Analysis of polyhedra morphology mutants of *Autographa californica* nuclear polyhedrosis virus: molecular and ultrastructural features

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Two new mutants of *Autographa californica* multiple nuclear polyhedrosis virus affected in the morphogenesis of their polyhedra, designated M276 and M934, were investigated. Marker transfer experiments demonstrated that the observed phenotype was due exclusively to alterations in the polyhedrin gene. M276 contained a 229 base insertion near the carboxyl terminus coding region which resulted in synthesis of a truncated protein; M934 had a point mutation substituting phenylalanine for leucine at amino acid 183. Both mutations occurred in highly conserved regions of the protein and prevented the occlusion of virus particles, but did not affect targeting for the intranuclear ring zone. M276 was distinct in that it had prominent cytosolic condensations of polyhedrin, although these were probably due to decreased protein solubility. M934 polyhedrin condensations associated prematurely with calyx material such that it became incorporated into the condensation rather than at the surface. Results confirm that occlusion size and shape are features inherent to the polyhedrin protein, and suggest that polyhedrin conformation may help regulate the occlusion process.

Introduction

The Eubaculovirinae subgenus multiple nucleocapsid polyhedrosis viruses (MNPVs) are characterized by the production of intranuclear occlusion bodies referred to as polyhedra (Wilson, 1991). These serve as the protective package for the virus particles outside the infected host and allow natural transmission of virus between insect hosts, assisting persistence in the environment. The major component of the polyhedral occlusion bodies is a single protein called polyhedrin, with an M₉ of about 29000. This protein forms the paracrystalline lattice of polyhedra and interacts with enveloped virions as well as other accessory molecules and structures to form a mature viral occlusion body (Derksen & Granados, 1988; Whitt & Manning, 1988). A carbohydrate-rich surface layer (Minion *et al.*, 1979), called the calyx (Whitt & Manning, 1988), is a distinct structural entity attached to the paracrystalline lattice (Harrap, 1972). Its presence seems important for the structural stability of the mature polyhedra (Williams *et al.*, 1989). Regulatory mechanisms controlling polyhedron lattice initiation and accumulation, occlusion of enveloped virions, and cessation of growth concomitant with attachment of calyx are unknown. However, characteristics such as polyhedron size and shape are heritable features of morphogenesis and presumably are intimately related to the tertiary and quaternary structure of polyhedrin (Gershenson, 1960; Brown *et al.*, 1980; Carstens *et al.*, 1986).

The high conservation of polyhedrin amino acid sequence amongst different nuclear polyhedrosis viruses (Kozlov *et al.*, 1981; Vlak & Rohrmann, 1985) suggests a high selection pressure to maintain structure based on function. We have been interested in defining functional domains of the polyhedrin protein by determining amino acid changes that result in alteration of polyhedra morphogenesis or morphology. Two such *Autographa californica* MNPV (AcMNPV) mutants have been characterized at the nucleotide level to date. M5, a mutant which synthesizes a single cubic polyhedron per infected cell (Brown *et al.*, 1980), carries a single point mutation at nucleotide 176 of the polyhedrin coding region replacing proline with leucine (Carstens *et al.*, 1986). The second mutant, M29, produces copious amounts of small particles within the nuclei of infected cells, but these particles have no obvious crystal lattice (Duncan & Faulkner, 1982; Duncan *et al.*, 1983). M29 carries a point mutation at nucleotide 253, substituting leucine with phenylalanine (Carstens *et al.*, 1987). Both the M5 and M29 mutations occur in amino acid domains highly conserved within at least four different NPVs whose amino acid sequences are known (Kozlov *et al.*, 1981). Reviews on the polyhedrin gene have been published (Vlak & Rohrmann, 1985; Rohrmann, 1986). The current study reports the nucleotide changes in two
other morphology mutants, designated M276 and M934 (Partington et al., 1990), and describes ultrastructural features of each.

Methods

Cell culture and virus. Spodoptera frugiperda IPLB-SF-21 (Sf) cells (Vaughn et al., 1977) were cultured at 28°C in Grace’s medium (Gibco/BRL) supplemented with 10% foetal calf serum. The AcMNPV strain HR3 (Brown et al., 1979), passage level three, was used as the wild-type (wt) for mutagenesis experiments. Mutagenization of replicating AcMNPV was carried out as previously described (Partington et al., 1990). Virus stocks were titrated by plaque assay (Brown & Faulkner, 1978). Morphology mutants were selected on the basis of aberrant polyhedra appearance in isolated plaques and were designated M followed by a plaque number. The other polyhedra morphology mutants M5 and M29 have been previously described (Brown et al., 1980; Duncan & Faulkner, 1982). All mutants were subsequently plaque-purified at least three times after the initial isolation.

Viral DNA purification, restriction enzyme analysis and cloning. Extracellular virus (ECV) was harvested from infected Sf cells at 48 h post-infection (p.i.) and purified by centrifugation (Tjia et al., 1979). Viral DNA was extracted by incubating the purified ECV preparations with proteinase K (0.4 mg/ml) for 15 min at 37°C followed by the addition of N-lauroylsarcosine to 0.8% and a further 2 h incubation at 37°C. The DNA preparations were extracted three times with Tris saturated phenol and dialysed extensively against 2 mM-trisodium citrate, 15 mM-NaCl pH 7.0.

Restriction enzyme digestion were carried out according to standard procedures as suggested by the manufacturers and DNA fragments were resolved on 0.7% agarose gels as previously described (Carstens, 1982). The EcoRI I fragments of M276 and M934 were purified by electroelution of the band from agarose gels and were then cloned into the EcoRI I site of the bacterial plasmid pUC19. Subclones using the BamHI and KpnI restriction enzyme sites were then generated.

Marker transfer experiments. Genetic transfer of the wt AcMNPV polyhedrin gene to the morphology mutant background was carried out by cotransfecting Sf cells with 1 to 2 μg of the cloned wt EcoRI I or the BamHI F fragment DNA and 500 ng of purified mutant viral DNA by the calcium phosphate precipitation method (Carstens et al., 1980, 1986). In addition, the cloned EcoRI I fragments of the two morphology mutants were cotransfected with purified wt AcMNPV DNA into Sf cells. The transfection supernatants were harvested at about 5 days post-transfection and were plaque-assayed. The resultant plaques were screened for reversion to wt polyhedra morphology or conversion to mutant polyhedra morphology.

DNA sequencing. Plasmid subclones were sequenced directly using universal primers complementary to pUC19 sequences directly upstream and downstream of the multicloning site. Routinely, 2 μg of purified plasmid DNA was denatured with 2 M-NaOH, neutralized and annealed to 0-8 μmol of primer. The inserts were then sequenced by the dideoxynucleotide chain termination method using a T7 DNA polymerase kit as described by the manufacturer (Pharmacia). The reaction mixtures were resolved on 6% denaturing polyacrylamide gels in the presence of 8 M-urea.

Protein PAGE. Cells infected with wt or mutant AcMNPV (m.o.i. of 10), were pulse-labelled with 25 μCi/ml of [35S]methionine in methionine-free medium for 1 h at 24 h p.i. Total cell extracts were prepared and the radioactive polypeptides were resolved on 12% polyacrylamide gels (Partington et al., 1990). The gels were dried and exposed to Kodak XAR film.

Cell preparation for electron microscopy. Infected Sf cells (m.o.i. of 10) were harvested at 26 and 48 h p.i. by centrifugation for 20 s at 14000 g. Cell pellets were rinsed in 0.06 M-PIPES/0.04 M-HEPES buffer pH 7.2 containing 10 mM-KCl, 2 mM-EGTA, 1.5 mM-MgCl2 (fix buffer) and fixed for 1 h with a mixture of 4% formaldehyde (from paraformaldehyde and glutaraldehyde (J.B. EM Services). Pellets were washed with fix buffer, then rinsed with 0.01 M-cacodylate buffer pH 6.8, and post-fixed for 40 min in 1% osmium tetroxide in cacodylate buffer. After washing in cacodylate buffer alone, the pellets were dehydrated to 70% ethanol at 4°C, and stained en bloc for 3 h with 1% uranyl acetate in 70% ethanol. Stained preparations were washed twice for 30 min in 70% ethanol, then dehydrated in absolute ethanol and embedded in Taab812 epoxy resin (Marivac). Thin sections were recovered on 300-mesh nickel grids and stained with uranyl acetate and lead citrate. Specimens were examined using a Hitachi H-7000 transmission electron microscope operating at 75 kV.

Results

The two polyhedra morphology mutants M276 and M934 were isolated on the basis of their aberrant cytopathic effect at late times p.i. (Partington et al., 1990). Examination of whole infected cells by phase contrast and dark field microscopy revealed that M276-infected cells had a homogeneous mass of material in the perinuclear region, on both the cytoplasmic and nuclear side of the nuclear boundary. M934 revealed roughly crescent-shaped inclusions located in a ring just within the nuclear boundary. Both mutants failed to produce polyhedra so the molecular basis of the mutations were investigated to identify genomic domains critical to proper functioning of the polyhedrin protein.

DNA analysis

Digestion of purified M276 viral DNA with various restriction enzymes revealed the presence of a DNA insert of approximately 200 bp within the BamHI F and HindIII V fragments (Fig. 1), a region known to code for the polyhedrin gene (Smith et al., 1983; Rohel et al., 1983; Hooft van Iddekinge et al., 1983). No alterations were observed in restriction patterns of M934 viral DNA (data not shown). To define the mutations precisely, the polyhedrin gene regions of both M276 and M934 were cloned and sequenced and their nucleotide sequences compared with that of wt polyhedrin (Hooft van Iddekinge et al., 1983) (Fig. 2). This analysis revealed that M934 carries a point mutation at nucleotide 547 changing cytidine to thymidine. This change results in phenylalanine replacing leucine at amino acid 183.

Analysis of the M276 polyhedrin coding region revealed the presence of a 229 nucleotide insertion after nucleotide 699 (Fig. 2). This insertion introduced two new amino acids (Phe and Ser) and then a premature
stop codon, resulting in a truncated polyhedrin polypeptide missing the terminal 12 amino acids. The inserted sequence produced a duplication of the sequence 5' GTTT 3'. This is similar to other types of insertion sequences which have been reported in baculoviruses where a four nucleotide duplication occurs at the insertion site (Carstens, 1987; Cary et al., 1989; Wang et al., 1989). There are three copies of the sequence 5' CATAATT 3' directly following the first stretch of 5' TTTT 3' at the insertion site. The insert sequence is 73% A/T-rich and includes a single copy of the baculovirus consensus very late transcription start site sequence 5' ATAAGTA 3'. However, there is no open reading frame downstream from this sequence. The origin of this short DNA sequence is unknown.

The effect of these mutations on polyhedrin gene expression was investigated by pulse-labelling infected cells at 24 h p.i. for 1 h with [35S]methionine and analysing the radiolabelled polypeptides by SDS gel electrophoresis (Fig. 3). The results clearly demonstrated that normal amounts of polyhedrin were produced in

![Fig. 1. Restriction enzyme digestion of M276 viral DNA. Purified M276 (lanes 2) and wt (lanes 1) AcMNPV viral DNA were digested with (a) HindIII or (b) BamHI, and the resulting fragments were separated on 0.7% agarose gels. The gels revealed the presence of a 200 bp insert in the HindIII V and BamHI F fragment of M276 (*). Lambda DNA is shown in lane L (a).](image)

![Fig. 2. Location of mutations on the AcMNPV polyhedrin gene sequence. The nucleotide sequence of the AcMNPV polyhedrin gene (Hooft van Iddekinge et al., 1983) and the computer-generated amino acid sequence are shown. The nucleotide changes in M5 (Carstens et al., 1986), M29 (Carstens et al., 1987) and M934 are indicated above the wt nucleotide sequence and the resulting amino acid substitutions are shown below the amino acid sequence. The sequence of the 229 nucleotide insert in M276 is underlined; the insertion point at nucleotide 699 is marked (*).](image)

![Fig. 3. Polyacrylamide gel electrophoresis of cell extracts. SF cells, mock-infected (lane M) or infected at 33 °C with ts8 (lane 1), wt (lane 2), M276 (lane 3) or M934 (lane 4), were pulse-labelled for 1 h with [35S]methionine at 24 h p.i. Total cell extracts were separated on 12% SDS-polyacrylamide gels, the gels were dried and exposed to film. The slightly faster mobility of M276 polyhedrin is indicated (*). The sizes of certain polypeptides are indicated to the right.](image)
M934-infected cells and a slight reduction was observed in the M276-infected cells. In addition, the M276 polyhedrin protein migrated slightly faster than either the wt or M934 polyhedrin. This observation is consistent with the sequence data, which indicated that a truncated polypeptide (27.8K as opposed to 28.6K) would be produced as a result of the insertion at the carboxyl-terminal region of the M276 gene.

**Marker transfer of morphology mutant phenotype**

Although specific nucleotide changes were detected within the polyhedrin coding sequence, there was a possibility that mutations elsewhere in the genome were responsible for the observed phenotype. A series of marker transfer experiments was done to show conclusively that only alterations within the polyhedrin gene...
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were necessary for these morphological alterations in polyhedra assembly.

Wild-type recombinant viruses were obtained when purified viral DNA from M934 or M276 was cotransfected with plasmid DNA carrying the wt genomic BamHI F fragment. The fragment includes a sequence coding for 185 amino acids at the carboxyl terminus of polyhedrin. The wt recombinants were detected by plaque assay of the supernatants from cotransfected SF cells, and polyhedra from plaque-purified isolates of these recombinants were examined at the electron microscopic (EM) level after negative staining of alkali-dissolved preparations. The presence of normal calyx bag-like structures (Harrap, 1972) and abundant occluded virus particles were the criteria for evaluating the viral occlusions. Polyhedra from recombinant wt stocks could not be distinguished from the wt reference stock. These results demonstrated that replacing the genomic BamHI F region of the morphology mutants could restore the normal polyhedra morphology.

Similar results were obtained when M934 viral DNA was cotransfected with the cloned genomic EcoRI I fragment from the previously described morphology mutant, M5 (Carstens, 1982). In this case, recombinants exhibited the cubic polyhedron morphology of M5. These experiments were corroborated by cotransfecting cells with wt AcMNPV DNA and the cloned genomic EcoRI I fragments of either M276 or M934. Recombinant plaques were obtained which exhibited the mutant polyhedra phenotype, confirming that the identified mutations within the polyhedrin gene were responsible for the altered morphologies.

**Ultrastructure of mutant virus-infected cells**

AcMNPV-infected SF cells contain numerous polyhedra and large fibrillar masses within the cell nucleus by 36 h p.i. (Fig. 4). Enveloped nucleocapsid bundles and occlusion body calyx are clearly visible within and on the polyhedra, respectively. Also, p10-containing fibrillar bodies (van der Wilk et al., 1987; Williams et al., 1989), and associated immature calyx, called fibrous sheet, are visible (Fig. 4).

The occlusions that formed in cells infected with the mutants M276 (Fig. 5a) and M934 (Fig. 6) are distinct from each other, and bear little resemblance to the wt structure (Fig. 4). M276-infected cells had small amorphous condensations of polyhedrin in both nucleus and cytoplasm (Fig. 5). These condensations had no discernible lattice and did not occlude virions (Fig. 5b and c), but the intranuclear mass did acquire a unilamellar calyx (Fig. 5c). The cytoplasmic polyhedrin masses were dispersed in roughly perinuclear clusters and were not membrane-bound (Fig. 5h), indicating formation in the cytosol and not in the endoplasmic reticulum. Combined 4,6-diamidino-2-phenylindole staining and dark-field microscopy confirmed that intranuclear masses of mutant polyhedrin, like that of wt, occurred primarily in the ring zone (Benz, 1986) surrounding the virogenic stroma. Thus, although some M276 polyhedrin did prematurely condense in the cytoplasm, it still retained the target specificity and transport competence for accumulation in the intranuclear ring zone.

M934-infected cells developed large amorphous masses of polyhedrin similar to those of M276 infection, in that a lattice was not seen and virions were not occluded within the matrix (Fig. 6). Fibrous sheet material associated with very small polyhedrin condensations (Fig. 6c), and although fibrous sheet was present in normal amounts at 26 h p.i. (Fig. 6b), it was rarely observed in infected cell nuclei by 48 h p.i. (Fig. 6a); in addition, polyhedrin condensations lacked a normal calyx layer (Fig. 6a). Very small polyhedrin condensations were seen to acquire patchy, discontinuous calyx profiles (Fig. 6d) that were indistinct and full of holes in EM preparations of purified occlusions dissolved in dilute alkali (data not shown). In contrast, M276-derived occlusions, when dissolved, released small sheets of otherwise normal calyx. Ontogeny of M934 calyx was apparently normal in the mutant-infected cells, since the polyhedra of all wt marker transfer recombinants had calyx indistinguishable from control wt calyx, as judged by EM examination of alkali-dissolved polyhedra.

**Discussion**

The two mutants described in this paper are morphologically indistinguishable from wt virus except with respect to polyhedron morphogenesis. The results from marker transfer experiments have established that the mutant phenotypes of both M934 and M276 resulted from alterations within the polyhedrin gene region and not from another locus elsewhere on the genome.

The most striking difference between M934- and M276-infected cells was that M934 polyhedrin conden-
sations were seen mostly in the nucleus, whereas M276 polyhedrin condensations were seen in both the nucleus and cytoplasm. Condensations of both mutant polyhedrins, usually situated in the perinuclear area, were faithful to the wt intranuclear partitioning (restricted to the ring zone and not usually infringing on the virogenic stroma). Accumulation of M276 polyhedrin in the cytoplasm is most likely the result of a change in biophysical properties such as protein solubility, caused by loss of the carboxyl terminus, since the protein retained the ability to localize properly. A potential nuclear localization sequence has been identified within the N-terminal 35 amino acids of polyhedrin (Jarvis et al., 1991) which is consistent with these observations.

An interesting aspect of M934 morphogenesis is the apparent exhaustion of calyx material relatively early in infection, before cessation of polyhedrin condensation. The alteration of polyhedrin amino acid sequence seemed to increase the affinity of the protein for calyx attachment (Fig. 6c and d), even to very small polyhedrin condensations, and to decrease affinity for virion bundles. In wt infection, polyhedrin and a distinct phosphoprotein designated pp34 are bound to the calyx by thiol linkages (Whitt & Manning, 1988), although the specific details of this association are unknown. Pp34 is required for morphogenesis of calyx as well as its precursor fibrous sheet structure (Zuidema et al., 1989), which is produced independently of the p10-containing fibrillar bodies (van Lent et al., 1990) or polyhedrin (G. V. Williams, personal observations). A temporal mechanism for polyhedra maturation might be expected, actively capping crystal growth and attaching the calyx. However, the high affinity of M934 polyhedrin for calyx precursor (fibrous sheet) and its low affinity for enveloped virus bundles, through a simple point mutation, suggests that the polyhedrin molecule has a role in regulating the morphogenesis process. Perhaps a shift in wt polyhedrin conformation acts as a switch in regulating virus particle occlusion versus calyx attachment. The heritable variation of polyhedrin conformation size between M5, M29 and M934 viruses, each having a simple point mutation, demonstrates that occlusion size and shape are ultimately encoded within the polyhedrin gene.

Each polyhedrin morphology mutant failed to occlude virus bundles, underscoring the stringent requirements for occlusion within the protein matrix. Cells infected with the mutant virus M5 contain a single large cuboidal occlusion, having an apparently normal paracrystalline array and surface calyx, and rarely encapsulating virions (Brown et al., 1980). In this mutant, a single base change within the polyhedrin gene replaced proline (amino acid 58) with leucine, and probably leads to an alteration in tertiary structure (Carstens et al., 1986).

By contrast, the small intranuclear particles formed in M29-infected cells lacked the obvious lattice structure, failed to occlude virus and did not acquire a calyx (Duncan et al., 1983). It is not clear whether these particles associate with calyx material. The point mutation of the polyhedrin gene, causing substitution of phenylalanine for leucine at amino acid 84, accounts for the change in phenotype (Carstens et al., 1987). Although the morphological effect is dramatic, the mutant polyhedrin protein cannot be distinguished from its wt counterpart by SDS–PAGE or mapping by limited proteolysis (Duncan et al., 1983). This is in contrast to M5 polyhedrin, which has a V8 protease map significantly different from that of the wt molecule (Brown et al., 1980), but with less perturbation of the biological structure. Clearly, the tertiary structure of polyhedrin is complex and encodes information vital for the proper morphogenesis of polyhedra.

These data do not shed light on the mechanism of virion occlusion into condensing polyhedrin matrix. The process must be considered strictly dependent on protein tertiary structure since virus occlusion was absent when the polyhedrin sequence was altered by a single amino acid. However, it is not known whether other aspects of polyhedra function, such as enzyme activity against a 68K peritrophic membrane glycoprotein (Derkson & Granados, 1988), is retained within the matrix of any of the mutant occlusions. Regions of protein–protein, protein–nucleic acid and protein–membrane interactions have been described for the polyhedrin molecule based on a theoretical analysis (Kozlov et al., 1986). If they exist, these foci would probably be discontinuous and more complex than the current model suggests. We postulate that polyhedrin must contain several functional domains and signal motifs within its peptide sequence, and probably encodes a great deal of the information required for polyhedra morphogenesis. Such signals would affect nuclear localization and accumulation in the ring zone, occlusion of enveloped virions, association with calyx, and polyhedra size and shape. These processes must be strictly controlled, perhaps through polyhedrin conformation.

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References


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