figs. 2C–2H, solid arrows). Most observations detected -E26 in membrane structures of small, discrete vesicles; however, it was also detected in more complex vesicles (Fig. 2D, arrows). Even in these more complex structures however, -E26 appeared to be membrane associated. A more rare observation was the detection of BV/ODV-E26 within cytoplasmic electron dense structures (Fig. 2B; arrowhead). Early in infection a consistent feature of -E26 localization was the detection of the protein within the nucleus and in association with electron dense regions (Fig. 2C; open arrows). Labeled cytoplasmic vesicles and intranuclear electron dense regions were not observed by 16 h p.i. (data not shown).

By 24 h p.i. -E26 was concentrated within virus-induced intranuclear microvesicles (Fig. 3B, open arrows), and ODV envelopes (Figs. 3C and 3D, arrows). BV/ODV-E26 was also detected in BV envelope and the plasma membrane (data not shown). The use of preimmune serum shows that background labeling in both cellular and viral structures is minimal (Figs. 2A and 3A).

Temporal analysis of transport of BV/ODV-E26 to the nucleus. At 6 h p.i., light microscopy, immunofluorescence label was concentrated predominately in punctate structures within the cytoplasm (Fig. 4A, 4a, arrow). The intracellular punctate structures correlate with the localization of -E26 in cytoplasmic membrane-bound vesicles as visualized by IEM (Fig. 2).

At 16 h p.i., -E26 was located in discrete foci within the nucleus (Fig. 4B, 4b, solid arrow). Also at 16 h p.i., a significant amount of -E26 was also detected in the cytoplasm (Fig. 4B, 4b; dashed arrow).

By 24 h p.i. -E26 was predominantly detected within the nucleus in discrete foci (Fig. 4C, 4c, arrow). This localization was consistent with IEM observations which show that -E26 located to intranuclear microvesicles (Fig. 3). Control experiments, including binding of preimmune serum against infected cells at all time points, and serum tested against uninfected Sf9 cells did not show detectable binding (data not shown).
BV/ODV-E26 is likely a peripheral membrane protein. Computer-assisted structural analysis of -E26 does not predict the existence of a membrane spanning domain in this protein (GenBank Accession No. L22858, nucleotides 13092–13767); however, -E26 clearly associates with membranes in the cytoplasm and nucleus. To test if -E26 is an integral or peripheral membrane protein, Triton X-114 detergent phase partition assay was performed. When infected cells (24 h p.i.) were separated into soluble and membrane fractions, the majority of -E26 partitioned with the membrane fraction and the three closely migrating protein bands of -E26 (~26 kDa) were easily visualized (Fig. 5A, lanes 1). When the membrane fraction (Fig. 5A, lane 2) was further analyzed and treated with Triton X-114, -E26 partitioned into the aqueous phase (Fig. 5B, lane 1). These data suggest that -E26 is a peripheral membrane protein.

BV/ODV-E26, FP25K, and actin may form a protein complex. To identify cellular or viral proteins potentially interacting with -E26, [35S]Met-labeled infected cell lysates (24 h p.i.) were immunoprecipitated with antibody to -E26 and immunoprecipitates analyzed by SDS–PAGE and Western blotting techniques. Approximately eight proteins were immunoprecipitated (Fig. 7A, lane 1) with -E26 being an abundant protein (Fig. 7A, lane 2). Previous work in this laboratory suggests that FP25K may effect the transport of some baculovirus ODV envelope-specific proteins (unpublished observations); therefore, we tested for the presence of FP25K in the immunoprecipitated sample. FP25K was detected among the proteins precipitated by the -E26 antibody (Fig. 7A, lane 3). Immunoprecipitation experiments performed with control preimmune antiserum did not reveal either FP25K or actin in the immunoprecipitated pellet (data not shown). To test for direct interaction between FP25K and -E26 a yeast two-hybrid assay was performed (FP25K, activation domain; -E26, binding domain) and a positive interaction between these proteins was demonstrated (Fig. 7B, iii.). Transfection of either plasmid alone did not result in color reaction product (data not shown) and control plasmids produced the predicted chromagenic reaction (Fig. 7B, i, ii).

Both FP25K and -E26 contain regions of similarity to the actin-binding proteins myosin and tropomyosin (BLAST/BEAUTY, Altschul et al., 1990) and examples of these homologies are shown in Fig. 6. In addition to the example shown for -E26 to tropomyosin, -E26 also had regions of homology to myosin (data not shown). FP25K contains regions of homology to myosin (Fig. 6B) and cytoplasmic dyneins (data not shown). In addition, FP25K contains a region that resembles the actin-binding helix present in gelsolins and profilins (Fig. 6C; McLaughlin et al., 1993). Because of the presence of these homologies in both -E26 and FP25K, we tested for the presence of actin in the -E26-immunoprecipitated preparation. A positive immunoreaction with monoclonal antibody to actin was obtained (Fig. 7A, lane 4; C4; Boehringer-Mannheim), indicating that actin could be part of a protein complex that includes -E26 and FP25K.

FIG. 5. Membrane association of BV/ODV-E26. (A) Western blot showing cofractionation of BV/ODV-E26 with the cellular membrane fraction. Lane 1, soluble fraction from 24 h p.i. Sf9; lane 2, membrane fraction. (B) Triton X-114 detergent phase partition assay. Lane 1, aqueous phase of Triton X-114-treated membrane fraction; lane 2, detergent phase.

FIG. 4. Light microscopy localization of BV/ODV-E26. Sf9 cells were infected and primary antibody to BV/ODV-E26 (No. 7554, 1:1000) was used along with FITC-labeled secondary antibody. DAPI staining was performed to visualize the nucleus. Data are presented either as FITC exposure alone (a,b,c) or FITC/DAPI dual exposure (A,B,C). (A, a) 6 h p.i. (B, b) 16 h p.i. (C, c) 24 h p.i.

FIG. 3. Immunological localization of BV/ODV-E26 in Sf9 cells infected with virus at the indicated times postinfection (p.i.).
The timing and amount of BV/ODV-E26 may be important for correct -E26 protein localization. Expression of genes cloned under the transcriptional regulation of the polyhedrin gene promoter (baculovirus expression vector system, BEVS) allows abundant expression of -E26 and expression in a different temporal pattern. The use of BEVS-expressed ODV-E66 showed that such an approach could be useful to detect potential sites of protein localization in the transport pathway (Hong et al., 1997).

Cells infected with a recombinant baculovirus expressing -E26 under the control of the polyhedrin promoter resulted in an unusual morphology of the virogenic stroma. At 24 h p.i. small regions within the virogenic stroma appeared more electron dense than normal (Fig. 8A; compare regions at small arrow vs large arrow); however, the appearance of enveloped virus was normal (Fig. 8A, open arrows). By 48 h p.i. varying degrees of unusual virogenic stroma morphology were detected. For example, Fig. 8B (large arrow) shows increased concentrations of the electron-dense material within the virogenic stroma, while in many cells the virogenic stroma was largely dispersed and exhibited a granular appearance (Figs. 8C and 8D, large arrows). In these cells -E26 was abundantly present in the virogenic stroma that exhibited the condensed granular appearance (Figs. 8C, and 8D, and 8H). Localization of -E26 in other cellular and viral structures was normal: It still located to intranuclear microvesicles; ODV envelope (Fig. 8E, m and open arrows); nuclear envelope; and plasma membrane (Fig. 8G, arrowheads). By 72 h p.i., the virogenic stroma was highly condensed (Fig. 8F, arrow).

**DISCUSSION**

The da26 ORF predicts a protein size of 26 kDa but the migration pattern on SDS-PAGE gels was more complex. SDS gels show that BV/ODV-E26 migrates as a single band of 26 kDa in cell extracts early in infection, but later in infection closely migrating bands at 25 kDa are detected; as well as several bands at 18 kDa and an additional band at 34 kDa (Fig. 1). While we don't understand the nature of the multiple immunoreactive species of proteins, immunoselection experiments indicate that these proteins species are highly immunorelated. It is possible that the differences in molecular weights are due to posttranslational modifications, protein processing, or protein degradation. However, -E26 is not glycosylated or phosphorylated, and the multiple protein forms are visible when protease inhibitors are used during sample preparation (data not shown). The proteins of closely related molecular weights of 26 and 18 kDa are present in purified virus; however, we note that the 26 kDa form is the predominant species present in BV. These data suggest that these multiple forms of -E26
may have structural or functional significance during viral infection or assembly.

We do not understand the nature or significance of the condensation of the virogenic stroma that results from abundant expression of BV/ODV-E26. Consistent with other observations of structural proteins expressed at levels higher than normal (unpublished observations), this result indicates that it is important for AcMNPV structural proteins to be present in cells in the proper stoichiometric amounts or have the proper temporal expression for normal interactions. Since -E26 has been shown to be required for late gene expression (in conjunction with da41; Guarino and Summers, 1988), we speculate that there is an association of -E26 with viral DNA or DNA-binding proteins. Such an interaction could account for the localization of -E26 in electron-dense regions within the nucleus early in infection (Fig. 2) and the structurally altered virogenic stroma upon abundant -E26 expression (Fig. 8).

Compared to previously described proteins that only locate to the BV or ODV envelope, -E26 is unique. Its incorporation into the envelope of BV would imply that the protein is transported to the cell plasma membrane early in infection (6 h p.i.) and immunogold experiments suggest that transport may be mediated through cytoplasmic membrane vesicles (Figs. 2 and 4). While it is possible that the labeled cytoplasmic vesicles could be the result of inoculum virus, time course immunofluorescence studies do not support this conclusion: The punctate pattern of label only occurs between 4 and 8 p.i., a time that is consistent with a BV envelope protein being transported to the cell surface. If these punctate patterns were the result of inoculum virus, one would predict that they would be present at very early time points, a result that is not visualized (data not shown). At later time points, -E26 is directed into the nucleus where it associates with intranuclear microvesicles and ODV envelope (Fig. 3). The pathway and regulation of nuclear transport of -E26 is unknown. All ODV envelope proteins characterized thus far contain at least one potential hydrophobic membrane-spanning domain (Hong et al., 1997); however, such a domain cannot be predicted from the primary amino acid sequence of -E26. BV/ODV-E26, however, does contain a sequence of basic amino acids that resembles a classical or bipartite nuclear localization sequence (HKKKLR X6 RKK; Dingwall and Laskey, 1991). The presence of a nuclear localization sequence and the apparent peripheral nature of membrane association of -E26 (Fig. 5) allows speculation that -E26 may be transported into the nucleus through the nuclear pores. Transport utilizing this pathway would suggest that once inside the nucleus, -E26 disassociates with transport
FIG. 8. Immunogold localization of BV/ODV-E26 in pVL1392-da26-infected cells. (A) 24 h p.i. (B–E, G–H) 48 h p.i. (I) 48 h p.i. Preimmune antisera. n, nucleus; c, cytoplasm; v, virogenic stroma; f, fibrillar structures; m, microvesicles; small arrows, normal virogenic stroma; large arrows, abnormal virogenic stroma; open arrows, ODV envelope labeling; arrowheads, labeling of plasma membrane and nuclear envelope.
nucleus in association with membranes or as a soluble protein. If -E26 utilizes the more defined pathway of transport through the nuclear pores, there must be a molecular process for -E26 to specifically associate with nuclear membranes after nuclear localization. The use of -E26 as a marker protein could assist in the identification of factors responsible for the regulation of -E26 interaction with membranes within the nucleus. If, however, -E26 traffics to membranes within the nucleus in association with other membranes, as has been proposed for ODV-E66 (Hong et al., 1997) and lamin B receptor (Smith and Blobel, 1993; Ye and Worman, 1994), then knowledge of the molecular mechanism of -E26 transport may provide insight on the factors necessary for regulation of this pathway. Considering that viruses are highly efficient at manipulating, exploiting, and/or redirecting normal cellular pathways, then insights provided by studies of AcMNPV of the regulation and pathway of trafficking of membrane proteins into the nucleus, may reveal pathways that might be extended to other species and cell types.

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REFERENCES
Hiller, G., Jungwirth, C., and Weber, Klaus (1981). Fluorescence micro-
AcMNPV ENVELOPE PROTEIN, BV/ODV-E26


