Morphological and molecular evidence that *Culex nigripalpus* baculovirus is an unusual member of the family *Baculoviridae*

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We present evidence that a newly discovered mosquito virus from *Culex nigripalpus* is an unusual member of the family *Baculoviridae*. Development of this virus was restricted to nuclei of midgut epithelial cells in the gastric caeca and posterior stomach. The globular occlusion bodies were not enveloped, measured around 400 nm in diameter, occurred exclusively in nuclei of infected cells and typically contained four, sometimes up to eight, virions. The developmental sequence involved two virion phenotypes: an occluded form (ODV) that initiated infection in the midgut epithelial cells, and a budded form that spread the infection in the midgut. Each ODV contained one rod-shaped enveloped nucleocapsid (40 \( \times \) 200 nm). The double-stranded DNA genome was approximately 105–110 kbp with an estimated GC content of 52%. We have sequenced approximately one-third of the genome and detected 96 putative ORFs of 50 amino acids or more including several genes considered to be unique to baculoviruses. Phylogenetic analysis of the amino acid sequences of *DNApol* and p74 placed this virus in a separate clade from the genera *Nucleopolyhedrovirus* and *Granulovirus*. We provisionally assign this virus in the genus *Nucleopolyhedrovirus*, henceforth abbreviated as CuniNPV (for *Culex nigripalpus* nucleopolyhedrovirus), and suggest that, awaiting additional data to clarify its taxonomic status, it may be a member of a new genus within the family *Baculoviridae*.

Introduction

The first modern report of a mosquito pathogenic virus was made in 1963 with the discovery of a cytoplasmic polyhedrosis virus isolated from *Culex tarsalis* (Kellen et al., 1963), now believed to be a densonucleosis virus (Federici, 1985). Additional viruses pathogenic to Culicidae have been reported from many different mosquito hosts by researchers in the United States, Europe and Russia (Federici, 1985). The first report of a mosquito baculovirus was from *Aedes sollicitans* (AesoNPV) (Clark et al., 1969). Natural infections with baculoviruses, all in the genus *Nucleopolyhedrovirus*, have been described for ten mosquito species representing the genera *Aedes*, *Anopheles*, *Culex*, *Psorophora*, *Uranotaenia* and *Wyeomyia* (Federici, 1985). The cytopathology and morphology of AesoNPV have been investigated (Federici & Anthony, 1972; Federici & Lowe, 1972; Federici, 1980; Stiles et al., 1983) but the molecular characteristics of mosquito baculoviruses or their relationship to baculoviruses from other insects is not known. Here we report on the cytopathology, morphology and molecular characteristics of a newly isolated baculovirus from the mosquito *Culex nigripalpus* and its relationship to other entomopathogenic viruses.

Methods

- **Virus production.** *Culex nigripalpus* larvae infected with a baculovirus were collected from a man-made settling pond of swine effluent located in Gainesville Florida (accompanying paper: Becnel et al., 2001). A laboratory culture of this baculovirus was initiated by amplification of field-collected virus from *C. nigripalpus* in colony *C. quinquefasciatus*. Virus concentrations were based on larval equivalents (LE). Approximately 3000 3-day-old *C. quinquefasciatus* larvae were exposed to 100 LE
of virus in 14 mM MgCl₂ (Becnel et al., 2001) and harvested 48 h post-inoculation (p.i.). Groups of 50 infected larvae were frozen in deionized water at −80 °C and used as inoculum for culture maintenance. Tissue culture production methods were unavailable and all studies were done in vivo.

**Infection of larvae for histopathology.** Groups of 100 3 to 4-day-old *C. quinquefasciatus* or *C. nigripalpus* larvae were exposed to 20 LE virus in 100 ml of water with 10 mM MgCl₂ plus 0.02% alfalfa and potbelly pig chow mixture (2:1). Groups without the addition of the virus served as controls. Exposed and unexposed larvae were removed at 30 min, 1, 2, 4, 8, 12, 13, 14, 15, 16, 18, 24, 32 and 48 h p.i. for histological studies.

**Histopathology.** The larvae of *C. quinquefasciatus* and *C. nigripalpus* have a relatively clear cuticle allowing the infected cells of the midgut to be detected with a dissecting microscope (Fig. 1a, b). Midguts were dissected from virus-infected and uninfected larvae in Ringer’s solution (Becnel, 1997). Cytopathological effects were determined by examination of mounted midguts by phase-contrast microscopy. Midguts were prepared for ultrastructural examination by primary fixation in 2.5% glutaraldehyde for 2 h, post-fixed in 2% osmium tetroxide, dehydrated in an ethanol series and embedded in Epon–Araldite. Thin sections, stained in uranyl acetate and lead citrate, were photographed at 75 kV. Suspensions of virions, released by alkaline treatment, were negatively stained in 1% phosphotungstic acid on coated grids and photographed at 75 kV.

**Heat stability.** Aliquots (5 LE) of crude viral suspensions or purified virus occlusion bodies (OBs) (approximately 1 x 10⁸ OBs/ml) in deionized water (0.5 ml final volume) were placed in microcentrifuge tubes, heated for 10 min in a water bath at temperatures from 35 to 60 °C in 5 °C increments and then chilled on ice. The heat-treated virus was fed to 3-day-old larvae in 10 mM MgCl₂ (following the standard protocol described above) and examined 2 days p.i. for evidence of infection.

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**Fig. 1.** Dissected midguts from *C. nigripalpus* third-instar larvae. (a) Healthy stomach and gastric caeca (GC) with Malpighian tubules (MT) attached. (b) Gross morphology of the stomach and gastric caeca infected with CuniNPV with Malpighian tubules removed. Infections of CuniNPV in the gastric caeca were primarily restricted to the proximal cells while cells in the distal portions are uninfected. The nuclei of virtually every cell in the posterior stomach (PS) were infected with CuniNPV while the cells of the anterior stomach (AS) were uninfected. Bars, 0.5 mm.
Characterization of a mosquito baculovirus

**Virus OB purification.** OBs were purified from 5-day-old (48 h p.i.) *C. quinquefasciatus* larvae, infected as described above. Larvae were ground with a Tekmar Tissuemizer in 0.1% aqueous SDS and filtered through polyester to remove large parts. The filtrate was spun at 1470 g for 5 min and the supernatant filtered through a 0.45 µm hydrophobic filter. The filtrate was further purified by differential centrifugation on a continuous Ludox gradient (Undeen & Alger, 1971) at 30% Ludox (30 min at 16,320 g). The OBs banded at a density of about 1.14–1.18 g/ml on the continuous gradient and pelleted in 30% Ludox. They were washed in 0.1 M NaOH, pH 10.0 (twice) followed by deionized water (twice) and stored at 4 °C. The OB concentration was estimated spectrophotometrically by establishing a standard curve at an OD of 260 nm. OBs were quantified initially with a Petroff–Hauser counting chamber and darkfield microscopy optics. The OD260 was measured for different concentrations, and a regression analysis done on the data. The resulting regression equation was used to calculate the OB concentration based on OD260.

**Protein sequencing.** A pure OB suspension (5 x 10^9 OBs/µl) was boiled in sample loading buffer (4 x buffer: 250 mM Tris–HCl, pH 6.9, 12% SDS, 20% β-mercaptoethanol, 40% glycerol) for 5 min to solubilize proteins. The proteins were separated on a standard SDS–PAGE gel (5% stacking gel, 10% separating gel) (Laemmli, 1970) of dimensions 11 x 18 x 0.75 cm overnight at 20 V and transferred to a PVDF membrane using standard Western blotting procedures. The N-terminal sequences of a major protein band, of size approximately 29 kDa, was determined by Edman degradation chemistry at the University of Florida’s Protein Chemistry Core Facility. Protein comparisons with entries in the GenBank, EMBL, PIR and SWISS-PROT databases were performed with BLAST (Altschul et al., 1990).

**Viral DNA preparation.** *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV) DNA was isolated directly from purified OBs. Because OBs do not dissolve readily at pH < 12 (unpublished results), we modified the standard alkali release procedure for lepidopteran baculoviruses. Briefly, the OB suspension was incubated for 5 min at pH 12.0 (raised pH to 12.0 with 0.01 M NaOH, pH 12.0), neutralized with 1/10 vol. of 1 M Tris–HCl, pH 8.0 and digested with Proteinase K (2 µg/µl) in 1% SDS and 200 mM β-mercaptoethanol overnight at 56 °C. The following day, a standard phenol–chloroform extraction and ethanol precipitation were performed (Sambrook et al., 1989).

**Genome size estimate.** Viral DNA was sized by pulsed-field gel electrophoresis and restriction enzyme analysis. Approximately 3 µg of viral DNA was restricted with *XbaI, SpeI* or *BglII*. The resulting fragments were separated by agarose gel electrophoresis (0.6% agarose gel in 1 x TAE buffer, 15 V/cm, 4°C, 30 h) and visualized by UV transillumination.

**Shotgun cloning and sequencing.** Viral DNA was restricted with *PstI, HindIII* or *EcoRI*; the resulting fragments were ligated into dephosphorylated plasmid pUC19 and grown in *E. coli* DH5a cells. Recombinant clones were selected by blue-white screening (Sambrook et al., 1989). Recombinant plasmid DNA was purified with the Qiaprep miniprep kit and restricted with the appropriate enzyme to estimate the size of the inserts. Additionally, dot-blot of the recombinant clones were probed with 32P-labelled or DIG-labelled viral DNA to confirm that viral DNA was cloned. DNA templates were sequenced at the University of Florida DNA Sequencing Core Laboratory using dideoxy chain terminator sequencing chemistries and an ABI PRISM 377 automated DNA sequencer. The sequences were assembled with Sequencher software.

**Nucleotide sequence analysis.** The sequence composition was determined with the Wisconsin Genetics Computer Group (GCG) software (Wisconsin Package version 10.0). DNA and protein comparisons with entries in the GenBank, EMBL, PIR and SWISS-PROT databases were performed with BLAST (Altschul et al., 1990). Two genes, a gene involved in DNA replication (*dnapol*) and a structural gene (*p74*), were selected for phylogenetic analysis.

A multiple amino acid sequence alignment of a conserved region of the DNA polymerase gene (Bulach et al., 1999) from eleven lepidopteran baculoviruses, one lepidopteran ascovirus, two entomopoxviruses (one from Orthoptera, one from Lepidoptera) and CuniNPV (see Fig. 6) was performed with GCG PileUp, CLUSTAL W (1.7) (Thompson et al., 1994) and PepTool (Wishart et al., 1997) computer programs and edited visually. Alignment files were analysed with PAUP 4β. The resulting sequence alignment was used to place confidence estimates on the groups contained in the optimal rooted tree. A multiple amino acid sequence alignment of nine lepidopteran baculovirus p74 proteins and the CuniNPV p74 protein was created and analysed in a similar fashion.

**Results**

A baculovirus pathogenic for the mosquitoes *C. quinquefasciatus* and *C. nigripalpus*, vectors of St Louis and Eastern encephalitis arboviruses, was isolated from mosquito larvae in agricultural wastewater in North Central Florida. We characterized this baculovirus in detail because it is highly virulent to these mosquito species.

**Gross pathology**

Virus infection affected development, behaviour and appearance of the *C. quinquefasciatus* and *C. nigripalpus* larvae. By 24 h p.i., infected larvae were stunted in size when compared to unexposed individuals. Larvae were feeding actively through about 48 h p.i. but by 72 h p.i. larvae were lethargic and often remained suspended at the water surface. By 48 h p.i., nuclei of most cells in the gastric caeca and posterior stomach appeared opaque to white in colour due to the proliferation of OBs (Fig. 1a, b). Cells in the anterior stomach rarely supported virus development (Fig. 1b). Death of the larvae usually occurred by 72–96 h p.i. at which time most nuclei in the posterior stomach and gastric caeca were infected (Fig. 1b).

**Histopathology**

Virus development, restricted to specific regions and cell types in the larval mosquito midgut, occurred primarily in resorbing/secreting cells found only in the proximal portions of the gastric caeca and the posterior stomach. Regenerative cells were infected while cells of the cardia were not infected. Cytopathological effects were first detected by 12 h p.i. in nuclei of epithelial cells in the gastric caeca and anterior portion of the posterior stomach (Fig. 2a, b). Healthy midgut epithelial
Fig. 2. Cytopathology of the larval midgut epithelium in third-instar of C. quinquefasciatus infected with CuniNPV. These are freshly dissected tissues examined by phase-contrast microscopy. (a, b) Transitional region between anterior (AS) and posterior stomach (PS) cells in healthy (a) and infected (b) larvae 14 h p.i. Infected cells (IN) in the posterior stomach (b) are hypertrophied with large nuclei and dispersed chromatin as compared to uninfected cells (UN) typified by a small nucleus with a well-defined nucleolus (a). FP, food plug; PM, peritrophic matrix. Bars, 0-1 mm. (c)–(h) Cytopathology of CuniNPV in epithelial cells of the posterior stomach. (c) Healthy cell demonstrating a nucleus with a well-defined centrally located nucleolus (arrow). (d) Infected cell (14 h p.i.) with margination of heterochromatin in the nucleus (arrow) and a disorganized and dense-appearing cytoplasm. (e) Infected nucleus with large, evenly dispersed areas of heterochromatin (arrow, 14 h p.i.). (f) Infected nucleus with OBs accumulated along the inner nuclear envelope (arrow, 16 h p.i.). (g) Infected nucleus (17 h p.i.) with numerous regions of highly refractile OB accumulation (arrow). Note the large cytoplasmic vacuoles (CV). (h) Nucleus filled with OBs (arrow, 24 h p.i.) Bars, 20 µm.
Fig. 3. Electron micrographs of a CuniNPV-infected posterior stomach epithelial cell during the first phase of virogenesis, which produces BV. (a) Infected nucleus with peripheral regions of heterochromatin and nucleocapsids dispersed throughout the nucleoplasm. Note the large accumulation of cytoplasmic vacuoles around the nucleus. Bar, 1.0 µm. (b) A single nucleocapsid in the process of budding through the nuclear envelope (arrow). The nucleocapsids (N) in the nucleoplasm are non-enveloped. Bar, 0.5 µm. (c) Higher magnification of the boxed region of (a) demonstrating a transport vesicle (TV) with several nucleocapsids after budding through the nuclear envelope (NE). Bar, 0.5 µm.

cells were characterized by distinct areas of cytoplasmic organelles and a nucleus with a dense, centrally located nucleolus (Fig. 2a, c). The first signs of infection were a rounding of the cell and a more granular and dense appearance of the cytoplasm (Fig. 2d). The centrally located nucleolus disappeared and heterochromatin accumulated along the inner
Ultrastructural features of virus development

Infected nuclei at various stages in the progression of virus development were found 24 h.p.i. Therefore, the ultrastructural observations reported here are principally from the examination of midgut tissues 24 h.p.i. and later. There were two phases of virus development, restricted to the nuclei of midgut epithelial cells: an early or first phase that produced budded virions (BV) and a later, second phase that produced occluded virions (ODV).

The initial signs of virus activity were cytoplasmic vacuolization, nuclear hypertrophy and margination of heterochromatin along the inner margins of the nuclear envelope (Fig. 3a). The early appearance of the virogenic stroma was as a loosely granular material dispersed throughout the nucleus (Fig. 3a). Empty capsids and nucleocapsids were located throughout the nucleoplasm and there was little or no evidence for nucleocapsid envelopment or occlusion (Fig. 3b, c). Rather, naked nucleocapsids budded through the nuclear envelope either singly or in groups (Fig. 3b, c). Once in the cytoplasm, transport vesicles (formed by the membranes of the nuclear envelope) released the nucleocapsids of the BV. BV accumulated at cell junctions, along the basement membrane and near the microvilli but we did not observe BV passing through any of these membranes (data not shown).

In the second phase, infected nuclei were characterized by a centrally located virogenic stroma with large parallel arrays of nucleocapsids, oriented primarily in one direction, at its periphery (Fig. 4a). Arrays of capsids and nucleocapsids were produced throughout the virogenic stroma (Fig. 4b, c). The stroma matured into dense structures located throughout the nucleoplasm that were involved in occlusion of virions (Fig. 5a). These areas were rich in capsids, nucleocapsids and virions in various stages of occlusion. OBs initially accumulated along the periphery of the nucleus and eventually filled the entire nucleoplasm of the infected nucleus (Fig. 5a). There was no evidence for the coalescence of the small globular OBs into larger OBs and the nuclear envelope remained intact throughout the process.

Mature OBs were globular in shape and uniform in size with a diameter of approximately 400 nm (Fig. 5b). The OBs lacked the envelope (the polyhedron envelope of NPVs) that is a characteristic feature of most other baculoviruses. Each OB typically contained four, sometimes up to eight, rod-shaped virions (Fig. 5a–c). Each virion consisted of one nucleocapsid, an intermediate layer and an outer envelope and measured approximately 200 × 40 nm (Fig. 5b).

Heat stability

After heat treatment of the viral suspension at 24 °C, the virus infection rate was 89.5 ± 3.3% (n = 6) and there was no significant difference between the crude or purified virus. Temperatures below 50 °C minimally affected virus infectivity. After heat treatment of the viral suspension at 50 °C, the infection rate was reduced to 6.4 ± 3.3% (n = 6) and at 55 °C it was 0.0% (n = 4).

Protein sequences

To further characterize this virus and determine its relationship to other baculoviruses, a major band of 29.7 kDa that we predicted might be the OB protein was isolated from purified OBs by SDS–PAGE (data not shown) and subjected to N-terminal sequence analysis. The N-terminal sequence, APQVK PRYRY AVAITH MDT, did not match any of the CuniNPV genome sequences or entries in the main databases using BLAST. It also showed no homology to the polyhedrin protein sequence of *Neodiprion sertifer* (Ns)NPV (published in Zanotto *et al.* (1993) with the permission of Robert D. Possee, Oxford, UK) or the N-terminal amino acid sequence of *Tipula paludosa* (Tp)NPV (Rohrmann *et al.*, 1981). These sequences are not in any of the major databases.

Genome size estimate

Based on restriction enzyme analysis and pulsed-field gel electrophoresis (results not shown), we estimated the CuniNPV genome size to be approximately 105–110 kbp. The BglII digest yielded five DNA fragments with estimated sizes of 6.2, 16.0, 23.0, 26.0 and 38.0 kbp; *SacI* digested the DNA into seven fragments of approximately 1.8, 2.6, 9.9, 18.0, 21.0, 25.0 and 31.0 kbp; *XbaI* digestion produced eight DNA fragments with estimated sizes of 3.5, 4.0, 4.8, 10.0, 11.8, 12.2, 25.0 and 33.5 kbp. A submolar band of 16.0 kbp in the *XbaI* digest may indicate the presence of more than one virus genotype.

Sequence data

To date we have sequenced approximately 33 kbp of the viral genome. The GC content of this sequence was 52%. We identified 96 CuniNPV potential ORFs of greater than 50 amino acids. Fifteen ORFs (∼16% of the predicted ORFs) were unique to CuniNPV and only eleven ORFs had a significant sequence similarity to the following lepidopteran baculovirus amino acid sequences: DNA polymerase, lef-1, lef-
Characterization of a mosquito baculovirus

Fig. 4. Electron micrograph of a CuniNPV-infected cell during the second phase of virogenesis, which produces ODV.
(a) A large centrally located virogenic stroma (VS) is surrounded by large parallel arrays of nucleocapsids (arrow). Note the margination of heterochromatin along the nuclear envelope and the accumulation of cytoplasmic vacuoles. Bar, 1·0 µm.
(b) Array of capsids (arrow) in the virogenic stroma. Bar, 0·25 µm.
(c) Cross-section of an array of nucleocapsids. Bar, 0·25 µm.

These sequences, several of which are considered to be unique to baculoviruses, clearly define CuniNPV as a member of the family Baculoviridae. The ORFs for p74 and p47 were

4, p47, p74, odv-e56, hypothetical 23·0 kDa protein, bro-a, p33, hypothetical 42·1 kDa viral capsid associated protein, and the product of Xestia c-nigrum granulovirus (XnGV) ORF33.

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overlapping. Orientations, sizes of the predicted ORFs and putative proteins are detailed in Table 1.

Sequence domains conserved among the α-like DNA polymerases and believed to be involved in 3′→5′ exo-nucleolytic activity, metal, DNA and dNTP binding (Blanco et al., 1991) are presented in the DNA polymerase amino acid sequence alignment (Fig. 6). The amino acid sequence of CuniNPV DNA polymerase contained regions Exo I, Exo II, Exo III and conserved regions I–IV (terminology of Blanco et al., 1991) in the same linear arrangement as the other baculoviruses. These regions correspond closely to those identified by Ahrens & Rohrmann (1996). The Exo I motif ‘DIET’ is preserved in all baculoviruses including CuniNPV. In region Exo II the aromatic residue Phe (F) always precedes the invariant Asp (D), except in CuniNPV where Asp (D) was preceded by Tyr (Y). In region Exo III, the motif ‘YN–D–L’ is preserved among all the baculoviruses including CuniNPV. The highly conserved motifs ‘D–SLYP’ (region I), ‘K–NS–YG’ (region II), ‘GR’ (region II) and ‘YGTDTDS’ (region III) were also present in the CuniNPV amino acid sequence. CuniNPV shared one invariant Glu (E) residue, two invariant Lys (K) residues and one invariant Tyr (Y) residue with the other baculovirus amino acid sequences in region IV. The DNA-binding motif Y–G(G/A), highly conserved among
family B DNA polymerases (Kähler & Antranikian, 2000), was also found in CuniNPV. Interestingly, in this alignment the CuniNPV DNA-binding motif YLGA was not located downstream of Exo III like the binding motif of the other baculoviruses but upstream at nucleotide positions 388–391. The DNA polymerase sequences showed considerable divergence (data not shown): Within the NPVs, sequence differences ranged from 0–59%, the GV sequence differed from the NPVs by about 62%, and the CuniNPV sequence differed from the NPVs by about 85% from the others. CuniNPV was basal to all the other baculoviruses but upstream at nucleotide positions 388–391.

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Table 1. Summary of BLAST amino acid search results: best matches to lepidopteran baculovirus amino acid sequences

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Discussion

This is the first report of a baculovirus from C. nigripalpus and only the third report from Culex (Federici, 1985). Both of the previously reported species were isolated in Louisiana, USA, one from C. salinarius (Clark & Fukuda, 1971) and the other from C. quinquefasciatus (Chapman, 1974). The only diagnostic information reported was that the NPV from C. quinquefasciatus had globular OBs, ranging from less than 1 to 5 µm in diameter (Federici, 1985). We also have isolated CuniNPV from field C. quinquefasciatus and C. salinarius in Florida (Becnel et al., 2001), and it cannot be ruled out that this is the same virus as in the earlier reports.

The only mosquito baculovirus that has been studied in any detail (AesoNPV) was isolated from Aedes sollicitans in Louisiana (Clark et al., 1969). Its pathology and morphology were investigated in several different mosquito hosts (Federici & Lowe, 1972; Federici & Anthony, 1972; Federici, 1980, Stiles et al., 1983). The baculovirus reported here as well as all other reported mosquito baculoviruses are restricted to larval midgut epithelial cells but there are differences in cell specificity. CuniNPV appears to selectively infect only the resorbing/secreting cells of the gastric caeca and the posterior stomach (Clements, 1992). The cells of the cardia are not infected and the cells of the anterior stomach are rarely infected. In AesoNPV the cardia, gastric caeca and the entire stomach supported virus development.

In studies with AesoNPV it was concluded that there was only a single replication cycle leading to the production of ODVs (Federici, 1980; Stiles et al., 1983). There was no evidence for the presence of BV or lateral transmission of the virus within the midgut. The cytopathology, virion formation and structure of the OBs for CuniNPV were similar to that detailed (AesoNPV) was isolated from Culex, and structure of the OBs for CuniNPV were similar to that reported for AesoNPV (Federici & Lowe, 1972; Federici & Anthony, 1972; Federici, 1980; Stiles et al., 1983). This sequence of events involved: hypertrophy of nuclei, frag-
mentation of the nucleolus followed by margination of heterochromatin to the nuclear envelope and the formation of the virogenic stroma. Rod-shaped nucleocapsids were formed within this stroma and finally enveloped and occluded. The production of OBs was similar in the CuniNPV and AesoNPV with a few notable differences. Both this virus and AesoNPV produced small OBs. These coalesced in AesoNPV to form large rugose ellipsoids (4–7 µm) and finally large smooth-surfaced spindles (up to 20 µm; Federici & Anthony, 1972). Although it did appear in fresh preparations of CuniNPV that
OBs coalesced to form larger OBs this was not confirmed with EM observations. At no point in development were OBs larger than approximately 0.5 µm observed in this new baculovirus. Another baculovirus from the mosquito Wyeomyia smithii also produced small OBs similar to this new virus (Hall & Fish, 1974). Those authors also observed that OBs appeared to coalesce in intact nuclei when viewed at the light level but could not confirm this with EM studies.

In insect baculoviruses, the primary site of infection is the midgut epithelium, where BV is produced that spreads the infection to other host tissues (Federici, 1997). We showed that spread of CuniNPV within the midgut apparently occurred by production of BV. Certain other NPVs from Hymenoptera, Thysanura, Trichoptera and Diptera (Federici, 1997) and one lepidopteran GV (Federici & Stern, 1990) also primarily infect the host’s midgut epithelium but the mechanisms by which the virus spreads from cell to cell in the midgut are not known. We surmise that a mechanism similar to the one described for CuniNPV involving BV is operating to disseminate the infection within the midgut.

We have presented evidence that virogenesis of this mosquito baculovirus involved two types of virions, ODV and BV. The first phase was initiated by ODVs that entered midgut cells through the plasmalemma of the microvilli. This initial replicative phase produced BV that disseminated the virus to other midgut cells. The nuclei of cells infected by BV may first...
Fig. 7. Phylograms derived from amino acid sequence analysis of DNA polymerases and p74 proteins. (a) Unrooted phylogram derived from analysis (maximum parsimony, PAUP 4* beta version 4.0b4a) of a polypeptide alignment of a conserved region of the DNA polymerase protein. The alignment was created with PileUp, CLUSTAL W and PepTool and was edited visually. CuniNPV branched from the tree prior to members of the genera Granulovirus and Nucleopolyhedrovirus. Similar phylogenies were obtained when rooting the tree with SAV and with distance-analysis methods. Bootstrap values on the rooted tree strongly supported the placement of CuniNPV into a taxon basal to XnGV and NPVs (not shown). For abbreviations see Fig. 6 legend. (b) Rooted phylogram generated from maximum parsimony analysis (PAUP 4* beta version 4.0b4a) of a polypeptide alignment of the p74 protein. The alignment was created with PileUp, CLUSTAL W and PepTool and was edited visually. Branch numbers indicate branch lengths (normal) and percentage of bootstrap replicates (italics) which contained that topology. The CuniNPV p74 protein was highly divergent from those of XnGV and the NPVs and branched first from the p74 gene tree. Abbreviations: SploNPV, Spodoptera littoralis NPV; see Fig. 6 legend.
produce additional BV for lateral transmission or more likely enter directly into the production of ODVs. This developmental cycle was similar to the cycles reported for other baculoviruses (Federici, 1997) except that the CuniNPV infections were confined to midgut tissues.

Unlike lepidopteran baculoviruses the OBs of CuniNPV were extremely resistant to dissolution at alkaline pH < 12·0. Federici & Anthony (1972) studied the chemical behaviour of OBs of AesoNPV. They reported that OBs rapidly dissolved when treated with 0·1 or 0·03 M NaOH (corresponding to pH values of approximately 13·0 and 12·3). They did not study the behaviour of OBs at lower alkaline pH.

The OB proteins of baculoviruses have been used extensively in phylogenetic analyses. The known polyhedrin and granulin baculovirus proteins are the predominant proteins in the NPVs and GVs and are approximately 29 kDa in size (Funk et al., 1997). By inference, we speculated that the 29·7 kDa protein band, which was a predominant polypeptide, was the CuniNPV OB protein. The only ‘polyhedrin’ sequence information for a dipteran baculovirus is that of TpNPV and studies on the relatedness of dipteran and lepidopteran OB proteins are limited (Guelpa et al., 1977; Rohrmann et al., 1981; Rohrmann, 1986). The N-terminal sequence of TpNPV showed no homology to the N-terminal sequence of the 29·7 kDa CuniNPV protein. Mosquito baculovirus OBs are adapted to persist in aquatic environments (C. nigripalpus is a freshwater mosquito, A. sollicitans a saltwater species), and it is not unexpected that these OB proteins would show low sequence homology to the granulins and polyhedrins of terrestrial insects.

We have sequenced approximately one-third of the CuniNPV genome and detected 96 ORFs of 50 amino acids or more, including several genes considered to be unique to baculoviruses. Its GC content of 52% was similar to that of Orgyia pseudotsugata (Op)MNVP (55%) and Lymantria dispar (Ld)MNVP (58%). In comparison, the GC content of Autographa californica (Ac)MNVP (41%) and Bombyx mori (Bm)NPV (40%) is much lower. On the assumption that this sequence information is representative of the whole genome of CuniNPV it can be inferred that it shares relatively few sequence homologies and a low degree of gene conservation with the other members of the family Baculoviridae (11/96 ORFs = ~ 11%).

We have identified four genes that are related to those in the baculovirus late expression factor (LEF) category (Lu & Miller, 1997): dna-pol, lef1, lef4 and p47. A number of domains of the dna-pol gene, involved in DNA replication, are highly conserved among different organisms. Among the baculoviruses, the dna-pol ORF was longest in CuniNPV (3417 nt). lef1 has been sequenced from seven lepidopteran baculoviruses and is essential for transient DNA replication in AcMNVP and OpMNVP. It has four conserved domains, three of which are also found in DNA primase genes, suggesting that lef1 may be a baculovirus primase gene (Lu et al., 1997). It is located adjacent to the egt gene in Charistoneura femorfera (Cf)MNVP, AcMNVP, OpMNVP, LdMNVP and Busura suppressaria (Busu)NPV, but not in Spodoptera exigua (Se)MNVP and the putative egt gene of CuniNPV. XnGV apparently does not have an egt gene.

lef4 and p47 are equimolar subunits of a DNA-dependent RNA polymerase that is required for transcription of viral late genes (Guarino et al., 1998) and their presence in CuniNPV suggest that the virus, like the other baculoviruses, encodes its own RNA polymerase. lef4 has been sequenced from AcMNVP, BmNPV, LdMNVP, OpMNVP, SeMNVP and XnGV, p47 has been sequenced from these viruses and also from BusuNPV and CinPV. In CuniNPV the ORFs of p47 and p74 overlapped, an unusual arrangement not found in any of the other baculoviruses studied so far. Promoter analysis of p74 and p74 revealed a possible p74 late promoter motif, TAAAT, at positions −15 to −12 upstream of the p74 start codon. A possible p47 start codon and promoter motifs were located at the 3′ end of the p74 ORF with the following nucleotide coordinates: 1932–1936 (CGTGC) at position 3). They did not study the ORFs of these two genes do not overlap but are immediately adjacent to each other. In the nucleopolyhedroviruses AcMNVP, BmNPV, CfMNVP, LdMNVP, OpMNVP and SeMNVP, the ORFs for p47 and p74 neither overlap nor are located next to each other (this information is not available for BusuNPV). The p74 gene encodes a structural protein associated with occluded viral envelopes (Funk et al., 1997). It is located adjacent to p10 in most of the NPVs including SeMNVP, AcMNVP, CfMNVP and OpMNVP but not in BusuNPV, Spodoptera litura (SpLi)NPV and XnGV. A p10 gene has not been identified on the CuniNPV sequence fragment that contains the p74 gene.

The odo-e56 gene encodes a structural protein of occluded viral envelopes (Funk et al., 1997). Its ORF is adjacent to the ORF of le-1 in AcMNVP, BmNPV, CfMNVP, LdMNVP and OpMNVP, but not in SeMNVP and XnGV where the two ORFs are distant from each other. odo-e56 did not appear to neighbour the putative le-1 in CuniNPV.

The study of gene arrangements in the phylogenetic analyses of baculoviruses in addition to gene homology has been discussed (Hu et al., 1998; Ikegami et al., 1999; Chen et al., 1999). Clearly, CuniNPV appeared to have a distinct gene arrangement and is highly divergent from the other known baculoviruses not only in gene homologies but also in gene order.

The CuniNPV DNApol and p74 amino acid sequence analysis suggested that CuniNPV is a member of the family Baculoviridae yet forming a clade separate from the lepidopteran NPVs and GVs. DNApol and p74 tree topologies among the NPVs and GV appeared to be similar to previously
published trees based on analysis of the DNA polymerase gene (Bulach et al., 1999), polyhedrin gene (Zanotto et al., 1993), lef2 gene (Chen et al., 1999), egt gene (Clarke et al., 1996) and gp64 gene (Liu & Maruniak, 1999). As discussed in Bulach et al. (1999), the DNA polymerase gene may be better suited for phylogenetic analysis than the polyhedrin gene because it has more informative characters and appropriate outgroup taxa are available.

Traditionally, the mosquito baculoviruses have been placed in the genus \textit{Nucleopolyhedrovirus}. Federici & Lowe (1972) suggested placement of the baculovirus from \textit{A. sollicitans} (AesoNPV) into a group separate from the NPVs and GVs, distinguished by the fusiform OBs (although based on our observations the use of fusiform OBs as a distinctive characteristic for the mosquito baculoviruses is not appropriate). The collective data available thus far from CuniNPV suggest that it is a member of a new genus within the family \textit{Baculoviridae} but more information is needed to resolve the taxonomic status of CuniNPV.

**Concluding remarks**

CuniNPV, a newly discovered mosquito virus, infects \textit{Culex} spp. with tissue specificity for gastric caeca and posterior stomach. The following morphological and molecular data place CuniNPV in the family \textit{Baculoviridae}. It has a double-stranded DNA genome (presumed to be circular) of approximately 105–110 kbp packaged into a rod-shaped enveloped capsid. The nucleocapsids average 40 nm in diameter and 200 nm in length. It has two virion phenotypes, an occluded form (ODV) that initiates infection in the midgut epithelial cells, and a budded form (BV) that spreads the infection within the midgut. This is in contrast to the other members of the family \textit{Baculoviridae}, where the BV is believed to infect other tissues. Each ODV contains one nucleocapsid. DNA replication occurs in the nucleus. Several genes diagnostic of members of the family \textit{Baculoviridae} have been identified. These include \texttt{lef1}, \texttt{lef4}, \texttt{odv-e56} and \texttt{p74}.

Like NPVs, the OBs are found exclusively in the nuclei of infected cells but unlike NPVs they are globular, not polyhedral (Blissard et al., 2000), in shape. They are similar in size (average diameter 400 nm) to GV OBs. Each OB typically contains four, sometimes up to eight, virions as opposed to GV that each OB contains one or two, rarely more virions. The CuniNPV gene order is distinct, and phylogenetic analysis of the p74 and DNA polymerase polypeptides placed the CuniNPV into a separate taxon basal to the NPVs and GV.

The data show that CuniNPV is a baculovirus with unusual characteristics. Pending additional data, we have elected to retain it in the genus \textit{Nucleopolyhedrovirus} but suggest that it may represent a new genus within the family \textit{Baculoviridae}.

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**References**


Characterization of a mosquito baculovirus


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