A 37-Kilodalton Glycoprotein from a Baculovirus of *Orgyia pseudotsugata* Is Localized to Cytoplasmic Inclusion Bodies†

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The gene encoding a 37-kDa glycoprotein (gp37) of *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus (OpMNPV) was located and sequenced. gp37 of OpMNPV was found to have 62 and 37% amino acid sequence identity with gp37 of *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) and with a protein reported to be a component of occlusion bodies from *Choristoneura biennis* entomopoxvirus, respectively. The mRNA start site of the OpMNPV gp37 gene was mapped within a late promoter sequence (TTAAG). A TrpE fusion protein containing 55% of the OpMNPV gp37 gene amino acid sequence was used to generate a monospecific antiserum. Western immunoblot analysis of OpMNPV-infected *Lymantria dispar* cells detected gp37 beginning at 24 h postinfection. Immunoelectron microscopy indicated that the protein is concentrated in cytoplasmic inclusion bodies late in infection. In contrast to gp37 of AcMNPV which was present in the matrix of occlusion bodies, OpMNPV gp37 was not observed in this location. Neither OpMNPV nor AcMNPV gp37 was associated with the polyhedron envelope.

Nuclear polyhedrosis viruses (NPVs) are members of the *Baculoviridae*, a family of large double-stranded DNA viruses with genomes of 88 to 160 kbp. NPVs are pathogenic to arthropods, primarily insects of the orders Lepidoptera, Hymenoptera, and Diptera. NPVs replicate in the host cell nucleus and are characterized by an infection cycle that involves the production of two structurally distinct virion phenotypes. The budded-virus (BV) phenotype, which is responsible for systemic infections of insects, is produced as nucleocapsids bud through and acquire their envelope from the host plasma membrane. In contrast, nucleocapsids of the polyhedron-derived virus phenotype are enveloped within the nucleus and are then occluded in polyhedron-shaped crystals (or polyhedra) composed primarily of a protein called polyhedrin. Polyhedra are surrounded by an electron-dense structure called the polyhedron envelope or calyx. Polyhedra are stable for extended periods in the environment and are responsible for the spread of virus between insects.

Recently, Yuen et al. (25) described a 38.5-kDa protein from occlusion bodies of an entomopoxvirus pathogenic to *Choristoneura biennis* (*C. biennis* entomopoxvirus [CbEPV]). Although their results suggested that this protein was the major inclusion body protein (called spheroidin), Hall and Moyer (8) have recently presented evidence that CbEPV spheroidin is a 115-kDa protein unrelated to the 38.5-kDa protein. The initial report of Yuen et al. (25) was followed by another study (22), which showed that the 38.5-kDa CbEPV protein has 39% amino acid sequence identity with an open reading frame (ORF) from *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV). This ORF encodes a 37-kDa glycoprotein (gp37) originally described by Wu and Miller (24). Using antibodies against the 38.5-kDa CbEPV protein in conjunction with

immunofluorescence microscopy and Western immunoblot analysis, Vialard et al. (22) suggested that AcMNPV gp37 was associated with the AcMNPV polyhedron envelope.

In this report, we describe the location, sequence, and transcriptional map of the gp37 gene from the NPV of *Orgyia pseudotsugata* (OpMNPV). In addition, a TrpE fusion protein of OpMNPV gp37 was constructed and used for the production of a monospecific antiserum. This antiserum was used to characterize the expression of gp37 by Western blot analysis and to localize gp37 by immunoelectron microscopy of infected cells and polyhedra from OpMNPV and AcMNPV.

MATERIALS AND METHODS

Viruses, insect cell lines, insects, and 38.5-kDa CbEPV protein antisera. The cloned isolate of OpMNPV used to infect *Lymantria dispar* cells (Ld-652Y) was described by Quant-Russell et al. (14). The E-2 strain of AcMNPV was used to infect *Spodoptera frugiperda* (SF9) cells. *L. dispar* and *S. frugiperda* cells were propagated in T flasks using TNM-FH medium (20) supplemented with 10% fetal bovine serum (FBS). For production of OpMNPV BV, *L. dispar* cells adapted to shaker culture (a gift from Stephan Weiss, GIBCO Laboratories) were grown in TNM-FH–10% FBS supplemented with 0.1% Pluronic-F68 (GIBCO Laboratories). The *O. pseudotsugata* fourth- and fifth-instar larvae were kindly provided by the USDA Forestry Sciences Laboratory (Corvallis, Oreg.). AcMNPV polyhedra were provided by John Kuzio. The 38.5-kDa CbEPV protein antisera were gifts from Jorge E. Vialard.

Purification of virus. For studies involving infected cells or BV, cells were infected with OpMNPV BV at a multiplicity of infection of 10 by following the procedures of Bradford et al. (2) unless otherwise indicated. For Western blot analysis, BV was isolated from two 150-ml shaker cultures of OpMNPV-infected *L. dispar* cells at 72 h postinfection (p.i.). After centrifugation to remove the cells ($2,000 \times g$ for 10 min), the supernatant was centrifuged at $80,000 \times g$ for 30

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FIG. 1. Location of the gp37 gene on the OpMNPV genome. The *Hind*III map of the OpMNPV genome is shown at the top and the positions of cosmid clones 47, 1, 54, 58, and 39 are indicated. The locations of the polyhedrin (PH), DNA polymerase (DNA pol), p39 capsid protein (capsid), BV envelope glycoprotein (gp64), polyhedron envelope protein (PE), and p10 protein are shown below the *Hind*III map. The expanded restriction map below the genome shows the locations of the gp37 gene and mRNA in relation to the DNA polymerase gene. Prominent restriction enzyme sites are indicated, and the shaded box shows the area sequenced. m.u., map units.

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AAGCGTATCCGCTGCGTTTGTTCGACGCGAAACCATGCGCATCAGTTGGCTCAAGCACATGACCATCTTAAACACTTTTATGAACGAGCTGCTAGAGATATTCGGGGACGAGCACAAGG 120
A Y P L R L F D A E T M R I S W L K H M T I L N T F M N E L L E I F G D E H K
ACGCGCTGGCCGAGTGCTACAGCGCCATCTCGAAAAGTACATGCAGCACCAAGCGTACGACAAAAGCGCGCCGCTGGTCAAATCGCCACGAAACGAAAGCGCCGAGCGGAGCGG 240
D A L A E C Y S A I L E K Y M Q H Q A Y D K K R A A L V K I A T K R K A P S A S
ACGCGTCCGGCAAACGCGCGCGCAAGGGGCGCGCCAGCGACGAGAGCGGCTCCAGCGAGGACGAAAGACGCGCGTGCAGCCCAAGTGCAGCAACACACGTTCAAGTTTGTGTC 360
D A S G K R A R K G A A P S D D E S G S S E D E D A P C E P K C A N N T F K F C
TGTACAAAGCACAATAAAGCAGTCGTTACTCAATTGTGTATTATTAAGCGCCCCACAAAATGTACAAACTTTGCGCGCTCTGTTTGGCTGGCGGTGCGCGCGTGCAGCCGACGCGC 480
L Y K A Q M Y K L C A V L F A L A V P A V R P H G
TACCTGTCCACGCGCCGTCGCCAGGCGAGTACAAATGCTTTGCGGACGCGCAACTTTTACTGCGCCGACAAACGCGCAGCGCTCCGACGAGGCGTGCAGCAACGCGTACAAAAAGTGTT 600
Y L S T P V A R Q Y K C F A D G N F Y W P D N G D G V P D E A C R N A Y K K V F
CACCGATACGCGCGCTGGCGCGCGCGCGGGGGAAGCGGCGCGCGCGCGGCGAGTACATGTTTTCAGCAGTACCGCGAGTACGCGCGGTGGCGCGCCCAACTACCGCGAGCTGGAAGT 720
H R Y R A V G A P P G E A A A A A Q Y M F Q Q Y A E Y A A V A G P N Y R D L E L
GTCAAGCGCGAAGTCTGCGGCACACGCTGTGCGGCGCGCGGCTAACGACCGCCACGCGTGTTCGGCGCAAGAGCGGCGATGGACGAGCGGTTCCACAACGCGCGCGGACGTTGCTG 840
V K R E V L P H T L C G A A A N D R H A L F G D K S G M D E P F H N W R P D V L
KpnI (trpE fusion site)
TATGTGAACCGCTACGAGCGCGCATCTCTTCAACGTGCACTTTTGCCTCCGCGCGTGCAGGACCCAGCTACTTCGAGGTGTACGTACCAAGTTTACGTGGGACCGCGCGAGCGCC 960
Y V N R Y Q R A H S F N V H F C P T A V H E P S Y F E V Y V T K F T W D R R S P
XhoI SstI
GTCACGTGGAACGAGCTCGAGTACATCGGCGGCAACGGCTCGGGTCTGGTGCCGAAACCGCGCGACGCGTTTTCGCCAGCGGCGAGCTTTACTCTATCCCGGTGTCGGTGCCGTACCGA 1080
V T W N E L E Y I G G N G S G L V P N P G D A F C A S G Q L Y S I P V S V P Y R
CCGGGCGCGTTTGTCTGTACGTGCGTGGCAGCGCATCGACCCGTTGGCGAAGGCTTCTACAACTGCGCGACCTCGTGTTCGGCACCGAGAACGACGAGTGCCGTTACGCGCGCGC 1200
P G P F V M Y V R W Q R I D P V G E G F Y N C A D L V F G T E N D E C R Y A R A
GCCAAGGCGGTGCGGACGAGTGCAGCAACAAACCTGTGCAATGATTGTGTTGAGGCGGGCCCCAAGAATCGTGCGGCCAACGCGCGCGCAACGCGCGCGCCACAATTATTGCGC 1320
A K A V R D Q L R Q Q N L C N D C V E A G P Q E S C A P T R P Q R R A H N Y L R
CGCGGCGCGCGCAGCACCAACAGGCGCAGGTGCCAGCGTGCAGGACATTGACGAGTTGTGAGCGTTCGGTCCGCGCATGATTATGCCAAGTGAGTCGTGTTAAAAACGCG 1437
R G G A H D Q Q A D G A S V R E T I D E L 321

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FIG. 2. Nucleotide sequence of the OpMNPV gp37 gene region. The amino acid sequences predicted for the C-terminal region of DNA polymerase (nt 1 to 374) and for the gp37 gene (nt 421 to 1383) are shown below the nucleotide sequence. The location of the 18-mer oligonucleotide primer used for primer extension analysis and the gp37 mRNA initiation site are indicated. The locations of major restriction sites, including the *Kpn*I site used for production of the TrpE-gp37 fusion protein, are also shown. Numbers to the right of the sequence indicate the positions of nucleotides and gp37 amino acids.

FIG. 4. Transcriptional mapping of OpMNPV gp37 transcripts. (a) Primer extension analysis of gp37 transcripts from different times p.i. The sizes (in nucleotides) of the primer extension products are indicated on the left and were determined by alignment with a p1,2,PEPCAT sequence ladder (7). (b) 3' mapping of the gp37 transcripts by S1 nuclease analysis. The size of the S1 nuclease-protected product was determined by comparison to a 32 P-labeled 1-kb ladder (Bethesda Research Laboratories) (M1) and an *Hae*III-digested ϕ X174 ladder (New England Biolabs) (M2) that were electrophoresed on the margins of the gel. Selected marker sizes (in nucleotides) are shown at the right. P, probe; U, uninfected cells.

labeled using a fill in reaction (18). RNA (30 μ g) from each time point was annealed to the end-labeled DNA. Annealing, hybridization, and S1 nuclease digestion were performed as described by Favaloro et al. (3).

gp37 antibody: construction, expression, and isolation of the TrpE-gp37 fusion protein. A 1.6-kb *Kpn*I-*Pst*I fragment, containing the C-terminal portion of OpMNPV gp37, was cloned into pBS and then excised with *Eco*RI and *Hind*III. The *Eco*RI-*Hind*III fragment was subcloned into the *trpE* expression vector pATH 1 (10) to construct an in-frame gene fusion containing the C-terminal 175 amino acids ($\approx$ 20 kDa) of the gp37 ORF. Recombinant plasmids were transformed into *E. coli* HB101 cells and expressed in modified M9 medium as previously described (6). The TrpE-gp37 fusion protein was isolated and used for antibody production in rabbits, as previously described (5).

Western blot analysis. To investigate the expression of gp37 in OpMNPV-infected cells, *L. dispar* cells from different times p.i. were lysed in 2 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (11). A sample equivalent to approximately 4.8×10^4 cells was electrophoresed per lane of an SDS-10% polyacrylamide gel (11). Western blot analysis was performed as previously described (14). The TrpE-gp37 antiserum was diluted 1:1,000.

N-linked glycosylation assay. To analyze for N glycosylation, *L. dispar* cells were infected at a multiplicity of infection of 10 and grown in the presence of 10 μ g of tunicamycin per ml. Cells were collected at 60 h p.i. and lysed in electrophoresis sample buffer (11). Proteins were separated by SDS-PAGE, and Western blot analysis was performed.

Immunoelectron microscopy. *L. dispar* cells were infected

with OpMNPV at a multiplicity of infection of 100. At various times p.i., cells were harvested, fixed in 2.5% glutaraldehyde, dehydrated, and embedded in LR White resin as previously described (17). The OpMNPV BV were injected into *O. pseudotsugata* larvae at approximately 50,000 50% tissue culture infective doses per insect. Fat bodies were removed from the insects at 4 days p.i., fixed in 2.5% glutaraldehyde, and prepared as described above. Immunoelectron microscopy was done as previously described (17). Goat anti-rabbit immunoglobulin G conjugated with 10-nm-diameter gold particles (Ladd Research Industries, Inc.) was used at a dilution of 1:20.

Nucleotide sequence accession number. The nucleotide sequence of the OpMNPV gp37 gene has been assigned accession number D13306 by EMBL, GenBank, and DDBJ.

RESULTS

Nucleotide sequence of the OpMNPV gp37 gene. In AcMNPV, the gp37 gene is located downstream of the DNA polymerase gene (24). Because of the colinearity of extensive regions of the OpMNPV and AcMNPV genomes (12), an area downstream of the OpMNPV DNA polymerase gene was cloned and sequenced. This region (nt 421 to 1383 [Fig. 2]), which encodes a putative protein of 321 amino acids with a predicted molecular mass of 36.1 kDa, has 62 and 37% amino acid identity with AcMNPV gp37 and CbEPV 38.5-kDa protein, respectively (Fig. 3). The five conserved regions previously identified by Vialard et al. (22) are also conserved in the OpMNPV gp37 protein (Fig. 3) and, except for region 2, show 80% or greater identity between the three proteins.

Transcriptional mapping and temporal expression of the

FIG. 5. Western blot analysis of OpMNPV gp37. (a) Expression of gp37 in OpMNPV-infected *L. dispar* cells. The numbers over the lanes indicate hours p.i. U, uninfected cells. (b) Western blot analysis of gp37 in purified OpMNPV budded and occluded viral phenotypes. I.C.S., infected-cell supernatant; OB, occlusion bodies. The capsid-associated p24 protein (23) was used as a positive control. (c) Analysis of N-linked glycosylation of gp37 in OpMNPV-infected *L. dispar* cells. The figure shows a Western blot of gp37 in cells not treated (-Tun) and treated with tunicamycin (+Tun). The sizes (in kilodaltons) of the protein standards are indicated to the left and the positions of protein (gp37 or p24) are indicated to the right of the Western blots.

gp37 gene. To locate the transcriptional start site and characterize the temporal expression of the gp37 gene, a 5'-end-labeled oligonucleotide was annealed to total RNA isolated from *L. dispar* cells at various times p.i. and elongated with reverse transcriptase. The sizes of the primer extension products were 58 and 59 nt, as determined by comparison with a DNA sequencing ladder (Fig. 4a). These products mapped to nucleotides 3 and 4 (nt 407 and 408 [Fig. 2]) in the promoter sequence TTAAG. The earliest primer extension products were detected at 24 h p.i., and mRNA was present through 72 h p.i., the last time point assayed (Fig. 4a).

To map the 3' end of the gp37 gene, S1 nuclease analyses were performed by using a plasmid containing the *KpnI-PstI* fragment (Fig. 1) that was linearized with *XhoI* and 3' end labeled. The 3'-end-labeled fragment was annealed to RNA samples isolated from representative times p.i. (Fig. 4b). At 24 h p.i. and thereafter, a major protected fragment of approximately 1.3 kb was observed, indicating that the 3' end of the gene is located approximately 890 nt downstream of the gp37 translational termination codon and maps to an unsequenced region (Fig. 1).

Characterization of gp37 expression and N-linked glycosylation. To generate antibodies specific for gp37, a gene fusion

was constructed in a pATH 1 bacterial expression vector. The TrpE-gp37 fusion protein was induced, purified, and used for antiserum production. This antiserum was used to detect gp37 in OpMNPV-infected *L. dispar* cells from various times p.i. by Western blot analysis. gp37 was first detected at 24 h p.i. and increased through 120 h p.i., the last time point assayed (Fig. 5a). Preparations of BV and occlusion bodies were also subjected to Western blot analysis. Although gp37 was present in infected-cell supernatants (Fig. 5b), it was not found in sucrose gradient-purified BV or occlusion body preparations (Fig. 5b). Antiserum against the virion-associated protein p24 (23) was used as a positive control in this analysis.

Western blot analysis of AcMNPV-infected Sf9 cells and occlusion bodies using both OpMNPV TrpE-gp37 and CbEPV 38.5-kDa protein antisera demonstrated the presence of the AcMNPV gp37 protein in cell extracts and occlusion bodies, confirming the report of Vialard et al. (22) (data not shown).

N-linked glycosylation of the OpMNPV gp37 was examined by Western blot analysis using TrpE-gp37 antiserum and 60-h p.i. extracts from cells that had been treated with or without tunicamycin. The greater mobility of the gp37 band from tunicamycin-treated cells compared with gp37 from untreated cells (Fig. 5c) indicates that OpMNPV gp37 is N glycosylated.

Immunoelectron microscopy of gp37. To determine the location of gp37, OpMNPV-infected *L. dispar* cells were examined by immunoelectron microscopy, using the OpMNPV TrpE-gp37 antiserum. Intense staining of gp37 was localized to cytoplasmic inclusion bodies late in infection (Fig. 6a and b). These inclusion bodies were located throughout the cytoplasm. No significant staining of polyhedra was seen at 84 h p.i. (Fig. 6c) or at other times during the infection (data not shown). However, in AcMNPV-infected Sf9 cells, the OpMNPV TrpE-gp37 antiserum localized gp37 to viral polyhedra (Fig. 6d). The staining was found distributed throughout the occlusion matrix and near embedded virions. The AcMNPV polyhedron envelope did not specifically stain (Fig. 6d). Similar results were observed when AcMNPV polyhedra were stained with CbEPV 38.5-kDa protein antiserum (data not shown). In AcMNPV-infected Sf9 cells stained with the 38.5-kDa CbEPV protein antiserum, cytoplasmic inclusion bodies similar to the ones seen in OpMNPV-infected cells were also observed (data not shown). Neither uninfected nor infected cells showed significant staining with TrpE-gp37 or TrpE antiserum, respectively (data not shown).

DISCUSSION

In this study, we identified a gene encoding gp37 in the baculovirus OpMNPV. In both OpMNPV and AcMNPV (24), the gp37 genes are located downstream of the DNA polymerase gene. The predicted OpMNPV gp37 amino acid sequence shows 37 and 62% identity with the CbEPV 38.5-kDa protein and AcMNPV gp37, respectively. Strong conservation between the OpMNPV and AcMNPV gp37 proteins was localized to the core of the protein, while the N- and C-terminal regions (13 and 51 amino acids, respectively) demonstrated lower degrees of similarity (38 and 18%, respectively). OpMNPV gp37 was shown to be N glycosylated and has one potential N-linked glycosylation site at amino acid 192, which is conserved among the three proteins (Fig. 3). The CbEPV 38.5-kDa protein and AcMNPV gp37

FIG. 6. Immunogold staining of OpMNPV- and AcMNPV-infected insect cells. (a) gp37 cytoplasmic inclusions in OpMNPV-infected *L. dispar* cells at 84 h p.i. (b) gp37 inclusion in *L. dispar* cells at 72 h p.i. (c) OpMNPV polyhedra from an *O. pseudotsugata* fat body cell. (d) AcMNPV polyhedra. The TrpE-gp37 antiserum was diluted 1:2,000 (panels a, c, and d) and 1:1,000 (panel b). Bars, 0.25 μ m.

each have an additional N-linked glycosylation site at amino acids 176 and 293, respectively (Fig. 3).

The 5' transcriptional start site was mapped to a late promoter consensus sequence 13 and 14 nt upstream of the gp37 ATG. The gp37 mRNA start site was located at the third and fourth nucleotides of TTAAG. Although late promoters most often have the sequence A/GTAAG (15), TTAAG has been reported as a late promoter for the AcMNPV gene encoding the 35,000-molecular-weight protein (13). In contrast to OpMNPV gp37, the AcMNPV gene contains two late promoter elements at 11 and 87 nt upstream of the translational start site (24).

Western blot analysis using a polyclonal antiserum produced against a TrpE-Op-gp37 fusion protein indicated that gp37 was expressed as early as 24 h p.i. but was not associated with the OpMNPV BV or occlusion bodies.

These results differ from those of Vialard et al. (22), who demonstrated the presence of gp37 in purified AcMNPV occlusion bodies by Western blot analysis. We confirmed their results using Western blots stained with OpMNPV TrpE-gp37 and CbEPV 38.5-kDa protein antisera and found gp37 associated with AcMNPV occlusion bodies. In addition, we found that both antisera recognized a 37-kDa protein from extracts of AcMNPV-infected *S. frugiperda* cells.

Immunoelectron microscopy was used to precisely locate gp37 in both OpMNPV and AcMNPV polyhedra and infected cells. gp37 was not found in OpMNPV polyhedra but was highly concentrated in cytoplasmic inclusion bodies late in infection. However, in AcMNPV polyhedra, gp37 was distributed within the polyhedron matrix with an apparent affinity for the periphery of occluded virions. There was no

specific localization to the polyhedron envelope, as previously suggested (22). Although no function has been determined for this protein in the baculovirus infection cycle, Wu and Miller (24) were unable to delete the gp37 gene from the AcMNPV genome, which suggests that gp37 may play an essential role in the infection cycle.

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