Transcription, Translation, and Cellular Localization of Three Autographa californica Nuclear Polyhedrosis Virus Structural Proteins: ODV-E18, ODV-E35, and ODV-EC27

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This paper identifies two structural proteins of the occluded derived viral envelope of Autographa californica nuclear polyhedrosis virus (AcMNPV): ODV-E18 and ODV-E35. In addition, we identify a protein, ODV-EC27, that is incorporated into the capsid of occluded virus, which is not detected in budded virus. The genes for these proteins reside within the IE0 intron. The intron was sequenced, and five open reading frames (ORF) were identified. ORF 3 (genomic ORF 143) codes for the ODV envelope protein, ODV-E18. ORF 4 (genomic ORF 144) codes for ODV-EC27, and Western blot analyses locate this protein to both the ODV capsid and envelope. Transcripts for both ODV-E18 and ODV-EC27 initiate from conserved TAAG motifs, and transcripts are detected from 16 through 72 hr p.i. Antiserum to ODV-E18 recognizes a band of 18 kDa on Western blots of extracts from infected cells and bands of 18 and 35 kDa on Western blots of proteins from purified ODV envelope. N-terminal amino acid sequencing reveals that both ODV-E18 and ODV-E35 contain the same N-terminus. Antiserum to ODV-EC27 recognizes a protein of 27 kDa on Western blots of extracts from infected cells and bands of 27 and 35 kDa on Western blots of proteins from purified ODV. Using immunogold labeling techniques, ODV-E18 and/or ODV-E35 are detected in viral induced intranuclear microvesicles and are not detected in the plasma membrane, cytoplasmic membranes, or the nuclear envelope. Immunogold labeling using antisera to ODV-EC27 detects this protein on both the ODV envelope and capsid.

INTRODUCTION

The nucleotide sequence and much of the genomic organization of Autographa californica nuclear polyhedrosis virus (AcMNPV) is known (Ayres et al., 1994). Approximately 60 of the potential 150 genes have been studied and based on direct evidence or computer assisted homologies, many of these gene products have been assigned an identity or function. Of all the genes and encoded proteins studied thus far, the immediate early protein IE0 is the only protein that is produced by an intron splicing event (Chisholm and Henner, 1988). The region of the genome that contains the IE0 intron is transcriptionally complex. Kovacs et al. (1991) demonstrated that multiple spliced transcripts are produced through this region both early and late in infection. The intron of IE0 contains five open reading frames, two with putative early transcription initiation motifs (CAGT) and three with late promoter motifs (TAAG). We have identified two ODV envelope proteins (ODV-E18, ODV-E35) and one structural protein which is a component of ODV capsid and envelope (ODV-EC27) and these are translated from two ORFs within the intron of IE0. In this study, we present evidence that at least one protein, ODV-E35, contains epitopes of proteins from two distinct open reading frames. While it is unclear what special biological significance genes within introns may represent, the data presented in this study increases our knowledge of the functional complexity of this intron region in the AcMNPV genome.

AcMNPV replication and assembly produces two infectious forms of progeny virus. The budded form of baculovirus (BV) matures early in infection and obtains its viral envelope from the plasma membrane. Virions present in viral occlusions which are called occlusion derived virus (ODV) mature late and obtain an envelope from unit membrane elements which assemble in the nucleoplasm. In infected cell nuclei, small microvesicles are induced which accumulate into foci. Three ODV envelope proteins, ODV-E25 (Russell and Rohrmann, 1993; and unpublished results), ODV-E56 (Braunagel et al., 1996), and ODV-E66 (Hong et al., 1994), are associated with these microvesicles. These data suggest that these microvesicles may be important intermediates in the envelopment of ODV. In this report we identify two additional proteins that are unique for the ODV envelope: ODV-E18 and ODV-E35. Like the other identified ODV envelope proteins, ODV-E18 and/or ODV-E35 locate to the baculo-
virus induced intranuclear microvesicles. Furthermore, we also describe another gene product, ODV-EC27, which is detected in the ODV but not BV capsid, the first example of a differentially localized capsid protein. All three of these structural proteins are translated from two of the five ORFs that are located in the IE0 intron.

We proposed that the transport of ODV-E56 into the nucleus of infected cells is membrane mediated. In the absence of a classical nuclear localization signal (ODV-E56), or a hydrophobic domain (ODV-E56-β-gal), the wild type and β-gal fusion proteins are incorporated into cytoplasmic membranes (presumably ER and/or Golgi) and the nuclear envelope (Braunagel et al., 1996). In this study we show that ODV-E18/35 are not detected in the plasma membrane, cytoplasmic membranes, or the nuclear envelope. The differences in the cytoplasmic and nuclear localization of AcMNPV ODV envelope proteins suggest that there are differences in the pathways of protein transport and assembly into the ODV envelope.

MATERIALS AND METHODS
DNA sequence determination and analysis

The sequence of the IE0 intron from AcMNPV (E2 strain) was determined on both strands and each base was sequenced an average of six times. All sequencing reactions were performed using oligonucleotides synthesized on a Model 391 PCR-MATE synthesizer (Applied Biosystems, Inc., Foster City, CA). Double-stranded DNA was sequenced using Sequenase 2.0 protocols (USB, Cleveland, OH) with [α-32P]dATP (DuPont, NEN Research Products, Boston, MA). Sequencing of cDNA was performed using cycle sequencing protocols of both Thermalbase Taq polymerase (Stratagene, La Jolla, CA) using [α-32P]dATP and SequiTherm Cycle Sequencing protocols (EpiCenter Technologies, Madison WI) utilizing SequiTherm Thermostable DNA polymerase and [α-32P]dATP. Sequence data were compiled and analyzed using the programs of the Genetics Computer Group (Devereux et al., 1984). The determined intron sequence was deposited with GenBank Data Library under Accession No. U09501.

Virus purification, N-terminal amino acid sequencing

AcMNPV viral occlusions, ODV, BV, viral envelope, and nucleocapsid fractions were prepared as described by Braunagel and Summers (1994). Viral preparations 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/10% separating gel that was prerun for 30 with 200 mM thioglycolic acid added to the upper buffer. The separated proteins were 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. Protein bands of 18 and 35 kDa were unique for the ODV envelope and N-terminal amino acid sequence for each protein was determined by The Biotechnology Instrumentation Facility (University of California, CA). Both proteins contained the same N-terminal amino acid sequence. The first six amino acids were used to design a degenerate oligonucleotide (ATGAT/C/G/AC/CT/G/ Ac/GA/;CC) and this was used to screen an AcMNPV genomic library.

RNA purification

SF9 cells (1.8 × 10⁶) were seeded into T150 flasks, allowed to attach for 1 hr, and infected with AcMNPV at a MOI of 20. Time zero for this and all subsequent experiments was defined at the addition of the virus. After 1 hr, the supernatant and unabsorbed viral inoculum were removed and replaced with TNM FH – 10% FBS. One T150 flask was harvested for each time point. Total cellular RNA was isolated using the method of Chirgwin et al. (1979) and purified using a cesium chloride gradients as described in Glisin et al. (1974).

Primer extension analysis

Transcript initiation was mapped using primer extension techniques (Sambrook et al., 1989). The oligonucleotides 5'-GCAATAATTACTACTACAGCCAAGATGG-3' (Fig. 2; oligo 1) and 5'-CATAGCTTTCTAAACTGC-3' (Fig. 2; oligo 2) were 5' end labeled with T4 polynucleotide kinase and probed against 20 μg of total cellular RNA. Extension products were generated using Superscript II-Reverse transcriptase (Gibco; BRL, Gaithersburg, MD) in the presence of 2.3 μg Actinomycin D, 28 units RNAsin, and 0.5 mM dNTPs for 60 min at 45°. Extension products were precipitated and digested with 0.1 N NaOH for 30 min at room temperature, resuspended in 80% formamide loading buffer, and boiled 3 min before loading onto a denaturing gel (7.0 M urea; 6% polyacrylamide; 100 mM Tris–borate; 20 mM EDTA, pH 8.3). Transcript initiation sites were identified by comparison with a concurrent DNA sequence using the same oligonucleotide primers as used for generating the extension products. The EcoRI B fragment of AcMNPV was used as template for the sequencing reactions.

Northern analysis

Twenty micrograms of total RNA was separated on a 1.2% agarose–formaldehyde gel and transferred to Zeta-Probe GT (Bio-Rad, Richmond, CA) using capillary transfer (50 mM NaOH, 6 hr). The same oligonucleotides described for primer extension analysis were 5' end labeled with 32P (Sambrook et al., 1989). Hybridization with Zeta-Probe immobilized RNA and labeled probe was per-
formed using 0.25 M Na₂HPO₄, 7% SDS (pH 7.2). After hybridization, the membrane was washed 2× (20 mM Na₂HPO₄, 5% SDS, pH 7.2), and 2× (20 mM Na₂HPO₄, 1% SDS, pH 7.2) for 30 min each wash. The blot was dried and labeled bands visualized by autoradiography. S1 nuclease analysis

Three prime S1 nuclease analysis was conducted as described by Favolaro et al. (1980). The probe was prepared by digesting a subclone containing ORFs 3 and 4 through the EcoRI site of the EcoRI B genomic fragment with BstEII. The DNA was 5′ end labeled at the BstEII site using [³²P]dATP (Sambrook et al., 1989) and then digested with EcoRI. The 5′ end-labeled fragment was separated and purified from an agarose gel. Forty thousand cpm of labeled probe was hybridized to 25 μg of total infected or uninfected cellular RNA (80% formamide, 40 mM Pipes, 0.4 M NaCl, and 1 mM EDTA, pH 6.4) at 42° for 16 hr before S1 nuclease digestion (BRL, Gaithersburg, MD). Protected fragments were analyzed on a 5% polyacrylamide–urea gel.

Heterologous protein expression and antibody production

Plasmids pGEX-E18 and pGEX-EC27 were designed to express GST fusion proteins in Escherichia coli for polyclonal antibody production. For pGEX-E18, oligonucleotide primers containing FLAG-epitope sequence after the ATG codon of ODV-E18 and the cloning sites for EcoRI and HindIII were synthesized (5′-CGGAATTCGCGGATCCATATGCGATACGGACGACGATGACAGATTCTACACTGATCCAC-3′ and 5′-CCCAAGCTTGCCCGCTCGAGCGGCTCTAGAGCTTTATTTAAGCC-ATGTATTTTGCTGAGCGC-3′). These oligonucleotides were used to amplify the E18 open reading frame by PCR. The PCR product was digested with EcoRI and HindIII and cloned into pUC 19 (pUC-E18). pGEX-E18 was constructed by digesting pUC-E18 with BamHI and HindIII, repairing the HindIII end with Klenow, and inserting the resulting fragment into pGEX-3 BamHI and SmaI sites. Thus, the amino acid sequence through the fusion site of GST was “MDYKKDDDDKITY...” (E18 sequence is in italics).

pGEX-EC27 was constructed by digesting the AcMNPV HindG fragment with Hpal and cloning this fragment into the Smal site of pUC 19. This construct was then further digested with BstEII and XbaI, Klenow repaired, and then cloned into pUC 19. Finally this construct was digested with BamHI and EcoRI and cloned into pGEX-3 using the same restriction sites. Thus, the fusion protein sequence for ODV-EC27 begins at the BstEII site with “VTE...”. Three prime S1 nuclease analysis was conducted as described by Favolaro et al. (1980). The probe was pre- and ODV-EC27 were induced in the bacterial strain JM83 using 0.1 mM IPTG for 3 hr at 37° (Frankel et al., 1991) through the EcoRI site of the EcoRI B genomic fragment and electroeluted from an SDS–PAGE gel.

Antiserum was produced in New Zealand White rabbits by three intramuscular injections at 4-week intervals. MPL-TDM-CWS (RIBI, Hamilton, MO) was used as adjuvant. The initial injection contained approximately 200 μg protein with each subsequent injection containing less antigen than previous injection. Eight days after the last injection, animals were terminally bled and sera collected.

In vitro translation

A Vsp1 restriction site flanks the region that contains both ORF 3 and ORF 4. The appropriate DNA was cut with Vsp1, filled with Klenow, and cloned into pBS (KS+/−) phagemid vector (Stratagene, LaJolla, CA) at the HincII site (pBS-43). To specifically translate ODV-E18, pBS-43 was digested with BstEII, transcribed by T7 RNA polymerase (Promega), ethanol precipitated, and resuspended with water. About 0.5 μg of the transcript was translated in rabbit reticulocyte lysate (Promega) containing [³⁵S]-methionine (DuPont, NEN). The translated product was separated on a 15% SDS–PAGE gel, dried, and visualized by autoradiography.

SDS–PAGE and Western blot analysis

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 1.5% SDS, 0.5% β-mercaptoethanol, 25 mM Tris-HCl (pH 6.8), and 7% glycerol for 15 min at 65°.
Western analysis was performed using protein blotted onto PVDF membrane (Immobilon-P; Millipore, Bedford, MA). The membranes were blocked with TBS-BLOTTO (150 mM NaCl, 10 mM Tris, 1% nonfat dry milk, 0.002% sodium azide, pH 8.0) containing 0.1% Tween 20. Antibody was bound overnight (ODV-E18 No. 7350, 1:1000; ODV-EC27 No. 7351, 1:1000), blots were washed twice with TBS-BLOTTO, and conjugating antisera (horseradish peroxidase-linked, anti-rabbit IgG, 1:10,000) was bound using a 3-hr incubation (TBS-BLOTTO; Tween 20). Blots were washed twice with TBS-BLOTTO (15 min), once with TBS (10 min), and color developed.

Immunogold electron microscopy

Sf9 cells were infected at a MOI of 20, virus was adsorbed for 1 hr, and then unabsorbed virus and media was removed and replaced with TNMFH, 10% FBS. At the designated time postinfection, cells were pelleted, fixed, and suspended in LR White. Ultrathin sections were prepared for antibody reaction as described by Hong et al. (1994). 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 (1:1000 dilution, 12 hr at 4°C), and washed (TBS; 4 x 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 (Bozzola and Russell, 1992) and lead citrate (Venable and Coggeshall, 1965) and were visualized with a Zeiss 10C transmission electron microscope.

RESULTS

Genomic organization, identification, and nucleotide sequences of genes encoding ODV-E18, ODV-E35, and ODV-EC27

To identify proteins unique to the ODV envelope, ODV and BV were purified and separated into envelope and nucleocapsid fractions. A comparison using SDS – PAGE analyses of the structural protein composition of the viral envelopes of both BV and ODV revealed two proteins of approximately 18 and 35 kDa that were unique to the ODV envelope. The N-terminal amino acid sequence of both proteins was determined: MIYTDPTTGATTSTDAP. A degenerate oligonucleotide was made to the first six amino acids and probed against an AcMN Pav genomic (9.5 vs 18 and 35 kDa). We considered the possibility ORF 3 and ORF 4 (Figs. 1 and 2) could be transcribed and translated as one open reading frame by an intron splicing event. To test this possibility, cDNA clones of this region were obtained from infected cells at both 24 and 32 hr p.i. Sequence analyses of these cDNA clones did not identify an intron (data not shown). To confirm that we did not have a sequencing error, the DNA sequence was determined independently (Gene Technolo-
FIG. 3. Northern analysis. Sf9 cells were infected and samples collected for RNA purification (hr p.i. is indicated on top of the figure). Twenty micrograms of total cellular RNA was separated on a 1.2% agarose/formaldehyde gel, transferred, and hybridized to labeled oligonucleotide (1 or 2). The sizes of RNA markers are indicated on the left. The sizes and predicted placement of detected transcripts for each oligo probe are indicated at the bottom of the figure.

ologies Laboratory, Texas A&M, College Station TX). For ORF 3, our sequence matched that reported by Ayres et al. (1994) with one discrepancy. This nucleotide change resulted in the identification by Ayres et al. of the ORF 3 start codon initiating further downstream as compared to our results (MFLTIL . . . see Fig. 2). Since the reported genomic sequence was of a different strain than ours, it may be that the nucleotide discrepancy simply reflect strain differences. The remaining sequence of ORFs 3, 4, and 5 matched the genomic reported sequence (ORF 143, 144, 146). Figure 1 cross-references the ORFs identified in GenBank entry No. U04051 with the genomic sequence provided by Ayres et al. (1994; GenBank No. L22858). Our GenBank release of this region of the genome preceded the release of the genomic sequence and there are some sequence discrepancies through ORFs 1 and 2. We have not performed further sequence analysis of these ORFs to resolve these differences.

Transcription analysis of ORF 3 and ORF 4

Northern analyses of infected cell total RNA using two strand-specific oligonucleotide probes revealed three transcripts. Oligonucleotide probe 1 (Figs. 2 and 3A) was designed to only recognize RNA transcribed through ORF 3. The major transcript identified by this probe was 1.5 kb, which is consistent with transcript initiation from an upstream TAAG sequence and ending within the ORF 4 sequence with a AATAAA polyadenylation sequence (Fig. 3A). Another minor transcript of 2.0 kb was identified. The size of this RNA is consistent with a transcript initiating from the TAAG sequence upstream of ORF 3 and terminating at the polyadenylation site for ORF 4 (Fig. 3A).

Oligonucleotide 2 was designed to identify transcripts that initiated from the TAAG sequence directly upstream of ORF 4 (Figs. 2 and 3B). Analyses using this oligonucleotide revealed two transcripts that were consistent with the results obtained from oligonucleotide 1, i.e., transcripts of 1.5 and 2.0 kb; and a third transcript of 1.2 kb was also detected (Fig. 3B). The size of this unique transcript correlates with transcript initiation at the TAAG immediately upstream of ORF 4 with termination at the polyadenylation site for ORF 4. Use of either oligonucleotide 1 or 2 shows that transcription through this region was detectable by 16 hr p.i. and maximal steady-state levels were attained at approximately 36 hr p.i. Transcription levels were still high at 72 hr p.i.

Primer extension analyses showed that the TAAG initiation sequences upstream of ORF 3 and ORF 4 were utilized (Figs. 2 and 4). Primer extension using oligonucleotide 1 showed four extension products corresponding to initiation from the three upstream TAAG sequences (Fig. 4A). Using oligonucleotide 2 as primer, there was one major product that corresponded to initiation from the TAAG sequence upstream of ORF 4 (Fig. 4B). Oligonucleotide 2 also detected four higher molecular weight extension products. We believe these correspond to the transcripts detected by oligonucleotide 1. Primer extension showed that transcripts initiated from ORF 3 at an earlier time than was detected by Northern analyses. These transcripts are detected at 8 hr p.i. (Fig. 4A). The primer extension results for ORF 4 matched those obtained by Northern analyses. Transcripts were detected at 16 hr p.i. with maximal steady-state levels at approximately 36 hr p.i.
FIG. 4. Primer extension analysis. Transcription initiation sites were mapped using 20 μg of total infected cell RNA (as in Fig. 3). Results obtained with oligo 1 revealed four transcription initiation sites, while oligo 2 revealed one additional unique site. The nucleotides recognized for transcript initiation are indicated below by arrows.

To map the 3' end of the transcripts, S1 nuclease analyses were performed on total RNA from infected cells. The labeled probe was hybridized to total RNA and treated with S1 nuclease (Figs. 2 and 4). These results revealed two protected fragments of 0.64 and 0.8 kb. The sizes of these protected fragments correspond to termination near the polyadenylation signals predicted for ORF 3 (0.64 kb) and ORF 4 (0.80 kb; Figs. 2 and 5). We noted that the polyadenylation consensus sequence for ORF 4 is within the extreme carboxy terminus of the coding sequence. While it is unclear whether it is possible for this sequence to be utilized, just downstream of the stop codon is an A/T rich region. Our data are consistent with polyadenylation occurring at or near the terminus of ORF 4. Use of strand-specific and two-sided probes showed similar results indicating that there is no detectable transcription on the noncoding strand (data not shown).

The data from the Northern, primer extension, and 3' S1 analyses showed that ORF 3 and ORF 4 are transcribed independently. There is also a larger transcript that clearly would include both ORFs 3 and 4. Transcripts are detectable at low levels as early as 8 hr p.i. (ORF 3; Fig. 4) with significant steady-state RNA levels by 16 hr p.i., maximal levels around 36 hr p.i., and high steady-state levels at 72 hr p.i. These data show that both ORF 3 and ORF 4 should be classified as baculovirus late genes.

Protein identification and temporal expression of ODV-E18, ODV-EC27, and ODV-E35

Polyclonal antibodies were produced separately to ORF 3 and ORF 4 as bacterial GST fusions. When infected Sf9 cell extracts were analyzed using SDS-PAGE and Western blot techniques, ORF 3 antibody
Western blot techniques, ODV-EC27 was detected in ODV but not in BV (Fig. 6C; lanes 3 and 4). We also noted that protein bands of higher molecular weights were present in BV which react with antibody to ORF 4 (Fig. 6; lanes 3 and 4). We do not have an explanation for these results. We considered the possibilities that ODV-EC27 is incorporated into BV in a different oligomerization state, in a modified form, or complexed with other proteins.

The Western blot results presented in Fig. 6C (ODV-E18; lanes 4 - 6; ODV-EC27, lanes 5 - 7) were from the same preparation of purified virus, envelope, and nucleocapsid fractions. These preparations were also blotted with antisera to gp67, p39, and ODV-E56 to assess the cross-contamination of the envelope and capsid fractions. We concluded that the envelope and capsid preparations contained little cross-contamination. Briefly, BV and BV envelope contained the expected major band of gp67; however, gp67 labeling was minimal in the nucleocapsid preparation. Purified ODV and ODV envelope preparations contained ODV-E56, while this protein was not detected in the nucleocapsid preparation. p39, the major capsid protein, was present in both ODV and BV purified nucleocapsid preparations but was not detected in the envelope preparation (data not shown). We were able to clearly detect the marker proteins using half of the amount of protein utilized for Fig. 6C. This difference may reflect a limited ability of ODV-E18 and ODV-EC27 antisera to recognize their respective denatured proteins or may indicate that these proteins are present in small amounts in purified virus. When the same fractions were reacted with ORF 3 and ORF 4 antisera (ODV-E18; ODV-EC27), a 35-kDa protein was detected in ODV and the ODV envelope fraction (Fig. 6C, ODV-E18, lanes 4 - 6; ODV-EC27, lanes 5 - 7). We know by amino acid sequencing that this protein (35 kDa) has the same N-terminal sequence as ODV-E18. This protein has been named ODV-E35.

Preimmune antisera for both ODV-E18 and ODV-C27 displayed a limited cross-reaction with both BV and ODV (Fig. 6C; lanes 1 and 2). Additionally, antisera to both ODV-E18 and ODV-EC27 did not react with proteins of uninfected Sf9 cells (Fig. 6B; M). Because antisera were generated to fusion constructs which also contained GST, Western blot analysis of BV, ODV, Sf9, and infected cell extracts was performed using antibodies to GST. Antibodies to GST detected at low levels several proteins in Sf9 cells and BV between 55 and 66 kDa (data not shown). When antibodies to GST were tested using IEM, no labeling was detected (data not shown).

Comparison of amino acid sequence of ODV-E18 and ODV-EC27 from different baculoviruses

A comparison of the amino acid sequence of ODV-E18 shows that this protein is highly conserved in at least
IDENTIFICATION OF ODV-E18, ODV-E35, AND ODV-EC27

FIG. 6. Protein analysis and identification of ODV-E18, ODV-EC27, and ODV-E35. (A) Antisera were produced to bacterial fusion proteins of ORF 3 and ORF 4 (B). Temporal accumulation of protein in infected Sf9 cells: 15 μg of total protein was loaded per lane, separated on a 12.5% gel, transferred to PVDF membrane, and reacted with antiserum to either ODV-E18 or ODV-EC27 (1:1000). (C) BV and ODV were purified and fractionated into envelope and nucleocapsid preparations. Ten micrograms of total protein was separated and reacted with antiserum as in (B). ODV-EC27, lanes 3 and 4 were loaded with 20 μg of total protein. ODV-E18 analysis included in vitro translation results and the translated product in indicated in ODV-E18, lane 7.

three baculoviruses (Fig. 7A). All of the baculovirus sequences have a conserved hydrophobic region (underlined) and a nearly identical carboxyl terminus. Figure 7B shows that AcMNPV and Bombyx mori nuclear polyhedrosis virus (BmMNPV) share 85.5% identity (90% similarity) and that both proteins share approximately 52% (51.8 – 53.6) identity with Heliothis zea nuclear polyhedrosis virus (HzSNPV).

The amino acid sequence of ODV-EC27 is highly conserved throughout the protein with only small regions of divergence (Fig. 7B). The sequence of the N-terminal 51 amino acids predicts a coiled-coil secondary structure and this region is conserved among the compared baculovirus proteins. The carboxyl region (amino acids 200–280) is highly conserved with several stretches of identity, while the extreme carboxyl terminal region is more divergent. Overall AcMNPV and BmMNPV are 94.9% identical with HzSNPV, sharing approximately 52% identity with AcMNPV ODV-EC27 (Fig. 7B).

Immunoelectron microscopy (IEM) of AcMNPV-infected S. frugiperda cells using antisera to ODV-E18 and ODV-EC27

Sf9 cells infected with AcMNPV were fixed and analyzed using IEM. Preimmune antisera for ODV-E18 displayed limited background reaction to baculovirus-induced structures including ODV, virogenic stroma, and viral occlusion and cellular structures including the nuclear and cytoplasmic membranes (Figs. 8A and 8B). ODV-E18 antisera specifically located ODV-E18 to the ODV envelope (Fig. 8C; arrows). It also labeled unit membrane structures that were associated with nucleocapsids apparently in the process of viral maturation (Figs. 8E and 8F; arrows). As with ODV-E66, ODV-E56, and ODV-E25, ODV-E18 labeling was enhanced in viral induced intranuclear microvesicles (Fig. 8D; m and inset). Labeling of the virogenic stroma was not above background (Fig. 8G; v). Additionally, cytoplasmic membranes
**FIG. 7.** Sequence homology analysis of ODV-E18 and ODV-EC27. (A) The predicted amino acid sequences of ODV-E18 and ODV-EC27 from AcMNPV were aligned with BmMNPV and HzSNPV. Boxed amino acids indicate 100% identity and shaded amino acids indicate similarity. The rules for similarity assignment were: G = A = S = T; V = L = I = M = F = Y = W; N = Q = D = E; and R = K = H. (B) Percentage identity and similarity (X) between these genes for the different baculoviruses. Comparisons were performed using the default parameters of the GAP program of UWGCG V8.0 (Devereux et al., 1984).

and the nuclear membrane are not labeled above background levels (Figs. 8D, 8E, and 8H).

ODV-EC27 preimmune sera also showed limited cross-reaction with infected cell structures including microvesicles, enveloped virus, polyhedra, and the cell nuclear envelope (Figs. 9A, 9B, and 9C). Figure 9H shows that ODV-EC27 is detected in both the ODV envelope (closed arrows) and capsid (open arrows). ODV-EC27 was detected in unit membranes associated with nucleocapsids in the process of viral maturation (Figs. 9E and 9F; closed arrows) and the viral induced intranuclear microvesicles (Fig. 9G, m). Open arrows in 9E, 9F, and 9I show that ODV-EC27 labeling was detected within maturing nucleocapsids and the virogenic stroma (Fig. 9I; v). We have shown that p39, the major capsid protein of both BV and ODV, located to empty capsid structures (Braunagel et al., 1996) and Fig. 9D demonstrates a similar pattern of label for ODV-EC27 (arrowheads).

Using IEM, antisera for ODV-E18 cannot distinguish between ODV-E18 and ODV-E35. ODV-EC27 antisera also cannot distinguish between ODV-EC27 and ODV-E35. The IEM labeling pattern of ODV-E18 and ODV-E35 is consistent with Western blot analysis (Fig. 6). We conclude that both ODV-E18 and ODV-E35 are present in the ODV envelope and least one, or both, of these proteins is present in intranuclear microvesicles. Western blot analysis of ODV-EC27 predicts that this protein is present in both ODV capsid and envelope and IEM methods detect a similar pattern of label. We cannot determine if the labeling of the viral-induced microvesicles using ODV-EC27 antisera represent ODV-E35 or ODV-EC27.

**DISCUSSION**

Several ODV envelope proteins have now been identified: ODV-E66, ODV-E56, and ODV-E25. Like the other
FIG. 8. Immunoelectron microscopy of AcMNPV infected Sf9 cells with antisera to ODV-E18. Infected Sf9 cells (72 hr p.i) were fixed and embedded in LR White for IEM analysis. After ultrathin sectioning, antisera to ODV-E18 were reacted and 30-nM gold conjugated to goat anti-rabbit IgG was used as secondary detection antibody. (A, B) Preimmune sera (7350; 1:1000). (C – H) Anti ODV-E18 (7350; 1:1000). Arrows indicate labeling of ODV envelope or membranes associated with maturing nucleocapsids; v, virogenic stroma; c, cytoplasm; n, nucleus; m, microvesicles. Bars, 1 μm.

identified envelope proteins, ODV-E18 and ODV-E35 are encoded by baculovirus late genes with transcription initiating from a TAAG motif (Fig. 4).

Our results predict that ODV-E18 exists as a dimer in the ODV envelope. In vitro translation produces a protein of approximately 10 kDa with no detectable 18-kDa protein (Fig. 6C; ODV-E18, lane 7). The determined N-terminal amino acid sequence of ODV-E18 contains the N-terminus predicted by ORF 3, and the excised band did not reveal a second N-terminal amino acid sequence. Additionally, when ODV-E18 is expressed under the strong polyhedrin promoter in a recombinant baculovirus, a protein of approximately 10 kDa is detected along with ODV-E18 in infected cell extracts (data not shown). We have not identified a 10-kDa protein in purified ODV, but rather see a diffuse band with an average molecular
FIG. 9. Immunoelectron microscopy of AcMNPV infected Sf9 cells with antisera to ODV-EC27. Infected cells were prepared as in Fig. 8 (72 hr p.i.). (A–C) Preimmune sera (7351; 1:1000). (D–I) Anti ODV-EC27 (7351; 1:1000). Arrow, ODV envelope and viral induced membranes; open arrows, nucleocapsids; arrowheads, empty capsids. m, microvesicles; v, virogenic stroma; n, nucleus; c, cytoplasm. Bars, 1 μm.
weight of 18 kDa. One would expect that SDS–PAGE techniques would denature and allow separation of most proteins into their monomeric form; however, for ODV-E18 this apparently does not occur. Glycophorin A is one example of a dimer that does not dissociate using SDS–PAGE techniques. Glycophorin A dimerizes into a parallel configuration with the transmembrane helices intimately associated along the carbon backbone (Adair and Engleman, 1994; Lemmon et al., 1994). Our results suggest that ODV-E18 exists in an oligomeric form that is resistant to SDS denaturing gel conditions.

In purified ODV, ODV-E18 exists as a broad band with an average molecular weight of 18 kDa (Fig. 6C). We do not have an explanation for this diffuse band in purified ODV and do not understand why ODV-E18 has a more concave banding pattern in infected cell extracts (Fig. 6B). We performed SDS–PAGE and Western blot analysis using a variety of acrylamide concentrations and protein solubilization techniques, and in all cases, ODV-E18 banding is diffuse in purified ODV and ODV envelope preparations. Preliminary data suggests that ODV-E18 is not N-glycosylated (data not shown). We are currently examining if ODV-E18 is posttranslationally modified by other types of glycosylation or other events.

Western blot analysis predicts that both ODV-E18 and ODV-E35 are structural proteins of the ODV envelope (Fig. 6C) and these analyses are consistent with IEM labeling. All ODV envelope proteins that have been described also are present in the viral-induced intranuclear microvesicles. Our IEM data indicate that ODV-E18 and/or ODV-E35 are also present in these structures.

We know that ODV-E35 and ODV-E18 have the same N-terminal amino acid sequence and ODV-E35 is recognized by polyclonal antibodies developed specifically to ODV-E18 and ODV-EC27. We also know by sequencing several cDNAs that there is no detectable intron splicing through this region (data not shown). Additionally, our transcriptional analysis of RNA shows a transcript that includes the reading frames of ORF 3 and ORF 4. To produce a full-length protein product from this transcript, the translation complex would have to shift by two nucleotides in the region between ORF 3 and ORF 4. We performed computer assisted RNA secondary structure modeling and these data predict that the RNA could form a stem-loop structure or pseudoknot, immediately downstream of the stop codon for ORF 3 (data not shown). This type of secondary structure when associated with “slip signals” can promote ribosomal jumping resulting in frameshifting events at high levels (Weiss, 1991). The slippery sequence UUUAAC is capable of promoting frameshifting 42% of the time when the coronavirus IBV is used as a model to study ribosomal frameshifting events (Brierley et al., 1992). A variety of “slippery sequences” have been identified and the region surrounding the predicted pseudoknot for the AcMNPV RNA near ORFs 3 and 4 contains several of these sequences (Brierley et al., 1992). Using the ODV-EC27 ATG start codon as nt 1, these slippery sequences include: UUUAAC (–76; IBV 42%), CCCUUA (–51; IBV 3%), AAAAAU (–5 and +58; IBV 10%). The combination of a predicted pseudoknot, in association with a cluster of “slippery sequences” and the recognition of ODV-E35 by ODV-E18 and ODV-EC27 antisera, supports the possibility that one or multiple “skipping” events result in a protein consisting of a fusion of the ORFs coding for ODV-E18 and ODV-EC27. Without direct evidence, however, we cannot rule out that ODV-E35 is an oligomer of ODV-E18 and ODV-EC27. There is an ammonium hydroxide cleavage site near the area where a frameshift would be predicted to occur, and we are currently attempting to purify sufficient quantities of ODV-E35 to directly determine the amino acid sequence through the region of the stop codons of ORF 3 and the start codon of ORF 4.

ODV-EC27 is the first baculovirus structural protein that is detected in both ODV nucleocapsid and envelope (Fig. 6C). The inability of the ODV-EC27 antibody to distinguish between ODV-EC27 and ODV-E35 in immunogold labeling experiments makes definitive fine structure localization of ODV-EC27 difficult. However, consistent with the Western blot analyses, IEM results detect ODV-EC27 in both the viral nucleocapsid and envelope. Empty capsids also label with ODV-EC27 antibody and this pattern of label is similar to that observed for p39 (Braunagel et al., 1996). Label is not observed in these empty capsid structures using ODV-E18 antisera. Therefore, we conclude that the labeling represents ODV-EC27 localization to the elongated empty capsid structures and nucleocapsids. These results lead to the speculation that ODV-EC27 may be incorporated into the capsid during assembly. Current experiments are designed to test the hypothesis that ODV-EC27 functions in the process of recognition or binding of nucleocapsids and precursor envelope membranes during the viral envelope matura- tion process.

All of the baculovirus capsid or nucleocapsid proteins studied to date are present in both BV and ODV. These include the major capsid protein, p39 (Theim and Miller, 1989; Pearson et al., 1988; Blissard et al., 1989), p87 (Muller et al., 1990; Lu and Carstens, 1992), putative DNA-binding protein of 6.5–7.5 kDa (Wilson et al., 1987; Russell and Rohrmann, 1990; Maeda et al., 1991), a 24- to 25-kDa protein that has been identified in both OpMNPV and AcMNPV (Wolgamot et al., 1993; Goh, 1993), the basic protein, vp12 (Tweeten et al., 1980), and the protein product of the 1629K gene (Pham et al., 1993; Vialard and Richardson, 1993). Based on the results of SDS–PAGE analyses of purified BV and ODV nucleocapsids, we hypothesized that the protein compositions of BV compared to ODV would be different (Braunagel and Summers, 1994).
# Table 1

<table>
<thead>
<tr>
<th>Localization (IEM)</th>
<th>ODV-E25 (p25)</th>
<th>ODV-E66</th>
<th>ODV-E56</th>
<th>ODV-E18/E35</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ODV</td>
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<td>+ + +</td>
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<tr>
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<tr>
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<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Inner nuclear membrane</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Endoplasmic reticulum/outer nuclear membrane</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Intranuclear electron dense regions</td>
<td>?</td>
<td>+</td>
<td>+ + +</td>
<td>-</td>
</tr>
<tr>
<td>Membranes associated with maturing nucleocapsids</td>
<td>+</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Virogenic stroma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note. In addition to the protein listed in this Table, p74 is also believed to be an ODV envelope-specific protein (Hill et al., 1993).

* Some of the localization's presented here represent unpublished observations of T. Hong and S. C. Braunagel.

ODV-EC27 is the first protein identified that is differentially incorporated into ODV vs BV capsids or nucleocapsids. We note that several proteins of a larger molecular weight react with ODV-EC27 antisera in BV (Fig. 6C; lanes 3 and 4). It is possible that ODV-EC27 may form a homo- or heterooligomeric complex in BV but not ODV. The N-terminal 58 amino acids of ODV-EC27 strongly predict a coiled-coil secondary structure, a structure that is commonly associated with protein–protein interactions or protein oligomerization. We are currently testing if ODV-EC27 oligomerizes or is associated with other proteins in BV.

A feature of ODV envelope proteins is that they are highly conserved among those of the baculoviruses studied. ODV-E56 is highly conserved even among divergent strains of baculoviruses (Theilmann et al., 1996). ODV-E66 displays 94% identity with BmMNPV and approximately 40% identity with LdMNPV. AcMNPV ODV-E25 shares 91% identity with BmMNPV and 63% with OpMNPV. ODV-E18 and ODV-EC27 are also highly conserved (Fig. 7).

The localization of ODV-E18 and ODV-E35 to cellular, viral, and viral-induced structures during infection shows similarities and differences when compared to other ODV envelope proteins (Table 1). All of the ODV envelope proteins show enhanced labeling in viral-induced intranuclear microvesicles, membranes that associate with maturing nucleocapsids and ODV envelope prior to, and after, incorporation into the viral occlusion. These data strongly support the hypothesis that these vesicles function as a precursor in the assembly of the ODV envelope.

There are significant differences in the cellular locations of the ODV envelope proteins during viral maturation (Table 1). Notably, ODV-E66 and ODV-E56 are present on the inner nuclear membrane and membranes juxtaposed to the cytoplasmic side of the nuclear envelope; presumably ER (Braunagel et al., 1996; Hong et al., unpublished results). ODV-E18 and ODV-E35 show different cellular localization as compared to ODV-E66 and ODV-E56. They are not detected in association with cytoplasmic membranes or the nuclear envelope (see Figs. 8D, 8E, and 8H). Even when these proteins are expressed at high levels under the control of the polyhedrin or p10 promoters, protein labeling is not detected in cytoplasmic membranes or the nuclear envelope (data not shown). We acknowledge that it is possible that ODV-E18 and ODV-E35 are present in cytoplasmic membranes and the nuclear envelope, but cannot be detected because the antibody epitopes are masked.

Our comparison of ODV envelope proteins during transport to the nucleus reveals significant differences in cellular location and these data are beginning to indicate that the pathways into the nucleus and incorporation in membranes are not only complex but may differ for some of the envelope proteins. The results with ODV-E56 and ODV-E66 led to the hypothesis that transport into the nucleus of ODV envelope proteins may be membrane mediated (Braunagel et al., 1996; Hong et al., manuscript in preparation). However, the inability to detect ODV-E18/E35 label in cytoplasmic membranes and the nuclear envelope leads us to consider that another pathway of protein nuclear import is also functioning. This would include import through the nuclear pores, with subsequent insertion into intranuclear membranes. We note, however, that ODV-E18 does not contain a recognizable nuclear localization signal. We speculate that ODV-E18 may either be complexed with other protein(s) during transport or it contains an unconventional nuclear local-
Our results from studies on ODV-E66 (Hong et al., manuscript in preparation) and ODV-E56 (Braunagel et al., 1996) predict that the hydrophobic domains in these proteins can function to target, sort, or retain proteins to viral induced intranuclear membranes. ODV-E18 (and presumably ODV-E35) also contains a hydrophobic domain (Fig. 2). We are currently using deletion mutations to identify if this domain functions as a target or retention signal for intranuclear microvesicles. It is our intent to systematically study each of the ODV envelope proteins to investigate the pathway of incorporation into membranes that reside in, or are induced, or amplified in the nucleus during baculovirus infection. The use of baculovirus envelope proteins as specific markers may reveal currently undefined transport signals, accessory proteins, or pathways of protein movement into the nucleus and incorporation or assembly into unit membranes required for viral maturation.

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