Evidence for the presence of a low-level, persistent baculovirus infection of *Mamestra brassicae* insects

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A laboratory culture of *Mamestra brassicae* insects (MbLC) harbours a latent or occult baculovirus that resembles *M. brassicae* multiple nucleocapsid nucleopolyhedrovirus (MbMNPV). Although conventional extraction techniques have failed to detect the presence of virus in MbLC, control virus-free insects (MbWS) died of an MbMNPV-like infection after being fed MbLC fat-body cells. This suggested that the MbLC cells harboured infectious MbMNPV, albeit at low levels. We have also demonstrated that fat-body cells from MbLC, but not from MbWS, contain mRNA specific for the polyhedrin gene and transcriptional factors that are capable of activating baculovirus late and very late gene promoters linked to a reporter gene encoding chloramphenicol acetyltransferase. Our data provide indirect evidence that the latent MbMNPV in the MbLC insects is maintained as a persistent infection, with the expression of viral genes at a low level.

An important factor contributing to the success of baculoviruses as biological control agents against insect pests is the ability of the virus to persist in the environment. This is achieved largely by protection of virus particles in proteinaceous occlusion bodies or polyhedra (Harrap *et al.*, 1977; Hunter *et al.*, 1984; Miller, 1996). Polyhedra are composed mainly of a single virus-encoded polypeptide, polyhedrin, which forms a quasi-crystalline lattice into which the virus particles become embedded (Rohrmann, 1986). Survival may be achieved by persistence of polyhedra in soil or decaying leaf matter, particularly during periods when the insect host is not available. There is evidence, however, that the virus may persist within the host insect population in an occult or latent state (Longworth & Cunningham, 1968; Jurkovicova, 1979; McKinley *et al.*, 1981; Burand *et al.*, 1992). Several reports have also shown that insects infected with one virus actually die from the activation of a supposedly latent virus rather than the applied virus (Longworth & Cunningham, 1968; Jurkovicova, 1979; McKinley *et al.*, 1981). However, the mechanisms of baculovirus latency and activation of occult viruses are poorly understood and, until recently, no-one had ever demonstrated the presence of a latent virus in an unchallenged insect population (Hughes *et al.*, 1993).

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Hughes *et al.* (1993) demonstrated that a laboratory culture of *Mamestra brassicae* insects (MbLC) harboured a latent or occult baculovirus infection. This virus was activated by feeding the *M. brassicae* larvae with either the closely related *Panolis flammea* multiple nucleocapsid nucleopolyhedrovirus (PaflMNPV), or the more distantly related *Autographa californica* (Ac) MNPV. DNA profiles from the activated virus showed that it was very closely related, if not identical, to *M. brassicae* (Mb) MNPV (Possee & Kelly, 1988). PCR amplification of polyhedrin gene sequences demonstrated that the MbMNPV-like virus was present in the egg, larval, pupal and adult stages of the insect life-cycle. Using PCR analysis of DNA isolated from dissected tissues of fourth instar MbLC larvae, virus sequences were only detected in the fat-body. We failed to detect the MbMNPV-like virus in a second culture of *M. brassicae* insects (MbWS), recently obtained from the environment and adapted to growth in laboratory conditions. These insects acted as important negative controls in all experiments.

There are two main mechanisms proposed to account for the latent state of virus in a host. The genetic material may integrate into the host cell genome as a provirus-like structure, as is found with hepatitis B virus (Howard, 1986), or may be maintained as independent, quiescent viral genetic material within the host cell nucleus, as in herpes simplex virus (Mellerik & Fraser, 1987). Alternatively, the virus may remain as a low level infection with the production of viral proteins, as has been found for measles virus (Cattaneo *et al.*, 1988). The latter is usually referred to as a persistent, rather than a latent, infection. In this report we provide indirect evidence to suggest that the MbMNPV-like virus is maintained in *M. brassicae* as a persistent infection.

Although Northern blotting experiments failed to detect virus-specific mRNA in MbLC larvae (data not shown), we have been able to detect polyhedrin-specific mRNA using the

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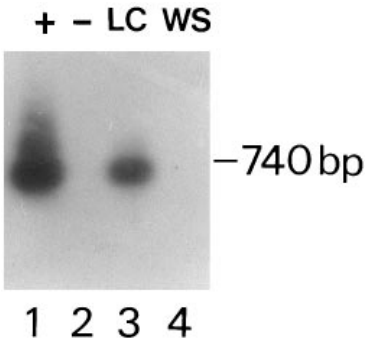

Fig. 1

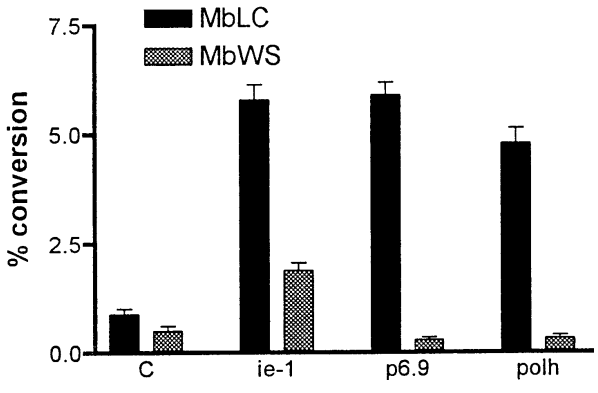

Fig. 2

Fig. 1. Autoradiograph showing RT–PCR amplification of polyhedrin-specific transcripts from *M. brassicae* total genomic RNA. RNA was isolated from the laboratory culture (LC, lane 3) and wild-stock (WS, lane 4) *M. brassicae* larvae and from MbMNPV-infected *M. brassicae* cells (+, lane 1). Following heating of the RNA (2 μg; 70 °C, 5 min), first strand cDNA synthesis was carried out in 50 mM Tris–HCl pH 8·3, 50 mM KCl, 10 mM $MgCl_2$, 0·5 mM spermidine, 10 mM DTT, 1 mM each dATP, dCTP, dGTP and dTTP, 25 units of RNasin ribonuclease inhibitor (Promega), 0·5 μg $pd(N)_6$ (Pharmacia) and 50 units of AMV reverse transcriptase (Promega). The reaction mixture was incubated (42 °C, 60 min) before removing one-third of the reaction volume for RT–PCR analysis. RT–PCR reactions used 0·5 μg cDNA together with 1·0 μM each primer in 50 mM KCl, 10 mM Tris–HCl, pH 8·8, 1·5 mM $MgCl_2$, 0·1% Triton X-100 and 0·2 mM each dNTP together with 2 units of *Taq* DNA polymerase (Promega). Each reaction consisted of 30 cycles of amplification using a step programme (94 °C for 1 min, 45 °C for 2 min, 74 °C for 2 min) followed by a 7 min final extension at 74 °C. The negative control (−, lane 2) contained water in place of template DNA. One-fifth (10 μl) of each reaction was resolved on a 1% agarose gel. PCR products were transferred to a nylon membrane and hybridized in 50% formamide to a ^{32}P-labelled AcMNPV *Hin*dIII'V' DNA probe, containing the 3' portion of the polyhedrin gene.

Fig. 2. Transient expression assays for identifying transcriptional factors from the persistent MbMNPV infection. Primary cultures of fat-body cells derived from MbLC or MbWS were transfected with 0·5 μg of reporter plasmid DNA (*ie-1*, *p6.9* or *polh*) as indicated. A control was included which contained the CAT gene with no promoter sequences (C). At 24 h (for C and *ie-1*) or 48 h (for *p6.9* and *polh*), cells were harvested, extracts prepared and CAT assays performed (Gorman *et al*., 1982; Passarelli & Miller, 1993). The percentage conversion of chloramphenicol to its acetylated products is shown ($n = 3$).

more sensitive technique of RT–PCR, in which mRNA is converted to cDNA prior to amplification. Total cellular RNA was extracted from third instar MbWS or MbLC larvae by grinding insect material (1·5 g) to a powder in liquid nitrogen before homogenization and extraction of RNA using the guanidium isothiocyanate method, essentially as described by King & Possee (1992) with the exception that the RNA was treated with DNase I prior to first strand cDNA synthesis. As a positive control RNA was similarly prepared from *M. brassicae* cells infected with MbMNPV and harvested at 24 h post-infection (p.i.). The RNA was treated with DNase I and first strand cDNA synthesis was performed as described in Fig. 1. PCR was then performed on samples of the cDNAs using 21-mer primers consisting of DNA complementary to the 5′ (5′ ACCCGTTACAGTTACAATCCG 3′; nt 5′–7 to 28–3′) and 3′ (5′ GGCGGGTCCGTTGTACAGAGG 3′; nt 3′–735 to 714–5′) regions of the 740 bp polyhedrin gene of MbMNPV (Cameron & Possee, 1989). A negative control consisting of amplification cocktail and sterile, deionized water *in lieu* of the template DNA was also included.

The PCR reactions were resolved on an agarose gel and specific products detected by Southern blotting and hybridization using ^{32}P-labelled polyhedrin gene sequences as the probe. As shown in Fig. 1, cDNAs derived from MbMNPV-specific transcripts were amplified from the MbLC larvae (Fig. 1, lane 3) but no baculovirus polyhedrin gene-specific sequences were detected in cDNA derived from MbWS RNA transcripts (Fig. 1, lane 4).

The presence of virus-specific polyhedrin mRNA suggested that a low level of virus replication was occurring in the MbLC larvae. Accordingly, we examined the MbLC larvae for the presence of transcriptional factors capable of transactivating baculovirus promoters in standard assays of transient chloramphenicol acetyltransferase (CAT) activity (Passarelli & Miller, 1993). Reporter plasmids were constructed containing early (*ie-1*), late (*p6.9*) and very late (*polh*) AcNPV gene promoters upstream of the *Escherichia coli* CAT gene. Preliminary experiments (data not shown), and the work of Cameron & Possee (1989), indicated that MbMNPV-infected cell extracts contained transcriptional factors capable of efficient activation of AcMNPV early, late and very late gene promoters. Primary cultures of fat-body cells (2×10^5 cells) derived from MbLC or MbWS were transfected with 0·5 μg of reporter plasmid DNA using Lipofectin (Gibco Life Technologies). At 24 h (*ie-1* or control promoter-less reporter plasmid) or 48 h (*p6.9* or *polh* promoter reporter plasmids), cells were harvested, lysates

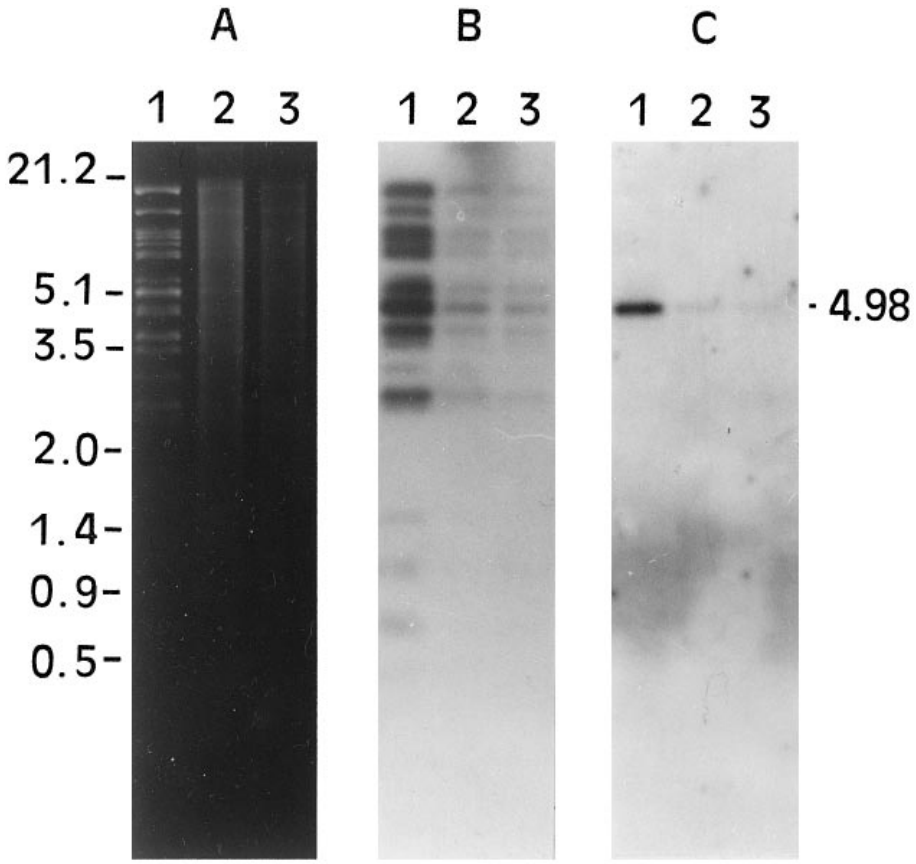

Fig. 3. Induction of polyhedrosis by inoculation of *M. brassicae* WS insects with MbLV cells. Typical results from individual *M. brassicae* WS larvae inoculated *per os* with MbLV1 (lane 2) or MbLV2 (lane 3) at a dose of 10^7 cells per larva are shown. After initial infection, polyhedra were harvested from larvae at 8–10 days p.i. before isolating and characterizing virus DNA. A virus DNA control of MbMNPV (lane 1) was previously amplified in *M. brassicae* WS. (*a*) Virus DNA (1 μg) was digested with *Hin*dIII and fractionated in a 0·6% agarose gel. λ-*Hin*dIII/*Eco*RI DNA markers (kbp) are shown on the left. (*b*, *c*) Southern blot hybridization analysis of the agarose gel shown in (*a*). Restriction fragments were transferred to a nylon membrane and hybridized in 50% formamide to a ^{32}P-labelled MbMNPV DNA probe (*b*) and with a radiolabelled AcMNPV *Hin*dIII'V' DNA probe, containing the 3′ portion of the polyhedrin gene (*c*). Filters were washed for 2 × 30 min in 0·1 × SSC, 0·1% SDS at 42 °C prior to autoradiography. The size (kbp) of the *Hin*dIII polyhedrin-containing fragment is indicated on the right.

prepared and CAT assays performed as previously described (Gorman *et al.*, 1982; Passarelli & Miller, 1993).

Transfection of primary cultures of MbLC fat-body cells with each of the vectors resulted in the detection of CAT production under the control of early, late and very late gene promoters (Fig. 2). No CAT activity was observed in the MbWS primary fat-body cells transfected with the reporter plasmids containing the *p6.9* or *polh* promoters. However, very low levels of CAT were observed in MbWS cells transfected with the *ie-1* reporter plasmid. This was consistent with the findings of Guarino & Summers (1988) who demonstrated low levels of CAT activity in non-infected cells transfected with plasmids containing the *ie-1* promoter. This demonstrated that early, late and very late gene expression factors were present, albeit at low levels, only in the MbLC fat-body cells and not in the fat-body cells derived from MbWS. Statistical analyses using a two-tailed *t*-test (Graph Pad Prism v2.0) indicated that the difference in the levels of CAT activity observed between the two insect hosts, MbLC and MbWS, was significant ($P < 0·05$).

In addition to the primary fat-body cultures, we have also established four independent cell lines (MbLV1–4) from fat-body tissue from MbLC larvae, all retaining latent or occult virus sequences when analysed by polyhedrin-specific PCR amplification (Hughes *et al.*, 1993). An *M. brassicae* cell line (MbC; King *et al.*, 1991) tested at the same time proved negative for virus-specific DNA sequences. As all attempts to detect virus proteins using conventional SDS–PAGE and Western blotting techniques failed (data not shown), we decided to use the MbLC cell lines to look for indirect evidence of virus production. Newly emerged, third instar MbWS larvae (200 per treatment or control; $n = 2$) were infected *per os* (King & Possee, 1992) with MbLV1–4 cells (10^7 cells per larva). Larvae, fed on diet inoculated with negative control MbC cells (King *et al.*, 1991) at a similar dose, were also included. All larvae were examined daily, and allowed to continue feeding

until death or pupation. Only those larvae which had died were frozen for later examination. Following pupation of the survivors, all larvae and pupae were individually smeared onto microscope slides and Giemsa staining was used to detect the presence of polyhedra and confirm virus-induced death. Smears showing nuclei containing polyhedra were classified as positive. The average number of positive larvae obtained from the four MbLV cell lines in two separate feeding experiments was 2% (±0·9). All the control larvae proceeded to pupation, and all were shown to be free of virus infection.

To confirm that MbMNPV was the cause of virus-induced deaths, DNA was extracted from individually infected larvae, as previously described (Hughes *et al*., 1993, 1994), and characterized by restriction endonuclease analysis and Southern blot hybridization. DNA from two individual larvae infected *per os* with cells isolated from MbLV1 and MbLV2 was cleaved with *Hin*dIII and fractionated in an agarose gel (Fig. 3 *a*, lanes 2–3, respectively). Purified MbMNPV DNA served as a control (lane 1). Discrete ethidium-bromide stained bands were detected in the DNA from the infected larvae. The smearing observed in these profiles can be assumed to represent cellular chromosomal DNA and suggests that not all the insect cells were infected with virus, as host cell DNA had not been completely degraded.

Southern hybridization analysis was employed to confirm that infection was due to an activated MbMNPV infection and not due to contamination with other, heterologous viruses. Restriction fragments were transferred to a nylon membrane by Southern blotting and hybridized to ^{32}P-labelled MbMNPV genomic DNA, which had been digested with *Hin*dIII and radiolabelled by random priming. The resultant autoradiograph, obtained after high-stringency washing of the filter, is shown in Fig. 3 (*b*). The MbMNPV DNA probe hybridized to the control, wild-type MbMNPV DNA (lane 1) and detected fragments of the same sizes in the DNA isolated from the individual infected larvae (lanes 2 and 3).

The MbMNPV probe was removed by boiling in 0·1% SDS and the membrane re-hybridized in 50% formamide to a ^{32}P-labelled polyhedrin-specific probe. The resultant autoradiograph, obtained after high-stringency washing of the filter, is shown in Fig. 3 (*c*). The polyhedrin-specific probe highlighted a 4·98 kbp polyhedrin-specific DNA fragment in control MbMNPV DNA (lane 1). Detection of a polyhedrin-specific DNA fragment of the same size in DNA isolated from the *M. brassicae* WS larvae which received MbLV1 (lane 2) or MbLV2 (lane 3) cells confirmed that virus infection was due to MbMNPV. These data suggest that the occult virus-containing fat-body tissue of *M. brassicae* LC, even after serial passage in cell culture, harbours MbMNPV-like virus particles or polyhedra which, in certain circumstances, can cause an overt infection in insect larvae. These results suggest, therefore, that the latent or occult MbMNPV may remain as a persistent infection, allowing the continuous expression of viral proteins at a low level. If the latent state was maintained as DNA alone, it would be very unlikely that the DNA would be able to initiate an infection via the insect gut.

Experiments designed to detect viable virus particles in the MbLV cell lines by inoculation of MbWS larvae with MbLV cells strongly suggested that the MbLV cells harbour persistent MbMNPV-like virus particles. Assays of transient reporter gene expression for identifying transcriptional factors from the persistent MbMNPV infection demonstrated that early, late and very late gene expression factors were present, albeit at low levels, in the fat-body cells. Baculovirus-specific mRNA was also detected in MbLC larvae by RT–PCR analysis of MbMNPV polyhedrin gene transcripts. These data strongly suggest that the cell cultures, established from the occult virus-containing fat-body tissue of MbLC, harbour MbMNPV-like virus particles/polyhedra which are able, in certain circumstances, to cause an overt infection in insect larvae. This suggests that the latent or occult MbMNPV remains as a persistent infection, allowing the continuous expression of viral proteins at a low level. This is analogous to the situation proposed to explain persistent measles virus infections (Cattaneo *et al*., 1988). It will be interesting to determine whether novel virus-encoded gene transcripts or proteins play a role in maintaining the low-level, persistent infection, as has been proposed for latent herpesvirus infections (Mellerik & Fraser, 1987) or whether the host cell plays a crucial role in maintaining the persistent state.

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References

Burand, J. P., Horton, H. M., Retnasami, S. & Elkington, J. S. (1992). The use of polymerase chain reaction and shortwave UV irradiation to detect baculovirus DNA on the surface of gypsy moth eggs. *Journal of Virological Methods* **36**, 141–150.

Cameron, I. R. & Possee, R. D. (1989). Conservation of polyhedrin gene promoter function between *Autographa californica* and *Mamestra brassicae* nuclear polyhedrosis viruses. *Virus Research* **12**, 183–200.

Cattaneo, R., Schmid, A., Eschle, D., Baczko, K., ter Meulen, V. & Billeter, M. A. (1988). Biased hypermutation and other genetic changes in defective measles viruses in human brain infections. *Cell* **55**, 255–265.

Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Molecular and Cellular Biology* **2**, 1044–1051.

Guarino, L. A. & Summers, M. D. (1988). Functional mapping of *Autographa californica* nuclear polyhedrosis virus genes required for late gene expression. *Journal of Virology* **62**, 463–471.

Harrap, K. A., Payne, C. C. & Robertson, J. S. (1977). The properties of three baculoviruses from closely related hosts. *Virology* **79**, 14–31.

Howard, C. R. (1986). The biology of hepadnaviruses. *Journal of General Virology* **67**, 1215–1235.

Hughes, D. S., Possee, R. D. & King, L. A. (1993). Activation and detection of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects. *Virology* **194**, 608–615.

Hughes, D. S., King, L. A. & Possee, R. D. (1994). Quantification of latent *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects using a PCR-scintillation proximity assay. *Journal of Virological Methods* **50**, 21–28.

Hunter, F. R., Crook, N. E. & Entwistle, P. F. (1984). Viruses as pathogens for the control of insects. In *Microbial Methods for Environmental Biotechnology*, pp. 323–347. Edited by J. M. Grainger & J. M. Lynch. New York & London: Academic Press.

Jurkovicova, M. (1979). Activation of latent virus infections in larvae of *Adoxophyes orana* (Lepidoptera: Tortricidae) and *Barathra brassicae* (Lepidoptera: Noctuidae) by foreign polyhedra. *Journal of Invertebrate Pathology* **34**, 213–223.

King, L. A. & Possee, R. D. (1992). *The Baculovirus Expression System: A Laboratory Guide*. London: Chapman & Hall.

King, L. A., Mann, S. G., Lawrie, A. M. & Mulshaw, S. H. (1991). Replication of wild-type and recombinant *Autographa californica* nuclear polyhedrosis virus in a cell line derived from *Mamestra brassicae*. *Virus Research* **19**, 93–104.

Longworth, J. F. & Cunningham, J. C. (1968). The activation of occult nuclear polyhedrosis viruses by foreign nuclear polyhedra. *Journal of Invertebrate Pathology* **10**, 361–367.

McKinley, D. J., Brown, D. A., Payne, C. C. & Harrap, K. A. (1981). Cross infectivity and activation studies with four baculoviruses. *Entomophaga* **26**, 79–90.

Mellerik, D. M. & Fraser, N. W. (1987). Physical state of the latent herpes simplex virus genome in a mouse model system: evidence supporting an episomal state. *Virology* **158**, 265–275.

Miller, L. K. (1996). Insect viruses. In *Fields Virology*, pp. 533–556. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott–Raven.

Passarelli, A. L. & Miller, L. K. (1993). Identification and characterization of *lef-1*, a baculovirus gene involved in late and very late gene expression. *Journal of Virology* **67**, 3481–3488.

Possee, R. D. & Kelly, D. C. (1988). Physical maps and comparative DNA hybridization of *Mamestra brassicae* and *Panolis flammea* nuclear polyhedrosis virus genomes. *Journal of General Virology* **69**, 1285–1298.

Rohrmann, G. F. (1986). Polyhedrin structure. *Journal of General Virology* **67**, 1499–1533.

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