ANALYSIS OF DEFECTIVE GENOMES OF BOMBYX MORI NUCLEOPOLYHEDROVIRUS GENERATED BY SERIAL UNDILUTED PASSAGE IN CELL CULTURE

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Summary. – Viral DNA was extracted from cells infected with bombyx mori nucleopolyhedrovirus (BmNPV) D1 strain after 34 serial undiluted passages (P34). P34 DNA was subjected to restriction analysis and Southern blot hybridisation using standard D1 DNA and P34 DNA of BmNPV as probes. Based on hybridisation profiles, the BmNPV DNA regions retained in the P34 DNA were localised on *Hin*dIII and *Pst*I restriction maps. Two regions of BmNPV DNA located at 0 – 12.8 and 40.2 – 65.0 map unit (m.u.) were highly conserved in P34 DNA. These regions contained two of three interspersed homologous sequences (*ihss*), but only one of five homologous regions (*hrs*). This suggests that *ihss* may have an essential role in BmNPV replication.

Key words: nucleopolyhedrovirus; Bombyx mori; serial undiluted passage; defective genome; BmN4 cell line

Introduction

Nucleopolyhedroviruses (NPVs) (genus *Nucleopolyhedrovirus*, family *Baculoviridae*) are enveloped viruses with a large circular, dsDNA genome (Murphy *et al.*, 1995). NPVs have been isolated mainly from insects and have a potential of insect pest control agents (Maeda, 1994). In addition, they are used extensively as vectors for high level expression of foreign proteins in insect cells and larvae (Maeda, 1994). However, a serial passage of NPVs in cell culture causes the accumulation of genetic alterations of NPVs, hence the ability to produce a foreign protein is quite reduced (Krell, 1996). This is a serious problem for a continuous expression system with NPVs *in vitro*.

In previous studies (Hashimoto *et al.*, 1993, 1995), we have subcultured a plaque purified strain (D1) of BmNPV in a *B. mori* BmN4 cell line without dilution of virus inocula. In the course of the passaging, the virus DNA synthesised in the cells became defective, the titre of standard non-occluded virus (NOV) decreased, and the subcultured virus interfered with the replication of NOV from parent BmNPV. These results indicated that the defective interfering particles (DIPs) were formed during the serial undiluted passage through the cultured cells.

In a study on autographa californica multinucleocapsid nucleopolyhedrovirus (AcMNPV), it was shown that undiluted serial passage of AcMNPV in *Spodoptera frugiperda* cells generated DIPs (Kool *et al.*, 1991, 1993; Wickham *et al.*, 1991; Lee and Krell, 1992). Also the viral DNA in the infected cells lost large parts of the parent virus genome (Lee and Krell, 1992; Kool *et al.*, 1993). These defective genomes (DGs) composed of specific regions of parent AcMNPV genome and plasmids containing these regions could function as an origin of viral DNA replication in *S. frugiperda* cells infected with AcMNPV (Kool *et al.*, 1993; Lee and Krell, 1994).

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Abbreviations: AcMNPV = autographa californica multinucleocapsid nucleopolyhedrovirus; BmNPV = bombyx mori nucleopolyhedrovirus; DG = defective genome; DIP = defective interfering particle; EDTA = ethylenediamine tetraacetate; *hrs* = homologous region; *ihss* = interspersed homologous sequence; m.u. = map unit; NOV = non-occluded virus; NPV = nucleopolyhedrovirus; PBS = phosphate-buffered saline; TnGV = trichoplusia ni granulovirus

In this study, we subjected the supercoiled viral DNA extracted from cells infected with BmNPV after 34 serial undiluted passages (P34 DNA) to restriction analysis and Southern blot hybridisation using standard D1 DNA and P34 DNA as probes. The regions retained in the P34 DNA were assigned to *Hin*dIII and *Pst*I restriction physical maps of BmNPV D1 genome.

Materials and Methods

Virus and cells. A plaque purified D1 strain of BmNPV was used as a standard virus (Hashimoto *et al.*, 1994). BmN4 cells derived from *B. mori* (Volkman and Goldsmith, 1982) were maintained in Grace's Insect Medium supplemented with 10% foetal calf serum (Gibco) at 27°C.

Serial passage of BmNPV in cell culture. BmN4 cells (7.5 x 10^6) in a 75 cm^2 flask (Falcon 3024) were initially infected with 2 ml of undiluted inoculum of BmNPV D1 at 27°C. After adsorption of the virus to the cells for 1 hr, the inoculum was replaced with 15 ml of fresh medium and the infected cells were incubated at 27°C for 70 hrs. Two ml of supernatant containing progeny NOV was used for the next virus passage performed as above. This cycle was carried out 34 times (Hashimoto *et al.*, 1995).

Isolation of viral DNA. BmNPV D1 was propagated using bi-voltine silkworm strains (Kinshu x Showa and Shunrei x Shogetsu). The polyhedra purification from infected larvae and isolation of genomic DNA from the polyhedra were performed as described by Hashimoto *et al.* (1994).

Isolation of viral DNA from the infected culture cells was performed as described in Hirt (1967) with some modifications. Briefly, after pelleting the cells at 250 x g for 10 mins, they were washed twice with 20 ml of phosphate-buffered saline (PBS) and lysed in 1 ml of 0.6% sodium dodecyl sulfate (SDS), 0.01 mol/l ethylenediamine tetraacetate (EDTA) pH 8.0 with 100 µg/ml proteinase K (Nacalai Tesque) overnight at 37°C. A solution of 5 mol/l NaCl was added to the cell lysate to a final concentration of 1 mol/l and the lysate was placed on ice for 2 hrs. After centrifugation at 10,000 x g for 10 mins at 4°C, the supernatant was saved and extracted by phenol-chloroform-isoamylalcohol (25:24:1). The DNA was ethanol precipitated, resuspended in TE buffer (10 mmol/l Tris.HCl pH 8.0, 1 mmol/l EDTA pH 8.0) and kept at 4°C overnight. To purify the supercoiled viral DNA, the DNA sample was subjected to isopycnic centrifugation in cesium chloride and ethidium bromide twice as described by Sambrook *et al.* (1989).

Restriction analysis of viral DNA. Viral DNA was digested with *Hin*dIII and *Pst*I under conditions recommended by the manufacturer (Takara), and the fragments were resolved by electrophoresis on a 0.7% agarose gel in a Tris-acetate-EDTA buffer. The gel was stained with 0.5 µg/ml ethidium bromide and photographed under ultraviolet light.

Southern blot hybridisation. Standard D1 DNA and P34 DNA of BmNPV digested with *Pst*I and *Xho*I were labelled with [α-^{32}P] dATP (6,000 Ci/mmol, NEN) using the Megaprime™ DNA Labeling System (Amersham) under conditions recommended by the manufacturer. The viral digests were electrophoresed, blotted to nylon membrane (Hybond-N+, Amersham) and hybridised in high stringency conditions as described by Maniatis *et al.* (1982). The blots were exposed to XAR X-ray film (Eastman Kodak) with an intensifying screen at -80°C.

Results

BmNPV D1 strain was subcultuered 34 times through BmN4 cells without dilution of the virus inocula. To analyse the BmNPV DGs, the supercoiled viral DNA was isolated from the infected cells at P34 and from standard virus D1. The DNA was digested with *Hin*dIII or *Pst*I and electrophoresed (Fig. 1A). Several restriction fragments of P34 DNA co-migrated with those of D1 DNA. However, the intensity of these fragments was not present in an equimolar ratio. This result indicated that viral DNAs of heterogeneous size were generated during the serial passage. P34 DNA fragments which co-migrated with *Hin*dIII E/F, J, K, M, N and Q fragments, in addition to *Pst*I I, J and K fragments of D1 DNA were more distinct than the other fragments. The bands representing larger fragments were too obscure to score their size. A Southern blot of D1 and P34 DNA restriction fragments was subjected to hybridisation with D1 probe (Fig. 1B). The probe hybridised with P34 DNA fragments and also with bands co-migrating with *Hin*dIII E/F, J, K, M, N and Q fragments, in addition to *Pst*I I/J, K, L, M and N fragments of D1 DNA (Fig. 1B). The larger fragments hybridised more intensively with the probe. Since shorter exposures of X-ray films to these blots did not help to distinguish the large DNA fragments, it is possible that the fragments not co-migrating with the D1 DNA fragments could be produced by extensive recombination. To determine which D1 DNA region was retained in P34 DNA, another blot of the gel was hybridised with P34 probe (Fig. 1C). The probe hybridised strongly with *Hin*dIII A, C, E/F and M/N fragments, in addition to *Pst*I C, E/F, G and K fragments of D1 DNA. This indicated that a part of these fragments was conserved specifically in P34 DNA. Hybridisation signals spread on whole lanes of P34 DNA digests could be caused by contaminating cellular DNA, which was not observed in other hybridisation profiles.

Based on the hybridisation profiles (Fig. 1B and 1C), BmNPV D1 DNA regions retained in P34 DNA were localised on *Hin*dIII and *Pst*I restriction maps of BmNPV D1 DNA (Hashimoto *et al.*, 1994; Shikata *et al.*, 1998) (Fig. 2A). The strongly hybridising regions which overlapped in the two maps were mapped to 0 – 12.8 and 40.2 – 65.0 m.u. Hashimoto *et al.* (1993) described the character of deletion of BmNPV genome during serial undiluted passage. They observed that intact *Hin*dIII A, E/F and M/N fragments were highly retained in BmNPV D1 DGs after 30 passages. Although they used only the parent viral DNA genome as a probe in Southern blot analysis, their findings likely support our results.

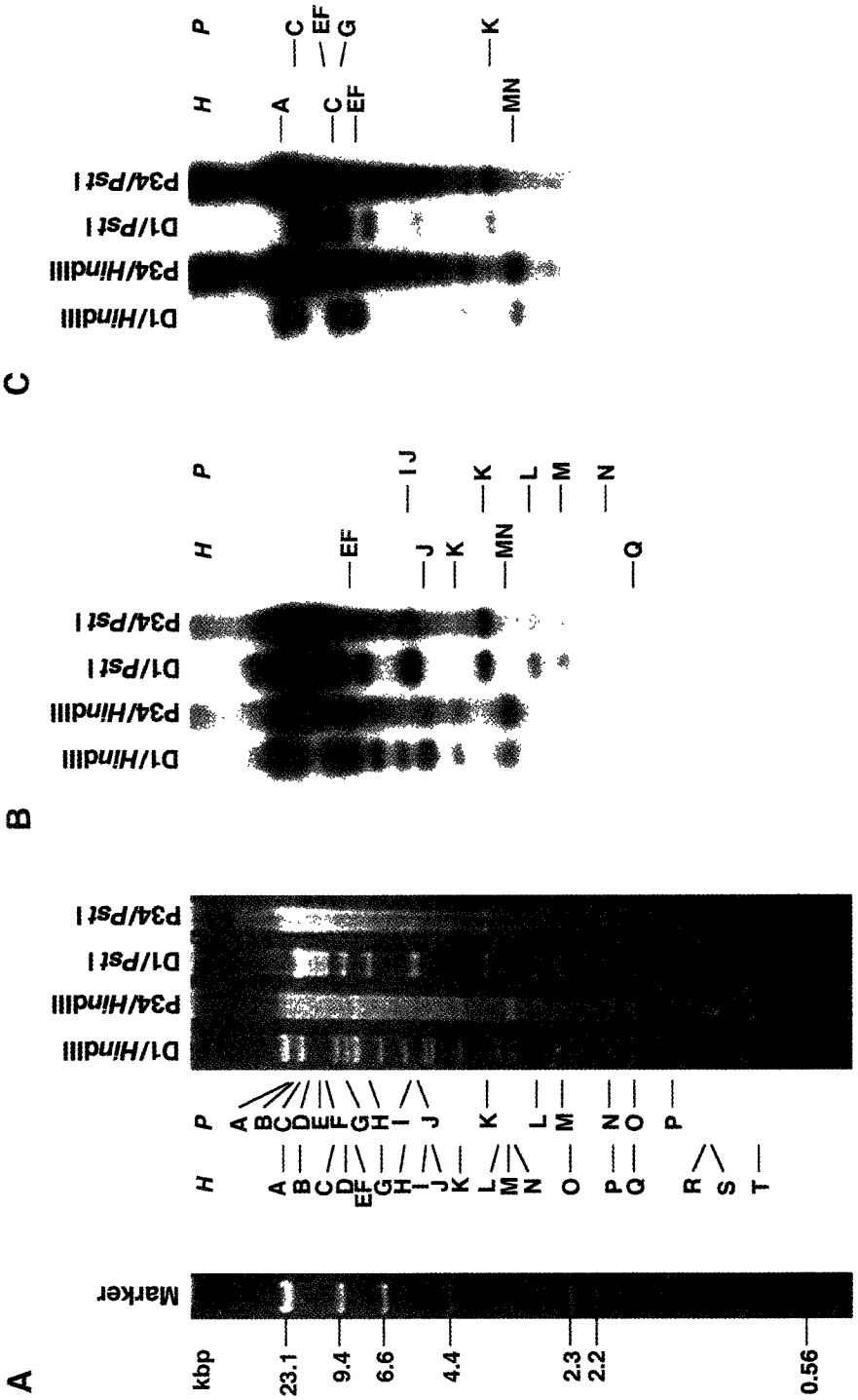

Fig. 1

Restriction (A) and Southern blot analyses (B,C) of BmNPV DNA

*Hin*dIII/lambda DNA size markers (A, left part). Letters to the left of the restriction profile (A) correspond to *Hin*dIII (*H*) and *Pst*I (*P*) restriction fragments of BmNPV D1. Southern blot analysis performed with BmNPV D1 (B) and BmNPV P34 (C) probes. Letters to the right of the Southern blots indicate positions of bands of BmNPV P34 DNA co-migrating with *Hin*dIII (*H*) and *Pst*I (*P*) fragments of BmNPV D1 DNA (B) and positions of bands of BmNPV D1 DNA co-migrating with *Hin*dIII (*H*) and *Pst*I (*P*) fragments of BmNPV P34 DNA (C).

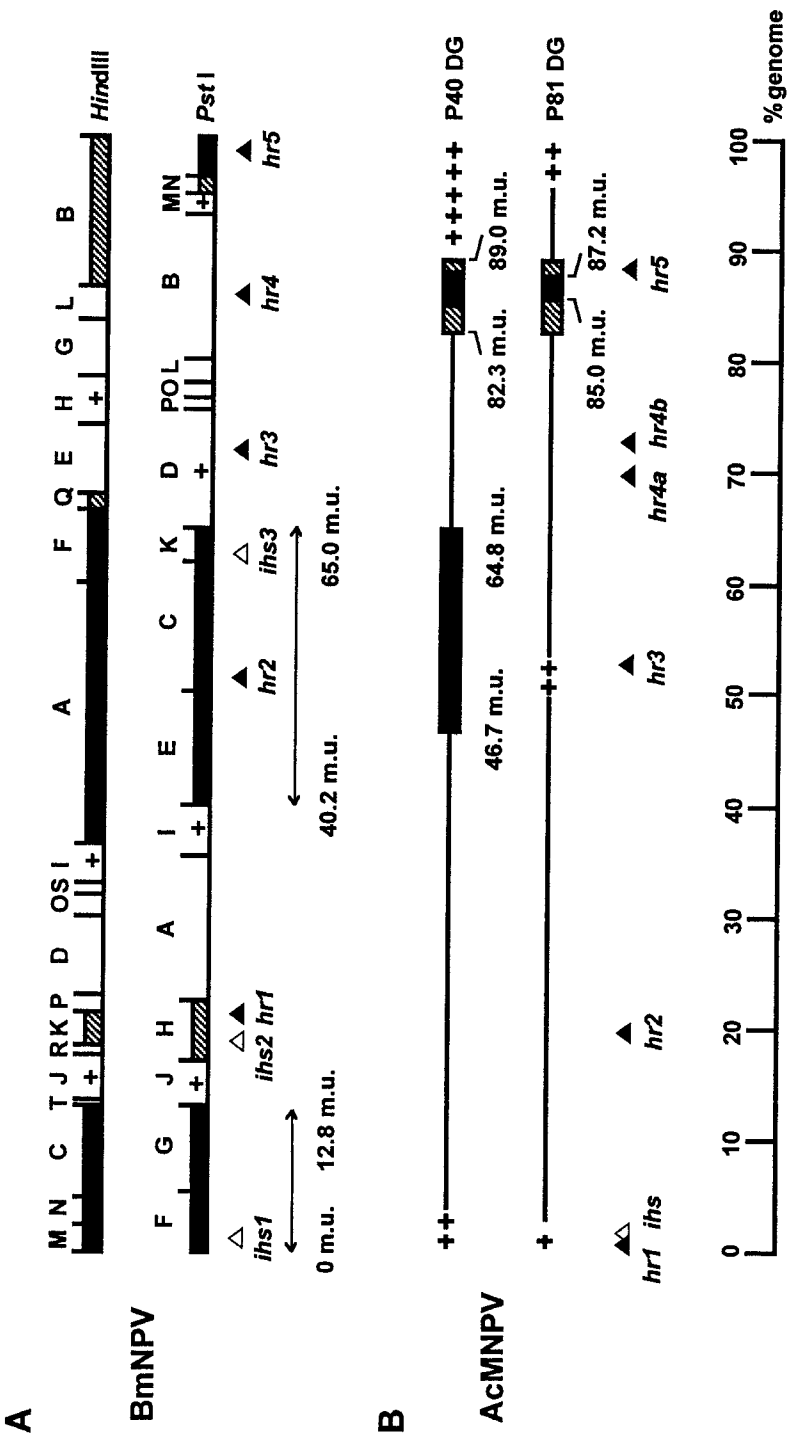

Fig. 2

Mapping of DGs of BmNPV (A) and AcMNPV (B) to their respective parental virus genomes

BmNPV D1 *Hin*dIII and *Pst*I fragments retained in the P34 DNA were identified by Southern blot analysis shown in Fig. 1. Black boxes indicate DNA fragments hybridising strongly while hatched boxes indicate fragments hybridising weakly. (+) denotes fragments hybridising very weakly. (↔) denotes overlapping regions of black boxes in the two maps. (Δ) and (▲) indicate *ihs*s and *hr*s, respectively. Maps of P40 and P81 DGs (B) are taken from Lee and Krell (1992). P40 = the 40th passage; P81 = the 81st passage.

Fig. 2B shows the mapping summary of the AcMNPV DGs at passages 40 and 81 (Lee and Krell, 1992). AcMNPV and BmNPV have a high homology and the types and positions of their genes are almost conserved (Majima *et al.*, 1993). When compared with the P40 DG of AcMNPV, the P34 contained a segment which corresponded to one (46.7 – 64.8 m.u.) of the two retained regions of P40 DG. However, the other region (82.3 – 89.0 m.u.), which was also maintained in the AcMNPV P81 DG, was not conserved in the P34 DNA.

Discussion

DGs within DIPs of herpes simplex virus and papovaviruses usually consist of reiterated copies of genomic DNA replication origins (Deiss and Frenkel, 1986; White and Fenner, 1995). Moreover, the regions retained in AcMNPV DGs may function as the origins of viral DNA replication in *S. frugiperda* cells infected with AcMNPV and the DGs most likely contain multiple repeats of these regions (Kool *et al.*, 1993; Lee and Krell, 1994). Kool *et al.* (1993) showed by restriction analysis of AcMNPV passage 40 DNA the presence of seven supermolar fragments flanking *hr*s which might function as NPV DNA replication origins. Lee and Krell (1992, 1994) showed that the DGs after 81 passages appeared to retain less than 2.8 kb of the parent AcMNPV genome derived from the segment 85.0 – 87.2 m.u. of the *Hin*dIII K fragment. Although this segment did not contain *hr* sequences, plasmids containing this sequence could replicate in *S. frugiperda* cells infected with the parent virus. It is of interest that the regions essential for the *Hin*dIII K replication origin function of AcMNPV are likely retained in BmNPV DNA (Kool *et al.*, 1994).

In this study, the restriction analysis and Southern blot hybridisation revealed that highly retained regions of P34 DNA contained only one of five *hr* sequences, *hr*2. In addition, the region corresponding to AcMNPV *Hin*dIII K replication origin was not present in supermolar amount in P34 DNA. Preliminary experiments showed that plasmids containing BmNPV *hrs* could not replicate in BmNPV-infected BmN4 cells (Hashimoto, unpublished data). Therefore, it is likely that *hr*s and the region corresponding to the AcMNPV *Hin*dIII K replication origin are not essential for the BmNPV DNA replication in BmN4 cells. In the retained regions of P34 DNA, two of three *ihs*s which had been identified in BmNPV isolates D1 and T3 (Hashimoto *et al.*, 1994) were present, namely *ihs*1 and *ihs*3. Moreover, the region containing *ihs*2 was relatively conserved in P34 DNA. Three *ihs*s have also been identified in trichoplusia ni granulovirus (TnGV) genome and they had about 50% homology with BmNPV *ihs*s (Hashimoto *et al.*, 1996). This finding suggested that *ihs*s may be conserved widely among baculoviruses and have a role as *cis*-acting elements. Although the function of BmNPV *ihs*s is not known, they may take part in the DNA replication of BmNPV as suggested previously (Hashimoto *et al.*, 1994; 1996). However, AcMNPV had only one *ihs* present in the *Eco*RI I fragment (0 – 5.9 m.u.) (Hashimoto *et al.*, 1996) and the region containing *ihs* was not conserved in the AcMNPV DGs (Lee and Krell, 1992).

This study suggests that the BmNPV DNA replication origin(s) may be different from those of AcMNPV. There is so far only a limited information on the BmNPV DNA replication origin(s). Further study of BmNPV DGs may provide a deeper insight in this problem.

Acknowledgements. We thank Dr. J. Y. Honda, San José State University, San José, CA, USA, for critical reading of the manuscripts and Dr. T. Kawarabata, Kyushu University, Fukuoka, Japan, for his encouragement and support. This work was partly supported by grants from the Dainippon Raw Silk Foundation (the Development of Vectors for Transgenic Silkworm) and the Ministry of Agriculture, Forestry and Fisheries of Japan (Integrated Research Programme on Development of Insect Technology).

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