INTRODUCTION

Nucleopolyhedroviruses (NPVs; family Baculoviridae) are rod-shaped double-stranded DNA viruses with a circular, supercoiled genome varying in size between approximately 80 and 180 kbp (Harrap and Payne, 1979). A characteristic feature of NPVs is the formation of paracrystalline structures called polyhedra. The polyhedra are large polyhedral-shaped proteinaceous bodies, which contain a number of virions and consist mainly of polyhedrin. The polyhedra range from 0.5 to 15 μm in diameter and have a variety of shapes: dodecahedra, cubes and angular forms of irregular polygonal shape, and their sizes and shapes are considered to be a characteristic of the virus isolate (Bergold, 1963; Summers, 1975). Mechanisms of how polyhedra form different shapes have not yet been well understood. Experiments on the self-assembly of polyhedra formed by the Bombyx mori NPV (BmNPV) indicated that the polyhedron component recrystallizes in vitro into several different shapes depending on the physicochemical conditions (Shigematsu and Suzuki, 1971). This experiment and others (Hukuhara, 1971; Bellonick, 1989) suggest that the shape of polyhedra is determined by differences in cellular and environmental factors instead of being directed by the virus itself. However, a later report indicated that interaction between other viral or cellular components during the crystallization of polyhedra is not necessary for supramolecular assembly of polyhedra into occlusion particles. Cheng et al. (1998) suggested that the polyhedrin gene determines the shape of the occlusion body formed during the supramolecular assembly of polyhedra. A number of polyhedrin mutation experiments showed differences in the polyhedra morphology of Autographa californica nucleopolyhedrovirus.
NPV (AcNPV) (Brown et al., 1980; Duncan and Faulkner, 1982; Duncan et al., 1983; Carstens et al., 1986, 1987, 1992). These studies suggest that specific regions or amino acids of polyhedrin are critical to interactions with polyhedrin and other viral molecules, and polyhedron morphology is likely to be determined by the amino acid sequence of the matrix protein.

We have reported that the morphology of polyhedra is controlled not only by the polyhedrin, but also by the host and/or viral factors other than polyhedrin (Woo et al., 1998). We also showed that substitution of the polyhedrin gene between NPVs results in a change in polyhedra morphology. Because of the importance of polyhedra in the transmission and maintenance of baculoviruses in nature, we were interested in studying factors that influence polyhedra morphogenesis.

In this study, we replace the polyhedrin gene of AcNPV with that of Spodoptera exigua NPV (SeNPV), and the morphological change of polyhedra is investigated in three cell lines, S. frugiperda (Sf9), S. exigua (Se301) and Trichoplusia ni (Hi5) cells. We also examine the polyhedra morphology of wild-type AcNPV and another recombinant virus, AcBmPol, which produces BmNPV polyhedrin instead of AcNPV polyhedrin and has been described previously (Woo et al., 1998). We suggest that the shape and uniformity of polyhedra depend on some viral gene other than the polyhedrin gene, and that the size of the polyhedra is influenced by a host cell factor.

MATERIALS AND METHODS

Cell culture and virus. Spodoptera frugiperda clone 9 (Sf9, Invitrogen) and Trichoplusia ni BTI-Tn-5B-4 (Hi5) cells, provided by Dr. P. V. Choudary (University of California at Riverside, U.S.A.), were maintained at 27°C in TC-100 medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL). Spodoptera exigua (Se301) cells, provided by Dr. T. Kawarabata (University of Kyushu, Japan), were maintained in IPL-41 medium (Gibco-BRL) supplemented with 10% FBS. The AcNPV C6 (Clontech), as a wild-type virus, and the recombinant virus, AcBmPol, were used for the comparison of polyhedra morphology. Routine cell culture maintenance and virus production procedures were carried out according to the published procedure (O’Reilly et al., 1992).

Construction of a transfer vector and generation of the recombinant. To generate the transfer vector pAcSePol, the polyhedrin-coding sequence of SeNPV was isolated from plasmid pGTSSeP (Choi et al., 1996) and ligated into an AcNPV transfer vector pBacPAK9 (Clontech). The pAcSePol was cotransfected with the genomic DNA of polyhedron-negative AcNPV (BacPAK6, Clontech) into Sf9 cells by a liposome-mediated method using the transfection reagent DOTAP (Boehringer Mannheim). The polyhedron-producing recombinant virus was purified by the end-point dilution method. The end-point dilution for purification and titration of virus stock was carried out according to published procedures (O’Reilly et al., 1992).

Light microscopy of virus-infected cells and polyhedra-positive cell rate. The various cells in the six-well plates were infected with wild-type AcNPV C6, or recombinant viruses, at a multiplicity of infection (MOI) of 1 plaque-forming unit (PFU)/cell. Cells were examined and photographed using a Hund D-35550 Wetzlar inverted microscope in culture dishes at 1, 2, 3, 4, and 5 d post-infection (p.i.). To quantify the rate of cells with polyhedra formation in the cell cultures, the numbers of polyhedra-positive cells were quantified using standard techniques (King and Possee, 1992).

Polyhedra yield and SDS-PAGE. Cells in the six-well plates were infected with each virus at a MOI of 1 PFU/cell. The cells infected with virus were floated by gentle pipetting and harvested at 1, 2, 3, 4, and 5 d post-infection (p.i.). To analyze the polyhedral protein, purified polyhedra were boiled in a protein sample buffer and then electrophoresed in a 12% SDS-polyacrylamide gel using a standard method (Sambrook et al., 1989).

Virus growth. Cells in the exponential phase were infected with viruses at a MOI of 1 PFU/cell in a 25 cm² culturing flask containing 5.0×10⁶ cells. Following a 1 h virus adsorption period, the cells were washed, supplied with fresh medium,
and incubated at 27°C. At 5 d after inoculation, the culture supernatant containing the extracellular virus was harvested, centrifuged at 1,000 rpm for 10 min, and the virus titer was determined using the end-point dilution method.

**Electron microscopy.** The polyhedra produced in the cells were collected by ultracentrifugation in linear 40 to 60% sucrose gradients, and examined under a scanning electron microscope (SEM). A drop of purified polyhedra solution of each virus was placed on grids and air-dried. The grids were coated with carbon and stained with gold. The samples were observed using the SEM, Jeol JSM-5410LV. For the specimen preparation of transmis-

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**Fig. 1.** Light micrographs of Sf9 (A–C), Se301 (D–F) and Hi5 (G–I) cells infected with wild-type AcNPV (A, D and G), AcBmPol (B, E and H) and AcSePol (C, F and I) at 5 d-p.i. Cells were infected with each virus at a MOI of 1 PFU/cell. The polyhedra shape is distinguished clearly only when cells infected with AcBmPol, which produces triangle-like polyhedra (arrows indicated), are present.
sion electron microscope (TEM), virus-infected cells were harvested by centrifugation at 3 d-p.i. Cell pellets were rinsed in 0.06 M PIPES/0.04 M HEPES buffer, pH 7.2, containing 10 mM KCl, 2 mM EGTA, and 1.5 mM MgCl2, and fixed for 2 h with a mixture of 2% paraformaldehyde and 2% glutaraldehyde. Pellets were washed with 0.05 M sodium cacodylate buffer, pH 7.2, and post-fixed for 1 h in 1% osmium tetraoxide in sodium cacodylate buffer. After washing in sodium cacodylate buffer alone, the pellets were stained for 30 min with 0.5% uranyl acetate. Samples were dehydrated through a standard ethanol series (25–100% ethanol, 15 min/step) and cleared with three washes in acetone. Tissues were infiltrated with EMBED 812 epoxy resin (Electron Microscopy Sciences). Resin blocks were dried in an oven at 70°C for 24 h. Thin sections were recovered and stained with 2% uranyl acetate for 3 min, followed by Reynold's lead citrate for 3 min. Sections were examined with a Jeol JEM1010 TEM.

RESULTS

Polyhedra appearance in various cells

The virus-infected cells were examined under a microscope, and it was found that the appearance of the polyhedra differed depending on the cell line (Fig. 1). The cells infected with AcNPV displayed typical features of NPV infection in all cell lines (Fig. 1A, D and G). AcBmPol, which produces BmNPV polyhedrin instead of AcNPV polyhedrin (Woo et al., 1998), produced characteristic triangle-like polyhedra in all the cell lines examined (Fig. 1B, E and H). AcSePol containing SeNPV polyhedrin genes instead of AcNPV polyhedrin genes produced many polyhedra that differed from both parental viruses and most abundant polyhedra in a cell when compared with other viruses.

SDS-PAGE was used to compare the polyhedrin proteins from the purified polyhedra of the recombinant viruses with those from wild-type AcNPV, BmNPV and SeNPV (Fig. 2). The results showed that the recombinant viruses expressed the polyhedrin proteins correctly and yielded polyhedra the same size as the polyhedra of BmNPV and SeNPV.

To further investigate the polyhedra morphology, the polyhedra were examined using SEM (Fig. 3) and TEM (Fig. 4). AcSePol produced almost regular tetrahedral polyhedra in all cell lines examined (Fig. 3C, F and I), however, the polyhedra shape produced in Hi5 cells was less angular than the others (Fig. 3I). Additionally, the polyhedra shapes produced by AcNPV and AcBmPol were not changed significantly in respect to the cell line. Only the polyhedra of AcBmPol were changed to a slightly more irregular shape in Hi5 cells (Fig. 3H). The size of polyhedra produced by each virus was greatest in Se301 cells, followed by Hi5 and Sf9 cells in decreasing order. The polyhedra of AcSePol produced in Se301 cells were larger (2.0–5.4 μm across) than those produced in Sf9 (1.5–3.7 μm across) and Hi5 (1.7–3.2 μm across) cells. The polyhedra of AcBmPol produced in Se301 cells were larger (3.5–10 μm across) than those produced in Sf9 (3.4–6.5 μm across) and Hi5 (5.6–9.1 μm across) cells. The polyhedra of AcNPV produced in Se301 cells were also larger (1.7–5.3 μm across) than those in Sf9 (1.5–3.9 μm across) and Hi5 (2.2–4.6 μm across) cells. TEM examination showed that the polyhedra from AcBmPol-infected cells contained a number of virions with multiple nucleocapsids as the polyhedra from AcNPV-infected cells (Fig. 4B, E and H), while the polyhedra from AcSePol-infected cells possessed little or no virions (Fig. 4C, F and I).

Polyhedra yield and virus production

To compare the yield of polyhedra from infected
Fig. 3. Scanning electron micrographs of the polyhedra of AcNPV (A, D and G), AcBmPol (B, E and H) and AcSePol (C, F and I) from Sf9 (A–C), Se301 (D–F) and Hi5 (G–I) cells. Regular tetrahedral polyhedra occurred in cells infected with AcSePol (C, F and I). Bar markers represent 1 μm.
cells, we calculated the number of polyhedra in each cell line (Fig. 5). Yields of polyhedra by each virus were greatest in Sf9 cells, followed by Hi5 and Se301 cells, in decreasing order. When Sf9 cells were infected with a virus, the yields of polyhedra by AcSePol were 6.0 and 1.7 times greater than yields by AcBmPol or AcNPV, respectively, at 5 d-p.i. We also determined the rate of polyhedra cell formation in a culture in an attempt to better understand the relationship between the amount of polyhedra production and the polyhedra-positive cell appearance (Fig. 6). Polyhedra-positive cells due to these viruses were again highest in the Sf9 cell line. The polyhedra-positive cell rate according to cell lines was similarly related to the polyhedra production. To compare the replication character of viruses in each cell line, we determined virus titers at 5 d-p.i. (Fig. 7). The titer of viruses was also determined to be the highest in the Sf9 cell line. However, the titer of the virus did not show any significant difference between each virus.
DISCUSSION

In a previous study, we suggested that polyhedral morphology is determined not only by amino acid sequence of polyhedrin, but also by host cells and/or viral factors other than polyhedrin. In this study, we show that the existence of some viral genes other than polyhedrin genes influencing polyhedral morphogenesis, and a host cell factor play a role in determining the size of the polyhedra.

The change in the AcSePol polyhedra from irregular to tetrahedral shape indicates that substitution of the polyhedrin gene influences the morphological change of polyhedra, but it is not the sole factor. This observation is consistent with a previously reported result for AcBmPol (Woo et al., 1998) and similar experiments performed by Gonzalez et al. (1989). The expression of the S. frugiperda NPV (SfNPV) polyhedron (which has 85% amino acid identity with the AcNPV polyhedron) in AcNPV resulted in small polyhedra that contain fewer virions than wild-type AcNPV polyhedra. The substitution of granulin from the Tri-
Choplusia ni granulosis virus for the polyhedrin of AcNPV also yielded few, but very large cuboidal inclusions having very few virions (Eason et al., 1998). These studies demonstrate that the substitution of occlusion body-related protein for polyhedrin in AcNPV changed the polyhedra shape and produced fewer virion occluded polyhedra. Our recombinant AcNPVs, however, showed several different features. The virions of polyhedra in AcBmPol did not differ from the wild-type AcNPV, but AcSePol produced small polyhedra containing fewer virions than did the wild-type polyhedra tested in all cell lines. The polyhedrin of AcNPV has an amino acid identity of 86% and 85%, which is similar to the BmNPV and SeNPV viral polyhedrins, respectively. This observation indicates that although the overall similarity of an amino acid composition of a polyhedrin may be able to affect polyhedra morphology, it does not relate to the occlusion of virions. Nearly 30 different polyhedrin genes of various baculoviruses have been sequenced, but very little is known about the role that the polyhedrin plays in the occlusion process. Virion occlusion and polyhedron formation are likely to involve complex processes involving a number of viral genes. It has been reported that a single amino acid point mutation in the polyhedrin of AcNPV results in abnormal polyhedra that lack virions (Lin et al., 2000). It has also been hypothesized that virion occlusion and polyhedral growth are initiated by specific interactions between polyhedrin molecules and the virion envelope (Harrap and Robertson, 1968). The occlusion of virions is speculated to involve at least three different proteins: polyhedrin, p10, and the polyhedron envelope protein (Chung et al., 1980; Whitt and Manning, 1988; Russell and Rohrmann, 1990; Russell et al., 1991; Rohrmann, 1992; van Oers et al., 1993; Gross et al., 1994). Although the polyhedron envelope protein and the p10 protein have significant roles in the formation of polyhedra, their functions are not clear. Our studies have suggested that few, or the absence of, virion occlusions is caused by a failure of interaction between the heterologous occlusion body molecule and the virion envelope as a result of their incompatibility. We also frequently observed fractured AcSePol polyhedra in cells (data not shown). Unsuccessful interactions between heterologous polyhedrin and AcNPV virions or the polyhedron envelope protein may account for the lack of virion occlusions and an unstable form of recombinant virus polyhedra. The difference of virion occlusion between AcBmPol and AcSePol, however, suggests the existence of some critical domain within the polyhedrin.

An interesting additional observation has been the change of polyhedra shape in AcSePol, from irregular to regular in all cell lines (Fig. 3C, F and I). Thus, while AcNPV had some regular polyhedra and SeNPV nearly irregular polyhedra, the polyhedra of AcSePol were more regular than that of AcNPV. This observation implies that the uniformity of polyhedra shape depends on viral factor(s) other than polyhedrin and the host cell.

The size of polyhedra produced by each virus was largest in the Se301 cells, followed by Hi5 and Sf9 cells, in decreasing order. We could not find any significant difference, however, in polyhedra shape according to the host cells. This result suggests that the host cells do not influence the shape of polyhedra, but rather influence their size. The results of the present study would be useful to define the factors of virion occlusion and polyhedra morphogenesis.

ACKNOWLEDGEMENTS

This work was supported by Korea Research Foundation Grant (KRF-2004-003-F00002).
REFERENCES


