# Characterization of the *Buzura suppressaria* single-nucleocapsid nucleopolyhedrovirus genome: a (phylo)genetic study

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# Characterization of the *Buzura suppressaria* single-nucleocapsid nucleopolyhedrovirus genome: a (phylo)genetic study

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proefschrift ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen
dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 8 september 1998
des namiddags te half twee in de Aula

The work presented in this thesis was carried out at the Laboratory of Virology, Department of Plant Breeding and Crop Protection, Wageningen University and Research Centre, Wageningen, the Netherlands, and the Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, People's Republic of China.

The work was initiated by a Marie Curie grant from EC and supported by the Wageningen University and Research Center (PhD sandwich grant). The research was also supported by grants from the Royal Academy of Sciences of the Netherlands, the Chinese Academy of Sciences and the National Natural Science Foundation of China.

Hu, ZhiHong

Characterization of the *Buzura suppressaria* single-nucleocapsid nucleopolyhedrovirus genome: a (phylo)genetic study - Z.H. Hu
Thesis Wageningen - with ref. - with summary in Dutch and Chinese
ISBN 90-5485-917-2
Subject headings: baculovirus

pNo8201, 2472

1. Single (S) and multiple (M) nucleocapsid nucleopolyhedroviruses do not represent distinct evolutionary groups.
*this thesis*

2. Gene homology and gene arrangement are independent parameters to study baculovirus phylogeny.
*this thesis*

3. The experiments by Fan *et al.* are not sufficient to conclude that protein kinase (PK-1) is a very late expression factor of baculoviruses.
*Fan et al., 1996. Virology 224, 1-9. McLachlin et al., 1998. Virology 246, 379-391.*

4. The complete genome sequence determination of the Gram-positive bacterium *Bacillus subtilis*, has not yet led to understanding the genetic difference between Gram-positive and Gram-negative bacteria.
*Kunst et al., 1997. Nature 390, 249-256.*

5. The *in vitro* interaction between RNA and P1 of cauliflower mosaic virus, described by Citovsky *et al.*, has no functional significance to explain the movement of this virus through plants.
*Citovsky et al., 1991. Proc. Natl. Acad. Sci. USA 88, 2476-2480. Pyott et al., 1993. Virology 195, 281-285.*

6. The introduction of protease inhibitors into plants may hamper the use of baculoviruses and *Bacillus thuringiensis* for insect control.
*Jongsma et al., 1996. Mededel. 14, 331-333.*

7. It may be a small planet but the worlds within are infinite.

8. Modesty helps one to go forward whereas conceit makes one lag behind.

9. In China tea will always be more popular than coffee.

10. Illiteracy is incorrectly considered to be a feminine virtue by Confucius.

11. Bureaucracy without system is as bad as a system without bureaucracy.

Stellingen behorend bij het proefschrift:

**Characterization of the *Buzura suppressaria* single-nucleocapsid nucleopolyhedrovirus genome: a (phylo)genetic study**

Wageningen, 8 September, 1998

Zhihong Hu

## PREFACE

The work presented in this thesis would not have been accomplished without the support and help of many people. I am grateful to my supervisor professor Just Vlak for giving me the opportunity to join his research group and for his excellent scientific guidance and discussions which elevated the standards of my research. I also wish to express my gratitude for establishing fruitful collaborations, which are much appreciated by my home institute. The Vlak family has been so hospitable and friendly, which made my stay in the Netherlands enjoyable right from the very first day. I thank professor Rob Goldbach for his continuous interest and support during the various phases leading to my PhD. It is an honour to have both of them as promotor.

I am very lucky to have enjoyed the wonderful friendships of many people from different countries. First of all I would like to thank Basil Arif, for science, humour, trips and swimming. Basil, without your help the thesis would not have been the same as it looks now. As a coach you can be proud of yourself because my swimming is significantly improved and this sport was one of my best friends during my thesis writing. I thank you and Elaine for showing me around the Europe and for the wonderful weekends we spent together. I am fortunate to be a friend of Johannes Jehle, a wonderful personality and with whom I had lots of helpful discussions. The time I spent with Basil, Johannes, Peter Krell, Liu Yi (Jenny), Mira Toister-Archituv and Delia Muñoz were days occupied with science, humour and the occasional cookies, when Peter was in a generous mood.

I have received lots of help from the friends and colleagues in the Department of Virology in Wageningen. Special thanks to Douwe Zuidema for his scientific input and continuous support; to John Martens for his valued supervision in the beginning, helpful discussions and friendship; to Monique van Oers, René Broer and Bep van Strien for their critical help throughout the study and writing of this thesis; to Joop Groenewegen for his excellent collaboration in my research and for repairing my Dutch bicycles. I have appreciated so much my interactions with everyone in the baculovirus group: Magda Usmany, Els Klinge-Roode, Felix Bianchi, Wilfred IJkel, Marielle van Hulten, Jozsef Hajos, and previous colleagues Lilian Colbers, Ruud Mans, Marcel Kool, Hans Flipsen, Jacco Heldens and Stan de Kort. My friends and office-mates, Lilian and Els, were so kind to allow me all the table space and computer time during my thesis writing. I wish to acknowledge and thank several students who contributed to my research: Jolanda Westerlaken, Thomas Luijckx and Leonie van Dinten. My gratitude also goes to the friends and colleagues outside the baculovirus group, in particular to Jan van Lent, Ine Derksen, Wout Rozeboom, Thea Bemmel, and Dick Peters for their help in and outside the lab.

The thesis would not have been finished without the solid support that I received from my home institute, the Wuhan Institute of Virology of the Chinese Academy of Sciences. I am indebted to the directors of the institute, professors He Tianfu, Li Xinge, Zhang Xianen, Gong Hanzhou and previous director professor Chai Yiquan for their support and understanding. I owe a great deal to my friends, colleagues and students in our group. Professor Jin Fen and Chen Xinwen took over all my responsibilities when I was in the Netherlands. Professor Jin

Feng, you were the first to initiate the molecular research on BusuNPV. Thank you and professor Zhang Yinlian for your forever warm support which means much to me. Xinwen your contributions are excellent and your friendship is warm. While in the Netherlands, my students back in China were also ably supervised by professors Peng Huiying, Wang LuMing, Wang Hanzhong, and Zu Bichun. I am greatful to them.Further I appreciate all my students, Li Mei, Luo Baojun, Wang Hualin and Liu Lan, for their understanding, hard work and good results. Special thanks to Li Mei for her excellent contribution to the research on HaSNPV. I thank my colleagues in Virology Section, specially professors Ke Lihua, Liang Bufeng and Ding Qingquan for their support and help. Many researchers in my home institute have done the pioneering work to develop BusuNPV as a successful biocontrol agent and it is their initial work that paved the way for me to conduct the research outlined in this thesis. These scientists earn respect and appreciation, in particular professor Xie Tianen who led all the projects and Liu Mingfu who made his early contribution to the molecular biology of BusuNPV. I also thank Sun Junsong for his contribution to the study and professor Liu Xinyuan, Li Minjin, Wang Zixuan and Lu Zaohai for their contribution to the initial molecular characterization of BusuNPV and the nice time I had in Shanghai.

Many people have made my stay in the Netherlands most comfortable and enjoyable. I thank Rinske and her family for my time in *La Ventana* in Bennekom. My Chinese friends in Wageningen made me feel at home, special thanks go to Yu Dazhao, Zhang Liru and Tom, Huang Jiang, He Qian and ToTo. Since one rarely gets opportunity to acknowledge old friends for what they have meant and continue to mean in one's life, I here acknowledge Zhongqiong, Feng Ming, Amy, Zhang Lu, Shuchan, Zeng Hong and Xiao Jie. And I am glad that I finally have an opportunity to thank my middle school teacher Huang Songsheng for initiating me to science.

My family plays the central role in my life and, therefore, lastly but most importantly, I am so very grateful to my husband Yiwu for his love, continuous support and understanding which served as guiding lights and fostered my motivation throughout my whole PhD period. I thank my father, mother and my grandmother Dai Xiuqing for teaching me the values of life that gave me strength through the difficult times and also showed me how to enjoy the beautiful life. My warm thoughts are for my sister Xiaorong and her family for their support. Yiwu's parents have given me love and understanding for which I am forever grateful.

To the people I love and to my home country,

Zhihong

Wageningen, July 1998

## CONTENTS

CHAPTER 1

# INTRODUCTION

## General

Baculoviruses are pathogens that often cause fatal diseases in insects, mainly in members of the families Lepidoptera, Hymenoptera, Diptera, and Coleoptera, but also in Neuroptera, Trichoptera, Thysanura, Siphonoptera as well as in crustaceans (Decapoda). More than 600 baculovirus isolates (Family Baculoviridae) have been described and classified into two genera: Nucleopolyhedrovirus (NPV) and Granulovirus (GV) (Murphy *et al.*, 1995). The viruses are designated SNPV or MNPV depending on the single (S) or multiple (M) packaging of the nucleocapsids in the virion. So far, nineteen relatively well characterized baculoviruses have received species status including *Autographa californica* (Ac) MNPV, *Spodoptera exigua* MNPV, *Lymantria dispar* MNPV, *Heliothis zea* SNPV and *Trichoplusia ni* GV. The other listed viruses are tentative species and their nomenclature has been derived from the first two letters of the Linnaean insect genus and species; e.g. BusuNPV for *Buzura suppressaria* NPV (Murphy *et al.*, 1995). The host families from which NPVs have been isolated are listed in Table 1.1 (adapted from Adams and McClintock, 1991).

Baculoviruses are host specific for insects and can cause epizootic in nature which appear to play a role in controlling insect populations. These viruses are, therefore, attractive biological control agents and offer an alternative to wide-spectrum chemical insecticides (FAO/WHO Report, 1973). Baculoviruses have been successfully used in most continents in the control of a variety of pest insects, including the cotton bollworm, velvetbean caterpillar, tea moth, codling moth, pine beauty moth, Douglas fir tussock moth, beet army worm and fall army worm. To date, a number of them have been registered as biological control agents in various countries. A few have been commercialized, such as the MNPV of the beet army worm *S. exigua* (Spodex$^{R}$, BioSys, USA) on cotton in the USA, on shallot, garden pea, grape and Chinese kale in Thailand, and on flowers and ornamentals in the Netherlands (Smits and Vlak, 1994; Kolodny-Hirsh and Dimmock, 1996). Others are produced through government sponsored agencies, such as Gypcheck$^{R}$ against *L. dispar* in the USA. In Brasil, 1,000,000 hectares of soybean are treated annually against the velvetbean caterpillar, *Anticarsia gemmatalis* (Moscardi and Sosa-Gómez, 1992). In China about 100,000 hectares of cotton and hot pepper are treated annually with the SNPV from *H. armigera* (Zhang, 1989; Zhang, 1994) and about 20 other baculoviruses have been developed as insecticides for pest control (Table 1.2). Baculoviruses are also well suited for use in Integrated Pest Management strategies because of their compatibility with other control agents, chemical or biological. They have a proven safety track record with minimal effects on non-target insects, such as honey bees. They do not appear to present safety or health hazards to humans and other vertebrates as encountered with chemical insecticides and, lastly, they do not invoke resistance in insects (Persley, 1996).

**Table 1.1.** *Nucleopolyhedroviruses isolated from Insect Species* *

| order | number | family | number |
|---|---|---|---|
| Coleoptera | 5 | | |
| | | Cerambycidae | 2 |
| | | Curulionidae | 1 |
| | | Dermestidae | 2 |
| Diptera | 27 | | |
| | | Calliphoridea | 1 |
| | | Chironomidae | 1 |
| | | Culicidae | 20 |
| | | Sciaridae | 3 |
| | | Tachinidae | 1 |
| | | Tipulidae | 1 |
| Hymenoptera | 31 | | |
| | | Argidae | 1 |
| | | Diprionidae | 19 |
| | | Pamphylidae | 3 |
| | | Tenthridinidae | 8 |
| Lepidoptera | 455 | | |
| | | Anthelidae | 2 |
| | | Arctiidae | 22 |
| | | Argyresthiidae | 1 |
| | | Bombyciidae | 4 |
| | | Brassolidae | 1 |
| | | Carposinidae | 1 |
| | | Coleophoridae | 1 |
| | | Cossidae | 1 |
| | | Cryptophasidae | 1 |
| | | Dioptidae | 1 |
| | | Gelechiidae | 3 |
| | | Geometridae | 63 |
| | | Hepialidae | 3 |
| | | Hesperidae | 5 |
| | | Lasiocampidae | 34 |
| | | Limacodidae | 11 |
| | | Lymantriidae | 49 |
| | | Lyonetiidae | 1 |
| | | Noctuidae | 107 |
| | | Notodontidae | 12 |
| | | Nymphalidae | 15 |
| | | Papillionidae | 6 |
| | | Pieridae | 9 |
| | | Plutellidae | 1 |
| | | Psychidae | 5 |
| | | Pyralidae | 23 |
| | | Saturniidae | 22 |
| | | Sphingidae | 14 |
| | | Thaumetopoeidae | 3 |
| | | Thyatiridae | 1 |
| | | Tineidae | 2 |
| | | Tortricidae | 26 |
| | | Yponomeutidae | 4 |
| | | Zygaenidae | 1 |
| Neuroptera | 2 | | |
| | | Chrysopidae | 1 |
| | | hemerobiidae | 1 |
| Siphonoptera | 1 | | |
| | | Pulicidae | 1 |
| Thysanura | 1 | | |
| | | Phaemachilidae | 1 |
| Trichoptera | 1 | | |
| | | Limnephilidae | 1 |

*From Adams and McClintock, 1991

**Table 1.2.** The baculoviruses that had been used as insecticides in China*

| baculoviruses | control pests of |
|---|---|
| *Apochemia cinerarius* NPV | forest |
| *Buzura suppressaria* NPV | tea, forest |
| *Buzura thibetaria* NPV | tea |
| *Dendrolimus punctatus* NPV | forest |
| *Ectropis obliqua* NPV | tea |
| *Euproctis pseudoconspersa* NPV | tea |
| *Euproctis similis* NPV | garden |
| *Gynaephora ruoergensis* NPV | grass |
| *Heliothis armigera* NPV | cotton, tabacco |
| *Hyphantria cunea* NPV | garden |
| *Lymantria dispar* MNPV | forest |
| *Lymantria xylina* NPV | forest |
| *Malacosoma neustria tesacea* NPV | forest |
| *Mamestra brassicae* NPV | vegetable |
| *Plusia agnata* NPV | vegetable |
| *Prodenia litura* NPV | vegetable |
| | |
| *Adoxophyes orana* GV | tea |
| *Agrotis segetum* GV | vegetable |
| *Andraca bipunctata* GV | tea |
| *Clostera anachoreta* GV | forest |
| *Pieris rapae* GV | vegetable |

*Adapted from D. Liang, 1991; Tan, Y. and Tao, T. (1991)

A major limitation to wider use of baculoviruses in insect control, is their relative slow speed of action, particularly in crops with low damage thresholds such as flowers and fruits. Normally, it takes a few days for the virus to induce feeding inhibition in larvae (Fig. 1.1) when a more immediate insecticidal effect is often desired. An other drawback is their relatively low virulence for the older instars (Table 1.3) which cause the majority of damage in crops. Finally, baculoviruses are often too specific (Table 1.4) to meet the commercial demands for broad spectrum activity.

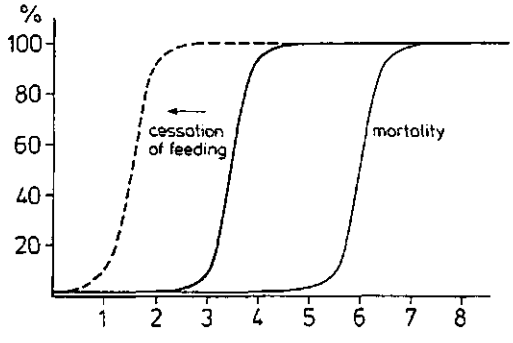


**Figure 1.1.** Relation between larval mortality (thin line) and cessation of feeding (thick line) in percentage versus time (days) after wild type baculovirus infection. The anticipated effect of baculoviruses with enhanced insecticidal activity is indicated by the dished line.

**Table 1.3.** Biological activity of *Mamestra brassicae* MNPV against *Mamestra brassicae* larval instars[a]

| Instar | $LD_{50}$[b] | 95% fiducial limits upper | lower |
|---|---|---|---|
| L1 | 7 | 5 | 11 |
| L2 | 947 | 796 | 1127 |
| L3 | 5420 | 4334 | 4777 |
| L4 | 59225 | 47629 | 73645 |
| L5 | 238370 | 184408 | 308123 |
| L5 (mid) | $>5 \times 10^7$ | - | - |

[a] from Evans (1981)
[b] polyhedra per insect of *Mamestra brassicae*

**Table 1.4.** Host range of selected baculoviruses in insects

| Virus species | Insect family | Insect species |
|---|---|---|
| *Autographa californica* MNPV | 13 | 73 |
| *Mamestra brassicae* MNPV | 4 | 36 |
| *Helicoverpa zea* SNPV | 1 | 7 |
| *Gilpinia hercyniae* NPV | 1 | 7 |
| *Cydia pomonella* GV | 1 | 4 |
| *Buzura suppressaria* SNPV | 1 | 1 |
| *Spodoptera litura* MNPV | 1 | 1 |
| *Spodoptera exigua* MNPV | 1 | 1 |
| *Euproctis chrysorrhoea* NPV | 1 | 1 |

Detailed knowledge on baculovirus gene structure, function and regulation has allowed the manipulation of the viral genome and the development of the baculovirus expression vector system (Smith *et al.*, 1983; O'Reilly *et al.*, 1992b; King and Possee, 1992). This technology has been exploited and tailored for the construction of baculoviruses with improved insecticidal properties (see Black *et al.*, 1997; Hu and Vlak, 1997; Miller, 1995 for review), such as increased speed of action (Wood and Granados, 1991; Vlak, 1993a, b; Miller, 1995; Bonning and Hammock, 1996, for review), enhanced virulence (Zuidema *et al.*, 1989; Ignoffo *et al.*, 1995) and extended host range (Thiem, 1997).

**Baculovirus structure and infection cycle**

The baculovirus phenotypes appear to have evolved to suit the unique features needed to cause horizontal and vertical infections of larvae. The replicate cycle is biphasic where two progeny phenotypes are produced, the occlusion-derived virus (ODV) and the budded virus (BV). ODV is encapsulated in a protein matrix composed predominantly of a single protein called polyhedrin (or granulin in GV). The budded form of virus (BV) is not occluded. ODV is responsible for the initiation of an infection in the insect. Infection is initiated when the alkali-sensitive occlusion body is ingested by a susceptible host and dissolves at the high pH of the insect midgut, thereby releasing virions. The midgut epithelial cells produce BVs which are transported via the hemolymph (Granados and Lawler, 1981) or the tracheal system

(Engeland *et al.*, 1994) to other tissues to cause a secondary infection. In the early stage of secondary infection, infected cells produce BVs which efficiently spread the infection from cell to cell within insect tissues. At later stage of secondary infection, virions are occluded into occlusion bodies in the infected cells. At the end of the infection, the cells and tissues of the dead insects are disintegrated and occlusion bodies are released into the environment. The next infection cycle starts again when the occlusion bodies are ingested by other susceptible insects (Fig. 1.2). The function of the occlusion body is two fold. One is offer a certain amount of protection to the virions against damaging environmental agents such as heat and decay. This may allow the virus to remain viable out side of the hosts for many years. The second function is to deliver the virus to the alkaline midgut where virus gets access to susceptible larval tissues. Although their nucleocapsids are similar in structure, ODVs and BVs are structurally distinct and have specific polypeptides (Fig. 1.3; see Funk *et al*, 1997, for review).

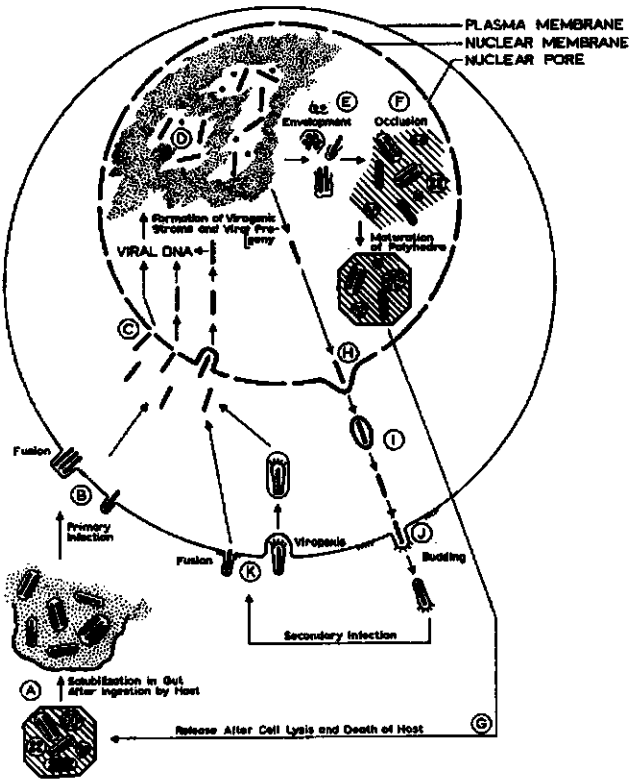


**Figure 1.2.** Schematic representation of the baculovirus infection cycle (from van der Beek, 1980; van Strien, 1997). Ingested polyhedra are solubilized in the midgut and virions are released (A). The envelopes of the virions fuse with the plasma membrane of the insect cell (B). After traversing the cytoplasm virions enter the nucleus, uncoat and release the viral DNA (C). Progeny viral nucleocapsids are synthesized in the virogenic stroma (D). Following envelopment in the nucleus (E), progeny nucleocapsids are initially released by budding (H, I, J). Budded virions infect adjacent cells by endocytosis (K). Nucleocapsids produced in later stages of an infection become occluded in polyhedral protein (F). Finally, the occlusion bodies are released by lysis of the infected cell.

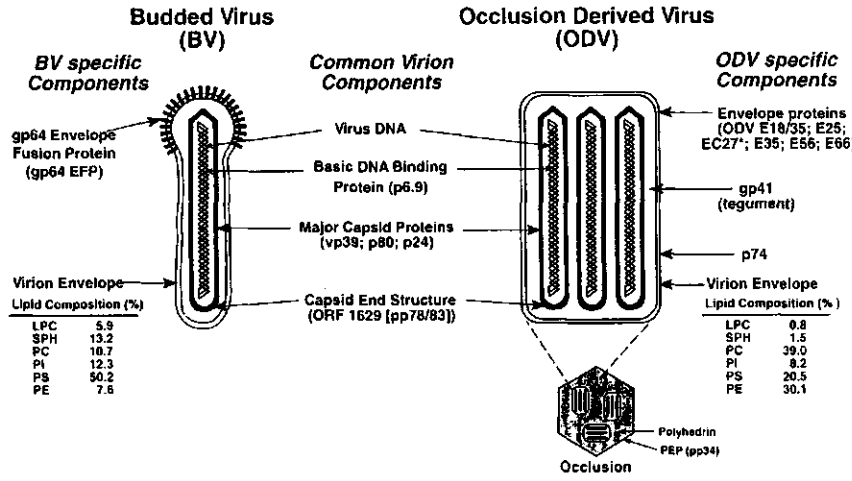


**Figure 1.3.** Structural comparison of the two baculovirus phenotypes, the budded virion, BV, and the occlusion derived virion (ODV) (from Blissard, 1996; Funk *et al.*, 1997). The ODV structure represents the MNPV subgroup. Proteins common to both virion types are indicated in the middle of the figure. Proteins specific to either BV or ODV are indicated on the left and right , respectively. The polar nature of the baculovirus capsid is indicated in the diagram with the claw-like structure at the bottom and the ring-like nipple at the top of the capsid. References for structural proteins are p6.9 (Wilson *et al.*, 1987); vp39 (Blissard *et al.*, 1989; Pearson *et al.*, 1988; Russell *et al.*, 1991; Thiem and Miller, 1989); p80 (Lu and Carstens, 1992); Müller *et al.*, 1990); pp78/83 (Russell *et al.*, 1997; Vialard and Richardson, 1993); polyhedrin (Hooft van Iddekinge *et al.*, 1983); PEP (PP34)(Gombart *et al.*, 1989; Russell and Rohrmann, 1990); ODV-E25 (Russell and Rohrmann, 1993); ODVE66 (Hong *et al.*, 1994); ODVE56 (Braungel *et al.*, 1996a; Theilmann *et al.*, 1996); ODVE18, E35, and EC27 (Braungel *et al.*, 1996b); gp41 (Whitford and Faulkner, 1992a, b); p74 (Kuzio *et al.*, 1989) and gp64 (Blissard and Rohramann, 1989; Whitford *et al.*, 1989). Lipid compositions of the BV and ODV envelopes derived from AcMNPV infected Sf9 cells (Braunagel and Summers, 1994) are indicated (LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phsophatidylinositol; PS, phosphatidylserine; PE, phosphatidylethonalamine).

## Genome organization and gene expression of baculoviruses

Baculoviruses have a circular, double stranded DNA genome varying in size from 90 to 160 kilobasepairs (kb) depending on the species. So far three genomes, that of AcMNPV, *Bombyx mori* (BmNPV) and *Orgyia psudotsugata* (OpMNPV), have been completely sequenced (Ayres *et al.*, 1994; S. Maeda, GenBank accession number L33180; Ahrens *et al.*, 1997). The genome of AcMNPV is composed of 133,894 base pairs (bp) potentially encoding 154 proteins, that of BmNPV is 128,413 bp in size and contains 136 putative genes, and the OpMNPV genome is 131,990 bp in size containing 152 putative genes. Sequence analyses of the three genomes have revealed a total of 184 different baculoviral ORFs. Among these ORFs, 119 are shared by the three genomes, 21 are present in two of the three genomes and 44 are unique to individual genomes (14 ORFs for AcMNPV, 4 ORFs for BmNPV and 26 ORFs for OpMNPV). Genomic comparisons indicated that, even though there are small inversions and insertions (or deletions), basically these three baculovirus genomes have a similar gene content and arrangement.

Baculovirus gene expression is organized in a sequential, cascade-like fashion. Each successive phase is dependent on the previous one (Blissard and Rohrmann, 1990). Regulation of baculovirus gene expression occurs at the transcriptional level. Three phases, **early**, **late** and **very late**, are distinguished during a baculovirus infection. Genes expressed during the **early** phase of the infection are transcribed by a host-cell encoded RNA polymerase. The products of early viral genes function to prepare the host cell for virus multiplication and to accelerate replicative events (Friesen, 1997). One of the important early genes is the immediate-early gene 1 (*ie-1*). Its product is involved in the transactivation of many genes that are expressed during the later phases of a baculovirus infection. The late stage of baculovirus infection is defined as those events that occur following the initiation of viral DNA replication and is usually subdivided into a **late** and a **very late** phase. These phases coincide with the production of BV and ODV, respectively (Lu and Miller, 1997). During the **late** phase, genes encoding structural proteins of the viral nucleocapsid, such as *vp39* and *p6.9*, the major capsid protein and basic core protein, respectively, are abundantly transcribed. Genes encoding occlusion-related polypeptides, such as polyhedrin or granulin, are transcribed primarily during the **very late** phase. Another abundantly expressed **very late** gene is p10, which encodes a small polypeptide that affects nuclear disintegration in the final phases of cell death.

***Buzura suppressaria* and BusuNPV**

*Buzura suppressaria* Guenée (Lepidoptera: Geometridae) is a polyphagous pest insect which causes damage to about 60 plant species including tea, tung-oil tree, metasequoia and Mandarin oranges. The insect has been reported from China, India, Burma and Japan. In China, it is widely distributed in thirteen provinces in the South-Central part including Hubei, Hunan, Jiangxi, Anhui, Jiangsu, Zhejiang, Shichuan, Guizhou, Guangxi, Guangdong, Fujian, Yunnan and Shanghai. In most regions the insect has two generations a year, but in the very south of China, such as the Guangdong, Guangxi and Fujian provinces, there are 3-4 generations per year (Institute of Tea, 1974). The *B. suppressaria* larvae can cause severe damage to the plants and chemical insecticides used to be the only means of control during its outbreaks.

In 1978, a single-nucleocapsid NPV of *B. suppressaria* (BusuNPV, also known as BsSNPV) was isolated from dead larvae (Xie *et al*, 1979; Gan, 1981; Chu *et al.* 1979). The virus was later developed as an insecticide to treat infested tea, tung-oil tree and metasequoia plants (Xie and Peng, 1980; Peng *et al.*, 1991; see report WIV *et al.*, 1985, for review). The virus has been used in Hubei, Jiangxi, Fujian, Hunan, Guangdong, Anhui, Guangxi, Guizhou province and Shanghai in a total area of about 300 hectares (4000 mu). The largest virus application was in 1989 in Chongming island off the coast of Shanghai where 100 hectares (1450 mu) of infested metasequoia forest was treated. The control was very successful in that the insect population was significantly reduced (Peng *et al.*, 1991) and more importantly, infestation by this pest insect has since not recurred in that area (H.Y. Peng, personal communication).

## The aim and outline of the thesis

Naturally existing baculoviruses is a potentially bountiful source of environmentally benign biocontrol agents. A detailed knowledge of genome organization, gene structure, function and regulation is an essential prerequisite to genomic manipulation and the generation of enhanced biocontrol agents. The major aim of this thesis was a detailed study of the genome organization of BusuNPV, and to provide knowledge for the future improvement of the viral insecticidal properties. In comparison to MNPVs, knowledge of the molecular biology and genetics of SNPVs is very limited. The study of the BusuNPV genome will allow the comparison of an SNPV with MNPVs both at the gene and the genome level.

**Table 1.5.** The NPVs isolated from Geomitridae insects*

| | |
|---|---|
| *Abraxas grossulariata* NPV | *Eupithecia annulata* NPV |
| *Acidalia carticcaria* NPV | *Eupithecia longipalpata* NPV |
| *Alsophila pometaria* NPV | *Hesperumia sulphuraria* NPV |
| *Amphidasis cognataria* NPV | *Hydriomena irata* NPV |
| *Anaitis plagiata* NPV | *Hydriomena nubilofasciata* NPV |
| *Anthelia hyperborea* NPV | *Hyperetis amicaria* NPV |
| *Apocheima cinerarius* NPV | *Jankowskia athleta* NPV |
| *Apocheima pilosaria* NPV | *Lambdina fiscellaria* NPV |
| *Biston betularia* NPV | *Lambdina fiscellaria lugubrosa* NPV |
| *Biston hirtaria* NPV | *Lambdina fiscellaria somniaria* NPV |
| *Biston hispidaria* NPV | *Melanolophia imitata* NPV |
| *Biston marginata* NPV | *Myrteta tinagmaria* NPV |
| *Biston robustum* NPV | *Nepytia freemani* NPV |
| *Biston strataria* NPV | *Nepytia phantasmaria* NPV |
| *Boarmia bistortata* NPV | *Nyctobia limitaria nigroangulata* NPV |
| *Bupalus piniarius* NPV | *Operophtera bruceata* NPV |
| *Buzura suppressaria* NPV | *Operophtera brumata* NPV |
| *Buzura thibtaria* NPV | *Opisthograptis luteolata* NPV |
| *Caripeta divisata* NPV | *Oporinia autumnata* NPV |
| *Clingilia catenaria* NPV | *Ouraptrgx ebuleata* NPV[3] |
| *Culcula panterinaria* NPV | *Paleacrita vernata* NPV |
| *Deileptenia ribeata* NPV | *Peribatodes simpliciaria* NPV |
| *Ectropis crepuscularia* NPV | *Pero behrensarius* NPV |
| *Ectropis excellens* NPV[1] | *Pero mizon* NPV |
| *Ectropis grisecens* NPV[2] | *Phigalia titea* NPV |
| *Ectropis obliqua* NPV | *Phthonosema tendinosaria* NPV |
| *Ennomos quercaria* NPV | *Protoboarmia porcelaria indicataria* NPV |
| *Ennomos quercinaria* NPV | *Ptychopoda seriata* NPV |
| *Ennomos subsignarius* NPV | *Scopula subpunctaria* NPV |
| *Enypia venata* NPV | *Selidosema suavis* NPV |
| *Erannis ankeraria* NPV | *Sucra jujaba* NPV[4] |
| *Erannis defoliaria* NPV | *Synaxis jubararia* NPV |
| *Erannis tiliaria* NPV | *Synaxis pallulata* NPV |
| *Erannis vancouvernsis* NPV | |

*Adapted from Martignoni, M.E. and Iwai, P.J. (1986) except references 1-4 (Chen *et al.*, 1989; Peng *et al.*, 1994; Zhang and Xing, 1986).

BusuNPV was chosen because it controls a major insect pest in China. By serendipity, it is also a SNPV and a representative of baculoviruses infecting members of the insect family Geometridae (Order Lepidoptera). This family hosts the second largest member of baculoviruses after those infecting Noctuidae (Table 1.1). So far, 67 baculoviruses have been described from geometrids (Table 1.5), including several important pest insects, such as the Eastern hemlock looper, *Lambdina fiscellaria*, in North America (Carrol, 1956; Raske *et al.*, 1995). When the study described in this thesis was initiated in 1993, none of the geometrid baculoviruses had been investigated at the molecular level to any detail. Recently, however, the polyhedrin sequences of *Lambdina fiscellaria fiscellaria* NPV, *Lambdina fiscellaria lugubrosa* NPV and *Ecotropis obliqua* NPV have been reported (Levin *et al*, 1997; Zhang *et al*, GenBank U95041). The research presented in this thesis is a significant step to the understanding of the genomic organization, gene structure and phylogenetic status of BusuNPV as a prelude to genetic modification of this virus.

Polyhedra (occlusion bodies) are the most prominent feature of baculoviruses infections. Polyhedrin, the major component of occlusion bodies, has been the subject of detailed investigations because it is important for the survival of the virus in the environment and also because it is the most conserved viral protein. The gene encoding polyhedrin is temporally regulated under the control of a powerful late promoter that led to the development of the successful baculovirus expression systems. More sequences for polyhedrins than for any other baculovirus protein have become available. Hence, on the basis of polyhedrin sequences, a phylogenetic tree of baculoviruses has been constructed (Zanotto *et al.*, 1993), which subdivides baculoviruses into several groups. Since the polyhedrins from SNPVs were under represented (only 2 included), it was necessary to identify and sequence this gene from BusuNPV (**Chapter 2**) and to verify the grouping of SNPVs in the phylogenetic tree (see Chapter 8).

The ecdysteroid UDP-glucosyltransferase (EGT) is a key enzyme in abrogating the insect host regulation of metamorphosis (O'Reilly and Miller, 1989). It conjugates ecdysteroids with sugars and hence blocks molting of the insect. Deletion of *egt* allows the insect to molt normally, and consume less foliage than wild type virus infected larvae. Also, larvae are killed quicker by an *egt* null virus (O'Reilly and Miller, 1991). So far, *egt* has only been identified in MNPVs and its presence in SNPVs has not been ascertained. The identification, sequence analysis and phylogeny of BusuNPV *egt* is described in **Chapter 3**. This gene locus may well be a future target to improve the insecticidal properties of BusuNPV. Knowledge of the sequences of polyhedrin and *egt* genes also allows the positioning BusuNPV into the phylogentic trees of baculoviruses.

The location of the *egt* relative to polyhedrin gene in the genome of the BusuNPV provided the first evidence for differences between the gene organization in this virus and that of AcMNPV, the baculovirus type species (Murphy *et al.*, 1995). Sequencing of a 3.2 kb *Hin*dIII fragment of the BusuNPV genome (**Chapter 4**) indicated that the genome organization of BusuNPV is unique and clearly distinct from those of other baculoviruses characterized so far.

The gene content and organization the BusuNPV genome was further delineated by sequencing a plasmid library either completely in both directions or only at the ends of the inserts ('sniff sequencing'). The sequence data (43.5 kb) facilitated a refinement of the physical map and allowed the generation of a partial gene map of the genome of BusuNPV (**Chapter 5**). The result confirmed that BusuNPV genome has a distinct gene arrangement. A novel method, called GeneParityPlot, was developed for comparing genomes in general and for identifying gene clusters that are conserved within baculoviruses genomes.

A second protein, P10, is also expressed abundantly late in infection but, unlike polyhedrin, the amino acid sequence of P10 is usually not conserved among baculoviruses. However, the structure of P10, in terms of the presence of important features such as the heptad repeats, coiled-coil formation is highly conserved (van Oers and Vlak, 1997). In **Chapter 6** the BusuSNPV *p10* gene was identified from sequence data. The coding region of the BusuNPV *p10* gene was swapped into an AcMNPV *p10* deletion mutant to test the functionality of the SNPV gene in an AcMNPV context (**Chapter 6**).

BusuNPV occludes single-nucleocapsid virions and AcMNPV occludes multiple-nucleocapsid virions. 'Gene swapping' was used to replace the AcMNPV polyhedrin gene with that from BusuNPV (**Chapter 7**). These experiments were undertaken to answer an intriguing question on the specificity of the virion occlusion process. Since the morphogenesis of the occlusion body is controlled by a number of factors, a polyhedrin swap could show whether a foreign polyhedrin can successfully function in concert with other factors resulting in virion occlusion and mature polyhedra.

In **Chapter 8**, the molecular genetics of BusuNPV derived from the studies carried out in this thesis as well as their impact on future engineering of the virus is discussed. In addition, the phylogenetic status of baculoviruses is discussed in detail. By comparison the 'single-gene' trees derived from polyhedrin, EGT and LEF-2 proteins, a phylogeny tree of the baculovirus is constructed. An attempt was made to figure out the evolutionary timescale of baculovirus by using the phylogeny tree derived from superoxide dismutase (SOD) proteins. Finally, the impact of baculovirus molecular genetics on taxonomy and phylogeny is discussed.

CHAPTER 2

# NUCLEOTIDE SEQUENCE OF THE *BUZURA SUPPRESSARIA* SINGLE NUCLEOCAPSID NUCLEOPOLYHEDROVIRUS POLYHEDRIN GENE


## SUMMARY

A portion of the genome of the *Buzura suppressaria* (Lepidoptera) single nucleocapsid nucleopolyhedrovirus (BusuNPV) containing the polyhedrin gene was sequenced. An open reading frame of 738 nucleotides was identified which encoded a protein of 246 amino acids and represented the polyhedrin gene. A conserved TAAG motif, associated with transcriptional start sites in other polyhedrin genes, was identified 51 nucleotides upstream of the BusuNPV polyhedrin gene. A putative polyadenylation signal, AATAAA, was found immediately downstream from the polypeptide termination codon. Comparison of the amino acid sequence of BusuNPV polyhedrin with other NPV polyhedrins and granulovirus granulins showed that the BusuNPV polyhedrin was most closely related to the polyhedrin of *Orgyia pseudotsugata* (Lepidoptera) SNPV and most distantly related to the polyhedrin of *Neodiprion sertifer* (Hymenoptera) SNPV.



The contents of this chapter have been published as : Hu, Z.H., M.F. Liu, F. Jin, Z.X. Wang, X.Y. Liu, M.J. Li, B.F. Liang and T.E. Xie. 1993. Nucleotide sequence of the *Buzura suppressaria* single nucleocapsid nuclear polyhedrosis virus polyhedrin gene. *Journal of General Virology* 74, 1617-1620. The nucleotide sequence data reported in this chapter have been deposited in GenBank under the accession number X70844 .

## INTRODUCTION

*Buzura suppressaria* (Lepidoptera:Geometridae), is the major pest insect of tea in China. This polyphagous insect also causes severe damage to tung oil, citrus and metasequoia plants. The single-nucleocapsid nucleopolyhedrovirus of *B. suppressaria* (BusuNPV) (Xie *et al.*, 1979. Gan, 1981; Chu *et al.*, 1979) has been successfully used for control of this insect (Xie & Peng, 1980; Peng *et al*, 1991). The double stranded viral DNA has been analyzed with restriction enzymes and a preliminary physical map of the viral DNA has been determined. The BusuNPV genome was found to be 129 kilobase pairs (kb) in size (Liu *et al.*, 1993).

The polyhedrin gene is the most extensively studied baculovirus gene. Polyhedrin is a major constituent of polyhedra, the large proteinaceous occlusion bodies (OBs) produced at the end of an infection. These OBs contain the rod-shaped virions with single (S) or multiple (M) nucleocapsids per virion. However, most of the information on polyhedrins has been derived from studies on MNPVs (Rohrmann, 1986). Only two SNPV polyhedrins have been sequenced, those from *Orgyia pseudotsugata* of the Lepidoptera (OpSNPV, Leisy *et al.*, 1986a) and *Neodiprion sertifer* of the Hymenoptera (NeseNPV, see Rohrmann, 1992). Since OpSNPV polyhedrin and NeseNPV polyhedrin have a low degree of amino acid sequence identity, sequencing of a third SNPV polyhedrin gene will likely be useful in understanding the relatedness among SNPV polyhedrins in particular and among polyhedrins and granulins (OB protein of granulovirus = GV) in general. In this paper we compared the BusuNPV polyhedrin gene and its flanking regions with those of other baculoviruses.

## MATERIALS AND METHODS

*Virus and DNA*

BusuNPV was propagated in *B. suppressaria* larvae and the occlusion bodies (OB) were purified by differential and rate zonal centrifugation (Xie *et al.*, 1979). The DNA was isolated directly from purified OBs by using a dissolution buffer (0.1M $Na_2CO_3$, 0.01M EDTA and 0.17M NaCl), followed by proteinase K and SDS treatment, phenol/chloroform extraction and dialysis. The purity of the DNA was determined spectrophotometrically.

*Cloning and sequencing of DNA fragments*

The *Autographa californica* (Ac) MNPV *Eco*RI-I fragment, which contains the polyhedrin gene (Vlak *et al.*, 1981), hybridized with a 2.0 kb *Kpn*I-I fragment of BusuNPV DNA (Liu *et al.*, 1992). The *Kpn*I-I fragment of BusuNPV DNA was cloned into a bacterial vector M13mp18 according to Maniatis *et al.* (1982). 'Erase-a-Base' (Promega) was used to generating subclones for sequencing. The vectors containing the 2.0 kb *Kpn*I-I fragment were digested with *Pst*I and *Bam*HI to produce 3' and 5' overhangs, respectively. The DNA was then treated with exonuclease III and nuclease S1 to produce overlapping fragments. These fragments were self-ligated and transformed to *E. coli* to obtain subclones from which DNA was isolated for sequencing (Maniatis *et al.*, 1982). DNA sequencing was carried out by using the dideoynucleotide chain-terminating method of Sanger *et al.* (1977), employing the T7 DNA polymerase Sequencing System (Promega). Since the polyhedrin gene sequence did not reside

totally within the *Kpn*I-I fragment, the adjacent *Kpn*I-*Hin*dIII fragment was cloned and the portion containing the 3' end of the polyhedrin gene sequenced.

*Computer analyses*
Sequences were analyzed with the aid of the UWGCG computer program; DNA and deduced amino acids sequences were compared with the updated GenBank/EMBL.

## RESULTS AND DISCUSSION

The 2.0 kb *Kpn*I-I fragment of BusuNPV DNA which hybridized with the AcMNPV *Eco*RI-I fragment was cloned into a M13mp18 vector and two kinds of clones were obtained which contain the *Kpn*I-I fragment in different orientations. By using 'Erase-a-Base' (Promega) a series of subclones containing overlapping sequences were obtained and from which the sequence of the both strands of the *Kpn*I-I fragment was generated. Sequence analysis showed that the 3' end of the polyhedrin gene did not reside within the *Kpn*I-I fragment, therefore, the adjacent *Kpn*I-*Hin*dIII fragment was cloned and sequenced to obtain the entire sequence of the polyhedrin gene.

A total of 2340 nucleotides of BusuNPV DNA was sequenced, which included the polyhedrin gene and its flanking regions (Fig. 2.1). The polyhedrin gene is 738 nucleotides long with the potential to encode a polypeptide of 246 amino acids. In the 5' non-coding region, a putative transcriptional start site is present with the canonical core sequence TAAG (Vlak and Rohrmann, 1985) at nucleotide position -51. In the 3' non-coding region, a poly (A) signal sequence AATAAA (Birnstiel *et al.*, 1985) was found 5 nucleotides downstream from the translational stop codon TAA. Our nucleotide sequence is slightly different from the partial 5' end sequence as reported previously for the BusuNPV polyhedrin gene (Zhang *et al.*, 1991), but this could be due to the existence of two different virus isolates.

The promoter region of the BusuNPV polyhedrin gene was found to be similar to those of other baculovirus polyhedrin genes (Fig. 2.2). These similarities include the A at position -3 (Kozak, 1983) and the TAAG motif involved in transcription initiation at position -51 (Rohrmann, 1986). The promoter region of the BusuNPV polyhedrin gene is most closely related to OpSNPV (Leisy *et al.*, 1986 a) with an identical length (51 nucleotides) and only 5 nucleotides difference in sequence. We found no homology between the sequences upstream of the BusuNPV polyhedrin gene promoter or downstream from the gene with those of AcMNPV (Possee *et al.*, 1992). No major open reading frames were observed in the upstream region.

The amino acid sequence of the BusuNPV polyhedrin was compared to twelve known other NPV polyhedrins (Fig. 2.3). The BusuNPV polyhedrin (246 amino acids) is similar in size to most other NPV polyhedrins including NeseNPV (246 amino acids) but not to GV granulins (size variation at N-terminus; data not shown). At amino acid residues 33-36 a KRKK sequence is present, which in AcMNPV polyhedrin served as a nuclear localization signal (Jarvis *et al.*, 1991).

```
GGTACCAATTCGAAACCAACGCAACAAGAATACGATACCAATATTGGCAATTGTAACAATAAAAATTTTGAAAATGTTAAACGTAATGAG    90
AATCCCGTAATAAAAAAATATTCGTATTTGTTTGAAGAAAAAACGTTGACGTTTGATAATCAGTTTTGTTTTGTGCTTCGGTATTTGATT   180
AAAAACGAAGAAATTTGGATGATTGGCTACGATCTCGGCCGCGGGAATCGGATTTGACAATCCCAACTTGGCCGTGAACCGATATGTTCA   270
ATTTACAGAATGGAAAAGTGTCAATCAATTGTTGTTTGATAAATTTACTGCTGACGACGGAATCAAATGTATCAATCGGAACGGCGCTCT   360
GCAACTGCTCAATAATATAGATTTTAAAAACAAGGCAGAATTTATTGCTTGTTTGTTGGAAACGTTTAACGAGATTGGAACACTTTCATA   450
AACCGTCTTCGACGTCTATAGTCAACGACGATGACAAATTCGATAAACTATTAGAAGCTATTGAGGCGATCAAGGTGTAACAACACACTT   540
TTGTTGGAAAATAACACTACATTTAAAACGCAAATTATTGACAAATTAGGCGCGTTTGAACTGCAATTTTCGCACCAAATTCAAGAGTTA   630
CACAATAAAATTATTCAATATGAAAATGTGGAACAATTATATACGCGTTTAAAAAAATACCATAAAACGTTAGAGGGTTCCGCGTTGGAC   720
GATTGCTGTTCCATTTTTAACGAACATCAAAATCGCTATGAAACCGTTAGATTTCCGCGCAATCCGTCCAAGTTTCCGAGGCTGGCAGTG   810
TACGTGAAACCCGACAATCAAGGCACTCAGCTGGCGATAATAGCTGGCCAACAGAAAAATATGCAAGCACGCAAACGCAAATACAAGGAT   900
ATGGAATTGGTGTATGACAGTGTTCATCCCAATCCTTTGTTGGCCGTCCATTGCATCAATGAAGAGTTGGATAATAAAAACTACAACTAT   990
AGTAAACGAGGCAAGCGTGTTTATCATATTCAATCTGATGTAAATACGGTTAAATCCTTTATAAATGAAAATGTTTAATAAAATATCTTC  1080
GATATAAATTGTTTTATTTTTTTACATTTATTGGATTACATCATTTATGAATAAAACATATTTAATATAATGCAAAAGCAATAAATACTG  1170
ATGCAATAATATTAGGAGTTTACAACAACAGAATATTAGTCACGCCGAGCGCTGTTGCATAACATCTTTTAATGCTGCCTGTTGCACATC  1260
GATCTGTTGTACATGTTGAAACGGATGAGATCTCAATATTTTGAACACCTGACGGCTTTACAGCATGTAATTCGATATACGATGTTTTAT  1350
                                                                                          M  Y
AAATTATCAAAATTTGTTCATTTACAATCTTCAATAAGTATTTTTTTCCTATTGTAAAACATTGTGAAAAATCAAATACAACATAATGTA  1440
  T  R  Y  S  Y  K  P  S  L  G  R  T  Y  V  Y  D  N  K  Y  Y  K  N  L  G  A  V  I  K  N  A
CACTCGTTACAGTTATAAGCCCTCTTTGGGGCGAACCTATGTCTACGACAACAAATACTACAAAAATTTAGGTGCAGTGATCAAGAACGC  1530
  K  R  K  K  H  E  I  E  H  E  V  E  E  R  T  L  D  P  L  D  K  Y  L  V  A  E  D  P  F  L
TAAACGCAAGAAGCACGAAATCGAGCATGAAGTGGAGGAGCGCACACTTGATCCGCTAGATAAGTATTTGGTCGCCGAAGACCCTTTTCT  1620
  G  P  G  K  N  Q  K  L  T  L  F  K  E  I  R  N  V  K  P  D  T  M  K  L  V  V  N  W  S  G
TGGACCCGGTAAAAACCAAAAACTTACTTTGTTTAAAGAAATTCGTAATGTTAAACCAGACACCATGAAATTGGTCGTTAACTGGAGCGG  1710
  K  E  F  L  R  E  T  W  T  R  F  M  E  D  S  F  P  I  V  N  D  Q  E  I  M  D  V  F  L  V
TAAAGAATTTCTGAGGGAAACTTGGACCCGCTTTATGGAGGATAGTTTTCCTATTGTGAACGACCAAGAAATCATGGATGTCTTTTTGGT  1800
  I  N  M  R  P  T  R  P  N  R  C  Y  R  F  L  A  Q  H  A  L  R  C  D  P  D  Y  V  P  H  E
AATTAACATGCGCCCAACCAGACCTAATCGTTGTTACAGATTTTTAGCTCAACACGCGCTCCGTTGCGACCCCGACTACGTGCCACACGA  1890
  V  I  R  I  V  E  P  S  Y  V  G  S  N  N  E  Y  R  I  S  L  A  K  R  G  G  G  C  P  V  M
GGTGATCCGAATTGTCGAGCCCAGCTACGTAGGTAGCAACAACGAGTATCGCATTAGTTTAGCCAAAAGAGGCGGTGGTTGCCCGGTAAT  1980
  N  L  H  S  E  Y  T  N  S  F  E  E  F  I  N  R  V  I  W  E  N  F  Y  K  P  I  V  Y  V  G
GAATCTGCATTCTGAATACACCAACTCGTTCGAGGAATTCATCAACAGAGTAATTTGGGAAAACTTTTACAAACCTATTGTGTACGTAGG  2070
  T  D  S  A  E  E  E  E  I  L  L  E  V  S  L  V  F  K  V  K  E  F  A  P  D  A  P  L  Y  T
TACCGATTCGGCAGAGGAAGAAGAAATTCTTCTTGAAGTTTCTTTGGTATTTAAAGTTAAAGAATTTGCGCCTGATGCCCCTCTATACAC  2160
  G  P  A  Y  *
AGGTCCTGCATATTAAATTCAAATAAATATATATATATATAAACCAACAATATTATAATATACTAAGCAAATTGAAGTATTGTGATTGAT  2250
GATGTTGCGCAAATTTTTTAGATAGGATTGAAATAGATCGTTCATTGTCGCATGCTGAGAGTAAATTTCCAATTCGTCATAAGCCAGCTG  2340
```

**Figure 2.1.** The nucleotide sequence of the BusuNPV polyhedrin gene and flanking regions. The predicted amino acids are indicated with one-letter code designations (start code ATG and stop code TAA are bolded). The TAAG consensus sequence for late baculovirus transcription initiation and a potential polyadenylation signal are underlined. The sites for the restriction enzyme *Kpn*I are shown in bold letters.

```
                -51                                                          -3
BusuNPV TTCA ATAAG TATTTTTTTCC TATTGTAAAA CATTGTGAAA AATCAAAT ACAAC  ATA
OpSNPV  CTCA ATAAG TATTTTTGTCC TTTCGTAAAA CATTGTGAAA TTTCAAAT ACACC  ATA
LdMNPV  TCCA ATAAG TATTTTATTCT TTTCGTAAAG ATTTTGGAAA AATCAAAT ACACCGTAAA

SfMNPV  AAAT GTAAG TAATTTTTTCC TTTCGTAAAA GATTGTGAAA AATAAAAT        ATA
SeMNPV  AAAT GTAAG TAATTTTTTCC TTTCGTAAAA GATTGTGAAA AATAAAAT        ATA
MbMNPV  AAAT GTAAG TAATTTTCTCC TTTCGTAGAA GATTGTGAAA AATAAAAT        ATA
PaflNPV AAAT GTAAG TAATTTTCTCC TTTCGTAGAA GATTGTGAAA AATAAAAT        ATA

AcMNPV  ATAA ATAAG TATTTTACTGT TTTCGTAACA GTTTTGTAAT AAAAAAAC CTATAA ATA
BmNPV   ATAT ATAAG TATTTTACTGT TTTCGTAACA GTTTTGTAAT AAAAAAAC CTATAA AT
OpMNPV  ATTA ATAAG TAATTTCCTGT TATTGTAACA ATTTTGTAAT AAAATTTC CTATAA CC
AgMNPV  GATA ATAAG TATTTTGCTGT TATTGTAGCA ACTTTGTAGT AAAATTTG CTATAA CT
```

**Figure 2.2.** Promoter sequences of baculovirus polyhedrin genes: BusuNPV, OpSNPV (Leisy *et al.*, 1986a),

*Lymantria dispar* MNPV (LdMNPV; Chang *et al.*, 1989), *Spodoptera frugiperda* MNPV (SfMNPV; Gonzales *et al.*, 1989), *S. exigua* MNPV (SeMNPV; Van Strien *et al.*, 1992), *Mamestra brassicae* MNPV (MbMNPV, Cameron and Possee, 1989), *Panolis flammae* MNPV (PaflNPV; Oakey *et al.*, 1989), AcMNPV (Hooft van Iddekinge *et al.*, 1983), *Bombyx mori* NPV (BmNPV; Iatrou *et al.*, 1985), *O. pseudotsugata* MNPV (OpMNPV; Leisy *et al.*, 1986b), *Anticarsia gemmatalis* MNPV (AgMNPV; Zanotto *et al.*, 1992). The transcription initiation motif TAAG is underlined. All NPV polyhedrin sequences start at +1. Only the numbering for BusuNPV is shown.

```
BusuNPV   MYTRYSYKPS LGRTYVYDNK YYKNLGAVIK NAKRKKHEIE HEVEERTLDP LDKYLVAEDP
OpSNPV    .......N.. .......... .......... .......Q.. ..A..H.... ..........
MbMNPV    .......N.. .......... ......S... ..N..R.Y.. ..L..K.... ..R.......
PaflNPV   .......N.. FP........ .......... ..N..NDF.. .QL..K.... ..R.......
SfMNPV    .......N.. .......... F.....S... .....E.LAL ..I........ .ER.V.....
SeMNPV    .......N.A .......... F.....S... .....E.LLQ ..I........ .ERYV.....
AcMNPV    .-PD...R.T I......... .......... .......FA. ..I..A.... ..N.......
OpMNPV    .-PD...R.T I......... ......S... .......LL. ..ED.KH... ..H.M.....
AgMNPV    .-PD...R.T I......... ......S... .......LL. .QE..KS..G ..H.I.....
CfMNPV    .-PD...R.T I......... ......S... .......LL. ..ED.KH... ..H.M.....
BmNPV     .-PN...N.T I......... ......GL.. .......L.. ..K..KQW.L ..N.M.....
LdMNPV    .HNF.N.S.A ..K....E.. ......T... Q...Q..LEQ ..I...S..H ..R.......
NeseNPV   MPNLAQG.QT. -AKS-I.... ...G..DI.N S..KR..DQD W.KHADQRRA .NGFILPLG. 60

BusuNPV   FLGPGKNQKL TLFKEIRNVK PDTMKLVVNW SGKEFLRETW TRFMEDSFPI VNDQEIMDVF
OpSNPV    .......... .......... .......... .......... .......... ..........
MbMNPV    .....J.... .......... .......... .......... .......... .....V....
PaflNPV   .....J.... .......... .......... .......... .......... .....V....
SfMNPV    .....J.... .......I.. .......... .......... .......... ..........
SeMNPV    .....J.... .......I.. .......... .......... .......... ..........
AcMNPV    .....J.... .......... ........G. K....Y.... .......... .....V....
OpMNPV    .....J.... .......... ......I... .......... ...V...... .....V....
AgMNPV    .....J.... .......... ......I... .......... ...V...... .....V....
CfMNPV    .......... .......... ......I... .......... ...V...... .....V....
BmNPV     .....J.... .....V.... ......I... .......... ...V...... .....V...Y
LdMNPV    .Y...J.... .......... .......... .......... .......... .....V..IY
NeseNPV   RT....HV.M VM.Q.V..I. AN....AI.. .S..Y...V. .T.I..T... N.Y.FT.... 120

BusuNPV   LVINMRPTRP NRCYRFLAQH ALRCDPDYVP HEVIRIVEPS YVGSNNEYRI SLAKRGGGCP
OpSNPV    .......... ...F...... ......E... .......... .......... ..........
MbMNPV    .......... ...FK..... .......... .......... .........V ..........
PaflNPV   .......... ....K..... ......E... .......... .........V ..........
SfMNPV    .......... ...F...... .......... .........V ...N...... ....K.....
SeMNPV    .......... ...F...... .......... .........V ...T...... ....K.....
AcMNPV    ..V....... ....K..... .......... .D........ W..........  ....K.....
OpMNPV    ..V....... ....K..... ...W.C.... .......... ...M...... ....K.....
AgMNPV    ....L..... ....K..... ...SHC.... .......... ...M...... ....K.....
CfMNPV    ..V....... ....K..... ...W.C.... .......... ...M...... ....K.....
BmNPV     ..A.LK.... ....K..... ...W.E.... ......M... ...M...... ....K.....
LdMNPV    .T..V..... ....K.V... .....EG... .......... T.E-...... ..........
NeseNPV   .E.RCT.NKS ..H....... G..M.M-ILC ..LPFALSNL CILRETPFLS V.L..D.... 180

BusuNPV   VMNLHSEYTN SFEEFINRVI WENFYKPIVY VGTDSAEEEE ILLEVSLVFK VKEFAPDAPL YTGPAY
OpSNPV    .....A.... .........H .......... .......... .......... I.......... .S.K..
MbMNPV    .......... .......... .......... .......... .......... I.......... .N....
PaflNPV   .......... .......... .......... .......... .......... I.......... .N....
SfMNPV    .........H .......... .......... .....G.... ....L..... I.......... .N....
SeMNPV    .......... .......... .......... .....G.... ....L..... I.......... .N....
AcMNPV    I......... ...Q..D... .......... I......... .......... .......... F.....
OpMNPV    I..I.A.... ...S.V.... .......... I....S.... ..I....... .......... F.....
AgMNPV    I..I...... ...S.V.... .......... I....S.... ..I....... .......... F.....
CfMNPV    I..I.A.... ...S.V.... .......... I....G.... M.I....... ........A. F.....
BmNPV     I..I...... ...S.V.... .......... I......... ..I....... I.......... F.....
LdMNPV    IR....A..T ...H.L.S.. .D........ ...T...... .......... I.......... FQ....
NeseNPV   ..KFRQ.FNEL.LD...I.IL .CH.HR.... I....G.... VVI.A..T.I I.....E..F VN..GM 246
```

**Figure 2.3.** Comparison of the amino acid sequences of twelve NPV polyhedrins. The one-letter code designation is used. Dots indicate identity with BusuNPV polyhedrin. The sources of the sequences are described in the legend to Fig. 2.2 except for those of *Choristoneura fumiferana* MNPV (CfMNPV) (B.M. Arif,

personal communication) and *Neodiprion sertifer* SNPV (NeseNPV, see Rohrmann, 1992).

Whereas MNPVs appear to be confined to Lepidopteran insects, SNPVs have been isolated from many other insect orders (Vlak & Rohrmann, 1985). Analysis of BusuNPV polyhedrin indicated that the encoded polypeptide is most closely related to that of OpSNPV with 80.7% nucleotide homology and 95.5% amino acid identity. Comparison to other MNPV polyhedrins indicated a nucleotide sequence homology and amino acid identity ranging from 70.6-80.6% and 82.9-94.3%, respectively. It is worth mentioning that a closer relatedness of BusuNPV polyhedrin was found to granulins (*Pieris brassicae* GV; Chakerian *et al.*, 1985 and *Trichoplusia ni* GV; Akiyoshi *et al.*, 1985) with nucleotide homology of 57.2-58.4% and amino acid identity of 56.2-57.1%, than to NeseNPV polyhedrin (see Rohrmann, 1992). These data give further credence to the hypothesis that NeseNPV evolved from the common ancestor of the Lepidopteran GVs and NPVs before GVs envolved from the Lepidopteran NPVs (Rohrmann, 1992). Although the BusuNPV polyhedrin was most related to that of OpSNPV, distinct lineages of MNPVs and SNPVs within the Lepidoptera are not evident.

Future research will focus on transcriptional mapping the 3' and 5' termini of the BusuNPV polyhedrin gene, and further sequencing its flanking regions. The generated data will give additional information on the organization of SNPV genomes in comparison to those of MNPVs.

## ACKNOWLEDGMENT

We are grateful to Dr. B.M. Arif for kindly providing the unpublished amino acid sequence of CfMNPV polyhedrin and helpful reviewing of the manuscript, and to Dr. Lu Zhaohai for excellent research assistance. This work was supported in part by National Foundation for Natural Sciences of China and the Director Foundation of Wuhan Institute of Virology.

CHAPTER 3

# CHARACTERIZATION OF THE ECDYSTEROID UDP-GLUCOSYLTRANSFERASE GENE OF A SINGLE NUCLEOCAPSID NUCLEOPOLYHEDROVIRUS OF *BUZURA SUPPRESSARIA*


## SUMMARY

A putative ecdysteroid UDP-glucosyltransferase (*egt*) gene was identified in the single nucleocapsid nucleopolyhedrovirus of *Buzura suppressaria* (BusuNPV). This is the first *egt* gene to be characterized in a SNPV, suggesting that *egt* genes are prevalent in nucleopolyhedroviruses and possibly in all baculoviruses. The open reading frame of the gene is 1539 nucleotides long, encoding a putative protein (EGT) of 513 amino acids with a $M_r$ of 58922. The 5' noncoding region contains three possible TATA boxes. A polyadenylation signal, AATAAA, was found downstream of the translation stop codon. A putative signal peptide of 16 residues was present at the N-terminus of the EGT. The BusuNPV *egt* gene has a high degree of nucleotide and amino acid sequence homology to multiple nucleocapsid (M) NPV *egt* genes, the highest being to the *Spodoptera exigua* MNPV *egt*. A phylogenetic tree of eleven known EGTs was constructed using maximum parsimony analysis.



The contents of this chapter have been published as: Hu, Z.H., R. Broer, J. Westerlaken, J.W.M. Martens, F. Jin, J.A. Jehle, L.M. Wang and J.M. Vlak. 1997. Characterization of the ecdysteroid UDP-glucosyltransferase gene of a single nucleocapsid nucleopolyhedrovirus of *Buzura suppressaria*. *Virus Research* 47, 91-97. The nucleotide sequence data reported in this chapter have been deposited in GenBank under the accession number U61154.

## INTRODUCTION

Insect baculoviruses are enveloped DNA viruses that are occluded into proteinaceous bodies (polyhedra) at the end of the viral replication cycle (Adams and McClintock, 1991). The Baculoviridae family is divided into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Murphy *et al.*, 1995). Several NPVs encode an ecdysteroid UDP-glucosyltransferase (EGT) enzyme which catalyzes the conjugation of sugars with ecdysteroids resulting in the inactivation of the hormone in infected larvae (O'Reilly, 1995; O'Reilly and Miller, 1989; O'Reilly *et al.*, 1992a). Active EGT prevents insect larvae from molting, which results in continued larval growth and increased yield of polyhedra. Infection of larvae by an *egt* null mutant of *Autographa californica* multiple nucleocapsid NPV (AcMNPV) results in increased speed of kill and reduced feeding (O'Reilly and Miller, 1991; Flipsen *et al.*, 1995). In effect, *egt* null mutants are enhanced viral insecticides. The strategy of deleting *egt* is now being adopted for the engineering of other baculoviruses of economically important insect pests.

So far, *egt* genes have been reported in multiple nucleocapsid (M) NPVs (O'Reilly, 1995; Clarke *et al.*, 1996) and recently in GVs (Smith and Goodale, 1996; Crook *et al.*, 1995). In this paper we report the identification, sequencing and comparative analysis of an *egt* gene of a single nucleocapsid (S) NPV i.e. of the tea moth *Buzura suppressaria* (Lepidoptera: Geometridae). This insect is a major pest of tea plantations in China and BusuNPV has been used as a successful biocontrol agent and a good alternative to chemical insecticides (Xie and Peng, 1980). So far, only limited characterization of BusuNPV has been achieved. The genome is approximately 130 kilobase pairs (kb) in size (Liu *et al.*, 1993) and the polyhedrin gene has been characterized (Hu *et al.*, 1993).

## MATERIALS AND METHODS

*Virus and DNA*

BusuNPV was propagated in *Buzura suppressaria* larvae and the occlusion bodies (OB) were purified by differential and rate zonal centrifugation. The DNA was isolated directly from purified OBs by using a dissolution buffer (0.1M $Na_2CO_3$, 0.01M EDTA and 0.17M NaCl), followed by proteinase K and SDS treatment, phenol/chloroform extraction and dialysis. The purity of the DNA was determined spectrophotometrically. The DNA was digested with various restriction enzymes and analyzed by agarose gel electrophoresis.

*Cloning and sequencing of DNA fragments*

Restriction enzymes digested BusuNPV DNA was shotgun-cloned into plasmid vector pTZ19R by using standard methods (Sambrook *et al.*, 1989). Southern blot hybridization was performed to confirm the authenticity of the cloned fragment as BusuNPV DNA. The termini of BusuNPV DNA inserts were sequenced using universal primers. The fragment showed partial putative *egt* sequence was hybridized to the library of BusuNPV DNA and a *Bgl*II fragment encompassing the entire *egt* gene was selected. Complete double stranded sequencing of the *egt* gene was achieved using 'primer walking' on the selcted *Bgl*II fragment.

*Computer analyses*

Sequences were analyzed with the aid of the UWGCG computer program. DNA and deduced amino acids sequences were compared with the updated GenBank/EMBL. Phylogeny analyses were performed with the PAUP 3.1 program (Swofford, 1993) using the PileUp program of the GCG program to produce input alignment of peptide sequences. GenDoc software was used for homology shading and scoring among the aligned sequences.

## RESULTS AND DISCUSSION

The putative BusuNPV *egt* gene was identified during random sequence analysis of a plasmid library of the viral genome. Part of the sequence was found in a 2.4 kb *Kpn*I fragment (*Kpn*I-H) of the viral DNA (Fig. 3.1) and showed a high degree of homology to other baculovirus *egt* genes. The *Kpn*I-H fragment is contained within a 6.2 kb fragment (*Bgl*II-D) which was fine-mapped and subcloned. The putative *egt* gene and its flanking regions were sequenced from both strands of subclones of *Bgl*II-D fragment by using universal and specific primers. The *egt* gene was located at map units (m.u.) 4.15 to 5.46 on the viral genome (Fig. 3.1).

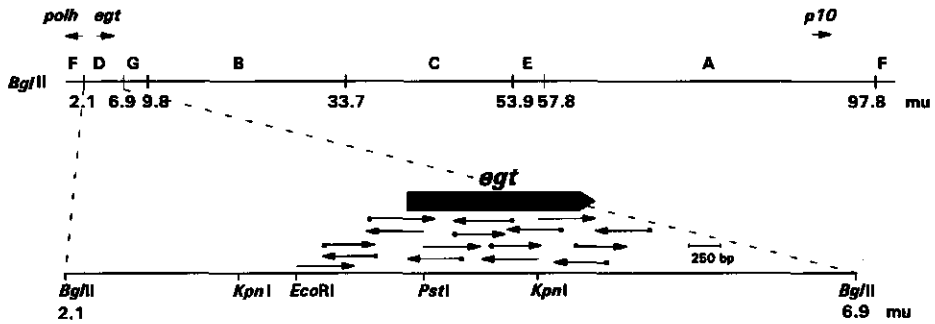


**Figure 3.1.** Location of the *egt* gene in the genome of BusuNPV. The gene is located in fragment *Bgl*II-D, which was mapped with respect to *Kpn*I, *Pst*I and *Eco*RI restriction enzyme sites. The position and direction of transcription of polyhedrin (*polh*), *p10* and *egt* genes are shown. For sequencing strategy, the arrows indicate the direction of sequencing and dots at the proximal end of the arrows indicate the use of specific primers.

The sequence data (Fig. 3.2) showed that the putative BusuSNPV *egt* gene has an open reading frame (ORF) of 1539 nucleotides (nt) encoding a protein of 513 amino acids (aa) with a predicted $M_r$ of 58922. The 5' noncoding region contains three possible TATA boxes located at 10, 77 and 135 nt upstream of the translational start codon. The TATA box is one of the core elements in baculovirus early promoters and is found functionally reiterated in some baculovirus early genes (Guarino and Smith, 1992). All identified baculovirus *egt* genes contain putative TATA boxes, and AcMNPV and *Lymantria dispar* (Ld) MNPV *egt* genes are transcribed beginning at 28 nt and 12 nt downstream of their TATA box, respectively (O'Reilly and Miller, 1990; Riegel *et al.*, 1994). No other early transcription motifs, such as a conserved cap site (CAGT) for mRNA transcription initiation, enhancer-like elements (search patterns: A(A/T)CGT(G/T); CGTGC; GATA; CACGTG), nor the TAAG motif for baculovirus late transcription, were found. Taken together, these founding suggest that the BusuNPV *egt* is likely to be an early gene transcribed by host

RNA polymerase II using one or more of the TATA boxes. There is a polyadenylation signal, AATAAA, 220 nt downstream of the translation termination codon TAA. BusuNPV EGT contained three potential *N*-linked glycosylation sites (Fig. 3.2) but it is not known if they are utilized.

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TGATACTGCTCTCGAAAAAATAACAATACAAGAATTAAATTACATTATAAATTAGAGGAAAAAAAAACGCGTTTAATGCAAATTAATTGA    90
CATTTGGTGACATATAAAAAGTGAGACGGCCCCAATGACCTCATTCAAGTGACCAACTTACTCGGGACTGTATCTGTTTCTATAAACAAC   180
ATGTATTTATTATAATAATTTTCTTGTGGTTAGCAAAACAATGTCACGGAGCAAAAATTTTGGCCGTAATGCCGACACCTTCATACAGCCAT   270
 M  Y  L  L  I  I  F  L  W  L  A  K  Q  C  H  G  A  K  I  L  A  V  M  P  T  P  S  Y  S  H
CAAAGCGTGTTCAAAGTGTATATTGAAAGTTTGGCGGAGCGCGGTCACACAATAGTGATCATCAAGCCGACTACTCGAGTGTTTTATGAT   360
 Q  S  V  F  K  V  Y  I  E  S  L  A  E  R  G  H  T  I  V  I  I  K  P  T  T  R  V  F  Y  D
GACCGATTCAGCGACAACATCACGGAAATTGACGCGACCATGTCGGAATTTTATTTTAGCGAATTATTTACAAATGCGTCGGTCTTTCGC   450
 D  R  F  S  D  N  I  T  E  I  D  A  T  M  S  E  F  Y  F  S  E  L  F  T  N  A  S  V  F  R
AAACGAGGAATAGTTGCCGATTCGAAAACGGTTACGTCTGCCAATTACCTTGGTTTGGTGCACATGATTAGTAACCAATTTAAACTGCCC   540
 K  R  G  I  V  A  D  S  K  T  V  T  S  A  N  Y  L  G  L  V  H  M  I  S  N  Q  F  K  L  P
                                PstI
GCCGTTAAACAACTGATTGAGCATCGCCATCGACTGCAGTTCGACGTGCTCATCACGGAAGCGTTTATTGATTATCCGTTGGTGTTTTCG   630
 A  V  K  Q  L  I  E  H  R  H  R  L  Q  F  D  V  L  I  T  E  A  F  I  D  Y  P  L  V  F  S
TATTTATTCGGCGATTTGCCTATCATTCAAATTTCCTCGGGCCACGGCGTGGCGGAGAATTTTGAAACAATGGGCGCGGTGAGCAGACAT   720
 Y  L  F  G  D  L  P  I  I  Q  I  S  S  G  H  G  V  A  E  N  F  E  T  M  G  A  V  S  R  H
CCACTTTACTATCCCAACATGTGGCGGGATCGTTTCACTAACCTCAACGTGTGGGAAACGATTAACGAAATTTACATGGAAATGCGTTTG   810
 P  L  Y  Y  P  N  M  W  R  D  R  F  T  N  L  N  V  W  E  T  I  N  E  I  Y  M  E  M  R  L
CAGAACGAGTTTAGCTTGCTGGCCGAAGAACAAAATAAACTGTTAGCGCATCAATTTGGCATTGGTGTACCAACCGTTCAAAAACTTCGC   900
 Q  N  E  F  S  L  L  A  E  E  Q  N  K  L  L  A  H  Q  F  G  I  G  V  P  T  V  Q  K  L  R
GACCGAGTCCAATTACTGTTGGTGAACACACACGCCGTGTTTGATAATAATCGCCCAGTGCCACCAAGCGTCCAATACATGGGTGGTTTA   990
 D  R  V  Q  L  L  L  V  N  T  H  A  V  F  D  N  N  R  P  V  P  P  S  V  Q  Y  M  G  G  L
CATTTGCACAAAAAAACCATAAAACCGATGAGTAACTATGTACAGAATTTTTTGGACAACTCGACGCGTGGCGTGGTGTACGTCAGTTTT  1080
 H  L  H  K  K  T  I  K  P  M  S  N  Y  V  Q  N  F  L  D  N  S  T  R  G  V  V  Y  V  S  F
GGATCGAGCATAACCTCCAAGAACATGGCGCCAGAATTTTTGAGCATGATTATTGAAACGTTTAAACTTGTACCCTACGATATAGCTTGG  1170
 G  S  S  I  T  S  K  N  M  A  P  E  F  L  S  M  I  I  E  T  F  K  L  V  P  Y  D  I  A  W
AAATTCGACATTGTGCCCGAAGTTAACAATTTGCCCGAAAATGTACTCATTCAAGATTGGTACGATCAATACAGCGTTTTGCATCACGTT  1260
 K  F  D  I  V  P  E  V  N  N  L  P  E  N  V  L  I  Q  D  W  Y  D  Q  Y  S  V  L  H  H  V
                                                        KpnI
AACGTTAAAGTTTTTGTTACTCAGGGTGGTGTGCAATCGACGGACGAGGCTATCGATGCTTTGGTACCTCTCGTTGGCGTACCCATGATG  1350
 N  V  K  V  F  V  T  Q  G  G  V  Q  S  T  D  E  A  I  D  A  L  V  P  L  V  G  V  P  M  M
GGAGATCAATTTTTCCACACAAACAAGTATGCGGAATTGAGCATCGGATGCGCAGTCGACACGCTCACCGTGAACAGTTTACAATTGATG  1440
 G  D  Q  F  F  H  T  N  K  Y  A  E  L  S  I  G  C  A  V  D  T  L  T  V  N  S  L  Q  L  M
AGAGCAATTGTCGATGCGGCCACGAGTGCCAAATATCGCAATGGATTGCGTCACCTTCGACAAATTATTAATCATCAACCTATGACGCCT  1530
 R  A  I  V  D  A  A  T  S  A  K  Y  R  N  G  L  R  H  L  R  Q  I  I  N  H  Q  P  M  T  P
TTACACAAGGCAATCTGGTATACGGAACACGTGATTCGCCACGGTAAAAACGATAATACATGGCTAAAAACAAAAGCGGCCAACGTTGGT  1620
 L  H  K  A  I  W  Y  T  E  H  V  I  R  H  G  K  N  D  N  T  W  L  K  T  K  A  A  N  V  G
TACAGTGATTACTTCATGATGTATATTTTATTTCCGCTAGTTTCGGTGACGGCCATGAATCAACTTCAGCAATTGATGCGCCTGACTTTT  1710
 Y  S  D  Y  F  M  M  Y  I  L  F  P  L  V  S  V  T  A  M  N  Q  L  Q  Q  L  M  R  L  T  F
TTTTCAATGTAAATTTATTGTTACTTTGTGACCTTGTTACATGAATTAAATTACACTTAAACATTTGCGTTTTACTTTTTTACTATTTTC  1800
 F  S  M  *
CGTAGGTAAATACTTATTTGCCAAAATTAGTCACGGCGCGCAGGTTATTACGCACGTCGTATAGTTATATACGAGTGTTGCTCTTTTTCA  1890
TTTCATTATAACGTCGAAACTGGCATGGTCAACATCATGTCTACTTTACGAAATAAATTATTGGTGAAAGACTTGCAGCGGTCGGCTAAC  1980
```

**Figure 3.2.** Nucleotide sequence of the BusuNPV *egt* gene and its flanking regions. The predicted amino acids are indicated by one-letter code designations below the nucleotide sequence. Putative transcription initiation and

termination signals are in bold face. The putative signal sequence is in bold italics and possible N-linked glycosylation sites (N-X-S/T) are underlined. The *PstI* and *KpnI* restriction enzyme sites are shown.

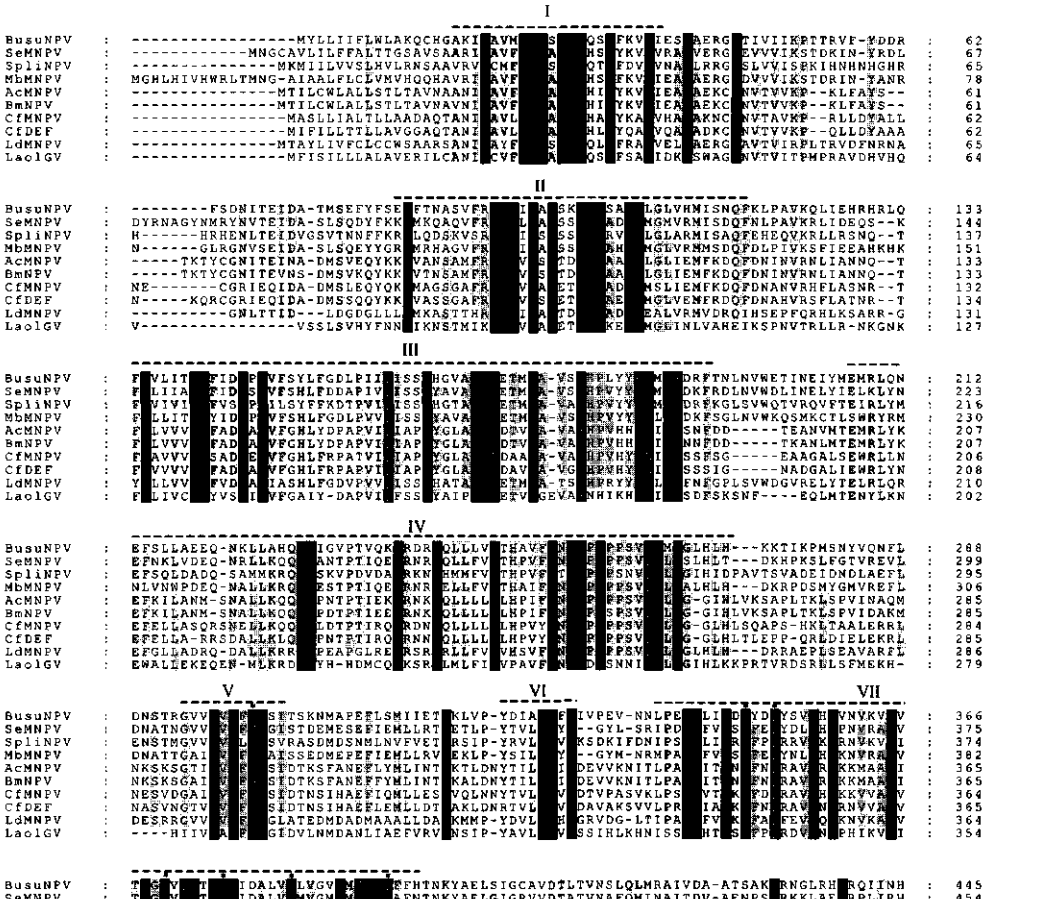



**Figure 3.3.** Alignment of the amino acid sequence of eleven baculovirus *egt* genes. The alignment was carried out by PileUp of the UWGCG computer program (release 8.0). GeneDoc software was used for homology shading. Two shading levels were set: black for 100% identity and grey for 70% identity. The conserved regions are indicated with Roman numerals (I-X). The sources of the sequences and the abbreviations of the viruses are given in the text. The seven absolutely conserved amino acids among all the UDP-glycosyltransferases we investigated are indicated by asterisks (*)

An *egt* gene has been reported from nine MNPVs and two GVs: AcMNPV (O'Reilly and Miller, 1990), *Bombyx mori* NPV (BmNPV; Genbank L33180), two NPVs from *Choristoneura fumiferana* (CfMNPV and CfDEF) (Barrett *et al.*, 1995), LdMNPV (Riegel *et al.* , 1994), *Spodoptera exigua* MNPV (SeMNPV; R.J.M. Mans, unpublished data), *S. littoralis* MNPV (SpliNPV; Faktor *et al.*, 1995) and *Mamestra brassicae* (Clarke *et al.*, 1996) and two GVs, *Lacanobia oleracea* GV (LaolGV; Smith and Goodale, 1996) and *Cydia pomonella* GV (CpGV; Crook *et al.*, 1995). The *Orgyia pseudotsugata* MNPV (OpMNPV) *egt* gene has been only partially sequenced (Pearson *et al.*, 1993). Comparison of the BusuNPV *egt* gene with MNPV *egt* genes indicated a nucleotide and amino acid sequence identity ranging from 45 to 59% and 39 to 57%, respectively. The BusuNPV *egt* gene is most closely related to that of SeMNPV with 59% nt identity and 57% aa identity. Like all identified EGTs, a putative signal peptide of 16 residues is present at the N-terminus of BusuNPV EGT (Fig. 3.2). Alignment of amino acid sequences shows that the baculovirus EGTs including that from BusuNPV display similarity throughout their sequences (Fig. 3.3). We have identified ten conserved regions (I-X) with an identity of at least 45% which is 10% higher than the lowest overall identity of 35% among EGTs (Fig. 3.3). Some of these domains have been reported before (Barrett *et al.*, 1995; Riegel *et al.*, 1994; O'Reilly, 1995). It is interesting to note that LaolGV lacks domain X (Smith and Goodale, 1996).

EGTs share sequence homology with UDP-glycosyltransferases from many species (O'Reilly, 1995). Alignment of EGTs with UDP-glycosyltransferases from mammalian, plant, nematode and bacterial origins (data not shown) indicated that only regions V and VII (Fig. 3.3) were conserved in all those investigated. Regardless of the enzyme origin, seven amino acids (BusuNPV EGT residues Gly301, Trp350, Gln353, Gly370, Glu376, Pro383 and Gln393) are absolutely conserved and all of these are located in either region V or region VII (Fig. 3.3). O'Reilly (1995) reported eight invariant amino acid residues among EGT/UDP-glycosyltransferases including a proline which also exists in BusuNPV EGT (Pro383).The latter is not conserved in a plant (UDP: indol-3-acetyl)-ß-D-glucosyl transferase (Szerszen *et al.*, 1994). EGTs also share homology with plant rhamnosyltransferases, however Pro383 is not conserved in the *Petunia hybrida* rhamnosyltransferase (Kroon *et al.*, 1994; Brugliera *et al.*,1994). O'Reilly (1995) suggested that the conserved amino acids are most likely critical for enzymatic activity and UDP-sugar binding. Among all the UDP-glycosyltransferases studied, baculovirus EGTs are most closely related to the mammalian enzymes. Regions VI, VIII, IX and X, and parts of regions I, III and IV are also conserved in the mammalian enzymes (data not shown). Conservation in region II and part of III and IV is restricted to baculovirus EGTs, suggesting that these areas are involved in processes specific for EGT (e.g. ecdysteroid recognition). The motifs identified in the gene and the sequence similarity of the protein to other EGTs indicate that the BusuNPV EGT is most likely functional during infection in *B. suppressaria* larvae. Transcriptional and enzymatic analysis should verify this point.

The phylogeny of eleven EGTs was analyzed with the aid of the PAUP 3.1 computer program (Swofford, 1993). The most parsimonious tree using LaolGV as an outgroup is shown in Fig. 3.4. Bootstrap analysis (100 replicates) of the data showed that the tree branches are well supported. Five baculovirus EGTs, from AcMNPV, BmNPV, CfMNPV, OpMNPV and CfDEF, consistently belonged to one group. Within this group two further subgroups could be distinguished. One

subgroup comprised EGTs from AcMNPV and BmNPV and the other constituted those from CfMNPV, OpMNPV and CfDEF. Results from bootstrap analysis indicated the inclusion of EGTs from BusuNPV, MbMNPV and SeMNPV into another group, but the support for this grouping was not as consistent as that for the group with AcMNPV. Using LaolGV as an outgroup SpliNPV EGT falls out as a separate group suggested that this EGT diverged considerably from the other NPVs. A better interpretation of this observation can be given when more NPVs and GV EGT sequences are available. Our results on the eleven EGTs further confirmed the earlier phylogenetic tree based on seven *egt* genes from MNPVs by Barrett *et al.* (1995). Except for SpliNPV the phylogeny derived from the baculovirus EGTs is similar to that based on polyhedrin genes of the same viruses (Zanotto *et al.*, 1993; Cowan *et al.*, 1994), suggesting that this phylogeny may reflect the natural evolution of the baculoviruses.

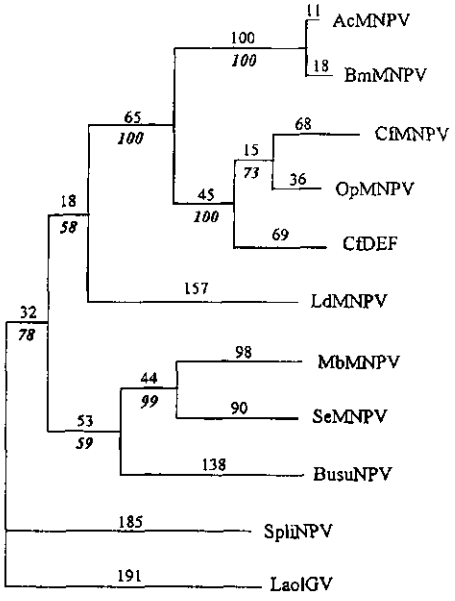



**Figure 3.4.** A phylogenetic tree of baculoviruses based on EGTs. The most parsimonious tree of the eleven baculovirus EGTs is shown. The tree was constructed by using the 'branch and bound' search program of PAUP 3.1 using LaolGV as an outgroup. For OpMNPV only the published N-terminal part was used. Numbers above the lines are the number of changes between the node and the species. Numbers below the line, in bold italics, indicate the frequency of that cluster after bootstrap analysis (100 replicates).

In summary, the BusuNPV *egt* is the first such gene to be discovered in SNPVs. The present findings clearly show that the BusuNPV *egt* has a high degree of similarity to MNPV *egt*s. The same motifs found in BusuNPV EGT as those found in MNPV EGTs suggests it is likely to be functional. Recently, *egt* genes have been reported from CpGV (Crook *et al.*, 1995) and LaolGV (Smith and Goodale, 1996). It is thus likely that *egt* genes from other SNPVs and GVs will be discovered. While BusuNPV is a promising biological agent for the control of *B. suppressaria* in tea plantations in China, deletion of its *egt* gene, and possibly introducing an exogenous gene deleterious to the pest, may further enhance the effectiveness of this virus (Bonning and Hammock, 1996).

**ACKNOWLEDGMENTS**

We are grateful to Basil M. Arif for critical review of the manuscript. We thank Douwe Zuidema and Ruud J.M. Mans for providing the SeMNPV *egt* sequence and Ian R.L. Smith for the LaoIGV *egt* sequence prior to publication. Special thanks to Elisabeth A. van Strien, Peter J. Krell and Douwe Zuidema for reviewing earlier versions of this manuscript. This research was supported by the National Natural Science Foundation of China (NSFC) grant 39370031. Z.H. Hu was a recipient of an EC Marie Curie fellowship and a PhD grant from the Wageningen Agricultural University.

CHAPTER 4

# GENETIC ORGANIZATION OF THE *HIND*III-J REGION OF THE SINGLE-NUCLEOCAPSID NUCLEOPOLYHEDROVIRUS OF *BUZURA SUPPRESSARIA*


## SUMMARY

In order to investigate the genomic organization of the single-nucleocapsid nucleopolyhedrovirus (SNPV) of *Buzura suppressaria* (BusuNPV), the *Hin*dIII-J fragment located at map units (mu) 26.6-29.4 of the viral genome was sequenced. The fragment contained two partial and three complete open reading frames (ORFs) representing the 3' end of a polyhedron envelope protein gene (*pep*), a homologue of the AcMNPV ORF117, a conotoxin-like protein gene (*ctl*), an inhibitor of apoptosis gene (*iap*) and a superoxide dismutase gene (*sod*), respectively. These five genes were identified for the first time in a SNPV. Sequence analysis further revealed that these ORFs have the same conserved motifs and gene structure as those observed in their homologues from other baculoviruses. Between *ctl* and *iap*, an intergenic region of about 700 basepairs with structure similar to non-*hr* origins of DNA replication was observed. The genomic arrangement of the ORFs in the BusuNPV *Hin*dIII-J fragment is very different from the arrangement of their homologues in the genome of *Autographa californica* multiple nucleocapsid (M) NPV and other baculoviruses to date. Our data suggest that on the basis of gene arrangement BusuNPV belongs to a distinct taxon within the Baculoviridae family, corroborating our previous conclusions derived from phylogeny analysis of several BusuNPV genes.



This chapter is a modified version of the paper: Hu, Z.H., B.M. Arif, J.S. Sun, X.W. Chen, D. Zuidema, R.W. Goldbach and J.M. Vlak. 1998. Genetic organization of the *Hin*dIII-I region of the single-nucleocapsid nucleopolyhedrovirus of *Buzura suppressaria*. *Virus Research* 55, 71-82. The nucleotide sequence data reported in this chapter have been deposited in GenBank under the accession number AF045936.

## INTRODUCTION

The Baculoviridae, a diverse family of more than 600 viruses including the nucleopolyhedroviruses (NPV) and granuloviruses (GV), are pathogenic for invertebrates, particularly insects of the order Lepidoptera (Murphy *et al.*, 1995). Baculoviruses have a circular, double stranded DNA genome, ranging in size between 90 and 160 kilobasepairs (kb). The complete genome of the baculovirus type species, *Autographa californica* multiple nucleocapsid NPV (AcMNPV), was sequenced and the size determined to be 134 kb, potentially encoding about 150 proteins (Ayres *et al.*, 1994). Recently, the nucleotide sequence of two other NPV genomes, that of *Bombyx mori* (BmNPV, S. Maeda, Genbank accession number L33180) and of *Orgyia pseudotsugata* (OpMNPV; Ahrens *et al.*, 1997) has been determined. Comparison of the OpMNPV and AcMNPV genomes indicates that, even though there are two inversions and three major insertion (or deletions), basically the two genomes have a similar gene organization (Ahrens *et al.*, 1997). As more genomic sequences become available from other baculoviruses, the phylogenetic relationships and the biodiversity of these viruses will be better understood. In this respect single-nucleocapsid (S) NPVs and granuloviruses are of considerable interest as only limited DNA sequence information is available from these viruses.

In this paper, we present the sequence analysis of a 3.2 kb region from the genome of the SNPV of *Buzura suppressaria* (BusuNPV) and make a comparison to the location of their homologues in the genome of AcMNPV. BusuNPV was originally isolated from a major pest, *Buzura suppressaria* Guenee (Lepidoptera: Geometridae), of tea plantations in China and the virus has since been used to control this pest (Xie *et al.*, 1979). To date, about 63 NPVs have been described from the Geometridae family (Adams and McClintock, 1991), but molecular characterization of these baculoviruses have been limited. Our previous studies on the polyhedrin (Hu *et al.*, 1993) and the ecdysteroid UDP-glucosyltransferase gene (*egt*) of BusuNPV (Hu *et al.*, 1997) indicated that this virus belongs to a separate phylogenetic group, distinct from that encompassing AcMNPV, BmNPV and OpMNPV. In order to further investigate the genomic organization and phylogenetic status of BusuNPV, the viral DNA was cloned as restriction fragments into a plasmid library and the inserts were terminally or totally sequenced. In this paper, we present the analysis of the *Hin*dIII-J region (3.2 kbp) located at map unit (mu) 26.6-29.4 of the 121 kb genome of BusuNPV.

### Materials and Methods

*Virus and DNA*

BusuNPV was propagated in *Buzura suppressaria* larvae and the occlusion bodies (OB) were purified by differential and rate zonal centrifugation. The DNA was isolated directly from purified OBs by using a dissolution buffer (0.1M $Na_2CO_3$, 0.01M EDTA and 0.17M NaCl), followed by proteinase K and SDS treatment, phenol/chloroform extraction and dialysis. The purity of the DNA was determined spectrophotometrically. The DNA was digested with restriction enzyme *Hin*dIII and analyzed by agarose gel electrophoresis.

*Cloning and sequencing of DNA fragments.*

The *Hin*dIII-J fragment of BusuNPV DNA was cloned into plasmid vector pTZ19R by using standard methods (Sambrook *et al.*, 1989). Restriction enzyme analysis and Southern blot hybridization were performed to confirm the authenticity and the location of the cloned fragment on the physical map of BusuNPV genome. Complete sequencing of both strands of the cloned fragment was performed using 'primer walking' at the Core Facility of Queens University (Kingston, Ontario, Canada).

*Computer analyses*

Sequences were analyzed with the aid of the UWGCG computer program (release 9.0); DNA and deduced amino acids sequences were compared with the updated GenBank/EMBL, SWISSPORT and PIR databases using FASTA and BLAST. Phylogeny analyses were performed with PAUP 3.1 program (Swofford, 1993) using the PileUp program of GCG to produce input alignment of peptide sequences. GeneDoc software was used for homology shading and scoring among the aligned sequences.

**Results and Discussion**

Restriction enzyme analysis and Southern blot hybridization confirmed that the cloned *Hin*dIII-J fragment was located at mu 26.6-29.4 of the BusuNPV genome. The nucleotide sequence, compiled from both strands, showed that the fragment contains 3248 bp. Searches of databases identified at least five open reading frames (ORFs) within *Hin*dIII-J. These ORFs encode the C-terminal part of a polyhedron envelope protein (PEP), a homologue of the AcMNPV ORF117, a conotoxin-like protein (CTL), an inhibitor of apoptosis (IAP), and part of a superoxide dismutase (SOD) respectively (Fig. 4.1). The nucleotide and the deduced amino acid sequence of the five ORFs together with putative transcription signals are presented in Fig. 4.2.

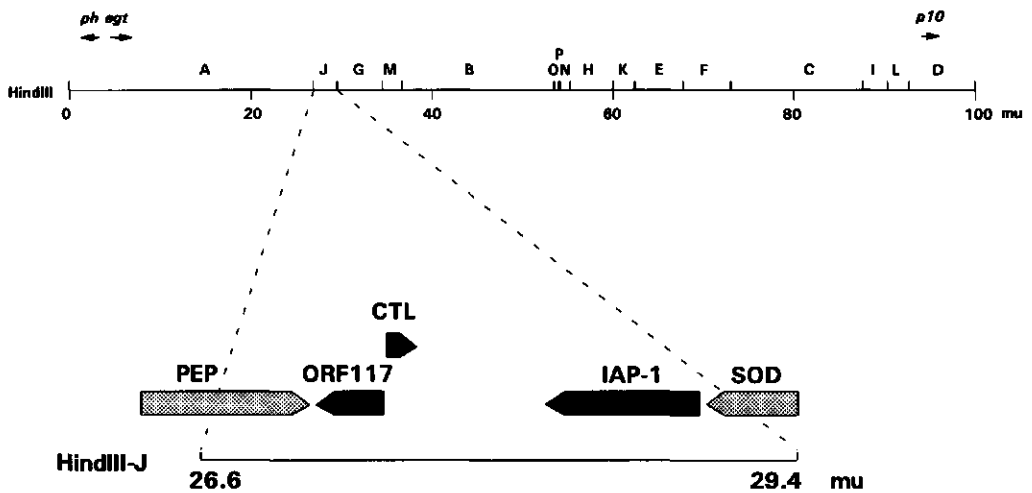
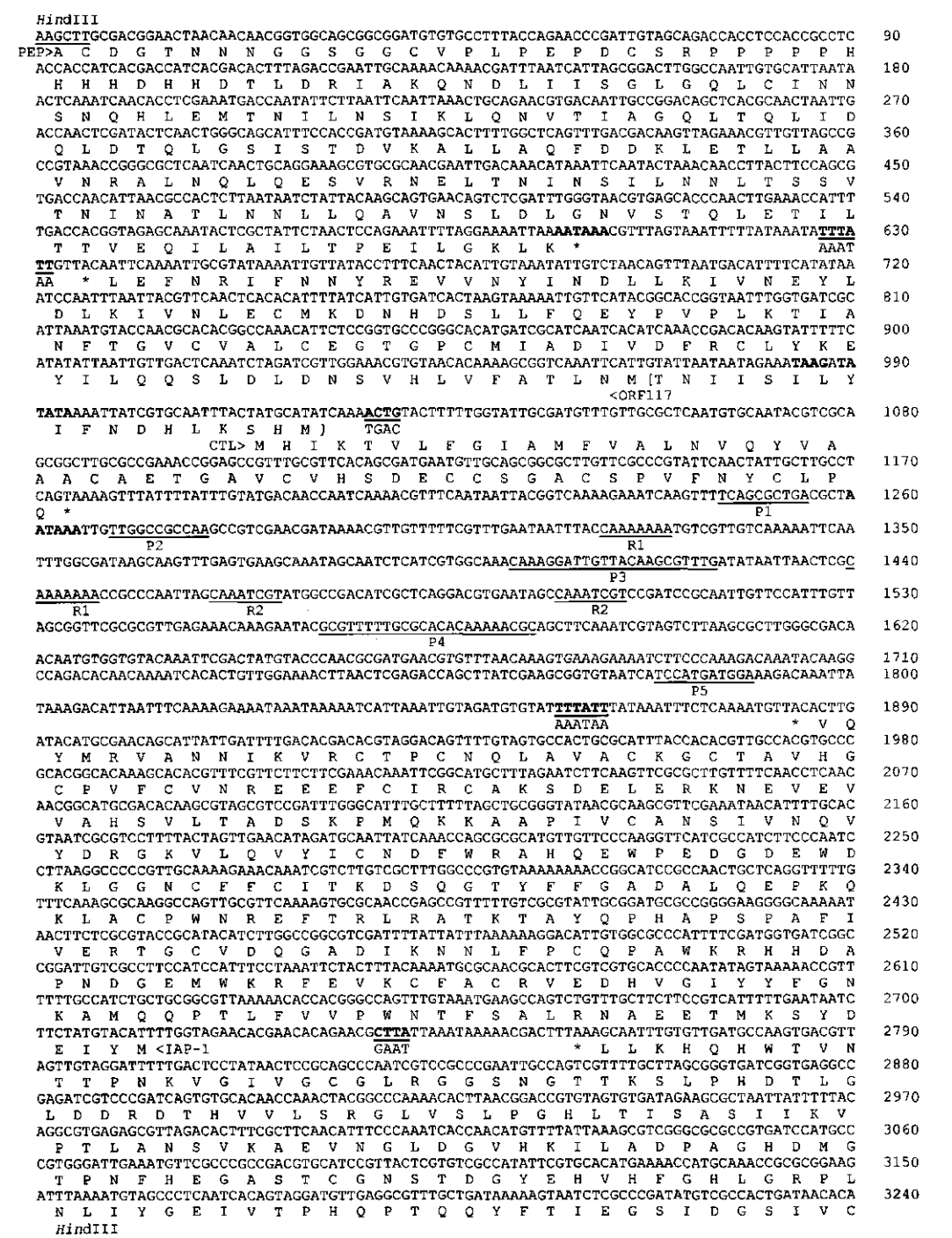



**Figure 4.1.** *Hin*dIII physical map of the BusuNPV genome and detailed gene organization of the *Hin*dIII-J fragment. The arrows represent the ORFs and point to their directions of transcription. *pep* and *sod* have been only partially sequenced (arrows shaded grey).


HindIII
AAGCTTGCGACGGAACTAACAACAACGGTGGCAGCGGCGGATGTGTGCCTTTACCAGAACCCGATTGTAGCAGACCACCTCCACCGCCTC 90
PEP>A C D G T N N N G G S G G C V P L P E P D C S R P P P P P H
ACCACCATCACGACCATCACGACACTTTAGACCGAATTGCAAAACAAAACGATTTAATCATTAGCGGACTTGGCCAATTGTGCATTAATA 180
H H H D H H D T L D R I A K Q N D L I I S G L G Q L C I N N
ACTCAAATCAACACCTCGAAATGACCAATATTCTTAATTCAATTAAACTGCAGAACGTGACAATTGCCGGACAGCTCACGCAACTAATTG 270
S N Q H L E M T N I L N S I K L Q N V T I A G Q L T Q L I D
ACCAACTCGATACTCAACTGGGCAGCATTTCCACCGATGTAAAAGCACTTTTGGCTCAGTTTGACGACAAGTTAGAAACGTTGTTAGCCG 360
Q L D T Q L G S I S T D V K A L L A Q F D D K L E T L L A A
CCGTAAACCGGGCGCTCAATCAACTGCAGGAAAGCGTGCGCAACGAATTGACAAACATAAATTCAATACTAAACAACCTTACTTCCAGCG 450
V N R A L N Q L Q E S V R N E L T N I N S I L N N L T S S V
TGACCAACATTAACGCCACTCTTAATAATCTATTACAAGCAGTGAACAGTCTCGATTTGGGTAACGTGAGCACCCAACTTGAAACCATTT 540
T N I N A T L N N L L Q A V N S L D L G N V S T Q L E T I L
TGACCACGGTAGAGCAAATACTCGCTATTCTAACTCCAGAAATTTTAGGAAAATTAAAATAAACGTTTAGTAAATTTTTATAAATATTTA 630
T T V E Q I L A I L T P E I L G K L K * AAAT
TTGTTACAATTCAAAATTGCGTATAAAATTGTTATACCTTTCAACTACATTGTAAATATTGTCTAACAGTTTAATGACATTTTCATATAA 720
AA * L E F N R I F N N Y R E V V N Y I N D L L K I V N E Y L
ATCCAATTTAATTACGTTCAACTCACACATTTTATCATTGTGATCACTAAGTAAAAATTGTTCATACGGCACCGGTAATTTGGTGATCGC 810
D L K I V N L E C M K D N H D S L L F Q E Y P V P L K T I A
ATTAAATGTACCAACGCACACGGCCAAACATTCTCCGGTGCCCGGGCACATGATCGCATCAATCACATCAAACCGACACAAGTATTTTTC 900
N F T G V C V A L C E G T G P C M I A D I V D F R C L Y K E
ATATATTAATTGTTGACTCAAATCTAGATCGTTGGAAACGTGTAACACAAAAGCGGTCAAATTCATTGTATTAATAATAGAAATAAGATA 990
Y I L Q Q S L D L D N S V H L V F A T L N M [T N I I S I L Y
<ORF117
TATAAAATTATCGTGCAATTTACTATGCATATCAAAACTGTACTTTTTGGTATTGCGATGTTTGTTGCGCTCAATGTGCAATACGTCGCA 1080
I F N D H L K S H M ) TGAC
CTL> M H I K T V L F G I A M F V A L N V Q Y V A
GCGGCTTGCGCCGAAACCGGAGCCGTTTGCGTTCACAGCGATGAATGTTGCAGCGGCGCTTGTTCGCCCGTATTCAACTATTGCTTGCCT 1170
A A C A E T G A V C V H S D E C C S G A C S P V F N Y C L P
CAGTAAAAGTTTATTTTATTTGTATGACAACCAATCAAAACGTTTCAATAATTACGGTCAAAAGAAATCAAGTTTTCAGCGCTGACGCTA 1260
Q *
ATAAATTGTTGGCCGCCAAGCCGTCGAACGATAAAACGTTGTTTTTCGTTTGAATAATTTACCAAAAAAATGTCGTTGTCAAAAATTCAA 1350
P2 R1
TTTGGCGATAAGCAAGTTTGAGTGAAGCAAATAGCAATCTCATCGTGGCAAACAAAGGATTGTTACAAGCGTTTGATATAATTAACTCGC 1440
P3
AAAAAAACCGCCCAATTAGCAAATCGTATGGCCGACATCGCTCAGGACGTGAATAGCCAAATCGTCCGATCCGCAATTGTTCCATTTGTT 1530
R1 R2 R2
AGCGGTTCGCGCGTTGAGAAACAAAGAATACGCGTTTTTGCGCACACAAAAACGCAGCTTCAAATCGTAGTCTTAAGCGCTTGGGCGACA 1620
P4
ACAATGTGGTGTACAAATTCGACTATGTACCCAACGCGATGAACGTGTTTAACAAAGTGAAAGAAAATCTTCCCAAAGACAAATACAAGG 1710
CCAGACACAACAAAATCACACTGTTGGAAAACTTAACTCGAGACCAGCTTATCGAAGCGGTGTAATCATCCATGATGGAAAGACAAATTA 1800
P5
TAAAGACATTAATTTCAAAAGAAAATAAATAAAAATCATTAAATTGTAGATGTGTATTTTATTTATAAATTTCTCAAAATGTTACACTTG 1890
AAATAA * V Q
ATACATGCGAACAGCATTATTGATTTTGACACGACACGTAGGACAGTTTTGTAGTGCCACTGCGGCATTTACCACACGTTGCCACGTGCCC 1980
Y M R V A N N I K V R C T P C N Q L A V A C K G C T A V H G
GCACGGCACAAAGCACACGTTTCGTTCTTCTTCGAAACAAATTCGGCATGCTTTAGAATCTTCAAGTTCGCGCTTGTTTTCAACCTCAAC 2070
C P V F C V N R E E E F C I R C A K S D E L E R K N E V E V
AACGGCATGCGACACAAGCGTAGCGTCCGATTTGGGCATTTGCTTTTTAGCTGCGGGTATAACGCAAGCGTTCGAAATAACATTTTGCAC 2160
V A H S V L T A D S K F M Q K K A A P I V C A N S I V N Q V
GTAATCGCGTCCTTTTACTAGTTGAACATAGATGCAATTATCAAACCAGCGCGCATGTTGTTCCCAAGGTTCATCGCCATCTTCCCAATC 2250
Y D R G K V L Q V Y I C N D F W R A H Q E W P E D G D E W D
CTTAAGGCCCCCGTTGCAAAAGAAACAAATCGTCTTGTCGCTTTGGCCCGTGTAAAAAAAACCGGCATCCGCCAACTGCTCAGGTTTTTG 2340
K L G G N C F F C I T K D S Q G T Y F F G A D A L Q E P K Q
TTTCAAAGCGCAAGGCCAGTTGCGTTCAAAAGTGCGCAACCGAGCCGTTTTTGTCGCGTATTGCGGATGCGCCGGGGAAGGGGCAAAAAT 2430
K L A C P W N R E F T R L R A T K T A Y Q P H A P S P A F I
AACTTCTCGCGTACCGCATACATCTTGGCCGGCGTCGATTTTATTATTTAAAAAAGGACATTGTGGCGCCCATTTTCGATGGTGATCGGC 2520
V E R T G C V D Q G A D I K N N L F P C Q P A W K R H H D A
CGGATTGTCGCCTTCCATCCATTTCCTAAATTCTACTTTACAAAATGCGCAACGCACTTCGTCGTGCACCCCAATATAGTAAAAACCGTT 2610
P N D G E M W K R F E V K C F A C R V E D H V G I Y Y F G N
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K A M Q Q P T L F V V P W N T F S A L R N A E E T M K S Y D
TTCTATGTACATTTTGGTAGAACACGAACACAGAACGCTTATTAAATAAAAACGACTTTAAAGCAATTTGTGTTGATGCCAAGTGACGTT 2790
E I Y M <IAP-1 GAAT * L L K H Q H W T V N
AGTTGTAGGATTTTTGACTCCTATAACTCCGCAGCCCAATCGTCCGCCCGAATTGCCAGTCGTTTTGCTTAGCGGGTGATCGGTGAGGCC 2880
T T P N K V G I V G C G L R G G S N G T T K S L P H D T L G
GAGATCGTCCCGATCAGTGTGCACAACCAAACTACGGCCCAAAACACTTAACGGACCGTGTAGTGTGATAGAAGCGCTAATTATTTTTAC 2970
L D D R D T H V V L S R G L V S L P G H L T I S A S I I K V
AGGCGTGAGAGCGTTAGACACTTTCGCTTCAACATTTCCCAAATCACCAACATGTTTTATTAAAGCGTCGGGCGCGCCGTGATCCATGCC 3060
P T L A N S V K A E V N G L D G V H K I L A D P A G H D M G
CGTGGGATTGAAATGTTCGCCCGCCGACGTGCATCCGTTACTCGTGTCGCCATATTCGTGCACATGAAAACCATGCAAACCGCGCGGAAG 3150
T P N F H E G A S T C G N S T D G Y E H V H F G H L G R P L
ATTTAAAATGTAGCCCTCAATCACAGTAGGATGTTGAGGCGTTTGCTGATAAAAAGTAATCTCGCCCGATATGTCGCCACTGATAACACA 3240
N L I Y G E I V T P H Q P T Q Q Y F T I E G S I D G S I V C
HindIII
TAAAGCTT 3248
L A <SOD

**Figure 4.2.** Nucleotide sequence of the *Hin*dIII-J fragment. The predicted amino acids are indicated by one-letter code designations below the nucleotide sequence. The genes, their direction of transcription and the *Hin*dIII restriction enzyme sites are indicated. Putative transcription signals such as the TATA and CAGT motifs for early transcription initiation; TAAG for late baculovirus transcription initiation and AATAAA for polyadenylation are bolded. Signals on the complementary strand of the DNA are underlined and their complementary sequences are shown. The palindromes (P1-5) and direct repeats (R1-2) found in the intergenic region are underlined.

*Polyhedron Envelope Protein, PEP*

At the left hand end of the *Hin*dIII-J fragment is a partial ORF, homologous to the 3' region of the baculovirus gene encoding the polyhedral envelope protein (PEP). The polyhedron envelope or calyx is an electron-dense and carbohydrate rich structure that surrounds the polyhedra (Minion *et al.*, 1979). It was identified that PEP is an integral component of the calyx (Gombart *et al.*, 1989; Lent *et al.*, 1990; Russell and Rohrmann, 1990; Whitt and Manning, 1988; Zuidema *et al.*, 1989). PEP has also been demonstrated to be a component of the electron-dense spacers found in baculovirus infected cells. It has been suggested that the protein may be associated with the carbohydrate residues of calyx via a thiol-glycosidic linkage through cysteine residues (Whitt and Manning, 1988; Gombart *et al.*, 1989).

So far, *pep* has been sequenced from five baculoviruses; AcMNPV (Oellig *et al.*, 1987), OpMNPV (Gombart *et al.*, 1989), BmNPV (S. Maeda, Genbank L33180), *Lymantria dispar* MNPV (LdMNPV) (Bjornson and Rohrmann, 1992) and *Choristoneura fumiferana* MNPV (CfMNPV) (A. Poloumienko and P.J. Krell, Genbank U53854). The *pep* sequence from the genome of BusuNPV represents the first for a SNPV. Alignment of the PEPs of baculoviruses (Fig. 4.3) shows that almost all the cysteine residues are clustered in a certain region of the sequence suggesting that this region might be important for the thiol-linkages. This also implies that the secondary structures of these proteins are similar. There was evidence indicating that PEP interacts with P10 fibrillar structures and that this interaction becomes more extensive when cells are infected with polyhedrin null mutants instead of wild-type virus (Russell *et al.*, 1991; Lee, *et al.*, 1996). The process of how calyx forms and the roles of PEP, P10 and polyhedrin in this process are still not clear, yet the overall conservation of the primary structure of baculovirus PEPs, and the clustering of the cysteine residues suggest that the structural conservation is important for PEP function.

*ORF117*

An ORF homologous to the AcMNPV ORF117 was located 31 nucleotides downstream and in the opposite orientation of *pep* (Fig. 4.1). Homologues of this ORF also exist in the genomes of OpMNPV and BmNPV (Ahrens *et al.*, 1997; S. Maeda, Genbank L33180). In the BusuNPV ORF117, there are two potential ATG start sites, one of which overlaps the ORF of *ctl* (Fig. 4.2). Such overlap is not very common for baculovirus gene organization. Also, alignment of the amino acid sequences predicted from ORF117s of other baculoviruses (Fig. 4.4a), suggests that the second ATG is the actual translation start codon. The function of baculovirus ORF117 is not known and no studies to date have been conducted for example to attempt to delete this ORF from the genome. However, the high conservation of the 14 N-terminal amino acids may imply that this region is important for the function of ORF117 proteins.

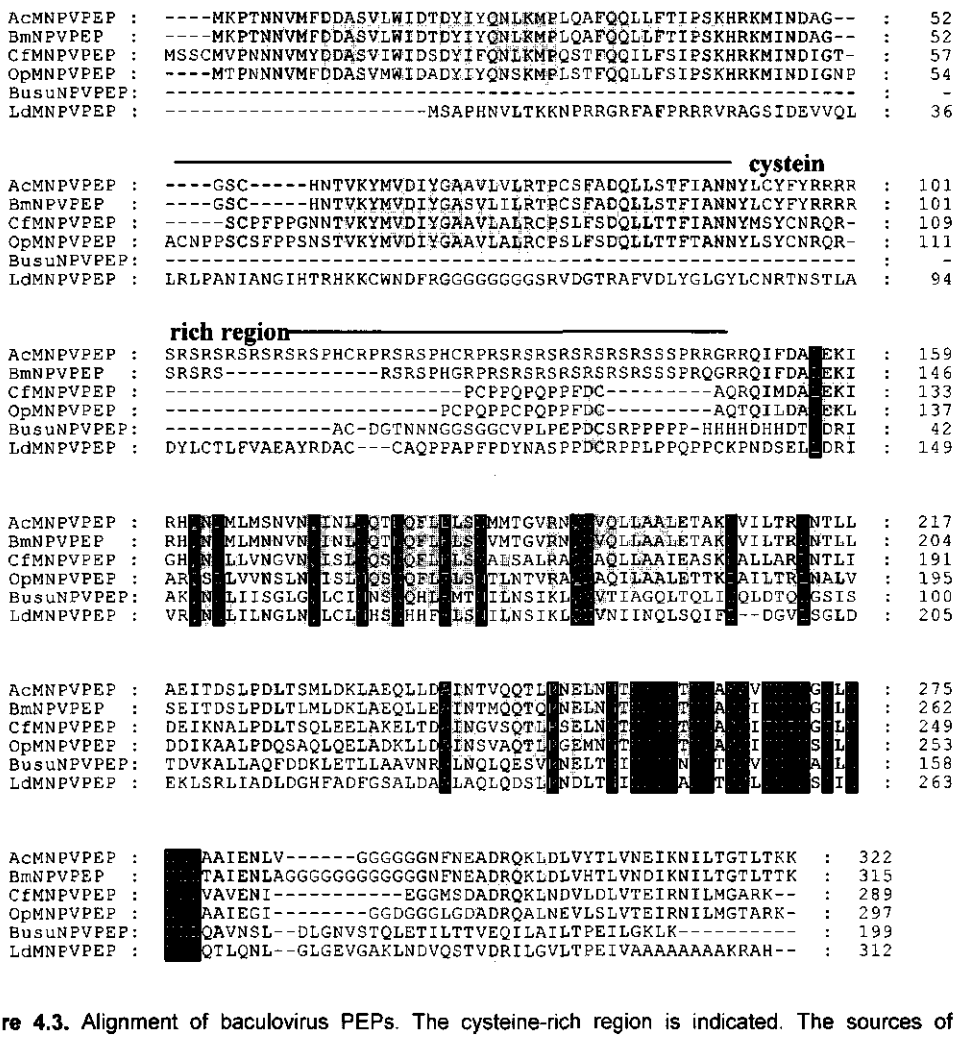


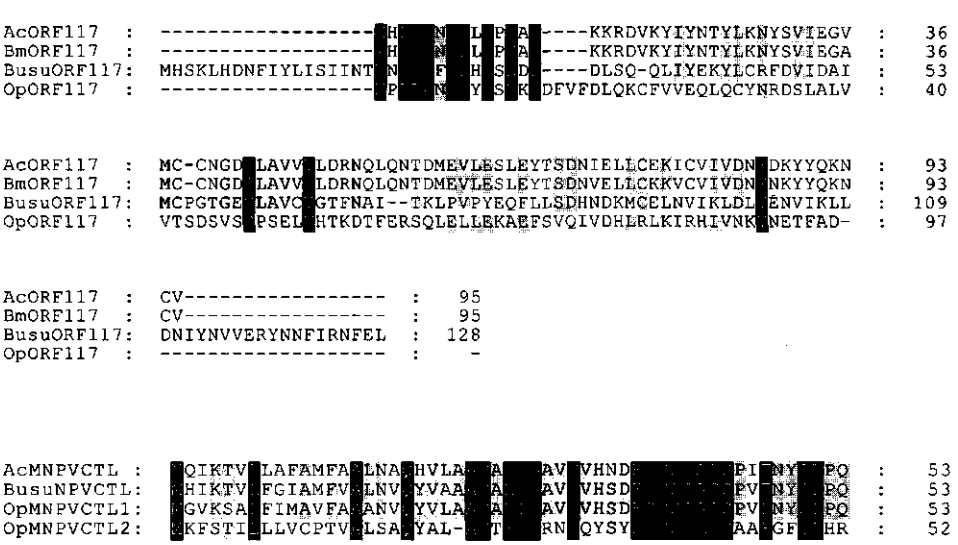

**Figure 4.3.** Alignment of baculovirus PEPs. The cysteine-rich region is indicated. The sources of the sequence are: Oellig *et al.* (1987) for AcMNPV; Poloumienko and Krell (Genbank U53854) for CfMNPV, Gombart *et al.* (1989) for OpMNPV; Maeda (Genbank L33180) for BmNPV, and Bjornson and Rohrmann (1992) for LdMNPV. Genedoc software was used for homology shading. Two shading levels were set: black for 100% identity and grey for 60% identity.

*Conotoxin-like protein, CTL*

The start of the gene encoding the conotoxin-like protein of BusuNPV is located 48 nt upstream of the ATG of ORF117 (Fig. 4.2). Conotoxins are small disulfide-rich ion channel antagonists isolated from the genus *Conus* (Olivera *et al.*, 1994). A single *ctl* is present in the genome of AcMNPV, whereas two (*ctl*-1 and *ctl*-2) are found in the genome of OpMNPV (Ahrens *et al.*, 1997). No *ctl* was found in the nucleotide sequence of the genome of BmNPV deposited in Genbank. The function of *ctl* in baculovirus infection is not understood. When

neonate or late instar *Spodoptera frugiperda* larvae were infected with an AcMNPV *ctl*-1 deletion mutant, no differences in mortality, motility, or weight gain were observed in comparison to larvae infected with wild-type virus (Eldridge *et al.*, 1992). Sequence alignment (Fig. 4.4b) indicates that the CTL of BusuNPV is most similar to that of AcMNPV CTL and OpMNPV CTL-1, whereas OpMNPV CTL-2 appears to be distinct suggesting that the latter belongs to a lineage different from the other CTLs.

(a)




(b)




**Figure 4.4.** Alignment of putative baculovirus ORF117s (**A**) and CTLs (**B**). The sources of the sequence are: Ahrens *et al.* (1997) for OpMNPV, Maeda (Genbank L33180) for BmNPV, Ayres *et al.* (1994) for AcMNPV ORF117 and Eldridge *et al.* (1992) for AcMNPV CTL. Black is 100% identity and grey 75% identity.

*Intergenic region*

Between *ctl* and the next ORF (*iap*) on the *Hin*dIII-J fragment of BusuNPV an intergenic region of about 700 bp is observed (Fig. 4.2). Several small ORFs, potentially capable to encode proteins of 63-69 aa, were found in this 700 bp area. However, these ORFs overlap with each other and none of these showed significant homology to any baculovirus or other DNA sequences in databases. Therefore, they were not included as ORFs in Fig.4.2

The intergenic region contains five perfect and imperfect palindromes (P1-5, Fig. 4.2) varying in size between 10 and 24 nt, and several direct repeats (R1-2). This cluster of palindromes and direct repeats is the common structure of non-*hr* origins of DNA replication found in AcMPNV (Kool *et al.*, 1994), OpMNPV (Pearson *et al.*, 1993) and *Spodoptera exigua* MNPV (SeMNPV) (Heldens *et al.*, 1997). Since a cell line permissive for BusuNPV is not available, it will not be possible at the present to test if this intergenic region serves as an origin of DNA replication in transient replication assays.

*Inhibitor of apoptosis, IAP*

An ORF homologous to the inhibitor of apoptosis (*iap*) genes was found about 700 bp downstream of the *ctl* in the genome of BusuNPV. Baculoviruses possess two different classes of genes with anti-apoptotic activity: *p35* and *iap* (see review by Clem *et al.*, 1996). So far, a single copy of *p35* has been found in the genome of AcMNPV (Clem *et al.*, 1991) and BmNPV (Kamita *et al.*, 1993), but none in the OpMNPV genome (Ahrens *et al.*, 1997). *Iaps* have been reported from *Cydia pomonella* granulosis virus (CpGV) (Crook *et al.*, 1993), OpMNPV (Birnbaum *et al.*, 1994; Ahrens *et al.*, 1997), AcMNPV(Birnbaum *et al.*, 1994; Ayres *et al.*, 1994) and BmNPV(S. Maeda, Genbank L33180). In contrast to the single copy of *p35*, *iaps* seem to belong to a gene family in baculovirus genomes. Both AcMNPV and BmNPV possess two *iap* homologues (*iap*-1 and *iap*-2), whereas OpMNPV has four *iap* homologues, *iap*1-4. The *iap*-4 of OpMNPV is a truncated sequence. Apart from *iap* reported here *(iap-1)*, two other *iap*-like ORFs were found in the BusuNPV genome in locations other than the *Hin*dIII-J fragment (Z.H. Hu, unpublished data). Although *iap*-1 of OpMNPV and the *iap* of CpGV were able to block apoptosis induced by an AcMNPV *p35-null* mutant in *S. frugiperda* cells, *iap*-1 of AcMNPV was non-functional in the same system (Crook *et al.*, 1993). It is, therefore, not clear why baculoviruses contain multiple *iap*-like copies in their genomes. It is conceivable that they are involved in a hitherto unknown process in the viral life cycle or that they have become redundant during the baculovirus evolution as evidenced by the truncated version of OpMNPV *iap*-4. Alternatively, they may be functional in other cell types, tissues or host insects.

Alignment of the sequences of known IAPs (Fig. 4.5a) indicates that the IAP of BusuNPV has similar structural features as other IAPs. For example, it contains the typical zinc finger motif $C_3HC_4$ (Freemont *et al.*, 1991) at the C-terminus. The baculovirus *iap* repeat (BIR, Birnbaum *et al.*, 1994) found in certain IAPs is also present at the N-terminus and near the centre of the IAP of BusuNPV. The alignment of the amino acid sequences was used to construct a phylogenetic tree of IAPs with the aid of PAUP 3.1 program. Exhaustive search resulted in a single most parsimonious tree (Fig. 4.5b) and showed that the baculovirus *iaps* are grouped into three classes. Class I contains *iap*-1 of AcMNPV, BmNPV and OpMNPV. Class II contains *iap*-2 of AcMNPV, BmNPV and OpMNPV. Class III contains *iap*-3 of OpMNPV, *iap* of CpGV and *iap*-1 of BusuNPV. Bootstrap analysis (100 replicates) of the data showed that the grouping of three classes is well supported. The grouping of *iap*-1 of BusuNPV together with the other two biologically active *iap* versions of CpGV and OpMNPV, suggests that the BusuNPV *iap*-1 might be able to complement *p35* in AcMNPV system. This, however, needs to be confirmed by further experiments. The subgrouping of *iap*-1 of BusuNPV and *iap* of CpGV within Class III is poorly supported by bootstrap analysis and therefore should not be considered as biologically significant.

*Superoxide dismutase, SOD*

The final gene found in the *Hin*dIII-J fragment is a Cu/Zn SOD like gene. This gene was initially found in the genome of AcMNPV (Tomalski *et al.*, 1991), but has now been shown to exist also in the genomes of OpMNPV and BmNPV. The amino acid sequences from the different baculoviruses appear to be highly conserved (Fig. 4.6). For example, the Cu-binding residues

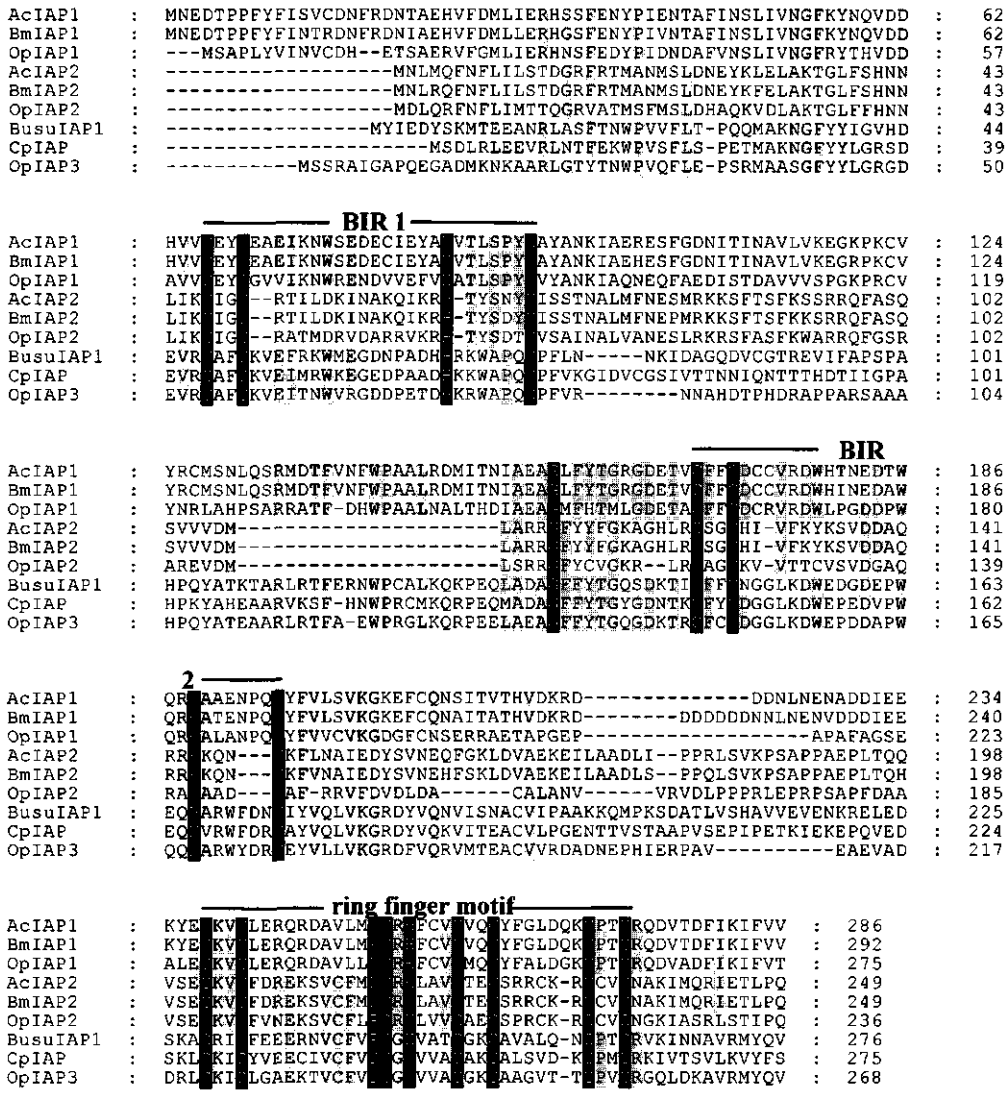



**Figure 4.5.** Alignment (**A**) and phylogeny (**B**) of baculovirus IAPs. The location and conservation of the zinc finger motif ($CX_2CX_{10}PCXHX_3CX_2CX_{6\text{-}7}CX_2C$) and the BIR motif ($CX_2CX_{14\text{-}16}HX_{3\text{-}6}C$) are shown in A, where X indicates any amino acid residue. The sources of the sequence are: Ayres *et al.* (1994) for AcMNPV; Maeda (Genbank L33180) for BmNPV; Ahrens *et al.* (1997) for OpMNPV and Crook *et al.* (1993) for CpGV. Black reflects 100% identity and grey 60% identity. The most parsimonious tree constructed using the 'exhaustive' search program of PAUP 3.1 is shown in B. Figures above the lines are the number of changes between the node and the species and those below the line, in bold italics, indicate the frequency of the cluster after bootstrap analysis (100 replicates). Three groups of baculovirus IAPs are indicated in the tree.

(b)

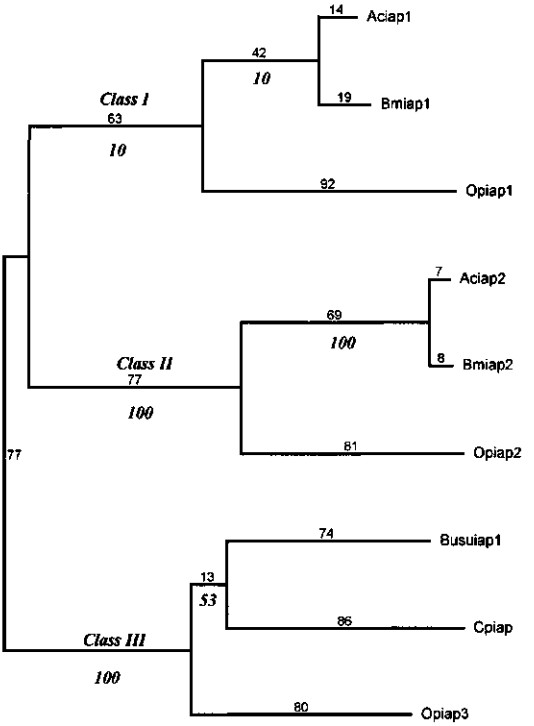



**Figure 4.5b.** (*Continued*)

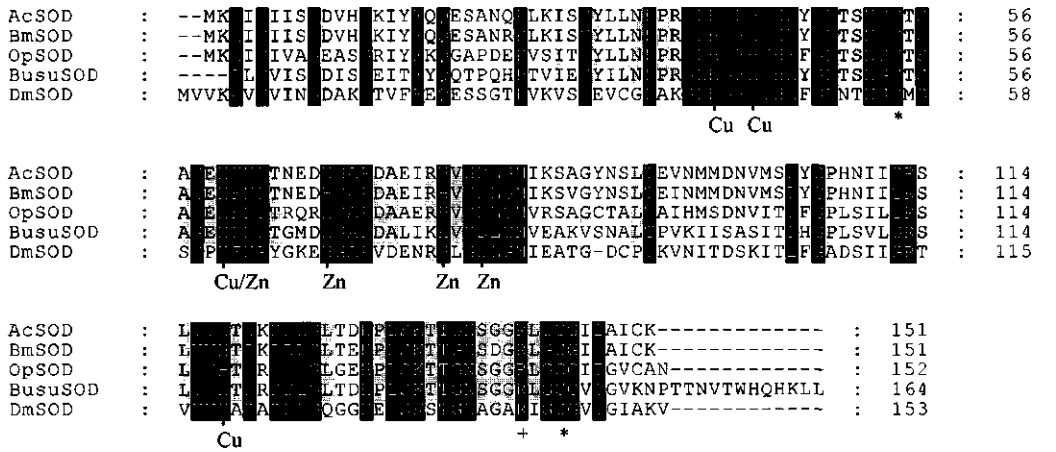



**Figure 4.6.** Alignment of baculovirus SODs and a SOD from *Drosophila melanogaster* (DmSOD). The sources of the sequence are: Tomalski *et al.* (1994) for AcMNPV; Ahrens *et al.* (1997) for OpMNPV; Maeda (Genbank L33180) for BmNPV and Seto *et al.* (1989) for DmSOD. Black is 100% identity and grey 75% identity. The element symbols (Cu and Zn) below the sequence designate the conserved metal-coordinating amino acids; asterisks align the conserved cysteines which form the intramolecular disulfide bond; + symbols align a vital and conserved arginine.

(His43, His60, and His118) and the Zn-binding residues (His60, His68, His77 and Asp80) (Richardson 1977, Richardson *et al.*, 1975) are totally conserved among all the proteins. Other conserved key residues are Arg141, critical for enzyme activity (Malinowski and Fridovich, 1979), Cys54 and Cys144 needed to form the intrasubunit disulfide bond (Abermethy *et al.*, 1974), and 18 of the 21-26 Gly residues which contribute to the sharp bends in the backbone of the polypeptides (Richardson, 1977). The function of the baculoviral *sod* is not known, but the virus-encoded SOD activity was not detectable in cells infected with AcMNPV (Tomalski *et al.*, 1991). Moreover, mutations in *sod* did not appear to affect the normal replication of the virus in cell culture and in insect larvae. The existence of *sod* in several baculoviruses and the high degree of conservation of SOD sequences are nevertheless suggestive of a specific role in the viral life cycle.

*Genome organization*

When the genomes of BusuNPV and the baculovirus type species AcMNPV are aligned, it can be observed that the genes clustered in the BusuNPV *Hin*dIII-J fragment are found dispersed in the AcMNPV genome (Fig. 4.7). In the latter virus, *pep, orf117, ctl* and *sod* are widely separated and located at different positions than in the genome of BusuNPV. For comparison several other genes which have been identified and sequenced in the genome of BusuNPV such as polyhedrin (Hu *et al.*, 1993), *egt* (Hu *et al.*, 1997), *p26* and *p10* (Oers *et al.*, 1998) are included. It is interesting to note that the *p26-p10* combination found in AcMNPV also exists in the genome of BusuNPV. However, *p74*, normally in close proximity and downstream of *p10* (*p26-p10-p74*) in the AcMNPV and several other baculovirus genomes, is found at a different location in the BusuNPV genome (Z.H. Hu, unpublished). Recent studies on the genome of another distantly related baculovirus, SeMNPV, indicated that in comparison to the genome of AcMNPV, a large region around the helicase gene has a highly conserved gene organization (Heldens *et al.*, 1998) whereas genes located in other regions, including those between polyhedrin and *p10*, are found dispersed (Strien *et al.*, 1997). This indicates that during evolution, some parts of the baculovirus genome remain as conserved gene clusters whereas other parts of the genome undergo considerable rearrangements.

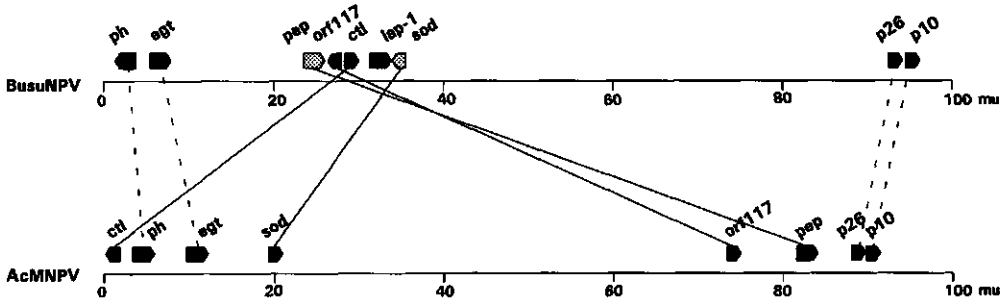



**Figure 4.7.** Comparison of the gene organization between the genomes of AcMNPV and BusuNPV. The arrows represent the ORFs and their directions of transcription. Gene homologues are linked by a line.

The family of baculoviruses is rather large containing more than 600 established and tentative species occurring in a variety of arthropod orders and families(Murphy *et al.*, 1995). It is, therefore, not surprising that these viruses show interspecies diversity in genomic organization. Such diversity also occurs in other large DNA viruses such as herpesviruses. Even though the mechanisms that generated the genomic diversity in baculoviruses during evolution are presently not understood, it should be possible to derive a phylogeny of baculoviruses based on the genome organization when more sequence data from different viruses become available (Strien *et al.*, 1997). It will be interesting to see if the phylogeny based on genome organization is similar to that based on individual genes. Our previously reported phylogeny analysis, based on baculovirus EGTs, corroborates the conclusion from this report that BusuNPV is distinct from the group of AcMNPV, OpMNPV and BmNPV. Molecular genetic studies on BusuNPV will further assist in our understanding of the diversity and evolution of baculoviruses as well as increase our knowledge of the gene organization and function of SNPVs.

**ACKNOWLEDGEMENTS**

We are grateful to Dr. J.A. Jehle and Dr. E.A van Strien for critical review of the manuscript. This research was supported in part by the National Natural Science Foundation of China (NSFC, grant 39370031) and a joint grant from the Chinese Academy of Sciences (CAS) and the Royal Netherlands Academy of Sciences (KNAW). Z.H. Hu is a recipient of a special grant from the chairman of CAS and a sandwich PhD grant from the Wageningen Agricultural University.

CHAPTER 5

# DISTINCT GENE ARRANGEMENT IN THE *BUZURA SUPPRESSARIA* SINGLE-NUCLEOCAPSID NUCLEOPOLYHEDROVIRUS GENOME


## SUMMARY

The genome organization of the *Buzura suppressaria* single-nucleocapsid (S) nucleopolyhedrovirus (BusuNPV) was largely elucidated and compared to those of other baculoviruses. A detailed physical map was constructed for the restriction enzymes *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Xba*I and *Xho*I. The 120.9 kilobase pairs (kb) viral genome was cloned as restriction fragments into a plasmid library from which about 43.5 kb of dispersed sequence information was generated. Fifty two putative open reading frames (ORFs) homologous to that of other baculoviruses were identified and their location in the genome of BusuNPV determined. Although the gene content of BusuNPV is similar to that of *Autographa californica* multiple-nucleocapsid NPV (AcMNPV), *Bombyx mori* (Bm) NPV and *Orgyia pseudotsugata* (Op) MNPV, the gene order is, however, significantly different from the other viruses, which have a high degree of collinearity. A new approach (GeneParityPlot) was developed to represent the differences in gene order among baculoviruses when limited sequence information is available and taking advantage of the high degree of gene conservation. The data obtained show that BusuNPV is a distinct baculovirus species and the analyses suggest that the gene distribution along baculovirus genomes may be used as a phylogenetic marker.



This chapter will be published as: Hu, Z.H., B.M. Arif, F. Jin, J.W.M. Martens, X.W. Chen, J.S. Sun, D. Zuidema, R. W. Goldbach and J.M. Vlak. 1998. Distinct gene arrangement in the *Buzura suppressaria* single-nucleocapsid nucleopolyhedrovirus genome. *Journal of General Virology, in press.*

## INTRODUCTION

The *Baculoviridae*, a diverse family of more than 600 viruses, contains two genera, the *Nucleopolyhedroviruses* (NPV) and the *Granuloviruses* (GV) (Murphy *et al.*, 1995). The virions are rod-shaped and contain a circular, double stranded DNA genome of 90 to 160 kilobasepairs (kb) depending on the species. Baculoviruses are pathogenic for arthropods, particularly insects of the order Lepidoptera. The current interest in the molecular biology of these viruses is fostered by their potential as alternatives to chemical insecticides in the control of agricultural and forest insect pests and also by their successful use as vectors for the expression of foreign proteins. To date, the complete sequence of the genomes of three baculoviruses, that of *Autographa californica* multiple-nucleocapsid NPV (AcMNPV), *Bombyx mori* (BmNPV) and *Orgyia psudotsugata* (OpMNPV) has been determined (Ayres *et al.*, 1994; S. Maeda, Genbank accession number L33180; Ahrens *et al.*, 1997). The genome of AcMNPV is 133,894 base pairs (bp) potentially encoding 154 proteins, that of BmNPV is 128,413 bp and contains 136 putative genes, and the OpMNPV genome is 131,990 bp and contains 152 putative genes. Genomic comparisons indicated that, even though there are small inversions and insertions (or deletions), basically these three baculovirus genomes have a similar gene content and a similar arrangement along the genome.

As more genomic sequences become available from other baculoviruses, their molecular biology will be better understood and their phylogenetic relationships and evolution will be more accurately determined. Until now, only limited DNA sequence information has been reported from single-nucleocapsid (S) NPVs and GVs. Consequently, our understanding of the gene content and organization of these viruses is rudimentary in comparison to that of MNPVs. In this paper, we present a study elucidating the genome organization of an SNPV infecting *Buzura suppressaria* Guenée (Lepidoptera: Geometridae). The virus (BusuNPV) was originally isolated from this major pest of tea plantations in China and it has since been used to control infestations (Xie *et al.*, 1979). So far, about 63 NPVs have been described from the Geometridae insect family (Adams and McClintock, 1991), but molecular characterization of these viruses has been limited. A study based on the amino acid sequences of baculovirus ecdysteroid UDP-glucosyltransferase (*egt*) genes has indicated that BusuNPV belongs to a phylogenetic group within the Baculoviridae family, which is distinct from that encompassing AcMNPV, BmNPV and OpMNPV (Hu *et al.*, 1997). Sequence analysis of a 3.2 kb *Hin*dIII fragment located at map unit (*mu*) 26.6-29.4 and the *p10* gene region (*mu* 94.0-95.3) revealed that the genome organization of BusuNPV might differ considerably from other baculoviruses investigated so far (Hu *et al.*, 1998; van Oers *et al.*, 1998).

In order to investigate the gene organization of BusuNPV and evaluate the phylogenetic status of this virus, a detailed physical map of the viral genome was constructed. A genomic library was made by cloning the viral DNA as restriction fragments into plasmid vectors and the partial or total nucleotide sequence of the inserts was determined. The sequence data were used to identify open reading frames (ORFs) and potential genes by comparison with databases. A genetic map describing the order of ORFs in the genome of BusuNPV was generated and compared to that of AcMNPV, BmNPV and OpMNPV using a novel approach

(GeneParityPlot).

## MATERIALS AND METHODS

*Virus and DNA*

The virus used in this study was originally isolated from an infestation by *Buzura suppressaria* larvae of a tea plantation in Hubei province in the P.R. China (Xie *et al.*, 1979). The virus was propagated in these larvae and occlusion bodies (OB) were purified by differential and rate zonal centrifugation. The DNA was isolated directly from purified OBs by dissolution in 0.1M $Na_2CO_3$, 0.01M EDTA and 0.17M NaCl, followed by proteinase-K and SDS treatment, phenol/chloroform extraction and dialysis. The purity of the DNA was determined spectrophotometrically.

*Restriction endonuclease analysis and cloning of viral DNA fragments in plasmid vectors*

Viral DNA was digested with various restriction enzymes (GIBCO/BRL) and the fragments were separated by electrophoresis in 0.6%, 0.7% and 0.8% agarose gels at 45V (1.5V/cm) for 14-20 hours. Lambda DNA digested with *Bam*HI-*Eco*RI-*Hind*III was used as molecular size standards.

Viral DNA restriction fragments were cloned into the plasmid vector, pTZ19R, by using standard techniques (Sambrook *et al.*, 1989). Firstly, the shotgun method was employed to generate a library of clones. Secondly, restriction fragments, absent from the shotgun library, were separated in agarose gels, purified by the freeze-squeeze method and cloned individually into pTZ19R. Restriction enzyme analysis and Southern blot hybridization were performed to confirm the authenticity and the location of the cloned fragments on the physical map of BusuNPV genome.

*Construction of the physical map of the BusuNPV genome*

The order of the restriction fragments on the viral genome was derived from single and double digestion of the cloned fragments with various enzymes, as well as by Southern blot hybridization. Sequence data of the cloned fragments were also used to establish and confirm the detailed map.

*DNA sequencing and computer analysis*

Plasmid DNA for sequencing was purified via Qiagen columns (Qiagen, Inc.). Partial sequencing was conducted on both ends of the cloned fragments by using the universal forward and reverse primers. Certain regions of the genome were selected for complete sequencing of both strands by using either a series of overlapping clones containing nested deletions or by the 'sequence walking' method with custom synthesized primers. The T7 DNA polymerase sequence system (Promega) was used for manual sequencing and the automatic sequencing was carried out at the Sequencing Facility in the Department of Molecular Biology of the Wageningen Agricultural University or at the Core Facility at Queens University (Kingston, Ontario, Canada). The generated sequences were analyzed with UWGCG computer programs (release 9.0). The DNA and the deduced amino acids sequences were

compared with the updated GenBank/EMBL, SWISSPORT and PIR databases using FASTA and BLAST. For studying baculovirus gene homology, the amino acid sequences of the selected homologues were aligned by the PileUp program of GCG. The resulting alignments were then imported into GeneDoc software from which the amino acid identities were calculated. The nucleotide sequences of the complete ORFs of BusuNPV have been deposited in Genbank and were assigned the following accession numbers: X70844 (polyhedrin; Hu *et al.*, 1993), U61154 (*egt*; Hu *et al.*, 1997), AF058928 (homologue of Ac111), AF060564 (late expression factor 2), AF045936 (homologue of Ac117, conotoxin-like gene, inhibitor of apoptosis like gene, superoxide dismutase gene; Hu *et al.*, 1998), AF058929 (cathepsin) and AF034410 (*p26, p10*; van Oers *et al.*, 1998). The partial sequences are available upon request.

*Gene order in baculovirus genome*

In order to facilitate comparisons of gene orders among BusuNPV, AcMNPV, BmNPV and OpMNPV, only those gene homologues identified so far in all genomes were selected. The identified BusuNPV ORFs were provisionally numbered 1-52 (Figure 5.3). To allow a computational comparison, the BusuNPV ORF homologues of AcMNPV, BmNPV and OpMNPV (Table 5.2 left) were renumbered manually starting with the polyhedrin gene as no. 1 and renumbering the other ORFs according to their sequential occurrence on the respective linearized genomes from left to right (Table 5.2 right). With these reassigned numbers as input data (see Table 5.2), the Chart Program packaged in Microsoft Excel software was then used to obtain a graphic comparison. The input data are sorted ascendant according to the gene order of one virus (e.g. AcMNPV). By choosing 'line' as chart type the GeneParityPlot is obtained showing the gene order as a line. The X-axis then represents the gene order of one given virus and the Y-axis the gene order of the other virus. This diagonal line (reference) is obtained when a given virus is both on the X-axis and Y-axis. From such GeneParityPlots the difference in gene organization between two baculoviruses can be conveniently compared.

If the gene arrangement of a given virus is collinear with that of AcMNPV, then a straight parity line will appear. A gene or gene cluster that is shifted to a different location will appear as a dot or a small line parallel to the parity line, with a distance from the diagonal dependent on the location. The direction of transcription of genes was also taken account to identify inversions. When the direction of one ORF is the same as that of its homologue in AcMNPV, an open symbol was used and when the direction is opposite, a solid symbol was used. Therefore, a typical inversion will be a solid line perpendicular to the diagonal. The resulting 'GeneParityPlot' shows the clustering of genes and their spatial distribution without knowing the entire sequence.

## RESULTS AND DISCUSSION

*Restriction endonuclease analysis of BusuNPV DNA*

Digestion of BusuNPV with *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Xba*I and *Xho*I, produced 10, 9, 7, 23, 16, 9, 5, 12 and 10 fragments, respectively. Restriction profiles of these digestions of BusuNPV genome DNA is shown in Fig. 5.1 and the size of the fragments is

summarized in Table 5.1. These sizes were estimated from the original agarose gel and adjusted according to the data obtained from the mapping and sequencing experiments. The total size of the BusuNPV is determined to be 120.9 kb which is about 8-13 kb smaller than the genomes of AcMNPV, OpMNPV and BmNPV (Ayres *et al.*, 1994; S. Maeda, Genbank L33180; Ahrens *et al.*, 1997).

A few submolar bands, such as *Bam*HI-F, *Eco*RI-Q and *Xba*I-G were observed in the restriction profiles indicating that the virus isolate used in this study was not homogeneous, but contained major and minor genotypic variants (Fig. 5.1). Comparison with a baculovirus isolate from *Buzura thibtaria* indicated that many of the submolar bands observed for BusuNPV were equimolar in ButhNPV (data not shown). *In vivo* cloning techniques (Smith and Crook, 1988) or plaque purification techniques using cultured cells (King and Possee, 1992) are required to isolate and characterize the genotypic variants of BusuNPV in more detail.

**Table 5.1.** Size of restriction endonuclease fragments (in kb) of BusuNPV DNA

| Fragment | *Bam*HI | *Bgl*I | *Bgl*II | *Eco*RI | *Hin*dIII | *Kpn*I | *Pst*I | *Xba*I | *Xho*I |
|---|---|---|---|---|---|---|---|---|---|
| A | 40.3 | 29.7 | 47.1 | **13.9** | 32.4 | 27.4 | 62.8 | 19.5 | 28.5 |
| B | 25.4 | 22.2 | 32.6 | 11.9 | 20.2 | 26.1 | 26.6 | 18.3 | 28.2 |
| C | 13.9 | 16.6 | 21.5 | **11.7** | **18.1** | 25.3 | 23.5 | **14.0** | 16.1 |
| D | 13.6 | 14.5 | **6.2** | 9.2 | **8.1** | 20.8 | **7.8** | 13.2 | 15.0 |
| E | **12.0** | 12.2 | 5.2 | **8.8** | **6.5** | 8.6 | **0.2** | **13.2** | 9.8 |
| F | 6.3 | 11.5 | 4.5 | 7.7 | **6.4** | **4.8** | | **12.7** | 8.7 |
| G | **4.2** | 6.0 | **3.8** | **7.3** | **6.1** | **3.4** | | **7.8** | 6.3 |
| H | **2.4** | 5.2 | | **5.2** | **5.8** | **2.4** | | 6.9 | 4.2 |
| I | **2.0** | 3.0 | | **5.2** | **3.6** | **2.1** | | **6.3** | 3.3 |
| J | **0.8** | | | **5.1** | **3.2** | | | 4.9 | 0.8 |
| K | | | | 4.9 | **2.9** | | | **2.2** | |
| L | | | | 4.4 | **2.7** | | | 1.9 | |
| M | | | | 4.4 | 2.6 | | | | |
| N | | | | 4.2 | **1.6** | | | | |
| O | | | | 3.9 | **0.6** | | | | |
| P | | | | **3.5** | **0.1** | | | | |
| Q | | | | 2.9 | | | | | |
| R | | | | **2.4** | | | | | |
| S | | | | **2.1** | | | | | |
| T | | | | 1.1 | | | | | |
| U | | | | 0.6 | | | | | |
| V | | | | **0.4** | | | | | |
| W | | | | **0.1** | | | | | |

*Note:* Bold indicates that the fragment is cloned in a plasmid vector and underlined indicates that the size of the fragment is confirmed by sequence analyses.

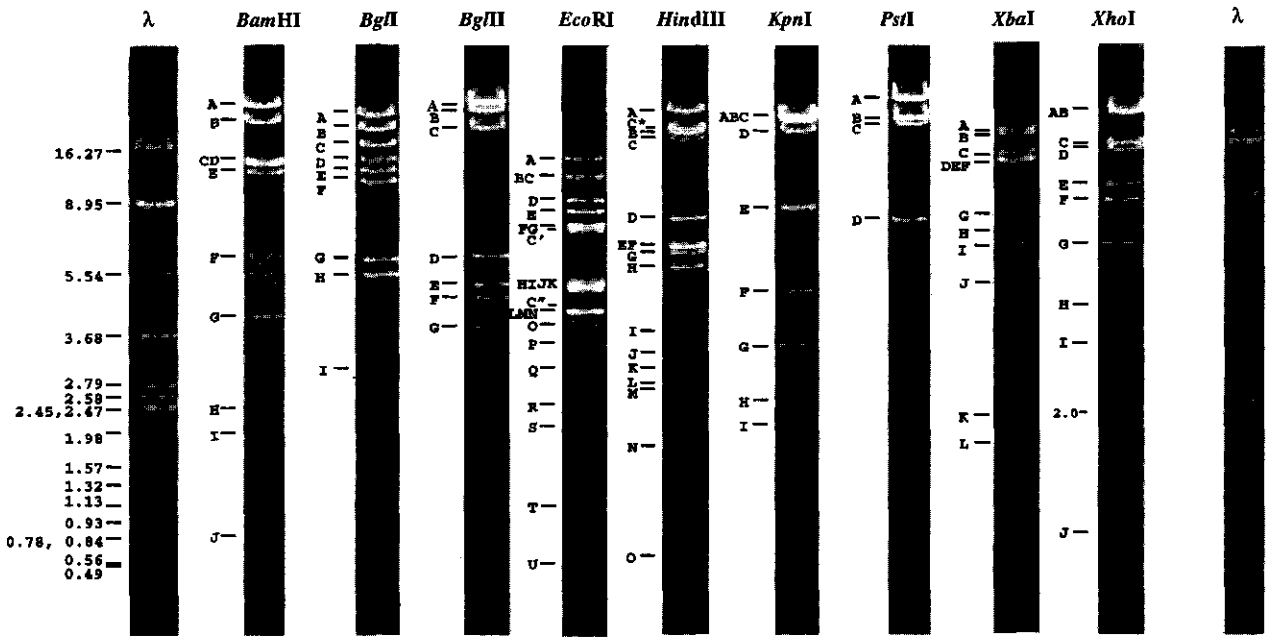



**Figure 5.1.** BusuNPV DNA digested with *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Xba*I and *Xho*I and electrophoretically separated in 0.6% agarose. The fragments are named alphabetically, starting with A for the largest fragment. Submolar bands, such as *Hin*dIII-C*, were also assigned. Lambda DNA digested with *Bam*HI-*Eco*RI-*Hin*dIII was used as molecular marker with their sizes indicated in kilobase pairs.

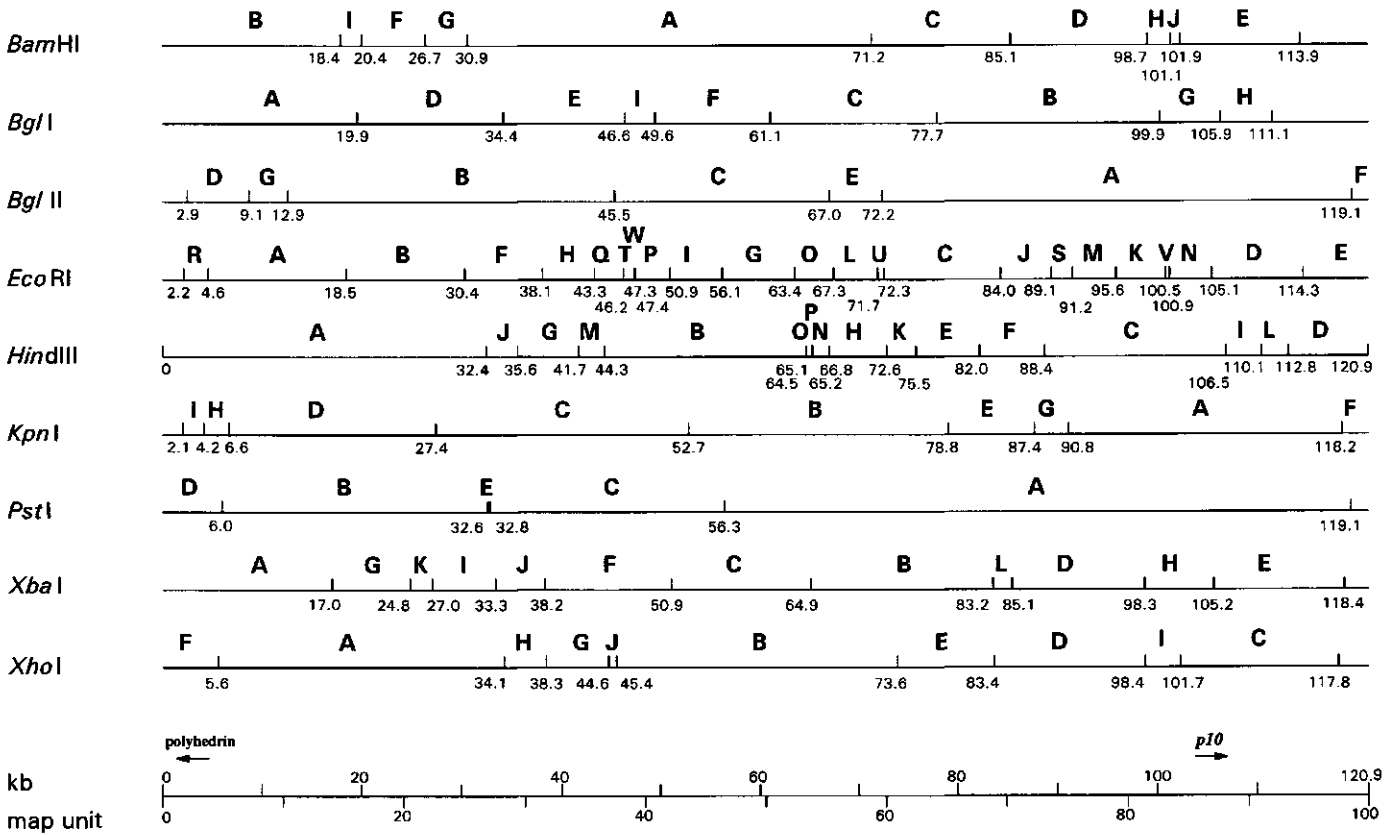



**Figure 5.2.** Linearized physical map of BusuNPV DNA with restriction sites for *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Xba*I and *Xho*I. The restriction sites are indicated in kb from the zero point. The genome size in kb (upper) and map units (lower) is indicated on a scale at the bottom. The location and the direction of transcription of polyhedrin and *p10* are shown by arrows.

*The restriction map of BusuNPV DNA*

With the aid of a genomic library of clones containing overlapping restriction fragments and additional extensive cross hybridization experiments, we were able to generate a physical map encompassing 103 restriction sites for *Bam*HI, *Bgl*I, *Bgl*II, *Eco*RI *Hin*dIII, *Kpn*I, *Pst*I, *Xba*I and *Xho*I for the major genotype (Fig. 5.2). Some fragments were submolar as a consequence of the presence of multiple genotypes in the isolate. Fragments *Eco*RI-Q and T were less than equimolar. A subpopulation of genotypes lacked an *Eco*RI site between these two fragments (Fig. 5.2) and formed a submolar band (Q+T) of about 4 kb. A similar situation existed for *Eco*RI-C (11.7 kb) which, in a subpopulation of genotypes, has an additional *Eco*RI site giving fragments *Eco*RI-C' and *Eco*RI-C" of 7.3 kb and 4.4 kb in size, respectively. Fragments *Hin*dIII-C and I are also less than equimolar, but they form a contig (*Hin*dIII-C*) in a subpopulation of genotypes. Fragments *Xba*I-G, *Eco*RI-B and *Bam*HI-F were also submolar, but this may be explained by the observation that these fragments cover a genomic region (m.u. 19), which is highly variable containing multiple sequence repeats in an intergenic region (data not shown). All submolar fragments were either found in our plasmid library and or identified by hybridization. The origin of a minor molar *Xho*I fragment of 2.0 kb was not further investigated.

Since the entire polyhedrin gene (*ph*) is contained within the fragment *Hin*dIII-A (Hu *et al.*, 1993), the linearized map was drawn in such a way that the zero point is located at the beginning of this fragment, as proposed by Vlak and Smith (1982). This also positioned *p10* (van Oers *et al.*, 1998) to the right-hand end of the map. The linearized map and its orientation reported in this paper differ from the preliminary map published previously (Liu *et al.*, 1993). Fragment *Hin*dIII-J (Fig. 5.2) has been previously assigned *Hin*dIII-I (Hu *et al.*, 1998).

*Sequence analyses of BusuNPV DNA*

In order to generate sufficient information to decipher the gross gene content and arrangement in the genome of BusuNPV, approximately 43.5 kb of the genome was sequenced. A total of 52 putative ORFs and genes homologous to those of other baculoviruses were identified by

comparison with data bases. These 52 ORFs were provisionally named Bs1 to Bs52 from left to right according to their sequential location on the physical map (Fig. 5.3). Of these 52 ORFs eleven are completely sequenced, including polyhedrin, *egt*, late expression factor 2 (*lef-2*), conotoxin-like peptide (*ctl*), superoxide dismutase (*sod*), cathepsin (*cath*), *p26* and *p10*.

Comparison of the overall gene content of divergent baculoviruses provides an initial view of the genes that are retained in all genomes and thus are likely to be essential for virus multiplication and survival. This comparison may also provide insight into the evolutionary origin of baculoviruses and their relatedness with the baculovirus family. Sequence analyses of AcMNPV, BmNPV and OpMNPV have so far revealed a total of 184 different baculoviral ORFs (Ayres *et al.*, 1994; S. Maeda, Genbank L33180; Ahrens *et al.*, 1997). Among these ORFs, 119 are shared by the three genomes, 21 are present in two of the three genomes and 44 are unique to individual genomes (14 ORFs for AcMNPV, 4 ORFs for BmNPV and 26 ORFs for OpMNPV). This indicates a large extent of gene conservation.

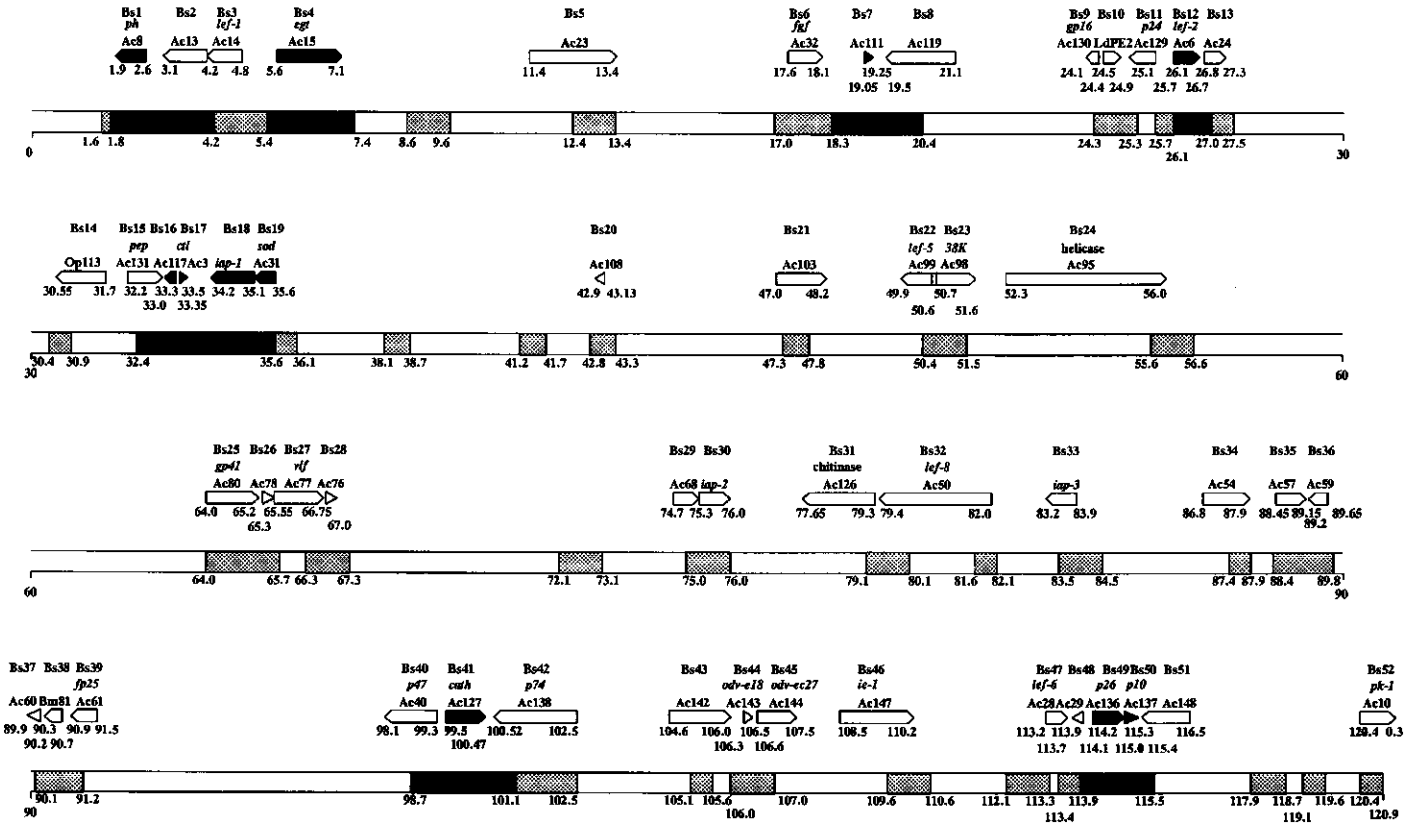



**Figure 5.3.** Genomic organization of the BusuNPV DNA. The upper part of each panel represents the location of putative ORFs; open arrows indicate the direction of transcription; completely sequenced ORFs are shown as solid arrows. The location of the ORFs, given in map units, is approximated. Names assigned to ORFs are referenced in the text and the homologues of AcMNPV ORFs (or other baculovirus when there is no homologue from AcMNPV) are shown. The location (in map units) of the 3' and 5' ends of the ORF were approximations based on the size of baculovirus homologs. The lower part of each panel represents an overview of the sequence information; black region indicates that the section has been sequenced from both strands and the shaded region indicates a sequence of one strand.

**Table 5.2.** Identified ORFs in BusuNPV and their homologues in AcMNPV, BmNPV and OpMNPV

| Busu ORF | Ac ORF | Bm ORF | Op ORF | Name | abbrev | Ac Order | Bm Order | Op Order | Busu Order | Ac dir | Bm dir | Op dir | Busu dir |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 8 | 1 | 3 | polyhedrin | *ph* | 1 | 1 | 1 | 1 | < | > | < | < |
| 2 | 13 | 5 | 12 | | | 3 | 3 | 3 | 2 | < | < | < | < |
| 3 | 14 | 6 | 13 | late expression factor | *lef-1* | 4 | 4 | 4 | 3 | < | < | < | < |
| 4 | 15 | 7 | 14 | | *egt* | 5 | 5 | 5 | 4 | > | > | > | > |
| 5 | 23 | 14 | 21 | | | 6 | 6 | 6 | 5 | > | > | > | > |
| 6 | 32 | 24 | 27 | fibroblast growth factor | *fgf* | 11 | 11 | 7 | 6 | < | > | > | > |
| 7 | 111 | 93 | 112 | | | 29 | 29 | 29 | 7 | < | < | < | > |
| 8 | 119 | 97 | 119 | | | 31 | 31 | 31 | 8 | > | > | > | < |
| 9 | 130 | 107 | 128 | | *gp16* | 35 | 35 | 35 | 9 | > | > | > | < |
| 10 | | | | LdMNPV PEP ORF2 | | | | | | | | | > |
| 11 | 129 | 106 | 127 | capsid protein | *p24* | 34 | 34 | 34 | 10 | > | > | > | < |
| 12 | 6 | 135 | 6 | late expression factor | *lef-2* | 45 | 45 | 2 | 11 | > | > | < | > |
| 13 | 24 | 15 | 44 | | | 7 | 7 | 11 | 12 | < | < | > | > |
| 14 | | | 113 | | | | | | | | | > | < |
| 15 | 131 | 108 | 129 | calyx protein | *pep* | 36 | 36 | 36 | 13 | > | > | > | > |
| 16 | 117 | 96 | 117 | | | 30 | 30 | 30 | 14 | > | > | > | < |
| 17 | 3 | | 136 | conotoxin | *ctl-1* | | | | | > | > | | > |
| 18* | | | | iap like gene | *iap-1* | | | | | | | | < |
| 19 | 31 | 23 | 29 | superoxide dismutase | *sod* | 10 | 10 | 8 | 15 | > | > | < | < |
| 20 | 108 | 91 | 108 | | | 28 | 28 | 28 | 16 | < | < | < | < |
| 21 | 103 | 87 | 104 | | | 27 | 27 | 27 | 17 | < | < | < | > |
| 22 | 99 | 83 | 100 | late expression factor | *lef-5* | 26 | 26 | 26 | 18 | > | > | > | < |
| 23 | 98 | 82 | 99 | | *38K* | 25 | 25 | 25 | 19 | < | < | < | > |
| 24 | 95 | 78 | 96 | helicase | | 24 | 24 | 24 | 20 | < | < | < | > |
| 25 | 80 | 66 | 83 | | *gp41* | 23 | 23 | 23 | 21 | < | < | < | > |
| 26 | 78 | 64 | 81 | | | 22 | 22 | 22 | 22 | < | < | < | > |
| 27 | 77 | 63 | 80 | very late factor | *vlf-1* | 21 | 21 | 21 | 23 | < | < | < | > |
| 28 | 76 | 62 | 79 | | | 20 | 20 | 20 | 24 | < | < | < | > |
| 29 | 68 | 56 | 73 | | | 18 | 18 | 18 | 25 | > | > | > | > |
| 30* | 71 | 58 | 74 | iap like gene | *iap-2* | 19 | 19 | 19 | 26 | > | > | > | > |
| 31 | 126 | 103 | 124 | chitinase | *chiA* | 32 | 32 | 32 | 27 | < | < | < | < |
| 32 | 50 | 39 | 54 | late expression factor | *lef-8* | 13 | 13 | 13 | 28 | < | < | < | < |
| 33* | | | | iap like gene | *iap-3* | | | | | | | | < |
| 34 | 54 | 43 | 58 | | | 14 | 14 | 14 | 29 | > | > | > | > |
| 35 | 57 | 46 | 61 | | | 15 | 15 | 15 | 30 | > | > | > | > |
| 36 | 59 | | 62 | | | | | | | < | < | | < |
| 37 | 60 | 48 | 63 | | | 16 | 16 | 16 | 31 | < | < | < | < |
| 38 | | 81 | | | | | | | | | > | | < |
| 39 | 61 | 49 | 64 | few polyhedra | *25K* | 17 | 17 | 17 | 32 | < | < | < | < |
| 40 | 40 | 31 | 45 | | *p47* | 12 | 12 | 12 | 33 | < | < | < | < |
| 41 | 127 | 104 | 125 | cathepsin | *cath* | 33 | 33 | 33 | 34 | > | > | > | > |
| 42 | 138 | 115 | 134 | | *p74* | 39 | 39 | 39 | 35 | < | < | < | < |
| 43 | 142 | 118 | 139 | | | 40 | 40 | 40 | 36 | > | > | > | > |
| 44 | 143 | 119 | 140 | ODV protein | *odv-e18* | 41 | 41 | 41 | 37 | > | > | > | > |
| 45 | 144 | 120 | 141 | ODV protein | *odv-ec27* | 42 | 42 | 42 | 38 | > | > | > | > |
| 46 | 147 | 123 | 145 | major transactivator | *ie-1* | 43 | 43 | 43 | 39 | > | > | > | > |
| 47 | 28 | 19 | 40 | | *lef-6* | 8 | 8 | 10 | 40 | > | > | < | > |
| 48 | 29 | 20 | 39 | | | 9 | 9 | 9 | 41 | < | < | > | < |
| 49 | 136 | 113 | 132 | | *p26* | 37 | 37 | 37 | 42 | > | > | > | > |
| 50 | 137 | 114 | 133 | fibrillin | *p10* | 38 | 38 | 38 | 43 | > | > | > | > |
| 51 | 148 | 124 | 146 | ODV protein | *odv-e56* | 44 | 44 | 44 | 44 | < | < | < | < |
| 52 | 10 | 3 | 1 | protein kinase | *pk-1* | 2 | 2 | 45 | 45 | > | > | > | > |

*Note:* The left columns represent the actual gene numbering derived for BusuNPV from Fig. 5.3 and for AcMNPV, BmNPV and OpMNPV from Ayres *et al.*, 1994; S. Maeda, Genebank L33180 and Ahrens *et al.*, 1997, respectively. The right columns represent the assigned ORF number after transformation and the direction of transcription of the ORFs in their respective genomes. *The *iap* genes of BusuNPV are numbered according to their location in the BusuNPV genome.

Table 5.2 shows the 52 ORFs of BusuNPV and their homologues in AcMNPV, BmNPV and OpMNPV. It is interesting to note that genes previously thought to be unique for OpMNPV (Op113) or BmNPV (Bm81), were also found in BusuNPV, as Bs14 and Bs38. A homologue of the ORF2 in the polyhedral envelop protein (PEP) region of *Lymantria dispar* MNPV (Bjornson and Rohrmann, 1992) was also found in BusuNPV as Bs10. Three inhibitor of apoptosis like genes (*iaps*) were identified in BusuNPV and they are numbered according to their relative location on the linearized BusuNPV genome (Fig. 5.3). So far the sequence information indicates that the *iap-2* of BusuNPV belongs to the lineage of *iap-2* of AcMNPV, BmNPV and OpMNPV, whereas the grouping of BusuNPV *iap-1* and *iap-3* is not clear (data not shown). Therefore only the counterparts of *iap-2* are shown in Table 5.2.

The 52 BusuNPV ORFs occupied about 70% of the sequenced region. Combined with the information from AcMNPV, BmNPV and OpMNPV, this suggests an extensive conservation of gene content in NPV genomes. The remainder of the sequenced BusuNPV regions consists of intergenic sequences between ORFs, as well as some potential ORFs that do not have significant homology to any sequence in Genbank. If the sequence information reported here represents a random distribution, it can be predicted that the unique ORFs occupy less than 25% of the BusuNPV genome. The high conservation of the gene content indicates that the NPVs have evolved from an ancestor whose genetic make up is not that dissimilar to the present day baculoviruses. In fact, this conservation is what defines a baculovirus including the genes essential for DNA replication, expression, assembly of progeny particles and occlusion at the end of the replication cycle. Some of these characteristic baculovirus genes have also been identified in the BusuNPV genome, such as immediate early gene 1 (*ie-1*), helicase, several of the late gene expression factor genes, *lef-1*, *lef-2*, *lef-5*, *lef-6*, *lef-8*, the 'very late factor' gene (*vlf-1*), as well as some structural genes known as *gp41, odv-e18, odv-ec27, odv-e56*, *ph*, *p10* and *pep* (Fig. 5.3, Table 5.2).

Apart from the above characteristic genes, baculoviruses also contain the so-called auxiliary genes. These genes are not essential for replication, but some provide the virus with selective advantages in nature (see O'Reilly, 1997 for review). Auxiliary genes, such as *egt*, *ctl*, *iap*, *sod*, *cath* and *chitinase* (*chiA*), were also found in BusuNPV. EGT delays larval moulting and allows the virus to produce large numbers of progeny particles. The *cath* and the *chiA* are needed for larval liquefaction and thus aid in the dissemination of the virus in nature (Hawtin *et al.*, 1997). It is plausible that an ancestral baculovirus contained some of the auxiliary genes and that the present baculovirus survived through evolution partly because of the advantages conferred by their encoded proteins. The conservation of these auxiliary genes in baculovirus genomes suggests that they are important to the viral life cycle.

*Baculovirus gene homology*

Comparison of gene homology among different baculoviruses provides an initial measure of gene conservation and an insight into function of these genes. Furthermore, based on the gene homology, such comparisons may also have an implication on the diversity of baculoviruses. Among the 52 ORFs of BusuNPV, eleven ORFs have been completely sequenced from both strands. These include Bs1 (polyhedrin), Bs4 (*egt*), Bs7 (Ac111), Bs12 (*lef-2*), Bs16 (Ac117), Bs17 (*ctl*), Bs18 (*iap*-1), Bs19 (*sod*), Bs41(*cath*), Bs49 (*p26*) and Bs50 (*p10*). Nine of the above 11 ORFs were used to study the gene homology among BusuNPV, AcMNPV, BmNPV and OpMNPV. The *ctl* was not included in the analysis because it does not exist in BmNPV. Similarly, the *iap*'s were not used because of multiple occurrence in all four genomes.

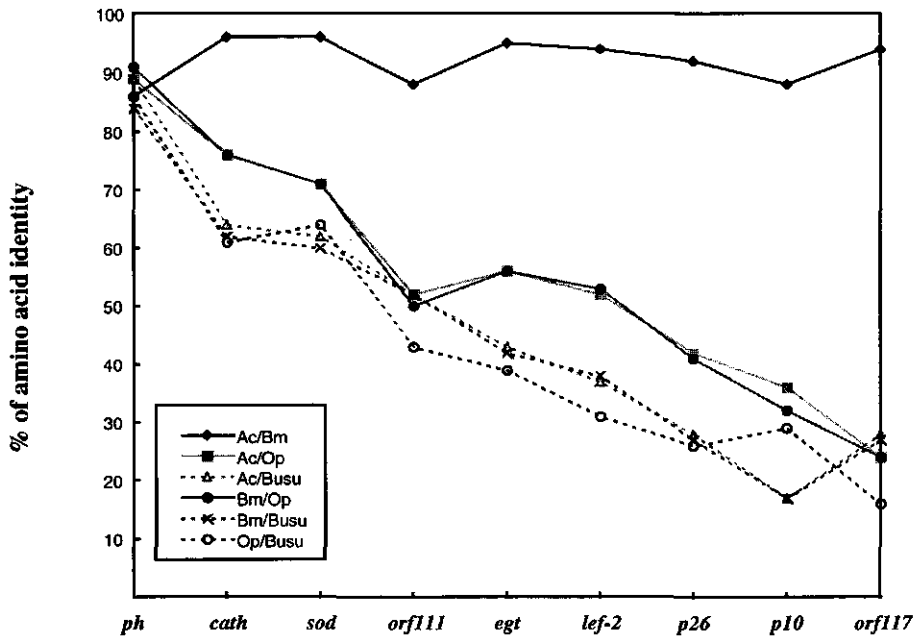



**Figure 5.4.** Pairwise homology of nine ORFs of AcMNPV (Ac), BmNPV (Bm), OpMNPV (Op) and BusuNPV (Bs). Gene nomenclature is according to Ayres *et al.*(1994).

Pairwise comparison of amino acid sequence identity revealed two kinds of conservation patterns (Fig. 5.4). For the closely related baculoviruses, such as AcMNPV and BmNPV, the homology is high for all the genes. Hence, when all genes are aligned, a conserved pattern with constantly high amino acid identity appeared. This pattern is characteristic of closely related baculoviruses and distinguishes these from the others. When less related viruses are compared, it is obvious that the extent of gene homology differs among individual genes. For example, the amino acid sequence identity ranks from in excess of 84% for polyhedrin to no higher than 28% for *orf117*. It is plausible that the extent of homology is related to the function of different genes. The pairwise comparison is useful to identify closely related baculoviruses when sequence information of not too highly conserved genes is available. In the case of OpMNPV and *Perina nuda* (Penu) MNPV, for example, the amino acid sequence identity is about 95% for *p10* (van Oers *et al.*, 1994) and 97% for *lef-2* (X.W. Chen, personal communication). Therefore, it is very likely that these two viruses are closely related.

Although the genome organization of OpMNPV is quite similar to that of AcMNPV and BmNPV as compared to BusuNPV (see below), the gene homology data show that OpMNPV is relatively distant from AcMNPV and BmNPV. The homology of BusuNPV ORFs to their counterparts of other viruses (dashed lines in Fig. 5.4) is slightly lower than the homology of OpMNPV ORFs to their counterparts of AcMNPV and BmNPV. This suggests that gene order and gene homology are two independent parameters in the study of baculovirus phylogeny.

*Distinct gene arrangement in the BusuNPV genome*

While the gene organization in AcMNPV, BmNPV, and OpMNPV are basically similar, we investigated whether the organization in the genome of BusuNPV is collinear with these viruses. The homologues of 45 ORFs which exist in all four genomes were chosen for comparison in the 'GeneParityPlot' analysis (see Methods). After transformation, the resulting relative order number and direction of the ORFs are listed in Table 5.2. The comparison of gene arrangement of the selected ORFs is shown in Fig. 5.5. Although the 45 ORFs represent only about 30% of the total ORFs in the baculovirus genome, the comparison of BmNPV and OpMNPV with AcMNPV (Fig. 5.5a, Fig. 5.5b) represents well the actual collinearity of these genomes (Ahrens *et al.*, 1997). The inversion in OpMNPV (Op10-Op21) in comparison to the AcMNPV genome (Ahrens *et al.*, 1997) is reflected in Fig. 5.5b as being perpendicular to the parity line. It is clear that the gene arrangement in BusuNPV is quite different from AcMNPV, BmNPV and OpMNPV, as evidenced from the dispersal of individual contig ORFs. This strongly suggests that BusuNPV is sufficiently distinct to receive a species status in the genus of Nucleopolyhedrovirus (Murphy *et al.*, 1995).

Attempts to find conserved gene clusters in the viral genomes may provide an initial insight in the evolution of baculovirus genomes. The method used in this paper provides a new approach to identify conserved gene clusters among baculovirus genomes. Despite differences in the gene arrangement, certain genes that are clustered in the genome of AcMNPV, BmNPV and OpMNPV, also remained together in the BusuNPV. Eight potential clusters have been identified so far (Fig. 5.5c): 1: Ac13-14-15-23; 2: Ac28-29; 3: Ac55-54-57-60-61; 4: Ac68-71, 5: Ac76-77-78-80-95-98-99-103; 6: Ac129-130; 7: Ac137-138 and 8:

Ac138-142-143-144-147. The numbers refer to the original ORFs in AcMNPV (Ayres *et al.*, 1994). These clusters can be refined when more sequence information of BusuNPV becomes available. Since the genome of OpMNPV is largely collinear with that of AcMNPV, when the order OpMNPV is used as a reference instead of that of AcMNPV, the GeneParityPlot pattern of BusuNPV remains similar (Fig. 5.5d).

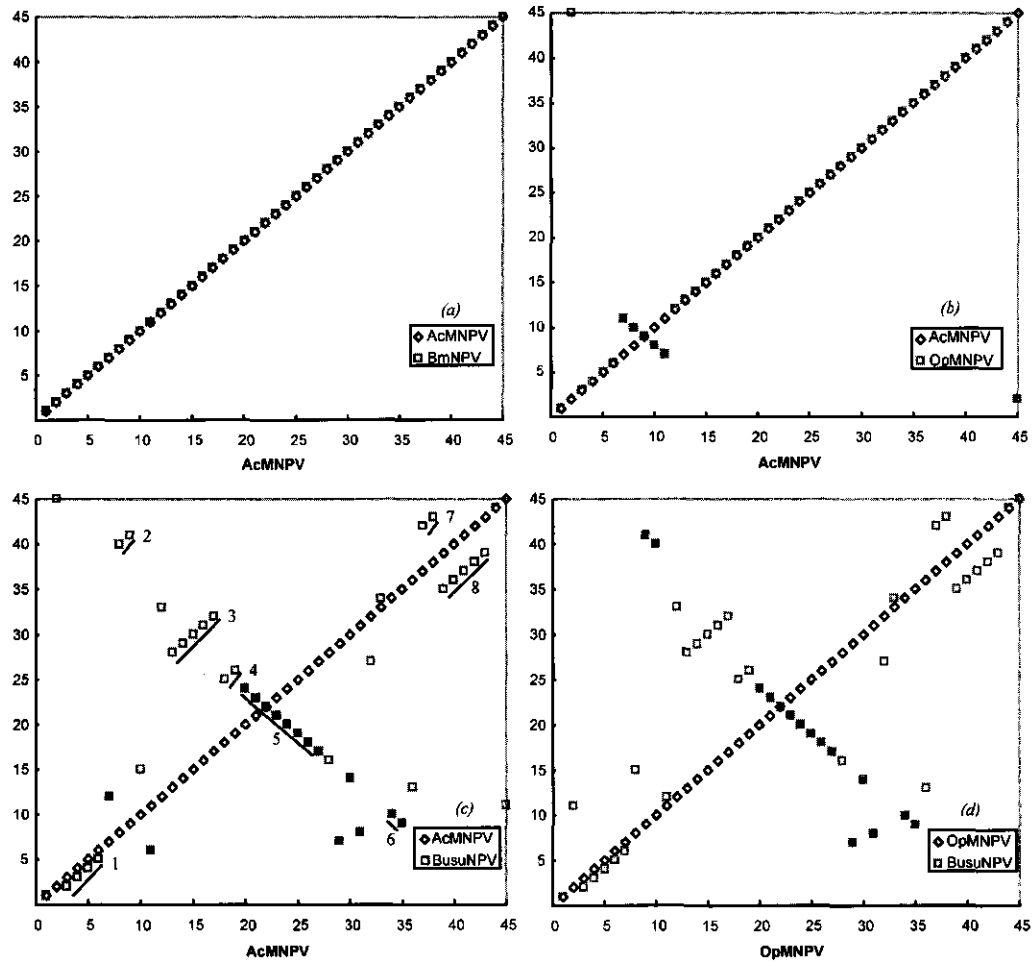



**Figure 5.5.** Graphic representation of the collinearity of baculovirus genomes obtained by GeneParityPlot analysis (see Methods and Table 5.2). *a* BmNPV versus AcMNPV; *b* OpMNPV versus AcMNPV; *c* BusuNPV versus AcMNPV; *d* BusuNPV versus OpMNPV. Eight putative gene clusters of the BusuNPV genome, which are similar to those of AcMNPV, were numbered and underlined. Solid symbols indicate that the direction of the transcription of an ORF is opposite to its homologues in AcMNPV, whereas the open symbols indicate same direction of transcription.

In addition to the common gene clusters described above, BusuNPV contains certain clusters which are also found in some other baculoviruses but not in AcMNPV, OpMNPV or BmNPV. For example, the cluster encompassing Bs47(*lef-6*)-Bs48(Ac29)-Bs49(*p26*)-Bs50(*p10*) (Fig. 5.3), which is absent in the genome of AcMNPV, OpMNPV or BmNPV, also exists in the genome of *Spodoptera exigua* (Se) MNPV (van Strien *et al.*, 1997). This indicates that such arrangement might have already existed before SeMNPV and BusuNPV diverged into different species. Another gene cluster, Bs31(*chiA*)-Bs32(*lef-8*) (Fig. 5.3), is also present in the genome of *Helicoverpa zea* (Hz) SNPV (Genbank U67265). The absence of Ac79 between Bs25 (Ac80; *gp41*) and Bs26 (Ac78) in cluster 5, is also observed in *Spodoptera frugiperda* (Sf) MNPV (Genbank U14725) and HzSNPV (Genbank L04747). It is noteworthy that *cathepsin* and *chitinase*, normally positioned in a head- to -head arrangement in all baculovirus genomes characterized so far, are located distally from each other in BusuNPV. This confirms a recent study (Hawtin *et al.*, 1997) that the function of theses two genes involved in larval melting is not dependent on their proximal position in many baculovirus genomes.

*Gene arrangements of baculovirus genomes as phylogenetic markers*

The study of molecular evolution of baculoviruses has so far been based on the phylogeny of single genes. Zanotto *et al.* (1993), for example, constructed a phylogenetic tree based on polyhedrin. Accordingly, most lepidopteran NPVs fall into two large groups, Group I and Group II. A similar grouping was observed using other baculovirus genes, such as *egt* (Chen *et al*, 1997b). Some of the NPVs mentioned in this paper such as AcMNPV, BmNPV and OpMNPV, belong to Group I, whereas SeMNPV, SfMPNV and HzSNPV (Cowan *et al.*, 1994) are positioned in Group II. BusuNPV is placed in Group II based on the studies of its polyhedrin and EGT sequences (Hu *et. al*, 1993, Hu *et al.*, 1997).

While the genome of AcMNPV, BmNPV and OpMNPV have collinear gene arrangement (Fig. 5.5), it is not known if this holds true for the other baculoviruses in Group I. It appears, however, that the genomic organization of another Group I virus, *Christoneura fumiferana* MNPV (CfMNPV), is basically similar to that of OpMNPV (B.M. Arif, personal communication). Therefore, it is likely that all the baculoviruses belonging to Group I have a similar gene arrangement.

BusuNPV has a quite different gene arrangement as compared to Group I viruses. A few of the different arrangements are shared by some of the group II viruses. Thus, gene arrangement may be used as a marker for phylogenetic study of baculoviruses as proposed by van Strien *et al.* (1997). When the phylogenetic trees based on single genes were evaluated by bootstrap analysis, it was shown that the clustering of group II is far less stable in comparison to group I (Cowan *et al.*, 1994; Hu *et al.*, 1997, Chen *et al*, 1997b). In other words, group II contains viruses that are more diverse than those in group I. Therefore, a collinear arrangement in the genomes of Group II viruses may be less likely. This is supported by the fact that the gene arrangement of SeMNPV, at least over a 20 kb region, is quite different from that of BusuNPV as well as from Group I viruses (van Strien *et al.*, 1997). By applying the GeneParityPlot method presented in this paper, it can be expected that other types and degrees of collinearity could be found when data of more genomes become available.

The mechanisms governing re-arrangement of baculovirus genes remain unclear. It is conceivable that a certain gene cluster might be in some way important for virus replication. For example, the region neighbouring the helicase gene appears to have a highly conserved gene order in baculoviruses as diverse as NPVs and GVs (Heldens *et al.*, 1998). The extensive gene re-arrangement in other regions of the BusuNPV genome apparently did not have adverse effects on virus replication and survival in the natural host. Since the baculovirus family comprises more than 600 members occurring in a variety of arthropod orders and families (Murphy *et al.*, 1995), it is likely that extensive gene re-arrangement has taken place in other species. The gene arrangement in baculoviruses may be, in addition to gene homology, a reflection of their evolutionary history. As more data on the genomic organization of baculoviruses become available, it should be possible to study the phylogeny based on gene arrangement (van Strien *et al.*, 1997) as has been shown with herpesvirus (Hannenhalli *et al.*, 1995). A phylogeny based on gene order is independent from that based on single genes, because the selection pressure for the sequence conservation of a certain gene is more linked to its structure-function relationship. Therefore, the studies of genome arrangement will explore the ancestral history of baculoviruses from a different point of view.

**ACKNOWLEDGEMENTS**

We are grateful to Dr. J.A. Jehle and Dr. E.A. van Strien for critical review of the manuscript. We thank Dr. S. Maeda for providing the order of BmNPV ORFs prior to its publication. This research was supported in part by the National Natural Science Foundation of China (NSFC, grant 39370031) and a joined grant from the Chinese Academy of Sciences (CAS) and the Royal Netherlands Academy of Sciences (KNAW). Z.H. Hu was a recipient of an EC Marie Curie fellowship, a special grant from the chairman of CAS and a sandwich PhD grant from the Wageningen Agricultural University.

CHAPTER 6

# THE SINGLE-NUCLEOCAPSID NUCLEOPOLYHEDROVIRUS OF *BUZURA SUPPRESSARIA* ENCODES A P10 PROTEIN


## SUMMARY

The *p10* gene of *Buzura suppressaria* single nucleocapsid nucleopolyhedrovirus (BusuNPV) was identified by virtue of its localization downstream from the *Autographa californica* (Ac) MNPV *p26* homologue. The BusuNPV *p10* gene encodes a protein of 94 amino acids. The amino acid sequence contains domains characteristic of baculovirus P10 proteins like a coiled-coil domain, a proline-rich motif and a positively-charged C-terminus. The highest amino acid homologies were found with the *Spodoptera littoralis* (Spli) NPV and *Spodoptera exigua* (Se) MNPV P10 proteins. An AcMNPV recombinant expressing the BusuNPV P10 formed fibrillar structures in the cytoplasm of *Spodoptera frugiperda* cells. BusuNPV P10 could not fully replace AcMNPV P10 in its nuclear disintegration function, since polyhedra were not efficiently liberated from infected cells late in infection. The BusuNPV *p26* gene encodes a protein of 263 amino acid residues with 70% amino acid similarity with SeMNPV P26. Downstream of the BusuNPV *p10* gene, the gene for the occlusion-derived virus protein ODVP-6e is located. This is unlike the situation in many other NPVs, including SeMNPV, where the *p10* gene neighbours the *p74* gene. The data presented here suggest that although the *p10* gene is not conserved in sequence, evolutionary pressure preserves the structure of P10 and hence, its function. This data also indicate that all NPVs, MNPVs as well as SNPVs, contain this gene.



This chapter has been published as: Oers, M.M. van, Z. Hu, B.M. Arif, E. A. van Strien, J.W.M. van Lent and Just M. Vlak. 1998. The single-nucleocapsid nucleopolyhedrovirus of *Buzura suppressaria* encodes a P10 protein. *Journal of General Virology* 79, 1553-1562. The nucleotide sequence data reported in this chapter have been deposited in GenBank under the accession number AF034410.

## INTRODUCTION

Polyhedrin and P10 are the major late proteins in insect cells infected with multiple-nucleocapsid nucleopolyhedroviruses (MNPVs). P10 is not a structural component of the virus, but is found as fibrillar structures in the cytoplasm and nucleus of infected insect cells (Croizier *et al.*, 1987; Vlak *et al.*, 1988; Williams *et al.*, 1989; van Oers *et al.*, 1994). It is involved in the release of polyhedra from infected cell-nuclei late in infection (van Oers *et al.*, 1993) and plays a role in polyhedron morphogenesis (Gross *et al.*, 1994; Lee *et al.*, 1996). Results obtained from both sequence and mutational analyses have led to a general model for structural and functional domains in P10 for which the name fibrillin was proposed (van Oers and Vlak, 1997). For the first domain, that comprises approximately the N-terminal half of P10, a coiled-coil structure was predicted (van Oers, 1994; Wilson *et al.*, 1995). This region is probably involved in intermolecular interactions leading to aggregation of P10 molecules. A second domain consists of a short proline-rich sequence that may be responsible for the liberation of polyhedra from the nuclei of infected cells. At the C-terminus, a positively-charged domain is present, that is necessary for the alignment of P10 aggregates into fibrillar structures (van Oers *et al.*, 1993).

So far, only *p10* sequences from MNPVs have been reported and the predicted P10 proteins show a high degree of variability in amino acid sequence (Kuzio *et al.*, 1984; Leisy *et al.*, 1986c; Chou *et al.*, 1992; Zuidema *et al.*, 1993, Hu *et al.*, 1994; Wilson *et al.*, 1995; Zhang *et al.*, 1995; Faktor *et al.*, 1997). The variability among *p10* genes complicates their identification in other baculoviruses. In this paper a *p10* homologue is described in the single-nucleocapsid nucleopolyhedrovirus (SNPV) of *Buzura suppressaria* (BusuNPV). This SNPV is a major pest insect in tea in China (Xie *et al.*, 1979) and a molecular analysis has been initiated. The sequence of the polyhedrin gene of this virus has been published previously (Hu *et al.*, 1993).

A gene coding for a protein of 26 kDa (P26) is located upstream of the *p10* gene in several MNPV genomes (Bicknell *et al.*, 1987; Zuidema *et al.*, 1993; Poloumienko and Krell, 1997; van Strien *et al.*, 1997). This conserved gene arrangement was used to locate a putative *p10* gene in BusuNPV. Once identified, the authenticity and functionality of this SNPV *p10* homologue were tested by expressing it from the AcMNPV genome.

## MATERIALS AND METHODS

*Sequence analysis*

The *Buzura suppressaria* NPV (BusuNPV) isolate, also known as BsSNPV, has been described by Xie *et al.* (1979). A DNA library was made by cloning *Hin*dIII, *Eco*RI, and *Bam*HI restriction fragments of the BusuNPV genome into pTZ19R. Random sequence analysis was performed with standard forward and reverse sequencing primers. The resulting sequences were compared with known baculoviral sequences by using UWGCG FASTA programs (versions 8.1 and 9.0). Based on these results, the sequence of the cloned *Eco*RI-E (pHZH50) and *Hin*dIII-D (pSH25) fragments (Fig. 6.1) was further analysed with specific primers to obtain the complete sequence of the putative *p26* and *p10* genes. The region upstream of *p26* was sequenced up to the *Bam*HI site in the *Hin*dIII-D fragment.

*Transfer of the BusuNPV p10 coding sequence to the AcMNPV genome*

The BusuNPV p10 coding sequence was obtained by PCR technology. The cloned *Eco*RI-E fragment (pHZH50) was used as template and the oligonucleotides 5' CGGGATCCATCATGTCGCAAAATATTTG 3' and 5' CGGGATCCTTATTTTTTCAATCCAGTG 3' as up- and downstream primers, respectively. In this way, the BusuNPV p10 sequence was amplified from nucleotide residue -3 to + 285, relative to the ATG translational start site and *Bam*HI restriction sites were introduced at both ends of this sequence (Fig. 6.4a). The resulting PCR product was digested with *Bam*HI and cloned into pTZ18R. The integrity of the cloned PCR product was confirmed by sequence analysis. Subsequently, it was recloned as a *Bam*HI fragment into plasmid pAcAS3 (Vlak *et al.*, 1990) downstream of the AcMNPV p10 promoter to generate transfer vector pAcMVO7. This transfer vector contains a gene cassette consisting of the *D. melanogaster* hsp70 promoter, the *E. coli* lacZ coding sequence and a SV40 transcriptional terminator, to enable the selection of recombinant viruses. The orientation of the insert was verified by restriction enzyme digestion with *Sph*I and *Ava*II (Fig. 6.4a) and by sequence analysis.

Plasmid pAcMVO7 was cotransfected into Sf21 cells by lipofectin-mediated transfection with DNA of the *p10*-negative parental virus AcMO21, that was linearized at the *p10* locus with *Bsu*36I (Fig. 6.4b; Martens *et al.*, 1995). Recombinant viruses were selected based on their □-galactosidase expression, plaque-purified and high-titre stocks were prepared using standard techniques (King and Possee, 1992). The identity of the resulting recombinant virus AcMVO7 was analysed by restriction enzyme analysis and by PCR using the oligonucleotide 5' GGTCTAGACTGTGTGCAATTGCCGTAC 3', that hybridizes upstream of the AcMNPV p10 promoter, and the downstream BusuNPV p10 primer mentioned above.

As a control in the experiments, recombinant AcMO16 was used (Fig. 6.4b; van Oers *et al.*, 1993). This recombinant contains the AcMNPV p10 sequence from -3 to +282 nt in the same up- and downstream context as the BusuNPV p10 sequence in the recombinant AcMVO7.

*Protein analysis*

*Spodoptera frugiperda* (Sf21) cells (Vaughn *et al.*, 1977) were grown and maintained in Hink's insect medium (Hink, 1970) supplemented with 10% fetal calf serum at 27□C. Cells were infected at a multiplicity of infection (m.o.i.) of 10 $TCID_{50}$ units with AcMNPV wild type (wt), the p10 deletion mutant AcMO21, the recombinant AcMVO7 encoding BusuNPV P10 and the control recombinant AcMO16. Infected cells were harvested at 48 h post infection (p.i.), washed twice with phosphate-buffered saline (PBS) and resuspended in PBS supplemented with 120 mM Tris-HCl, pH 6.8; 1.25% SDS, 425 mM ß-mercaptoethanol, 6% w/v Ficoll and 0.001% w/v bromophenol blue. The protein pattern was analysed in a 16.5 % Tricine-SDS PAGE system, according to Schägger and Von Jagow (1987), enabling the separation of polypeptides ranging from 10 to 40 kDa.

*Phase-contrast and electron microscopy*

Sf21 cells were infected with the recombinants AcMO16, AcMO21 and AcMVO7 at a m.o.i. of 10 $TCID_{50}$ units and incubated at 27□C. Infected insect-cell cultures were examined at 5 days p.i. with a Leitz Labovert phase-contrast microscope. For electron microscopy, infected cells were harvested at 48 h p.i. and embedded as described by van Lent *et al.* (1990). Ultrathin sections

were cut with a Reichert-Jung Ultracut microtome and examined with a Philips CM12 electron microscope.

## RESULTS

*The BusuNPV p26 gene*

Randomly cloned restriction fragments of BusuNPV DNA were partially sequenced with standard primers hybridizing to vector sequences. In this way, the 3' sequence of the *p26* gene was found at one end of the 8.8 kb *Eco*RI-E fragment by comparison with known baculovirus sequences (Fig. 6.1). Previously, the other end of this fragment was shown to harbour polyhedrin gene sequences (Hu *et al.*, 1993). The sequence of the 3' end of the *p26* gene was completed with specific primers hybridizing internally in *p26*. The 5' end of *p26* and its upstream flanking region were obtained by sequencing the *Hin*dIII-D fragment up to the internal *Bam*HI site (Fig. 6.1).

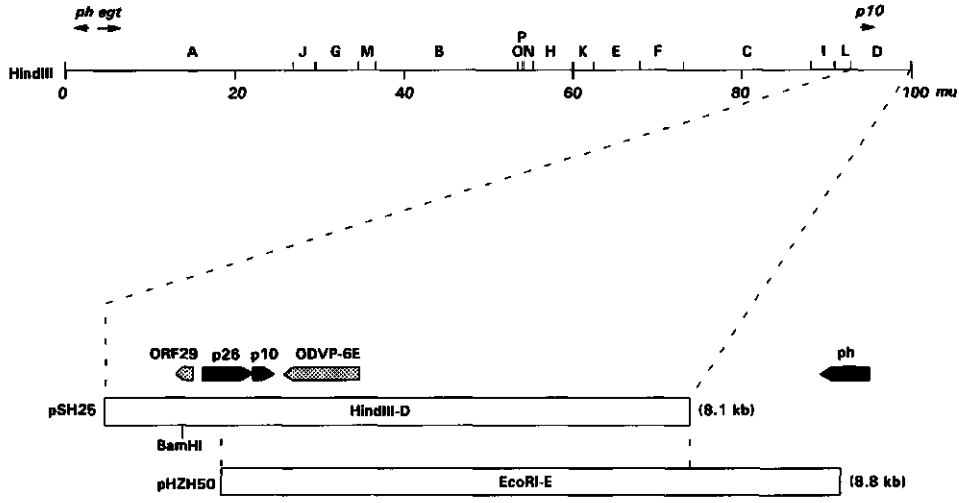



**Figure 6.1.** Linear physical map of the BusuNPV genome for restriction endonuclease *Hin*dIII. Below the cloned *Hin*dIII-D (pSH25) and *Eco*RI-E (pHZH50) fragments are indicated and the genes identified thus far on these fragments. ph = polyhedrin.

The BusuNPV *p26* gene has an open reading frame of 789 nt, potentially coding for a protein of 263 amino acids with a putative mass of 30.7 kDa (Fig. 6.2). Upstream of the p26 ORF, a TAAG motif characteristic for baculovirus late promoters, is located at -15 nt relative to the putative ATG start codon. Further upstream at position -141 a CAGT motif is found, characteristic of baculovirus early gene mRNA start sites (Blissard *et al.*, 1992; Pullen and Friesen, 1995). The amino acid sequence of the predicted P26 protein was aligned with other known P26 sequences (data not shown) and was most homologous to SeMNPV P26 (69.7 %). Several amino acid sequences are well conserved in P26, like the HQFPGV, GAPI, LVSVVT and QLPY motifs (Fig. 6.2, printed in bold). The function of P26 in the infection process is not clear yet, but it is non-essential for

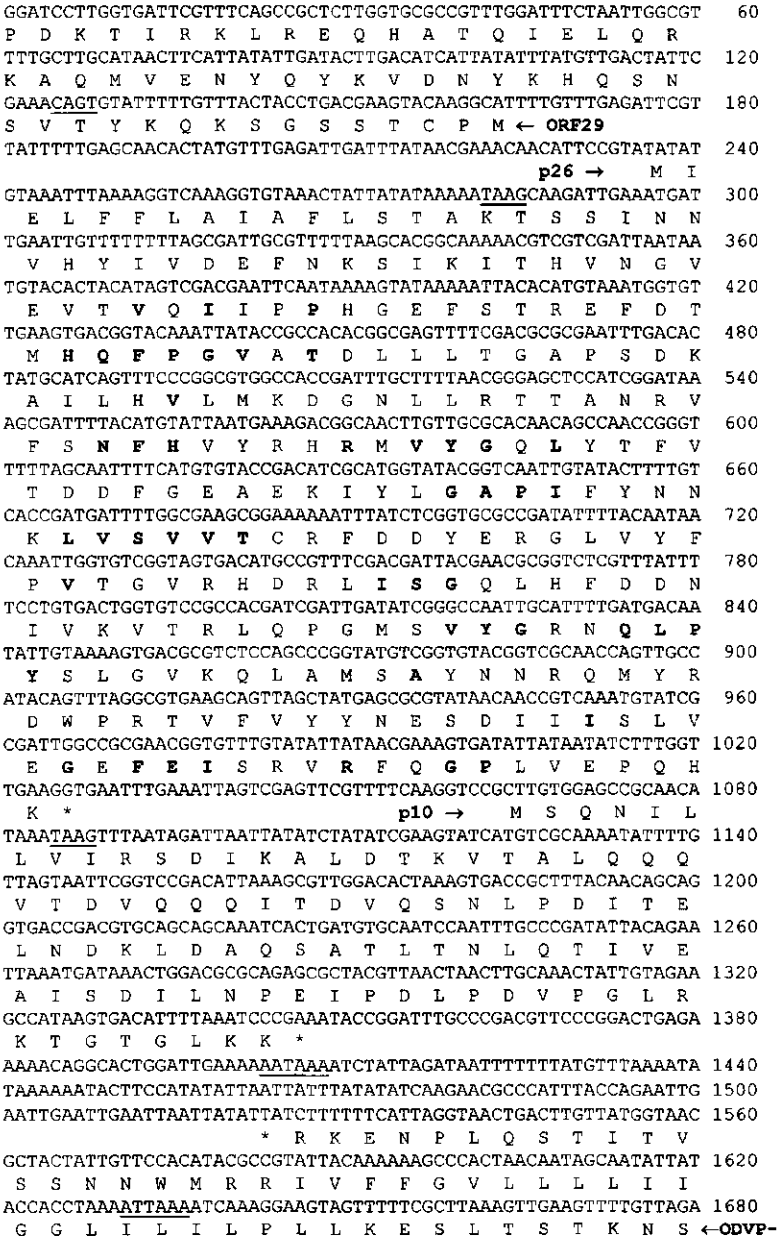



**Figure 6.2.** Nucleotide sequence of a 1680 bp region of the BusuNPV genome, starting from the *Bam*HI site in the *Hin*dIII-D fragment and containing the *p26* and *p10* genes. The p26 ORF starts at residue 296 and terminates at residue 1085. Conserved amino acid residues in the P26 protein are printed in bold. The p10 coding sequence starts at residue 1123 and ends at residue 1405. Underlined are the CAGT, TAAG and putative poly(A) motifs. The BusuNPV *odvp-6e* homologue is located downstream of *p10* in opposite orientation.

AcMNPV replication in cell culture (Rodems and Friesen, 1995). Screening the EMBL and GenBANK databases for sequences homologous to either the complete P26 or the conserved motifs mentioned above did not provide further information as to the possible function of P26. Upstream of the *p26* gene, a partially-sequenced ORF is located in the opposite orientation that extends beyond the *Bam*HI site and shows homology with the AcMNPV ORF 29 (Ayres *et al.*, 1994).

*The BusuNPV p10 gene*

In order to find the BusuNPV *p10* gene, the region downstream of the *p26* gene was sequenced. This strategy was based on the collinear arrangement of *p26* and *p10* genes in several MNPVs. An ORF of 282 nt was found downstream of *p26* on the *Eco*RI-E fragment (Fig. 6.1) encoding a putative protein of 94 amino acids with a predicted molecular mass of 10.2 kDa. It is most homologous to the SpliNPV (63.2 % identity) and SeMNPV (61.9 % identity) P10 proteins (Fig. 6.3). The amino terminal half of the predicted protein contains several heptad-repeat sequences, in which the first and fourth amino acid are occupied by hydrophobic amino acids, as has been found in other P10 proteins (Fig. 6.3, dark shading). Like P10 proteins of MNPVs, the BusuNPV P10 protein has a proline-rich domain (PEIPDLPDVP; Fig. 6.3, shaded light grey), where prolines are surrounded by hydrophobic and negatively charged residues and a positively-charged carboxy-terminal domain (RKTGTGLKK; Fig. 6.3, shaded deep grey).

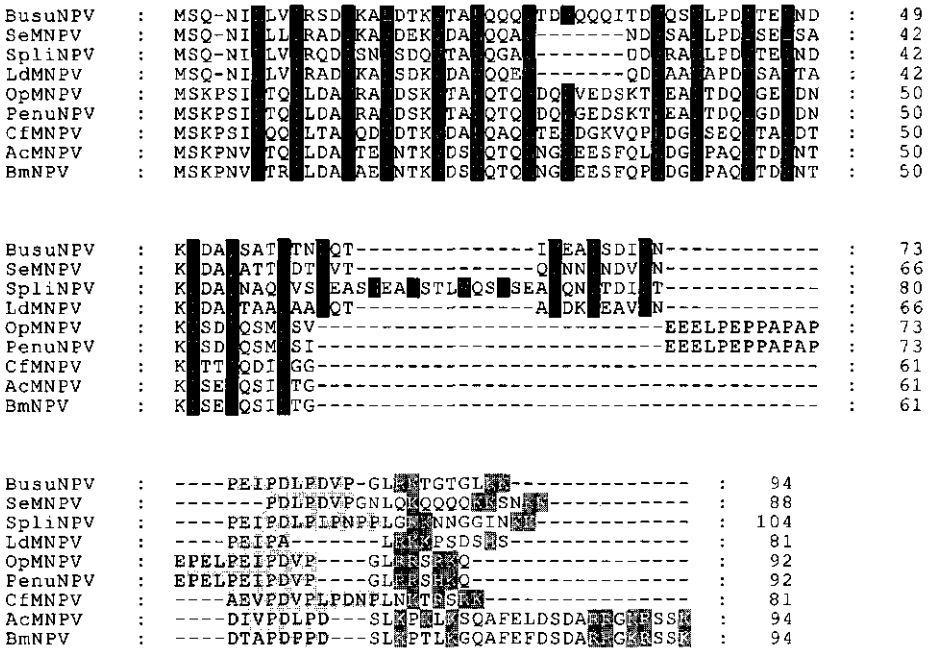



**Figure 6.3.** Sequence alignment of BusuNPV P10 with the P10 proteins of other nucleopolyhedroviruses. Black shading is used to indicate positions 1 and 4 in heptad-repeat sequences in the amino-terminal domain, light grey shading points towards a conserved proline-rich domain and dark grey is used to show the positively-charged residues in the C-terminal region of the various P10 proteins. The abbreviations of the various baculoviruses are explained in the text.

The 5' flanking region of BusuNPV p10 contains a consensus baculovirus late promoter motif TAAG, that overlaps with the translational stop codon (TAA) of the *p26* open reading frame. Thirty-five nucleotides separate the TAAG motif and the ATG start codon. The use of the TAAG motif as transcriptional start site would result in a p10 transcript with a short, unstructured 5' untranslated region (UTR) with a GC content of approximately 20%. At the 3' end, a putative polyadenylation signal (AATAAA) was found encompassing the translational stop codon. An alternative signal (ATTAAA) is present 233 nt downstream of the TAA stop codon. This ATTAAA motif is followed by GT-rich sequences, commonly found 30-40 nt downstream of active poly(A) signals (Edwalds-Gilbert *et al.*, 1997). The two ATTAAA motifs present in the BusuNPV p10 ORF are not followed by such GT-rich sequences.

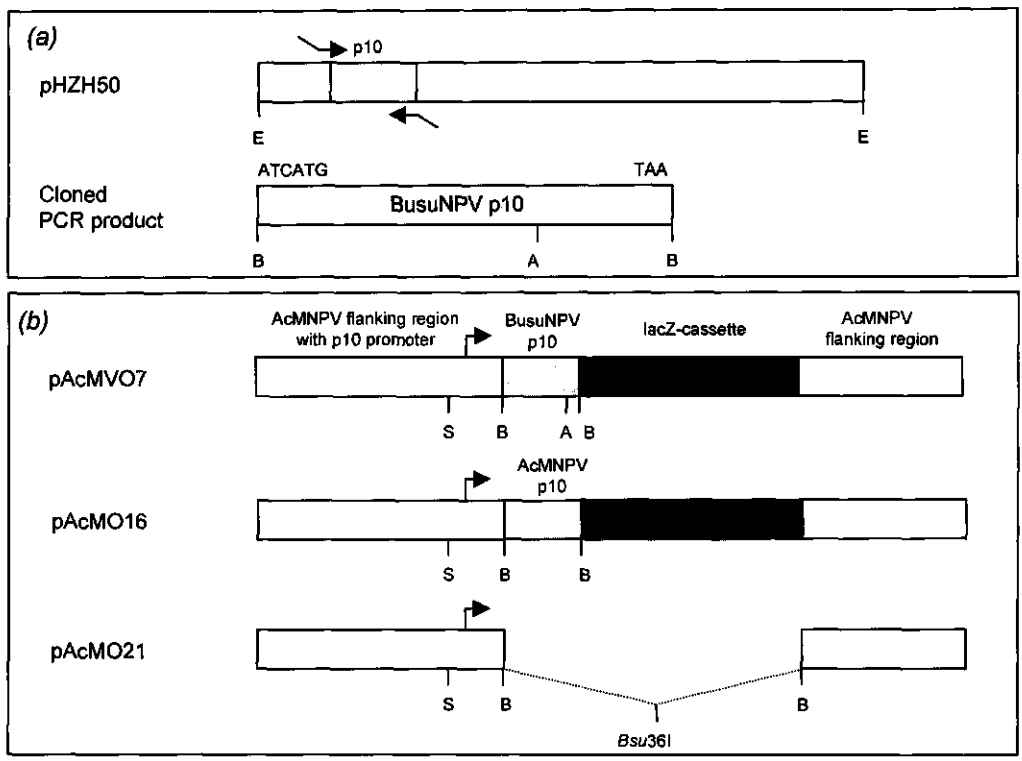
Another ORF (Fig. 6.1) with homology to the AcMNPV 'occlusion derived viral protein' gene (*ODVP*-6e) (Theilmann *et al.*, 1996) was found downstream of the *p10* gene (Xinwen Chen, personal communication). This ORF is oriented in opposite direction to *p10* and is separated from the p10 coding sequence by 100 nt. This means that BusuNPV differs from AcMNPV, BmNPV, CfMNPV, OpMNPV and SeMNPV in that in the genome the *p10* gene is not followed by the *p74* gene (Kuzio *et al.*, 1989; Leisy *et al.*, 1986c; Hill *et al.*, 1993; Zuidema *et al.*; 1993; Palhan and Gopinathan, 1996). The BusuNPV *p74* gene was found in another part of the genome (Hu Zhihong, personal communication).

*Functionality of BusuNPV P10*

Due to the absence of a system to generate BusuNPV recombinants or deletion mutants, the functionality of the BusuNPV P10 protein was studied in Sf21 cells by replacing the AcMNPV *p10* coding sequence with that of BusuNPV. To this aim the BusuNPV *p10* coding sequence was obtained by PCR technology and cloned under control of the AcMNPV p10 promoter in the transfer vector pAcAS3 (Vlak *et al.*, 1990). The recombinant virus AcMVO7 was made (Fig. 6.4b) by recombination with the AcMNPV p10 deletion mutant AcMO21 (Martens *et al.*, 1995). As a positive control, the recombinant AcMO16 (van Oers *et al.*, 1993) was used, in which the AcMNPV p10 ORF is in the same up- and downstream sequence context as the BusuNPV p10 ORF in AcMVO7. Infection of Sf21 cells with the recombinant AcMVO7 led to the synthesis of a protein of approximately 10 kDa (Fig. 6.4c, lane 5) that was not observed in cells infected with the *p10* deletion mutant AcMO21 (lane 4). The BusuNPV P10 protein is comparable in size to AcMNPV P10 (lanes 2 and 3), as was expected from the sequence data (Fig. 6.2, 6.3).

Sf21 cells infected with the recombinant AcMVO7 were analysed by electron microscopy to see whether P10-specific structures were formed. Fibrillar structures were observed in the cytoplasm (Fig. 6.5a) and resembled fibrillar structures found in AcMNPV and AcMO16- infected cells (Fig. 6.5d). In the nucleus of AcMVO7-infected cells, large amorphous structures were formed (Fig. 6.5b). These structures differed significantly from those in the cytoplasm and from the nuclear, fibrillar structures known from AcMNPV P10. The amorphous, nuclear structures were clearly distinct from the virogenic stroma and appeared to interact with electron-dense spacers (Fig. 6.5b) like fibrillar structures in AcMNPV-infected cells. Both fibrillar and amorphous structures, must be induced by the BusuNPV P10 homologue, since they were absent from cells infected with the p10 deletion mutant AcMO21 (data not shown). The polyhedra of the recombinant are surrounded by

polyhedral envelopes (Fig. 6.5c) as in wild type AcMNPV infections.




(c)

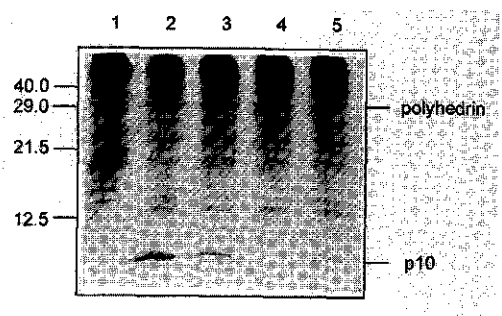



**Figure 6.4. (a)** Schematic overview of the amplification of the BusuNPV p10 coding sequence with the cloned *Eco*RI-E fragment used as template in the PCR. The arrows indicate the positions of the primers. **(b)** Representation of the *p10* locus in the recombinant AcMVO7, encoding BusuNPV P10, the AcMNPV P10 encoding recombinant AcMO16 and the p10 deletion mutant AcMO21. A=*Ava*II, B=*Bam*HI, E=*Eco*RI, S=*Sph*I. **(c)** 16.5% Tricine-SDS-PAGE of Sf21 cells (1) infected for 48 h with wild type AcMNPV (2), AcMO16 (3), AcMO21 (4) and AcMVO7 (5). Each lane contains the equivalent of 3.5 x $10^4$ cells. Molecular weight markers are indicated in kDa.

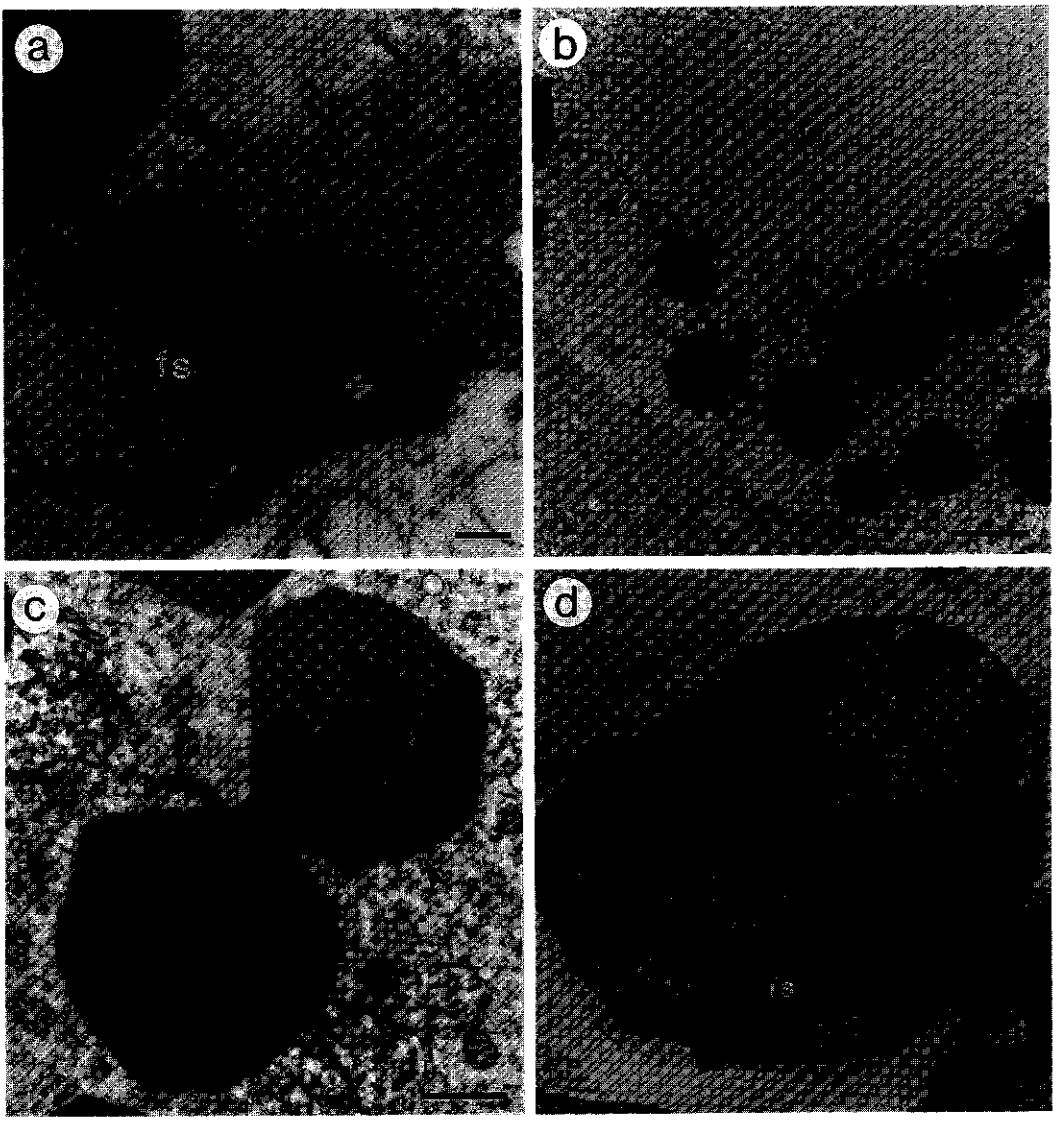



**Figure 6.5.** Electron microscopic images of Sf21 cells 48 h p.i. with AcMVO7, expressing BusuNPV P10, and AcMO16, expressing AcMNPV P10. **(a)** Cytoplasmic fibrillar structure in AcMVO7-infected cells. **(b)** Amorphous nuclear structure induced by AcMVO7; **(c)** AcMVO7 polyhedra surrounded by polyhedron envelopes. **(d)** Nuclear and cytoplasmic fibrillar structures in Sf21 cells infected with AcMO16. fs = f ibrillar structure; as = amorphous structure; arrows indicate electron dense spacers (b) and (d) or the polyhedral envelope (c). The bars correspond to 0.2 μm, 1 μm, 0.5 μm and 2 μm in (a), (b), (c) and (d), respectively.

One of the functions ascribed to P10 is the release of occlusion bodies from the nuclei of infected cells (van Oers *et al.*, 1993). In order to study this function of BusuNPV P10, Sf21 cells were infected with AcMVO7, AcMO16, and with the p10 deletion mutant AcMO21. Five days p.i. cells were examined for the release of polyhedra (Fig. 6.6). Cells infected with AcMO16 (Fig. 6.6a) released large numbers of polyhedra due to the presence of wild type P10 protein. Cells infected with the p10 deletion mutant AcMO21 (Fig. 6.6b) did not release polyhedra. The recombinant AcMVO7 expressing BusuNPV P10 (Fig. 6.6c) released very few polyhedra from infected cell nuclei, as compared to AcMO16. Even at 10 days p.i., the majority of polyhedra were still captured within the nuclei (data not shown).

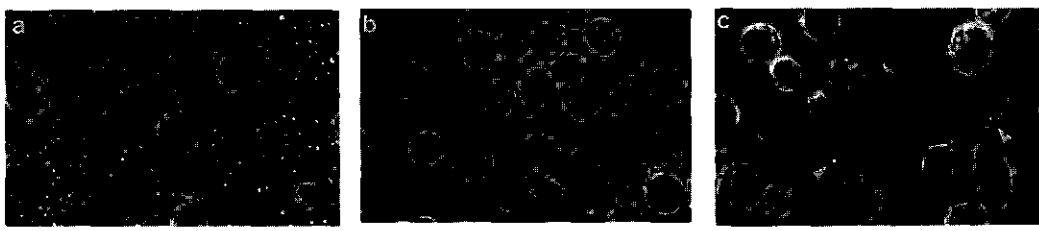



**Figure 6.6.** Phase-contrast images of Sf21 cells infected with **(a)** the recombinant AcMO16, expressing AcMNPV P10, **(b)** the p10 deletion mutant AcMO21 or **(c)** recombinant AcMO7, expressing BusuNPV P10 at five days p.i. Arrows indicate polyhedra released from AcMVO7-infected cells.

## DISCUSSION

P10 proteins accumulate to high levels at the very late stage of an MNPV infection. In general, the amino acid sequence homology among the P10 proteins is low, as the consequence of highly diverged nucleotide sequences. This precludes the detection of *p10* in other baculovirus genomes by nucleic acid hybridisation. In the genomes of AcMNPV, CfMNPV, OpMNPV, BmNPV and SeMNPV (Liu *et al.*, 1986; Bicknell *et al.*, 1987; Zuidema *et al.*, 1993; Wilson *et al.*, 1995; Palhan and Gopinathan, 1996; Poloumienko and Krell, 1997) the *p10* gene is preceded by the *p26* gene. The conserved gene arrangement of *p26* and *p10* was used to identify the *p10* gene of an SNPV, namely that of BusuNPV. Random sequence analysis of restriction fragments of BusuNPV DNA led to the identification of *p26*, which is located on the *Hin*dIII-D fragment. Sequencing downstream of *p26* revealed an ORF of 282 nt potentially encoding a protein with features characteristic for a P10 protein: N-terminal heptad-repeat motifs, a proline-rich domain and a positively-charged C-terminal domain (see review van Oers and Vlak, 1997). This strongly suggest that this ORF encodes a P10 homologue. This is the first report of a *p10* gene in an SNPV and it may suggest that such a gene is preserved in all NPVs.

Downstream of the *p10* gene the genomic map of BusuNPV is different from MNPVs and shows an ORF homologous to the *ODVP-6e* gene of OpMNPV and *Cydia pomonella* granulovirus (Theilmann *et al.*, 1996). In the genomes of AcMNPV, BmNPV, CfMNPV, OpMNPV and SeMNPV, the *p74* gene has been found at this position (Kuzio *et al.*, 1989; Leisy *et al.*, 1986c; Hill *et al,* 1993; Zuidema *et al.*; 1993; Palhan and Gopinathan, 1996).

The putative BusuNPV P10 protein formed fibrillar structures when expressed under control of the AcMNPV p10 promoter in recombinant AcMVO7. This observation confirms that the 282 nt ORF encodes the BusuNPV P10 protein. The nuclear structures induced by this protein had a less fibrillar appearance than AcMNPV nuclear fibrillar structures. The fact that the nuclear structures interacted with electron-dense spacers, as do AcMNPV nuclear fibrillar structures, and that polyhedral envelopes were formed around AcMVO7 polyhedra, indicate that BusuNPV P10 assists in each aspect of AcMNPV polyhedron morphogenesis. Previously, it has been shown that SeMNPV P10 could replace its AcMNPV homologue in this function (van Oers *et al.*, 1994). The present result confirms the value of the 'swapping' assay for the functional analysis of putative P10 proteins.

In AcMNPV-infections P10 is responsible for the disintegration of the nuclei at the final stage of infection and thus for the dissemination of separate polyhedra into the environment (Williams *et al.*, 1990; van Oers *et al.*, 1993). BusuNPV P10 protein released very few polyhedra when present in an AcMNPV environment (Fig. 6.6c). This parallels a previous observation (van Oers *et al.*, 1994) that SeMNPV P10 protein could not perform this function when present in an AcMNPV environment, even though the cells were permissive to SeMNPV. On the other hand, SeMNPV P10 was perfectly able to release polyhedra from the same cells when they were infected with SeMNPV (van Oers *et al.*, 1994). The results obtained here further support the view that P10 proteins show specificity in performing this function and that other viral factors seem to contribute to this process. However, we can not rule out the possibility that the amount of BusuNPV P10 protein produced during infection with the recombinant AcMVO7 is insufficient to achieve an efficient disintegration of the cell nuclei.

**ACKNOWLEDGMENTS**

We thank George Rohrmann for providing *Lymantria dispar* MNPV p10 sequence prior to publication. The authors acknowledge Xinwen Chen for his contribution to the sequence analysis and Magda Usmany for technical assistance in insect cell culture.

CHAPTER 7

# SPECIFICITY OF POLYHEDRIN IN THE GENERATION OF BACULOVIRUS OCCLUSION BODIES


## SUMMARY

The role of polyhedrin gene in the occlusion of virions was studied by swapping two heterologous polyhedrin coding sequences, one from a multiple nucleocapsid (M) nuclepolyhedrovirus (NPV) of *Spodoptera exigua* (Se), and one from a single-nucleocapsid (S) NPV of *Buzura suppressaria* (BusuNPV) into the genome of *Autographa californica* (Ac) MNPV. Both heterologous polyhedrin genes were highly expressed and polyhedra were produced in the nuclei of cells infected with the respective recombinant AcMNPVs. Polyhedra produced by the recombinant with BusuNPV polyhedrin showed normal occlusion of multiple-nucleocapsid virions and were equally infectious to *S. exigua* larvae as wild type AcMNPV. This indicates that virion occlusion is not specific with respect to whether the virions or polyhedrin are from an SNPV or MNPV. Polyhedra produced by the recombinant containing the SeMNPV polyhedrin, had an altered morphology being pyramidal rather than polyhedral in shape and with much fewer virions occluded. These occlusion bodies were less infectious to *S. exigua* larvae than those of wild-type AcMNPV. These results indicate that the virion occlusion is a fine-tuned process, which is to some extend specific to the polyhedrin involved, and may also require other viral or host factors for optimal morphogenesis.



This chapter has been submitted for publication as: Hu, Z.H., T. Luijckx, L. van Dinten, M.M. van Oers, J.P. Hajós, F.J.J. A. Bianchi, J. W.M. van Lent, D. Zuidema and J.M. Vlak. Specificity of polyhedrin in the generation of baculovirus occlusion bodies.

## INTRODUCTION

Baculoviruses are a large group of viruses which are pathogens for arthropod, mainly insect, and which occlude their virions in large, proteinaceous capsules or occlusion bodies (OBs). The Baculoviridae family is composed of two genera, Nucleopolyhedrovirus (NPV) and Granulovirus (GV) (Murphy *et al.*, 1995). In GV a single virion is occluded in an OB, whereas in NPVs many virions are occluded. According to the number of nucleocapsids occluded in virions, NPVs are distinguished in single-nucleocapsid (S) NPVs and multiple-nucleocapsid (M) NPVs. The genetic basis for the difference in occlusion strategy is unknown.

The baculovirus replicative cycle is biphasic generating two distinct progeny phenotypes; the budded virus (BV) needed for the dissemination of infection within insect tissues and the occlusion derived virus (ODV) for spreading the infection to other susceptible larvae. The function of the OB is most likely to protect the ODVs against physical and (bio)chemical decay and to allow the virions to retain their biological activity outside the host even for many years. Furthermore, OBs might protect ODVs against proteolytic decay during the last stages of infection. OBs are surrounded by a carbohydrate-rich structure which is called calyx (Minion *et al.*, 1979). The major protein component of the calyx is the polyhedron envelope protein (PEP) which seems to be associated with the carbohydrate residues of the calyx via a thiol-glycosidic linkage through cysteine residues (Whitt and Manning, 1988; Gombart *et al.*, 1989). The calyx is important in preventing polyhedron aggregation and further protecting polyhedra from mechanical damage (Zuidema *et al.*, 1989; Gross *et al.*, 1994).

The factors directing the ODV occluded into OBs are not well known. Polyhedrin and ODVs are likely to be the two main entities directly involved in ODV-occlusion. Furthermore, it has been shown that a mutation in the 25K protein results in abnormal ODV envelopment, lack viral occlusion and reduced OB formation (Harrison and Summers, 1995a, *ibid.* 1995b). Since the 25K protein is not a component of ODVs and a mutation in the 25K gene resulted in reduced polyhedrin synthesis and nuclear localization (Jarvis *et al.*, 1992), the 25K protein may play an indirect role in the virion occlusion process (Harrison and Summers, 1995a, *ibid.* 1995b).

Polyhedrin has been the subject of several reviews because of its unique properties and its pivotal role in the dissemination and survival of the virus (Vlak and Rohrmann, 1985; Rohrmann, 1986, *ibid.* 1992). So far, about 30 polyhedrin genes of different baculoviruses have been sequenced and they exhibited a highly conserved structure with amino acid identity of at least 70% among lepidopteran NPVs. Research on the polyhedrin gene of *Autographa californica* (Ac) MNPV has revealed a nuclear localization signal (KRKK) located at amino acids 32 to 35 and a domain required for assembling into occlusion-like structures at amino acid 19 to 110 (Jarvis *et al.*, 1991). In contrast to the extensive sequence information, little is known on how polyhedrin plays its role in the occlusion process. For example, what determines the size and form of polyhedra, and are polyhedrins specific in their occlusion of single or multiple ODVs? Although polyhedrin is a highly conserved protein, it is not known whether all its functional domains and recognition signals are universal to all NPVs.

In this study, we analysed the role of two polyhedrins, one is from *Spodoptera exigua* (Se) MNPV (van Strien *et al.*, 1992) and the other is from an SNPV, *Buzura suppressaria* (Busu)NPV (Hu *et al.*, 1993), in the occlusion process. The question was addressed whether an SNPV polyhedrin would occlude ODVs from a MNPV and whether there are other viral specific factors involved in the occlusion process. To this end recombinant AcMNPVs were constructed by replacing the AcMNPV polyhedrin coding sequence with the homologous sequence of either BusuNPV or SeMNPV. As SeMNPV OBs are considerably smaller on average than AcMNPV, the study should also shed light on to what extent the size and shape of OBs are determined by the polyhedrin itself or by other viral or host factors. The resulting recombinants were examined biochemically, by electron microscopy and in bioassays.

## MATERIALS AND METHODS

*Cells and viruses*

The *Spodoptera frugiperda* cell line IPLB-Sf21-AE (Sf21) (Vaughn *et al.*, 1977) was maintained in TNM-FH medium (Hink, 1970) supplemented with 10% fetal calf serum at 27° C. The C6 strain of AcMNPV (Ayres *et al.*, 1994) was used as wild-type control; its polyhedrin-negative mutant AcMNPV-PAK6 (Kitts and Possee, 1993) was used as parental virus for construction of recombinants. Sf21 cells were infected with a multiplicity of 10 TCID50 units per cell as described previously (van Oers *et al.*, 1994).

*Generation of transfer vectors and recombinants*

Transfer vector pAc-BsPH was constructed containing the polyhedrin gene of BusuNPV (Hu *et al.*, 1993) in an AcMNPV transfer vector. The ORF was amplified by PCR from plasmid pBsPD containing the 7.8 kb *Pst*I-D fragment of BusuNPV DNA (Hu *et al.*, 1998). PCR was carried out using 5' GG*AGATCT* ATA ATG TAC ACT CGT TAC 3' as the forward primer and 5' GG*AGATCT* TTA ATA TGC AGG ACC TGT 3' as the reverse primer. The PCR product carried *Bgl*II (shown in italics in the primers) sites at either end and was cloned into pAcJR1 (Roosien *et al.*, 1986) to give pAc-BsPH (Fig. 7.1).

Transfer vector pAc-SePH was constructed using the polyhedrin gene of SeMNPV as template (van Strien *et al.*, 1992). The coding sequence was isolated by PCR from plasmid pSeSDN containing the 3.6 kb *Nru*I fragment derived from the *Sph*I-D fragment of SeMNPV DNA. PCR was carried out using 5' GG*AGATCT* ATA ATG TAT ACT CGC TAC 3' as the forward and 5' GG*AGATCT* TTA ATA GGC GGG TCC GTT 3' as the reverse primers, respectively. The PCR product carried *Bgl*II sites at either end and was cloned into pAcJR1 to give pAc-SePH (Fig. 7.1).

As a control, the AcMNPV polyhedrin coding sequence, was amplified by PCR from the plasmid pAcMK10 containing the 7.2 kb *Eco*RI-I fragment of AcMNPV DNA. PCR was carried out using the forward primer 5' GG*AGATCT* AAT ATG CCG GAT TAC TCA 3' and the reverse primer 5' GG*AGATCT* TTA ATA CGC CGG ACC AGT 3'. The PCR product carried *Bgl*II sites at either end and was cloned into pAcJR1 to give pAc-AcPH.

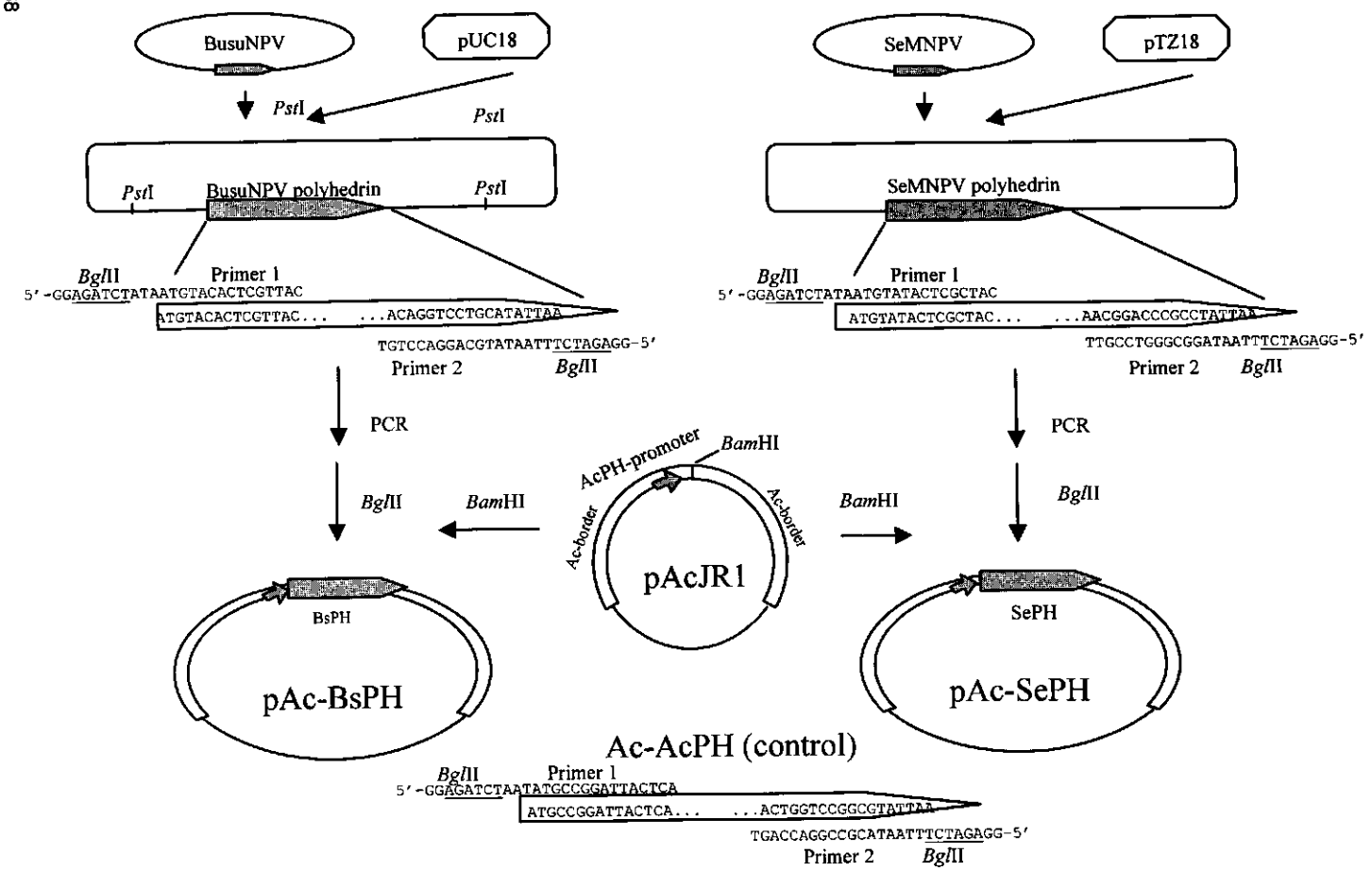



**Figure 7.1.** Construction scheme for transfer vectors pAc-BsPH, pAc-SePH and pAcAcPH, using parental AcMNPV transfer vector pAcJR1 and PCR products from BusuNPV, SeMNPV and AcMNPV polyhedrin genes. The open reading frames of the various polyhedrin genes was cloned downstream of the AcMNPV promoter. The primer sequences and restriction sites are indicated.

Sf21 cells (2 x $10^6$) were transfected with 5 μg of transfer vector and 1 μg of *Bsu*36I-linearized AcMNPV-PAK6 (Kitts and Possee, 1993) DNA using Lipofectin™ (GIBCOBRL/Life Technologies). The recombinant viruses AcMNPV-AcPH, AcMNPV-BsPH and AcMNPV-AcPH were purified from polyhedra-containing plaques and their DNA was analysed by restriction enzyme digestion and gel electrophoresis. To ensure that the inserted polyhedrin did not contain mutations, PCR was performed using recombinant virus DNA as template with the same primers that were used in the construction of the transfer vectors. The PCR products were cloned into pTZ19R and sequenced.

*Protein analysis*

Sf21 cells were infected with wild-type AcMNPV (C6) or with one of the recombinant viruses AcMNPV-AcPH, AcMNPV-BsPH and AcMNPV-SePH at a multiplicity of infection (m.o.i.) of 10 $TCID_{50}$ units/cell and were harvested at 48 h p.i. The protein pattern was analysed by SDS-PAGE in a 12.5% polyacrylamide gel according to van Oers *et al.* (1994). Purified polyhedra of wild-type AcMNPV, SeMNPV and BusuNPV were loaded on the same denaturing gel for comparison of the polyhedrin sizes.

*Electron microscopy*

Sf21 cells infected with wt AcMNPV or with the recombinant viruses AcMNPV-AcPH, AcMNPV-BsPH and AcMNPV-SePH at a m.o.i. of 10 TCID50 units/cell were harvested at 52 h p.i. and processed for electron microscopy as described previously (van Lent *et al.*, 1990).

*Insect bioassays*

Polyhedra from wt AcMNPV (Ayres *et al.*, 1994) and recombinants were fed to late third-instar *S. exigua* larvae to obtain passage 1 polyhedra for the bioassay. Polyhedra isolated from the infected insects were then used in a bioassay using the modified droplet-feeding method as previously described by Hughes and Wood (1981). Suspensions of $10^4$, $10^5$, $3x10^5$, $10^6$, $10^7$ and $10^8$ polyhedra per ml were chosen for wt-AcMNPV and AcMNPV-AcPH, and suspensions of $10^4$, $10^5$, $10^6$, $10^7$, $10^8$ and $3x10^8$ polyhedra per ml were chosen for AcMNPV-BsPH and AcMNPV-SePH. For each concentration, 36 early third-instar *S. exigua* larvae were tested. Larvae were incubated at 28° C and mortality was recorded seven days later. Median doses of ingested polyhedra were calculated with the median volume ingested by third instar *S. exigua* larva (0.55 μl) (F.J.J.A. Bianchi, unpublished results) and the polyhedra concentrations of the suspensions. The dose-mortality data were analysed by probit analysis, using the computer program POLO (Russell *et al.*, 1977).

## RESULTS

*Generation of recombinants*

To generate AcMNPV recombinants carrying the polyhedrin gene from either BusuNPV or SeMNPV, the transfer vector plasmids pAc-BsPH and pAc-SePH (Fig. 7.1) containing the BusuNPV and SeMNPV polyhedrin coding sequence, respectively, in an AcMNPV transfer vector were constructed. Sequence analyses confirmed that the polyhedrin genes were inserted correctly into the various transfer vectors. Sf21 cells were co-transfected with either vector and

the AcMNPV-PAK6 (Kitts and Possee, 1993) by using lipofection. AcMNPV-PAK6 lacks the polyhedrin gene and recombinants can thus be easily recognized by the appearance of polyhedra. Recombinant AcMNPV-AcPH was made where the native polyhedrin coding sequence was re-introduced into the AcMNPV-PAK6 and used as a control to assess any cloning artefacts. The recombinants were plaque-purified and their DNA were analyzed by restriction enzyme digestion and agarose electrophoresis. The data (not shown) confirmed that the polyhedrin coding sequences were inserted at the correct location and orientation in AcMNPV-PAK6. The polyhedrin genes were recloned as PCR product from the respective recombinant viruses and the sequences were found to be identical to the published sequences (Hooft van Iddekinge *et al.*, 1983; van Strien *et al.*, 1992; Hu *et al.*, 1993).

Sf21 cells infected with AcMNPV-AcPH, AcMNPV-BsPH and AcMNPV-SePH were harvested at 72 h after infection. Protein of the cells were analyzed by SDS/PAGE and compared to polyhedrin protein from wild-type AcMNPV, BusuNPV and SeMNPV (Fig. 7.2). The data showed that the polyhedrin genes of BusuNPV, SeMNPV and AcMNPV were correctly expressed by the recombinants and had the same size as the polyhedrin present in wild-type BusuNPV, SeMNPV and AcMNPV polyhedra. For example, the size of wt SeMNPV polyhedrin is about 2 kDa smaller than that of wt AcMNPV (Caballero *et al.*, 1992), this is also the case for the proteins expressed by the recombinants (Fig. 7.2). The level of polyhedrin expression in all recombinants was high, although slightly less than that in wild-type AcMNPV-infected cells.

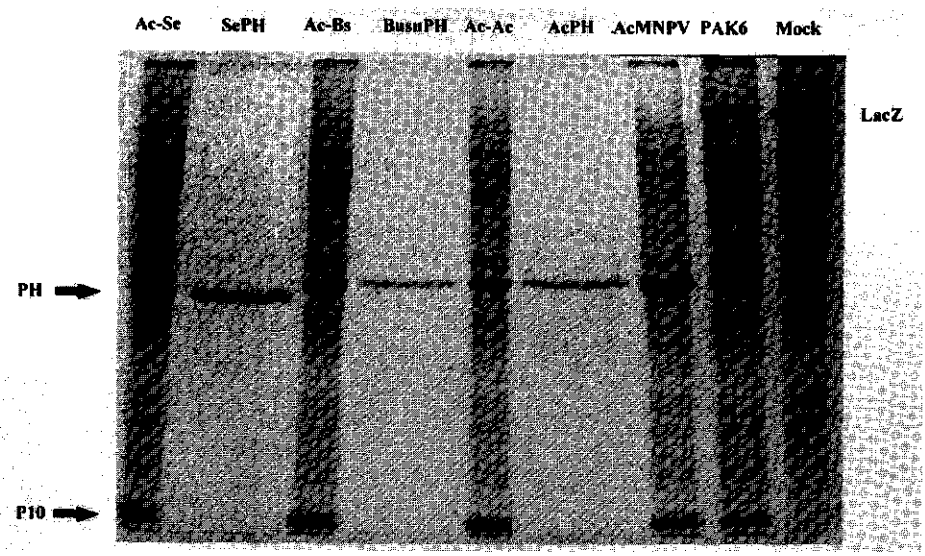



**Figure 7.2.** SDS-PAGE of protein from uninfected Sf21 cells (Mock), Sf21 cells infected (48 h p.i.) with parental virus AcMNPV-PAK6 (PAK6), wild-type AcMNPV-C6 (AcMNPV), AcMNPV-AcPH (Ac-Ac) and AcMNPV-BsPH (Ac-Bs) and polyhedra of wild-type AcMNPV (AcPH), BusuNPV (BsPH) and SeMNPV (SePH). Protein equivalent to $5x10^4$ cells or $1x10^5$ polyhedra was loaded per lane. The gel was stained with coomassie Briliant Blue.

*Phase-contrast and electron microscopy*

When Sf21 cells were infected with different AcMNPV recombinants, polyhedra could be easily seen in the nuclei by phase contrast microscopy (Fig. 7.3). No significant difference in the size and shape of polyhedra could be observed in cells infected with the AcMNPV wild-type control (Fig. 7.3a), AcMNPV-AcPH recombinant control (Fig. 7.3b) and AcMNPV-BsPH (Fig. 7.3c). The AcMNPV-SePH polyhedra (Fig. 7.3d) were, however, smaller in size and had a triangular shape distinct from the wild type AcMNPV or SeMNPV polyhedra. Polyhedra were released from the infected cells at the end of the infection (data not shown), indicating that the expression of a foreign polyhedrin did not affect nuclear disintegration.

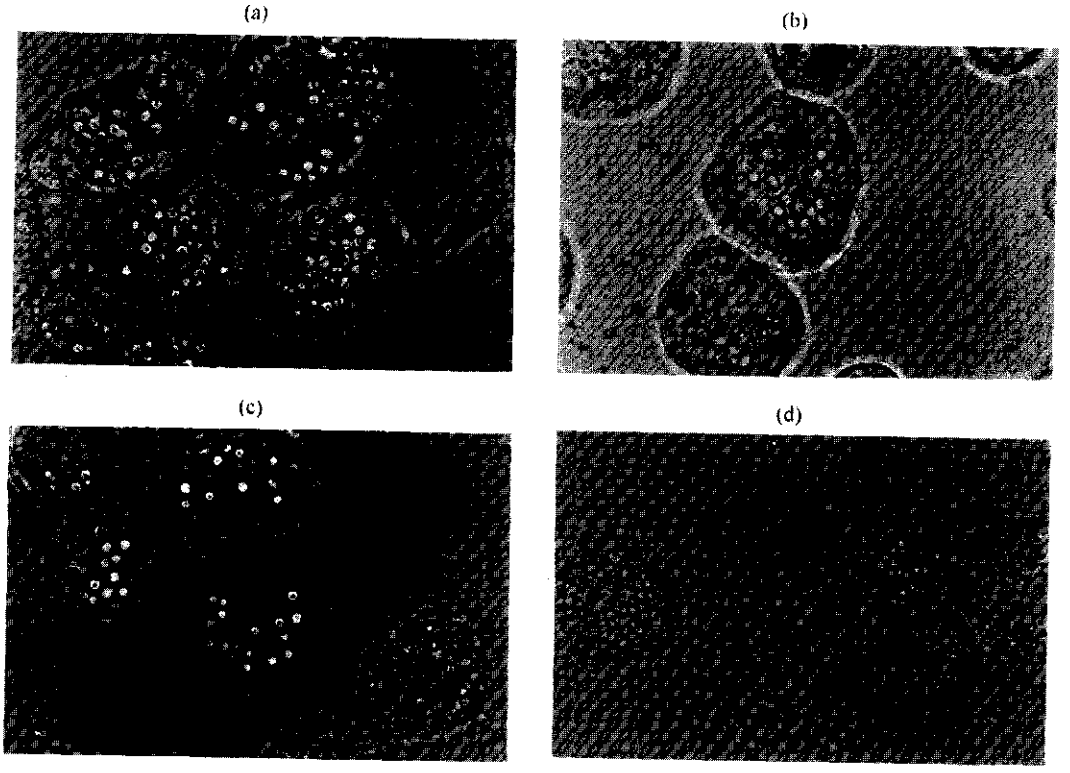



**Figure 7.3.** Phase contrast microscopy of Sf21 cells infected with wild-type AcMNPV (*a*), AcMNPV-AcPH (*b*), AcMNPV-BsPH (*c*) and AcMNPV-SePH (*d*) at 52h p.i.

Polyhedra produced by wild-type AcMNPV (Fig. 7.4a) and by AcMNPV-AcPH (Fig. 7.4b) and AcMNPV-BsPH (Fig. 7.4c) were of normal shape, size and structure as seen by electron microscopy. Numerous multiple-nucleocapsid virions were observed in sections of those polyhedra and the polyhedra were completed with a calyx. AcMNPV-BsPH did not select for

single-nucleocapsid virions. The polyhedra produced by AcMNPV-SePH, had distinct (often triangular) shapes in thin sections and appeared to contain only few multiple-nucleocapsid virions (Fig. 7.4d). Scanning electron microscopy confirmed the pyramidal shape of these polyhedra. These polyhedra also appeared to be completed with a calyx. Apart from occlusion, other pathogenic features or processes associated with baculovirus infection appeared to be indistinguishable irrespective whether recombinants or wild-type AcMNPV were used (Fig. 7.5).

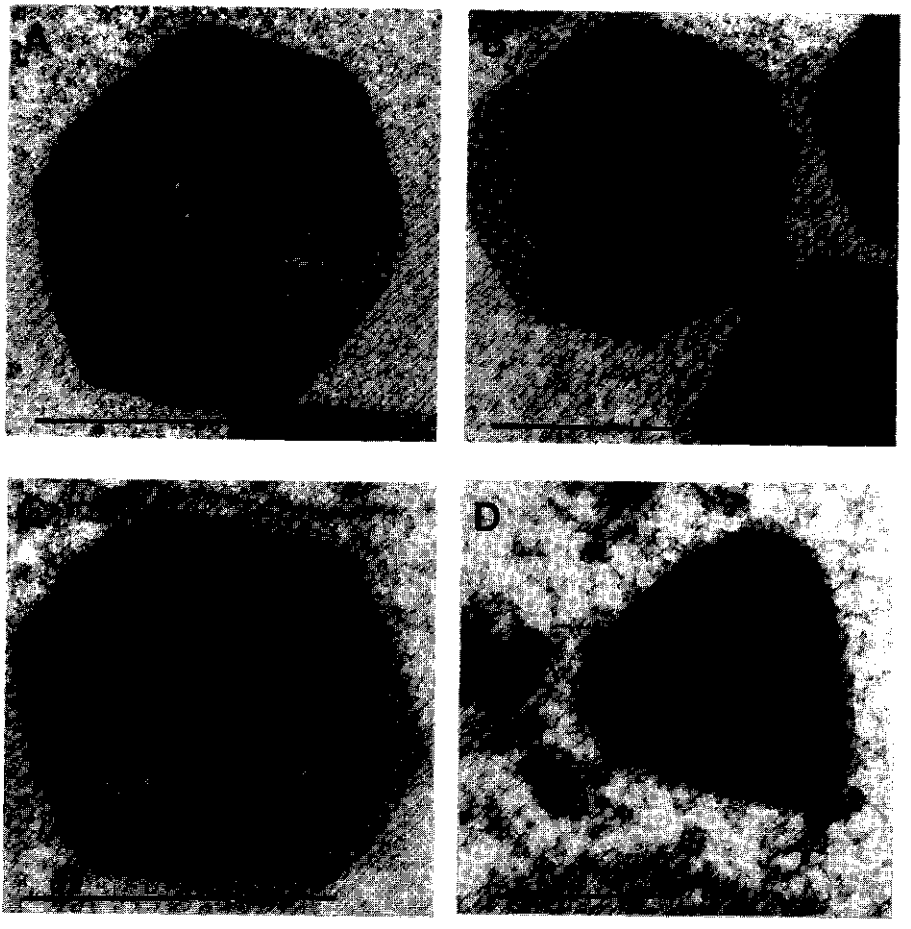



**Figure 7.4.** Electron microscopy of ultrathin sections of polyhedra produced by wild-type AcMNPV (*a*), AcMNPV-AcPH (*b*), AcMNPV-BsPH (c) and AcMNPV-SePH (*d*). Bars represent 1 μm.

*Physical and biological activity*

The alkali - sensitivity was tested in an *in vitro* assay following the dissolution of polyhedra over time (Zuidema *et al.*, 1989). No difference in the alkali - sensitivity between the wild-type and recombinant AcMNPVs was observed (data not shown). Polyhedra from all recombinant NPVs dissolved readily in weak alkali and released ODVs. The remaining polyhedron calyx or 'bag' could be seen from recombinant as well as wild type AcMNPV.

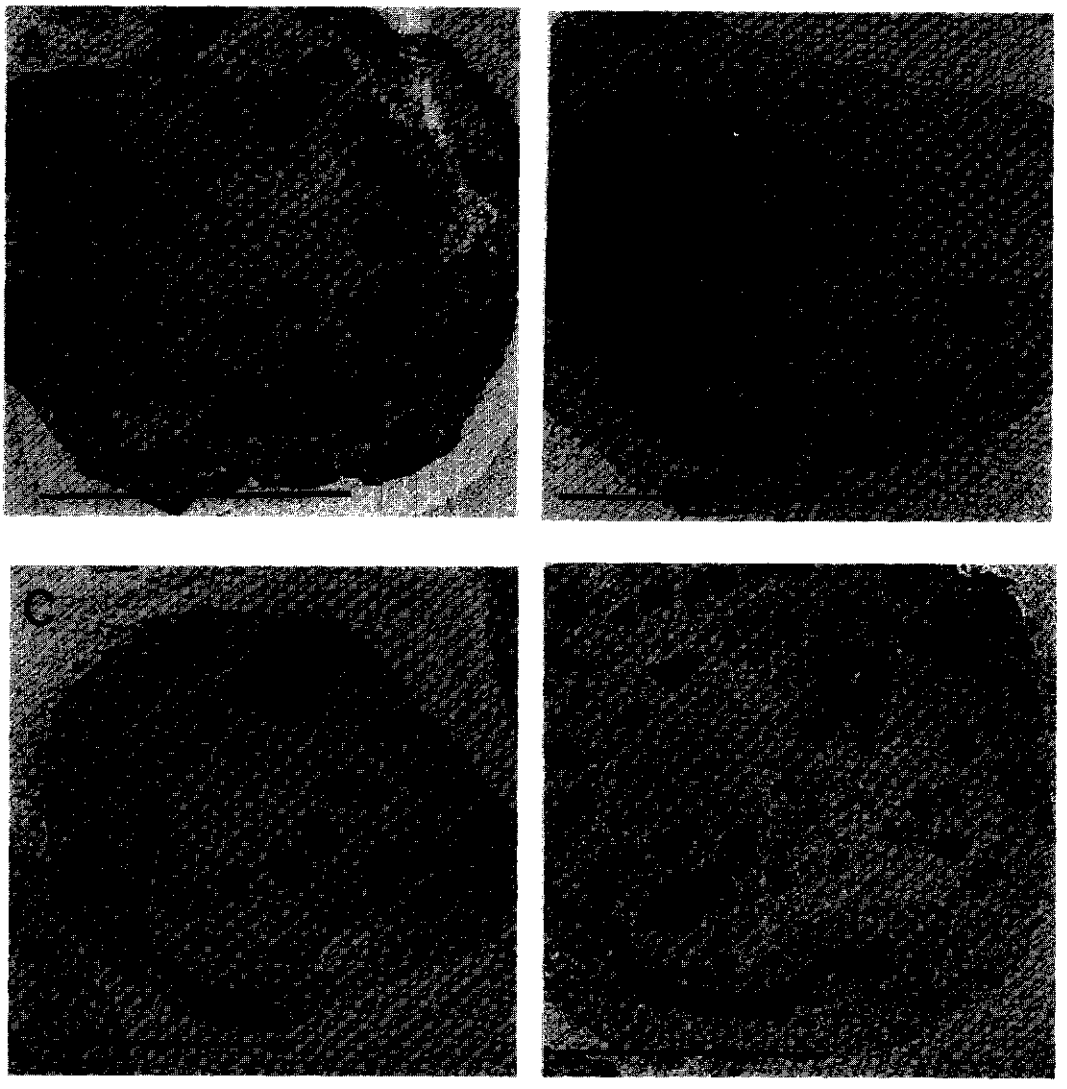


***Figure 7.5.*** *Electron microscopic images of ultrathin sections of Sf21 cells infected with wild-type* AcMNPV (*a*), AcMNPV-AcPH (*b*), AcMNPV-BsPH (*c*) and AcMNPV-SePH (*d*). Bars represent 10 μm.

The infectivity of the various recombinant viruses was determined with a droplet-feeding bioassay using third instar *S. exigua* larvae as host and the result of these assays is shown in Table 7.1. The various viruses were passaged once in *S. exigua* larvae prior to the assay to eliminate any cell culture effects. The median lethal dose ($LD_{50}$) of AcMNPV-AcPH was not significantly different from that of the wild-type virus AcMNPV-C6 (the parental virus of AcMNV-PAK6) indicating that the cloning process had not affected the biological activity of the virus. The $LD_{50}$ of AcMNPV-BsPH was slightly higher than that of wild-type AcMNPV, whereas the $LD_{50}$ of AcMNPV-SePH was significantly higher than the other recombinants and wild-type AcMNPV.

**Table 7.1.** Dose-mortality relationship of wild-type and recombinant AcMNPV for early-third instar *S. exigua* larvae

| Virus | $LD_{50}$ (polyhedra) | 90% confidence interval | | slope |
|---|---|---|---|---|
| | | lower | upper | |
| wt-AcMNPV | $3.5x10^3$ | $1.4X10^3$ | $1.2X10^4$ | 0.933±0.111 |
| AcMNPV-AcPH | $5.3x10^3$ | $2.1x10^3$ | $2.0x10^4$ | 0.785±0.100 |
| AcMNPV-BsPH | $1.3x10^4$ | $4.0x10^3$ | $4.8x10^4$ | 1.054±0.125 |
| AcMNPV-SePH | $1.4x10^5$ | $4.1x10^4$ | $3.8x10^6$ | 0.783±0.143 |

*Note* The dose-mortality data presented here were from probit analysis using POLO computer program.

## DISCUSSION

Our study indicates that heterologous polyhedrin ORFs, either from SNPV or MNPV can be highly expressed in an AcMNPV-Sf21 expression system, and that the polyhedrins are also correctly translocated into the nucleus to form polyhedra. These recombinant polyhedra showed the characteristic paracrystallin lattice structure and were correctly surrounded by a polyhedron envelope (calyx). Jarvis *et al.* (1991) showed that the AcMNPV polyhedrin protein contains domains responsible for nuclear localization and supramolecular assembly. It seems that those domains in the polyhedrin proteins of SeMNPV and BusuNPV are also functional in the AcMNPV-Sf21 cell system, a heterologous genetic setting. Similar results were observed by Roosien *et al.* (1986) and Gonzalez *et al.* (1989) when polyhedrin gene of *Mamestra brassicae* MNPV and *S. fugiperda* MNPV, respectively, were introduced into an AcMNPV-polyhedrin deletion mutant by heterologous recombination rather than targeted insertion. Therefore, the nuclear localization and supramolecular assembly signals of the polyhedrin are likely to be recognized by the viral or host factors of a different baculoviral system. In the two cases studied, the envelopment of the heterologous OBs with a calyx appeared to be unimpeded. Our results further showed that the expression of a foreign polyhedrin did not affect nuclear disintegration, which appears to be a process requiring the interaction of P10 with at least one virus-specific factor (Van Oers *et. al.*, 1994). Therefore, polyhedrin is not likely to be the virus-specific factor.

Our results contrast with those of Zhou *et al.* (1998), where the granulin gene of *Trichoplusia ni* GV was used to replace the polyhedrin gene of *Bombyx mori* (Bm) NPV. The resulting recombinant BmNPV produced polyhedron-like OBs, which did not have paracrystalline lattices and had fragments of the polyhedron envelope wrapped inside. The amino acid conservation between granulin and lepidopteran polyhedrin is much lower than that between two lepidopteran polyhedrin proteins (e.g. 53% amino acid identity for that of BmNPV/TnGV; 84% for SeMNPV/AcMNPV and 89% for BusuNPV/AcMNPV). It is, therefore, conceivable that granulin has already diverged to such an extent from polyhedrin that it has lost its property to interact with calices or ODVs of NPVs. The observation of Zhou *et al.* (1998) that granulin forms polyhedron-like OBs in NPV background may indicate that the supramolecular assembly mechanism in NPVs and GVs were developed from a common system.

The occlusion process does not appear to be dependent on the viral morphotype since OBs derived from recombinant AcMNPV producing BusuNPV polyhedrin appear to occlude multiple nucleocapsid virions of AcMNPV quite efficiently and with no apparent loss of infectivity. This suggests that a polyhedrin from an SNPV can clearly recognize ODVs or other MNPV proteins involved in NPV occlusion and OB morphogenesis. It has been clearly shown that a heterologous MNPV polyhedrin from an MNPV (SeMNPV) does not necessarily function better than that from an SNPV (BusuNPV) in an AcMNPV/Sf21 environment.

Polyhedra produced by AcMNPV-SePH are pyramidal in shape and differ profoundly from those produced by wild type AcMNPV and SeMNPV, which are polyhedral in shape and differ in size. Since it has been reported before that alteration of a single amino acid in sequence can cause a major morphological change of polyhedra (Carstens *et al.*, 1986, Carstens *et al.* ,1987, Carstens *et al.*, 1992), several control experiments were carried out to ensure that in our case the correct SeMNPV polyhedrin gene was inserted in the recombinant AcMNPV-SePH. First, the insert polyhedrin sequence in transfer vector pAc-SePH was sequenced and found to be correct proving that no errors were introduced by the PCR amplification. Second, the transfection and the plaque purification procedures to generate AcMNPV-SePH were repeated and several plaques were picked and purified separately. In all cases the AcMNPV-SePH polyhedra showed the same, altered morphology. Finally, the polyhedrin gene was amplified from AcMNPV-SePH by using PCR and sequenced. The sequence data confirmed that the polyhedrin gene of in AcMNPV-SePH recombinant was identical to that of wild-type SeMNPV (van Strien *et al.*, 1992). Therefore, the explanation for the altered polyhedral morphology of the recombinant AcMNPV-SePH must be found in the expression of the SeMNPV polyhedrin in an AcMNPV/Sf21 environment. When AcMNPV-SePH was amplified in an *S. exigua* cell line (Se-IZD2109) or in *S. exigua* larvae (after injection), the resulting polyhedra still showed an aberrant morphology, suggesting the involvement of a virus specific factor in the morphogenesis of polyhedra. When *S. exigua* cells were co-infected with AcMNPV-SePH and wt SeMNPV in a ratio of 50:1 (finial MOI = 10), most of the resulting polyhedra showed size and shape of wt SeMNPV (data not shown), suggesting the existence of such a factor.

The infectivity of AcMNPV-BsPH was similar to wild-type AcMNPV indicating that BusuNPV polyhedrin can functionally replace AcMNPV and that the differences in amino acid sequence between AcMNPV and BusuNPV (Fig. 7.6) have a neutral effect. The infectivity of AcMNPV-SePH for *S. exigua* is low in comparison to wild-type AcMNPV as reflected by a higher $LD_{50}$. There appeared to have been fewer virions occluded in the recombinant polyhedron containing SeMNPV poyhedrin (Fig. 7.4). The less efficient occlusion of AcMNPV virions by SeMNPV-polyhedrin in comparison to BusuNPV-polyhedrin, suggests that virion occlusion is a complex and fine-tuned process which maybe to some extent specific to the polyhedrin protein involved. Amino acid sequence comparison indeed shows that the polyhedrin of BusuNPV is more closely related to that of AcMNPV than to that of SeMNPV (Fig. 7.6). Recent experiments expressing the more distantly related granulin gene by BmNPV (Zhou *et al.*, 1998) showing the formation of improperly occluded OBs, supports this view.

Although there are about 30 polyhedrin genes sequenced to date and the fact that the polyhedrin protein is highly conserved, little is known about the structure-function relationship; e.g. its role in crystalline matrix formation and how it interacts with the ODV and with the calyx. Amino acid residues that are conserved in all the baculovirus polyhedrin proteins are likely to be functionally important. Often a single amino acid mutation in polyhedrin can alter the morphology of polyhedra (Carstens *et al.*, 1986; Carstens *et al*, 1987; Carsten *et al.*, 1992). The alterations caused by these mutants are all occurred in the conserved regions of polyhedrin (Fig. 7.6). The host cell type also seems to contribute to the size of polyhedra (Bonning *et al.*, 1995). Our results, especially those obtained with AcMNPV-SePH, suggest that amino acid residues which are not conserved among baculoviral polyhedrin proteins as well as other viral factors may play a role in the morphogenesis of OBs and in the efficiency of virion occlusion.

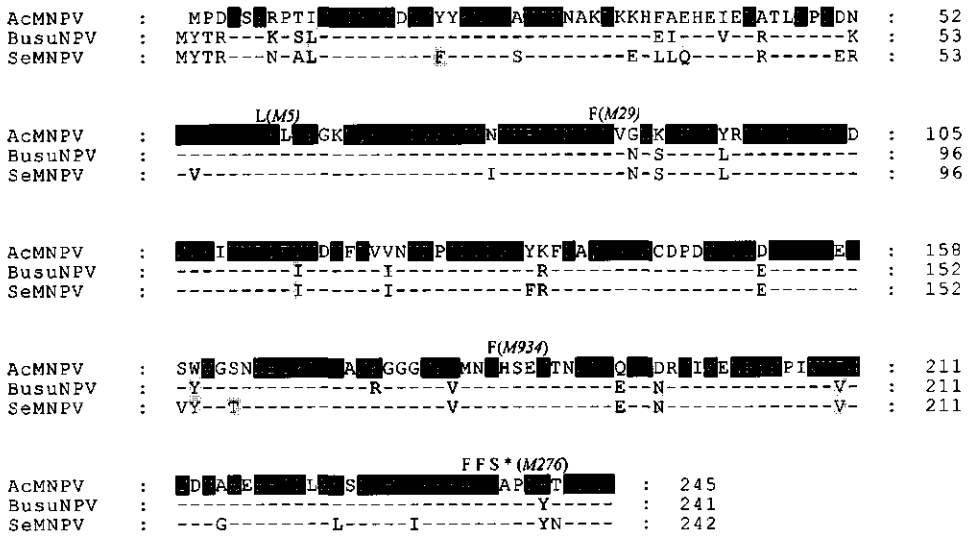



**Figure 7.6.** Alignment of amino acid sequence of the polyhedrin gene of AcMNPV, SeMNPV and BusuNPV. Black shading in the AcMNPV sequence indicated the conserved amino acid sequence among so far identified polyhedrins (data derived form nucleotide sequences of polyhedrin available in Genbank). The sequence similarity of BusuNPV and SeMNPV to that of AcMNPV are shown: dashes indicating amino acid identity and grey for similarity. The location of the four mutations in AcMNPV, that of M5 (Carstens *et al.*, 1986), M29 (Carstens *et al.*, 1987), M934 and M276 (Carstens *et al.*, 1992), are also indicated.


## ACKNOWLEDGMENTS

We are grateful to Joop Groenewegen for expert technical assistance in electron microscopy. We thank Rob Goldbach and Basil Arif for their continuing interest and critical review of the manuscript.

CHAPTER 8

# GENERAL DISCUSSION AND PHYLOGENY OF BACULOVIRUSES

## INTRODUCTION

Baculoviruses are a potentially bountiful natural source of environmentally safe biocontrol agents to combat pest insects in agriculture and forestry. More than 600 baculovirus isolates have been described mainly from the insect order Lepidoptera. Nineteen relatively well-investigated baculoviruses have so far obtained species status (Murphy *et al.*, 1995). Based on occlusion body morphology, baculoviruses are classified as nucleopolyhedroviruses (NPVs) or granuloviruses (GVs). NPVs are designated as SNPV or MNPV depending on either the single (S) or multiple (M) packaging of nucleocapsids in the occluded virion. Knowledge of the genetic and molecular biology of baculoviruses has so far been mainly derived from only a few well-studied species such as AcMNPV, BmNPV and OpMNPV, and may not be representative for all baculoviruses. Therefore, genetic information of other baculoviruses, particularly that of SNPVs and GVs, is required. This kind of information is not only relevant for further application of baculoviruses as insect pest control agents, including the engineering of baculoviruses with enhanced insecticidal properties, but also to study baculovirus phylogeny and evolution history of baculoviruses.

*Buzura suppressaria* SNPV (BusuNPV) is a representative of SNPVs and was isolated from one of the major pests, *Buzura suppressaria* Guenee, of tea plants (Xie *et al*, 1979; Gan, 1981; Chu *et al.* 1979). *B. suppressaria* is a polyphagous pest insect which also causes damage to about other 60 plant species. In the last 20 years, intensive research has been carried out in China for developing BusuNPV as a bio-pesticide against this pest insect. The research encompassed analysis of the infection process and morphogenesis of BusuNPV in the host insect (Xie *et al.*, 1982), initial characterization of the viral DNA and viral proteins (Li *et al*, 1983; Zhang, 1985), biological activity (Zeng *et al.*, 1983) and epidemiology (Peng *et al.*, 1986). Two *B. suppressaria* cell lines were established for studying the replication of BusuNPV *in vitro* (Xie and Wang; 1985; Liu and Xie, 1985; Xu and Xie, 1992; Hu and Xie, 1991) but were lost over time. A technique for large scale production of BusuNPV from larvae and formulation as an insecticide was also developed (Xie *et al.*, 1992). The virus has been successfully applied to control *B. suppressaria* on tea, tung-oil tree and metasequoia plants (Xie and Peng, 1980; Peng *et al.*, 1988; Peng *et al.*, 1992).

At the start of the research presented in this thesis, knowledge of the molecular biology of BusuNPV was rather limited and consisted of preliminary reports on the location of the polyhedrin gene and a simple physical map (Liu *et al.*, 1992; *Liu et al.*, 1993). **The first part of this chapter** discusses the molecular genetics of BusuNPV derived from the studies carried out in this thesis, as well as the impact of these studies on possible future engineering of the virus. Intricacies of the genetics and molecular biology of baculoviruses have given us much

appreciation of their complexity and evolution. Ironically, this knowledge has been gained from only a few well-studied species, mostly MNPVs. The data described in this thesis embody the first detailed study on an SNPV and establish BusuNPV as a distinct viral species (proposed name BsSNPV). **The second part of this chapter** focuses on the phylogeny of baculoviruses, which is not only relevant for a taxonomic purpose, but also for understanding the relationship between the viruses and their hosts and for defining biologically relevant characters such as host range and specificity.

**Molecular genetic map of BusuNPV**

Among the baculoviruses, only a few MNPVs have been investigated to a significant detail. The data presented in this thesis now allow a meaningful comparison of the genome organization between the MNPVs and a representative of the SNPVs, BusuNPV. It is clear from the genetic map of BusuNPV that the gene content of an SNPV is rather similar to that of the MNPVs identified so far. The size of the BusuNPV genome is 121 kb, which is not that much smaller than that of *Autographa californica* (Ac) MNPV (134 kb, Ayres *et al.*, 1994), *Bombyx mori* (Bm) NPV (128 kb, S. Maeda, Genbank accession number L33180) and *Orgyia psudotsugata* (Op) MNPV (132 kb, Ahrens *et al.*, 1997). By sequencing the ends of BusuNPV plasmid inserts ('sniff sequencing'), we were able to identify and localize 52 ORFs on the BusuNPV genome. These included many known genes associated with DNA replication and transcription, such as the immediate early gene 1 (*ie-1*), DNA helicase, several of the late expression factor (*lefs*) genes, *lef-1, lef-2, lef-5, lef-6, lef-8,* the 'very late factor' gene (*vlf-1*), as well as some virion structural genes known as *gp41, odv-e18, odv-ec27, odv-e56,* polyhedrin, *p10* and the polyhedral envelop protein. The similarity in genetic content between the MNPVs and BusuNPV suggests that they, and possibly all the NPVs, have a similar multiplication strategy.

**Baculovirus gene arrangement and 'GeneParityPlot' analysis**

Comparison of AcMNPV, BmNPV and OpMNPV showed that baculovirus genomes may vary due to deletions, insertions and inversions (Ayres *et al.*, 1994; S. Maeda, Genbank accession number L33180; Ahrens *et al.*, 1997). The results described in this thesis revealed that an even more distinctly organized BusuNPV genome and indicates that gene translocations may have happened at a high frequency during baculovirus evolution (see Chapter 5). The mechanisms underlying these rearrangements are still unclear. The wild-type baculovirus isolates are a collection of slightly heterogeneous viral strains that may serve as a gene pool to act optimally in different environments. Also, the generation of defective interfering particles (DIPs) during *in vitro* replication suggests that wild-type viruses already contain deletion mutants or that these mutants are easily generated (Kool *et al*, 1991). Furthermore, many baculoviruses contain homologous repeat sequences (*hrs*) dispersed throughout the genome (Ayres *et al.*, 1994; Cochran and Faulkner, 1983; Garcia-Maruniak *et al.*, 1996; Theilmann and Stewart, 1992; Xie *et al.*, 1995; Pearson *et al.*, 1995; Broer *et al.*,1998). There is evidence that the *hrs* are related to the occurrence of the different baculovirus genotypes (Arif and Doerfler, 1984; Garcia-Maruniak *et al.*, 1996; Muñoz *et al.*, 1996) suggesting that *hrs* may serve as 'hot spots' where genomic rearrangements can easily take place. Finally, several baculoviruses have been

shown to contain transposable elements that may well play an important role in the evolution of the viral genome (Friesen, 1993; Jehle, 1996). The organization of baculovirus genomes may therefore be a reflection of their evolutionary history.

Comparison of the genome organization in baculoviruses is only possible when sufficient sequence information is available. As baculoviruses have a relatively large genome (90-160 kb), an overall comparison of gene organization is often difficult when only partial sequence information is available. To overcome this difficulty, a new method was developed (**'GeneParityPlot'**, see Chapter 5) for generating meaningful genomic comparisons. By this method, taking the polyhedrin gene as the first gene on a linearized genome and ordering all other genes according to their sequential appearance in their respective genomes, allowed a direct comparison of genome organization. This approach proved useful to find conserved gene clusters and their distribution along the genome which may provide an initial insight into the evolution of baculoviruses. By applying this method, seven potential gene clusters were identified in the genome of BusuNPV.

**The genetic determinants of the SNPV and MNPV phenotypes**

So far the molecular genetic mechanisms determining the assembly of the virus into an SNPV or MNPV morphotype are still unclear. It could be that one or more SNPV (versus MNPV)-unique genes are responsible for this morphological effect. Approximately one third of the BusuNPV genome has been sequenced, and we were able to identify a few unique ORFs of BusuNPV (data not shown) which might be SNPV-specific. Alternatively, the alternation in one or more ODV-specific genes could be responsible for the SNPV/MNPV morphotype. The large gene content and the high similarity of the SNPVs and MNPVs genomes makes it difficult to resolve this conundrum by sequencing alone.

In this research, the functionalities of the BusuNPV polyhedrin and p10 genes in an AcMNPV context were studied by a 'gene swapping' method (Chapter 6 and 7). By swapping genes between heterologous viruses, it should be possible to analyze if additional factors are needed for the function of a specific gene product. Since the AcMNPV/Sf21 cell system is well characterized, this method can assist to elucidate gene functions from viruses that do not have a permissive cell line. Eventually, such a system could be utilized in identifying genes determining the SNPV or MNPV morphotype. By using this method it was demonstrated that both the polyhedrin and p10 genes of a SNPV were functional in a MNPV environment and therefore, they are not the key proteins determining the SNPV morphotype.

**The impact of molecular genetics on future engineering of BusuNPV**

Understanding the molecular properties of BusuNPV provides a crucial basis for site-directed manipulation and improvement of the virus as biocontrol agent. The identification of the *egt* gene of BusuNPV in Chapter 3, for instance, will assist to generate *egt*-minus recombinant viruses with the expected improved insecticidal properties relative to wild-type virus. It has been shown that infection by an AcMNPV *egt*-null mutant results in an increased speed of kill and

reduced larval feeding (O'Reilly and Miller, 1991; Flipsen *et al.*, 1995) which in effect, generates an enhanced viral insecticide. This strategy has been adopted for the engineering of several other baculoviruses of economically important insect pests such as LdMNPV, CfMNPV, HzSNPV and HearNPV. Moreover, an *egt*-null mutant of BusuNPV could also be engineered to contain an exogenous gene deleterious to the host, such as insect-specific toxins (McCutchen *et al.*, 1991). It is expected that these genetic modifications will result in an improved product for applications on economically important infested crops such as tea plantations. A number of BusuNPV-specific promoters are available to regulate the expression of exogenous genes. Over-expression of exogenous and insecticidal genes could be driven by a very late promoter such as that of polyhedrin or p10 gene. A strong early promoter may also be useful in transgenic viruses. Furthermore, the system can be optimized by altering the codon usage of the exogenous gene to that of BusuNPV. The lack of a susceptible cell line of BusuNPV, however, is one of the main shortages that need to be resolved for generating a genetically improved BusuNPV insecticide.

**Molecular phylogeny of baculoviruses**

Baculoviral phylogeny studies based on the sequence divergence of the occlusion body proteins (polyhedrins and granulins) have illustrated three main points.

1. Different lepidopteran NPVs have evolved from a common ancestral virus rather than being evolved by cross-infection from NPVs of other insect orders.
2. GVs have evolved from a lepidopteran NPV early in the adaptive radiation of lepidopteran baculoviruses (Rohrmann *et al.*, 1981, Vlak and Rohrmann, 1985; Rohrmann, 1992; Zanotto *et al.*, 1993).
3. Most lepidopteran NPVs can be classified into two distinct groups referred to as groups I and II (Zanotto *et al.*, 1993, Cowan *et al.*, 1994).

Scanty information on polyhedrin sequences from SNPVs precluded confident classification of this morphotype and, the question whether they formed a separate group in the phylogeny tree remained unanswered.

The following sections address a number of important points related to the phylogeny and taxonomy of baculoviruses. First, a phylogenetic tree based on currently available polyhedrin and granulin genes was constructed. The derived phylogenetic data may be helpful in answering some basic questions, such as, how are the baculoviruses interrelated? Did baculoviruses co-evolve with their hosts, and do SNPVs and MNPVs form separate groups? Second, by constructing phylogenetic trees based on two other genes, *egt* and *lef-2*, and then comparing them to that based on the occlusion body proteins, a proposed phylogenetic tree for baculoviruses was built. Third, a tentative timescale of baculoviruses evolution was conceived. Finally, the impact of molecular genetics on taxonomy and phylogeny is discussed.

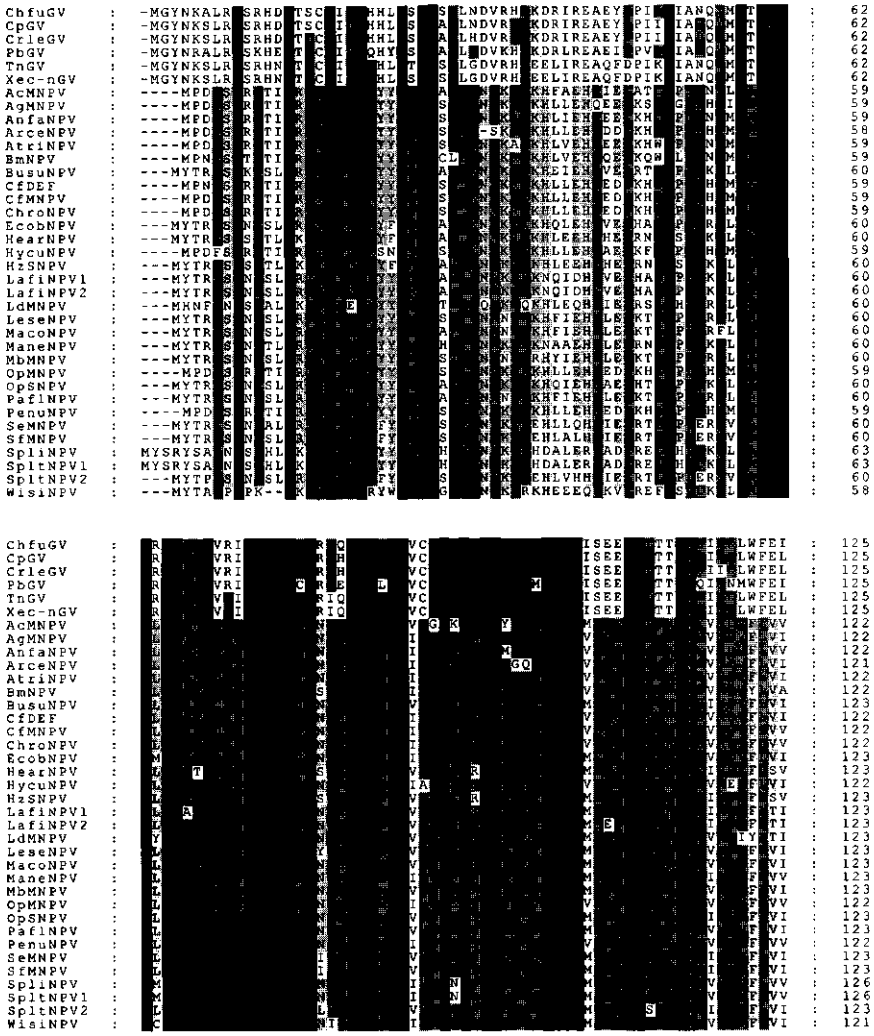
**Phylogeny of baculoviruses based on the polyhedrin and granulin genes**

Polyhedrin, the major component of occlusion bodies, has been the subject of detailed investigations because it is abundantly expressed under the control of a powerful promoter and

because of its importance for the survival of the virus in the environment. Polyhedrin genes are readily identified by cross hybridization and their sequences are now the most abundant in baculovirus literature (Table 8.1). Also, convention commands that the fragment containing the polyhedrin gene be placed as the start point of the physical map. More polyhedrin and granulin sequences have become available since the reporting of the BusuNPV polyhedrin gene in 1993 (Chapter 2) and they are listed in Table 8.1. The amino acid sequences of the proteins were aligned by Pileup GCG program (Fig. 8.1) and used for phylogeny analysis with the aid of PAUP 3.1 program (Swofford, 1993). Amino acid sequences obtained entirely by peptide sequencing methods were not included in Table 8.1 or used in the alignment because of the inherent errors associated with these procedures.

**Table 8.1**. List of sequenced polyhedrin/granulin genes (as of June, 1998)

| Virus | Abbreviation | accession | Host family | S/M | References |
|---|---|---|---|---|---|
| *Autographa californica* MNPV | AcMNPV | K01149 | L:Noctuidae | M | Hooft van Iddekinge *et al.*, 1983 |
| *Anticarsia gemmatalis* MNPV | AgMNPV | JQ1607* | L:Noctuidae | M | Zanotto *et al.*, 1992 |
| *Anagrapha falcifera* MNPV | AnfaNPV | U64896 | L:Noctuidae | M | Federici and Hice, 1996 |
| *Archips cerasivoranus* NPV | ArceNPV | U40834 | L:Tortricidae | | Rieth *et al.*, Genbank |
| *Attacus ricini* NPV | AtriNPV | S68462 | L: Saturniidae | | Hu *et al.*, 1993 |
| *Bombyx mori* NPV | BmNPV | L33180 | L: Bombyciidae | M | S. Maeda, Genbank |
| *Buzura suppressaria* SNPV | BusuNPV | X70844 | L: Geomitridae | S | Hu *et al.*, 1993 |
| *Choristoneura fumiferana* defective NPV | CfDEF | U78194 | L:Tortricidae | M | Jamieson and Arif, Genbank |
| *Choristoneura fumiferana* MNPV | CfMNPV | | L:Tortricidae | M | Arif, personal comunication |
| *Choristoneura rosaceana* MNPV | ChroNPV | U91940 | L: Tortricidae | M | Lucarotti and Morin, Genbank |
| *Ecotropis obliqua* SNPV | EcobNPV | U95014 | L: Geomitridae | S | Zhang *et al.*, Genbank |
| *Helicoverpa armigera* SNPV | HearNPV | U97657 | L: Noctuidae | S | Chen *et al.*, 1997a |
| *Hyphantria cunea* MNPV | HycuNPV | D14573 | L:Arctiidae | M | Isayama *et al.*, Genbank |
| *Helicoverpa zea* SNPV | HzSNPV | Z12117 | L:Noctuidae | S | Cowan *et al.*, 1994 |
| *Lambdina fiscellaria fiscellaria* MNPV | LafiNPV1 | | L:Geomitridae | M | Levin *et al.*, 1997 |
| *Lambdina fiscellaria lugubrosa* MNPV | LafiNPV2 | | L:Geomitridae | M | Levin *et al.*, 1997 |
| *Lymantria dispar* MNPV | LdMNPV | M23167 | L:Lymantriidae | M | Smith *et al.*, 1988 |
| *Leucania separata* MNPV | LseNPV | U30302 | L: Noctuidae | M | Wang *et al.*, 1995 |
| *Mamestra brassicae* MNPV | MbMNPV | M20927 | L: Noctuidae | M | Cameron and Possee, 1989 |
| *Mamestra configurata* MNPV | MacoNPV | U59461 | L: Noctuidae | M | Li *et al.*, 1997 |
| *Malacosoma neustria* NPV | ManeNPV | X55658 | L: Lasiocampidae | | Vladimir and Kavsan, Genbank |
| *Orgyia pseudotsugata* MNPV | OpMNPV | M14885 | L:Lymantriidae | M | Leisy *et al.*, 1986a |
| *Orgyia pseudotsugata* SNPV | OpSNPV | M32433 | L:Lymantriidae | S | Leisy *et al.*, 1986b |
| *Panolis flammea* MNPV | PaflNPV | D00437 | L: Noctuidae | M | Oakey *et al.*, 1989 |
| *Perina nuda* MNPV | PenuNPV | U22824 | L: Lymantriidae | M | Chou *et al.*, Genbank |
| *Spodoptera exigua* MNPV | SeMNPV | X67243 | L: Noctuidae | M | van Strien *et al.*, 1992 |
| *Spodoptera frugiperda* MNPV | SfMNPV | J04333 | L: Noctuidae | M | Gonzalez *et al.*, 1989 |
| *Spodoptera litoralis* MNPV | SpliNPV | D01017 | L: Noctuidae | M | Croizier and Croizier, 1994 |
| *Spodoptera litura* MNPV | SpltNPV1 | AF037262 | L: Noctuidae | M | Wei, *et al.*, Genbank |
| *Spodoptera litura* MNPV | SpltNPV2 | X94437 | L: Noctuidae | M | Bansal *et al*, Genbank |
| *Wiseana signata* SNPV | WisiNPV | AF016916 | L:Hepialidae | S | Sadler *et al.*, Genbank |
| *Choristoneura fumiferana* GV | ChfuGV | U87621 | L: Tortricidae | | Bah *et al.*, 1997 |
| *Cydia pomonella* GV | CpGV | Y09478 | L: Tortricidae | | Crook *et al.*, 1997 |
| *Cryptophlebia leucotreta* GV | CrleGV | X79596 | L:Tortricidae | | Jehle and Backhaus, 1994 |
| *Pieris brassicae* GV | PbGV | X02498 | L: Pieridae | | Chakerian *et al.*, 1985 |
| *Trichoplusia ni* GV | TnGV | K02910 | L: Noctuidae | | Akiyoshi *et al.*, 1985 |
| *Xestia c-nigrum* GV | Xec-nGV | U70069 | L: Noctuidae | | Goto and Maeda, Genbank |



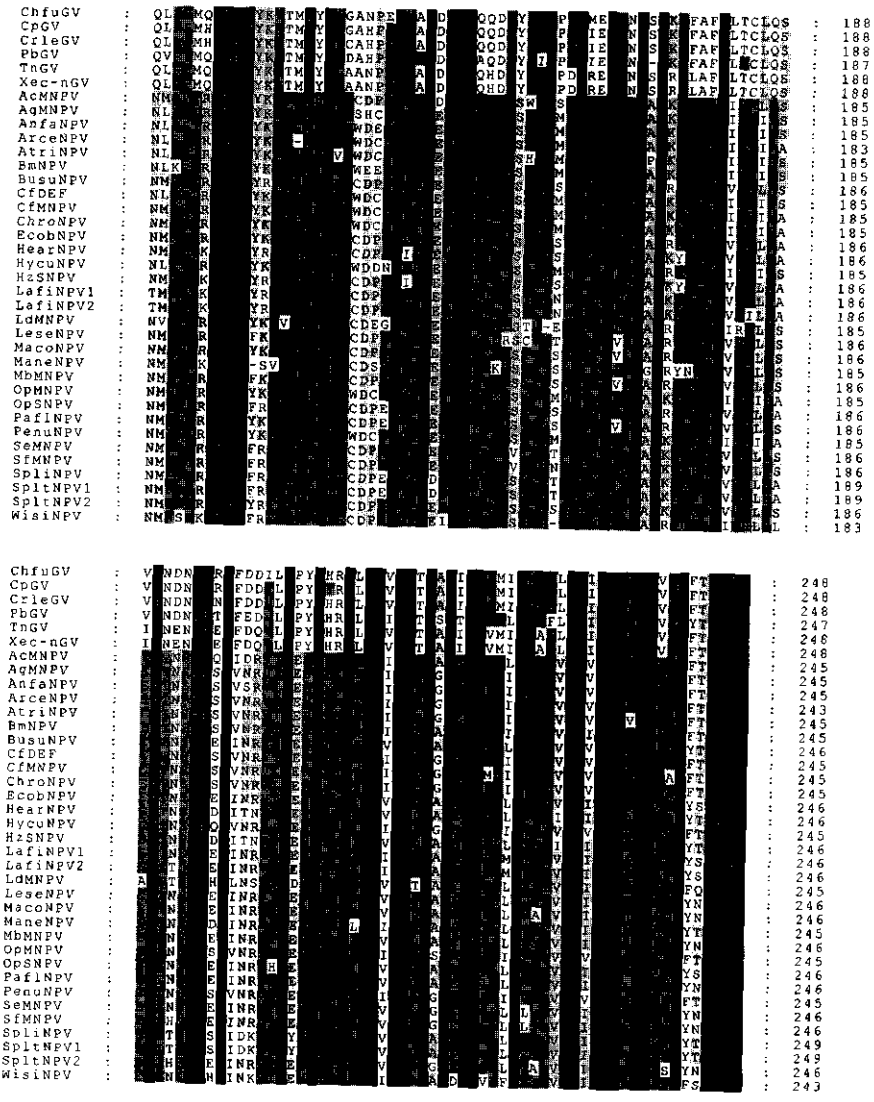


**Figure 8.1.** Alignment of the amino acid sequences of baculovirus polyhedrins/granulins. The sources of the sequences are listed in Table 8.1. PileUp program of GCG was used for constructing the alignment and GeneDoc software was used for homology shading. Black represents 100% identity, deep grey 80% identity, and light grey 60% identity.

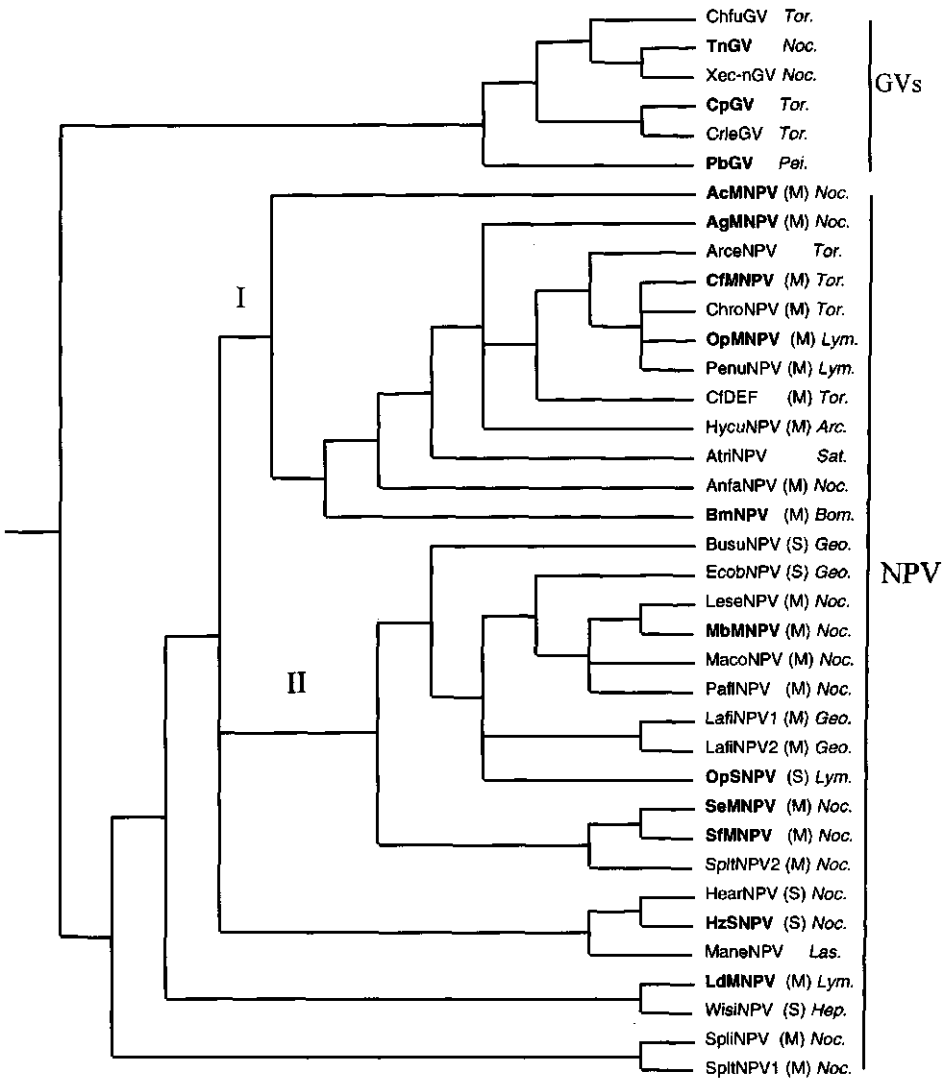



**Figure 8.2.** Phylogenetic tree based on the amino acid sequences of polyhedrin/granulin. The alignment used for the analysis was shown in Fig. 8.1. The computer-condensed most parsimonious tree was constructed using the heuristic search program of PAUP 3.1. The group of GVs, NPVs, and the subgroup of NPV (Group I and II) are indicated. The host families (all from the order Lepidoptera) and the SNPV or MNPV phenotypes are indicated. S=SNPV; M=MNPV; *Arc.*=Arctiidae; *Bom.*=Bombyciidae; *Geo.*=Geometridae; *Hep.*=Hepialidae; *Las.*=Lasiocamp; *Lym.*=Lymantriidae; *Noc.*=Noctuidae; *Pei.*=Pieridae; *Sat.* =Saturniidae; *Tor.*=Tortricidae. The baculoviruses that have obtained species status are indicted in bold.

A heuristic search with the above alignment resulted in 810 most parsimonious trees with the length of 473. Computer condense was performed and resulted in the tree shown in Fig. 8.2. The rooting was done with all the granulin sequences as an out-group. Note that the tree shown in the Fig. 8.2 is not a phylogram tree and, therefore, the branch lengths do not represent the distance between the nodes. According to these analyses, the lepidopteran baculoviruses are divided into the GV group and NPV Group, and the NPV Group is further divided into Groups I and II. The tree in Fig. 8.2 is basically the same as the one presented by Zanotto *et al.* (1993) except that the present one includes more polyhedrins. Several NPVs are not clustered in either Group I or Group II, including the groups of HzSNPV (HearNPV, HzSNPV ManeNPV), of LdMNPV (LdMNPV and WisiNPV), and of SpliNPV (SpliNPV and SpltNPV1). However, Cowan *et al.* (1994) was able to include the group of HzSNPV and of SpliNPV into group II by using the nucleotide sequence instead of amino acid sequence and by using LdMNPV as an outgroup for the analysis.

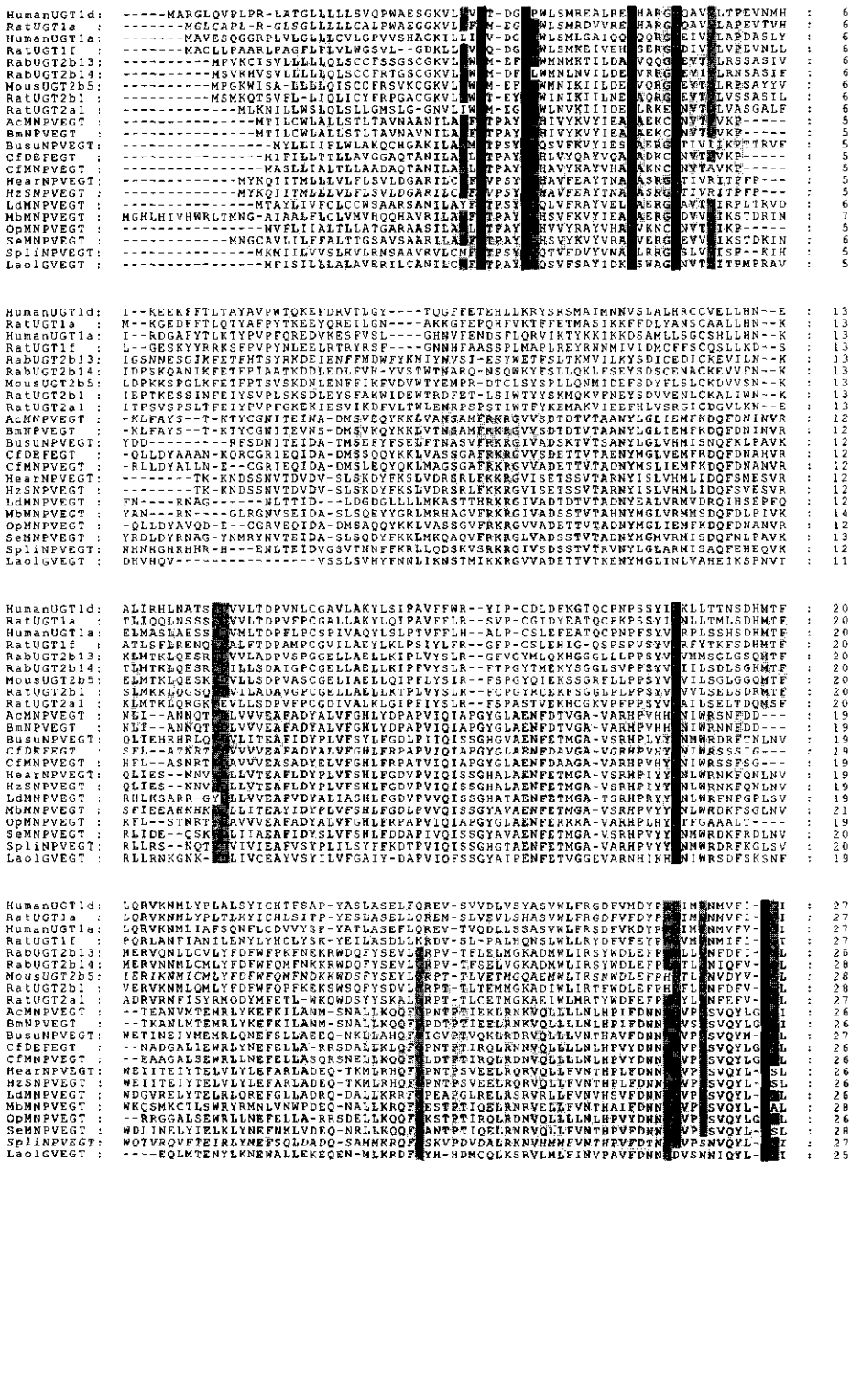
It can be concluded from Fig. 8.2 that the SNPVs do not form a separate group and that Group I appears to favour only MNPVs. Viruses from different insect host families appear to be clustered in the same group. On the other hand, viruses from the same insect family are seen in different groups which may suggest that some lepidopteran baculoviruses did not co-evolve with their insect host species.

The conclusion about the relationship of baculoviruses and their insect hosts has to be tempered by a number of factors. Firstly, it is known that some baculoviruses, such as AcMNPV, infect more than one insect family and the given baculovirus name represents only one of its hosts. Secondly, at the present time our knowledge of baculovirus DNA sequences has been derived only from viruses infecting lepidopteran insects (Table 8.1). Baculoviruses have been isolated from seven other insect orders such as Hymenoptera, Diptera, Coleoptera, as well as from the Decapoda order of Crustacea. Molecular data on viruses from these other insect orders are, therefore, necessary for more meaningful phylogeny analysis. It has been previously suggested that the NPVs have either evolved along with, or have had an ancient relationship with their insect hosts and that the infectivity of baculoviruses has been confined to their respective order since the divergence (Vlak and Rohrmann, 1985). Thirdly, the molecular phylogeny needs to be performed on the host species, particularly on genes that can act as molecular markers and are common to both the virus and the host, such as the superoxide dismutase (SOD) gene. Therefore, the phylogenetic relationship between baculoviruses and their hosts will be further understood when more data become available and our knowledge of both the virus and the host is more comprehensive.

**Baculovirus phylogeny based on single gene trees**

Baculoviruses have a relatively large genome containing more than 100 ORFs, less than half of which have been functionally characterized (Miller, 1997). In this context, phylogeny based on a single gene may not be an accurate representation of their evolution. In other words, do different genes produce similar or different phylogenetic trees? Therefore, two other baculovirus genes, *egt* and *lef-2*, of which reasonable amount of sequence information is

**Figure 8.3.** Alignment of the amino acid sequences of some mammalian UGTs and baculovirus EGTs



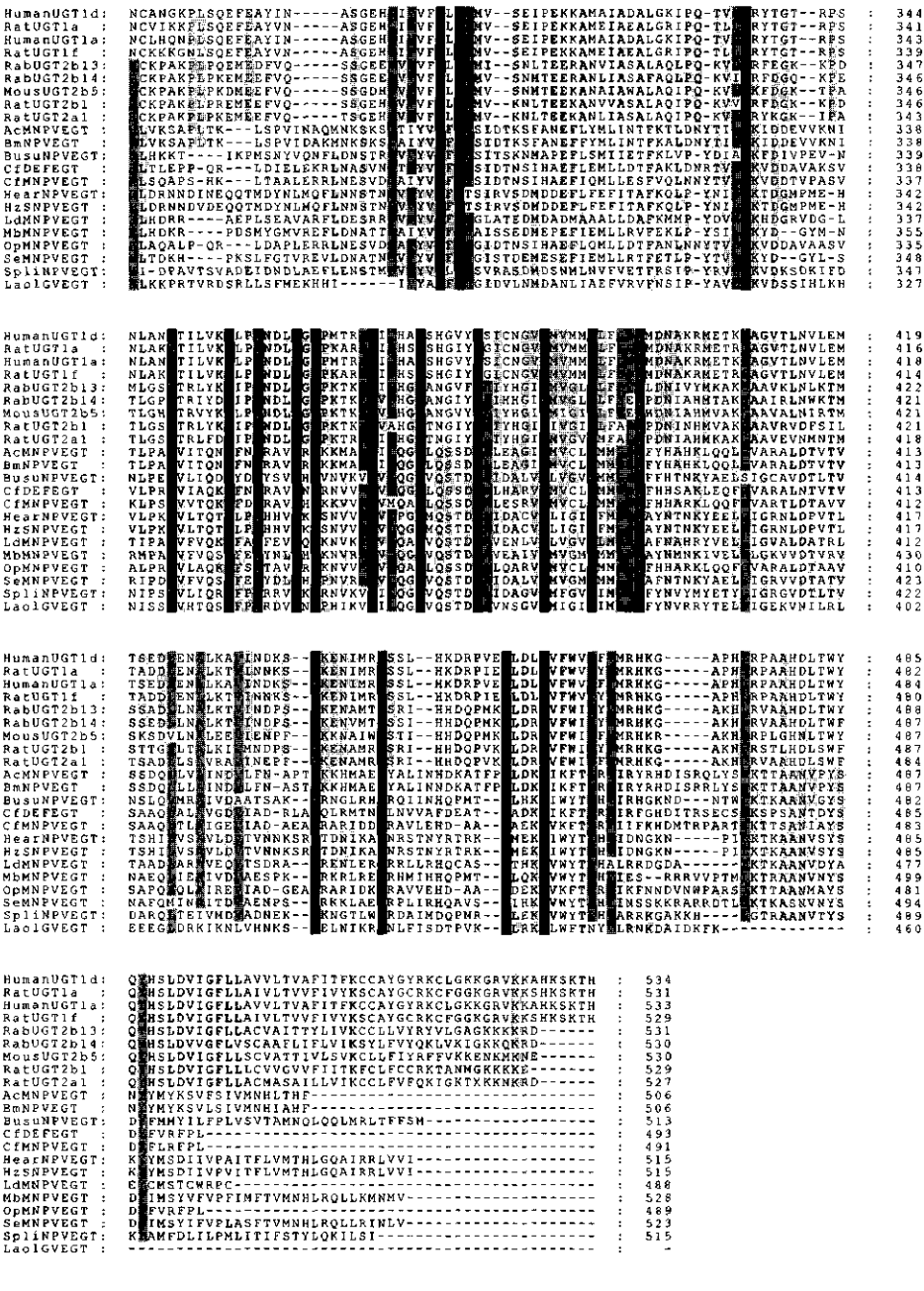


**Figure 8.3.** Alignment of the amino acid sequences of some mammalian UGTs and baculovirus EGTs. The sources of the UGT sequences are:GenBank P22310 for human UGT1d; P20720 for rat UGT1a; P22309 for human UGT1a; P08430 for rat UGT1f; P36512 for rabbit UGT2b13 (RabUGT2b13); P36513 for rabbit UGT2b14 (RabUGT2b14); P17717 for mouse UGT2b5 (MousUGT2b5); P09875 for rat UGT2b1; P36510 for rat UGT2a1. The sources for EGT sequences are: O'Reilly and Miller, 1990, for AcMNPV; GenBank L33180 for BmNPV; Hu *et al*, 1997, for BusuNPV; Barrett *et al.*, 1995, for CfMNPV and CfDEF; Chen, *et al.*, 1997b for HearNPV; Popham *et al.* 1997 for HzSNPV; Riegel *et al.*, 1994, for LdMNPV; Clarke *et al.*, 1996, for MbMNPV; Ahrens *et al.*, 1997, for OpMNPV; R.J.M. Mans (personal communication) for SeMNPV; Faktor *et al.*, 1995, for SpliNPV; and Smith and Goodale, 1998, for LaolGV. PileUp program of GCG was used for making the alignment and GeneDoc software was used for homology shading. Black represents 100% identity, deep grey 80% identity, and light grey 40% identity.

available, were analyzed to provide further information on viral phylogeny. EGT abrogates the larval metamorphosis to maximize baculovirus yield and *lef-2* is one of the genes involved in viral DNA replication (Kool *et al.*, 1994). It is possible that the evolutionary pressure to conserve these proteins is different.

Unlike polyhedrin, which does not appear to have homologues in organisms other than baculoviruses, EGT shares sequence homology with a variety of UDP-glycosyltransferases (see O'Reilly, 1995 for review). Several mammalian UDP-glucuronosyltransferases (UGTs) were aligned with the baculovirus EGTs (Fig. 8.3) and used for phylogeny analysis. The generated most parsimonious tree with midpoint rooting is shown in Fig. 8.4. Bootstrap analysis (100 replicates) shows that the monophyletic baculovirus EGT branch is very well supported. The two trees based on EGT and occlusion body protein agree with each other specially when the polyhedrin of the Hymenopteran SNPV (*Neodiprion sertifer*) was included in the analysis (Zanotto *et al.*, 1993), where the Lepidoptera NPVs and GVs form a monophyletic group. The grouping of LaolGV with SpliNPV in the EGT tree was not supported by the bootstrap analysis and therefore unlikely to reflect true relatedness of the two viruses. The inner topology of the baculovirus branch of the EGTs is similar to that of the phylogenetic tree based on the polyhedrin gene. The members of Group I viruses were similar in both EGT and polyhedrin tree, and with the relatively lower conservation of EGT sequences, two subgroups (IA and IB) within Group I are further identified.

The baculoviral late essential factor-2 (*lef-2*) is needed for DNA replication and late gene expression (Kool *et al.*, 1994; Passarelli and Miller, 1993; Merrington *et al.*, 1996). The availability of eleven *lef-2* genes (aligned in Fig. 8.5) allows the establishment of a phylogenetic relationship among LEF-2 proteins (Fig. 8.6). The most parsimonious tree with midpoint rooting was calculated with the PAUP heuristic search algorithm and reliability of phylogeny was further evaluated by bootstrap analysis. Based on LEF-2 sequences, baculoviruses can be divided into three groups; group I NPV, group II NPV and GV, which corroborates the phylogenetic analysis based on polyhedrin / granulin. The detailed topology of the subgroups IA and IB, is almost identical when the tree from LEF-2 is compared with that from EGT. The long branch lengths of the group II NPVs indicate that group II contains distinctly related NPVs and that further (sub)grouping can occur when more viruses are studied.

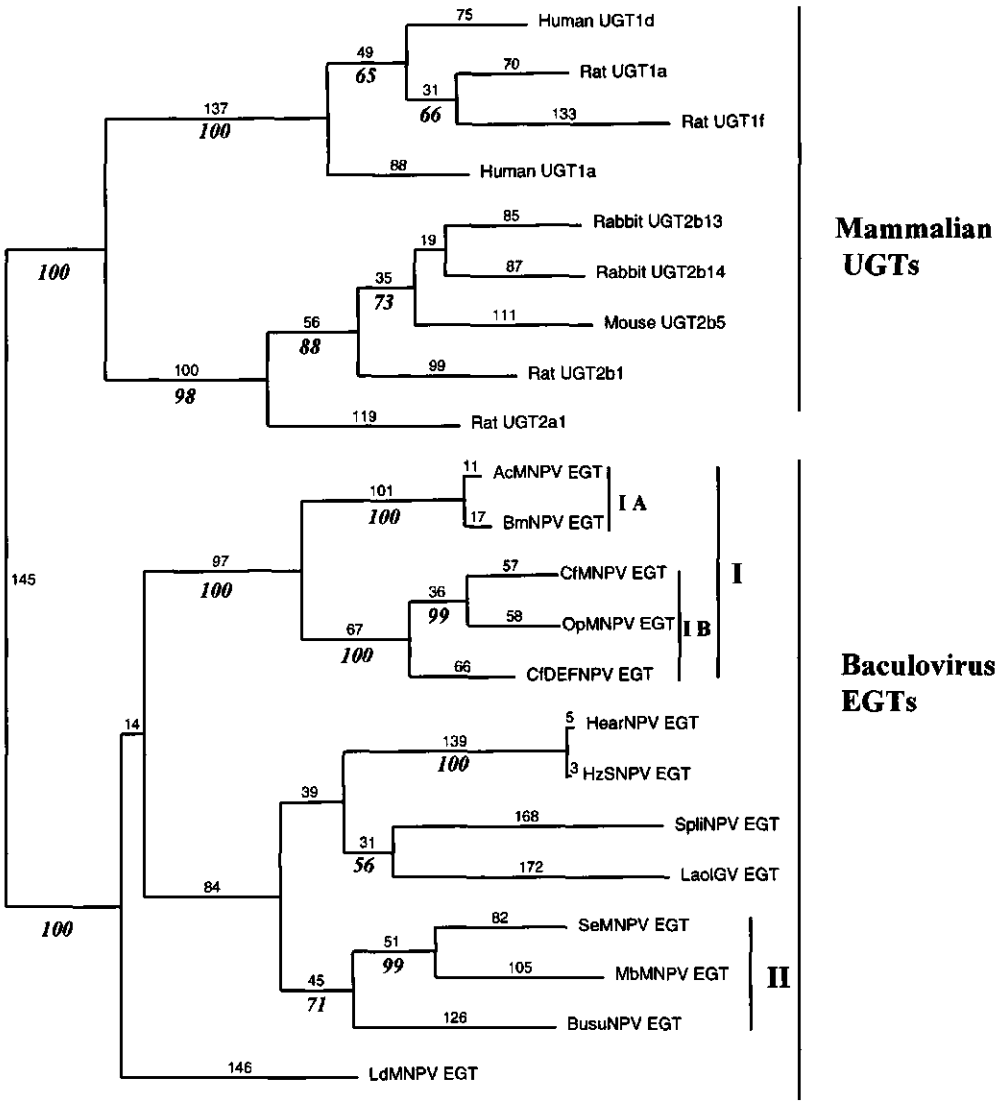



**Figure 8.4.** Phylogenetic tree of the mammalian UGTs and baculovirus EGTs. The alignment used for the analysis was shown in Fig. 8.3. The most parsimonious tree was constructed by using the heuristic search program of PAUP 3.1. Figures above the lines are the number of changes between the node and the species and those below the line, in bold italics, indicate the frequency of the cluster after bootstrap analysis (100 replicates). The group of mammalian UGTs, baculoviral EGT and NPV group I (subgroup IA and IB) and II are indicated.


**Figure 8.5.** Alignment of the sequences of 11 baculovirus LEF-2 proteins. The sources of the sequences are: AcMNPV (Passarelli *et al.*, 1993); AgMNPV (P. Zanotto, personal communication); AnfaNPV (Federici and Hice, 1997); BmNPV (GenBank L33180); BusuNPV (GenBank AF060564); CfMNPV (C. Dominy, personal communication); LdMNPV (G. F. Rohrmann, personal communication), OpMNPV (Ahrens *et al.*, 1997); PenuNPV (Z. F. Lou, personal communication); SpliNPV (O. Faktor, personal communication) and CpGV (Jehle *et al.*, 1997). PileUp program of GCG was used for making the alignment and GeneDoc software was used for homology shading. Black represents 100% similarity, deep grey 80%, and light grey 60% similarity.

In conclusion, the single gene trees based on polyhedrin/granulin, *egt* and *lef-2* genes corroborate each other to a large degree and exhibit a similar topology. Based on these trees, a proposed lepidopteran baculovirus evolution is shown in Fig. 8.7 and which reads as follows:

All baculoviruses are derived from a common ancestor. GVs separated from the lepidopteran NPVs during the early events of the evolution. Lepidopteran NPVs further diverged into Group I and II. Soon thereafter, the radiation of Group II NPVs took place. The radiation of Group I viruses came either later or was slower than that of group II. Group I diverged further into two subgroups; IA containing AcMNPV, AnfaNPV and BmNPV and IB containing AgMNPV, ArceNPV, CfMNPV, CfDEF, ChroNPV, OpMNPV. The predicted relationship of Group IB viruses is shown on the Fig. 8.7. AtriNPV and HycuNPV are predicted to be Group I viruses, but their lineage within group I is not yet certain. The radiation of Group II seems to be greater than that of Group I and the inner relationships of Group II viruses are so far not clear. Several possible subgroups are shown in Fig. 8.7, including the one containing MbMNPV, SeMNPV, SfMNPV and SpltNPV2, and the one containing HearNPV, HzSNPV and ManeNPV. It is likely that Group II contains several distinct subgroups with at least LdMNPV and SpliNPV

representing two of these. It is attempted to use the branch length in Fig. 8.7 to reflect the evolution history, but the reality need to be better defined when more data become available.

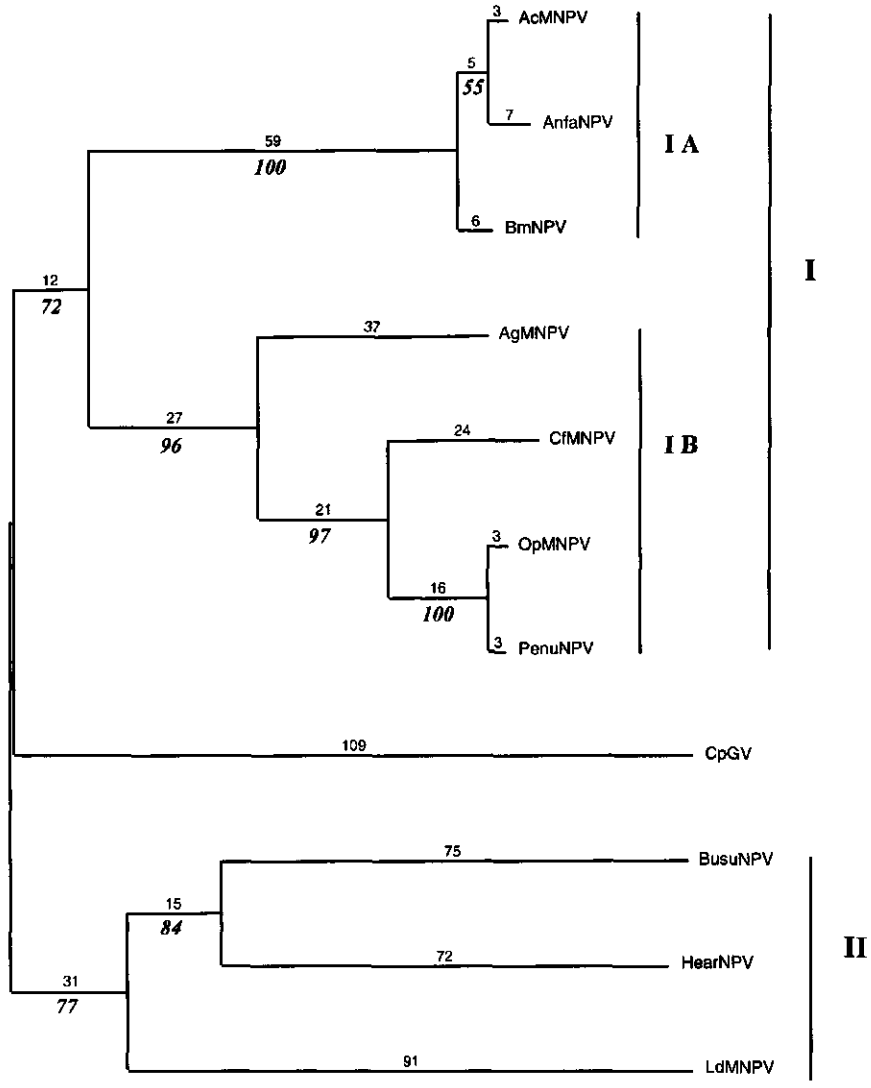



**Figure 8.6.** A phylogenetic tree of baculovirus LEF-2 proteins. The alignment used for the analysis was shown in Fig. 8.5. The most parsimonious tree was constructed by using the heuristic search program of PAUP 3.1. Figures above the lines are the number of changes between the node and the species and those below the line, in bold italics, indicate the frequency of the cluster after bootstrap analysis (100 replicates). The NPV group I , II and the subgroups of I (IA and IB) are indicated.

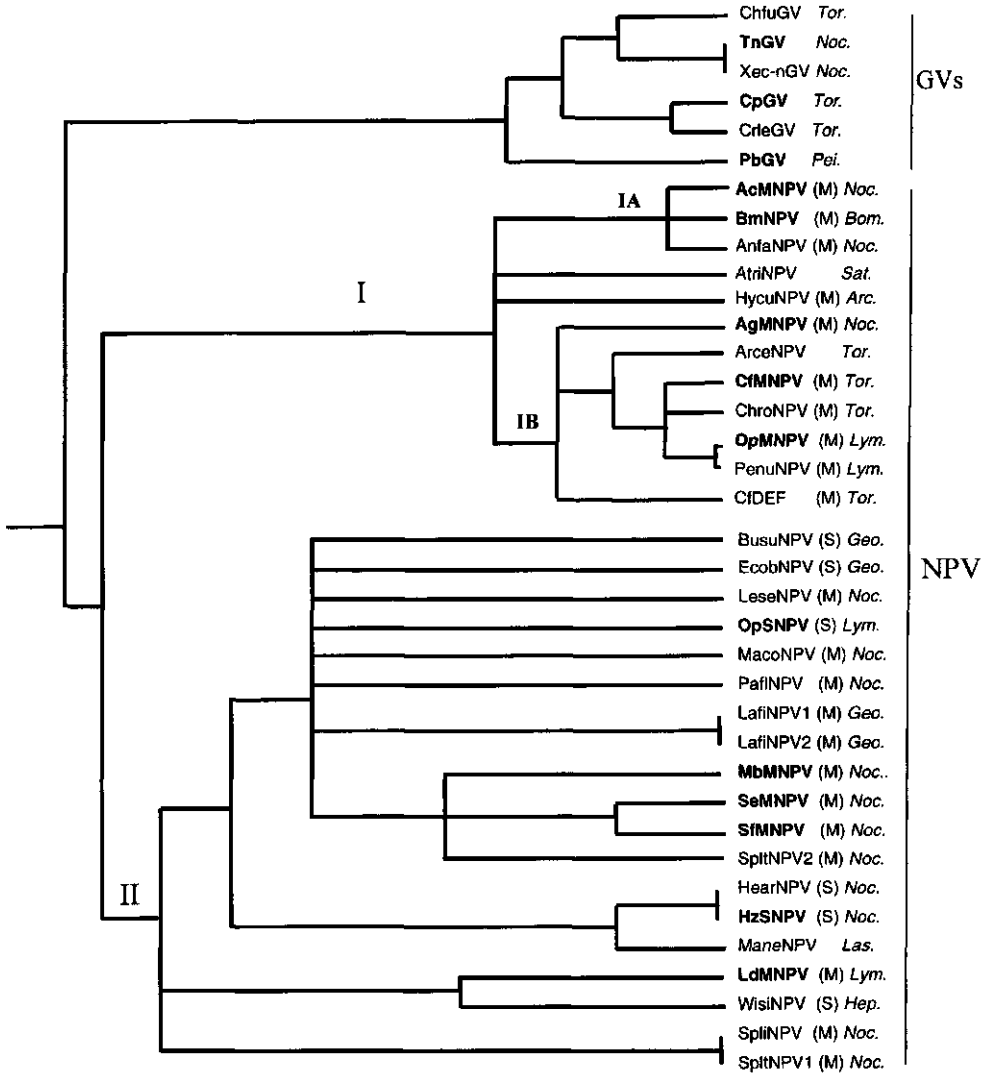



**Figure 8.7.** Proposed phylogeny of lepidopteran baculoviruses based on the trees from polyhedrin/granulin, EGT and LEF-2 proteins.

## Timescale of baculovirus evolution

The timescale of evolutionary events is important in understanding the phylogenetic history of any organisms. Since fossil records of baculoviruses do not exist and since they may or may not have co-evolved with their hosts, the most plausible timescale may, therefore, be derived from the molecular phylogeny of their proteins. The viral genome contains genes common to other organisms. Some of these genes have available molecular clocks derived from comparative studies of those other organisms. Here, we choose one such gene, the Cu/Zn superoxide dismutase (SOD) gene, for timescale analysis of baculoviruses divergence.

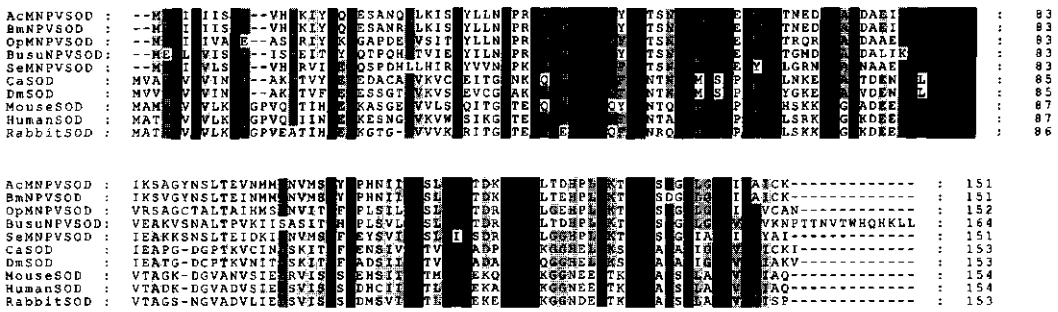



**Figure 8.8.** Alignment of the sequences of several SOD proteins. The sources of the sequences are: Tomalski *et al.* (1994) for AcMNPV; Maeda (Genbank L33180) for BmNPV, Ahrens *et al.* (1997) for OpMNPV; Hu *et al.* (1998) for BusuNPV, Kwiatowski *et al.* (1992) for CaSOD, Seto *et al.* (1989) for DmSOD, Bewley, G.C. (1988) for mouse SOD, Hallewell *et al.* (1985) for human SOD and Jankson and Ho (GenBank Z22644) for rabbit SOD. PileUp program of GCG was used for making the alignment and GeneDoc software was used for homology shading. Black represents 100% similarity, deep grey 80%, and light grey 60% similarity.

The phylogeny and molecular evolution of SOD has been well documented in many organisms. The evolutionary rate of this protein among Drosophila species has been calculated to be $16.2*10^{-10}$ replacements per site per year, which is similar to those among mammal species ($17.2*10^{-10}$) and species of insect dipteran families ($15.9*10^{-10}$). The evolutionary rate is estimated to be much slower between animal phyla ($5.3*10^{-10}$) and even slower among the three animal subkingdoms ($3.3*10^{-10}$) (Ayala, 1997).

So far, SODs from five baculoviruses, AcMNPV (Tomalski *et al.*, 1991), BmNPV (S. Maeda, Genbank L33180), OpMNPV (Ahrens *et al.*, 1997), BusuNPV (Hu *et al.*, 1998) and SeMNPV (W.F.J. IJkel, personal communication) have been identified. The baculovirual SODs were shown to be highly homologous to SODs of cellular organisms, with all the key amino acid residues being conserved (Hu *et al.*, 1998). In figure 8.8, the baculovirus SODs were aligned with several SODs from other organism, including two from Drosophilidae (*Drosophila melanogaster,* Dm; and *Chymomyza amoena, Ca*) and three from mammalia (mouse *Mus musculus*; human *Homo sapiens*; rabbit *Oryctolagus cuniculus*). Exhaustive search by PAUP

3.1 computer program resulted in two most parsimonious trees (A, B) shown in Fig. 8.9 (A, B). An average evolution rate ( R ) of $17*10^{-10}$/site/year (Ayala *et al.*, 1996) was used to calculate the divergence in time among dipterans or among mammal species and a rate of $5*10^{-10}$/site/year was used to calculate the divergence in time among different animal phyla. An average length of 150 amino acids of SODs was used for calculation. By assuming that the evolutionary rate among the baculoviruses was similar to that for dipterans or mammals ($17*10^{-10}$/site/year), the estimated divergence in time was calculated and presented in Fig. 8.9 (A,B). For example, AcMNPV and BmNPV diverged from their common ancestor about 4 million years (Myrs) and 20 Myrs ago, respectively.

The trees of SOD (Fig 8.9 A,B) are not a good representation of the evolution history of the baculoviruses where BusuNPV is grouped into Group I instead of Group II. The grouping is also poorly supported by the bootstrap analysis. The same uncertainty of grouping appears to occur for the mammalian SODs, that they are grouped differently in trees A and B. However, the divergence time of the mammals does not change considerably when tree A and B are compared. Therefore, these trees can still be used to explore the divergence time of baculoviruses. If the assumption of baculovirus SOD evolutionary rate ($17*10^{-10}$/site/year) is correct, Fig. 8.9 indicates the early divergence of the baculoviruses occurred from a common ancestor more than 100 Myrs ago. Adopting the hypothesis that the lepidopteran NPVs have evolved at the time when lepidopteran insects emerged (Vlak and Rohrmann, 1985), this result conflicts with an earlier theory based on fossil records that lepidopteran insects diverged about 40-60 Myrs ago (Reik, 1970). However, a recent report (Friedrich and Tautz, 1997) suggests that the Lepidoptera insects diverged about 250 million years ago, which is more in line with the hypothesis that baculoviruses may co-evolved with their insects hosts (Vlak and Rohrmann, 1985). Needless to say, more genes are need for analysis to elucidate baculovirus evolution more thoroughly.

**Taxonomy of GV versus NPV and SNPV versus MNPV**

Phylogenetic analyses of baculoviruses infecting lepidopteran insects indicate that the taxonomic separation of GVs from NPVs, initially based on morphology, fits with their evolutionary history. So far, GVs have been isolated only from the order Lepidoptera. The relationship between NPVs isolated from other insect orders, such as dipterans, hymenopterans and coleopterans, with those of lepidopteran NPVs and GVs is not yet known.

Although the S and M morphology is a stable character for each NPV, so far the underlying molecular genetic basis of this distinction has not been determined. Since the MNPV morphotype only exists in viruses isolated from Lepidoptera, whereas other insect orders appear to harbor only SNPVs, it has been suggested that the MNPVs have evolved from an ancient ancestral SNPV, when the Lepidoptera had emerged as a distinct insect order (Rohrmann 1986, Vlak and Rohrmann, 1985). From the phylogenetic trees presented in this chapter, it is clear that MNPVs are not a monophyletic group except Group I. Therefore, the MNPV versus SNPV morphotype can not be regarded as phylogenetic marker within lepidopteran NPVs. Instead, molecular genetic characters of these viruses, such as gene order

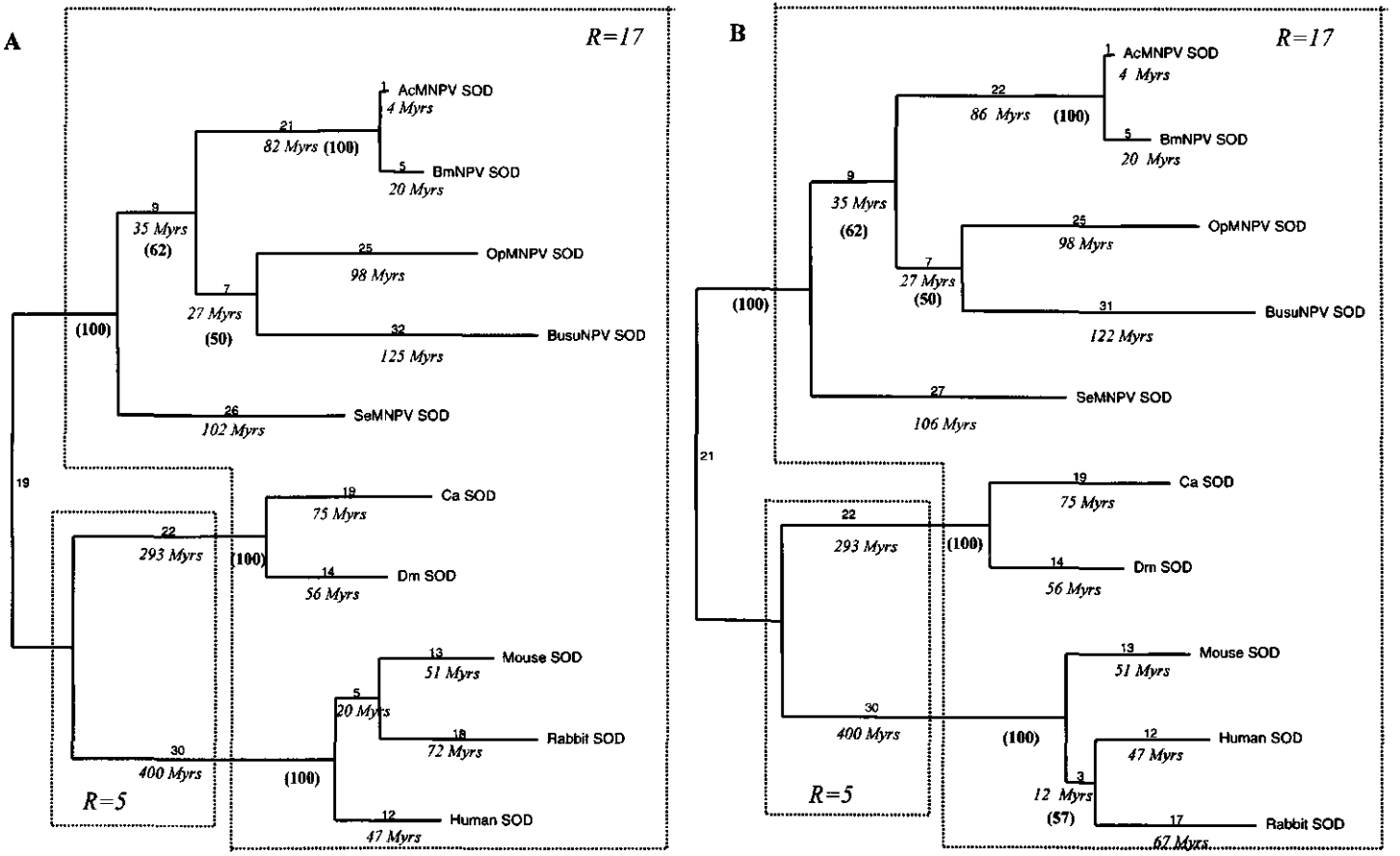



**Figure 8.9.** Timescale of SOD protein evolution: A test for baculovirus evolution. The alignment used for the analysis was shown in Fig. 8.8. The two most parsimonious trees constructed using the exhaustive search program of PAUP 3.1 are shown in A and B. Figures above the lines are the number of changes between the node and the species. The numbers below the line, in italics, represent the calculated divergence in time by using the evolution rate (R, $x10^{-10}$ /site/year) indicated in the top or bottom corners of the dished squares. Bootstrap analysis results above 50% majority (out of 100 replicates) were indicated in brackets.

and sequence should be used to determine a more accurate taxonomic status for baculoviruses. In this respect, the taxonomy of Group I and II forms a good starting point.

**Host range and taxonomy**

Most baculoviruses are host specific indicating that they have adapted to their respective hosts for optimal production. The mechanisms that determine the baculovirus host range or specificity are not very clear at the moment, but several genes have been identified that are somehow involved in baculovirus host range (see Thiem, 1997, for review). These include genes involved in viral DNA replication and gene expression such as helicase (Maeda *et al.*, 1993; Croizier *et al.*, 1994), *lef-7* (Lu and Miller, 1995; Chen and Thiem, 1997), host cell specific factor-1 (*hcf-1*, late expression factor; Lu and Miller, 1995; *ibid.*, 1996); genes involved in overcoming host cellular defense system such as the inhibitor of apoptosis (IAP) and p35 (see review by Clem *et al.*, 1996); as well as genes involved in global protein synthesis such as host range factor-1 (*hrf-1*, Thiem *et al.*, 1996). A recent study suggests that some sort of an immune response is implicated in the insect restriction of baculoviral systemic infection (Washburm *et al.*, 1996). Thus, the factors involved in viral specificity and their mode of action are still enigmatic.

Baculoviruses are usually named after the insect from which they were first isolated (Murphy *et al.*, 1995). The drawback of this system is that a virus with a broad host range, such as AcMNPV, could be isolated from a number of different insect species. On the other hand, distinct viruses have been isolated from the same insect species such as OpMNPV and OpSNPV from *O. pseudotsugata* and, CfMNPV and CfDEFNPV from *C. fumiferana*. Therefore, baculovirus taxonomy based on host range may not be supported by molecular genetics and phylogeny.

**Baculovirus genome organization and phylogeny**

Based on the genomic characteristics of BusuNPV, AcMNPV, BmNPV and OpMNPV, it can be concluded that the genome contents of lepidopteran baculoviruses are very similar that approximately 80% of their genes are conserved. Since the sequence information of baculovirus isolated from insect orders other than Lepidoptera is not yet available, it is not known for certain that the similarity in gene content is universal for the whole baculovirus family. One can, however, assume that the similarity is universal because of the structural and pathological properties that define a baculovirus. The high conservation of gene content of the lepidopteran viruses may also indicate that most genes are essential for multiplication and survival. The distinct genomic organization of BusuNPV suggests that gene arrangement can be used as an independent marker to determine baculovirus phylogeny. The impact of gene arrangement on phylogeny will likely be more profound in the near future when more data from other baculovirus genomes become available.

**What are the molecular genetic criteria that define a distinct baculovirus species?**
It was shown in Chapter 5 that closely and distantly related baculoviruses are distinguished from each other using homology analyses of their genes (Fig. 5.4). It is therefore tempting to

define a homology level at which two viruses are assumed to be the same species. The amino acid identity at this hypothetical level will have to be 90% or higher since the so called close-related baculoviruses (e.g. AcMNPV and BmNPV) are already at that level. These two viruses have been assigned distinct species status presumably because of their different gene content and distinct host range. The divergence time predicted in Fig. 8.9 also indicates that they diverged from each other about at least 4 million years ago.

Among the relatively well characterized baculoviruses (Table 8.1.), HearNPV and HzSNPV are two candidates that could be the same species. They both have the same host range and their identified proteins have high amino acid identity, such as 97% for chitinase and 99% for EGT. Another pair of candidates is OpMNPV and PenuNPV, that share 94% amino acid identity for P10 and 97% for LEF-2, although their host range needs to be identified. Three other pairs of candidates, LafiNPV1/LafiNPV2, SpliNPV /SpltNPV1 and TnGV/Xex-nGV, with about 100% amino acid identity for occlusion protein, also appear to belong to three species (Fig. 8.7). Therefore, the 37 separate baculoviruses listed in Table 8.1 and Fig 8.7 are likely to represent 32 different species. As more molecular and biological data become available, the taxonomy and phylogeny of baculoviruses will be more redefined.

CHAPTER 9

## SUMMARY

Baculoviruses are attractive biological alternatives to chemical insecticides for insect pest control. So far, more than 600 baculoviruses have been isolated from different insect species which provide a rich resource for developing new viral insecticides. Most baculoviruses are host specific. Baculovirus nomenclature is based on the host from which the virus was first isolated and the taxonomy is based on the morphology of the occlusion body, polyhedra (nucleopolyhedroviruses = NPVs) or granula (granuloviruses = GVs). Until 1995, the NPVs had been further subdivided into single-nucleocapsid (S) and multiple-nucleocapsid (M) NPVs. Phylogeny studies based on the polyhedrin gene indicated that lepidopteran NPVs can be classified into two groups (I and II). Group II NPVs were found to encompass both SNPVs and MNPVs, whereas group I NPVs were composed of only MNPVs. Three Group I NPVs, *Autographa californica* (Ac) MNPV, *Orgyia pseudotsugata* (OpMNPV) and *Bombyx mori* (Bm) NPV, have been entirely sequenced and their genome organization was found to be very similar. Such detailed sequence information from other baculoviruses is scare particularly from SNPVs and GVs. The subject of this thesis is the *Buzura suppressaria* (Busu) NPV which was isolated from a Geometrid insect (tea moth) and has been used successfully as a bio-insecticide in China. Significant amount of sequencing was conducted on a genomic library of this virus which allowed us to conduct comparative investigations on the genome organization and phylogeny.

First, the most conserved baculovirus gene, the polyhedrin gene, was identified by hybridization and then sequenced (Chapter 2). The location of the polyhedrin gene was used to set the zero point of the physical map of the viral genome. Phylogeny research based on the polyhedrin sequence placed BusuNPV into the Group II baculoviruses (see Chapter 8).

Ecdysteroid UDP-glucosyltransferase (EGT) is an enzyme encoded by baculoviruses which delays larval molting and allows the virus to extend the production of large numbers of progeny virus. Deletion of the *egt* gene from the genome of baculoviruses results in an increased speed of kill and a reduced larval feeding activity and therefore enhances viral insecticidal activity. The strategy of deleting *egt* is now being adopted for the engineering of baculoviruses of economically important insect pests. In Chapter 3, the identification, sequencing and comparative analysis of the *egt* gene of BusuNPV is reported. This is the first *egt* gene characterized in a SNPV. Maximum parsimony analysis based on the amino acid sequence of baculoviruses EGTs confirmed that BusuNPV belongs to Group II baculoviruses.

In order to study the genome organization of BusuNPV, a plasmid library covering the entire genome was constructed and the inserts were terminally or completely sequenced. In Chapter 4, the sequence analysis of the *Hin*dIII-J region (3.2 kb) of the BusuNPV genome revealed five ORFs (*pep*, *ORF117*, *ctl*, *iap* and *sod*). The genomic arrangement of these ORFs in BusuNPV

turned out to be significantly different from that of AcMNPV and other characterized baculoviruses. This indicates that BusuNPV may have a gene arrangement distinct from other baculoviruses identified so far.

To further investigate the genomic organization and phylogenetic status of BusuNPV, a detailed physical map was constructed by extensive restriction enzyme analysis and Southern blot hybridization (Chapter 5). The size of the viral genome was estimated to be approximately 120.9 kb. About 43.5 kb of dispersed sequence information was generated from the plasmid library. Fifty-two ORFs, homologous to those of other baculoviruses, were identified and their location on the BusuNPV genome determined (Chapter 5). Although the gene content of BusuNPV, based on these 52 ORFs, is similar to that of AcMNPV, BmNPV and OpMNPV the arrangement is, however, significantly different from the latter, highly co-linear Group I NPVs. A new approach (GeneParityPlot) was developed to represent the differences in gene order among baculoviruses when limited sequence information is available. The method is useful to identify potential conserved gene clusters. The data obtained show that BusuNPV is a distinct baculovirus species and suggest that group II viruses may have a genome organization distinct from Group I viruses. It is concluded that the gene distribution along baculovirus genomes may be used as an independent parameter to study baculoviral phylogeny.

Protein P10 is a major component of the fibrillar structures found in the cytoplasm and nucleus of infected insect cells. This small protein is involved in the release of polyhedra from infected cell-nuclei late in infection and plays a role in morphogenesis of the occlusion body. A putative p10 gene was identified for the first time in a SNPV (BusuNPV) based on its size and domain structures. Its function was determined in a 'swap assay' by using an AcMNPV p10 deletion mutant as an acceptor for the putative BusuNPV p10 gene (Chapter 6). The AcMNPV recombinant expressing the BusuNPV P10 formed fibrillar structures in the cytoplasm of *Spodoptera frugiperda* cells, but was unable to induce nuclear disintegration. This supports the current hypothesis that additional viral factors are required for this process.

Polyhedrin is the major component of the occlusion body, which encapsulates the virions and protects them against physical and chemical decay. The gene swap assay was applied to investigate the specificity of the polyhedrin gene in the generation of occlusion bodies (Chapter 7). The BusuNPV polyhedrin gene expressed by AcMNPV resulted in the formation of normal occlusion bodies containing multiple-capsid virions. The occlusion bodies were as infective as normal AcMNPV suggesting that the occlusion process is not SNPV or MNPV specific. However, the recombinant AcMNPV expressing the polyhedrin gene of *Spodoptera exigua* (Se) MNPV, had an altered polyhedron morphology, pirmidal in shape, with few occluded virions. The infectivity was also lower than that of wild-type AcMNPV. These data suggest that the occlusion process is fine-tuned and may be dependent on one or more factors in addition to polyhedrin, that determine the size, shape and occlusion of virions.

The genetic analysis compiled in this thesis shows that BusuNPV is a distinct baculovirus species. Alignment of various BusuNPV ORFs with baculovirus homologues and phylogenetic analyses indicated that SNPVs do not form a monophyletic group within the phylogenetic trees. The gene content of BusuNPV is very similar to that of AcMNPV, BmNPV and OpMNPV, suggesting that baculoviruses may have most of their genes in common. In addition to gene homology genome organization may also reflect the evolutionary history of baculoviruses. The molecular genetic studies of BusuNPV and their impact to future engineering of the virus for improved insecticidal activity are discussed (Chapter 8).

## SAMENVATTING

Baculovirussen vormen een aantrekkelijk alternatief voor chemische middelen voor de bestrijding van schadelijke insecten. Tot dusverre zijn er meer dan 600 baculovirussen geïsoleerd bij uiteenlopende insectensoorten en deze vormen een rijke bron om nieuwe bestrijdingsmiddelen te ontwikkelen op basis van baculovirussen. De naamgeving is bij baculovirussen gebaseerd op de gastheer, waaruit ze zijn geïsoleerd, en op de verschijningsvorm van het virus, polyeders in het geval van de kernpolyedervirussen (nucleopolyhedrovirussen = NPV) en granula bij de granulovirussen (= GV). Tot voor kort werden de NPV's onderverdeeld in SNPV's en MNPV's, die respectievelijk één (single = S) of meer (M) staafvormige nucleocapsiden hebben ingesloten per virion. Fylogenetische studies gebaseerd op het polyedereiwit, dat de matrix vormt van de polyeder waarin de virions zijn opgesloten, gaven aan dat de NPV's die bij schubvleugelige insecten (Lepidoptera) voorkomen in twee groepen kunnen worden onderverdeeld (I en II). Groep II omvat zowel MNPV's als SNPV's, terwijl groep I baculovirussen alleen MNPV's omvat en de vraag is in hoeverre SNPV en MNPV twee monofyletische groepen zijn. Van drie baculovirussen uit groep I (*Autographa californica* MNPV, *Orgyia pseudotsugata* MNPV en *Bombyx mori* NPV) is de volledige DNA basenvolgorde bekend en de organisatie van hun genoom bleek onderling zeer verwant te zijn. Gedetailleerde informatie van andere baculovirussen, zoals SNPV's en GV's is nog zeer schaars. Dit proefschrift beschrijft de karakterisering van het genoom van *Buzura suppressaria* SNPV (BusuNPV) en de fylogenetische relatie met andere baculovirussen. BusuNPV is met succes gebruikt bij de bestrijding van thee-mot (Geometridae) in de Volksrepubliek China.

Als eerste werd het zeer geconserveerde baculovirus-gen, polyhedrine, geïdentificeerd via nucleïnezuurhybridisatie en de basenvolgorde bepaald (Hoofdstuk 2). The locatie van dit gen op het DNA van BusuNPV werd gebruikt om het nulpunt van de circulaire fysische kaart te bepalen. Fylogenetische analyse van polyhedrine gaf aan dat BusuNPV behoort tot de 'groep II' baculovirussen, maar ook dat dit virus meer verwant is met een MNPV dan met andere SNPV's (Hoofdstuk 2).

Ecdysteroid UDP-glucosyltransferase (EGT) is een door baculovirussen gecodeerd enzym, dat de larvale ontwikkeling ontregelt ten voordele van de productie van grote hoeveelheden polyeders. Insecten, die geïnfecteerd zijn met baculovirussen zonder het gen voor EGT (*egt* deletiemutanten) gaan sneller dood en veroorzaken minder vraatschade. Via deletie van het *egt* gen kan de insecticide-werking van baculovirussen worden verbeterd. Deze strategie wordt nu toegepast om baculovirussen te ontwikkelen voor bestrijding van insecten voor veel gewassen van economisch belang. De identificatie, moleculaire organisatie en fylogenie van het *egt* gen van BusuNPV wordt in Hoofdstuk 3 beschreven. Dit is het eerste *egt* gen dat bij SNPV's is gevonden. De fylogenetische analyse van EGT bevestigde dat BusuNPV tot de 'groep II' baculovirussen behoort.

Om de genetische organisatie van het BusuNPV genoom nader te bestuderen werd een plasmidenbibliotheek gemaakt, die het gehele virale genoom omvat (Hoofdstuk 4). De

basenvolgorde van de virale segmenten, die in de plasmiden waren opgenomen, werd veelal vanaf de uiteinden en soms in zijn geheel bepaald. Aldus werd ondermeer de basenvolgorde van het *Hin*dIII-I fragment van BusuNPV (3,2 kilobasenparen) geanalyseerd en een vijftal open leesramen (open reading frames = ORF's) geïdentificeerd (*pep*, ORF117, *ctl*, *iap* en *sod*), die ook al in AcMNPV en OpMNPV DNA voorkomen. De plaats van deze ORF's op het genoom van BusuNPV is significant verschillend ten opzichte van AcMNPV en OpMNPV en dit zou er op kunnen wijzen dat SNPV's een andere gen-organisatie hebben.

Om de genetische organisatie van het genoom en de fylogenetische positie van BusuNPV verder te onderzoeken werd een gedetailleerde kaart gemaakt van restrictie-enzym-knipplaatsen op het genoom via restrictie-enzymanalyse en nucleïnezuurhybridisatie (Hoofdstuk 5). De afmeting van BusuNPV DNA werd aldus bepaald op circa 120,9 kb. Ongeveer 43,5 kb aan genetische informatie werd verkregen via het willekeurig bepalen van de basenvolgorden van inserties van plasmiden uit de plasmidenbank. Tweeënvijftig ORF's werden geïdentificeerd, waarvan een homoloog gen aanwezig is in AcMNPV, OpMNPV en BmNPV. De positie van deze 52 ORF's op het genoom van deze drie baculovirussen is vrijwel gelijk, maar verschilt significant van die van BusuNPV. Een nieuwe methode (GeneParityPlot) werd ontwikkeld om de verschillen tussen baculovirussen in genoomorganisatie zichtbaar te maken, met name in die gevallen waarbij maar beperkte basenvolgorde-informatie beschikbaar is. Deze methode kan wellicht later gemathematiseerd en geschikt gemaakt worden voor fylogenetische studies. Uit basenvolgorden-analyse van BusuNPV DNA blijkt in elk geval dat BusuNPV een unieke baculovirussoort is, duidelijk te onderscheiden van 'groep I' baculovirussen, en dat de verdeling van de ORF's over het genoom een fylogenetische parameter zou kunnen zijn, onafhankelijk van de fylogenie op basis van individuele gen-analyse.

Het P10 eiwit is een belangrijke component van fibrillaire structuren in de kern en het cytoplasma van geïnfecteerde cellen. Dit kleine eiwit is betrokken bij de vrijmaking van polyeders uit de celkern laat in infectie en speelt een rol bij de morfogenese van polyeders. In Hoofdstuk 6 werd een mogelijk *p10* gen in het BusuNPV genoom geïdentificeerd op basis van de grootte en de domeinstructuur van het eiwit en zijn functie werd verder onderzocht door uitwisseling van het AcMNPV p10 gen met het mogelijke BusuNPV *p10* gen. De verkregen AcMNPV recombinant vormde normale fibrillaire structuren in het cytoplasma van *Spodoptera frugiperda* cellen, maar was niet in staat de desintegratie van de celkern en vrijmaking van polyeders te bewerkstelligen. Dit resultaat ondersteunt de hypothese dat er nog andere virale factoren bij dit proces zijn betrokken. Dit is het eerste p10 gen, geïdentificeerd bij SNPVs en op functionaliteit getoetst.

Polyhedrine is de belangrijkste component van de polyeders, die de virions omsluiten en deze beschermen tegen fysische en chemische afbraak. De specificiteit van het insluitingsproces van MNPV's versus SNPV's werd in Hoofdstuk 7 verder bestudeerd door het AcMNPV polyhedrine-gen uit te wisselen met het BusuNPV polyhedrine-gen. De aldus verkregen polyeders vertoonden normale opsluiting van virions en waren biologisch net zo actief als wild-type AcMNPV. Dit zou aangeven dat het insluitingsproces niet MNPV of SNPV-specifiek is.

AcMNPV voorzien van het polyhedrine-gen van *Spodoptera exigua* (Se) MNPV daarentegen maakte piramidevormige polyeders waarin maar weinig virions waren opgenomen. Deze polyeders waren tevens minder biologisch actief dan wild-type AcMNPV. Deze resultaten geven aan dat er wellicht toch nog een of meer virale/cellulaire factoren bij de vorming van polyeders is betrokken, die de grootte, de vorm en de mate van virion-insluiting bepalen.

Genetische analyse, zoals beschreven in dit proefschrift gaf aan dat BusuNPV een onderscheidelijk baculovirus is. Vergelijking van diverse BusuNPV ORFs met homologe ORF's van andere baculovirussen en fylogenetische analyse gaf aan dat SNPV's geen monofyletische groep vormen. De waarneming, dat AcMNPV, OpMNPV en BusuNPV voor 80% dezelfde set van genen bevatten, suggereert dat baculovirussen een groot aantal genen gemeenschappelijk hebben. De volgorde waarin genen op het baculovirusgenoom voorkomen kan, evenals de verwantschap van de genen onderling, een afspiegeling zijn van de evolutie van baculovirussen. Een mogelijk mechanisme om de virus-specifieke volgorde van deze kennelijk essentiële genen te verklaren en de mogelijkheden om, met de in dit proefschrift opgedane kennis, BusuNPV genetisch te veranderen voor een effectiever bestrijdingsmiddel te krijgen worden in Hoofdstuk 8 bediscussieerd.

# 总结

杆状病毒杀虫剂可替代化学农药杀灭害虫，减少环境污染，具有广泛应用前景. 目前国际上已分离出600多种杆状病毒，为筛选和制备杀虫剂提供了丰富的资源. 杆状病毒科包含核型多角体病毒属(NPV)及颗粒体病毒属(GV). 依据病毒粒子的包埋形态，NPV可分为单粒包埋(S)和多粒包埋(M)两种类型. 杆状病毒的分子进化研究表明，鳞翅目NPV可分为两大组(I和II)，其中I仅包含MNPV，而II则有SNPV和MNPV. 目前仅有AcMNPV，OpMNPV 及BmNPV 3种病毒的全序列报道. 这3种病毒均属组I，序列分析显示它们的基因组结构十分类似. 与MNPV相比，SNPV及GV的序列少有报道. 因此，本论文选择在中国成功用于防治茶树害虫的油桐尺蠖SNPV(BusuNPV)进行详尽的基因组结构分析，并与其它杆状病毒基因组进行比较，从而研究杆状病毒的分子分类及分子进化.

第二章介绍了BusuNPV的多角体蛋白基因. 多角体蛋白是包涵体的主要组成成份，多角体蛋白基因是杆状病毒中最保守的基因之一. 该章报道了多角体蛋白基因的定位、克隆和序列测定，为绘制BusuNPV基因组物理图谱及确定其分类地位奠定了基础.

第三章介绍了BusuNPV的脱皮激素乙酰基转移酶(EGT)基因. EGT是由杆状病毒编码并作用于宿主昆虫的基因. 该酶通过阻滞昆虫脱皮达到大量繁殖子代病毒的目的. 从基因组中删除egt基因，不仅加快杆状病毒的杀虫速度，而且减少宿主昆虫的进食量，从而提高杆状病毒的杀虫性能并减轻宿主昆虫对植物的损害. 一些重要杆状病毒杀虫剂均利用了缺失egt基因的方法进行重组改良. 该章报道了BusuNPV egt基因的序列测定及比较分析. 这是在SNPV中首次发现egt基因. EGT分子进化分析将BusuNPV分类定位在NPV组II.

为分析BusuNPV的基因组结构，首先构建并筛选了该病毒的基因文库. 用这些克隆片段进行末端或整体序列分析. 第四章是其中一个3.2 kb的HindIII酶切片段的序列分析结果. 该片段编码*pep*, *orf117*, *ctl*, *iap*及*sod* 等5个基因. BusuNPV基因组上这5个基因的排列方式与AcMNPV和其它已知杆状病毒有明显不同，表明该病毒可能具有独特的基因组结构.

第五章报道了BusuNPV的精细物理图谱和基因图，并对BusuNPV的基因组结构及其在杆状病毒中的分类地位进行更深入的分析. 首先通过大量的内切酶分析和Southern杂交构建了BusuNPV的物理图谱，结果表明该病毒的基因组大小为120.9kb. 利用已构建的基因文库，测定了基因组中43.5 kb的核苷酸序列，对这些序列的比较分析确定了BusuNPV基因组中52个杆状病毒同源基因. 结果表明，BusuNPV虽然与已知的AcMNPV，BmNPV及OpMNPV的基因组成相似，但基因排列分布则与上述其它病毒有明显差异. 在第五章中我们还在国际上首次报道了一种比较基因组排列方式的新方法 - 基因对等排列图(GeneParityPlot)，该方法以简单明了的线性方式显示不同病毒的基因排列，不仅可用于完整的基因组间的比较，而且还可用于不完全基因组间的比较，这对寻找进化保守的基因群尤其有效. 研究显示BusuNPV是一个与其它杆状病毒显著不同的新种，预测NPV组II的基因排列可能与组I有明显差异，文章最后指出基因排列方式可作为杆状病毒分子进化分析的新参数.

第六章介绍了BusuNPV的P10基因. P10基因是杆状病毒在感染晚期高度表达的基因, 其表达产物构成被感染细胞的细胞质与细胞核中独特的纤维状结构, 并参与多角体的释放和包涵体的形成. BusuNPV的P10基因是通过其序列结构特征发现的, 这是在SNPV中首次报道P10基因. 为进一步证实该基因, 我们将它与AcMNPV中的P10基因重组置换, 结果表明, 重组病毒可在 Sf 细胞质中形成纤维状结构, 但形成的多角体不能释放, 从而证实该基因具有P10基因的功能, 同时揭示病毒多角体的释放需要其它病毒因子参与.

第七章研究多角体蛋白基因与被包含的病毒粒子之间的相互关系. 多角体可保护病毒粒子免受外界物理和化学因素降解. NPV有多粒包埋(M)及单粒包埋(S)两种形式, 但至今这两种形态的产生机理仍未阐明. 该章通过将BusuNPV(SNPV)及SeMNPV的多角体蛋白基因与AcMNPV基因组中的多角体蛋白基因进行置换, 研究多角体蛋白在病毒粒子包埋及多角体成熟过程中的作用. 结果表明当BusuNPV的多角体蛋白置换了AcMNPV的多角体蛋白时, 重组AcMNPV所形成的多角体呈多粒包埋类型, 并且这些多角体也具有良好的生物活性. 因此, 多角体蛋白在选择被包埋的病毒粒子时并无明显的单粒或多粒倾向性. 但当以SeMNPV的多角体蛋白替代了AcMNPV中的多角体蛋白时, 重组AcMNPV形成的多角体具有与野生型AcMNPV或SeMNPV所不同的表型结构. 这些多角体中所包含的病毒粒子比野生型的少, 其生物活性也明显低于野生型多角体, 表明包含体的形成过成是一个复杂而精细的过程, 与多角体及其它病毒因子等因素有关.

第八章最后总结BusuNPV的遗传分析及其对改良杀虫性能的潜在作用; 并着重讨论了杆状病毒的分子进化. 该论文的研究表明BusuNPV代表一个独特的杆状病毒种. 通过比较多角体蛋白基因、egt基因及lef2基因, 绘制了杆状病毒分子进化的模拟进化树. 研究表面SNPV在进化树中没有明显的独立分支. 通过对SOD蛋白的进化分析研究, 首次推测了杆状病毒的进化时间. 该章同时对杆状病毒分子分类等有关内容进行了探讨.

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## CURRICULUM VITAE

Hu ZhiHong was born on March 23, 1966 in Wuhan, China. She completed her BSc study at the Department of Virology and Molecular Biology, Wuhan University, in 1986 and continued her MSc study at the Wuhan Institute of Virology (WIV) of the Chinese Academy of Sciences (CAS) in Wuhan. During the latter study she was supervised by professor Xie Tianen and studied the baculovirus of *Buzura suppressaria* (BusuNPV). In 1989 she received her MSc degree and became a research scientist at the WIV. Guided by professor Jin Feng she participated in a NSFC-sponsored project on the molecular biology of BusuNPV. Her scientific and technical skills were further improved by working as a visiting scientist for about one year in the laboratory of professor Liu Xinyuan at the Shanghai Institute of Biochemistry of CAS. In 1992, she was appointed as assistant-professor at the WIV. In 1993 she obtained a Marie Curie grant from EC which enabled her to work as a visiting scientist in the baculovirus research group of professor Just Vlak at the Department of Virology, Wageningen Agricultural University (WAU), the Netherlands, for one year. This stay initiated a PhD study on the genome organization of BusuNPV under supervision of professor Just Vlak. The study was carried out at the WAU (July 1995 - September 1996; August 1997 - September 1998) and at the WIV. The results of this research are described in this thesis.

Hu Zhihong was appointed as associate-professor in 1994 and professor in 1997 at the WIV. She is currently the head of the Laboratory of Virology, WIV, CAS and intends to continue her research in Virology.