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Characterization of Two Morphology Mutants of *Autographa californica* Nuclear Polyhedrosis Virus with Large Cuboidal Inclusion Bodies

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SUMMARY

Two morphology mutants designated *m-5* and *m-6*, of *Autographa californica* nuclear polyhedrosis virus (NPV) were isolated from virus grown in the presence of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Infected cells contained single, large cuboidal inclusion bodies with a crystalline lattice ultrastructure and envelope similar to that of wild-type (wt) polyhedra. The inclusion bodies had low infectivity when fed to *Trichoplusia ni* larvae. The paracrystalline lattice structure of the mutants was similar to wt, but occluded viruses were rarely found within the mutants. The *m-5* polyhedrin (mol. wt. 30000) could be distinguished from wt and *m-6* polyhedrin on the basis of migration in SDS–PAGE gels. Peptide maps of polyhedrin were obtained following digestion with chymotrypsinogen or *Staphylococcus aureus* V8 protease. They were identical for *m-6* and wt polyhedrin but *m-5* polyhedrin gave a different pattern. Thus, the altered morphology may be due to a change in polyhedrin composition not detectable in *m-6* polyhedrin by the methods used here, or it may be the result of a mutation affecting a protein not yet identified.

INTRODUCTION

Inclusion bodies of nuclear polyhedrosis virus (NPV, family *Baculoviridae*) range in diam. from 0·5 to 15 μm and are found in a variety of shapes: dodecahedra, tetrahedra, cubes and angular forms of irregular polygonal shape (Bergold, 1963*a*), and size and shape are considered by some to be specific for the virus in a particular insect host (Summers, 1975). Heritable variations in morphology can occur spontaneously in nature and have been described for inclusion bodies of *Antherea pernyi* (Gershenson, 1960), *Galleria mellonella* (Stairs, 1964) and *Lymantria dispar* (Skatulla, 1977) baculoviruses. These viruses produced cuboidal variants and their phenotype was preserved when they were passaged in insects. Selection of cuboidal forms may also occur when baculoviruses are passaged extensively *in vitro* (Hirumi *et al.* 1975). This report concerns the characterization of two morphology mutants of *Autographa californica* NPV with cuboidal inclusion bodies which were isolated following treatment of the virus with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). The ultrastructure and biochemical characteristics of the inclusions were investigated. Studies on these and other morphology mutants derived from cloned virus may be of value in interpreting inclusion body morphogenesis and function.

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METHODS

Cells and virus. Spodoptera frugiperda (fall armyworm) cells were from a continuous uncloned line (IPLB-SF-21) derived from pupal ovarian tissue (Vaughn *et al.* 1977). *Trichoplusia ni* (cabbage looper) cells were from a continuous uncloned line (TN-368) derived from minced larval ovarian tissue (Hink, 1970). Both *T. ni* and *S. frugiperda* cells were grown at 25 °C in BML-TC/10 culture medium (Gardiner & Stockdale, 1975) containing gentamicin (50 μg/ml) and 10% heat-inactivated foetal calf serum and were subcultured at 3 and 5 day intervals respectively.

The virus designated as wild-type (wt) was a plaque-purified MP strain of *A. californica* NPV (Potter *et al.* 1976) which was further plaque-purified at 33 °C (Brown *et al.* 1979). The two morphology mutants were designated *m-5* and *m-6* according to the nomenclature system outlined previously (Brown *et al.* 1979). They were isolated from a preparation of wt virus which had been grown in the presence of NTG (3 μg/ml) (Brown *et al.* 1979) and plaque-purified three times at 25 °C (Brown & Faulkner, 1978), then grown to passage 3 as working stocks, using an input m.o.i. of 0·01. Wild-type virus was also used in these experiments at passage level 3.

Isolation of inclusion bodies. Inclusion bodies were released from infected cells by treatment with 0·3% Triton-X and 3·3% sodium deoxycholate (DOC) (Faulkner & Henderson, 1972) and were purified by centrifugation through a 30 ml sucrose gradient (45 to 62% in distilled water) at 6500 ***g*** for 2 h (McCarthy & Liu, 1976). The band of inclusion bodies was collected and diluted in distilled water. Inclusion bodies were pelleted at 3000 ***g*** for 10 min, resuspended in 0·05 M-sodium chloride and used for infectivity tests.

Bioassay. Infectivity of inclusion body preparations was determined in feeding experiments done by Dr R. P. Jaques, Agriculture Canada Research Station, Harrow, Ontario. Inclusion body concentration was determined by counting using phase-contrast microscopy. The preparations were diluted and 5 μl amounts were air dried on to discs (0·8 cm^2) of collard leaf which had been washed in Tween 20 diluted 1:100. Discs were fed to groups of third instar *T. ni* larvae (12 to 18 mg) which were reared individually in vials. Those which had consumed 75% of the leaf disc 24 h post-inoculation were reared on artificial diet at 25 °C and mortality due to inclusion body disease was recorded 13 days post-inoculation. No mortality was observed in insects fed on control (mock-infected) leaf discs.

Electron microscopy. Infected cells containing inclusion bodies were centrifuged at 250 ***g*** for 10 min and resuspended in 1·75% glutaraldehyde in 0·1 M-cacodylate buffer (pH 7·2) chilled to 4 °C. The samples were washed three times, post-fixed in 1% osmic acid (pH 7·2), washed and dehydrated in graded concentrations of ethanol (50%, 80%, 95%, 100%). Pellets of cells were embedded in Epon by the procedure of Luft (1961). Blocks were cut using a Reichert OMU2 ultramicrotome with a diamond knife. Sections were stained with lead citrate and uranyl acetate and examined with a Philips 200 electron microscope at 80 kV.

Purification of polyhedrin. Inclusion bodies were released from infected cells by treatment with DOC and Triton-X (final concentration 1% each) for 30 min at room temperature. They were rinsed with distilled water and purified by centrifugation through 30 ml sucrose gradients (35 to 65% in distilled water) at 6500 ***g*** for 2 h. Inclusion body-containing bands were removed and the inclusion bodies were washed with distilled water by repeated cycles of centrifugation and suspended in a small volume of distilled water.

Protein concentration was calculated from the u.v. absorption at 260 and 280 nm (Dawson *et al.* 1969) or by the method of Lowry *et al.* (1951). Inclusion bodies were dissolved by incubating a sample of an inclusion body preparation (less than 5 mg/ml protein) with an equal volume of lysis solution (0·016 M-Na_2CO_3 plus 0·1 M-NaCl, pH 10·6) for 5 to 10 min at room temperature. After dissolution of polyhedra as observed

under a microscope, the reaction was stopped by lowering the pH to 8·5 to 9 with 0·05 M-tris (pH 8) and the mixture centrifuged at 100000 *g* for 30 min to pellet debris. The supernatant was harvested taking care not to disturb the pellet and the protein concentration was measured. The polyhedrin was then lyophilized.

Polyacrylamide gel electrophoresis. Protein samples were analysed in 10 and 12% polyacrylamide slab gels with 3·5% spacer gels using the discontinuous SDS gel systems of Laemmli (1970) and the general apparatus and methodology described by Studier (1973). Lyophilized samples were dissolved in electrophoretic sample buffer (ESB: 0·0625 M-tris-HCl, pH 6·8, 1% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0·001% bromophenol blue) and aliquots adjusted to contain 10 μg protein/well were heated at 100 °C for 2 min before electrophoresis. For the analysis of peptides generated by proteolysis, 15% polyacrylamide gels were used. Gels were stained overnight in a mixture of acetic acid:methanol:water (7:20:73, by vol.) containing 0·1% Coomassie brilliant blue and destained in the same solution without Coomassie brilliant blue.

Peptide mapping by limited proteolysis. The relationship of *m-5* and *m-6* polyhedrin was investigated following limited proteolysis (Cleveland *et al.* 1977). Inclusion body proteins obtained by dissolution of polyhedrin in lysis solution were heated in ESB and resolved by polyacrylamide gel electrophoresis. The gels were stained and destained as briefly as possible. Polyhedrin bands at mol. wt. 30000 were cut from the gel (slices of 4 mm width) and soaked for 30 min in TSE buffer (0·125 M-tris-HCl pH 6·8, 0·1% SDS and 1 mM-EDTA). The gel slices (containing 10 to 30 μg of polyhedrin) were inserted into the wells (containing TSE buffer) of a second slab gel which consisted of a 3 cm stacking gel (3·5% acrylamide, pH 6·8) and a 16 cm resolving gel (15% acrylamide, pH 8·8). *Staphylococcus aureus* V8 protease (Miles Laboratories, Elkhart, Ind., U.S.A.) was diluted in TSE buffer containing 10% glycerol and 0·001% bromophenol blue and 10 μl amounts containing 0, 0·5 and 2·5 μg of protease were added to the wells. The samples were subjected to electrophoresis (75 V) until the bromophenol blue band had almost reached the bottom of the stacking gel. The power was shut off for 60 min to allow digestion of the polyhedrin and then electrophoresis was continued (100 V) until the dye reached the bottom of the resolving gel. The gels were stained, destained and photographed.

RESULTS

Isolation and growth characteristics of m-5 *and* m-6

Two morphology mutants were isolated from separate *A. californica* NPV preparations grown in the presence of NTG (3 μg/ml) during screening procedures to identify *ts* mutants (Brown *et al.* 1979). Both morphology mutants induced the formation of single, cuboidal crystals (10 to 15 μm in one dimension) in the nuclei of infected *S. frugiperda* cells (Fig. 1 *a*, *b*). Cuboidal inclusion bodies were also formed in infected *T. ni* cells. Comparative yield experiments done in *S. frugiperda* cells at 25 and 33 °C indicated that the morphology mutants were not *ts*. The ratio of non-occluded virus (NOV) yield at 33 °C to NOV yield at 25 °C for *m-5* and *m-6* was $(5{\cdot}8 \pm 2{\cdot}7) \times 10^{-2}$ and $(1{\cdot}4 \pm 6{\cdot}5) \times 10^{-2}$ respectively, in the same order as that observed for wt virus $(8{\cdot}4 \pm 4{\cdot}3) \times 10^{-2}$. However, the characteristic crystal formation at 25 °C was variable at 33 °C as some cells contained a single crystal, whereas others showed a rather granular c.p.e. Both *m-5* and *m-6* were considered to be morphology mutants; their phenotype was unaltered after four passages in culture. No wt revertants were observed in culture, and based on end-point $TCID_{50}$ titrations (Brown & Faulkner, 1975) the reversion frequency was below the detectable limit, $3{\cdot}3 \times 10^{-5}$.

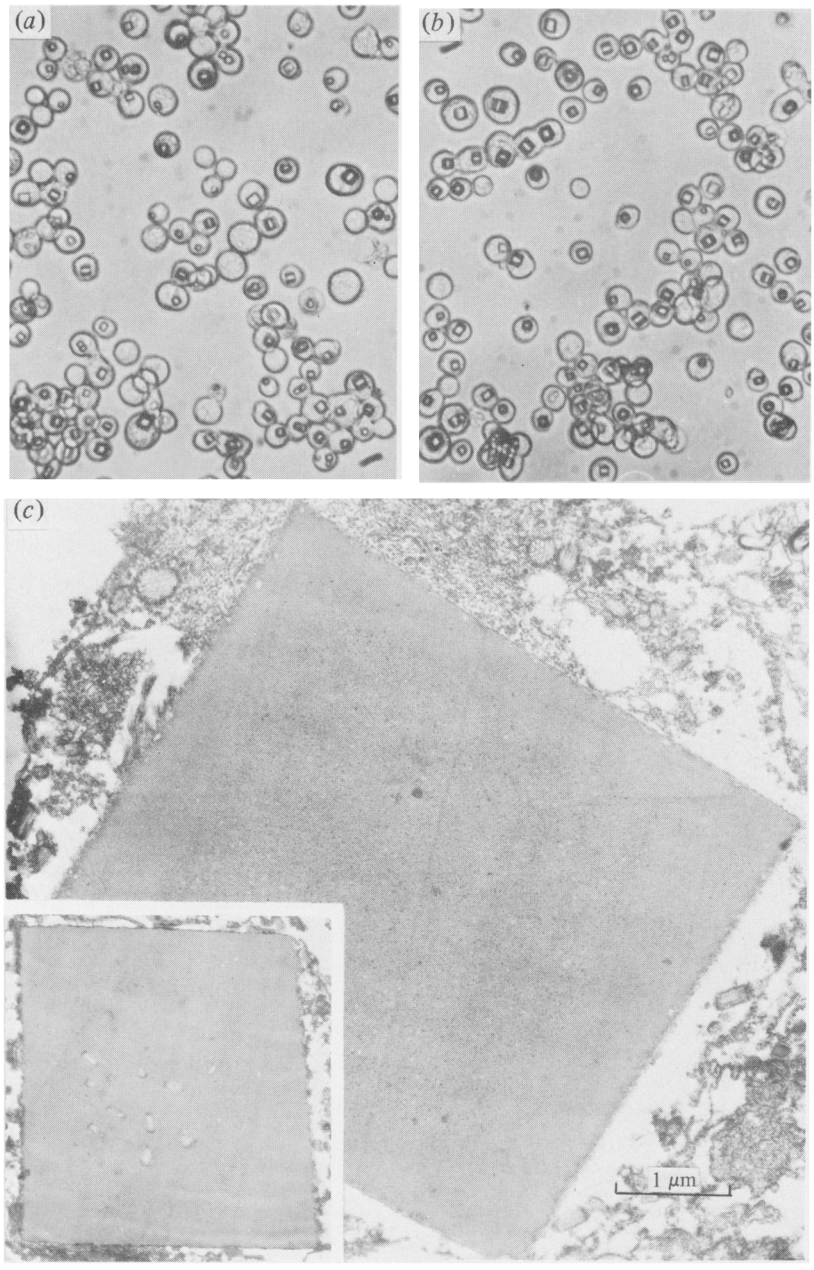

Fig. 1. Crystalline nuclear inclusion bodies in *S. frugiperda* cells infected with morphology mutants. (*a*) *m-5*; (*b*) *m-6*; (*c*) thin section of *S. frugiperda* cell infected with *m-5*. Inset: occluded virus bundles within a cuboidal inclusion body.

Ultrastructure of inclusion bodies

Thin sections of the cuboidal inclusion bodies *m-5* and *m-6* were examined in the electron microscope. Unlike wt inclusion bodies, the majority of cuboidal inclusion bodies did not contain occluded virus (Fig. 1*c*). Very rarely ($< 1{\cdot}0\%$) solitary bundles of apparently normal nucleocapsids were occluded (Fig. 1, inset). A crystalline lattice structure and envelope typical of baculovirus inclusion bodies (Bergold, 1963*b*; Hughes, 1978; Chung *et al.* 1980) was seen in wt polyhedra and in both mutant inclusion bodies (Fig. 2).

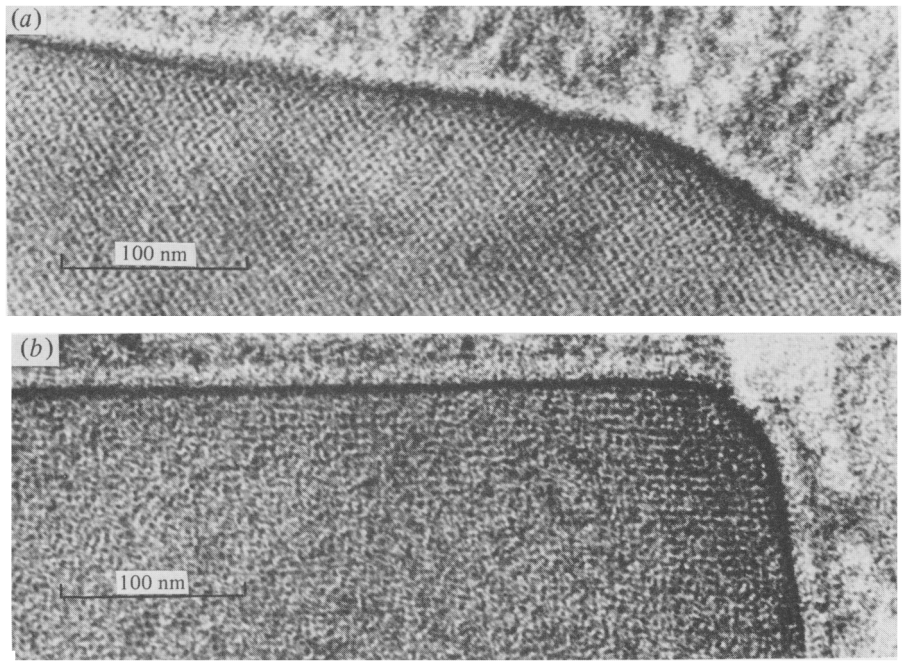

Fig. 2. Internal lattice structure of (*a*) wt and (*b*) *m-5* inclusion bodies.

Table 1. In vivo *infectivity of* m-5, m-6 *and wt inclusion bodies*

Sample	Dose (inclusion bodies/larva)	Number of test larvae killed	% Mortality
wt	2·5	2/21	9·5
	5	2/22	9·1
	10	15/115	13·1
	50	35/111	31·5
	100	41/90	46·5
	250	51/88	58·0
m-5	100	0/42	0
	250	0/44	0
	2500	0/18	0
	10000	1/17	5·9
m-6	100	1/24	4·2
	250	0/23	0
	500	0/21	0
	2500	3/21	14·3
	10000	12/24	50·0

Infectivity of m-5 *and* m-6 *inclusions*

Infectivity of the *m-5* and *m-6* inclusion bodies was tested by feeding purified preparations to third instar *T. ni* larvae (Table 1). The large crystals had low infectivity when compared with wt polyhedra; doses of 10000 inclusions per larva gave rise to 5·9 and 50% mortality for *m-5* and *m-6* respectively, whereas 50% mortality was achieved with 100 to 200 wt polyhedra.

Biochemical studies of wt and mutant inclusion body protein

During the purification of *m-5* and *m-6* inclusion bodies, it was observed that treatment of infected cells with SDS (1%) or with sodium lauryl sarcosine (3·3%) to release the inclusion bodies caused dissolution of the crystals while wt polyhedra were not affected. This suggested that there were alterations in biochemical properties of the large crystals.

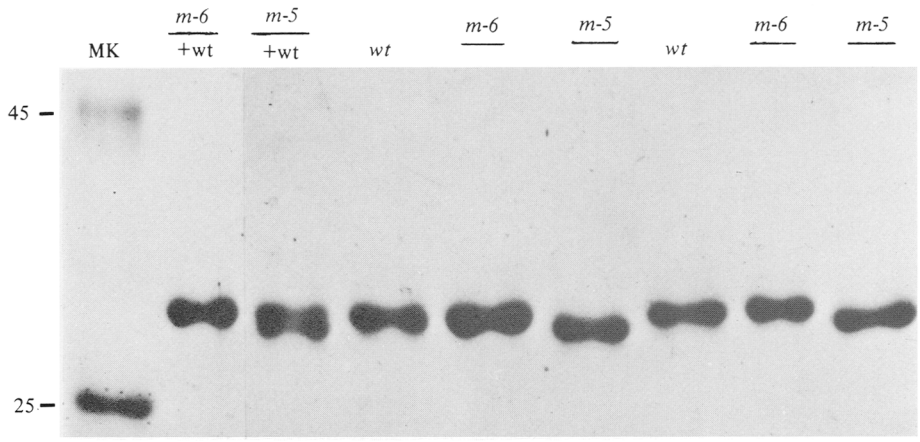

Fig. 3. SDS–PAGE of polyhedrin from wt and mutant inclusion bodies. Markers (MK) had mol. wt. of 25000 and 45000.

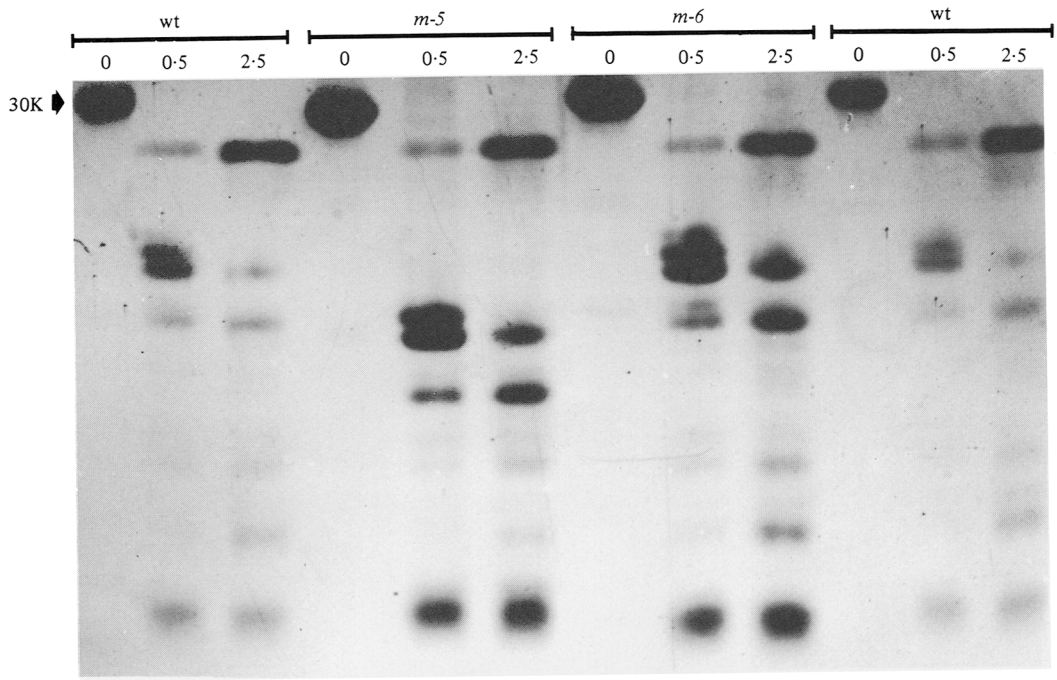

Fig. 4. Peptide analysis following limited proteolysis of polyhedrin from wt and mutant inclusion bodies. The *m-5*, *m-6* and wt polyhedrin was purified by SDS–PAGE. The bands were excised and subjected to proteolysis.

Polyhedrin released from wt, *m-5* and *m-6* inclusion bodies was compared using SDS–PAGE (Fig. 3). The *m-6* and wt polyhedrin migrated the same distance on the gel, whereas *m-5* polyhedrin migrated slightly ahead. Mixtures containing *m-5* and either wt or *m-6* polyhedrin could be resolved into two components, whereas mixtures of wt and *m-6* polyhedrin migrated as a single band. Thus, *m-5* polyhedrin appeared to be altered with respect to wt and *m-6* polyhedrin.

Peptide maps of *m-5*, *m-6* and wt polyhedrin were compared using the limited proteolysis technique of Cleveland *et al.* (1977) (Fig. 4). The electrophoretic patterns of polyhedrin digests generated by *S. aureus* V8 protease were similar for wt and *m-6* polyhedrin, but the pattern of *m-5* polyhedrin was different. In *m-5* polyhedrin samples treated with *S. aureus* V8 protease, one of the larger peptides was missing and a peptide of lower mol. wt. appeared, which was not represented in wt and *m-6* polyhedrin. Limited proteolysis with chymotrypsinogen also resulted in similar electrophoretic patterns for wt and *m-6* polyhedrin which were different from the pattern observed with *m-5* polyhedrin (data not shown).

DISCUSSION

Two mutants of *A. californica* NPV, with a defect in polyhedra formation, were isolated. The inclusion bodies formed in cells infected with these mutants were large cuboidal crystals (Fig. 1) with a crystalline lattice structure and a polyhedron envelope indistinguishable from that of wt polyhedra (Fig. 2). Occluded virus was rarely found in thin sections of the cuboidal inclusion bodies (Fig. 1). This observation appears to be consistent with the low but measurable infectivity of the large crystals in feeding experiments with *T. ni* larvae (Table 1). Although inclusion body formation in cells infected with the two mutants was less efficient at 33 than at 25 °C, inclusion body formation at 33 °C was variable and was not considered to be temperature-sensitive.

The identity of the mutants *m-5* and *m-6*, as derivatives of *A. californica* baculovirus strain HR-3 is inferred since they were derived from a parental stock that had been plaque-purified. Both mutants were able to complement restricted functions of *ts* mutants representing four complementation classes (Brown *et al.* 1979) of their common parent HR-3 (data not presented). The *m-6* polyhedrin fingerprint was identical to that of HR-3 (Fig. 4). We considered that *m-5* and *m-6* may be deletion mutants of HR-3 since in previous experiments we failed to detect genetic recombination between them (Brown & Faulkner, 1980) and in this study the reversion frequency was below the limit of detection.

Although *m-5* and *m-6* express the same morphological phenotype, several differences between them have been identified: (i) the yield of inclusion bodies in cells infected with *m-5* is lower than that in cells infected with *m-6* (data not presented); (ii) *m-5* inclusion bodies have lower infectivity *in vivo* than do *m-6* inclusion bodies (Table 1); (iii) *m-5* polyhedrin migrates slightly faster in SDS–polyacrylamide gels than does *m-6* polyhedrin (Fig. 3); and (iv) *m-5* polyhedrin yields a different peptide pattern following digestion with chymotrypsinogen and *S. aureus* V8 protease (Fig. 4). The differences between *m-5* and wt polyhedrin may not be responsible for the alteration in polyhedra morphology since the same morphological phenotype is expressed by *m-6* whose polyhedrin was indistinguishable from that of wt in these experiments. However, it is possible that more sensitive methods than the Cleveland technique used here are necessary to detect differences between *m-6* and wt polyhedrin. Thus, the assembly of *m-5* and *m-6* polyhedrin into large cuboidal crystals may be due to alterations in the *m-5* and *m-6* polyhedrin *per se* or may be due to an altered function not yet identified.

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