

## Polyhedrin sequence determines the tetrahedral shape of occlusion bodies in *Thysanoplusia orichalcea* single-nucleocapsid nucleopolyhedrovirus

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A nucleopolyhedrovirus (NPV) isolated from the looper *Thysanoplusia orichalcea* L. (Lepidoptera: Noctuidae) (ThorNPV) is occluded in a tetrahedral protein matrix. The ORF of the ThorNPV polyhedrin gene contains 738 nt which code for 246 amino acids of the putative polyhedrin protein with an estimated molecular mass of 28778 Da. The promoter of this gene is similar in length to the promoter of *Spodoptera frugiperda* NPV (SfMNPV), with a 5 nt deletion before the start codon compared to those of other NPVs. When the polyhedrin gene of *Autographa californica* NPV (AcMNPV), whose occlusion bodies (OBs) are polyhedral, was replaced by the polyhedrin gene of ThorNPV, which produces

tetrahedral OBs, tetrahedral polyhedra with properly occluded virions were produced. This work establishes the importance of the polyhedrin protein sequence in determining OB shape. Leucine at position 43 of ThorNPV polyhedrin was identified as responsible for the tetrahedral shape of ThorNPV OBs by PCR-based site-directed mutagenesis. Susceptibility to alkaline buffer of OBs formed by recombinant AcMNPV (RECAcV) carrying the polyhedrin gene of ThorNPV was slightly greater than that of native ThorNPV OBs. The LD<sub>50</sub> of RECAcV for third-instar beet armyworm (*Spodoptera exigua*) was significantly lower than that of AcMNPV (253 and 31 OBs per larva, respectively).

### Introduction

The distinct paracrystalline matrix of baculovirus occlusion bodies (OBs) as seen under thin-section transmission electron microscopy (TEM) is composed of polyhedrin protein. This protein primarily functions to protect the virions and viral DNA from environmental decomposition and inactivation by UV light (Blissard & Rohrmann, 1990). Polyhedrin is the most highly conserved baculovirus protein so far characterized. There is extensive identity among the nucleotide ( $\geq 70\%$ ) and amino acid ( $\geq 80\%$ ) sequences of polyhedrin proteins from different lepidopteran nucleopolyhedroviruses (NPVs) (Rohrmann, 1986). The polyhedrin protein has attracted much attention due to its high level of expression and function in virus survival (Rohrmann, 1986). Of the total alkali-soluble protein present in infected insects,  $> 17\%$  is polyhedrin (Quant *et al.*, 1984). It is this high level of expression from the polyhedrin gene promoter that makes recombinant baculovirus

expression vector systems (BEVS) very useful in foreign protein production (Smith *et al.*, 1983; Pennock *et al.*, 1984).

OBs formed by most NPVs infecting insects and crustacea are polyhedral (Federici, 1986). Investigations of how OBs form different shapes are antithetic. Experiments on the self-assembly of polyhedra formed by the NPV infecting *Bombyx mori* L. (BmNPV) indicate that the 6·26S polyhedron component recrystallizes *in vitro* into several different shapes depending on the physicochemical conditions (Shigematsu & Suzuki, 1971). These experiments and others (Hukuhara, 1971; Belloncik, 1989) suggest that the shape of OBs is determined by differences in cellular and environmental factors instead of being directed by the virus itself. When the granulin gene of *Trichoplusia ni* granulosis virus (TnGV) was substituted for the polyhedrin gene of BmNPV, and was expressed in insect cells, no granules were formed. Based on this, Zhou *et al.* (1998) suggested that the granulin sequence could not determine OB morphology. A contradictory scenario is suggested by point mutation and recombination experiments (Carstens *et al.*, 1986) with the polyhedrin gene of *Autographa californica* NPV (AcMNPV), whose OBs are polyhedral. Formation of cuboid polyhedra with virtually no occluded virions occurred when a single point mutation resulted in substitution of leucine for

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proline at amino acid 58 of the polyhedrin protein of the AcMNPV M5 morphology mutant. This mutated gene was used to replace the native polyhedrin gene of AcMNPV. The new mutant, AcM5polyI, produced cuboid and pyramidal polyhedra with few or no occluded virions. These point mutation and recombination experiments suggest that the shape of the polyhedron is determined by the polyhedrin gene itself.

An NPV was isolated from *Thysanoplusia orichalcea* L. (Lepidoptera: Noctuidae) collected in Indonesia. This virus (ThorNPV) was found to infect the soybean looper, *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae) (unpublished results). Initial observation of ThorNPV showed it to be a single-nucleocapsid virus with tetrahedral OBs. This paper reports the sequences of the polyhedrin gene and protein of ThorNPV. The polyhedrin gene was cloned into an AcMNPV BEVS in order to test the hypothesis that the tetrahedral shape of the OB of ThorNPV is governed only by its polyhedrin gene. An amino acid responsible for the tetrahedral shape of ThorNPV was identified. The infectivity and alkaline susceptibility of recombinant AcMNPV were also tested.

## Methods

**Insect rearing.** Soybean loopers were from a laboratory colony established using eggs obtained from the United States Department of Agriculture Agricultural Research Service (USDA-ARS) Southern Insect Management Laboratory, Stoneville, MS. Beet armyworms (*Spodoptera exigua*) (Lepidoptera: Noctuidae) were kindly provided by John J. Hamm (USDA-ARS, Tifton, GA). Larvae were individually reared on pinto bean artificial diet (Burton, 1969) at  $27 \pm 1$  °C, 75% relative humidity and 16:8 h (light:dark) photoperiod.

**Propagation of ThorNPV.** Infected *T. orichalcea* collected from carrots in Indonesia in 1992 and frozen at  $-20$  °C were transported to the United States where they were thawed and macerated in a glass homogenizer containing 0.1% SDS. After filtering the homogenate through four layers of cheesecloth, the virus suspension was subjected to differential centrifugation (Harrap *et al.*, 1977). The semi-purified OBs were used to prepare an inoculum (0.07 ml at  $1 \times 10^7$  OBs/ml) that was spread on the surface of the diet on which third-instar soybean loopers were feeding. Infected larvae showing swollen bodies and pale colour were collected and stored at  $-20$  °C.

**Purification of OBs, virions and genomic DNA.** Purification of ThorNPV OBs and virions from infected soybean loopers followed the procedure described by Harrap & Longworth (1974) and Harrap *et al.* (1977). DNA from pure virions was isolated using the resin-based genomic DNA isolation kit 100/G (Qiagen), following the 'cell culture' protocol of the kit.

**Restriction endonuclease digestion and Southern analysis.** Genomic DNA from ThorNPV was digested with *Hind*III, *Eco*RI, *Pst*I or *Xba*I (Promega) according to the manufacturer's recommendation. Fragments were fractionated by electrophoresis in a 0.6% agarose gel and transferred to a nylon membrane (Hybond-N+, Amersham) by the standard alkaline method (Southern, 1975; Chomczynski, 1992).

A plasmid containing the polyhedrin gene from *Orgyia pseudotsugata* MNPV (OpMNPV) was kindly provided by G. F. Rohrmann (Oregon State University, Corvallis, OR). A 1.85 kbp *Xba*I fragment containing

this polyhedrin gene was prepared as a probe by random labelling with digoxigenin according to the manufacturer's protocol (Genius I, Boehringer Mannheim).

Hybridizations were performed overnight at 50 °C with 20 ng/ml digoxigenin-labelled probe. The blot was washed twice for 5 min in  $2 \times$  SSC containing 0.1% SDS at 22 °C, and twice for 15 min in  $0.1 \times$  SSC containing 0.1% SDS at 62 °C. Chromogenic detection (Genius I) was used to visualize the hybridized probe.

### Cloning and nucleic acid sequencing of polyhedrin gene.

The hybridization pattern shown in Fig. 1 was used to deduce the position in the gel of fragments that might contain the polyhedrin gene of ThorNPV. These fragments were ligated into pBluescript II SK(+) (Stratagene) and transformed into *Escherichia coli* DH5 $\alpha$  by electroporation. Recombined plasmids were digested with *Bam*HI and *Hha*I, followed by Southern analysis, and subcloned to obtain shorter fragments (about 400 bp) for sequencing. An ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) was used with universal T3 and T7 or M13 forward and M13 reverse primers to produce sequences of about 400 nucleotides of both the first and second strands of subcloned restriction fragments. Comparison of sequence information with nucleotide sequences for NPV polyhedrin genes was conducted with the DNA analysis program GCG V.8 (Genetics Computer Group).

### Expression of the ThorNPV polyhedrin gene by AcMNPV.

The BEVS MaxBac 2.1 (Invitrogen) was used to express the polyhedrin gene of ThorNPV in insect cells. The gene was first amplified by PCR from the clone designated pXB-1. The forward (5' ctgctcgagATGT-ATACACGTTAC 3') and reverse (5' tgctctagaTGAGAGCTATCGTTG 3') primers were commercially synthesized (Integrated DNA Technologies). Lower-case letters denote additional restriction sites (*Xho*I and *Xba*I) and clamping sequences used to facilitate cloning of the PCR product and control its orientation for correct expression (Fig. 2). Purified PCR product was cloned into a T-vector prepared from pBluescript II SK(+) according to Marchuk *et al.* (1991). Restriction and sequence analyses verified that one of these clones, designated pB5, contained the ORF of the ThorNPV polyhedrin gene. This clone was digested with *Xho*I and *Xba*I and the resulting fragment was inserted into pBlueBac4.5 which was used to vector insertion of the ThorNPV polyhedrin gene ORF into Bac-N-Blue AcMNPV. Transfection of log phase *Spodoptera frugiperda* cells (Sf9) and isolation of recombinant AcMNPV (RECAcV) followed the manufacturer's protocol (Invitrogen), with progress of infection monitored daily. Insertion of the ORF for the ThorNPV polyhedrin gene into AcMNPV was confirmed by PCR. Scanning electron microscopy (SEM) and TEM were used to determine the shape of polyhedra formed by RECAcV.

**Electron and light microscopy.** Viral OBs were purified from infected Sf9 cells as described above. For SEM, OB pellets were suspended in distilled water, spread on a glass slide, dehydrated at 37 °C for 30 min and coated with gold. For TEM, OB pellets were processed following the procedure of Granados & Lawler (1981). An Olympus inverted microscope was used for monitoring progress of cell infection.

**Site-directed mutagenesis of the ThorNPV polyhedrin gene.** DNA site-directed mutagenesis was carried out by PCR (Ausubel *et al.*, 1994), with clone pB5 as the template. Tyrosine at position 38 was mutated to phenylalanine and leucine at position 43 was mutated to isoleucine (Fig. 3). Phenylalanine and isoleucine are found at positions 38 and 43 in AcMNPV polyhedrin. Appropriate combinations of the mutated fragments, designated M1, M2 and M3, were cloned into pBlueBac4.5 which was used to vector insertion of these fragments into Bac-N-Blue AcMNPV, followed by subsequent infection of Sf9 cells and two rounds of plaque purification. OBs produced by these recombinant

AcMNPVs, designated AcM1, AcM2 and AcM3, were observed by SEM and TEM as above.

**■ Alkaline susceptibility of ThorNPV, AcMNPV and RECAcV.** Aliquots (250  $\mu$ l) of ThorNPV, AcMNPV and RECAcV, at  $5 \times 10^7$  OBs/ml, were mixed with equal amounts of alkaline buffer (0.1 M  $\text{Na}_2\text{CO}_3$ , pH 10.7) and incubated at 22 °C with gentle agitation. After incubation for 5, 10 and 15 min, 40  $\mu$ l samples were withdrawn and mixed with 40  $\mu$ l  $2 \times$  SDS sampling buffer and placed on ice. Aliquots (40  $\mu$ l) of each sample were loaded on a 10% SDS-PAGE discontinuous gel and electrophoresed with protein standard markers at 150 V for 1.5 h. The gel was then stained in Coomassie blue.

**■ Infectivity of AcMNPV and RECAcV to beet armyworm.** Because the soybean looper colony was contaminated with a cypovirus, an alternative species, beet armyworm, was used for the infectivity test. The method for bioassay followed Wang & McCarthy (1993) with the following modification. Lettuce leaf disks (4 mm) were substituted for the diet disks (Wang & McCarthy, 1993) and 50 third-instar beet armyworms were given each virus dose. The doses were 0.29, 2.9, 29, 290 and 2900 OBs per larva for AcMNPV, and 0.36, 3.6, 36.6, 366 and 3660 OBs per larva for RECAcV. Larvae were exposed to the virus-contaminated leaf disks for 15 h before being transferred to artificial diet.  $\text{LD}_{50}$ s were estimated by the computer program POLO-PC (Le Ora Software).

## Results

### Restriction and Southern analyses

Restriction enzyme digestion of ThorNPV genomic DNA by *Hind*III, *Eco*RI, *Pst*I or *Xba*I produced discrete bands with a broad size range (Fig. 1A). The labelled polyhedrin probe hybridized to only one fragment produced by each enzyme tested (Fig. 1B). All four fragments were cloned, and the 4 kbp *Xba*I fragment (pXB-1) was chosen for subcloning and sequencing.

### Nucleotide sequence

The ThorNPV polyhedrin gene had a single ORF of 738 bp encoding a deduced 246-amino-acid protein with a calculated molecular mass of 28 778 Da (Fig. 2). A polyadenylation signal was found 291 bp downstream of the translation stop codon (Fig. 2).

The nucleotide sequence of the ORF of the ThorNPV polyhedrin gene has 81% identity with those of AcMNPV and SfMNPV. The deduced amino acid sequence has 95% identity with that of OpSNPV (Fig. 3). A putative transcription start site with the core sequence TAAG (Rohrmann, 1986) was located 46 bp upstream of the translation start codon of the polyhedrin ORF (Fig. 2). This putative promoter region shared 85% of its nucleotide sequence with the same region from SfMNPV. Both ThorNPV and SfMNPV polyhedrin promoters showed a 5 nt deletion when compared with other NPV polyhedrin gene promoters.

### Expression of the ThorNPV polyhedrin gene

Compared to healthy Sf9 cells, the cells and nuclei of infected Sf9 cells started enlarging 24 h after co-transfection

with Bac-N-Blue AcMNPV and pBlueBac4.5 plasmids containing the ORF of the ThorNPV polyhedrin gene. OBs were observed in infected cells 3 days after transfection. About 20% of the Sf9 cells were infected 5 days after transfection, and the medium was collected for plaque purification and assay for blue RECAcV. Blue plaques were first visible 4 days after plating with the number increasing for a further 2 days. A second round of plaque purification and assay was performed to exclude non-recombinant AcMNPV.

The ORF of the ThorNPV polyhedrin gene was amplified by PCR from DNA of blue plaque-producing RECAcV from the second round of plaque purification. OBs formed by native Bac-N-Blue AcMNPV appeared polyhedral when observed by SEM (Fig. 4 B1) and TEM (Fig. 4 B2), but tetrahedral OBs were formed by ThorNPV (Fig. 4 A1, A2) and recombinant Bac-N-Blue AcMNPV containing the ORF of the ThorNPV polyhedrin gene (Fig. 4 C1, C2). OBs of RECAcV contained occluded virions with multiple nucleocapsids, but occluded virions seemed less numerous than in wt AcMNPV. Some virions were not entirely enclosed within the OB (Fig. 4 C2).

### Identification of the amino acid responsible for the tetrahedral shape of ThorNPV OB

Amino acids at positions 38 (tyrosine) and 43 (leucine) were found to be unique to the deduced amino acid sequence of the ThorNPV polyhedrin gene (Fig. 3). Therefore, it was assumed that one of these amino acids or both were responsible for the tetrahedral shape of the ThorNPV OB. To test this, three recombinant AcMNPVs were constructed as follows: leucine at position 43 was mutated to isoleucine (AcM1); tyrosine at position 38 was mutated to phenylalanine (AcM2); AcM3 carried both mutated amino acids.

Under SEM, AcM1 and AcM3 showed polyhedral OBs, while OBs from AcM2 were tetrahedral. This indicates that when leucine at position 43 of the ThorNPV polyhedrin protein was changed to the corresponding isoleucine from AcMNPV (in AcM1 and AcM3), the tetrahedral OB shape was changed to polyhedral. Therefore, leucine at position 43 of the ThorNPV polyhedrin amino acid sequence was responsible for the tetrahedral shape of ThorNPV OBs. When tyrosine at position 38 of the ThorNPV polyhedrin protein was mutated to the corresponding phenylalanine from AcMNPV (AcM2), OB shape remained tetrahedral. Therefore, tyrosine at position 38 of the ThorNPV polyhedrin amino acid sequence does not influence the tetrahedral OB shape.

### Alkaline susceptibility

OBs produced by RECAcV were slightly more soluble in alkaline buffer than those of native ThorNPV. Both ThorNPV and RECAcV produced more alkali-soluble polypeptides than AcMNPV. Polypeptide profiles of ThorNPV, AcMNPV and RECAcV were similar, with a dominant polypeptide of 25 kDa. The 30 kDa polypeptide in ThorNPV and RECAcV was

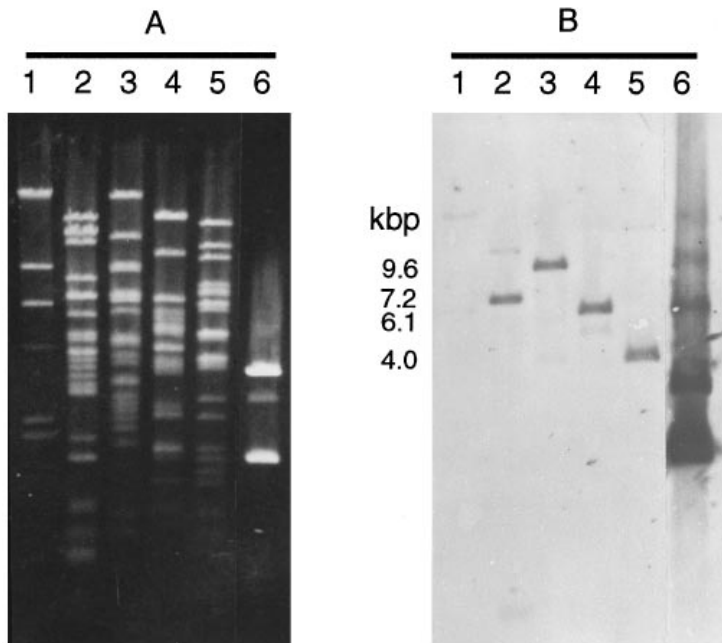


Fig. 1. Restriction enzyme digestion of ThorNPV genomic DNA (A) and Southern analysis using the OpMNPV polyhedrin gene as a probe (B). Lane 1, *Hind*III-digested  $\lambda$  phage DNA. Lanes 2–5, ThorNPV genomic DNA digested with *Hind*III, *Eco*RI, *Pst*I and *Xba*I, respectively. Lane 6, *Xba*I-digested plasmid containing OpMNPV polyhedrin gene.

probably undegraded polyhedrin protein, p29. However, this polypeptide was barely seen after the 15 min incubation period for AcMNPV. The most abundant polypeptide in RECAcV was one of 36 kDa. The nature of this polypeptide is unknown. Under these alkaline conditions at least four polypeptides with molecular masses below 30 kDa were produced, which were considered to be degraded polyhedrin protein.

### Infectivity test

RECAcV showed significantly less virulence than AcMNPV for third-instar beet armyworm ( $P < 0.01$ ). The  $LD_{50}$ s of RECAcV and AcMNPV against third-instar beet armyworm were 253 and 31 OBs per larva, respectively, which is about an eightfold difference (Table 1). OBs formed by RECAcV in beet armyworm showed tetrahedral shape and proper occlusion of virions, as verified by SEM and TEM (data not shown).

### Discussion

This study of ThorNPV arose in part from the discovery of tetrahedral and polyhedral OBs in soybean loopers infected with the original semi-purified NPV isolate from infected *T. orichalcea* collected in Indonesia (unpublished data). Although reports of NPVs with tetrahedral shape exist (Smith, 1976; Young & Yearian, 1983), little was known about the molecular biology of these NPVs and what determined OB shape. Research reported here provides information on the molecular biology of genes encoding polyhedrin proteins and the role of these genes in directing OB shape.

The complete nucleotide sequences were determined for the polyhedrin gene and the upstream, putative promoter from ThorNPV. The identity alignments between the nucleotide and deduced amino acid sequences of the ThorNPV polyhedrin gene and those from other insect NPVs confirm that this gene is a member of the baculovirus family of polyhedrin protein genes. The nucleotide sequences of the ThorNPV polyhedrin gene and putative promoter have greatest identity to those of SfMNPV. However, the deduced amino acid sequence of the ThorNPV polyhedrin protein showed the highest identity to that of OpSNPV (Fig. 3).

Nucleocapsid morphology has been used to separate the genus NPV into the subgenera SNPV and MNPV (Francki *et al.*, 1991). Neither nucleotide nor amino acid sequence identities to polyhedrin genes and proteins from other NPVs appear to provide a discrete way of classifying ThorNPV into a subgenus, because the polyhedrin gene and its promoter have more identity to those of multiple-nucleocapsid NPVs. This disagreement between molecular (polyhedrin gene and amino acid sequences) and morphological information in the classification of SNPV and MNPV morphotypes was also observed by Zanotto *et al.* (1993). Therefore, in the present (sixth) ICTV report (Murphy *et al.*, 1995), the use of single or multiple nucleocapsid as a taxonomic character has been dropped. Viruses are still named after the host from which they were isolated (Matthews, 1982; Francki *et al.*, 1991; Murphy *et al.*, 1995), and taxonomic classification of baculoviruses is still based on virus morphology and structure, because morphology is the first character observed and molecular information is either unavailable or not easily obtained. The shape of OBs can be an important morphological marker, especially when working with a mixed virus infection (Smith,

TAGTTTTCTTATGCGATTGGTTATTTTATCAAGCTTAAAGCGGTTCAACGAGTCCGCTTCG  
 HindIII  
 TTTAAACAACAACATGATGAGACATTTACTCTGCGTCCGTGTAGATGTAAGTTCCCGGT  
 HhaI  
 GAATGTGTAATCTTGTGCGACAGGCTAGTATTATATTCATGAGATCGCGCAGAGTCA  
 GTTGACATTCGCGTGTATTATATTAGGAACAGTTCAAAATAAAGATTGTATTGGAACCGG  
 TAGGTTTTTATTGTAAGTATTTTTCGCTTTTGTAAACAGATTGTGAAAAATCAAAATATA  
 -46  
 \*M Y T R Y S Y S P S L G R T Y V Y D N K  
 ATGTATACAGTTACAGCTACAGCCCGTCTGGCCGACCTACGTATACGATAACAG 60  
 Y Y K N L G A V I K N A K R K K H Y A R  
 TATTACAAAAATTTAGGTGCTGTCAATAAAGAACGAAACGCAAGAAGCCTACGCCGAG  
 HindIII  
 .H...L...E E A T L D P L D N Y L V A E D P  
 CATGAALTAGAAGAAGCCACTTTAGATCCCTAGATAACTACCTAGTTCGAGGATCCT 120  
 Primer RP  
 F L G P G K N Q K L T L F K E I R N V K  
 BamHI  
 TTCTTGGTCCCGGTAAGAACCAGAACTCCTTTGTTCAAGAAATCCGTAATGTAAAG  
 P D T M K L V V N W S G K E F L R E T W  
 CCTGATACCATGAAGCTTCTCGTTAACTGGACCGTAAAGATTCTCGAGGAACTTGG 300  
 HindIII  
 T R F M E D S F P I V N D Q E I M D V F  
 ACCGTTTCATGGAAGACAGCTTTCCTATTGTTAACGACCAGGAAATATGGATGCTTT 360  
 L V I N M R P T R P N R C F K F L A Q H  
 CTTGTAATAAACATGCGCCCAACCAGACCAACAGATGCTCAAGTTCGACAGCAT 420  
 HhaI  
 A L R C D P D Y V P H E V I R I V E P S  
 GCTCTGCTGCGACCCCTGACTACGTTCCCTCATGAAGTCATCCGCAATGTCGAGCTTCT 480  
 W V G S N N E Y R I S L A K K G G G C P  
 TGGTGGGCGACCAACAAGATACCCGATCAGCTTGGCTAGAAGGGCGGTGCTGCCCA 540  
 I M N L H S E Y T N S F E E F I N R V I  
 ATCATGAACCTTCATTCGAGTACACCACTCTTGGAGGATTCATCAACCGTGTGATC 600  
 W E N F Y K P I V Y V G T D S A E E E E  
 TGGGAGAAGCTTCTACAAACCCATTTGTTATGTAGGACCCGACTCTGCCAAGAGAGGAA 660  
 I L L E V S L V F K I K E F A P D A P L  
 ATCCTCTGTAGGTGCTTTAGTGTCAAAATCAAAAGATTTCGCCCCAGACCGCTTTA 720  
 HhaI  
 Y S G P A Y \*  
 TACTCTGGCCCGCATACTAAGCCGTTGATTCGTTTCAACGATAGCTCTCTTTTGTGTA  
 AGATATTGATCCAATAATTTTTTCTCGCCCGGTTTTTATTAGGTTTATTGACTAGACGC  
 GAAGCTGTATAGATATAGAGCTTAACTCGTCTGTTGTAATGTTTTCCTGTGAATTTTA  
 TTCATGAGGTTAGTGTAAATCGACAGAACCTGTAACAGTTTTCGCTGTCGCTTAAACGACCG  
 TTATCGTCTCGGTTACTGAAGATTCTTCGCTGCTGAGTTTCTCGATTCTAAACGTTTTT  
 TTAACACGTTTAAATAAGGATTAATGTTGATATAGGTTTTTATTGACGCTTCCCTCCGTC  
 +291  
 ATGATTTTTTTTTTACTTTTTTAAAGTTGATCTTTACGTTATAGCTTCCATGAGCTC  
 BamHI  
 AGATCGAATGCCATGACAGCGGAAGAAGTTTGTGTTGTTGCTGACTGCTGG

Fig. 2. Nucleotide sequence of the ThorNPV polyhedrin gene and adjacent flanking regions. The deduced amino acid sequence of the ORF is also shown. Putative transcription start and polyadenylation sites are shown in bold. \*, Translation start and stop codons. Sequences in solid boxes indicate positions of primers used for PCR amplification of the ThorNPV polyhedrin gene. Sequences in dotted boxes were used for primers for site-directed mutagenesis. Lower-case letters within primer sequences indicate bases to be mutated. Arrows denote direction of synthesis from primers in PCR. Slanted arrows above the sequence point to introduced amino acids.

1976). The tetrahedral shape of ThorNPV made it easy to identify in a mixed infection with another polyhedral-shaped NPV in soybean loopers during the initial isolation of ThorNPV (unpublished results). The disagreements between molecular and morphological classifications observed here and by Zanotto *et al.* (1993) are interesting in the light of the present investigation, which found genes encoding polyhedrin proteins to be important in determining polyhedron shape.

OB formation by AcMNPV was altered from its normal polyhedral shape to the tetrahedral shape of ThorNPV by recombination of the polyhedrin gene from ThorNPV into linearized Bac-N-Blue AcMNPV. Moreover, the tetrahedral OBs formed by the recombinant RECAcV contained occluded virions. This recombinant tetrahedral RECAcV was isolated

AcMNPV	MPD-YSYRPTIGRTYVYDNKYKLNGLGAVIKNAKRKHFAEH	40
BmNPV	.N...T.....CL.....ILV..	40
OpMNPV	.....S.....LL..	40
SfMNPV	.YTR..N.SL.....F.....S.....LL..	41
OpSNPV	.YTR..N.SL.....QI..	40
ThorNPV	.YTR...S.L.....A.....Y..	41
AcMNPV	EIEEATLDPLDNYLVAEBPFLGPGKNQKLTFLKEIRNVKPD	81
BmNPV	.O..KQW.L...M.....S...	81
OpMNPV	.ED.KH.....H.M.....	81
SfMNPV	..R.....ER.V.....I..	82
OpSNPV	.A.H.....K.....	81
ThorNPV	.L.....	82
AcMNPV	TMKLVVWGKGEFYRETWTRFEMDSFPPIVNDQEVMDVFLVV	122
BmNPV	...I.N.S...L...V.....Y..A	122
OpMNPV	...I.N.S...L...V.....	122
SfMNPV	...N.S...L...V.....I..I	123
OpSNPV	...N.S...L...I.....I..I	122
ThorNPV	...N.S...L...I.....I..I	123
AcMNPV	NMRPTRPNRCYKFLAQHALRCDPDYVPHDVIRIVEPSWVGS	163
BmNPV	.LK.....WEE...E...M...Y..M	163
OpMNPV	.....W.C...E...E...M	163
SfMNPV	.....E...E...VY..N	164
OpSNPV	.....FR.....E...E...Y..	163
ThorNPV	.....F.....E.....	164
AcMNPV	NNEYRISLAKKGGGCPIMNLHSEYTNFSFEQFIDRVIWENFY	204
BmNPV	.....I.....S.VN.....	204
OpMNPV	.....A.....S.VN.....	204
SfMNPV	.....V.....H...E..N.....	205
OpSNPV	.....R...V...A...E..N..H.....	204
ThorNPV	.....E..N.....	205
AcMNPV	KPIVYIGTDSAEEBEILEVSLVFKVKEFAPDAPLFTGPAY	245
BmNPV	.....I.....I.....	245
OpMNPV	.....S.....I.....	245
SfMNPV	...V...G...L...I...YN..	246
OpSNPV	...V...G...L...I...YS..	245
ThorNPV	...V...G...L...I...YS..	246

Fig. 3. Comparison of the deduced amino acid sequence of ThorNPV polyhedrin with those of polyhedrins from AcMNPV, BmNPV, OpMNPV, OpSNPV and SfMNPV. Dots indicate identity to AcMNPV and dashes represent gaps introduced to maximize alignment. The dotted box shows a proline residue that was altered to leucine in the AcMNPV M5 morphology mutant (Carstens *et al.*, 1986). Solid boxes indicate the unique amino acids of ThorNPV polyhedrin protein that were hypothesized to be responsible for tetrahedral shape determination.

from infected Sf9 cells and fed to soybean loopers, which subsequently became infected, died and yielded only tetrahedral OBs. However, the LD<sub>50</sub> of RECAcV for beet armyworm was significantly higher than that of AcMNPV (Table 1). Roosien *et al.* (1986) inserted the polyhedrin gene of *Mamestra brassicae* MNPV into AcMNPV and produced a recombinant AcMNPV infectious to beet armyworm. However, the recombinant had a much higher LD<sub>50</sub> than AcMNPV. Gonzalez *et al.* (1989) also produced a recombinant AcMNPV by insertion of the polyhedrin gene from SfMNPV which yielded properly occluded virions. However, the expression of SfMNPV polyhedrin in AcMNPV was only one-quarter that of wt AcMNPV. The high LD<sub>50</sub> of RECAcV for beet armyworm is possibly caused by the lower number of virions occluded (Fig. 4 C2). There was not enough difference in susceptibility to alkaline buffer of OBs from ThorNPV, AcMNPV and RECAcV to consider this a factor contributing to reduced infectivity of RECAcV. Other studies report the production of normally and abnormally shaped OBs by AcMNPV recombined with mutant polyhedrin genes from AcMNPV (Carstens *et al.*, 1986). However, these OBs contained few or no occluded virions. The polyhedrin gene not

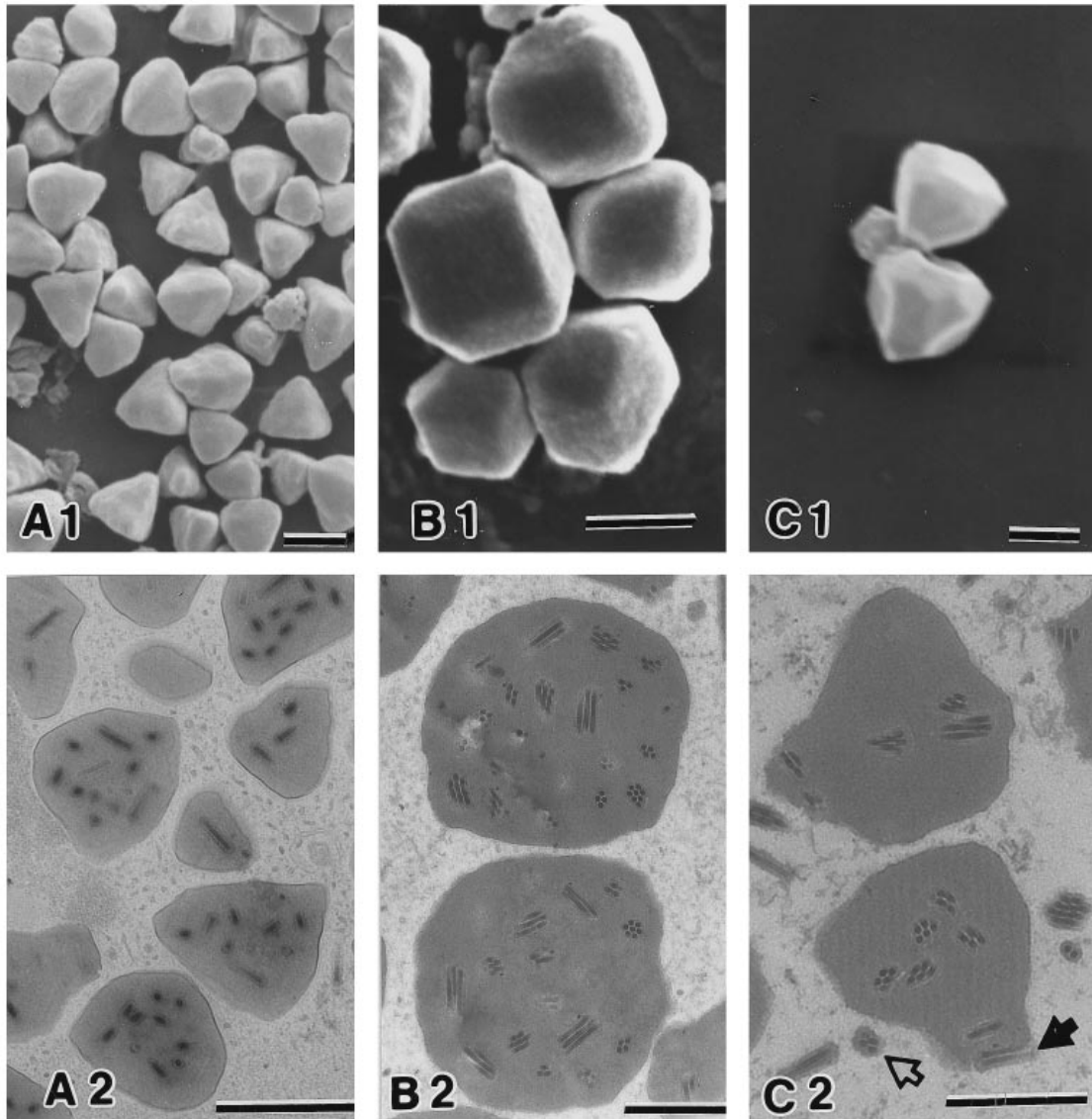


Fig. 4. Scanning (1) and transmission (2) electron micrographs of polyhedra formed by ThorNPV (A), Bac-N-Blue AcMNPV (B) and RECAcV (C). Bars represent 1 μm. Solid arrow indicates protruded virions. Open arrow indicates free virions not occluded.

**Table 1.** Calculated LD<sub>10</sub>, LD<sub>50</sub>, LD<sub>90</sub> and associated statistics for AcMNPV and RECAcV

LD<sub>10</sub>, LD<sub>50</sub>, LD<sub>90</sub> and associated statistics were estimated using the computer program POLO-PC (Le Ora Software). Each dose was applied to 50 third-instar beet armyworms.

Virus	Dose (OBs per larva)			Slope	Intercept
	LD <sub>10</sub>	LD <sub>50</sub>	LD <sub>90</sub>		
AcMNPV	2.9 (1.3, 5.2)*	30.9 (19.9, 48.4)	328.0 (182.7, 742.9)	1.250	-1.863
RECAcV	20.3 (11.5, 84.7)	253.1 (51.2, 1782.3)	3156.1 (680.8, 1.26 × 10 <sup>6</sup> )	1.169	-2.810

\* Numbers in parentheses represent lower and upper 95% confidence limits.

only appears to direct the shape formed during supramolecular assembly of the polyhedron, but appears to determine whether or not virions are occluded within.

Jarvis *et al.* (1991) found the domain between amino acids 19 and 110 of AcMNPV polyhedrin to be important for supramolecular assembly into OBs. The importance of this domain is supported by Carstens *et al.* (1986) whose recombinant AcM5poly1 virus had leucine substituted for proline at position 58 of wt AcMNPV polyhedrin (Fig. 4) and produced cuboid and pyramidal OBs with few or no occluded virions. We have shown by PCR-mediated site-directed mutagenesis that leucine at position 43 of ThorNPV polyhedrin controls the tetrahedral shape. When leucine was mutated to isoleucine, the tetrahedral shape of RECAcV was lost. The difference between leucine and isoleucine is in the position of a methyl group. The mutagenesis study suggests that moving this methyl group may lead to changes in protein secondary structure, and eventually in quaternary structure.

Interactions between other viral or cellular components during crystallization of ThorNPV polyhedrin produced by RECAcV were not considered in the current investigation. Jarvis *et al.* (1991) suggested that such interactions are not necessary for supramolecular assembly of polyhedrin into occlusion particles. Nakazawa *et al.* (1996) used AcMNPVs recombined with polyhedrin genes from four BmCPV strains to show that BmCPV virions and other BmCPV proteins are not needed for supramolecular assembly, and that assembly of polyhedra only requires the correct polyhedrin protein. Therefore, it is probable that the shape of the OB formed during supramolecular assembly of polyhedrin is determined by the amino acid sequence of the polyhedrin protein.

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## References

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1994). *Current Protocols in Molecular Biology*. New York: John Wiley.
- Belloncik, S. (1989). Cytoplasmic polyhedrosis viruses – *Reoviridae*. *Advances in Virus Research* **36**, 173–209.
- Blissard, G. W. & Rohrmann, G. F. (1990). Baculovirus diversity and molecular biology. *Annual Review of Entomology* **35**, 127–155.
- Burton, R. L. (1969). Mass rearing the corn earworm in the laboratory. *Agriculture Research Service ARS-134*.
- Carstens, E. B., Krebs, A. & Gallerneault, C. E. (1986). Identification of an amino acid essential to the normal assembly of *Autographa californica* nuclear polyhedrosis virus polyhedra. *Journal of Virology* **58**, 684–688.
- Chomczynski, P. (1992). One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Annals of Biochemistry* **201**, 134–139.
- Federici, B. A. (1986). Ultrastructure of baculoviruses. In *The Biology of Baculoviruses*, vol. 1, pp. 61–88. Edited by R. R. Granados & B. A. Federici. Boca Raton: CRC Press.
- Francki, R. I. B., Fauquet, C. M., Knudson, D. L. & Brown, F. (editors) (1991). Classification and Nomenclature of Viruses. Fifth Report of the International Committee on Taxonomy of Viruses. *Archives of Virology, Supplementum 2*, 1–119.
- Gonzalez, M. A., Smith, G. E. & Summers, M. D. (1989). Insertion of the SfMNPV polyhedrin gene into an AcMNPV polyhedrin deletion mutant during viral infection. *Virology* **179**, 160–175.
- Granados, R. R. & Lawler, K. A. (1981). *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. *Virology* **108**, 297–308.
- Harrap, K. A. & Longworth, J. F. (1974). An evaluation of purification methods for baculoviruses. *Journal of Invertebrate Pathology* **24**, 55–62.
- Harrap, K. A., Payne, C. C. & Robertson, J. S. (1977). The properties of three baculoviruses from closely related hosts. *Virology* **79**, 14–31.
- Hukuhara, T. (1971). Variations in cytoplasmic polyhedrosis virus. In *The Cytoplasmic Polyhedrosis Viruses of the Silkworm*, pp. 61–78. Edited by H. Aruga & Y. Tanada. Tokyo: Tokyo University Press.
- Jarvis, D. L., Bohlmeier, D. A. & Garcia, A. (1991). Requirements for nuclear localization and supramolecular assembly of a baculovirus polyhedrin protein. *Virology* **185**, 795–810.
- Marchuk, K., Drumm, M., Saulina, A. & Collins, F. S. (1991). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Research* **19**, 1154.
- Matthews, R. E. F. (1982). Classification and nomenclature of viruses. *Intervirology* **17**, 1–99.
- Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A. & Summers, M. D. (editors) (1995). *Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses*. Vienna & New York: Springer-Verlag.
- Nakazawa, H., Kendirgi, F., Bellonick, S., Ito, R., Takagi, S., Minobe, Y., Higo, K., Sumida, M., Matsubara, F. & Mori, H. (1996). Effect of mutations on the intracellular localization of *Bombyx mori* cytoplasmic polyhedrosis virus polyhedrin. *Journal of General Virology* **77**, 147–153.
- Pennock, G. D., Shoemaker, C. & Miller, L. K. (1984). Strong and regulated expression of *Escherichia coli*  $\beta$ -galactosidase in insect cells with a baculovirus vector. *Molecular and Cellular Biology* **4**, 399–406.
- Quant, R. L., Pearson, M. N., Rohrmann, G. F. & Beaudreau, G. S. (1984). Production of polyhedrin monoclonal antibodies for distinguishing two *Orgyia pseudotsugata* baculoviruses. *Applied and Environmental Microbiology* **48**, 732–736.
- Rohrmann, G. F. (1986). Polyhedrin structure. *Journal of General Virology* **67**, 1499–1513.
- Roosien, J., Usmany, M., Klinge-Roode, E. C., Meijerink, P. H. S. & Vlak, J. M. (1986). Heterologous recombination between baculoviruses. In *Fundamental and Applied Aspects of Invertebrate Pathology*, pp. 389–392. Edited by R. A. Samson, J. M. Vlak & D. Peters. Wageningen: Foundation of the Fourth International Colloquium of Invertebrate Pathology.
- Shigematsu, H. & Suzuki, S. (1971). Relationship of crystallization to the nature of polyhedrin protein, with reference to a nuclear-polyhedrosis virus of the silk-worm, *Bombyx mori*. *Journal of Invertebrate Pathology* **17**, 375–382.
- Smith, K. M. (1976). *Virus–Insect Relationships*. New York: Longman.

**Smith, G. E., Summers, M. D. & Fraser, M. J. (1983).** Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molecular and Cellular Biology* **3**, 2156–2165.

**Southern, E. M. (1975).** Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.

**Wang, S. & McCarthy, W. J. (1993).** Purification and characterization of the *Platynota idaeusalis* baculovirus pathogenic to the tufted apple bud moth. *Journal of Invertebrate Pathology* **62**, 37–46.

**Young, S. Y. & Yearian, W. C. (1983).** Pathology of a nuclear

polyhedrosis virus of *Rachiplusia nu* in *Rachiplusia ou* (Lepidoptera: Noctuidae). *Journal of Invertebrate Pathology* **42**, 410–412.

**Zanotto, P. M. A., Kessing, B. D. & Maruniak, J. E. (1993).** Phylogenetic interrelationships among baculoviruses: evolutionary rates and host association. *Journal of Invertebrate Pathology* **62**, 147–164.

**Zhou, C. E., Ko, R. & Maeda, S. (1998).** Polyhedron-like inclusion body formation by a mutant nucleopolyhedrovirus expressing the granulin gene from a granulovirus. *Virology* **240**, 282–294.

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