Establishment of an insect cell clone that harbours a partial baculoviral genome and is resistant to homologous virus infection

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After serially undiluted passage of Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV), persistently infected Se301 cells were established. A cell strain, in which no polyhedra or viral particles were observed, was cloned and designated P8-Se301-C1. The P8-Se301-C1 cells are morphologically similar to but grow slower than Se301 cells and they can homologously interfere with SeMNPV. PCR analysis showed that SeMNPV ie-0 and polyhedrin genes were present but DNA polymerase and orf67 genes were absent in P8-Se301-C1, suggesting that the cells harbour incomplete SeMNPV genomes. Dot-blot analysis demonstrated that 0.32 ± 0.16 ng SeMNPV DNA was present in 1.25×10^6 P8-Se301-C1 cells. A quantitative real-time PCR assay showed that there were 13.2 ± 4.3 copies of the SeMNPV polyhedrin gene in each cell. Nested RT-PCR demonstrated the presence of SeMNPV polyhedrin transcripts in P8-Se301-C1 cells. The fact that P8-Se301-C1 cells carry low levels of partial viral genome but do not produce viral progeny suggests a latent-like viral infection in the cells.

Baculoviruses are a large family of circular, double-stranded DNA viruses that specifically infect insects (Rohrmann, 2008). Canonical baculovirus life-history is different from that of many other viruses as there are two highly divergent virion morphotypes involved: occlusion-derived virus (ODV) and budded virus (BV) (Rohrmann, 1992). Baculoviruses are generally thought to be transmitted horizontally from host to host via the proteinaceous occlusion bodies (OBs) containing ODVs.

OBs permit baculoviruses to persist in a viable state in the environment, particularly during periods when the insect host is not available (Cory & Myers, 2003; Fuxa, 2004). It was suggested that baculoviruses cannot rely on the strategy of persistent or latent infection due to the dramatic physiological changes occurring in the short life span of their hosts (Slack & Arif, 2007). However, reports have provided evidence that laboratory insect cultures (Hughes et al., 1993, 1997) and natural populations (Burden et al., 2003, 2006) may harbour persistent baculoviruses (Il'inykh & U'yanova, 2005). The persistent infection can be transformed to a replicative and infective state caused by stress factors such as overcrowding or ingestion of heterologous viruses (Cooper et al., 2003; Fuxa et al., 1999; Podgwaite & Mazzone, 1986; Steinhaus, 1958). Detailed experiments designed to understand the molecular mechanisms of persistent infections are limited (Hughes et al., 1997). Establishment of cells persistently infected with baculovirus has been described only rarely (Crawford & Sheehan, 1983; Lee et al., 1998; McIntosh & Ignoffo, 1981), and there is a lack of virus cell models available to investigate the mechanism of baculovirus persistence. Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) is mono-specific and highly virulent for S. exigua (Gelernter & Federici, 1986a). Cell line Se301 is derived from S. exigua (Hara et al., 1995) and infection of Se301 with SeMNPV results in cell cytolysis and cell death (Gelernter & Federici, 1986b). In this report, a persistent SeMNPV infection in Se301 cells was established. From the persistently infected cells, a cell clone was isolated in which no infectious virus was produced but which contains incomplete SeMNPV genomic DNA and some SeMNPV gene transcripts.

Firstly, SeMNPV was serially passaged undiluted in Se301 cells, as described previously (Pijlman et al., 2001), and the infectivity of SeMNPV was reduced gradually. Upon infection with the attenuated SeMNPV at passage 8, a few cells survived; these continued to grow and could be subcultured. The recovered cells, designated P8-Se301, showed a persistent infection phenotype because polyhedra emerged consistently in some cells. Subsequently, a cell clone designated P8-Se301-C1 was isolated from P8-Se301 cells using a limiting dilution method as described previously (Crawford & Sheehan, 1983). The kinetics of cell growth was measured by recording cell densities for...
6 days with the trypan blue method. Based on the exponential phase from 24 to 96 h post-incubation (Fig. 1a), P8-Se301-C1 cells have a population doubling time of 37–39 h and grow slower than Se301 cells, which have a population doubling time of 29–31 h.

P8-Se301-C1 cells were morphologically similar to Se301 cells. Electron microscopy was performed as described previously (Li et al., 2005), and signs of viral infection including virogenic stroma, viral particles and OBs were not observed (Fig. 1b), which confirmed that no virus particles were present in the cells. When the supernatant of P8-Se301-C1 cell culture was examined with a TCID$_{50}$ endpoint dilution assay (O’Reilly et al., 1994), no infectious virus was detectable, indicating that no infectious BVs were released from the cells.

To confirm that P8-Se301-C1 cells are virus-free, total cellular DNA was extracted as described previously (Pijlman et al., 2002). PCR was performed on 100 ng total DNA to detect the presence of SeMNPV genome sequences. Primers were designed according to the genome sequence of SeMNPV deposited in GenBank (Ji et al., 1999). Primers SeMNPV-ie-0-131937f (5’-CTATAGCTGACGCTGGTG-3’) and SeMNPV-ie-0-132447r (5’-ATCCTCTTGATACCCGGAG-3’) were used to amplify a 511 bp fragment of the SeMNPV immediate-early region (ie-0) gene; primers SeMNPV-dnapol-90450f (5’-CAACGGCACCAGACTGTAAAC-3’) and SeMNPV-dnapol-90871r (5’-AGCACGCATCACTGAGAAT-3’) were used to amplify a 422 bp fragment of the SeMNPV early DNA polymerase gene (dnapol); primers SeMNPV-orf67-64186f (5’-GTGCGGACGATGACGACA-3’) and SeMNPV-orf67-64708r (5’-AGCACGACTTTGGGCGACTT-3’) were used to amplify a 523 bp fragment of the SeMNPV orf67 gene (ac38K); primers SeMNPV-polh-88f (5’-AAAAACGCAACAGCAAGGACAT-3’) and SeMNPV-polh-443r (5’-ATGCTGGGTACGCAACGGAGAGC-3’) were used to amplify a 356 bp fragment of the SeMNPV very late polyhedrin gene (polh). As shown in Fig. 2(a), the PCR products of the ie-0 and polh genes were detected in both SeMNPV-infected Se301 cells and P8-Se301-C1 cells. The PCR products were cloned for sequencing and the results proved that the DNA sequences of the products were identical in both cells. The products of the dnapol and orf67 genes were detected in the SeMNPV-infected Se301 cells but not in the P8-Se301-C1 cells (Fig. 2a), although both genes are essential for virus replication of Autographa californica multiple nucleopolyhedrovirus (AcMNPV) (Vanarsdall et al., 2005; Wu et al., 2006). No products could be amplified from the mock-infected Se301 cells using any of these primer sets, excluding viral DNA contamination in the samples. This suggested that SeMNPV-specific DNAs were present in P8-Se301-C1 cells; however, there were no intact SeMNPV genomes.

Because we do not know the size of the SeMNPV genomic sequence in P8-Se301-C1 cells, we cannot determine the number of incomplete SeMNPV genome copies precisely. Therefore, a dot-blot analysis was performed to estimate the relative abundance of the SeMNPV genome content in P8-Se301-C1 cells. Total DNA was extracted from equal amounts (1.25 × 10$^5$ cells) of P8-Se301-C1 or Se301 cells and subjected to dot-blot hybridization as described previously (Wu et al., 2006). SeMNPV genomic DNA was extracted from polyhedra as described previously (Simon et al., 2005) and used as a probe after being labelled with digoxigenin according to the manufacturer’s instructions (Roche). The blots were scanned and quantified using a Quantity One 1-D analysis software (Bio-Rad). The SeMNPV DNA content in the cells was determined by interpolating data from a standard curve, which was obtained with increasing amounts of SeMNPV genomic DNAs. As shown in Fig. 2(b), although the signals in P8-Se301-C1 cells were faint, they were slightly higher than the background as measured in Se301 cells. Based on the relative intensity of SeMNPV genomic DNA, we estimated that 0.32 ± 0.16 ng of SeMNPV genomic DNA was present in 1.25 × 10$^5$ P8-Se301-C1 cells.

Subsequently, a quantitative real-time PCR was carried out to assess the number of copies of SeMNPV polh gene in P8-Se301-C1 cells. Primers SeMNPV-polh-495f (5’-CAACGAATACCGCATCA-3’) and SeMNPV-polh-645r (5’-CACTCCCTCCCTACCGCC-3’) were used and real-time PCRs were carried out on an iQTM5.0 thermal cycler (Bio-Rad) for 45 cycles (95 °C for 30 s, 60 °C for 20 s and 72 °C for 20 s) after an initial 15 min at 95 °C. Quantification was relative to a standard curve, which was generated by 10-fold serial dilutions of plasmid

![Fig. 1. P8-Se301-C1 isolated from persistently infected Se301 cells. (a) Growth curves for P8-Se301-C1 (○) and Se301 (■) cells. Data points represent means ± SD from three independent experiments. (b) Electron microscopy showed that the P8-Se301-C1 cells had no features characteristic of virus infection, such as virogenic stroma, viral particles and OBs. Bar, 0.1 μm.](image-url)
containing the SeMNPV \textit{polh} gene. The result showed that 13.2 ± 4.3 copies of the SeMNPV \textit{polh} gene were present in each of P8-Se301-C1 cells.

Then, RT-PCR was performed to detect SeMNPV transcripts in P8-Se301-C1 cells. Total cellular RNA was prepared according to the manufacturer’s instructions (RNeasy mini kit, Qiagen) and 1 μg total RNA was treated with RNase-free DNase (Promega). First strand cDNA was synthesized using an oligo (dT) primer according to the manufacturer’s protocol [TaKaRa RNA-PCR kit (AMV) version 3.0]. RNA isolated at 96 h post-infection (p.i.) from Se301 cells infected with SeMNPV at an m.o.i. of 0.1 was used as positive control; RNA from mock-infected Se301 cells was used as negative control. PCR was carried out with the primers for the SeMNPV \textit{polh} or \textit{ie-0} genes (given above). Neither \textit{polh} nor \textit{ie-0} gene transcripts were detected in the P8-Se301-C1 cells (Fig. 2c). In contrast, in the SeMNPV-infected Se301 cells, transcripts of the \textit{polh} gene were detected, but transcripts of the \textit{ie-0} gene were not. To avoid the possibility that the gene transcripts could not be detected because of low abundance, 1 μl amplification product from the first round PCR was used as a template for an additional amplification round of nested PCR. Nested primer pairs SeMNPV-\textit{polh}-116f (5’-TGCAA-CATGAAATTTGAAGAG-3’) and SeMNPV-\textit{polh}-323r (5’-AAGCTGTCCTCCATGAAACG-3’) were designed to target a 208 bp fragment inside the first PCR product of \textit{polh}; nested primer pairs SeMNPV-\textit{ie-0}-131957f (5’-CTGA-GTTACGTTCCATGAAACG-3’) and SeMNPV-\textit{ie-0}-132206r (5’-TATATGCACGTCATCAAACT-3’) targeted a 250 bp fragment inside the first PCR product of \textit{ie-0}. The products of SeMNPV \textit{polh} were detected for both P8-Se301-C1 cells and SeMNPV-infected Se301 cells, but not in mock-infected Se301 cells (Fig. 2c). All the RNA samples prepared in this study were subjected to PCR and nested PCR with the same primers. The negative results indicated that DNA contamination was absent.
Homologous interference is the ability of an established virus infection to interfere with infection by an identical or closely related virus (Adams & Brown, 1985). To investigate the homologous interference ability of P8-Se301-C1 cells with infection by SeMNPV, 2 x 10^5 P8-Se301-C1 cells or Se301 cells were infected in triplicate with SeMNPV at an m.o.i. of 0.1. No morphological differences were observed between the two cell cultures at 0 h p.i. However, at 48 h p.i., polyhedra appeared in the SeMNPV-infected Se301 cells; in contrast, no polyhedra were observed in the SeMNPV-infected P8-Se301-C1 cells. At 96 h p.i., more than 50% of the infected Se301 cells contained OBs and some OBs were released into the culture medium. In contrast, the infected P8-Se301-C1 cells showed less cytopathogenic effects, one of which is that most of the cells remained attached to the culture vessel and less than 10% of the cells contained OBs. In a parallel series of experiments, the virus progeny yields of the infections were quantified. At various time points after infection, the cell pellets were harvested and the OBs were counted using a haemocytometer following release of the OBs, as described previously (Kelly et al., 2008). The virus titres of BV-containing supernatants were calculated by a TCID_{50} end-point dilution assay on SeUCR1 cells. As shown in Fig. 3(a), OB amounts increased sharply in the infected Se301 cells at 48 h p.i. In contrast, there was only a minor increase in the number of polyhedra in the infected P8-Se301-C1 cells. When comparing the peak values in the two cell cultures at 120 h p.i., the amount of polyhedra produced was 1.9-fold lower in the P8-Se301-C1 cells than in the Se301 cells (t-test, P<0.01) (Fig. 3a). The kinetics of BV production (Fig. 3b) indicated that the number of BVs produced in the infected P8-Se301-C1 cells were consistently 10-fold lower than in the infected Se301 cells (t-test, P<0.05). However, the interference of P8-Se301-C1 cells did not occur when the cells were challenged with the heterologous AcMNPV. The BV yields in AcMNPV-infected P8-Se301-C1 cells were comparable to those of AcMNPV-infected Se301 cells (Fig. 3c). The results demonstrated that P8-Se301-C1 cells possess the capacity to interfere with the homologous SeMNPV.

In conclusion, with the passaged, attenuated SeMNPV, a cell line P8-Se301 was established which continuously produces progeny viruses and shows a typical persistent infection. P8-Se301-C1 was cloned from the P8-Se301 cells. The P8-Se301-C1 cells do not produce infectious virions and viral particles are not observed (Fig. 1b). However, a small portion of SeMNPV-specific DNA (e.g. polh and ie-0) and SeMNPV-specific transcripts (e.g. polh) are present in the cells (Fig. 2). This suggests that P8-Se301-C1 cells show some characteristics of latent infection. In a typical latent infection, e.g. latent infection of herpesviruses in humans, viral genomes and limited transcripts are detected in cells, but no infectious virus is produced (Stevens, 1989). Although, in our hands, various treatments such as thermal shock, exposing cells to heterologous viruses (AcMNPV or Spodoptera litura nucleopolyhedrovirus) have been performed, reactivation of infectious virus in P8-Se301-C1 cells failed (data not shown). So far, we suggest that SeMNPV resides in P8-Se301-C1 cells in a latent-like infection status.

There was interference with replication of the homologous SeMNPV in P8-Se301-C1 cells, but none with the heterologous AcMNPV (Fig. 3). Some previous studies have shown that the cell clones derived from cells persistently infected by vertebrate viruses have the capacity of homologous interference, but they are virus-free or lack viral RNA or DNA (Ahmed et al., 1981; de la Torre et al., 1988; Dermody et al., 1993; Zhong et al., 2006). However, in the present study, P8-Se301-C1 cells were shown to harbour a low abundance of SeMNPV genomes by using PCR, dot-blotting and real-time PCR. So far, the mechanism of homologous interference remains unclear. In general, homologous interference of a persistent infection or latent infection might be caused by a number of factors, including competition for intracellular host factors or cell surface receptors (Adams & Brown, 1985;
Karpf et al., 1997; Zhong et al., 2006), and the production of defective interfering viral genomes from the first infecting virus (Kirkwood & Bangham, 1994). The role of the SeMNPV-specific DNA and the viral transcripts in P8-Se301-C1 cells in homologous interference needs further investigation. Recently, we observed that when P8-Se301-C1 cells were infected with SeMNPV, persistent infection was established constantly (data not shown). This cell clone would be a promising and workable experimental system to investigate the mechanisms of baculovirus persistence in insects.

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References


