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Polyhedra Productions of Recombinant *Autographa californica* Nucleopolyhedroviruses Containing Additional Polyhedrin of *Autographa californica, Bombyx mori* or *Spodoptera exigua* Nucleopolyhedrovirus

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The role of polyhedrin in the polyhedra production in baculovirus *Autographa californica* Nucelopolyhedrosisvirus (AcNPV) was studied by over-expression of AcNPV polyhedrin or heterologous polyhedrin from *Bombyx mori* (Bm) NPV or *Spodoptera exigua* (Se) NPV. The transfer vectors containing additional polyhedrin from AcNPV, BmNPV, or SeNPV were constructed and co-transfected with bacmid bApGOZA into Sf9 cells. The resulting recombinants, designated as vApAcPol, vApBmPol, and vApSePol were constructed, and the polyhedra production of the recombinant was characterized. All of the recombinants produced polyhedra in the nucleus, and the polyhedrin was over-expressed. Among three recombinants, vApAcPol and vApBmPol were discriminated by their larger polyhedra size than that of wild type AcNPV, and vApSePol also produced larger polyhedra than wild type SeNPV polyhedra.

Key words : AcNPV, BmNPV, SeNPV, vApGOZA, Polyhedra, Polyhedrin

Introduction

Baculoviruses are a diverse group of viruses found mostly in insects. Although interest in baculoviruses originally centered on their natural ability to control insect pest populations, baculoviruses have been used as expression vectors for the production of proteins for medical research and biotechnology (Blissard and Rohrmann, 1990; O'Reilly *et al.*, 1992). Most baculoviruses produce occlusions with a characteristic size and shape, but little is known about the specific interactions between the occlusion matrix protein and other viral components that control various occlusion and occlusion body morphology (Eason *et al.*, 1998). This characteristic structure, occlusion body stabilizes virions, allowing them to remain viable for long periods in the environment.

Baculoviridae is composed of two genera, Nucleopolyhedrosisvirus (NPV) and Granulosisvirus (GV) (Murphy *et al.*, 1995). Especially, NPV has polyhedron-shpaed occlusion bodies of 1 to 15 um in diameter composed of a protein called polyhedrin. Based on the number of nucleocasids enveloped in virions, NPVs can be distinguished as single-nucleocapsid (S) NPVs or multiple-nucleocapsid (M) NPVs (OReilly *et al.*, 1992; Rohrman 1992). GV has small inclusion bodies (0.25 to 0.5 μm). The inclusion body of GV normally contains a single nucleocapsid, which is enveloped by a protein related to polyehdrin called granullin.

Polyhedrin has been reviewed because of its unique property and its pivotal role for dissemination and survival of the virus (Rohrmann, 1986, 1992; Vlak and Rohrmann, 1985). In NPVs, it has been suggested that virion occlusion and polyhedral production are initiated by a specific interaction between polyhedrin molecule and virion envelope (Harrap and Robertson, 1968). Ultrastructural evidence suggested that polyhedron formation is initiated by nucleation of polyhedrin in the virus surface (Eason 1998; Federici 1980, 1986). So far, about 30 polyhedrin genes from different baculoviruses have been sequenced, and the sequences showed a highly conserved structure, with amino acid identity of at least 70% among lepidopteran

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NPVs (Hu *et al.*, 1999; Jarvis *et al.*, 1991). In contrast to the extensive sequence information, little is known about how polyhedrin plays its role in the occlusion process. In this study, we constructed the recombinant AcNPVs which expresses AcNPV polyhedrin or heterogeneous polyhedrin from BmNPV or SeNPV and characterized their recombinant polyehedra structure.

Materials and Methods

Cells and viruses

Sf9 cells (Summers and Smith, 1987) were maintained in TC-100 medium (GIBCO BRL, USA) supplemented with 10% fetal bovine serum (GIBCO BRL, USA). The Ac NPV-C6, BmNPV-K1 (Hong *et al.*, 2000), and SeNPV-K1 (Choi *et al.*, 1996) were used as wild type viruses throughout these experiments. Virus stocks were generated, propagated and titered on Sf9 cells as described in O'Reilly *et al.* (1992).

Construction of the transfer vectors

The schemes for the construction of the transfer vectors are summarised in Fig. 1A. The polyhedrin-based vector pBacPAK8 or 9 (Clontech. Co., USA) was used. The transfer vectors containing each polyhedrin gene from AcNPV, BmNPV, and SeNPV were constructed as follows: the ORF of each polyhedrin gene was amplified by PCR from AcNPV, BmNPV, or Se NPV genome as template. The primer sequences of the primers used in this study are listed in Table 1. To construct transfer vector containing AcNPV polyhedrin, pBac8-Ac, polyhedrin gene was amplified from AcNPV C-6 strain viral genome with Ac-F as forward and Ac-R as reverse primers. The PCR product carried *Xho*I sites (shown in underline) at either end and was cloned to pBacPAK8 which was digested by *Xho*I and treated by CIP (alkaline phosphatase of Calf Intestinal; TaKaRa Biomedicals, Japan).

In constructing transfer vectors with BmNPV and Se NPV polyhedrin, pBacPAK9 plasmid was used (Fig. 1A). The polyhedrin gene of BmNPV and SeNPV was amplified from BmNPV-K1 and SeNPV-K1 viral genome with Bm-F/R and Se-F/R primers, respectively. The amplified BmNPV polyhedrine was digested by *Kpn*I and inserted to *Kpn*I site of pBacPAK9 vector and was designated pBac-Bm. The PCR product from SeNPV polyhedrin also was restricted by *Sac*I and was subcloned to *Sac*I site of pBacPAK9 vector and resulted in pBac-Se plasmid. The construction of each transfer vector was confirmed by restriction enzyme digestion (Fig. 1B) and DNA sequence analysis (data not shown).

Table 1. Primers used in this study

Primers		Sequences
AcNPV	Ac-F	5-AAGGATTCATGCCGGATTATTCA
	Ac-R	5-TTGGATTCTTAATACGCCGGACC
BmNPV	Bm-F	5-AAGGTACCATGCCGAATTATCCA
	Bm-R	5-TTGGATTCTTAATACGCCGGACC
SeNPV	Se-F	5-AAGAGCTCATGTATACTCGCTAC
	Se-R	5-TTGAGCTCTTATATGGCGGGTCC

Generation of recombinant baculoviruses

Thirty-five mm dishes were seeded with 1×10^6 Sf9 cells and incubated at 27°C for 2 to 24 hrs to allow the cells to attach. 100 ng of viral DNA, ApGOZA (Je *et al.*, 2001) and 500 ng of each transfer vector containing polyhedrin of AcNPV, BmNPV, or SeNPV and sterile water (to a final volume of 50 μl) were mixed in a polystyrene tube. An equal volume of 100 ng/ml Lipofectin was gently mixed with the DNA solution and the mixture was incubated at room temperature for 30 min. The cells were washed twice with 2 ml serum-free TC-100 medium and fed with 1.5 ml serum-free TC-100. The Lipofectin-DNA complex was added dropwise to the medium covering the cells while the dish was gently swirled. After incubating at 27°C for 5 hrs, 1.5 ml of TC-100 medium containing 50 units of penicillin and 50 μg streptomycin per ml, and 10% FBS was added to the dish and continued incubation at 27°C continued. At 5 days post-transfection, the recombinant viruses were isolated by screening for occlusion positive plaques. To ensure the recombination, PCR was performed with recombinant virus DNAs as templates with Bac 1 primer (Clontech Co., USA) and their sequences was also analysed (data not shown).

Light microscopy

After purification, each recombinant virus was infected to 1×10^6 Sf9 cells at an MOI (multiplicity of infection) of 5 PFU (plaque forming units) per cell. After incubation for 5 days, microscopy of recombinant polyhedra produced by each recombinant baculovirus was performed using light microscopy (Axiophot Universal Microscope, Zeiss)

Protein analysis

Protein synthesis in recombinant virus-infected cells was analysed by SDS-PAGE. 1×10^6 Sf9 cells were mock infected or infected with recombinant virus at an MOI of 5 PFU per cell. After incubation for 5 days, cells were harvested and washed twice with PBS. Total cellular lysates were prepared and subjected to 10% SDS-PAGE (Laemmli 1970).

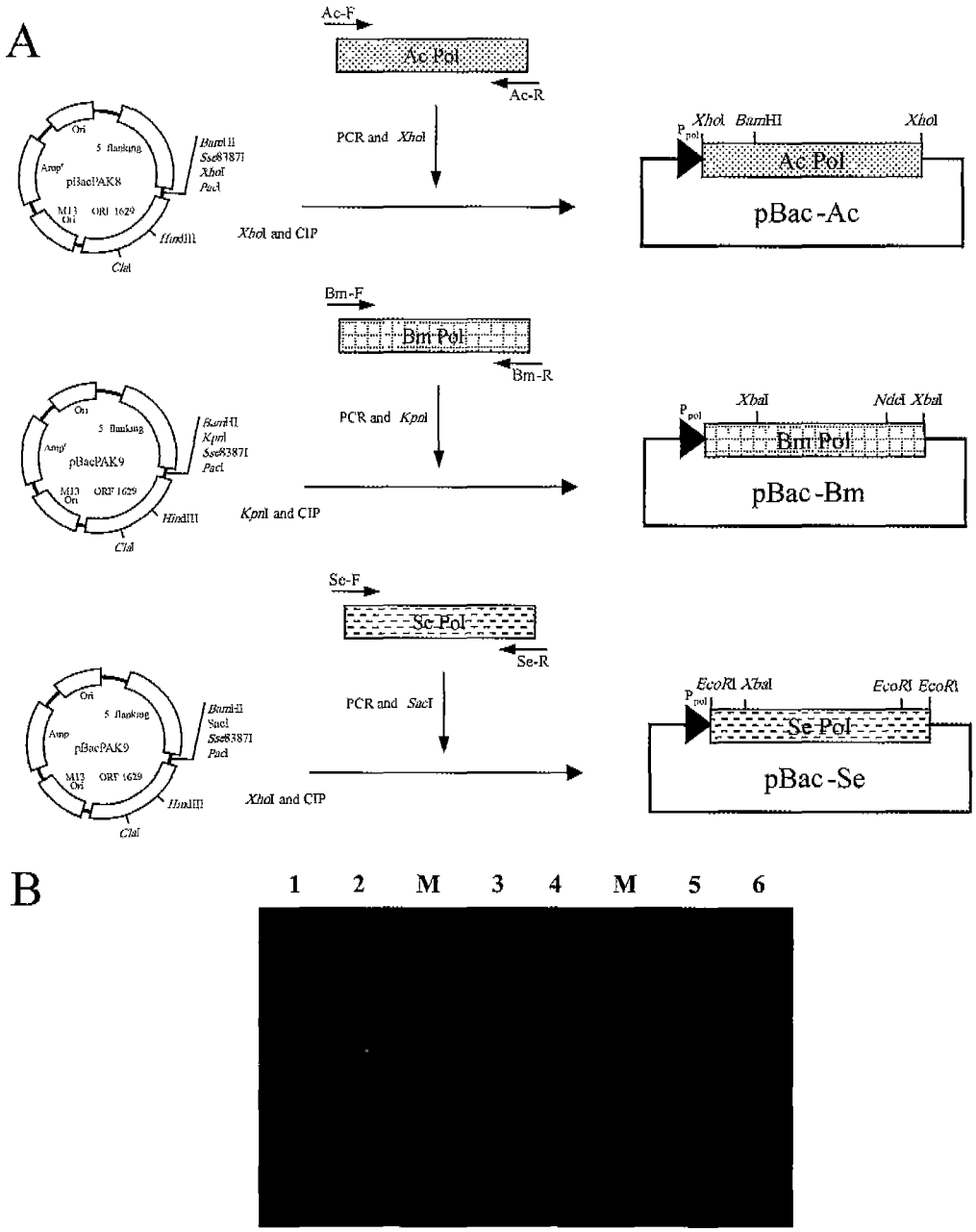

Fig. 1. Schematic outline for the construction of transfer vectors (A) and restriction enzyme digestion analysis of transfer vectors electrophoresed on 0.9% agarose gel (B). Lane M, Lambda DNA digested with *Hin*dIII; 1 and 2, pBac-Ac digested with *Bam*HI and *Xho*I; 3 and 4, pBac-Bm digested with *Nde*I and *Xba*I; 5 and 6 pBac-Se digested with *Eco*RI and *Xba*I, respectively.

Results

Construction of the transfer vectors

The transfer vector pBac-Ac, pBac-Bm, and pBac-Se were comprised in each polyhedrin gene under the control of polyhedrin promoter (Fig. 1A). The insertion of polyhedrin in 738 bp from AcNPV, 738 bp from BmNPV, and 741 bp from SeNPV was analysed by DNA sequencing analysis and the structure was confirmed by restriction enzyme digestion (Fig. 1B). The sequence of each polyhedrin is the same previous reports (Choi *et al.*, 1996; Possee *et al.*, 1991; Woo, 1996) and the structure is also coincident to the expectation.

Generation of recombinant baculoviruses

In this study, we used bacmid bApGOZA as a parent viral genome to construct recombinants. The newly devised viral genome bApGOZA employs a modified baculovirus

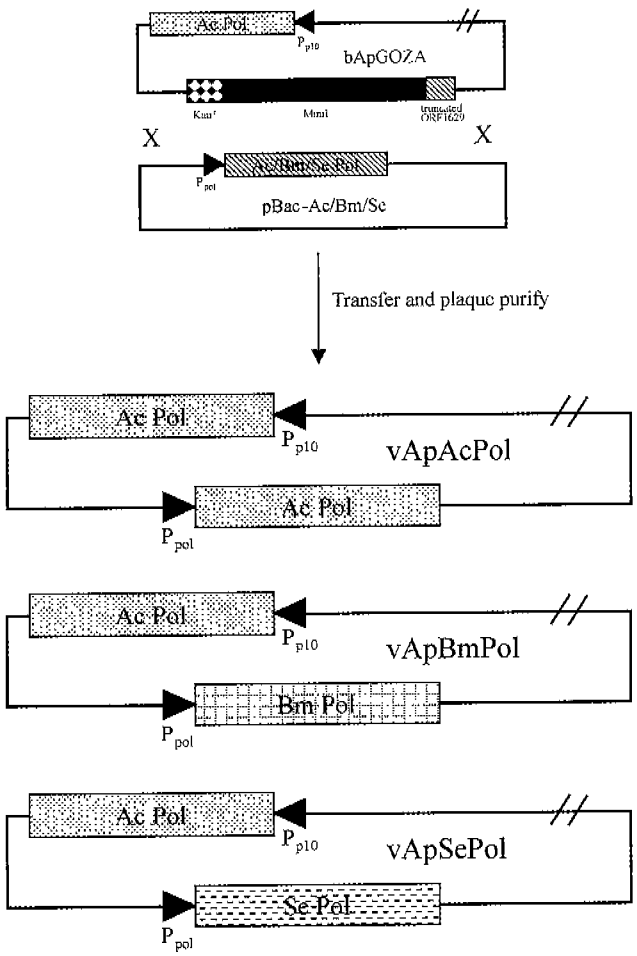

Fig. 2. Schematic outline of the generation of recombinants, vApAcPol, vApBmPol and vApSePol.

genome that lacks a part of ORF1629 but includes a mini-F replicon, and makes only recombinant virus obtained for its defectiveness (Je *et al.*, 2001). The bacmid bApGOZA inherently carries AcNPV polyhedrin under p10 promoter that can be attributed to the occlusion-positive trait of recombinants.

To assess the difference between polyhedrins, pBac-Ac, pBac-Bm, and pBac-Se plasmids were co-transfected with bApGOZA and the respective recombinants vApAcPol, vApBmPol, and vApSePol were purified as described in OReilly *et al.* (1992) (Fig. 2). The construction of recombinants was detected by PCR and DNA sequence analyses (data not shown).

As shown in Fig. 3, the polyhedra morphology in Sf9 cells infected by vApAcPol, vApBmPol, and vSePol can be easily discriminated in size. All recombinants produced polyhedra as many as wild type AcNPV but differed in their magnitude. The recombinants, vApBmPol and vApAcPol, made dramatically expanded polyhedra than wild type AcNPV. Especially in Sf9 cells infected with vApBmPol, the extraordinarily large polyhedra were observed without rareness. In Fig. 4, we could also assure the expression of each polyhedrin which were about 30 kDa in size.

Discussion

In this study, over-expression of native AcNPV polyhedrin or heterogeneous polyhedrins either from BmNPV or SeNPV, can be obtained and the correct translocation of polyhedra containing one or more kind of polyhedrin into the nucleus was also occurred.

As a previous report (Jarvis *et al.*, 1991), AcNPV polyhedrin protein contains domains functional to nuclear localization and supramolecular assembly. Besides AcNPV polyhedrain, BmNPV and SeNPV polyhedrin also can applicable in AcNPV system and successfully implement the polyhedra production. Similar results were observed by Hu *et al.* (1999) in that showed the substitution of polyhedrin gene from AcNPV to SeNPV and *Buzura suppressaria* (Busu) NPV. This substitution also didnt affect the nuclear localization and characteristic paracrystalline lattice structure. In Hu *et al.* (1999) experiment, the recombinant virus containing SeNPV polyhedrin instead of AcNPV polyhedrin in AcNPV produced profoundly different polyhedra in size and morphology. They suggested that this morphological difference in SeNPV polyhedrin-derived polyhedra of AcNPV was stemmed from virus specific factor, and this unusual morphology could be restored to that of SeNPV by co-infection with wild type SeNPV.

In our study, we introduced foreign polyhedrin in addition to indigenous AcNPV polyhedrin. Although the nucleic acid and amino acid similarity between AcNPV polyhedrin and Bm or Se NPV polyhedrin are over 75% and 84%, respectively (Choi *et al.*, 1996; Hu *et al.*, 1999), the amplified size was held in BmNPV polyhedrin which is belonged to SNPV. And in the previous report (Hu *et al.*, 1999), only Busu NPV-polyhedrin substituted recombinant showed the magnification of polyhedra size. This Busu NPV also is belonged to SNPV. From these results, some viral factor related only to SNPV seems to exist and can unknown function in polyhedra construction.

Considering MNPV polyhedrin in vApAcPol and vAp SePol, wild type AcNPV polyhedrin also seemed to be differentiated in the polyhedra structure by expanding size or shaping from wild type AcNPV. Compared with the polyehdra morphology of SeNPV polyhedrin-substituted recombinant from Hu *et al.* (1999) and our vApSeNPV, vApSePol produced quite regular form of polyhedra, similar to wild type AcNPV in size and shape. This change

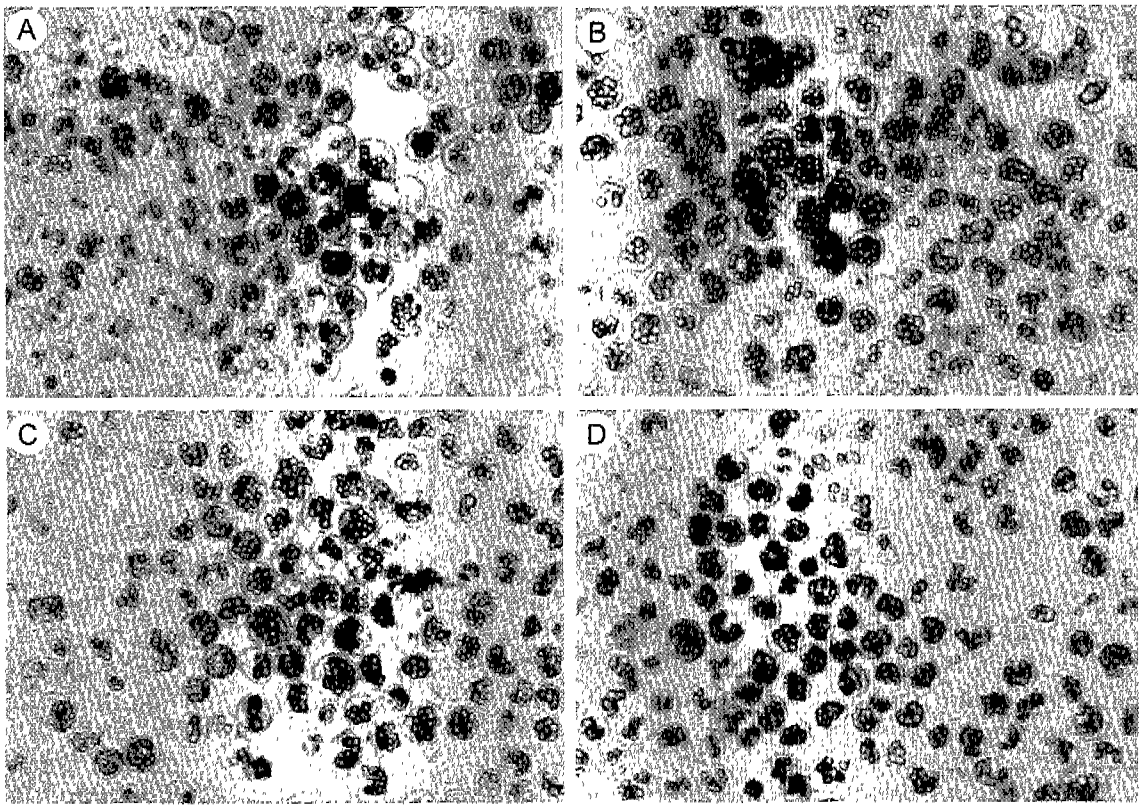

Fig. 3. Microscopy of Sf9 cells infected with recombinants. Sf9 cells infected with wild-type AcNPV (A), bApAcPol (B), bApBmPol (C) and vApSePol(D) were observed by light microscope (× 400).

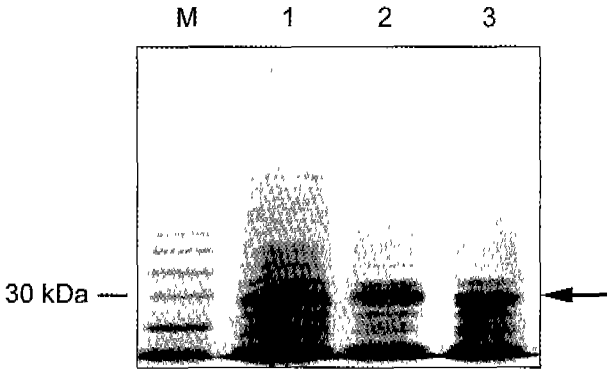

Fig. 4. SDS-PAGE analysis of polyhedrin expression in Sf9 cells infected with recombinants. Lane M, 10 kDa protein ladder (Gibco BRL, USA); 1, vApAcPol; 2, vApBmPol; 3, vAp SePol. The expression of polyhedrin is indicated on the right of panel.

might be related to the expression of wild type Ac NPV polyhedrin. This wild type Ac NPV polyhedrin also suggested to take apart in expanding polyhedra in vAcApPol. The over-expression of AcNPV polyhedrin in vApAcPol than wild type AcNPV could also promote the production of polyhedra in size and the concentration. From these observations, strain specific factors in MNPV also seem to have efficiency in polyhedra production.

From these results, we can suggest some viral factor to control the polyhedra production, especially in size. If the core region or site of these operation can be detected and used, we can make much larger or massive polyhedra than wild types, and this manipulation can be efficiently applied to BEVS or bio-engineered biopesticide production.

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