Analysis of a Mutant of *Autographa californica* Nuclear Polyhedrosis Virus with a Defect in the Morphogenesis of the Occlusion Body Macromolecular Lattice

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SUMMARY

A mutant (m-29) of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) grew in *Spodoptera frugiperda* and *Trichoplusia ni* cells but did not form typical intranuclear occlusion bodies (OB); instead, small particles (95 to 180 nm diam.) were produced in copious amounts within nuclei. Ultrastructural studies showed that the particles did not occlude enveloped nucleocapsids and that they lacked a macromolecular paracrystalline lattice and a structure equivalent to the occlusion body envelope. The particles within nuclei stained in an immunofluorescence test with anti-polyhedrin antibody and when extracted from cells the major polypeptide of a particle preparation was indistinguishable from polyhedrin when examined on SDS-polyacrylamide gels and had an identical peptide pattern following proteolysis with V8 protease. Other elements believed to be implicated in OB morphogenesis such as a proliferation of intranuclear membranes, enveloped bundles of nucleocapsids, patches of fibrous material and fibrous sheets were present in normal amounts. No alteration in the synthesis or processing of polypeptides was seen in mutant-infected cells. Analysis of m-29 DNA with *BamHI*, *EcoRI* and *HindIII* restriction endonucleases revealed that the *HindIII* restriction site at the F/V junction of viral DNA was absent in the mutant. No other modifications in the restriction patterns were detected. It is proposed that an alteration in the amino acid sequence of polyhedrin towards the -NH2 terminus of the polypeptide may account for the growth characteristics of the mutant.

INTRODUCTION

The occlusion bodies (OB) of baculoviruses are proteinaceous granules containing virus particles and are structures which transmit the virus in nature. In OB of the nuclear polyhedrosis viruses (NPV), DNA-containing virus nucleocapsids are enveloped either singly (SNPV) or in bundles (MNPV) and lie within a macromolecular paracrystalline protein lattice (Bergold, 1962; Harrap, 1972; Hughes, 1978) whose major component is a polypeptide, polyhedrin, of mol. wt. about 31000 (Summers, 1975). OB of NPV range in size up to 15 μm and may carry several hundred nucleocapsids. In the microscope they are seen to be angular and are most commonly described as having polyhedral or cuboidal shapes. OB are bordered by a glycoprotein envelope (Minion *et al.*, 1979) which is considered to be derived from fibrous structures seen in infected cell nuclei (Chung *et al.*, 1980). Ultrastructural studies on the morphogenesis of OB indicate that occlusion of enveloped virus occurs by condensation of fibrous material in nuclei to form a lattice which surrounds the virus bundles (Chung *et al.*, 1980; Yamamoto *et al.*, 1981) and genetic complementation with temperature-sensitive (ts) mutants has indicated that expression of several genes is necessary in the final assembly of OB (Brown *et al.*, 1979; Lee & Miller, 1979; Duncan & Faulkner, 1982).

Because of the importance of OB in the transmission and maintenance of baculoviruses in nature, we are interested in identifying genes required in OB morphogenesis and studying their
function. The analysis of ts and morphology (morph) mutants is one means of studying this phenomenon. A class of ts and morph mutants that produce essentially wild-type (wt) amounts of budded non-occluded virus (NOV) and polyhedrin, but are defective in the formation of OB, has been reported (Brown et al., 1979; Duncan & Faulkner, 1982), and in this paper we describe biochemical and biophysical properties of a morph mutant, m-29, of this type. The virus produced large amounts of a polypeptide which could not be differentiated from wt polyhedrin but which associated into small, stable intranuclear particles lacking a crystalline structure and containing no embedded occluded virus (OV). The mutant lacked a HindIII restriction site within the gene coding for polyhedrin.

METHODS

**Cells and viruses.** *Spodoptera frugiperda* (SF) cells (IPLB-SF-21; Vaughn et al., 1977) were grown and maintained in culture medium (BML-TC/10; Gardner & Stockdale, 1975) supplemented with 10% foetal bovine serum (FBS) and 50 mg gentamicin sulphate/l. The wt virus was strain HR-3 of AcMNPV (Brown et al., 1979). The morphology mutant m-29 was isolated from a bromodeoxyuridine (BUdR)-mutagenized wt stock and plaque-purified on SF cells (Duncan & Faulkner, 1982).

**Electron microscopy.** Techniques for fixation, embedding and cutting of thin sections have been described previously (Chung et al., 1980). For negative staining of occlusions a drop of suspension was deposited on a Formvar-coated copper grid and stained as described by Horne & Brenner (1960).

**Isolation of occlusion bodies.** These were released from infected cells and fractionated on gradients as described by Brown et al. (1980).

**Comparison of polypeptides by proteolysis.** Mutant and wt polyhedrin were examined using limited proteolysis (Cleveland et al., 1977) followed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as previously described (Brown et al., 1980). To obtain polyhedrin polypeptide, infected cells were lysed in electrophoresis sample buffer (0·05 M-Tris–HCl pH 6·8, 1% SDS, 1% 2-mercaptoethanol, 10% glycerol), then forced through a small gauge hypodermic needle several times. After heating the mixture for 2 min in a boiling water-bath the polypeptides were separated on discontinuous 12·5% SDS–polyacrylamide gels (Laemmli, 1970). The gels were briefly stained and destained, the prominent polyhedrin polypeptide band at 31 000 mol. wt. (p31) was excised and the protein electroeluted from the gel. The purified p31 was digested with *Staphylococcus aureus* V8 protease (0·05 and 0·5 μg of enzyme) and the polypeptides were analysed on 15% polyacrylamide gels (Brown et al., 1980).

**Immunofluorescence.** An indirect immunofluorescence (IFF) assay using rabbit antiserum prepared against gel-purified polyhedrin and commercially available goat anti-rabbit IgG conjugated with fluorescein isothiocyanate was performed as described previously (Duncan & Faulkner, 1982).

**Pulse, pulse–chase and NOV release studies.** Twelve-well Linbro plates were seeded with 7·5 × 10^5 SF cells/well and were infected with mutant or wt virus at an m.o.i. of 10. At 2 h post-infection the inoculum was removed and the cells were incubated in complete medium at 25 °C. At various times the medium was removed from one well, the monolayer was rinsed three times with 0·5 ml leucine-free culture medium (and lacking FBS) and the cells were pulsed for 1 h with 20 μCi [3H]leucine (New England Nuclear) in 0·4 ml leucine-free labelling medium (culture medium lacking leucine and FBS, and containing 1/10 of the standard amount of tryptose phosphate). After the pulse the cells were scraped into the labelling medium and washed once. The cell pellet was suspended in 200 μl of electrophoresis sample buffer, heated for 2 min in a boiling water-bath and stored at −20 °C until all had been similarly processed. Equal volumes of samples from each time period were analysed on 12·5% SDS–polyacrylamide gels (Laemmli, 1970). The gels were impregnated with En3Hance (New England Nuclear), dried under vacuum and exposed on Kodak X-OMAT AR X-ray film at −70 °C.

For chases, the [3H]leucine medium was removed after pulsing, the cell monolayers were rinsed three times with culture medium and then incubated for 12 h in culture medium supplemented with 10 times the amount of unlabelled leucine present in BML-TC/10 medium before harvesting the cells.

**DNA analysis.** Mutant and wt DNA were extracted from virions and purified as previously described (Cochran et al., 1982). Viral DNA was digested with the restriction endonucleases *BamHI, EcoRI and HindIII*, and the fragments were labelled by replacement synthesis with [32P]dCTP using T4 DNA polymerase (O'Farrell et al., 1980). The labelled fragments were separated on 0·7% agarose gels, and the gels were dried and exposed on Kodak X-OMAT AR X-ray film. For probing of DNA, unlabelled viral DNA was digested with HindIII, the fragments were separated on 0·7% agarose gels and blotted onto nitrocellulose sheets using a modified Southern (1975) technique as described by Tjia et al. (1979). The probe was the AcMNPV HindIII-V fragment inserted into plasmid pUC8. It was labelled with [32P]dCTP by nick-translation (Rigby et al., 1977) and hybridized with the blotted viral DNA as described by Wahl et al. (1979). The pUC8 plasmid with a HindIII-V insert was a gift from M. Cochran and was obtained by subcloning from plasmid pAcEcoI16 (Cochran et al., 1982).
AcMNPV mutant with a defect in OB lattice

RESULTS

Isolation of mutant m-29

Mutant m-29 was isolated from a stock of AcMNPV obtained by mutagenesis of strain HR-3 with BUdR (Duncan & Faulkner, 1982) and formed plaques in monolayers of S. frugiperda cells. At 4 days post-infection nuclei of cells infected with wt virus contained typical baculovirus OB (Fig. 1 a). The m-29-infected cells displayed nuclear hypertrophy but OB were not seen at 4 days post-infection. Instead, the nuclei contained an amorphous material which was opaque under the light microscope (Fig. 1 b). The phenotype was observed at 25 °C and 33 °C; thus, the virus was designated a morph mutant. A similar cytopathic effect was seen in T. ni monolayers infected with m-29. Cells infected with m-29 released amounts of NOV comparable to wt at both temperatures and the estimated reversion frequency (4.98 × 10^-8) indicated that the mutant was genetically stable (Duncan & Faulkner, 1982).
An indirect immunofluorescence (IIF) test was done using antiserum prepared against purified OB protein. Mutant-infected cells produced abundant amounts of a protein that cross-reacted with polyhedrin even though no OB were present in the cells. The IIF staining was localized within nuclei and was seen as a pinpoint fluorescent granular pattern (Fig. 1d). The granular material consisted of particles much smaller than OB seen in wt infections (Fig. 1c).
Ultrastructural studies on particles within infected cells

No major morphological changes were seen in cells infected with m-29 at 12 h post-infection, but at 24 h post-infection virogenic stroma was observed within nuclei (Fig. 2) and a small number of nucleocapsids were seen within the stroma. Patches of fibres and membranous material (Chung et al., 1980) were also present in nuclei. By 36 h spherical particles were seen within the periphery of the nucleus (Fig. 3). Masses of fibrous material were now present in the vicinity of the particles and virus-specific membrane had also begun to accumulate.
After 5 days, infected nuclei contained large numbers of particles 95 to 180 nm in cross-section (Fig. 4a). No macromolecular crystalline lattice was seen in the particles nor was a structure similar to the polyhedron envelope seen (Fig. 4b). By this time there was considerable accumulation of membranous material within nuclei which resembled virus envelopes. Enveloped virus bundles (Chung et al., 1980) were also present in nuclei but were not occluded within the particles.

The particles were released from infected monolayers with detergent, fractionated on a sucrose gradient and examined in the electron microscope. Particles similar to those observed in thin sections of mutant m-29-infected cells were seen (Fig. 5). SDS–PAGE indicated that the principal polypeptide had a mol. wt. of 31000 (p31) and co-migrated with polyhedrin from AcMNPV OB.
**AcMNPV mutant with a defect in OB lattice**

To characterize further the m-29 mutant, the p31 polypeptides from AcMNPV and m-29-infected cultures were compared as it was possible that the inability of the mutant to produce OB may have been due to the production of an altered polyhedrin incapable of crystallization. *S. frugiperda* cells were infected with mutant m-29 or wt AcMNPV. Five days post-infection cells were harvested into electrophoresis sample buffer and the polypeptides separated by SDS-PAGE. The major polypeptide in both samples had a mol. wt. of 31 000. They were excised from the gels and subjected to limited proteolysis (Fig. 6). Undigested p31 and *S. aureus* V8 protease-generated polypeptides from both wt and mutant strains co-migrated in gels, indicating that the mutant protein and polyhedrin had very similar properties. In addition, both wt and mutant p31 polypeptides co-migrated in non-equilibrium two-dimensional gels (data not shown) indicating that they were similar in size and charge.

**Protein synthesis in infected cells**

Protein synthesis was compared in cells infected with wt and the m-29 mutant and pulsed at intervals with [3H]leucine (Fig. 7). With both viruses there was a gradual reduction of host cell

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**Fig. 7.** Polypeptide synthesis in cells infected with wt (H) or mutant (M) AcMNPV. Cells were pulsed for 1 h with [3H]leucine at the indicated times post-infection, then cell extracts were heated in electrophoresis sample buffer and analysed by SDS-PAGE on 12.5% gels. Lane U was loaded with extract from uninfected cells. Numbers on left-hand side show mol. wt. × 10⁻³ of virus-induced polypeptides. Bars in figure (−) indicate earliest detection of a polypeptide in a culture infected with wt virus and seen later in mutant m-29 infection.
protein synthesis with concurrent appearance of virus-specific proteins as the infection progressed. Repeated attempts to detect differences in the polypeptide profiles of mutant- and wt-infected cells pulse-labelled with $[^{35}S]$methionine or $[^{3}H]$leucine failed. There was, however, a reproducible lag of 3 to 6 h in the appearance of at least 14 out of 29 detected virus-induced polypeptides in cells infected with mutant m-29 (Fig. 7). For instance p78, p69, p39, p31.5, p30, p16, p15.5 and p12 all appeared at 18 h post-infection in wt-infected cells but not until 21 h post-infection in m-29-infected cells. Although the exact time of appearance of specific polypeptides varied somewhat between different experiments the same pattern was evident and there was no indication of a missing or altered protein in the m-29 profiles.

There was clear evidence of post-translational modification of virus-induced proteins in both mutant- and wt-infected cells. Polypeptides p78, p39, p25.5, p15.5 and p15 each decreased in intensity whereas p16.5 and p12 increased in intensity following the 12 h chase (Fig. 8). Polypeptides p69, p61 and p31.5 disappeared following the chase. The only new polypeptides
detected after chasing were p60 and p31. The p31 polypeptide co-migrated with polyhedrin from $^{35}$S-labelled OB (data not shown) and appeared to be derived from the slightly larger p31.5 polypeptide present in the 24 h pulse. The change in mobility of p31.5 following the chase was slight but reproducible and could only be detected in the 24 h pulse–chase series when the polypeptides were present in smaller amounts. By 36 h post-infection polyhedrin was synthesized in copious amounts and p31.5 could not be resolved. These results confirm a previous report that polyhedrin may be derived from cleavage of a slightly larger intracellular precursor (Carstens et al., 1979). As with the pulse experiments no difference was detected in the pattern produced in mutant-infected versus wt-infected cells other than the previously mentioned temporal lag in mutant protein synthesis.

Alterations in genomic DNA

$[^{32}P]$dCTP-labelled BamHI, EcoRI and HindIII fragments of mutant and wt DNA were analysed on 0.7% agarose gels. Differences were only seen in the HindIII digestion profile. These were the absence of HindIII-F and HindIII-V fragments in mutant DNA and the appearance of a new fragment which co-migrated with the HindIII-D/E doublet (Fig. 9),
indicating the loss of the HindIII-F/V restriction site on the physical map of the genome (Cochran et al., 1982). No insertions or deletions were detected in the m-29 genome. To confirm this alteration, unlabelled HindIII fragments of mutant and wt DNA were separated on 0.7% agarose gels, blotted onto nitrocellulose sheets and probed with nick-translated, cloned HindIII-V. The probe hybridized to the HindIII-V fragment of the wt DNA but it also hybridized to a fragment which co-migrated with the HindIII-D/E doublet of m-29 DNA (Fig. 9) and thus confirmed the loss of the HindIII-F/V restriction site in the m-29 genome.

DISCUSSION

Two morph mutants of AcMNPV have been described in which the size, shape and packaging of virions are altered, but the typical paracrystalline structure of condensed polyhedrin was preserved (Brown et al., 1980). No lattice was seen in the small granular particles within cells infected with mutant m-29 even though the principal structural protein (p31) had biochemical properties similar to polyhedrin and cross-reacted with anti-polyhedrin serum. The particles did not occlude virus, although bundled nucleocapsids were present in the nuclei. The particles were produced in copious amounts in cells as a late event in the infection cycle. Fibrous material and condensed fibrous sheets, structures seen in cultures infected with AcMNPV and T. ni MNPV, were also present in seemingly normal amounts in m-29 infections.

The major polypeptide (p31) of the granular particles could not be distinguished from AcMNPV polyhedrin on the basis of limited proteolysis using S. aureus V8 protease (Fig. 6) or chymotrypsin (data not shown). However, differences in amino acid sequence could have gone undetected by these methods and the absence of a lattice may be due to an alteration in chemical composition of polyhedrin. The studies on DNA restriction enzyme sites in the genome of m-29 reinforce the proposition that the amino acid sequence of p31 polypeptide of the virion is different from the AcMNPV polyhedrin. When m-29 DNA was cleaved with BamHI, EcoRI and HindIII, elimination of the HindIII-F/V site in the mutant was the only alteration observed in the restriction pattern in comparison with AcMNPV viral DNA (Fig. 9). A consensus physical map for AcMNPV has been established by workers from several laboratories (Vlak & Smith, 1982). The polyhedrin gene of AcMNPV is located within the EcoRI-I segment of DNA (Smith & Summers, 1980) and recently polyhedrin mRNA was found, in hybridization selection studies, to bind to the 930 base pair HindIII-V DNA fragment and not to adjacent HindIII-F or -T fragments, although Smith et al. (1982) consider that the 5' terminal region of the RNA may extend a short distance into the HindIII-F locus. Polyhedrin mRNA of AcMNPV is 1100 to 1400 nucleotides in length (D. Z. Rohel, M. A. Cochran & P. Faulkner, unpublished results) and Rohrmann et al. (1982) have shown that polyhedrin of Orgya pseudotsugata is produced by an unspliced mRNA and has a short leader sequence. If the same properties hold true for AcMNPV polyhedrin mRNA the mutation in m-29 DNA at the HindIII-F/V junction probably occurs near the 5' terminus of the polyhedrin message and thus could alter the amino acid sequence towards the –NH₂ end of the polypeptide. Conservation of the –NH₂ terminus of the polypeptide may thus be crucial for lattice formation by polyhedrin. No insertions or deletions were detected in m-29 DNA, but we cannot rule out the possibility that mutations may have occurred at sites other than the HindIII-F/V junction and that the phenotype of the granular particles results from several alterations in the genome.

Promoter sequences lying upstream from the polyhedrin message do not seem to be modified in the mutant since the sequential appearance of polypeptides was similar in pattern to wt virus and large amounts of late polypeptides were synthesized which could not be distinguished from wt. There appeared to be a decrease in the overall rate of polypeptide synthesis (Fig. 7) in m-29-infected cells but eventually all polypeptides seen with wt infections were detected. Although most recent studies in polypeptide synthesis in baculovirus-infected cells have confirmed that some undergo post-translational modification conflicting results have been reported concerning the synthesis of a polyhedrin precursor. Carstens et al. (1979) considered that polyhedrin arose from a slightly larger precursor but the finding was not confirmed in other reports (Dobos & Cochran, 1980; Wood, 1980; Maruniak & Summers, 1981; Vlak et al., 1981). Using leucine-
labelled cells, we found that p31.5 could be chased into a late polypeptide that co-migrated with polyhedrin and was produced in large amounts both in wt- and mutant-infected cells (Fig. 8) and thus we consider that a more refined analysis is necessary to resolve the question.

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REFERENCES


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