DEVELOPMENT OF A BACULOVIRUS INSECTICIDE EXPLOITING THE *BACILLUS THURINGIENSIS* INSECTICIDAL CRYSTAL PROTEIN

John Martens

Promotor: Dr. R.W. Goldbach,
Hoogleraar in de Virologie

Co-promotoren: Dr. J.M. Vlak,
Universitair hoofddocent
Vakgroep Virologie

Dr. D. Bosch
hoofd sectie Genexpressie
CPRO-DLO

John Martens

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Stellingen

1. Neurotoxines zijn beter geschikt om de werkingssnelheid van baculovirussen te verhogen dan toxines van *Bacillus thuringiensis*.

   Dit proefschrift.

2. Toxines geproduceerd door *Bacillus thuringiensis* zijn in staat, zonder tussenkomst van een receptor, poriën in membranen van cellen te maken.

   Dit proefschrift.

   Slatin, L.S., *et al.* 1990. Delta-endotoxins form cation-selective channels in planar lipid bilayers. Biochem. Biophys. Res. Commun. **169**:765-772.

3. Extracellulaire virusdeeltjes zijn, in tegenstelling tot wat Morris en Miller (1992) beweren, niet geschikt om de risico's van verspreiding van recombinante baculovirussen te toetsen.

   Morris, T.D. and L.K. Miller. 1992. Promoter influence on baculovirus-mediated gene expression in permissive and nonpermissive insect cells. J. Virol. 66:7397-7405.

4. Choma and Kaplan (1990) en Choma et al. (1991) hebben ten aanzien van de conformatie verandering die mogelijk optreedt tijdens de activatie van het protoxine maar in één van hun hieronder genoemde tegenstrijdige artikelen gelijk.

   Choma, C.T. and H. Kaplan. 1990. Folding and Unfolding of the protoxin from *Bacillus thuringiensis*: Evidence that the toxic moiety is present in an active conformation. Biochemistry **29**:10971-10977.

   Choma, C.T., *et al.* 1991. The toxic moiety of the *Bacillus thuringiensis* protoxin undergoes a conformational change upon activation. Biochem. Biophys. Res. Commun. **179**:933-938.

5 Het gebruik van pre-immuunserum (Ryerse *et al.*, 1990) is niet de juiste controle om te bepalen of de waargenomen binding aan de microvilli van het middendarmepitheel specifiek is.

   Ryerse, J.S., *et al.* 1990. Light microscope immunolocalization of *Bacillus thuringiensis kurstaki* delta-endotoxin in the midgut and Malpighian tubules of the tobacco budworm, *Heliothis virescens*. J. Invertebr. Pathol. **56**:86-90.

6. Zanotto *et al.* (1992) denken hun hypothese, dat het A/TTTGTA motief in de polyhedrine en de p10 promoter verantwoordelijk is voor de binding van RNA polymerase III, waarschijnlijker te maken door promoters die niet aan de hypothese voldoen niet in hun vergelijking mee te nemen.

   Zanotto, P.M. de A., *et al.* (1992). The *Anticarsia gemmatalis* nuclear polyhedrosis virus polyhedrin gene region: sequence analysis, gene product and structural comparison. J. Gen. Virol. **73**:1049-1056.

7. Een met "multiple alignment" gegenereerd dendogram wordt vaak ten onrechte gebruikt voor het demonstreren van evolutionaire verwantschappen.

Yamamoto T. and G.K. Powell. 1993. *Bacillus thuringiensis* crystal proteins: Recent advances in understanding insecticidal activity. *In* L. Kim (ed.), Advanced engeneered pesticides. New York.

Kalman S. *et al.* 1993. Cloning of a novel cryIC-type gene from a strain of *Bacillus thuringiensis* subsp. *galleriae*. Appl. Environm. Microbiol. **59**:1131-1137.

8. De conclusie van Matzuk *et al.* (1992) dat het α-inhibine gen een tumorsuppressor gen is is voorbarig.

Matzuk M.M. *et al.* 1992. α-inhibin is a tumour-suppressor gene with gonadal specifity in mice. nature **360**:313-319.

9. Ook voor de gewasbescherming geldt: "voorkomen beter is dan genezen".

10. Het feit dat er vaak met nucleotidenvolgorden van nieuwe genen geen homologie wordt gevonden met genen in de databank wil niet zeggen dat er geen homologie is, maar dat het gebruikte algoritme niet in staat is deze te vinden.

11. Review artikelen mogen wel wat strenger gereviewed worden.

12 Een sterke promotor-activiteit is van wezenlijk belang voor het goed tot expressie komen van een proefschrift.

13. Het schrijven van een proefschrift leidt tevens tot een onnodige verhoging van de stapel oud papier.

14. Darwin heeft het nooit durven beweren, maar religies evolueren ook.

15. "Always" is wat overdreven voor een tijdelijk probleem.

16. Ten aanzien van rechts-extremisme geldt "spreken is zilver" maar "zwijgen is fout"!

Stellingen behorende bij het proefschrift:

Development of a baculovirus insecticide exploiting the *Bacillus thuringiensis* insecticidal crystal protein

Wageningen, 2 december 1994 John Martens

VOORWOORD

Persoonlijk voel ik er wel wat voor om het voorwoord wat eigenlijk een nawoord is, want alle arbeid is al achter de rug, kort te houden (ik houd niet zo van typen en dat heb ik de laatste tijd al iets te veel gedaan), maar ik denk niet dat dat erin zit. Ik zou daarmee veel mensen te kort doen en er later ook spijt van krijgen. In het bijzonder gaat mijn dank uit naar mijn kamergenoten van het oude virologie gebouw (that's were it all happened): Marcel, Monique, en Bep; de zwoegende AIOs. Opgeteld waren we altijd meer dan de afzonderlijke delen. Daarnaast wil Just bedanken voor het begeleiden en het sturen van het onderzoek en Rob voor het kritisch beoordelen van alles wat uit de pen is gekomen. Zo ben ik binnen afzienbare tijd op dit proefschrift afgestoomd. Daarnaast wil ik alle baculovirologen die direct bij mijn proefschrift betrokken waren bedanken: Magda, Els, Douwe, Ine, Jan Roosien, Ruud, Peter Roelvink, René, Jacco en Fons. De EM afdeling: Hans, Joop en Jan van Lent, die altijd dingen zien die wij willen zien. Verder wil ik onze tegenhangers, de plantevirologen, niet vergeten. Ik noem de belangrijkste: Frank, Richard, Ineke, Peter de Haan, Marcel Prins, Rene van de Vlugt, Hans van den Heuvel, de BRAIO's Renato en Antonio en Jawaid.

Mijn speciale dank gaat verder uit naar de *B.t.* werkgroep van het CPRO: Bert Visser, Guy Honée (in de begin periode) en Dirk Bosch (aan het eind). Met plezier heb ik met jullie van gedachten gewisseld over het werkingsmechanisme van dit verdraaid interessante toxine.

A special word of honour to all our foreign guests: Miguel Medina, Primitivo Caballero (Figura dell'arte), Jordi Cairo (Mister Barcelona), Hu Zhi-Hong (alias Rose), Peter Kulczar (the Mussle man) and of course Basel Arif. With all of whom I could get along quite well. Thanks a lot all of you.

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John alias Dr. Bob T.

En tot slot wens ik jullie

CONTENTS

"Success is to be measured not so much by the position that one has reached in life as by the obstacles which one has overcome while trying to succeed" Booker T. Washington.

CHAPTER ONE

INTRODUCTION TO THE THESIS

BACULOVIRUSES IN PEST CONTROL

Insect larvae cause enormous losses in agriculture and forestry and for several decades chemical insecticides have been used at a large scale to reduce these losses. Chemical insecticides, however, usually destroy not only the harmful insects but also harmless and beneficial insects and other organisms. Nevertheless, chemical insecticides are cheap and can be used against a number of different insect pests and have, therefore, been preferentially used for pest control over other alternatives. The idea about these "ideal" insecticides has changed in the last thirty years mainly for two reasons (Carson, 1964). Firstly, a number of pest insects showed resistance to chemical pesticides and, therefore, higher amounts of pesticides had to be applied or new expensive pesticides had to be developed. Secondly, most chemical insecticides are very persistent and hence pollute surface water, soil, and remain in the treated area for a long time and accumulate in the food chain of higher organisms (Bohmfalk, 1986). This resulted in a call for altenative, biologically safe insect-specific agents, such as predatory organisms and parasites like bacteria, viruses, fungi, nematodes and protozoa. Viruses are one of the most promising for reasons outlined below (Payne, 1988; Cunningham, 1988).

There are a number of viruses which can be used for insect control but from these, members of the family Baculoviridae are specific for arthropods. Actually, most baculoviruses can only infect insects and often a particular baculovirus can infect only one or a few closely related insect species (Granados and Federici, 1986). For example the *Spodoptera exigua* nuclear polyhedrosis virus (*Se*NPV) exclusively infects *Spodoptera exigua* larvae (Smits, 1986). Representatives of other virus groups, such as the Reoviridae, Poxviridae, Picornaviridae, Densoviridae, Rhabdoviridae, Orthomyxoviridae and Iridoviridae occur in other phyla including vertebrates as well. Since baculoviruses are very specific and do not infect vertebrates or plant species, they are currently considered to be best suited among viruses for insect pest control, even more, as no resistance to baculoviruses has been documented (Briese, 1986). In addition, the biology (Granados and Federici, 1986) and the molecular genetics (Blissard and Rohrmann, 1990) of baculoviruses have been studied in detail and this has led to the opportunitiy to modify their genome and to exploit them for foreign gene expression (Smith *et al.*, 1983b; Luckow and Summers, 1988; Vlak *et al.*, 1990) as well as to improve their insecticidal properties (Payne, 1988).

In nature baculoviruses cause epizootics, which reduce the size of susceptible insect populations. This was first observed in 1911 by Reiff, who suggested that the gypsy moth wilt disease, which was in fact a baculovirus infection, could be used as a control agent (Glaser and Chapman, 1913; Steinhaus, 1953). This feature has been exploited and baculoviruses have been accepted and used as natural pest control agent (Entwistle and Evans, 1985). About 39 pest species might be effectively controlled by appropriate baculovirus insecticides (Entwistle and Evans, 1985). From these, thirty-two are pests caused by Lepidoptera, six by Hymenoptera and one by a Coleopteran; sixteen of these involve pests in forestry and twenty-three pests in agriculture.

Table 1.1. Baculoviruses as Pest Control Agents.

Advantages	Disadvantages
Environmental safety	Slow speed of action
Narrow host range	High registration costs
Safe for humans and other vertebrates	Restricted market size
Natural agent	Low virulence to older instar larvae
	Sensitivity to UV-light

Payne, 1988; Cunningham, 1988.

Although baculoviruses seem suitable for insect pest control, they are not widely used for this purpose, since a number of features mean that baculoviruses are not competitive with chemical insecticides (Table 1.1). A major disadvantage is that the infection process occurs very slowly; for the first five days no symptoms of a virus infection and no decrease of damage can be observed. Secondly, the high costs of pesticide registration combined with the small market potential due to high specifity of these baculoviruses, makes their production much more expensive than their environmentally more hazardous chemical competitors. Thirdly, older instar larvae are far less susceptible than early instar larvae (Briese, 1986) and, therefore, only young larvae can be effectively controlled. Finally, baculoviruses are not very persistent in the environment as they are rapidly inactivated. Through genetic

engineering some of these negative properties might be improved (Payne, 1988). Genes which affect metabolism (toxins or hormones), host-range or virulence are potential candidates for this approach. In order to discuss these options in more detail some knowledge about the biology of baculovirus infection and methods used to manipulate their genome is essential and will be discussed in the next paragraphs.

CLASSIFICATION OF BACULOVIRUSES

Baculoviruses contain a double stranded DNA genome varying in size between 80 and 200 kilobase pairs (kb), which can encode at least 100 to 150 proteins. The genome is packaged in rod-shaped (baculum = rod) virus particles, called polyhedron derived virus particles (PDVs), which in turn are embedded into large proteinaceous occlusion bodies (OBs), also called polyhedra or granula (for review see Blissard and Rohrmann, 1990). For stability a calyx, which is composed of carbohydrates and the calyx protein, pp34, is attached via thiol-linkages to the polyhedron (Whitt and Manning, 1988; Zuidema *et al.* 1989). The baculovirus family is subdivided into two genera: the genus *Nucleopolyhedrovirus* and *Granulovirus* (22nd triennial meeting of the Executive Committee of the ICTV, 1993, Glasgow). This subdivision is based on morphological, serological and on genetical information (Phylogenetic interrelation; Zanotto *et al.* 1993). The baculovirus *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV), the type member of the *Nucleopolyhedrovirus* genus, is used in the present study and will be discussed in more detail.

THE BACULOVIRUS INFECTION PROCESS

The infection cycle of the baculovirus *Autographa californica* nuclear polyhedrosis virus

In nature, OBs are orally ingested by a larva during feeding (See Fig. 1.1 for a schematic representation). In the midgut, these OBs dissolve and the virus particles present in the OB are released into the midgut lumen. These particles enter the midgut columnar cells by membrane fusion (Harrap, 1970) after recognizing a specific receptor (Horton and Burand, 1993). Thereafter, the nucleocapsids are transported to the nucleus most likely via filamentous F-actin cables (Charlton and Volkman, 1993). The cable formation is presumably

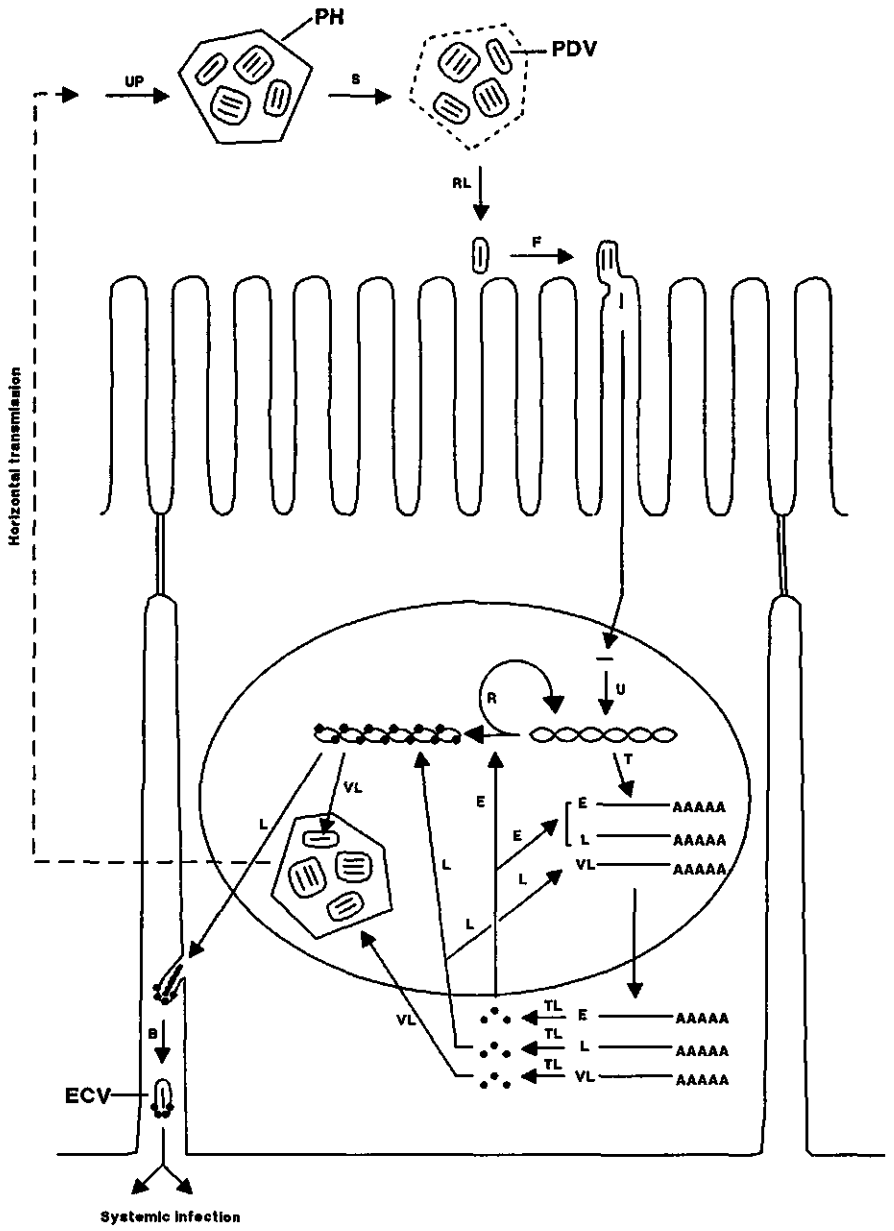

Figure 1.1. A typical primary baculovirus infection. PH = polyhedra; PDV = Polyhedron derived virus particle; ECV = extracellular virus; UP = oral uptake; S = solubilization of PH in the midgut; RL = release of the PDV from PH; F = fusion; U = uncoating of the viral DNA; T = transcription; TL = translation; E,L,VL = early, late or very late expressed; R = replication; B = budding; O = occlusion; N = nucleus and M = microvilli.

induced by a protein kinase present in the nucleocapsid. In the nucleus the viral DNA is uncoated (Granados and Lawler, 1981), most likely after phosphorylation of the p6.9 protein (Wilson and Consigli, 1985), a very basic arginine-rich protein which is tightly associated with viral DNA in the nucleocapsids. In the nucleas, the viral DNA is transcribed resulting in the on-set of viral DNA replication in the "virogenic stroma".

After the start of the replication (8 h p.i.) the assembly of nucleocapsids occurs, resulting in progeny virus particles that are released at the basal side of the infected midgut cell into the haemolymph. These extracellular virus particles (ECVs), subsequently, infect cells facing the haemolymph such as haemocytes, connective tissue, fat body, tracheal elements, muscle cells and Malpighian tubules (Keddie *et al.*, 1989) via receptor-mediated endocytosis (Volkman and Goldsmith, 1985). After internalization of the virus particles in the newly-infected cell, the virus particles are delivered in the endosome. The low pH in de endosome activates the glycoprotein gp64 present in the envelop of ECV, which catalyzes membrane fusion, thereby allowing the nucleocapsids to enter the cytosol (Blissard and Wenz, 1992). The subsequent released nucleocapsids then undergo another round of replication.

In the second phase of the replication cycle (after 12h p.i.), virus particles are no longer released into the haemolymph but are, instead, embedded into newly-made polyhedra in the nucleus of primary and secondary infected cells. Finally, the larva is filled with polyhedra, succumbs and liquifies due to the activity of a virus-encoded chitinase and cathepsin proteases, releasing large numbers of polyhedra into the environment (about 10^9 - 10^{10} per larva).

ECVs can be propagated *in vitro* in tissue culture. This has been most extensively studied with the baculovirus *Ac*NPV in *Spodoptera frugiperda* (*S.f.*) cells (Vaughn *et al.*, 1977; Adams *et al.*, 1977). The ability to promote virus infection *in vitro* has considerably increased the knowledge about the regulation of viral gene expression, and replication and has extended the possibilities to engineer the virus for several purposes.

Regulation of viral gene expression during the infection cycle

During the course of infection four phases of viral gene expression can be distinguished. Originally, these separate phases were recognized during time course analysis of pulse-labelled infected cells after adding inhibitors of protein synthesis (cyclohexamide)

and DNA replication (aphidicolin). Furthermore, viral RNA synthesis was monitored in time by performing northern blotting, S1 mapping or primer extension (reviewed by Friesen and Miller, 1986). Later, these observations were supported by showing that early transcription is performed by RNA polymerase II and that late transcription only occurred after the onset of viral DNA replication by a virus-specific RNA polymerase of which the recently identified protein lef-8 might be part (Passarelli *et al.*, 1994). Furthermore, it was shown that early transcription is trans-activated by virus-encoded factors making viral gene expression independent of the host regulatory elements (reviewed by Blissard and Rohrmann, 1990). The switch from late to very late gene expression is less confined yet. Below, the present knowledge on viral gene expression is briefly outlined.

Immediately after the release of the viral DNA into the nucleus, "immediate early" (IE) genes are transcribed by host RNA polymerase II (Fuchs *et al.* 1982). IE genes contain promoter elements which are very similar to that of host promoters, such as a TATA box, a CAGT transcriptional start site, upstream elements and enhancer sequences (e.g. GATA and MLTF elements: Krappa *et al.* 1992; Kogan and Blissard, 1994). All these IE genes, such as IE-0 (Kovacs *et al.*, 1991), IE-1 (Guarino and Summers, 1986), IE-N (Carson *et al.*, 1988), PE-38 (Krappa and Knebel-Mörsdorf, 1991), ME-53 (Knebel-Mörsdorf *et al.* 1993), CG30 (Thiem and Miller, 1989) and HE65 (Becker and Knebel-Mörsdorf, 1993) most likely produce transcription factors, that transactivate the transcription of the other early genes, the "delayed early" (DE) genes (Guarino and Summers, 1986; Carson *et al.*, 1988; Lu and Carstens, 1993). Thus far trans-activation has only been shown for IE-1, IE-N and PE-38. For the other genes a similar function is presumed since they contain serine-threonine rich regions (IE-N), poly-glutamine tracts (IE-N), zinc-fingers (PE38, ME53, CG30) or leucine zipper motifs (PE-38, CG30) reminiscent of other known transcription factors (Carson *et al.*, 1988).

The products of a number of IE and DE genes are essential for (1) the inhibition of virus-induced apoptosis, (2) the down regulation of host gene expression by RNA polymerase II, (3) the on-set of viral replication and (4) late gene expression. These four processes and probably a few more are essential for a successful virus infection. Apoptosis is one of the host defence mechanisms against a virus infection. Recently, it was shown the early viral 35 kDa gene plays a key role in preventing apoptosis in baculovirus-infected *S.f.* cells (Clem *et al.* 1991). Host transcription is also rapidly down regulated by a mechanism which is still

poorly understood. Presumably, host transcription interferes with replication and synthesis of viral structural proteins and is, thus, undesirable during the late phase of the virus infection. For the replication of the baculovirus genome, six early genes have shown to be essential (DNA polymerase, DNA helicase, IE-1, lef-1, 2 and 3)(Kool *et al.* 1994). Three other early genes (p35, IE-N, PE-38) stimulate the replication. These genes are also required for late gene expression since it is dependent on viral DNA replication. Furthermore, additional early genes (lef-4, 5, 6, 7 and 8) are required for the onset of late gene expression (Passarelli and Miller, 1993a,b,c; Li *et al.*, 1993; Passarelli *et al.*, 1994), whereas others (etl, da26, da24, p47) are presumed to have only a stimulatory effect (Crawford and Miller, 1988; Guarino and Summers, 1988). A number of early genes, such as orf453, orf2070, p94, ets and p39, remain uncharacterized but they presumably a play part in other as yet unknown functions controlling host cell regulation.

The infection is completely controlled by the virus after the on-set of viral DNA replication (8h p.i.). Viral replication continues during the late phase of the infection. During this phase (the γ phase) the late (L) genes are transcribed from a special promoter containing a $^{A}/_{G}$TAAG sequence motif, the "Rohrmann" box (Rohrmann, 1986), by a virus-encoded RNA polymerase. Most of the late genes are structural genes required for the ECV or PDV. The basic DNA binding protein (p6.9; Wilson *et al.*, 1987), the capsid protein (CP; Pearson *et al.*, 1988, p80 (Lu and Carstens, 1992) and orf1629 (Vialard and Richardson, 1993) are present in virus particles of both ECV and PDV. Proteins p74 (Kuzio *et al.* 1989) and gp41 (Whitford and Faulkner, 1992) are only present in PDVs. The protein p74 is essential for a successful primary infection of the insect midgut collumnar cells. The basic DNA binding protein is presumably involved in neutralizing the viral DNA required for condensation of the genome. Together with the capsid protein the condensed genome forms the nucleocapsids. Soon after the onset of DNA viral replication, the ECV peplomer gp64 is abundantly synthesized and transported to the cell membrane possibly directing the virus particles to bud from the cell. Later at the end of the infection cycle when the nucleus is filled with nucleocapsids (the δ phase (18h p.i.)), the "very late" (VL) genes are transcribed. These genes, such as calyx protein pp34 (Whitt and Manning, 1988), and polyhedrin (PH), are involved in the formation of polyhedra. Furthermore, genes required for the release of the polyhedra from the host are then produced, such as cathepsin (Kuzio and Faulkner, 1991), chitinase (Hawtin *et al.*, 1992) and the highly expressed protein p10 (Van Oers, 1994).

GENETIC ENGINEERING OF BACULOVIRUSES

Baculoviruses as expression vectors

For a decade baculoviruses have been used as eucaryotic expression vectors of foreign genes (for review see, Luckow and Summers, 1988). This is mainly due to the strength of the promoter of two baculovirus VL genes, PH and p10. These genes are not essential for virus replication *in vitro* and their open reading frame can be easily replaced by others. The foreign genes are usually highly expressed, correctly post-translationally modified (phosphorylated, glycosylated, amidated etc.), highly immunogenic and often biologically active. This expression system can be exploited to improve the properties of baculoviruses as insecticides by exploiting genes that might improve the insecticidal properties of baculoviruses.

The genome of baculoviruses is too large to allow direct insertion of the foreign gene, which is therefore introduced into the genome via homologous recombination (Fig. 1.2). To this end, the gene of interest is inserted in a so-called transfer vector. In general such a vector contains one of the strong, very late baculovirus promoters followed by one or more unique restriction sites in which the foreign gene can be introduced. The promoter and the cloning sites are flanked by sequences of the viral genome where the insertion has to occur. After co-transfection of viral DNA with this transfer vector DNA, recombination will occur between the homologous sequences of the virus and the transfer vector resulting in the insertion of the foreign gene at the expected location in the genome. To facilitate screening for the recombinant viruses a marker gene such as polyhedrin (Emery and Bishop, 1987), ß-galactosidase (Zuidema *et al.*, 1990; Vlak *et al.*, 1990), ß-glucuronidase (Roelvink, unpublished) or luciferase (Oker-Blom *et al.*, 1993) is usually present in these transfer vectors. Using this approach an increasing number of foreign genes has already been successfully expressed in insect cells.

Genetic engineering for pest control

As stated at the beginning of this chapter baculoviruses are excellent agents for biologically safe control of insect pests. One of the major limitations of a wide-spread use of these viruses, however, is their slow speed of action. Several suggestions have been made to improve the insecticidal properties of baculoviruses through genetic engineering (Payne,

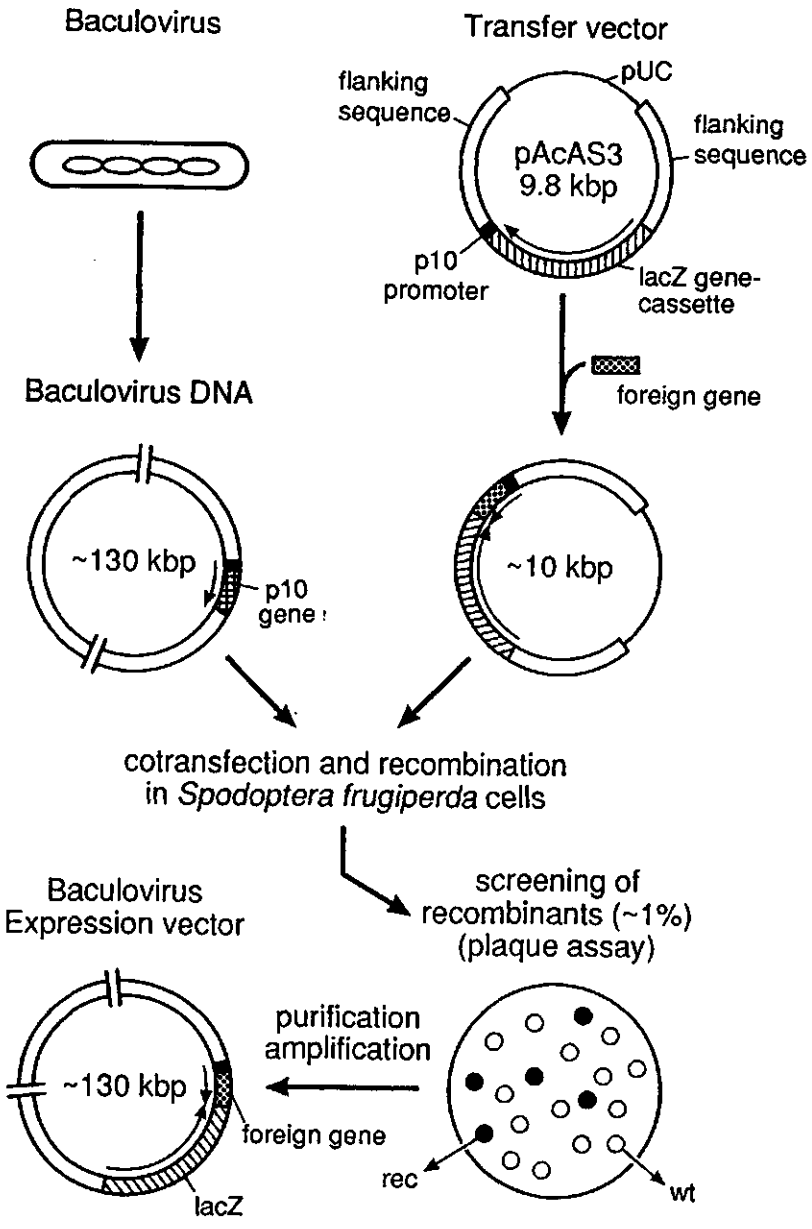

Figure 1.2. Construction of p10-based recombinant baculoviruses using homologous recombination (Vlak *et al.*, 1990). The transfer vector pAcAS3 contains the p10 promoter (black box), a ß-galactosidase selection marker (dashed box) and the p10-gene-flanking sequences for homologous recombination. After a co-transfection, the p10 gene can be replaced by the foreign gene (stippled box). In a plaque assay, recombinant viruses can be visualized after the addition of X-gal and subsequently purified.

1988). Expression of genes coding for proteins that interfere with insect metabolism and behavior, such as hormones (prothoracicotropic hormone and eclosion hormone), enzymes (juvenile hormone esterase), growth and water balance regulators (diuretic hormone), or genes encoding insect-specific toxins (neurotoxins or insecticidal crystal proteins). At the start of the research described in this thesis the highly specific insecticidal crystal proteins (ICPs), produced by the bacterium *Bacillus thuringiensis*, were well characterized and their cloned genes were readily available. Therefore, these ICPs were chosen to explore their possibility of improving the insecticidal properties of baculoviruses.

Insecticidal crystal proteins of *Bacillus thuringiensis*.

Bacillus thuringiensis (*B.t.*) is a soil bacterium which produces large insecticidal inclusions (for review see Höfte and Whiteley, 1989; Gill *et al.*, 1993; Honée *et al.*, 1993). The bacterium was first isolated from diseased silkworm larvae in 1901 (Ishiwata, 1901). Since then, more than 1000 different strains have been isolated which are currently subdivided into at least 34 serovars (de Barjac and Frachon, 1990). Thus far, most of these strains are toxic to larvae from Lepidoptera, Diptera and Coleoptera and, therefore, potentially interesting as pest control agents. The inclusions mainly consist of proteins (ICPs) with insecticidal activity which accumulate in large amounts during sporulation and are deposited in the environment together with the spore. All known ICPs are produced as protoxins (Fig. 1.3 A). Activation by proteolytic enzymes only occurs after dissolution of these crystals at a specific pH, alkaline or acidic, depending on the midgut environment of the host insect (Lilley *et al.*, 1980; Nagamatsu *et al.*, 1984), the obvious target for the action of these activated ICPs. However, injection of activated toxins in the haemolymph does also result in mortality of insect larvae (Lilley *et al.*, 1980; Nagamatsu *et al.*, 1984; Knowles *et al.*, 1984). The toxic fragment is located at the N-terminus of the protoxin (Fig. 1.3 A) and is completely responsible for the toxic effect observed after the ingestion of a *B.t.* suspension. The activation of the ICPs is mediated by trypsin-like proteases such as the one recently purified from *Choristoneura fumiferana* (Milne and Kaplan, 1993).

The current view about the mode of action of the activated ICPs is schematically represented in Fig. 1.3 B and C. The mature toxin contains three functional domains (Fig. 1.3 A and B; Li *et al.*, 1991). Domain I consists of a bundle of 7 α-helices; the central helix, α_5, is hydrophobic and is surrounded by the six other amphipathic helices (Fig. 1.3;

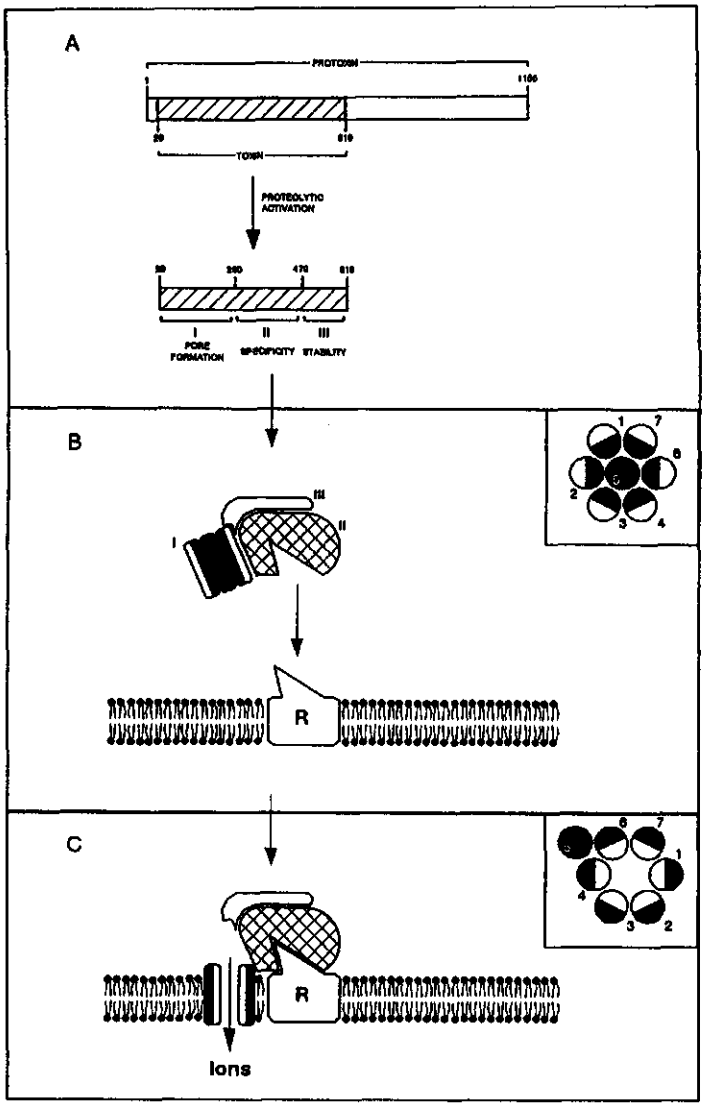

Figure 1.3. Mode of action of insecticidal crystal proteins of *Bacillus thuringiensis*.
A) Activation of protoxin. In the midgut the protoxin (70 to 130 kDa) is degraded by proteolytic enzymes to a mature toxin of approx. 55 kDa. The presumed activation of CryIA(b) protoxins is presented here. Domains I, II and III represent functional domains of the mature toxin. B and C) Presumed cytolytic effect of the mature toxin. After receptor (R) recognition by domain II of the mature toxin, domain I is forced into the membrane midgut epithelial cell. *Inset* B,C) The orientation of the 7 α helices of domain I (top view). Before penetration the 6 amphipathic α helixes surround helix 5 (B); after penetration when a pore is formed, the helices are rearranged possibly by a mechanism as suggested in C.

Inset B). The hydrophilicity pattern of domain I is conserved among all ICPs and since these helices are long enough to span a membrane they are believed to play a major role in pore formation (Li *et al.*, 1991; Walter *et al.*, 1994; Chapter 4). Domain II is the most variable region and is most likely involved in receptor binding (Ge *et al.*, 1989). Domain III interfaces between domain I and II; its C-terminus (ß-strand 23; Li *et al.*, 1991) is highly conserved and probably important for the correct conformation of the mature toxin and, thus, for its sensitivity for proteolytic degradation (Höfte *et al.*, 1986; Chapter 4).

The protoxin becomes active only after proteolytic removal of a small N-terminal peptide of 25 to 60 amino acids and the C-terminal amino acids until the conserved ß-strand 23 (Nagamatsu *et al.*, 1984; Höfte *et al.*, 1986; Chapter 4). Only this mature toxin is able to bind to a specific receptor (Hofmann *et al.*, 1988) localized in the microvilli of the midgut cells (Bravo *et al.*, 1992a,b; Denolf *et al.*, 1993a,b; Chapter 3). Receptor recognition leads to close contact between domain I and the cell membrane. Upon insertion of domain I into the membrane the amphipathic 6 α-helixes possibly turn their inside out removing helix α_5 from their center (Fig. 1.3; *Inset* B,C) resulting in a pore of about 1 nm (Knowles and Ellar, 1987). This pore causes an influx of ions, presumably K^+ ions, in the cell resulting in swelling and subsequent cell lysis (English *et al.*, 1991).

AIM OF THE RESEARCH

The aim of the research was to investigate whether *Bacillus thuringiensis* insecticidal crystal proteins can be exploited to improve the insecticidal properties of the baculovirus, *Autographa californica* NPV. The cryIA(b) gene isolated from *B.t. aizawai* was selected for this study, since the host-specificity of this well-characterized ICP largely overlaps with that of *Ac*NPV (Höfte *et al.*, 1988; Payne,1986). *In nature* ICPs are produced as protoxins, while activation and receptor recognition occurs in the midgut, where both the required environment and the proteolytic enzymes are available. Furthermore, receptors have thus far only been detected on the exterior of insect cells (Murphy *et al.*, 1976; Johnson, 1981; Hofmann *et al.*, 1988). Hence, ideally the baculovirus-expressed protoxin should be secreted into the midgut. Alternatively, the mature toxin can be expressed from a truncated cryIA(b) gene and secreted as such into the hemocoel. For both strategies it was necessary to determine first whether a baculovirus-expressed CryIA(b) protoxin is biologically active

(Chapter 2). Subsequently, the expression and secretion of both the protoxin and the mature toxin from insect cells by recombinant baculoviruses was accomplished (Chapter 6). To enable expression of mature toxin the borders of the mature CryIA(b) toxin were determined (Chapter 4). To obtain more insight into the exact location of receptors in the larva, a cytochemical assay for midgut sections was developed (Chapter 3). This assay was also used to study the biological activity of the baculovirus expressed cryIA(b) protoxin (Chapter 3). Additionally, an improved method to construct recombinants in the p10 locus was developed (Chapter 5) which considerably facilitated the construction of recombinant viruses. Finally, the results obtained were evaluated and compared with alternative strategies to genetically improve baculovirus insecticides (Chapter 7).

CHAPTER TWO

INSECTICIDAL ACTIVITY OF THE *BACILLUS THURINGIENSIS* CRYIA(b) CRYSTAL PROTEIN EXPRESSED BY A BACULOVIRUS RECOMBINANT IN INSECT CELLS

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SUMMARY

Baculoviruses are insect pathogens with a relatively slow speed of action and this limits their use as control agents of insect pests. Introduction into baculoviruses of genes, which code for proteins interfering specifically with insect metabolism or metamorphosis, such as toxins, hormones and enzymes, may enhance the pathogenicity of these viruses. The complete cryIA(b) insecticidal crystal protein (ICP) gene of *Bacillus thuringiensis* (*B.t.*) *aizawai* 7.21 was engineered into the nuclear polyhedrosis virus of *Autographa californica* (*Ac*NPV) in place of the polyhedrin gene. In infected *Spodoptera frugiperda* (*S.f.*) cells the cryIA(b) gene was expressed at high level without interference with *Ac*NPV production. The ICPs were found in the cytoplasm of *S.f.* cells, mainly as large crystals with an ultrastructure similar to that of *B.t.* crystals. Infected-cell extracts inhibited feeding of the large cabbage white, *Pieris brassicae*. The toxicity of the ICP expressed by *Ac*NPV recombinants was comparable with that of the ICP expressed by a corresponding *Escherichia coli* recombinant.

INTRODUCTION

Baculoviruses are pathogenic to a number a lepidopteran insects (Granados and Federici, 1986) and are attractive biological agents for the control of agriculturally important insect pests. In nature, these pathogens regulate the size of insect populations in a variety of ecosystems (Martignoni, 1984). A major drawback for a more wide-spread use of these viruses is their relatively slow speed of action. It may take five to ten days after infection before feeding is reduced. Through genetic engineering it may be possible to improve the insecticidal properties of these viruses by introduction of genes, whose products interfere with insect metabolism or morphogenesis (Keeley and Hayes, 1987). Insecticidal crystal protein genes of *Bacillus thuringiensis* (*B.t.*) may be good candidates for this purpose.

B. thuringiensis is a Gram-positive soil bacterium that produces one or more crystals during sporulation (Krieg, 1986). These crystals contain proteins, which are processed upon ingestion by gut alkaline proteases into proteins that are toxic to insect larvae. There are five main classes of *B.t.* isolates and one of them, CryI, produces crystals that are toxic to larvae of Lepidoptera (for review see Höfte and Whiteley, 1989). The crystals are bipyramidal in shape and contain one or several toxic proteins with a molecular weight of approximately

130 kDa (Bulla *et al.*, 1977). Each of these proteins appears to have its own specific toxicity spectrum with respect to insect hosts (Höfte *et al.*, 1988; Visser *et al.*, 1988).

In order to investigate whether the CryIA(b) ICP is potentially useful to improve the pathogenicity of baculoviruses, it is necessary to establish that this protein can be expressed in insect cells by a recombinant baculovirus while maintaining its toxicity for larvae of Lepidoptera. To this end we introduced an ICP gene, isolated from *B.t. aizawai* strain 7.21 and classified as cryIA(b) (Höfte and Whiteley, 1989), into the genome of the *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV). The host-range specificity of the cryIA(b) gene of *B.t. aizawai* strain 7.21 (Höfte *et al.*, 1988) overlaps with that of *Ac*NPV (Payne, 1986) and this provides the possibility to improve pathogenicity of *Ac*NPV by the addition of this *B.t.* gene. The CryIA(b) ICP was produced at high level in recombinant *Ac*NPV-infected cells and showed high toxicity to a susceptible insect.

MATERIALS AND METHODS

Bacteria, insect cells and viruses

The *Bacillus thuringiensis aizawai* strain 7.21 was supplied by Dr. H. de Barjac, Institute Pasteur, Paris. *E. coli* strain DH5α and JM101 were used for transformation. The recombinant *E. coli* (7.21A) containing the plasmid p7.21A and expressing the cryIA(b) gene from *B.t. aizawai* strain 7.21 (Honée *et al.*, 1990), and a recombinant *E. coli* harboring pBR322, were used for comparison.

The *Spodoptera frugiperda* (*S.f.*) cell line IPLB-SF-21 (Vaughn *et al.*, 1977) was used and maintained in plastic tissue culture flasks in TNM-FH medium (Hink, 1970) supplemented with 10% fetal bovine serum.

*Ac*NPV, strain E2 (Smith and Summers, 1978), was used as *wild-type* (*wt*) virus. Recombinant *Ac*NPV/DZ1 which has a deletion of the polyhedrin gene and instead an insertion of the LacZ-gene screening cassette was used as negative control (Zuidema *et al.*, 1990). The infectivity of the extra-cellular virus (ECV) was determined using the endpoint dilution method (Vlak, 1979) and the titres were expressed as $TCID_{50}$ units per ml. In case of recombinants the endpoint was determined using 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) (25μg/ml) as ß-galactosidase activity indicator.

Antibodies

Crystals of *B.t. aizawai* strain 7.21 were solubilized in 0.01 M sodium phosphate pH 7.1, 6 M urea and 2% ß-mercaptoethanol and subsequently dialyzed for 48 h against 1 x SSC. One hundred μg of ICP was injected into a rabbit, followed by two boosts each (100μg) given after 4 and 6 weeks, respectively. The rabbit was bled after 7 weeks and its serum was used as polyclonal anti-CryIA(b) serum.

Plasmids and transfer vectors

Plasmid p7.21A contains a 7.5 kb *Bam*HI-*Pst*I insert which was isolated from total DNA of *B.t. aizawai* 7.21 and which contains the entire cryIA(b) gene. The plasmid p109.2, derived from p7.21A, contains only the coding sequence of the cryIA(b) gene flanked by a *Bam*HI- and a *Bgl*II-site (Honée *et al.*, 1990). These sites were used to insert the cryIA(b) gene in the *Ac*NPV transfer vector pAcDZ1.

Transfer vector pAcDZ1 (Fig. 2.1) contains a lacZ gene cassette, a unique *Bam*HI cloning site downstream the polyhedrin promoter to insert foreign genes, and polyhedrin gene-flanking sequences to facilitate recombination (Zuidema *et al.*, 1990). The lacZ gene cassette consists of the *Drosophila melanogaster* heat shock promoter hsp70, the bacterial lacZ gene and the SV40 dual transcription terminator sequence.

Construction of the *Ac*NPV transfer vector pAcJM3

Plasmid p109.2 was digested with *Bam*HI and *Bgl*II, and the fragment containing the coding sequence of the ICP gene (3.5 kb) was isolated from a 0.7% agarose gel. This fragment was ligated into the transfer vector pAcDZ1, which had been digested with *Bam*HI and dephosphorylated. This resulted in transfer vector pAcJM3 (Fig. 2.1). The junction between the polyhedrin promoter and the cryIA(b) gene construct had the following sequence: 5'-acctataaatC*GGATCC*GTATG-3', where the 3' t is the end of the polyhedrin leader, *GGATCC* is the BamHI cloning site and ATG the start of the protoxin. Digestion, ligation and transformation procedures were carried out as described in Maniatis *et al.* (1982). Plasmid DNA was recovered from transformed *E. coli* cells by the alkaline lysis method of Birnboim and Doly (1979) followed by CsCl centrifugation.

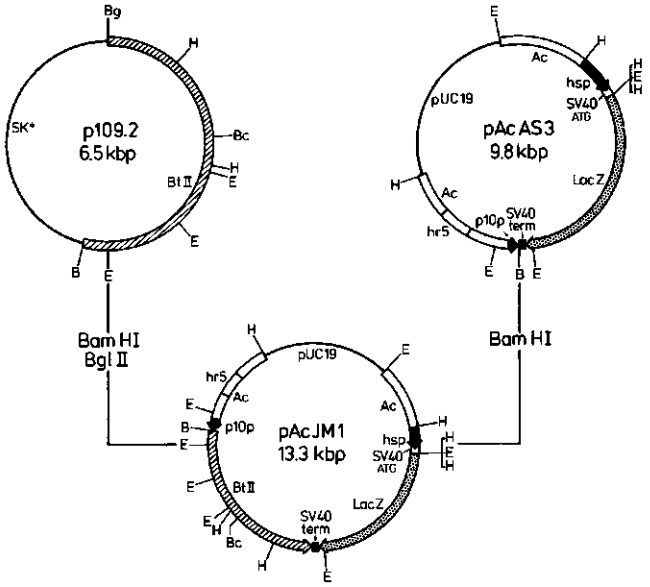

Figure 2.1. Construction of transfer vector pAcJM3. B = *Bam*HI; Bc = *Bcl*I; Bg = *Bgl*II; E = *Eco*RI and H = *Hind*III. kbp = kilobase pairs; Ac = *A. californica* NPV; SV40 = Simian Virus 40; term = transcription terminator; php = polyhedrin promoter; hsp = *D. melanogaster* heat shock promoter hsp70; LacZ = bacterial ß-galactosidase. The arrows indicate the direction of transcription.

Infection and transfection of cells

S.f. cells were co-transfected with DNA of *wt Ac*NPV and the plasmid pAcJM3 using the calcium phosphate precipitation technique essentially as described by Smith *et al.* (1983c) with some minor modifications (Vlak *et al.*, 1988). Recombinant plaques were recognized by blue color development upon addition of X-gal (25 μg/ml) and by the absence of polyhedra in these cells. The putative recombinants were plaque-purified four times to reach genetic homogeneity. Infection of cells was carried out with the ECV form of the virus at a multiplicity of infection (MOI) of 10 $TCID_{50}$ units per cell.

DNA and protein analysis

DNA obtained from plasmids or ECVs of *wt Ac*NPV, *Ac*NPV/DZ1 and *Ac*NPV/JM3 was subjected to restriction enzyme analysis as described previously (Vlak *et al.*, 1988). The DNA was analyzed on 0.7% agarose gels as described by Maniatis *et al.* (1982).

For protein analysis, uninfected and infected *S.f.* cells as well as *E. coli* cells with and without the cryIA(b) gene were gently washed three times with excess phosphate buffered saline pH 7.5 (PBS), and analyzed by SDS-PAGE on 12.5% gels according to Laemmli (1970) using a BioRad Protean II apparatus. The gels were stained with Coomassie Brilliant Blue or subjected to immunoblotting.

The amount of 130 kDa ICP present in a protein sample was estimated from a PAGE gel using a LKB Ultrascan XL laser densitometer followed by integration of the peaks with a LKB 2400 gelscan XL software package.

For immunoblotting the gels were transferred in 25 mM Tris, 39 mM glycine, 0.0375% (w/v) SDS and 20% (v/v) methanol to nitrocellulose filters by electroblotting for 1 h at 0.8 mA/cm^2 gel using a semi-dry blot apparatus (BioRad). The blot was treated with 2% (w/v) milk powder in TBS (50 mM Tris/HCl, pH 7.4, 200 mM NaCl) for 2 h at room temperature to prevent a-specific antibody binding. The blot was then incubated for 1 h at room temperature in 0.2% (v/v) milk-powder in TBS to which a 1:2000 dilution of the polyclonal antiserum against the ICPs of *B.t. aizawai* strain 7.21 was added. To remove the antiserum the blot was washed three times for 10 min with excess TBS. The blot was further incubated with a second antiserum (swine anti-rabbit immunoglobulins conjugated with horse-radish peroxidase) in a 1:2000 dilution in 0.2% (v/v) milk-powder in TBS at room temperature for 1 h. The blot was stained in TBS containing 16.6% (v/v) methanol, 0.018% (v/v) peroxide and 0.5 mg/ml 4-chloro-1-naphthol. After 20 min the reaction was stopped with distilled water and the blot was dried.

Electron microscopy and immunogold labelling

S.f. cells were infected with a MOI of 10 $TCID_{50}$ units per cell of either *wt Ac*NPV or *Ac*NPV/JM3 and harvested 48 h p.i. The cells were further processed for electron microscopy as described previously (Van der Wilk *et al.*, 1987). Ultrathin sections were treated with colloidal protein-A gold as described by Van Lent *et al.* (1990).

Bioassays

S.f. cells infected with *wt Ac*NPV, *Ac*NPV/DZ1 and *Ac*NPV/JM3 at a MOI of 10 $TCID_{50}$ units per cell were harvested 48 h p.i. and washed twice with excess PBS. The cells were finally resuspended in 500 μl PBS giving a final concentration of 3.0 x 10^7 cells per ml.

Mock-infected cells were treated similarly. *E. coli* cells were cultured overnight and concentrated 50 times giving a final concentration of 1.8 x 10^9 cells per ml.

For a qualitative assay 5 μl of infected or uninfected insect cells, (1.5 x 10^5 cells), or bacteria (9.0 x 10^6 cells), were spread on a 1.75 cm^2 leaf disk of cabbage. The disk was placed on top of a humidified cotton plug in a 24-well tissue culture plate (Costar). For each assay 32 second instar *P. brassicae* larvae were used. After two days at 25°C the assay was analyzed for insect mortality and feeding damage.

To determine the dose-response relationship, 6 dilutions of *Ac*NPV/JM3-infected *S.f.* cells and *E. coli* 7.21A cells were tested on second instar larvae of *P. brassicae*. Per dilution 32 larvae were tested. Each of these larvae was placed on a 1.75 cm^2 leaf disk on which 5 μl of each dilution was spread. Insect mortality was scored after two days of incubation at 25° C. The mortality data were processed using the probit analysis methods described by Finney (1971) as detailed elsewhere (Smits and Vlak, 1988). The LC_{50} values for each sample were expressed as cells per cm^2 leaf disk.

RESULTS

Construction of the transfer vectors pAcJM3 and the recombinant virus *Ac*NPV/JM3

The first step in the engineering of a baculovirus recombinant was the construction of a transfer vector in which the coding sequence of the polyhedrin gene was replaced by the coding sequence of the cryIA(b) gene of *B.t. aizawai* strain 7.21. The transfer vector pAcDZ1 contains, in addition to a unique cloning site downstream from the polyhedrin promoter, a gene cassette containing lacZ, which is expressed under the control of the *D. melanogaster* heat shock promoter hsp70 (Zuidema *et al.*, 1990). This promoter is constitutively expressed in infected insect cells and drives the expression of the marker gene. The plasmid p109.2 contains the complete cryIA(b) gene starting one basepair upstream of the ATG codon and ending at a *Dra*I site 10 nucleotides downstream of the stop codon. The cryIA(b) gene was inserted as a 3.5 kb *Bam*HI-*Bgl*II fragment from p109.2 into the transfer vector pAcDZ1 and resulted in the transfer vector pAcJM3 (Fig. 2.1).

S.f. cells were co-transfected with pAcJM3 and *wt Ac*NPV DNA. Recombinant viruses were distinguished from *wt* virus in a plaque assay (Summers and Smith, 1987) by the absence of polyhedra and by their blue coloration after the addition of X-gal. This marked

the replacement of polyhedrin gene in the recombinants. Five "blue" plaques were purified to genetic homogeneity. The restriction pattern of these viruses was analyzed and all the recombinant viruses appeared to have inserted the cryIA(b) gene correctly.

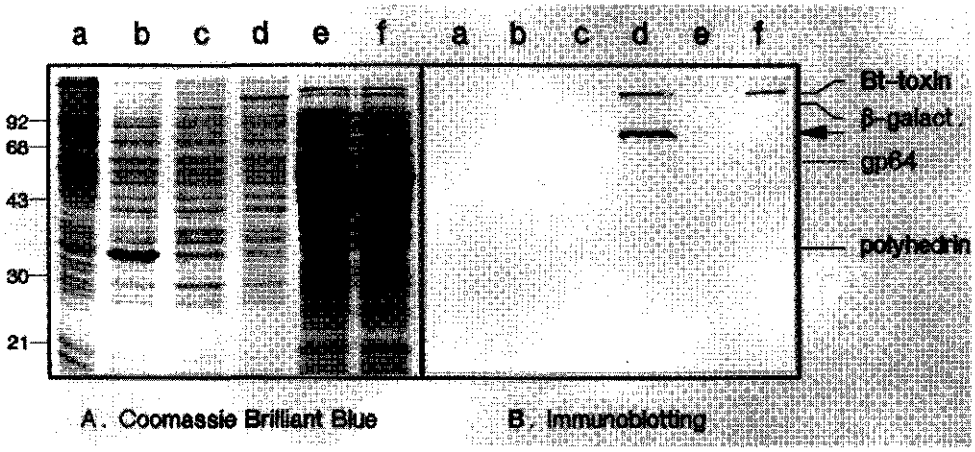

Figure 2.2. SDS-PAGE of *S. frugiperda* cells mock-infected (a) or infected with *wt* *Ac*NPV (b), *Ac*NPV/DZ1 (c) and *Ac*NPV/JM3 (d). Cells were infected with a multiplicity of 10 $TCID_{50}$ units per cell. Each lane contained approximately 1 x 10^4 cells. Extracts of *E. coli* (4.5 x 10^6 cells), containing the plasmids pBR322 or p7.21A respectively were included (e and f) as controls. Gels were stained with Coomassie Brilliant Blue (A). A duplicate gel was subjected to immunoblot analysis using polyclonal serum against dissolved CryIA(b) ICPs purified from *B.t. aizawai* strain 7.21 (B).

Protein synthesis in *S. frugiperda* cells infected with *Ac*NPV/JM3

S.f. cells, infected with *wt* *Ac*NPV, *Ac*NPV/DZ1 and *Ac*NPV/JM3, were harvested 48 h p.i. and analyzed by SDS/PAGE and immunoblotting (Fig. 2.2). In cells infected with recombinant *Ac*NPV/JM3 polyhedrin was absent; instead a protein of approximately 130 kDa was observed, the size expected for the CryIA(b) ICP (Fig. 2.2 A, lane d). Beta-galactosidase expressed by the heat shock promoter, was present as a band of about 116 kDa in cells infected with recombinants *Ac*NPV/DZ1 (Fig. 2.2 A, lane c) and *Ac*NPV/JM3 (Fig. 2.2 A, lane d). Analysis of *E. coli* 7.21A cells, expressing the cryIA(b) gene also showed a 130 kDa protein band (Fig. 2.2 A, lane f), which was absent in *E. coli* cells harboring pBR322 (Fig. 2.2 A, lane e). The 130 kDa protein was superimposed on a minor 130 kDa *E. coli* protein (Fig. 2.2 A, lanes e and f). That these 130 kDa bands were indeed cryIA(b)

gene products was demonstrated by immunoblotting (Fig. 2.2 B). The antiserum raised against dissolved crystals of *B.t. aizawai* strain 7.21 reacted with the 130 kDa protein in *Ac*NPV/JM3-infected cells (Fig. 2.2 B, lane d) and in *E. coli* 7.21A cells (Fig. 2.2 B, lane f). In *Ac*NPV/JM3-infected cells an additional band of about 73 kDa also reacted strongly with the antiserum in *Ac*NPV/JM3-infected cells (Fig. 2.2 B, lane d).

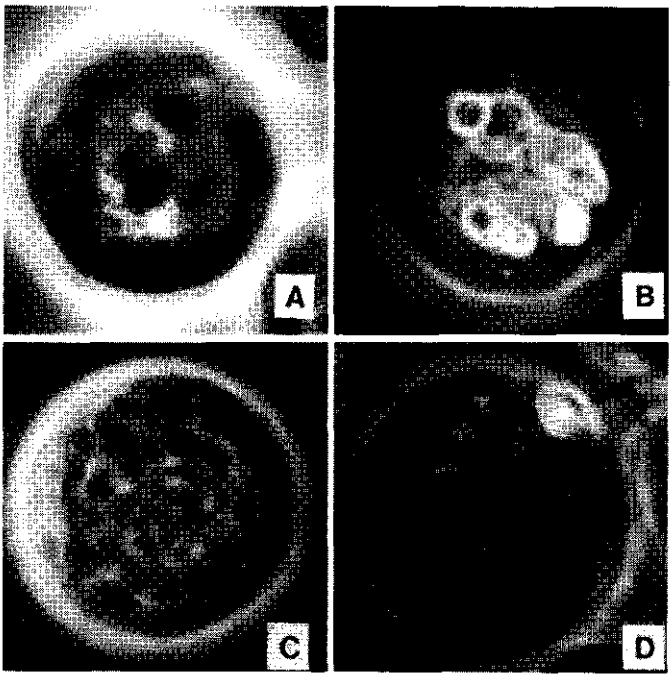

Figure 2.3. Phase-contrast microscopy of uninfected *S. frugiperda* cells (a) and *S. frugiperda* cells infected with *wt Ac*NPV (b), *Ac*NPV/DZ1 (c) and *Ac*NPV/JM3 (d). The arrow indicates *B.t.* crystals. The bar represents 2 μm.

Phase-contrast and electron microscopy of *S. frugiperda* cells infected with *Ac*NPV/JM3

Uninfected, *wt Ac*NPV-, *Ac*NPV/DZ1- and *Ac*NPV/JM3-infected *S.f.* cells were studied by phase-contrast and electron microscopy. It was observed in the phase-contrast microscope that in nuclei of cells infected with the recombinants *Ac*NPV/DZ1 and *Ac*NPV/JM3 polyhedra were absent as a result of the deletion of the polyhedrin gene (Fig. 2.3, c and d). In the cytoplasm of *Ac*NPV/JM3 infected cells large crystals, often bipyramidal in shape,

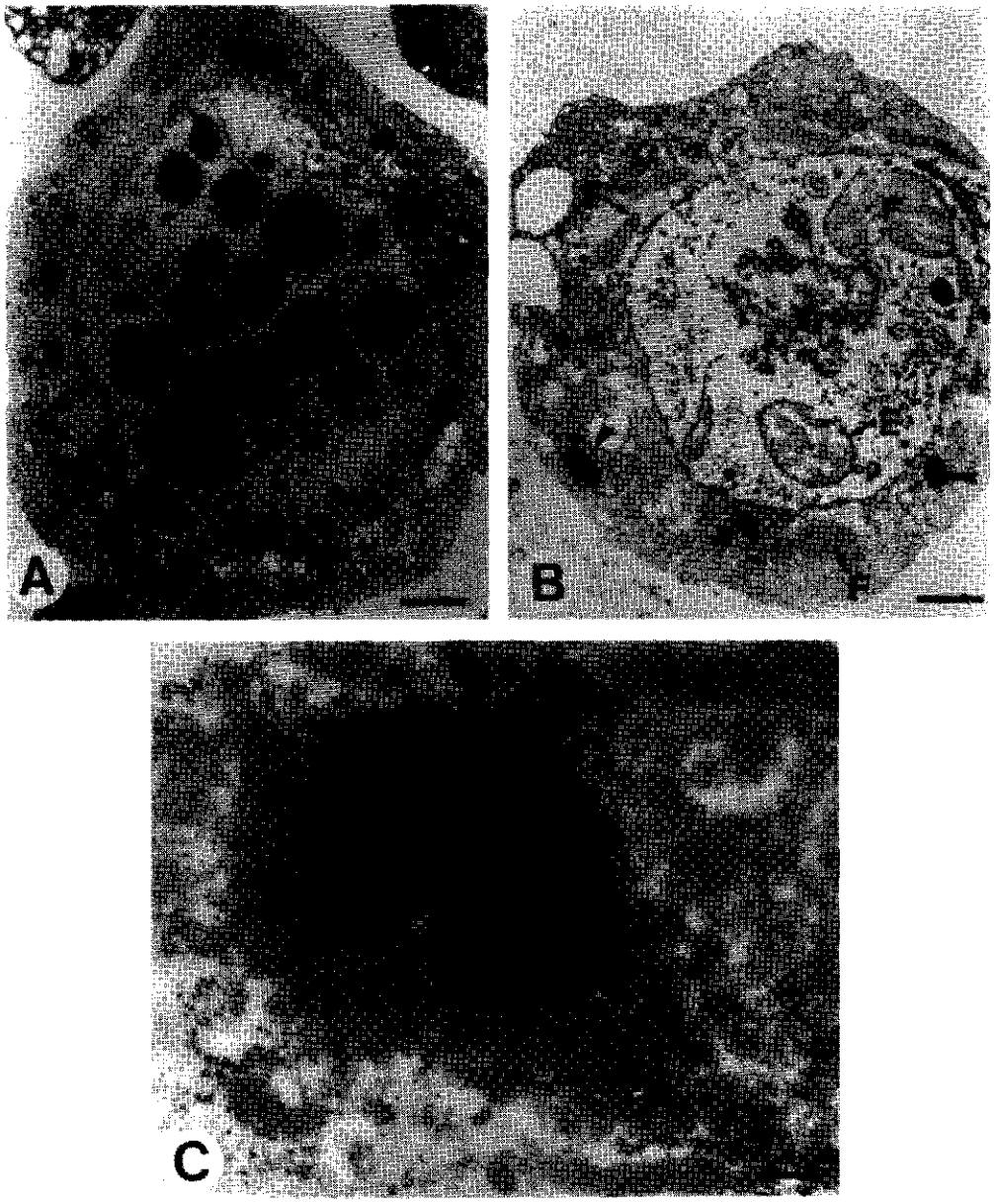

Figure 2.4. Electron microscopy of an ultrathin section of *S. frugiperda* cells infected with *wt Ac*NPV (A) and *Ac*NPV/JM3 (B and C), at 48 h p.i. Sections of these infected cells treated with the antiserum mentioned in the legend of Fig. 2.2. E = electron-dense spacer; F = fibrillar structures; P = polyhedron ; V = virogenic stroma. Arrowheads indicate *B.t.* crystals. The bars indicate 2 μm (A and B) and 0.2 μm respectively (C).

were observed. These crystals appeared similar to those observed in *B.t.*, as described by Krieg (1986). They were not found in uninfected, *wt Ac*NPV- and *Ac*NPV/DZ1-infected cells (Fig. 2.3, a, b and c). The presence of *B.t.* crystals and proteins in the cytoplasm was confirmed by immuno-fluorescence (not shown).

In ultrathin sections of *Ac*NPV/JM3-infected *S.f.* cells large electron-dense, granular, paracrystalline inclusions were observed in the cytoplasm (Fig. 2.4 B). These inclusions were different in appearance from the large fibrillar structures found in the nucleus and cytoplasm of *wt Ac*NPV-infected cells as described by Van der Wilk *et al.* (1987). The nucleus contained multiple nucleocapsids and other structures indicative of *Ac*NPV infection, e.g. fibrillar structures, electron-dense "spacers" and virogenic stroma. Polyhedra were present in *wt Ac*NPV-infected cells (Fig. 2.4 A). At 72 h p.i. comparatively smaller crystals were observed in the nucleus, sometimes surrounded by electron-dense 'spacers' only (not shown).

To show that these paracrystalline inclusions in the cytoplasm were indeed equivalent to the CryIA(b) crystals and contained CryIA(b) ICPs, ultrathin sections of *Ac*NPV/JM3-infected *S.f.* cells were labelled with antiserum against dissolved crystals of *B.t. aizawai* strain 7.21 and visualized using protein-A gold (Fig. 2.4 C). The gold particles were mainly found in association with the electron-dense inclusions and confirmed that these contained CryIA(b) ICP. Some gold particles were randomly distributed over the cytoplasm suggesting that not all the ICPs were present in a crystallized form. This was not a background signal because these labels were not found in nucleus of *Ac*NPV/JM3 infected cells and not in *Ac*NPV/DZ1 or *wt Ac*NPV-infected cells.

The biological activity of the insecticidal crystal protein expressed by *S. frugiperda* cells infected with *Ac*NPV/JM3

In a bioassay the biological activity of the ICP expressed by *S.f.* cells infected with *Ac*NPV/JM3 was evaluated against *P. brassicae* and compared with the ICP produced by *E. coli* 7.21A cells (Table 2.1). Mock-infected, *wt Ac*NPV- and *Ac*NPV/DZ1-infected *S.f.* cells and *E. coli* cells, transformed with pBR322, served as negative controls. Larvae of *P. brassicae* were chosen for this assay because they are non-susceptible to *Ac*NPV (Vaughn *et al.*, 1977), but highly sensitive to the CryIA(b) ICP from *B.t.* (Höfte *et al.*, 1988). Hence, it should be possible to measure the effect of the CryIA(b) ICP without the effect of virus infection on mortality. Samples equivalent to 1.5×10^5 infected *S.f.* cells or 9.0×10^7 *E. coli*

cells were deposited on cabbage leaf disks (1.75 cm^2) and the effect on the mortality of second instar larvae of *P. brassicae* was measured. *S.f.* cells infected with *Ac*NPV/JM3 and *E. coli* 7.21A cells that expressed the cryIA(b) gene caused larval mortality (Table 2.1) and only superficial feeding marks were observed on these leaf disks. In contrast, when cells were used in which the cryIA(b) gene was absent, the leaf disks had been almost completely consumed after two days.

Table 2.1. Qualitative comparison of CryIA(b) ICP from *E. coli* cells and baculovirus-infected *S. frugiperda* cells against second instar larvae of *P. brassicae*.

Source	Mortality*
E. coli	0
E. coli (7.21A)	100
S.f. cells	3
S.f. cells + *Ac*NPV	0
S.f. cells + *Ac*NPV/DZ1	3
S.f. cells + *Ac*NPV/JM3	97

* Mortality was scored two days after application of cell samples and is presented as percentage of the total number of larvae used per assay (n=32).

In order to compare the relative toxicity of the ICPs produced by *Ac*NPV/JM3-infected *S.f.* cells and *E. coli* 7.21A cells, dilutions of *Ac*NPV/JM3-infected *S.f.* cells and *E. coli* 7.21A cells were tested in a dose response assay (Table 2.2). An administration level of 3.4 x 10^2 infected *S.f.* cells and 2.7 x 10^4 *E. coli* cells per cm^2 leaf disk caused 50% mortality, respectively. The slopes of the two regression lines were not significantly different indicating that the larvae reacted similarly to the different amounts of ICP.

Dilutions of *Ac*NPV/JM3-infected *S.f.* cells and *E. coli* 7.21A cells that gave the same mortality were analyzed by PAGE and the amount of 130 kDa protein was approximated by laser densitometry. It appeared that dilutions that were equally toxic contained similar amounts of 130 kDa ICP. This suggested that the toxicity of the *Ac*NPV-derived and *E. coli*-

derived CryIA(b) ICP was in the same order of magnitude. The 73 kDa product found in the immuno-blot was disregarded in this calculation because it could not be detected by laser-scan densitometry indicating that this protein is present in low amounts in CBB-stained gels.

Table 2.2. Quantitative comparison of CryIA(b) ICP from *E. coli* cells and baculovirus-infected *S. frugiperda* cells against second instar larvae of *P. brassicae*.

Source	LC_{50}	95% fiducial limits	
		lower	upper
E. coli (7.21A)	2.7×10^4	1.7×10^4	4.6×10^4
S.f. cells + *Ac*NPV/JM3	3.4×10^2	1.9×10^2	5.9×10^2

* LC_{50} is presented in cells/cm^2 leaf disk.

DISCUSSION

Baculoviruses are capable of expressing foreign genes under the control of the major late polyhedrin promoter (Luckow and Summers, 1988; Maeda, 1989b; Miller, 1988 and 1989). The polyhedrin gene is dispensable for virus replication and the expression of a foreign gene is based on the allelic replacement of the polyhedrin gene (Smith *et al.*, 1983a). Recombinants are usually recognized by their polyhedron-negative appearance. Using a novel transfer vector, pAcDZ1 (Zuidema *et al.*, 1990), the screening for recombinants containing the cryIA(b) gene was facilitated by the co-expression of ß-galactosidase as a marker. All 'blue' recombinant viruses analyzed showed identical DNA patterns and had a correct insertion of the CryIA(b) ICP gene. The fact that these recombinants were easily retrieved suggests that the 130 kDa ICP is not toxic when produced within *S.f.* cells.

Cells infected with recombinant *Ac*NPV/JM3 expressed the CryIA(b) ICP at the correct size of 130 kDa at relatively high level (approximately 5 % of the total cell protein). The protein was visible in gels by Coomassie Brilliant Blue stain (Fig. 2.2 A) and its identity as

the CryIA(b) ICP was confirmed by immunoblotting (Fig. 2.2 B). In addition to the 130 kDa ICP a product of 73 kDa was observed using immunoblotting (Fig. 2.2 B). Although this band reacted equally strongly with antiserum as compared to the 130 kDa ICP, it was not visible in the Coomassie Brilliant Blue-stained gel (Fig. 2.2 A). The 73 kDa band is most likely a breakdown product of the *B.t.* protein since no cross-reaction of the antiserum with other insect cell samples was detected (Fig. 2.2 B, lanes a, b and c). It is possible that one or more epitopes become exposed as a result of proteolytic breakdown in the cytoplasm or during isolation.

The ICP was found predominantly in the cytoplasm of *S.f.* cells as large, paracrystalline, electron-dense inclusions, sometimes bipyrimidal in shape (Fig. 2.4, B and C). These structures are reminiscent of the crystals found in *B.t.* itself (Krieg, 1989). Although these crystals were shown in prokaryotic recombinants such as *Bacillus* (Shivakumar *et al.*, 1986) and *E. coli* species (Oeda *et al.*, 1989), such crystalline structures have not been demonstrated in eukaryotic cells. Some ICP was found spread over the cytoplasm as concluded from immuno-electron microscopy (Fig. 2.4). The presence of breakdown products, that were not able to crystalize into large inclusions, may explain this observation.

The ICP produced in *S.f.* cells was highly toxic to *P. brassicae* larvae (Table 2.1 and 2.2). This provided further support that the ICP made in *S.f.* cells is authentic and that it is most likely cleaved by the alkaline proteases in the insect gut to give an active 60 kDa toxin (Höfte and Whiteley, 1989). In vitro assays using insect gut juices containing proteases did substantiate this (not shown). The ICP from infected *S.f.* cells was equally toxic to *P. brassicae* larvae as the ICP expressed by *E. coli*. In this calculation the breakdown products, present in low amounts as indicated by the scanning of PAGE gels, were disregarded. It is interesting to note that a baculovirus-expressed C-terminal truncated version of the CryIA(b) crystal was equally toxic for *P. brassicae* larvae. This recombinant, *Ac*NPV/JM4, contains only the first 645 codons of the ICP gene which includes the entire sequence coding for the active 60 kDa toxin (Honée *et al.*, 1990). The truncated ICP was expressed from the polyhedrin promoter at high levels too. The toxicity of the truncated protein was similar to the full-length protoxin. In insect cells, this protein did not produce crystal-like structures. All this indicates that the biological characteristics of these two recombinant proteins are the same (J.W.M. Martens, unpublished results).

Baculoviruses are usually found occluded in large protein capsules (polyhedra) and mainly in this form infectious for insects. *Ac*NPV/JM3 lacks the polyhedrin gene and produces only multiply-enveloped virions that are not occluded (Fig. 2.4). The amount of such virions is difficult to quantify and, on a particle basis, at least a five-fold less infectious for insects when infected orally (Volkmann and Summers, 1977). Therefore, the effect of the introduction of the cryIA(b) gene into the *Ac*NPV genome on the pathogenicity against *Ac*NPV-susceptible hosts cannot easily be assessed. Besides, the application of polyhedron-negative viruses in the field is impractical since they are quickly inactivated (Bishop, 1989; Entwistle and Evans, 1986). *Ac*NPV recombinants that maintained the polyhedrin gene and thus produce polyhedra, are more suitable to assess a dose and time mortality-relationship. Therefore, *Ac*NPV recombinants that have a *B.t.* ICP gene inserted, e.g. in the p10 gene locus under the control of the p10 promoter are now being constructed (Chapter 6). Alternatively, the polyhedrin-negative recombinant *Ac*NPV/JM3 can be co-occluded with *wt* *Ac*NPV into one polyhedron (Kuroda *et al.*, 1989; Price *et al.*, 1989) and used to determine the biological activity. The fact that the CryIA(b) ICP produced in baculovirus-infected insect cells is biologically active provides the basis for a strategy using *B.t.* ICP genes to enhance baculovirus pathogenicity.

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CHAPTER THREE

A CONVENIENT NON-RADIOACTIVE ASSAY TO DEMONSTRATE *BACILLUS THURINGIENSIS* INSECTICIDAL CRYSTAL PROTEIN-RECEPTOR INTERACTIONS

Authors: J.W.M. Martens, J.T.M. Flipsen, A.J.A. Groffen, D. Bosch and J.M. Vlak.

SUMMARY

An immuno-cytochemical technique was developed to identify specific binding of activated insecticidal crystal proteins (ICPs) to cryo-sections of midguts of insect larvae. Mildly fixed cryo-sections were incubated with activated ICPs. Bound IPCs were visualized by immunofluorescence. Specific binding of *E. coli*- or baculovirus-produced CryIA(b) toxin to midgut sections of third instar larvae of both *Manduca sexta* and *Spodoptera exigua* was determined. The toxin bound to the microvilli of both insect species over the whole length of the midgut epithelium. No binding was observed to the peritrophic and basal membrane nor to the cavity of the goblet cells. The dipteran-specific toxin CryIVD was unable to bind to these sections indicating that the binding was specific and correlated with toxicity. The improved assay described here is rapid and easy and can be used to predict the toxicity spectrum of new ICPs especially to those which are difficult to assay *in vivo*.

INTRODUCTION

Insecticidal crystal proteins of *Bacillus thuringiensis* (*B.t.*) are toxic to larvae of different insect and nematode species. Thus far, 5 different classes of ICPs are known: CryI proteins toxic to Lepidoptera; CryII proteins toxic to both Lepidoptera and Diptera; CryIII proteins toxic to Coleoptera, CryIV proteins toxic to Diptera and a last class, CryV proteins, toxic to nematodes (Höfte and Whiteley, 1989; Feitelson *et al.*, 1992). The toxicity of these ICPs is determined by the solubility of the crystals (Aronson *et al.*, 1991), the activation of the protoxin by proteolytic enzymes present in the midgut (Haider *et al.*, 1986; Ogiwara *et al.*, 1992), the retention of the toxins by the peritrophic membrane (Bravo *et al.*, 1992a; Denolf *et al.*, 1993b), the binding to susceptible cells (Hofmann *et al.*, 1988) and the penetration and subsequent formation of pores into membranes (Knowles and Ellar, 1987). Most research has been focussed on binding of ICPs to possible receptors present on the midgut epithelial cells, since binding to putative receptors correlates well with toxicity of the ICPs to larvae *in vivo*. (Hofmann *et al.*, 1988; Van Rie *et al.*, 1989; Van Rie *et al.*, 1990a). In addition, resistance to ICPs found in several insects (McGaugy and Whalon, 1990) is a result of loss of receptors in the midgut epithelial cells (Ferré *et al.*, 1991; Van Rie *et al.*, 1990b). Putative receptor proteins are identified in a binding assay to Western-blot containing

brush-border membrane vesicles (BBMV) proteins (Oddou *et al.*, 1991; 1993). The results together indicate that proteinaceous ICP-specific receptors are present in the midgut epithelial cells.

The toxicity spectrum of ICPs is usually determined in a bioassay (Schesser *et al.*, 1977; Krieg, 1986). Alternatively, binding studies to BBMVs can be used to identify the specificity (Hofmann *et al.*, 1988). Both techniques are time consuming, require a considerable number of insects and, in the latter case, radioactively labelled toxin molecules. Here, we developed a histochemical technique to detect specific binding of activated ICPs to midguts of insect larvae. This method, based on similar technique developed by Ryerse *et al.* (1990), is much faster and less laborious than conventional approaches. Most importantly, the binding observed correlates with toxicity *in vivo*. The method described here was tested on *Manduca sexta* and *Spodoptera exigua* larvae using lepidopteran- and dipteran-specific ICPs. As a source of ICPs both baculovirus- and *E. coli*-expressed ICPs were used.

MATERIALS AND METHODS

Recombinant proteins, larvae and antibodies

The CryIA(b) (Höfte *et al.*, 1986) and CryIVD (Donovan *et al.*, 1988) proteins were derived from *E. coli* recombinants. Alternatively, the baculovirus-derived CryIA(b) proteins were produced in *S. frugiperda* IPLB-SF-21 (*S.f.*) cells infected with *Ac*NPV/JM3, which expresses the cryIA(b) gene from the polyhedrin promoter (Chapter 2). *E. coli* cells expressing the CryIA(b) or CryIVD protein were grown overnight, harvested and lysed as described by Sambrook *et al.* (1989). *S.f.* cells infected with *Ac*NPV/JM3 were harvested after 72 h and lysed in PBS containing 1% Nonidet P40. The CryIA(b) and CryIVD ICPs were dissolved and activated similarly as described by Höfte *et al.* (1986) and Dai *et al.* (1993), respectively. Third instar *M. sexta* and *S. exigua* larvae were used in this experiment. The polyclonal antibodies used were raised in rabbits against *B.t.* supsp. *aizawai* ICPs for CryIA(b) (Chapter 2) and against *B.t. israelensis* ICPs for CryIVD.

Preparation of midguts

The midguts were collected from *M. sexta* or *S. exigua* larvae immobilized in ice-cold PBS (= 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 137 mM NaCl, 2,7 mM KCl, pH 7.4). The

midguts were fixed in 2% paraformaldehyde; 0.01% glutardialdehyde; 0.1 M Na_2HPO_4; 9.7 mM citric acid (pH=7.2) and 1.5 mM $CaCl_2$ for 2 h on ice and washed 3 times for 15 min in PBS, containing 0.001% NaN_3 (PBS-az). Subsequently, the midguts were embedded in Tissue-Tek embedding solution (Miles, USA) and immediately frozen (-20°C). Using a Jung Frigocut 2800E Cryotome, transverse sections of 6 μm were produced and attached to gelatinchroomaluin coated microscopical glass slides and stored at -80°C until further use.

Binding assay

The sections were treated with 1% glycine; 0.02% $NaBH_4$ in PBS in the dark for 15 min at room temperature to remove aldehyde polymers and to block free aldehyde groups. Then, the sections were incubated with 1% bovine serum albumin in PBS-az (PBS-BSA) for 1 h to block aspecific binding. Subsequently, each mounting glass was treated for 1 h with 7,5 μg Cry protein in 0.5 ml PBS-BSA ($\pm$300 nM). Unbound toxin was removed by washing the sections three times for 10 min with PBS-az. The bound toxin was labelled for 1 h using 0.5 ml of a primary antibody (1:300) either against CryIA(b) or CryIVD. The unbound antibody was removed by washing the sections three times for 10 min with PBS-az. In the dark, the bound primary antibody was labelled for 1 h with 0.5 ml a secondary antibody conjugated with FITC (1:100) (Nordic). At the same time the nuclei of the midguts were stained with 0.001% 4,6-diamidino-2-phenylindole (DAPI) (1:5000). Unbound secondary antibody and DAPI were removed by washing the sections as described above. The sections were embedded in cyti-fluor (Agor Aids) before examination under a Leitz Labolux S Fluorescence Microscope. DAPI staining was detected as a blue color, whereas FITC staining was green.

RESULTS

A rapid and convenient method was developed to detect specific binding of ICPs to midgut of insects. The protocol is fast since time-consuming de-hydration and impregnation of the embedding medium as described by Ryerse *et al.*, (1990) could be avoided. Instead, midgut sections were rapidly frozen for cryo-sectioning keeping the whole procedure including labelling and examinations limited to a single day.

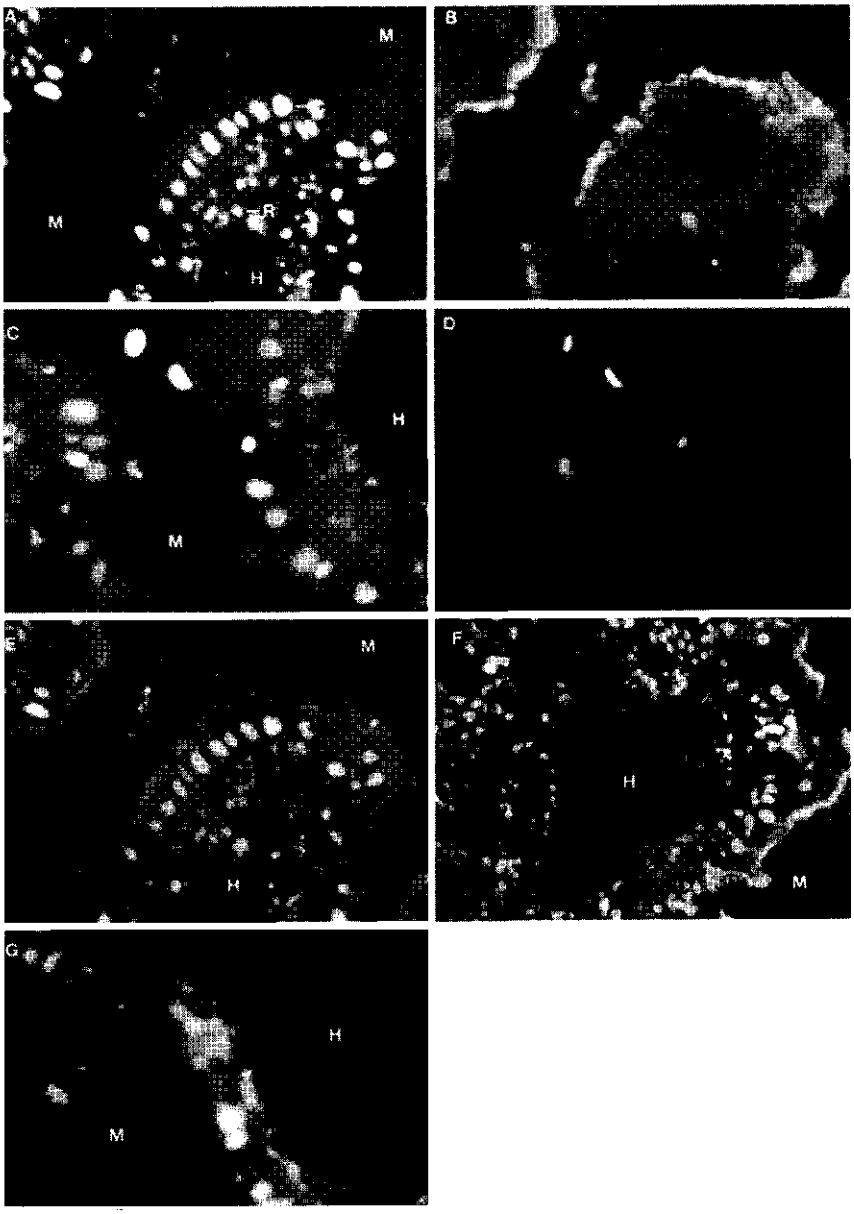

Figure 3.1. Specific binding of ICPs to midgut cryo-sections of *M. sexta* larvae (all except F) and *S. exigua* (F) incubated with (A,B,E,F) or without (C,D) activated CryIA(b) toxin or with CryIVD toxin (G). In each section the nuclei of the cells were stained with DAPI and the bound toxin was labeled with FITC-labeled antibodies. DAPI staining is shown in blue, while FITC labelling is in green. C = nucleus columnar cell, R = nucleus regenerative cell, M = midgut lumen and H = haemolymph.

Trypsin-activated CryIA(b) protein (=CryIA(b) toxin) derived from both baculovirus-infected *S.f.* cells and *E. coli* cells were used to perform binding assays both on *M. sexta* and *S. exigua* longitudinal midgut sections. The binding was detected with a polyclonal antibody specific for the ICP used, followed by a FITC-conjugated secondary antibody. Additionally, the midgut sections were stained with DAPI, which specifically stains the nuclei of the cells. DAPI staining of a *M. sexta* section is shown in figure 3.1A. The nuclei of columnar cells (NC) facing the midgut lumen (ML) are much larger than the nuclei of the regenerative cells (NR), which are close to the basal membrane and face the haemolymph (HL). The CryIA(b) toxin, derived from an *E. coli* recombinant in this case, was detected in the same section by fluorescein-labelling and was bound on the apical side of the midgut cells (Fig. 3.1 B). No staining and hence no binding was present on the basal and basolateral side of the midgut epithelium, on the peritrophic and the basal membrane and in the cavity of the goblet cells.

The intensity of the fluorescent signal was maximal at toxin concentrations of 30 nM or higher indicating that the receptors present were then saturated with toxin. When lower concentrations were used a decrease of the fluorescent signal was observed (not shown). Closer examination revealed that the binding was restricted to microvilli present on the columnar cells (not shown). The baculovirus-expressed CryIA(b) proteins showed a similar distribution as compared to the *E. coli* protein (not shown). Control midgut sections were incubated with PBS-BSA alone to detect a-specific binding of the primary and secondary antibodies. In these *M. sexta* sections (Fig. 3.1 C and D; showing DAPI and FITC staining, respectively) no aspecific binding was observed.

In order to ascertain that the binding observed correlates with specificity *in vivo*, the affinity of CryIA(b) to *M. sexta* sections was compared with that of CryIVD, a dipteran-specific protein. From this experiment only the double labelling (DAPI and FITC) of sections incubated with CryIA(b) (Fig. 3.1 E) and CryIVD (Fig. 3.1 G) is shown. In contrast to CryIA(b) (Fig. 3.1 E), no binding to *M. sexta* sections was observed when the dipteran specific CryIVD protein was used (Fig. 3.1 G). The inability of CryIVD to recognize receptors of *M. sexta*, in contrast to CryIA(b), strengthens the hypothesis that the specific binding observed here reflects the specificity *in vivo*.

The applicability of the assay for other insect larvae was examined by determining the affinity of the CryIA(b) toxin for receptors on midguts of *S. exigua* which is also highly

susceptible to CryIA(b). As in *M. sexta*, binding was observed on the apical side of the columnar cells of *S. exigua* (Fig. 3.1 F). This signal was not a result of an aspecific reaction of the antibodies since no signal was observed in sections in which incubation with the CryIA(b) toxin was omitted (not shown).

DISCUSSION

A quick and easy method was developed to determine specific binding of activated ICPs to midgut sections of insect larvae. To test the protocol, a lepidopteran (CryIA(b)) and a dipteran-specific (CryIVD) ICP were used on midgut sections of *M. sexta* and *S. exigua*. The CryIA(b) toxin derived from either *E. coli* or baculovirus-infected *S.f.* cells showed specific binding to microvilli of midgut section of both *M. sexta* and *S. exigua* and no difference was observed between binding characteristics of the CryIA(b) toxins of different origin. This confirms that the baculovirus-expressed CryIA(b) protein produced in *S.f.* cells is biologically active (Chapter 2). As in *M. sexta*, binding was observed on the apical side of the columnar cells of *S. exigua* proving for the first time that receptors for CryIA(b) in *S. exigua* are also present only on the apical side of columnar cells. The CryIVD protein was unable to bind to these sections. So, for these two ICPs the observed binding correlates with their toxicity spectrum (Höfte and Whiteley, 1989). Binding assays on midgut sections of dipteran larvae (e.g. *Anopheles stephensi*) could mirror the results with the lepidopteran midguts obtained here. Additionally, more closely related CryI proteins which are not toxic to these larvae can be tested to confirm the specificity of the binding.

The fluorescent signal was maximal at a toxin concentration of 30 nM or higher. Lower toxin concentrations resulted in a decrease of the signal which suggests that these concentrations are not saturating. This is in line with the observation of Hofmann *et al.* (1988) who observed saturating binding of CryIA(b) to BBMVs of *M. sexta* (K_d = 0.43 nM) at toxin concentration higher than 10 nM.

Ryerse *et al.* (1990) previously developed a similar histochemical technique to detect specific binding of the CryIA(c) toxin to midgut sections of *H. virescens*. They observed binding of the CryIA(c) toxin to *H. virescens* midgut, but in their experiment no control larvae were shown in which incubation with toxin was omitted which made their conclusions rather premature.

Table 3.1. Correlation between binding to midgut section and toxicity *in vivo*.

Type of larvae	Type of gene	Toxicity	Binding to brush-border	References
Manduca sexta	IA(b)	T	$+^{P}$	1,5,10
	IA(c)	T	$+^{Tr}$	1,5
	IB	T	$+^{P}$	1,5
	IIIA	NT	$-^{P}$	1,5
	IVD	NT	-	2,10
Ostrinia nubilalis	IA(a)	T	+	7
	IA(b)	T	+	7
	IA(c)	T	$+^{PBM}$	7
	IB	T	$+^{P}$	7
	ID	NT	-	7
	IE	NT	-	7
Heliothis virescens	IA(c)	T	+	2,3
Spodoptera exigua	IA(b)	T	+	9,10
Plutella xylostella	IA(a)	T	+	6
	IA(b)	T	$+^{P}$	4,5
	IA(c)	T	+	6
	IB	T	$+^{P}$	4,5
	ID	NT	-	4,6
	IE	NT	-	4,6
Plutella xylostella (resistant)	IA(b)	NT	$-^{P}$	4,5
	IB	T	$+^{P}$	4,5
Leptinotarsa decemlineata	IA(b)	NT	-	5
	IB	NT	-	5
	IIIA	T	$+^{P}$	5
Anopheles gambiae	*B.t.i.*	T		8
	IVD		+	8
	cytA		+	8

1 = Hofmann *et al.*, 1988; 2 = Hofte and Whiteley, 1989; 3 = Ryerse *et al.*, 1990; 4 = Ferré *et al.*, 1991; 5 = Bravo *et al.*, 1992b; 6 = Denolf *et al.*, 1993b; 7 = Denolf *et al.*, 1993a; 8 = Ravoahangimalala *et al.*, 1993; 9 = Honée, 1992; 10 = this chapter; T = toxic, NT = not toxic, + = binding to brush border, - = no binding, P,B,Tr,M = binding to peritrophic membrane, to basal membrane, to tracheal element and to malpighian tubules, respectively.

Recently, other groups (Bravo *et al.*, 1992a,b; Denolf *et al.*, 1993a,b and Ravoahangimalala *et al.*, 1993) developed protocols to detect specific binding to midgut sections. The results of these experiments are summarized in Table 3.1. In all cases the binding to the microvilli of the midgut epithelial cells correlated with toxicity. The binding to tracheal cells and the peritrophic and basal membrane, which was occasionally observed, did not. The binding to the micrivilli of the Malpighian tubules seems to be specific, but this interaction is most likely physiologically irrelevant because these binding sites are not directly exposed to toxins after oral uptake of ICPs.

Although all these bindings studies are similar, they differ from each other in four aspects: (i) the way of application of the toxin (*in vivo* by applying the toxin orally or *in vitro* after sectioning); (ii) the length of fixation and type of embedding used (Paraplast or polywax for normal sectioning or tissue TEK for cryo-sectioning); (iii) the type of detection (alkaline phosphatase, horse-radish peroxidase or fluorescent labelling) and (iv) the larval stage used (L3 to L5). All these parameters influence the speed, sensitivity and specificity of the binding. The method described in this paper is the fastest because the tissues are only mildly fixed and time consuming dehydration and impregnation of the embedding medium can be omitted. Therefore, sections can be prepared, labelled and examined within one day. Using this mild fixation also as many epitopes as possible will be preserved. So, the binding more closely resembles the *in vivo* situation. Additionally, thus far no aspecific binding has been observed using this method.

The method developed here is useful to identify new ICPs, which are now characterized by laborious bioassays or binding assays to BBMVs. The latter methods require a large number of midguts and radioactively labelled ligands. In the present protocol at least 20 analyses can be done with only a single midgut of a third instar *M. sexta* larvae. The method can be used for competition assays, using biotinylated *B.t.* toxins (Denolf *et al.*, 1993b), to determine if the different ICPs recognize the same or a distinct receptor. Biotin labelled toxins are of interest since antibodies against the ICP are then no longer required in the binding assay. This is especially relevant for determining the toxicity spectrum of new IPCs for which usually no antibodies are available. This technique is useful to separate the activation process from receptor recognition since binding assays with protoxins can also be performed. Furthermore, mutant or engineered IPCs can be rapidly tested for their ability to bind to receptors on midgut sections. Finally, due to the fixation procedure, the method

might allow the separation of ICP binding from pore formation and the implementation of realistic competition experiments.

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CHAPTER FOUR

MAPPING AND CHARACTERIZATION OF THE ENTOMOCIDAL DOMAIN OF THE CRYIA(b) PROTOXIN

This chapter was submitted for publication as: **Martens, J.W.M., B. Visser, J.M. Vlak and D. Bosch.** Mapping and characterization of the entomocidal domain of the *bacillus thuringiensis* CryIA(b) protoxin. To Mol. Gen. Genetics.

SUMMARY

The precise borders of the toxic domain of the CryIA(b) protoxin were determined by deletion and site-directed mutagenesis. At the C-terminus the introduction of stop codons in the cryIA(b) gene behind codons Arg601, Phe604 or Ala607 showed that only a polypeptide running from Met1 to Ala607 (p607) possessed insecticidal activity, demonstrating that *in vivo* activation by midgut proteases takes place distal from Ala607. The two slightly further truncated polypeptides were prone to proteolytic degradation and, therefore, biologically inactive. At the N-terminus the requirement for proteolytic activation was investigated by removing of the first 28 amino acids of p607. This protein, p29-607, was of the same size as the *in vitro*-released mature toxin (56 kDa), and appeared to be toxic not only to susceptible insect larvae, such as *M. sexta* and *H. virescens*, but also to *E. coli* cells. This suggests that the first 28 amino acids keep the CryIA(b) protein inactive. This hypothesis was supported by results obtained with another mutant protein, p1-607RD, in which only the putative cleavage site, Arg28, of p607 was replaced by Asp. p1-607RD was resistent to trypsin treatment and had indeed reduced toxicity for *M. sexta*. An additional mutant protein (p29-429) that contains only amino acid 29 to 429 of the CryIA(b) toxin and, consequently, lacks part of the receptor-binding domain, was also toxic for *E. coli* cells. This suggests that the toxicity of p29-607 and p29-429 to *E. coli* is most likely not receptor mediated and that only amino acid 29 to 429 of the CryIA(b) protoxin are sufficient for the pore formation.

INTRODUCTION

Insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis* (*B.t.*) are among the best studied biological insecticides (Höfte and Whiteley, 1989). These proteins, synthesized as large crystals during sporulation of the bacterium, are highly active against many important pest insects and dipteran vectors of human diseases, such as malaria and yellow fever. When ingested by larvae, these crystals solubilize in the midgut and release proteins (protoxins) ranging from 70 to 130 kDa and are, subsequently, activated by gut proteases into mature toxins of approximately 55 to 65 kDa (Lilley *et al.*, 1980; Nagamatsu *et al.*, 1984; Milne and Kaplan, 1993). These mature toxins bind receptors present on epithelial cells of the

midgut of susceptible insects (Hofmann *et al.*, 1988) and probably force pores in the membrane of these cells (Knowles and Ellar, 1987; Slatin *et al.*, 1990), leading to cell lysis and finally to death of the larvae.

Recently, the 3-D structure of the mature toxin of the coleopteran-specific CryIIIA ICP has been resolved (Li *et al.*, 1991) and three functional domains within the mature toxin were proposed (Fig. 4.1 A): (i) domain I, containing 7 α-helixes, is involved in pore formation; (ii) domain II, containing 3 ß-sheets, mediates the specific binding to the receptor, and (iii) domain III, containing a ß-sandwich, is involved in stability and protection of the mature toxin against midgut proteases.

The process of activation of ICPs by midgut proteases has been studied for several ICPs. For the CryIA(b) protein, the N-terminal amino acid of the mature toxin of both trypsin- and midgut-juice-activated protoxins is Ile29 (Höfte *et al.*, 1986; Ogiwara *et al.*, 1992). The C-terminus of the CryIA(b) toxin, as determined by Bal31 deletion mutagenesis, is located in between amino acid 599 and 607 (Höfte *et al.*, 1986). The amino acids sequence in this area, IDRIEF, is highly conserved among all ICPs that have been sequenced thus far (Box 5 in Fig. 4.1 A). Höfte *et al.* (1986) and Nakamura *et al.* (1992) proposed that this sequence was either involved in proper cleavage after the conserved trypsin cleavage site, Arg601, or involved in stabilizing the mature toxin and protecting it against proteolytic degradation. Our aim was to determine the precise borders of the toxic fragment of the CryIA(b) protoxin and determine which steps of the proteolytic activation are required for the release of the mature toxin. Therefore, stop codons were introduced downstream of codon 601, 604 or 607. These mutants could clarify if the conserved sequence IDRIEF is involved either in proteolytic activation at the potential trypsin cleavage site, Arg601, or in stabilizing the C-terminus of the mature toxin. The necessity of proteolytic activation at the N-terminus was studied either by removing the potential cleavage site, Arg28, or by deleting the amino acid residues preceding the N-terminus of the mature toxin.

MATERIALS AND METHODS

Cells and plasmids

JM101 recA$^-$ or JM105 cells were used for transformation. The vectors Bluescript pSK+ and pKS+ (Stratagene), pTZ18R and pTZ18U (Pharmacia) were used for cloning.

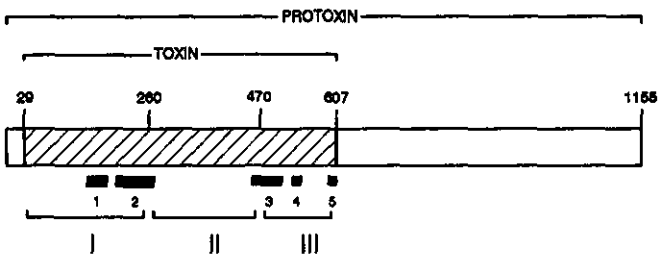
pTZBg was derived from pTZ18R by inserting a *Bgl*II linker (5'-CAGATCTG-3') in the *Sma*I site. pSKBg was constructed from pSK+ by inserting this *Bgl*II linker in the blunted *Pst*I site of pSK+. pKS+ was modified by inserting a *Nco*I linker (5'-CCCCATGGGG-3') in the *Eco*RV site resulting in pKSNco. pBD10, a derivative of pKK233-2 (Pharmacia), was used for the expression of the cryIA(b) constructs in *E. coli* under control of the *tac* promoter (Bosch *et al.*, in preparation). For the expression of the full-length cryIA(b) gene the plasmid pBD140 was used (Bosch *et al.*, in preparation). For the construction of the deletions, a 3'-truncated cryIA(b) gene was taken from p109.2δ (Honée *et al.*, 1990). General molecular techniques were performed as described by Sambrook *et al.* 1989.

Modification of the 3' of the cryIA(b) gene (Fig. 4.1 B)

For the construction of 1-601, 1-604 and 1-607, the 0.6 kbp *Sst*I-*Bgl*II fragment of p109.2δ, containing 3' part of the truncated cryIA(b) gene from nucleotide (nt) 1350 to 2075, was cloned in pSKBg to form pJM17. This plasmid was used as template for PCR to generate two C-terminal deletions of the cryIA(b) gene. For the construction of 1-601 primer Bt601 (5'-GC*AGATCT*ATCGATCTATATAAACT-3') was used as forward primer while for 1-607 primer Bt607 (5'-GC*AGATCT*ATGCCGGAACAAATTCA-3') was used. As reverse primer in both cases the reverse sequencing primer (Biolabs) was used. Primers Bt601 and Bt607 insert a translation stop codon and a *Bgl*II site in the cryIA(b) ORF immediately downstream of codon 601 and 607, respectively. For PCR reaction 200 pg of PJM17 was used as template DNA together with 50 pmol of each primer, 200 μM of each dNTP, 10 μl of PCR reaction buffer (100 mM Tris-HCl, pH 8.3, 500 Mm KCl, 15 mM $MgCl_2$ and 0.01 % w/v gelatin) and 2.5 units of *Taq* DNA polymerase in a total volume of 100 μl. The sample was subsequently incubated for 1 min at 94°C, 1.5 min at 50°C, 2 min at 72°C. This incubation was repeated 30 times in a PCR apparatus (Perkin-Elmer Cetus). The 450 bp PCR fragments were cut with *Sst*I and *Bgl*II and cloned into pSKBG resulting in pJM25 for primer Bt601 and in pJM26 for primer Bt607.

The third C-terminal deletion mutant was constructed as follows: the 0.4 kb *Eco*RI-*Bgl*II fragment of pJM17, containing the C-terminus of the truncated cryIA(b) gene from nt 1577 to 2075, was cloned into pTZBg, resulting in pJM19. This plasmid was digested with *Xba*I and partially with *Xmn*I. The *Xba*I site was filled in with *Klenow*. A 3.1 kb *Xmn*I-*Xba*I fragment, in which *Xmn*I had cut in the cryIA(b) gene at position 1811, was isolated from

A) Functional domains of the CryIA(b) Protoxin.



B) Restriction maps of the deletion mutants.

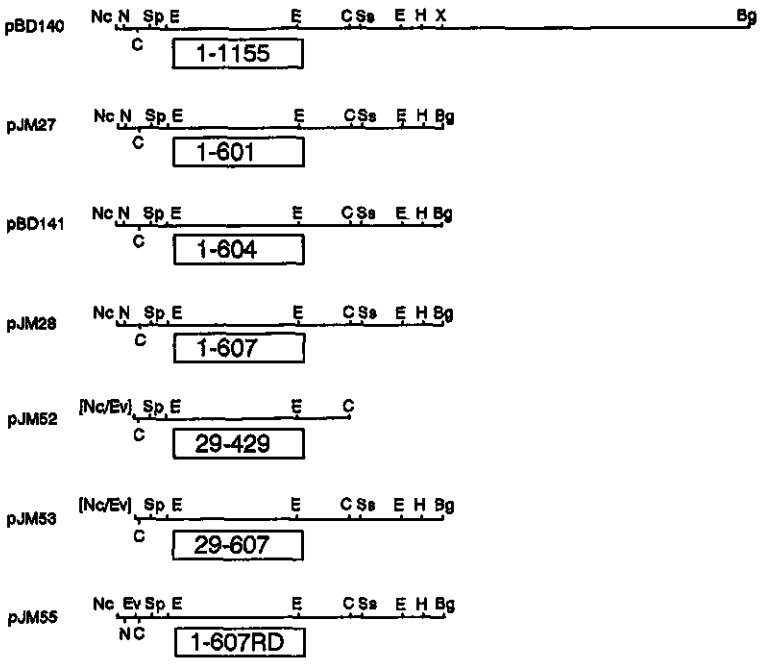


Figure 4.1. A) Functional domains of the CryIA(b) protoxin of *Bacillus thuringiensis*. Domains I, II and III represent functional domains of the mature toxin. The boxes 1 to 5 represent the conserved blocks described by Höfte and Whiteley, 1989. The amino acids bordering the domains were determined by alignment with the CryIIIA protein. B) Restriction maps of the deletion mutants used in this study. Restriction sites used in the cloning steps are included in the drawings. The size of the constructs corresponds exactly with the size of the CryIA(b) protein drawn in A. The name of the plasmid is indicated on the left of each construct. Underneath each construct the name of each construct derived from the first and the last amino acid of the construct, is given. Nc=*Nco*I, N=*Nsi*I, C=*Cla*I, Sp=*Spe*I, E=*Eco*RI, Ev=*Eco*RV, Ss=*Sst*I, H=*Hind*III, X=*Xmn*I and Bg=*Bgl*II. [Nc/Ev]= is constructed by ligating the blunted *Nco*I containing the start codon with the *Eco*RV site.

gel and closed with T4 ligase resulting in pJM20. pJM20 contains a TAG stopcodon downstream of codon 604. The 0.25 kb *Eco*RI-*Sal*I from pJM20 was cloned back into pJM17 resulting in pJM21. pJM21 was subsequently linearized with *Xba*I; filled in with *Klenow* and religated by inserting a *Bgl*II linker (5'-AGATCT-3') resulting in pJM21Bg.

pJM25, pJM21Bg and pJM26 were checked by sequencing and subsequently used to construct the C-terminal deletion mutants of the cryIA(b) gene. To this purpose the C-terminal *Sst*I-*Bgl*II fragment of pBD140 was replaced by a 450 bp *Sst*I-*Bgl*II fragment of either pJM25, pJM21Bg or pJM26, resulting in pJM27, pBD141 and pJM28, respectively. These plasmids express CryIA(b) derivatives running from codon 1 to 601, 1 to 604 or 1 to 607 and were called construct 1-601, 1-604 and 1-607, respectively.

Modification of the 5' end of the cryIA(b) gene (Fig. 4.1 B)

For construction of 29-607, 1-607RD and 29-429, plasmid pJM15 was used, which contains the 5' end of the cryIA(b) gene from 2 nucleotides upstream of the start codon to nt 1350, as a *Bam*HI-*Sst*I fragment subcloned from p109.2δ in pTZ18U. In pJM15, the primer Bt28-29 (5'-TGGAGAA*GATATC*GAAACTG-3') was introduced using site directed mutagenesis (Kunkel, 1985), resulting in pJM16. This plasmid contains an additional *Eco*RV site which cleaves in between codon 28 and 29 and changes Arg 28 into Asp. The other codons remain unchanged. A 300 bp *Bam*HI-*Eco*RI fragment of pJM16, containing the mutation was checked by sequencing. The 1.4 kb *Bam*HI-*Sst*I fragment of pJM16 was cloned into pSKNco resulting in pJM31. The 120 nt *Nco*I-*Cla*I fragment containing the 5' mutated part of the gene was cloned in pBD140 replacing the 3,5 kb full-length cryIA(b) gene, resulting in pJM50. pJM50 was cut with *Nco*I and *Eco*RV; made blunt using *Klenow* and religated again resulting in pJM51. In the *Cla*I site of pJM51, the adjacent 1.2 kb *Cla*I fragment of the cryIA(b) gene was inserted resulting in pJM52, construct 29-429. The construct 29-607 was completed by replacing the 1.1 kb *Spe*I-*Sal*I fragment of pJM52 for a 1.7kb *Spe*I-*Sal*I fragment of pJM28. This resulted in pJM53.

For the construction of 1-607RD, the 1.3 kb *Nsi*I-*Sst*I fragment (nt 30 to 1350) of pJM28 was replaced by the corresponding 1.3 kb *Nsi*I-*Sst*I fragment of pJM16 resulting in pJM54. To ensure that no other mutations were present in the construct, the 1,7kb *Spe*I-*Sal*I fragment of pJM54 was replaced by the 1,7kb *Spe*I-*Sal*I fragment of pJM28. The resulting plasmid was called pJM55 and contains construct 1-607RD.

Protein analysis

From each *E. coli* recombinant an overnight culture (OD_{660} = ±1.6-2.0) was grown at 37°C at 200 rpm in an orbital shaker. Subsequently, the cells were washed two times with PBS and concentrated in lysis buffer (10 mM Tris-HCl pH=8, 5 mM EDTA, 100 mM NaCl) to an OD_{660} of 10. One ml of each of these samples was used for the purification of ICPs and subsequent trypsin treatment. Therefore, 200 μg lysozyme and PMSF (50 μM final concentration) was added to each sample and incubated at room temperature for 20 min. to lyse the cells. After this 5 μg of sodium deoxycholate (DOC) and 5 μl DNAse (10 mg/ml) was added followed by an incubation of the sample for 30 min at 37°C to solubilize the membranes and to degrade the bacterial DNA. To solubilize possible aggregates and crystals, the pH of the samples was increased by adding 1/10 volume of 10x carbonate buffer (0.5 M Na_2CO_3, 100 mM DTT and 1 M NaCl; pH=10) and incubating them at 37°C for 2h. Subsequently, the pH of the samples was neutralized by dialyzing overnight (at 4°C) in excess 50 mM Tris-HCl pH 8.5; 200 mM NaCl. Part of the sample was used directly for protein analysis, whereas the rest was treated with 1:10 (v/v) trypsin (10 mg/ml) for 1 h at 37°C. Both untreated and trypsin-treated samples were analyzed in a 12.5% SDS-PAGE followed by a Western analysis (Sambrook *et al.*, 1989). For the immune-detection, a polyclonal antiserum raised against total ICPs of *B.t.* subsp. *aizawai* 7.21 was used (1:1000), followed by an incubation with a secondary antibody conjugated with alkaline phosphatase (1:1000; Tago). For color development the substrates NBT and BCIP were added (Gibco BRL).

Bioassays

Bioassays were done mainly as described by Schesser *et al.* (1977). Serial dilutions of the different *E. coli* recombinants were layered in wells (1.7 cm^2) on top of artificial diet (100 μl/well). In each well one neonate *Manduca sexta* or *Heliothis virescens* larva was placed. The larvae were subsequently incubated at 28°C and a humidity of 70% for 5 days. After the incubation the larvae were weighed. For each recombinant 5 or 6 concentrations were tested and for each concentrations 24 larvae were used.

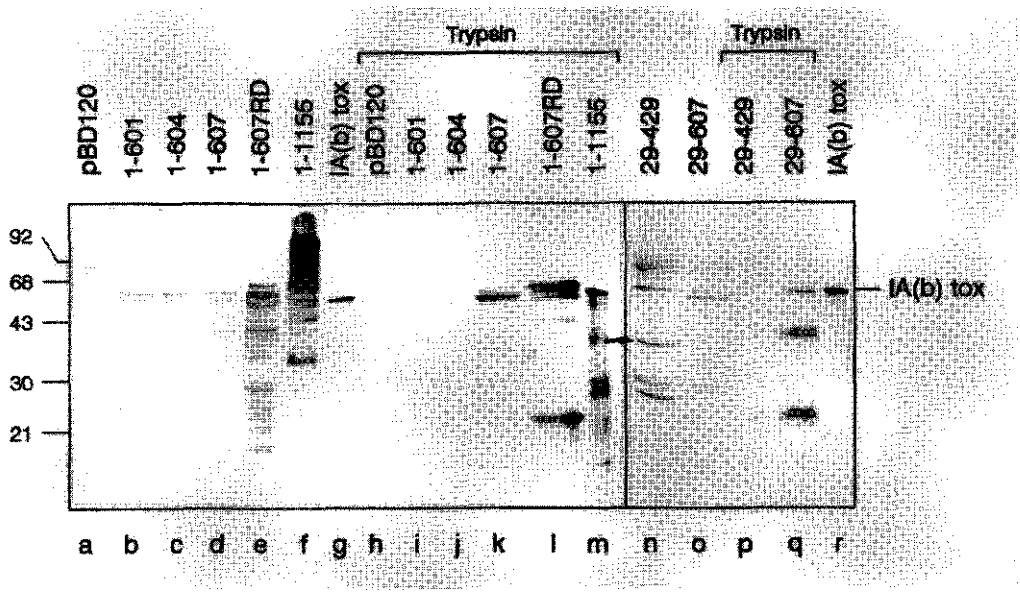


Figure 4.2. Western blot of total protein from the deletion mutants. In the left panel in each lane $1.7*10^7$ *E. coli* cells were loaded; in the right panel $1*10^8$ *E. coli* cells. The samples of the right part of each panel were treated with trypsin. For the detection a polyclonal antibody against ICPs of *B.t. aizawai* was used. In lane g and r 50 ng of trypsin-activated CryIA(b) protein was loaded.

RESULTS

Construction and analysis of the C-terminal deletion mutants of the cryIA(b) gene.

To map the C-terminal border of the CryIA(b) mature toxin, stop codons were introduced into the cryIA(b) gene as described in Materials and Methods. This resulted in constructs 1-601, 1-604 and 1-607. The open reading frame (ORF) of construct 1-601 terminates immediately downstream of a potential trypsin cleavage site, Arg601, present in the conserved sequence, IDRIEF; the ORF of construct 1-604 contains this sequence completely and that of construct 1-607 includes the complete ß-strand 23 (Li *et al.*, 1991). The last construct is similar to the longest toxic deletion mutant constructed by Höfte *et al.* (1986), except that the ORF of 1-607 terminates in a proper termination codon and has,

therefore, no additional amino acids derived from the vector. All constructs were expressed in *E. coli* from the *tac* promoter of pBD10.

The amount of the truncated CryIA(b) proteins (p601, p604, and p607, respectively) produced by each recombinant was analyzed in SDS/PAGE followed by Western-analysis using a CryI-proteins-specific polyclonal antibody (Fig. 4.2). From the blot it is apparent that the amount of CryIA(b)-specific protein expressed by the three C-terminal deletion mutants is comparable (Fig. 4.2, lanes b, c and d), since equivalent amounts of *E. coli* cells were loaded. As compared to recombinant expressing the *wild-type* (*wt*) protoxin (1-1155, lane f), however, the amount of CryIA(b) protein expressed was far less. As expected, the size of the truncated proteins (about 59 kDa) slightly increased with the increasing length of the construct.

The biological activity of the three different *E.coli* recombinants to neonate *Manduca sexta* larvae (Fig. 4.3, panel A) was determined and compared with the recombinant expressing the *wt* cryIA(b) gene (1-1155). Only the recombinant which expressed p607 was toxic, whereas the other two recombinants expressing slightly smaller CryIA(b) polypeptides were not. Compared with the recombinant expressing the *wt* protoxin, the recombinant expressing p607 was far less toxic. This difference is most likely caused by a difference in the amount of ICP per *E. coli* cell between these recombinants and not by a difference in toxicity of the proteins itself. Similar results were obtained in bioassay done with *Heliothis virescens* larvae (data not shown).

To address why the p601 and p604 were not toxic, their susceptibility to trypsin was compared with p607. Incubation of *wt* CryIA(b) protoxin with trypsin results in a characteristic trypsin resistant mature toxin of about 56 kDa (Fig. 4.2, lane g). Incubation of the truncated CryIA(b) proteins with trypsin revealed that p601 and p604 are highly susceptible whereas p607 is resistant to this enzyme (Fig. 4.2, lane i, j and k). The difference in toxicity of these truncated proteins is, therefore, most likely caused by a difference in susceptibility to digestive midgut enzymes of insects. In summary, these results show, firstly, that p607 is the smallest biologically active C-terminal truncated CryIA(b) protein and, secondly, that proteolytic activation of the CryIA(b) protoxin *in vivo* occurs C-terminal of Ala607.

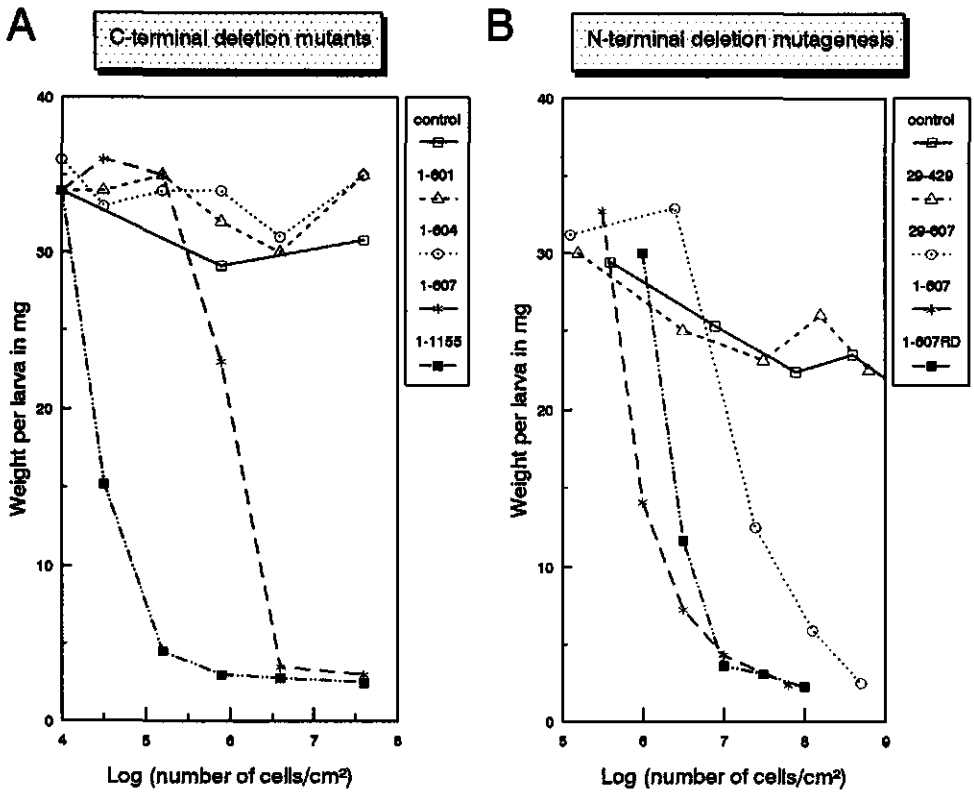


Figure 4.3. Bioassay against neonate *M. sexta* larvae of cell extracts of *E. coli* recombinants expressing different deletion constructs. The toxicity was determined by growth reduction of the larvae after a five days incubation on surface contaminated diet. The average weight of a larva was plotted against the dose in number of *E. coli* cells per cm².

Construction and analysis of N-terminal mutants of the cryIA(b) gene.

At the N-terminus, both the significance of the amino acids residues preceding the N-terminus of the mature toxin and the necessity of proteolytic activation was investigated. Initially two mutants were designed. In the first one, 29-607, a translation start codon was introduced into construct 1-607 in front of Ile29. In the second one, 1-607RD, the N-terminal trypsin cleavage site was removed by replacing Arg28 for Asp. In contrast to the recombinant expressing 1-607, the recombinant expressing construct 29-607, produced very small colonies on solid medium and grew poorly in liquid media (data not shown). This

indicated that the protein expressed (p29-607) was toxic to *E. coli*. An additional recombinant expressing only amino acids 29 to 429 of the cryIA(b) gene (p29-429) showed the same severe growth retardation. Despite the poor growth of both of these recombinants, CryIA(b)-specific proteins could be detected in these samples (Fig. 4.2, lane n and o). For the recombinant expressing p29-607, a specific protein of 56 kDa was detected which co-migrates with the *in vitro*-released mature toxin (Fig. 4.2, lane r); in the other recombinant a protein of 38 kDa was observed (see arrow). Other bands observed on the blot were caused by a-specific binding of the primary antibodies with *E. coli* proteins since they were present in both lanes. Compared to the recombinant expressing p607 (Fig. 4.2, lane d) the amount of p29-607 and p29-429 expressed was far less, since ten times more protein had to be loaded to visualize these proteins. The recombinant expressing 1-607RD, showed normal growth and produced a CryIA(b)-specific protein, p607RD, of similar size (59 kDa) as recombinant expressing p607, but the amount produced was significantly higher (Fig. 4.2, lane d and e). On the blot an additional minor band representing a protein of 63 kDa, was observed. This protein was also detected in the recombinants expressing p601, p604 and p607, when more total *E. coli* protein was loaded (not shown), but not in the control lane. The origin of this band remains unclear.

The toxicity of the three recombinants to *M. sexta* larvae was determined and compared with the recombinant expressing p607 (Fig. 4.3 B). The recombinant expressing p29-607 was toxic, although to a lesser extent than the one expressing p607; the recombinant expressing p29-429 was not. The difference in toxicity between the recombinant expressing p29-607 and the one expressing p1-607 is caused by a difference in CryIA(b)-specific protein production. The recombinant expressing p1-607RD was toxic for *M. sexta* larvae too (Fig. 4.3 B), but slightly less than the recombinant expressing p607. Thus, despite the higher CryIA(b)-specific protein production, recombinant 1-607RD was less toxic than the recombinant 1-607. Removal of the trypsin cleavage site from p607, therefore, interferes with toxicity *in vivo*, but does not abolish it.

Trypsin treatment showed that p29-607 was resistant whereas p29-429 was sensitive to trypsin (Fig. 4.2, lane o, q, n and p). This is why p29-429 lacks toxicity *in vivo*. P607RD appeared also to be resistent to trypsin and of the same size as the untreated protein (Fig. 4.2, lane e and k). This indicates that in p607RD the only N-terminal trypsin cleavage site present in the CryIA(b) protoxin has been removed.

DISCUSSION

Comparison of three C-terminal deletion mutants of the CryIA(b) protoxin demonstrated that amino acids C-terminal of ß-strand 23 (Ala607) are not required for insecticidal activity. Removal of only three amino acids from this ß-strand, as was done for p604, completely abolished the activity. ß-strand 23, is part of the inner sheet of domain III and is embedded inside the protein and interacts with amino acids both in domain II and III (Li *et al.*, 1991). Apparently disturbance of this ß-strand severely affects normal folding and renders the polypeptide susceptible to proteolytic enzymes. P601 and p604, although possibly still able to bind to receptors and to make pores in membranes, are rapidly degraded by midgut proteases and, therefore, no longer toxic to *M. sexta* and *H. virescens* larvae. These results are in line with experiments performed with other ICPs. In all cases the mutants containing a complete ß-strand 23 retain insecticidal activity whereas the others do not (Schnepf and Whiteley, 1985; Adang *et al.*, 1985; Höfte *et al.*, 1986; Sanchis *et al.*, 1989; Widner and Whiteley, 1989; Yoshida *et al.*, 1993; Ward and Ellar, 1988).

The results further demonstrate that the highly conserved Arg601 is not susceptible to trypsin but, on the contrary, highly protected against cleavage. Nakamura *et al.* 1992 replaced this Arg for Gln in the cryIA(b) protoxin, which resulted in a protoxin that was not toxic to *M. sexta* and highly sensitive to midgut proteases. Removal or alteration of only one or a few amino acids in ß-strand 23, therefore, changes the stability of domain III making it susceptible to proteolytic enzymes.

Since ß-strand 23 is absolutely required for toxicity *in vivo*, the final activation by midgut proteases at the C-terminus most likely occurs at the first available trypsin cleavage site C-terminal of ß-strand 23. Nakamura *et al.* (1992) replaced in the protoxin each of these sites (Arg619; Lys622 or Lys637), individually or combined, by Gln residues. In all four cases the mutant proteins were still toxic. Interestingly, the mutant protoxin in which all three residues were replaced by Gln residues, produced a mature toxin after *S. littoralis*-midgut-juice treatment which was slightly larger than that of the *wt* cryIA(b) protoxin. This shows that indeed one of these residues is involved in the final cleavage and that the final cleavage at the C-terminus is performed by a trypsin-like protease. Such a protease has recently been purified from gut juices of *Choristoneura fumiferana* (Milne and Kaplan, 1993). Furthermore, the replacements done by Nakamura *et al.* (1992), prove that the mature toxin

can be extended at least until Lys637 without loss of toxicity. For this reason, it is likely that ICPs that produce 65-70 kDa protoxins, like CryII, III, IVC and IVD proteins, and that terminate almost immediately C-terminal of ß-strand 23 do not need proteolytic activation at their C-terminus.

Since the C-terminal activation occurs C-terminal of Ala607 and the N-terminal cleavage in front of Ile29, p29-607 does not require further processing by midgut proteases to be functional. Surprisingly, and in contrast to p1-607, this protein was not only active against lepidopteran larvae, but also to *E. coli* (this study) and *Spodoptera frugiperda* cells (Chapter 6). Possibly, the first 28 amino acids prevent the CryIA(b) toxin from inserting into membranes irrespective of the presence of receptors. The observation that p29-429, which lacks a part of the receptor binding domain II, is similarly toxic for *E. coli* supports the hypothesis that the toxicity of p29-607 to *E. coli* is not receptor-mediated. Receptor-independent insertion of *in vitro* released toxins in phospholipid vesicles has been reported before and is only observed if high concentrations of toxin are used (Haider and Ellar, 1989; English *et al.*, 1991). The biological function of the first 28 amino acids of the cryIA(b) protoxin, therefore, might be to keep the toxin in an inactive stage during its synthesis in *B.t.*. Whether this is a general feature for all the ICPs remains to be determined but this seems plausible since all ICPs that have been studied thus far contain a stretch of 25 to 60 amino acids in front of domain I of the toxin that is removed by proteolytic enzymes (Höfte *et al.*, 1986; Höfte and Whiteley, 1989; Carroll *et al.*, 1989; Dai and Gill, 1993). Expression of this and other mature toxins in *B.t.* and *E. coli* might show whether this is indeed the case.

To determine whether the removal of the first 28 amino acids is essential for toxicity *in vivo*, the trypsin target site, Arg28, was replaced by Asp. This prevented p607RD protein from being cleaved by trypsin *in vitro* (Fig. 4.2, lane l) and lowered its toxicity to *M. sexta* larvae (Fig. 4.3 B). Hence, trypsin cleavage at Arg28 is not essential for toxicity. The remaining activity of this mutant protein might be explained by assuming that *M. sexta* midgut proteases are able, although less efficient, to activate CryIA(b) *in vivo*. Alternatively, if midgut protease are unable to remove the N-terminal amino acid residues, these residues might reduce the toxicity, because they are interfering with pore formation.

The toxicity of p29-429 for *E.coli* cells indicates further that only this part of the toxin is required for pore formation. Recently, Walters *et al.* (1993) have observed that a 21.5 kDa peptide containing only the N-terminal part of the mature toxin, was still able to produce cation-specific channel in lipid bilayers. Both results confirm that the N-terminal of the mature toxin alone is capable of the formation of pores as suggested by Li *et al.* (1991).

In conclusion, these results presented here show that the borders of the CryIA(b) toxin are not fixed. Toxicity is guaranteed as long as α-helix 1 and ß-strand 23 are kept intact. Additional sequences to both the N- and the C-terminus are allowed without loss of toxicity.

CHAPTER FIVE

A BACULOVIRUS VECTOR THAT FACILITATES THE GENERATION OF P10-BASED RECOMBINANTS

This chapter has been accepted for publication in a slightly modified form as: **Martens, J.W.M., M.M. Van Oers, B.D. Van de Bilt and J.M. Vlak.** A baculovirus vector that facilitates the generation of p10-based recombinants. J. Virol. Methods.

SUMMARY

A baculovirus vector was developed which facilitated the retrieval of recombinants in the p10 locus by introducing a unique *Bsu*36I restriction site at this location. Linearization of the viral DNA significantly reduces the number of parental virus produced in a co-transfection with a p10 transfer vector. This increases the percentage of recombinants from about 0.5% to 25% which greatly facilitates the isolation of recombinant viruses by reducing the number of plaque purification from four to only one or two.

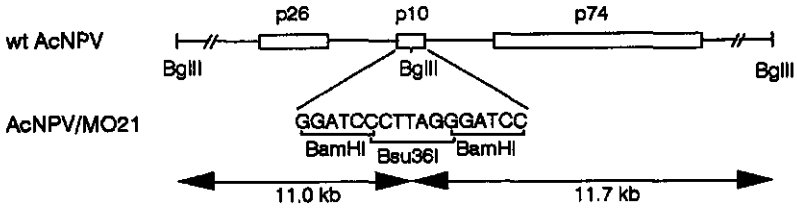
INTRODUCTION

Baculoviruses are routinely used as vectors for the high level expression of foreign proteins in insect cells (Luckow and Summer, 1988). The strong promoters of two very late genes, polyhedrin (PH) and p10, are exploited to drive the expression of these foreign genes (Smith *et al.*, 1983; Vlak *et al.*, 1990). The recombinant proteins produced are near authentic and often biologically active. Baculoviruses are successfully used in basic research and in human and veterinary medicine, and form a good alternative for bacterial, yeast and mammalian expression systems.

Recombinant viruses are usually produced in insect cells by homologous recombination between parental viral DNA and transfer vector DNA (See Fig. 1.2). The frequency of recombination is low, varying from 0.1 to 2%. Plaque morphology (occlusion body-negative), enzymatic activity (ß-galactosidase, ß-glucuronidase and luciferase expression), immuno-screening, hybridization and PCR have been routinely used for the screening and subsequent isolation of recombinants. The purification of recombinants, however, is often difficult and remains time consuming since several rounds of plaque purification are required due to the low percentage of recombinants. Linearization of viral DNA that contains a unique *Bsu*36I restriction site in the polyhedrin locus efficiently reduced the amount of parental virus produced after a co-transfection (Kitts *et al.*, 1990). This increased the proportion of recombinants from an average of 1% to about 25% and reduced the time required for plaque purification considerably .

Polyhedrin and p10 are both dispensable for viral replication and for recombinant protein production in insect cells. Polyhedra are, however, important for efficient production

A) Schematic representation of the p10 locus



B) Restriction analysis of AcNPV/MO21

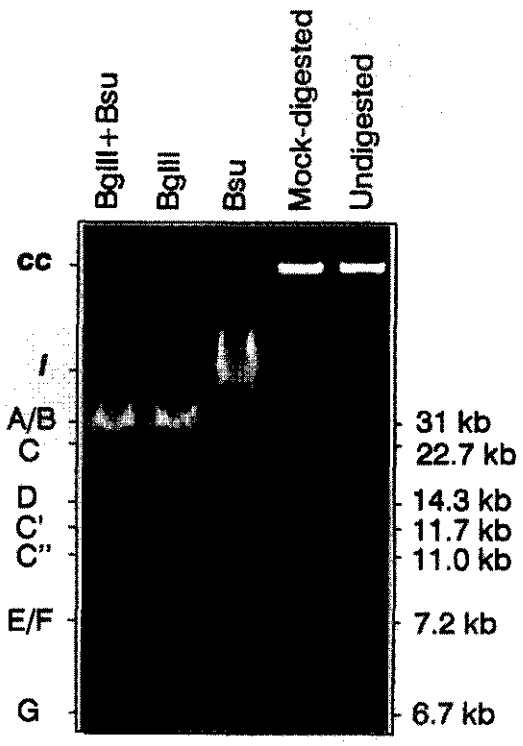


Figure 5.1. A) Schematic representation of the p10 locus of both wild-type *Ac*NPV and the recombinant *Ac*NPV/MO21. B) Restriction enzyme analysis of *Ac*NPV/MO21. The arrows in A indicate the two *Bgl*II-*Bsu*36I fragments (C' and C'') containing the up and downstream sequences of the p10 locus of *Ac*NPV. The size of the fragments is indicated in kilobase pairs. *cc* = closed circular supercoiled DNA; *l* = linear DNA. BglII = *Bgl*II; Bsu = *Bsu*36I.

of recombinant proteins in larvae and for the production of recombinant viruses for pest control. In these cases the polyhedrin gene needs to be maintained and either duplication of one of the very late promoters (Emery and Bishop, 1987; Weyer *et al.*, 1990) or the exploitation of the p10 locus (Vlak *et al.*, 1990) is required. P10-minus recombinant viruses are also biologically contained, since during the infection the nuclear disintegration of such recombinants is impaired, making them presumably safer for release in the environment (Van Oers *et al.*, 1993).

RESULTS AND DISCUSSION

In this paper, an improved procedure for the retrieval of p10-based *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) expression vectors is described. A recombinant virus was constructed in which the p10 coding sequence was replaced by a *Bsu*36I restriction site (Fig. 5.1 A). To this end, a *Bsu*36I linker was cloned into the transfer vector pAcAS2 (Vlak *et al.*, 1990), which contains the flanking sequences of the p10 gene and a *Bam*HI cloning site. The resulting transfer vector, pAcMO21, was used in a co-transfection and thereby introduced a unique *Bsu*36I restriction site into the p10 locus of *Ac*NPV. The recombinant virus, *Ac*NPV/MO21, was plaque-purified and checked by digesting its viral DNA with *Bsu*36I and *Bgl*II (Fig. 5.1 B). The *Bgl*II C fragment (22.7 kb) of *Ac*NPV/MO21, containing the p10 locus, was cut by *Bsu*36I in two fragments of 11.7 kb (C') and 11.0 kb (C''). This indicated that the *Bsu*36I site was inserted at the correct position. Using *Bsu*36I alone, the viral DNA was converted efficiently from a closed circular supercoiled (*cc*) into a linear (*l*) molecule (Fig. 5.1 B). A part of the mock-digested viral DNA was also linear, which is most likely due to random shear during the isolation of the viral DNA (Fig. 5.1 B). The biological activity (LT_{50} and LD_{50}) of *Ac*NPV/MO21 for second instar larvae of *Spodoptera exigua* was determined and found to be the same as wild-type *Ac*NPV.

To determine the frequency of recombination of both linear and circular viral DNA, *Ac*NPV/MO21 DNA was digested with *Bsu*36I or mock-digested, and co-transfected as described elsewhere (King and Posse, 1992) with two different transfer vectors, pAcAS3 and pAcMKn1. These plasmids both contain p10 flanking sequences and a ß-galactosidase marker gene cassette (Vlak *et al.*, 1990). This cassette is required for visual screening of p10

recombinants. Transfer vector pAcMKn1 contains the ß-glucuronidase gene downstream of the p10 promoter. The efficiency of recombination was calculated from the total amount of progeny virus (polyhedra-positive) and the amount of recombinant virus (blue color upon addition of X-gal) (Table 5.1). For both transfer vectors linearization of the parental viral DNA resulted in an increase of the percentage of recombinants from about 2.9 to 26%. A similar increase (from 1.3% to 26%) was found after linearization of the parental virus for the polyhedrin locus (Kitts *et al.*, 1990).

Table 5.1. Percentage of recombinants[1] in co-transfection in *S.frugiperda* 21 cells using *Ac*NPV/MO21 as parental viral DNA and pAcAS3 derivatives as transfer vector DNA.

DNA source	Percentage of recombinants[1]	
Mock-digested + pAcAS3	3.0	(1.5)
Mock-digested + pAcMKn1	2.8	(1.3)
*Bsu*36I-digested + pAcAS3	30.0	(9.9)
*Bsu*36I-digested + pAcMKn1	22.3	(12.6)

[1] 1 µg *Ac*NPV/MO21 DNA was co-transfected with 0.5 µg transfer vector DNA and after 5 days the titer of both the total progeny and recombinant virus was determined using an end point dilution assay (King and Possee, 1992). The titer of recombinant virus was determined after the addition of X-gal to the assay. The results are the average of nine separate transfection assays. Between brackets the standard deviation is given.

The frequency of recombination measured for all other recombinants produced using this method was about 25% (not shown) except for the recombinant viruses *Ac*NPV/E2, *Ac*NPV/SG2 and 4. From the last two recombinants which express the mature form of *Bacillus thuringiensis* CryIA(b) insecticidal crystal protein (ICP)(See also chapter 6), the frequency of recombination was only 0.5% (n=3) which is significantly different ($p<0.05$) from all the others. The cytotoxicity of the mature ICP for *S.f.* 21 cells most likely explains the poor progeny virus production of these two recombinants. The frequency of recombination for the recombinant *Ac*NPV/E2 which expresses the E2-glycoprotein of the Hepatitis C virus, was 6.1% ± 3.5% (n=7) which is also significantly different ($p<0.05$).

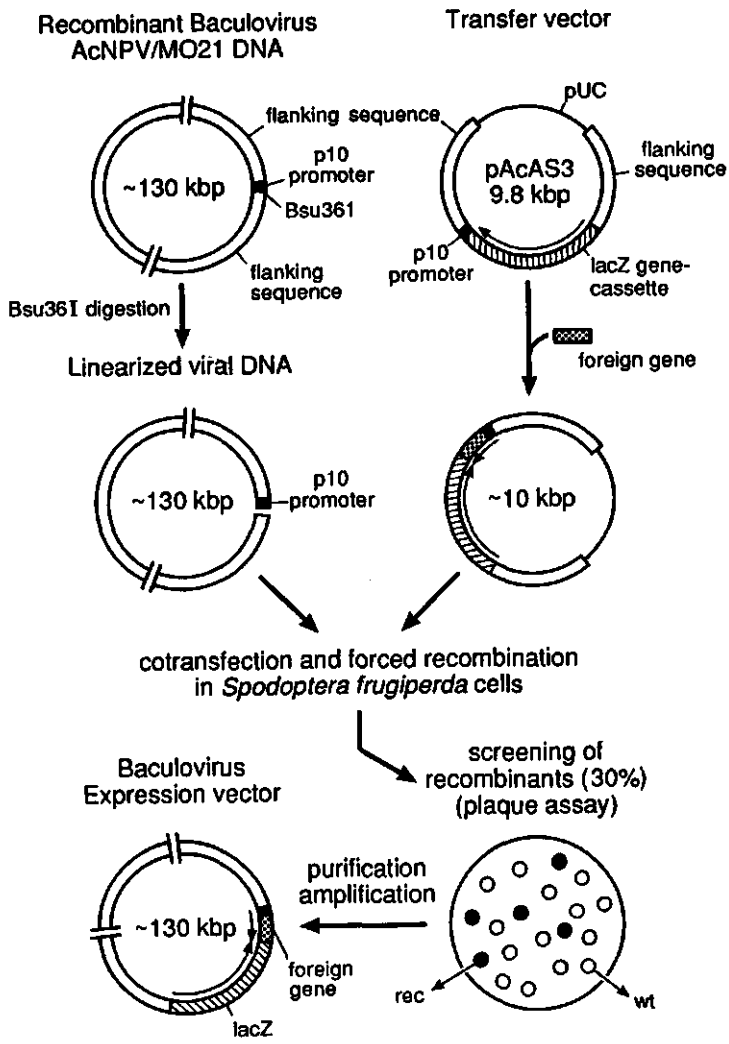


Figure 5.2. Construction of p10-based recombinant baculoviruses using baculovirus DNA which has been linearized in the p10 locus with the restriction enzyme *Bsu*36I. The transfer vector pAcAS3 contains the p10 promoter (black box), a ß-galactosidase selection marker (dashed box) and the p10-gene-flanking sequences for homologous recombination (Vlak *et al.*, 1990). After a co-transfection, the foreign gene is introduced into the p10 locus (stippled box). In a plaque assay, recombinant viruses can be visualized after the addition of X-gal and subsequently purified. Using linearized viral DNA the number of parental virus is efficiently reduced resulting in a percentage of recombinants of 25 to 30%.

The E2-glycoprotein, which is highly expressed by this baculovirus recombinant (not shown), most likely interferes with the production of extra-cellular virus after the co-transfection, although this remains to be determined. These three observations show that the recombination frequency after a co-transfection is a marker for interference of the expressed protein with the baculovirus infection.

In conclusion, the use of a parental viral vector which can be linearized at a unique restriction site present in the p10 locus, significantly increases the percentage of recombinant virus produced after a co-transfection and, therefore, greatly facilitates the recovery of p10-based recombinants (Schematically shown in Fig. 5.2). Insertion of a visible screening marker, such as ß-galactosidase, along with the foreign gene into progeny virus allows the convenient positive screening and titration of p10-based recombinants and reduces the time required for their purification from five to only two weeks. The use of transfer vectors that insert selectable markers, such as antibiotic resistance genes or genes essential for baculovirus infection, together with the foreign gene may further facilitate the retrieval of baculovirus p10 recombinants (e.g. p35 (Rodems and Friesen, 1993) and orf1629 (Kitts and Possee, 1993; Possee *et al.*, 1991).

ACKNOWLEDGEMENT

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CHAPTER SIX

CHARACTERIZATION OF BACULOVIRUS INSECTICIDES EXPRESSING TAILORED *BACILLUS THURINGIENSIS* CryIA(b) INSECTICIDAL CRYSTAL PROTEIN CONSTRUCTS

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SUMMARY

Full-length, truncated and mature forms of the CryIA(b) insecticidal crystal protein gene of *Bacillus thuringiensis* (*B.t.*) were engineered into the p10 locus of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV). A signal sequence of *Heliothis virescens* juvenile hormone esterase was introduced at the N-terminus of these constructs to induce secretion. All recombinants, except those containing the mature toxin, produced high levels of CryIA(b) ICPs in insect cells. Thirty percent of the intracellular protoxin was N-glycosylated suggesting the protoxin was translocated across the ER membrane. Secretion into the medium, however, was limited. The production of the mature toxin was poor as a result of its cytotoxicity to insects cells. In a bioassay against second instar *Spodoptera exigua* larvae, using a recombinant expressing the *Androctonus australis* scorpion toxin gene in the same p10 locus as a positive control, the median survival time of *Ac*NPV recombinants expressing the various *B.t.* CryIA(b) ICP constructs was not significantly different from *wild-type Ac*NPV. This suggests that production and/or secretion of *B.t.* (pro)toxins by *Ac*NPV p10 recombinant viruses does not increase insecticidal activity, since (i) the protoxins produced are inactive and not likely to be activated *in vivo*; (ii) secretion of the *B.t.* protoxins is poor and (iii) production of the mature toxins results in cytotoxicity.

INTRODUCTION

Baculoviruses have a relatively slow speed of action compared to chemical insecticides and this has limited their wide-spread use as biocontrol agents of insect pests (Payne, 1988). Production during the infection of proteins that interfere specifically with insect metabolism or metamorphosis, such as hormones, enzymes and toxins might enhance the pathogenicity of these viruses (Wood, 1991; Vlak, 1993). The production of a diuretic hormone (Maeda, 1989a), a mite toxin (Tomalski and Miller, 1991 and 1992), a scorpion toxin (Stewart *et al.*, 1991; McCutchen *et al.*, 1991; Maeda *et al.*, 1991) or a modified juvenile hormone esterase (JHE) (Hammock *et al.*, 1993) enhances biological activity of baculoviruses and so these are the first examples of genetically-improved baculovirus insecticides.

Production of insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis* (*B.t.*), might also improve the speed of action of baculoviruses. *B.t.* produces entomocidal protein crystals

during sporulation (Höfte and Whiteley, 1989). When ingested by an insect larva, these crystals dissolve due to the high pH of the midgut. The proteins (67 to 130 kDa) released from these crystals are further cleaved by proteolytic enzymes to active toxins (about 55 kDa). These mature toxins disrupt the midgut epithelium which results in a rapid death of the insects (see Gill *et al.*, 1993, for review).

In previous experiments it has been shown that large amounts of fully active ICPs could be produced from the strong late polyhedrin and p10 promoter of the baculovirus *Autographa californica* Nuclear Polyhedrosis Virus (*Ac*NPV) (CryIA(c): Merryweather *et al.*, 1990; CryIA(b): chapter 2; CryIA(b,c): Ribeiro and Crook, 1993; CryIVD: Pang and Federici, 1992). Although the produced protoxins were fully active against susceptible insects when administered *per os*, the production of these protoxins or truncated protoxins did neither improve the virulence of *Ac*NPV (Merryweather *et al.*, 1990) nor speed up its activity towards insect larvae (Ribiero and Crook, 1993). In order to be active in the insect midgut, hemocoel or other tissues the ICPs need further processing by proteolytic enzymes (Lilley *et al.*, 1980; Nagamatsu *et al.*, 1984). In nature, activation only occurs in the midgut lumen. The main reason for the inability of the ICPs that have been used thus far to improve the biological activity of baculoviruses is that they are produced as protoxins inside insect cells, where they are not readily accessible for protease-mediated activation.

Production of the mature toxin to circumvent the activation process or relocation of both the protoxins or mature toxins towards the midgut lumen or to the hemocoel might result in an improved baculoviral insecticide. After secretion and activation in case of the protoxin, the toxin can reach and bind to its receptor, which is present on midgut cells (Hofmann *et al.*, 1988) and, possibly, on other susceptible cells of the insect (Murphy *et al.*, 1976, Johnson, 1981). Recently, we have determined the borders of the mature toxin of CryIA(b) ICP (chapter 4). A polypeptide containing amino acids 29 to 607, can be regarded as a mature toxin. This peptide does not require further proteolytic processing to be fully active.

In this paper we report on the engineering of full-length, truncated and mature *B.t.* ICP genes into the p10 locus of *Ac*NPV. The constructs were also preceded by a signal sequence to allow secretion required for maturation and subsequent binding of the toxin to its receptor. The recombinant viruses were first analyzed for the production and secretion of the recombinant ICPs. Subsequently, the biological activity of these recombinant viruses was

tested against *Spodoptera exigua* larvae and compared to wt *Ac*NPV. As a positive control a recombinant virus was used that expresses an insect-specific toxin gene (*Aa*IT) from the scorpion *Androctonus australis* in the same locus. Production of such a toxin enhances the speed of action of *Ac*NPV (Stewart *et al.*, 1991).

MATERIALS AND METHODS

Plasmids

P109.2, p109.2δ and pSG3 contain the full-length protoxin (1155 codons), the 3'-truncated protoxin (first 646+4 codons) and the mature toxin (codon 29 to 607 preceded by a start codon) of the cryIA(b) genes as a *Bam*HI-*Bgl*II fragment (Honée *et al.*, 1990; unpublished results) in Bluescript. pJM55 contains the mature cryIA(b) toxin gene (codon 29 to 607 without a start codon) as a *Eco*RV-*Bgl*II fragment; pTZ-AaHIT contains the bombyxin signal sequence followed by coding sequence of the *Aa*IT toxin from the scorpion *Androctonus australis Hector*, flanked by *Bam*HI sites (McCutchen *et al.*, 1991).

pAcAS3 was used as basic transfer vector for the construction of p10 recombinants (Vlak *et al.*, 1990). This vector contains a *Bam*HI cloning site downstream of the p10 promoter and a ß-galactosidase reporter gene cassette for convenient screening (Fig. 6.1).

The signal sequence of the JHE of *Heliothis virescens* (Hanzlik *et al.* (1989) was synthetically made, using two oligomer primers:
5'-GCATGCTGATC ATG ACT TCA CAC GTA CTC GCG CTC GCG-3' and
5'-GGATCCTAGATCT GCC GGC CAG CGC TGT GCA CGC GTG TAG AAG GAA CGC GAG CGC GAG-3'

Insect cells and viruses

The *Spodoptera frugiperda* cell line IPLB-SF-21 (Vaughn *et al.*, 1977) was maintained in TNM-FH medium (Hink, 1970) supplemented with 10% fetal bovine serum and cultured at 27°C. The E2 strain of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) (Summers and Smith, 1978) was used as parental virus for the production of recombinants and also as control virus. Recombinant *Ac*NPV/AS3, which contains the beta-galactosidase reporter gene cassette in the p10 locus (Vlak *et al.*, 1990), was used as p10-negative control.

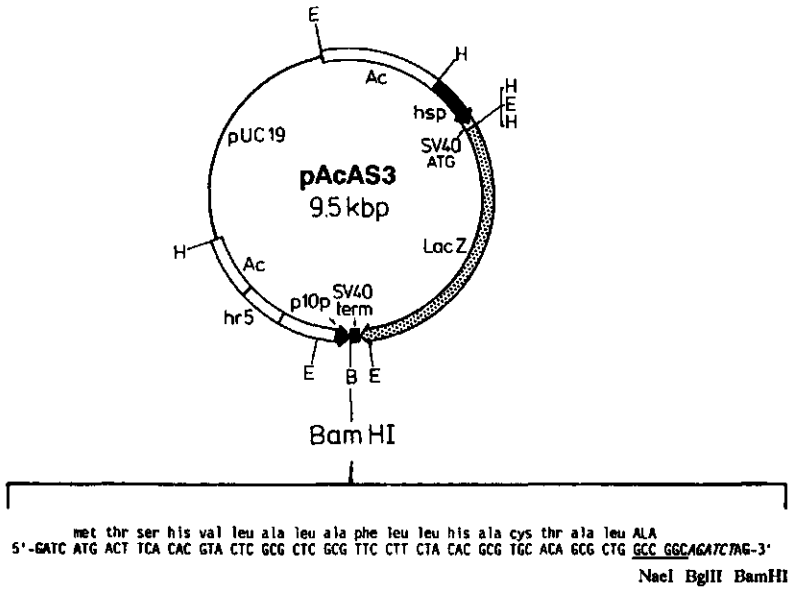

Figure 6.1. A) Restriction map of the expression vector pAcAS3 and B) the *BclI-BamHI* fragment containing the JHE signal sequence which was cloned into the *Bam*HI cloning site of pAcAS3 to give pAcFW1. The *Nae*I, *Bam*HI and *Bgl*II cloning sites are shown underneath the sequence.

For the efficient retrieval of recombinant viruses *Ac*NPV/MO21 (chapter 5) was used, which contains a unique *Bsu*36I restriction site in the p10 locus. Co-transfection with *Bsu*36I linearized *Ac*NPV/MO21 viral DNA results in a ten times higher proportion of recombinants (25%). *Ac*NPV/*Aa*IT (McCutchen *et al.*, 1991) contains the toxin from the scorpion *A. australis* Hector preceded by a p10 promoter inserted in front of the polyhedrin gene.

Construction of transfer vectors

The signal sequence of JHE was synthesized by using the two primers described above, which are complementary at their 3' ends for 12 nucleotides (nt). These primers were annealed, made double stranded using Klenow DNA polymerase, digested with *BclI* and *Bam*HI and inserted into the *Bam*HI site of pAcAS3 resulting in the JHE signal sequence containing transfer vector pAcFW1 (Fig. 6.1). This vector contains a *Nae*I, *Bam*HI and *Bgl*II cloning site for the insertion of foreign genes in three open reading frames.

The full-length protoxin (1155 amino acids (aa)) was inserted as a 3.5 kbp *Bam*HI-*Bgl*II fragment from p109.2 into the unique *Bam*HI sites of pAcAS3 and pAcFW1 to give transfer vectors pAcJM1 and pAcFW2. In pAcFW2, a spacer of six amino acids (GRSRFC) is

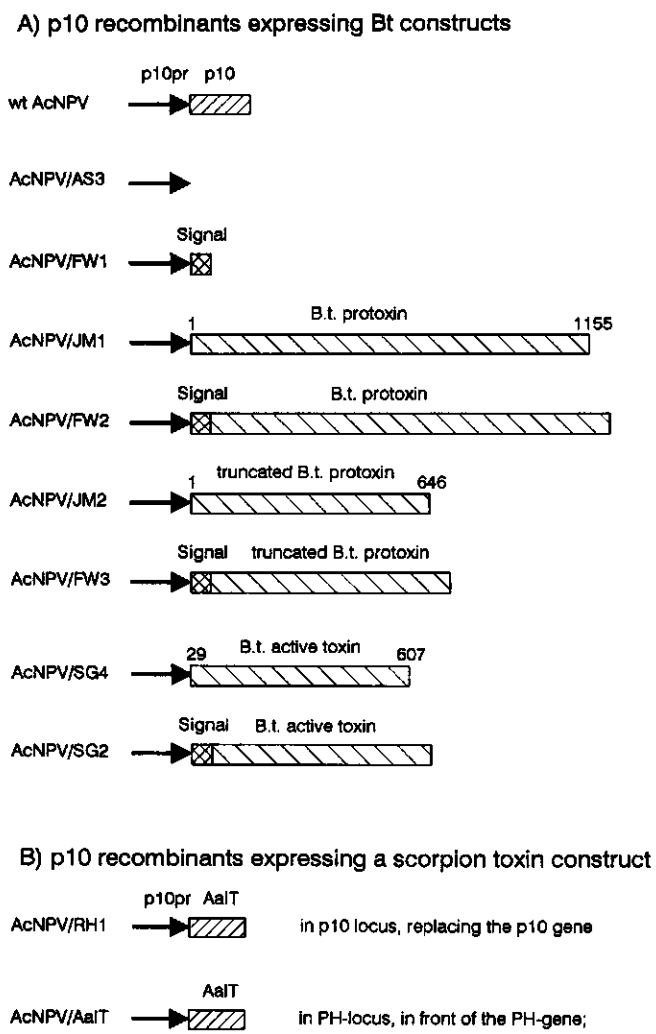

Figure 6.2. Constructs introduced in the p10 locus of the different recombinant viruses expressing *Bacillus thuringiensis* CryIA(b) (pro)toxins (A) and *Androctonus australis* scorpion toxin constructs (B). Indicated are the p10 promoter (p10pr), the *H. virescens* juvenile hormone esterase signal peptide (signal). For the *B.t.* constructs the first and last amino acid present in the construct are indicated. *Aa*IT = the scorpion toxin constructs.

present between the C-terminal Ala of the JHE signal peptide and N-terminal methionine of the protoxin. The truncated protoxin (646 aa) was inserted as a 1.9 kbp *Bam*HI-*Bgl*II fragment from p109.2δ into the *Bam*HI sites of pAcAS3 and pAcFW1 to give transfer vectors pAcJM2 and pAcFW3. The latter contains the same spacer between the signal peptide and the truncated protoxin as present in pAcFW2 (Fig. 6.2).

The mature toxin (aa 29 to 607) was inserted as a 1.7 kbp *Bam*HI-*Bgl*II fragment from pSG3 downstream of the p10 promoter in pAcAS3 resulting in pAcSG4. The N-terminal Met was directly followed by Ile29 of the protoxin. pAcSG2 was constructed by inserting a blunt-ended *Eco*RV-*Bgl*II fragment containing the toxin (aa 29 to 607) into pAcFW1, which was digested with *Nae*I and *Bgl*II (Fig. 6.1 and 6.2). The C-terminal Ala of the signal peptide was followed by Ile29 of the toxin.

The *Androctonus australis* toxin gene (*Aa*IT) was cloned as a *Bam*HI fragment from pTZ.*Aa*IT (Mc Cutchen *et al.*, 1991) into pAcAS3 resulting in transfer vector pAcRH1.

Isolation of recombinant viruses

Recombinant viruses were obtained by co-transfecting either wt *Ac*NPV/E2 DNA or *Bsu*36I-linearized *Ac*NPV/MO21 DNA with the various transfer vectors using the lipofectin method (King and Possee, 1992). Recombinant viruses were visualized after the addition of X-gal and plaque purified (Vlak *et al.* 1990). The DNA profile of the recombinant viruses was checked using various restriction enzymes.

Protein analysis and immunoblot analysis

For protein analysis *S.f.* 21 cells were infected with a multiplicity of infection of 10. After 1 h the inoculum was removed and replaced by either Hink's or EX-CELL (JR Scientific, Inc.) medium. Serum free EX-CELL medium was used when the protein content of the medium was analyzed. After 48 h the infected *S. frugiperda* cells were gently washed twice with excess phosphate buffered saline, pH 7.5, (PBS), and analyzed in 12.5% SDS/PAGE according to Laemmli *et al.* (1970) using a BioRad Protean II apparatus. To analyse the (secreted) CryIA(b) proteins, the medium was harvested at 48 h p.i. and concentrated 10 times by TCA precipitation. The gels were stained with Coomassie Brilliant Blue or used for immunoblotting as described by chapter 2. For immunoblot analysis a

1:1000 dilution of the polyclonal antiserum against the ICPs of *B.t. aizawai* strain 7.21 was used (Chapter 2).

N-linked glycosyl groups present on proteins were removed with N-glycosidase F, according to the protocol provided by the manufacturer (Boehringer Mannheim). The proteins were subsequently subjected to 12.5% SDS/PAGE and to immunoblot analysis as described above.

Biological assay

For each recombinant late-third instar *Spodoptera exigua* larvae were infected and polyhedra were isolated as described by Caballero *et al.* (1992). The virulence (LD_{50}) was determined by the droplet-feeding method as previously described by Smits and Vlak (1988). Suspensions of $3.3*10^6$, $1.0*10^6$, $3.3*10^5$, $1.0*10^5$, and $3.3*10^4$ polyhedra per ml were used. For each concentration 48 second instar *Spodoptera exigua* larvae were taken. After six days of incubation at 28°C the mortality was determined. The median lethal concentration (LC_{50}) was determined from a probit mortality analysis plot (Finney, 1971). In addition the volume of liquid ingested (about 0.1μl) by these larvae during the bioassay was determined. Using these data, the median lethal dose (LD_{50}) was calculated from the LC_{50}. For the determination of the median survival time (ST_{50}) 48 second instar *Spodoptera exigua* larvae were subjected to a LD_{99} dose. From about one day before until one day after the approximate median survival time (ST_{50}), the mortality was scored every three hours. The ST_{50} was determined using a probit analysis plot as described by Finney *et al.* (1971).

RESULTS

Expression of *B.t.* protoxin constructs in *S.f.* 21 cells

The full-length (1155 aa) and a C-terminal truncated (646 aa) form of the *B. thuringiensis* CryIA(b) protoxin were inserted into the p10 locus of *Ac*NPV under the control of the p10 promoter (Fig. 6.2). Both the full-length (*Ac*NPV/JM1) and the truncated protoxin (*Ac*NPV/JM2) were produced in large amounts in *S.f.* 21 cells at the expected size of 130 kDa and 68 kDa, respectively (Fig. 6.3, lanes 4 and 6). In cells infected with both types of recombinants polyhedra were made as expected and in similar amounts as compared to *wild-type Ac*NPV (Fig. 6.3, lanes 1, 4 and 6). P10 protein, which is present in wt *Ac*NPV-

infected cells, is not observed in cells infected with *Ac*NPV/AS3 (p10 negative control), JM1 or JM2 (Fig. 6.3, lanes 1, 2, 4 and 6). In cells infected with the recombinant producing the full-length protoxin (Fig. 6.3, lane 4) a 73 kDa CryIA(b)-specific protein was also observed. This protein is presumably a degradation product of the full-length protoxin. The amount and the size of the CryIA(b) protoxins produced from the p10 promoter were comparable to that from the polyhedrin promoter (chapter 2).

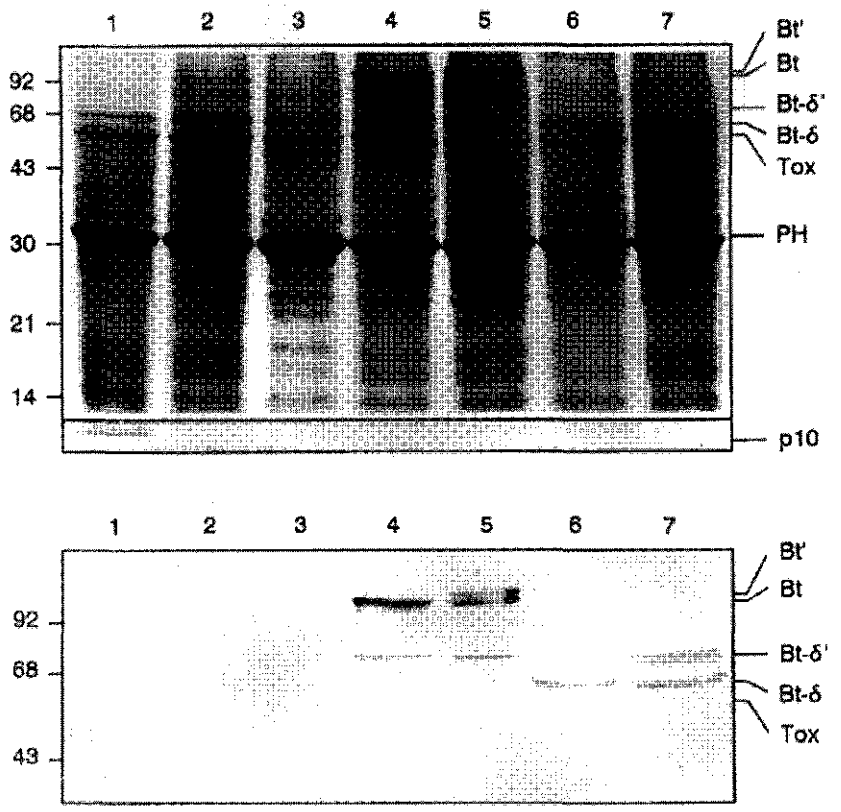

Figure 6.3. SDS/PAGE of 5*10^4 *S. frugiperda* 21 cells infected with wt *Ac*NPV (1), *Ac*NPV/AS3 (2), *Ac*NPV/FW1 (3), *Ac*NPV/JM1 (4), *Ac*NPV/FW2 (5), *Ac*NPV/JM2 (6), *Ac*NPV/FW3 (7) with a multiplicity of infection of 10 $TCID_{50}$ units per cell, and harvested at 48 h p.i. Top. Coomassie brilliant blue-stained gel; Bottom. Immunoblot using polyclonal CryIA(b) antiserum and peroxidase for detection. Bt, Btδ, Bt', Btδ' represent proteins that react with the CryIA(b) antiserum; Tox represents the mature CryIA(b) toxin; PH and p10 represents the polyhedrin and the p10 protein, respectively.

Secretion of *B.t.* protoxins from *S.f.* 21 cells

Processing of *B.t.* protoxins is required to activate their insecticidal activity and hence secretion of the protoxin from infected cells especially into the midgut might be important to achieve enhancement of the biological activity of recombinant viruses producing such a protoxin. Therefore, both protoxin constructs were fused to the secretion signal of the *H. virescens* JHE (Hanzlik *et al.*, 1989). This enzyme is produced in large amounts and efficiently secreted from insect cells both *in vitro* and *in vivo* by baculovirus polyhedrin and p10 expression vectors (Hammock *et al.*, 1990; Roelvink *et al.*, 1992; Bonning *et al.*, 1992). A synthetic linker containing the signal sequence was inserted into the transfer vector pAcAS3 downstream of the p10 promoter, resulting in the transfer vector pAcFW1 (Fig. 6.1). Suitable restriction sites were present to allow in frame fusion with the coding sequence of the two protoxin constructs. Using the transfer vector pAcFW2 containing the full-length and pAcFW3 containing the truncated protoxin gene the constructs were introduced into the genome of *Ac*NPV resulting in *Ac*NPV/FW2 and *Ac*NPV/FW3 (Fig. 6.2).

In cells infected with the respective recombinants *Ac*NPV/FW2 and *Ac*NPV/FW3, CryIA(b)-specific 130 kDa and 68 kDa products were found in similar quantities (Fig. 6.3, lanes 5 and 7) as for the corresponding recombinants *Ac*NPV/JM1 and *Ac*NPV/JM2 lacking a secretion signal (lanes 4 and 6). Additional CryIA(b)-specific products of 140 kDa and 73 kDa were observed in *Ac*NPV/FW2 and *Ac*NPV/FW3 infected cells, respectively.

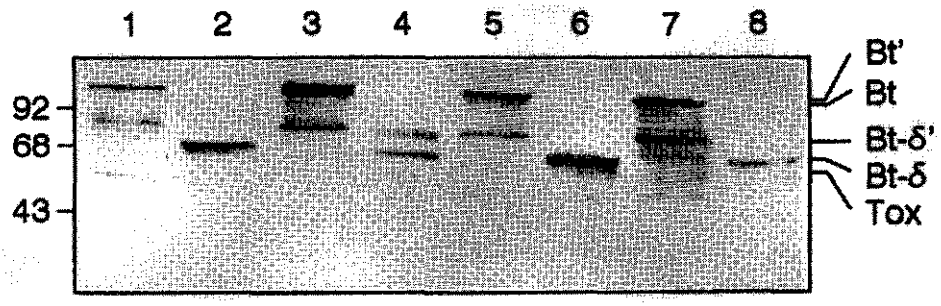

Figure 6.4. Immunoblot of SDS/PAGE of $5*10^4$ *S. frugiperda* 21 cells infected with *Ac*NPV/JM1 (1, 5), *Ac*NPV/JM2 (2, 6), *Ac*NPV/FW2 (3, 7) and *Ac*NPV/FW3 (4, 8) with a multiplicity of 10 $TCID_{50}$ units per cell, and harvested at 48 h p.i.. Samples 5 through 8 were treated with N-glycosidase F. Immunoblotting and legend as described in Fig. 6.3.

To study whether these larger products were the result of N-linked glycosylation, the protein samples of the recombinant *Ac*NPV infected cells were treated with the enzyme N-glycosidaseF which specifically removes N-linked glycosyl groups from proteins. The mobilities of the protoxins in cells infected with recombinants *Ac*NPV/JM1 and *Ac*NPV/JM2 (Fig. 6.4, lanes 5 and 6) was unaltered as compared to mock-treated samples (Fig. 6.4, lanes 1 and 2). However, in the case of *Ac*NPV/FW2 and *Ac*NPV/FW3 the 140 kDa and 73 kDa bands present in mock-treated samples were absent in the N-glycosidaseF-treated samples (compare Fig. 6.4, lanes 3 and 4 with 7 and 8). About 20% of the recombinant CryIA(b) protoxin was glycosylated and thus translocated across the ER membrane as a consequence of the presence of the signal sequence.

Secretion of *B.t.* protoxin into the medium

To test whether the protoxins were secreted from the *S.f.* 21 cells, infections were carried out in serum-free medium with recombinants *Ac*NPV/JM1 and *Ac*NPV/JM2 (without signal) and *Ac*NPV/FW2 and *Ac*NPV/FW3 (with signal). At 48 h p.i., the proteins present in this medium were analyzed by SDS-PAGE followed by immunoblotting (Fig. 6.5, lane 1 to 4). The majority of the ICPs present in the medium from *Ac*NPV/FW2 and *Ac*NPV/FW3-infected cells (Fig. 6.5, lanes 2 and 4) was glycosylated but also some unglycosylated protein was observed. The presence of the unglycosylated protoxins was most likely due to lysed cells, since they were also observed in similar amounts in the medium of *Ac*NPV/JM1 and *Ac*NPV/JM2-infected cells, where the *B.t.* ICPs lack a signal peptide (Fig. 6.5, lane 1 and 3). The amount of glycosylated CryIA(b) protoxin in the medium was at least 50 times lower than inside cells.

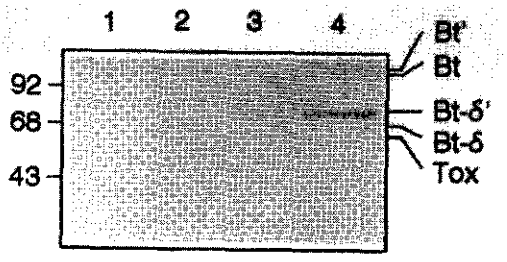

Figure 6.5. Immunoblot of SDS/PAGE of medium in which $5*10^5$ *S. frugiperda* 21 cells infected with *Ac*NPV/JM1 (1), *Ac*NPV/FW2 (2), *Ac*NPV/JM2 (3) and *Ac*NPV/FW3 (4) have grown, harvested 48 h p.i. The medium was concentrated 10 times by TCA precipitation prior to loading. Immunoblotting and legend as described in Fig. 6.3.

Production of the mature CryIA(b) toxin

A second possibility to enhance the insecticidal activity of baculoviruses by *B.t.* ICPs is the production and/or secretion of the mature toxin. For such a toxin biological activity is not dependant on further proteolytic cleavage. For the CryIA(b) protoxin, a mature toxin contains amino acid 29 until 607 (chapter 4). The coding sequence for this toxin was inserted into a p10 transfer vector with (pAcFW1) and without (pAcAS3) a signal sequence, resulting in pAcSG2 and pAcSG4, respectively. In pAcSG2, the C-terminal Ala of the JHE signal sequence was fused to the N-terminal amino acid (Ile29) of the mature toxin. Using these transfer vectors two recombinant baculoviruses, *Ac*NPV/SG2 and *Ac*NPV/SG4, were constructed (Fig. 6.2).

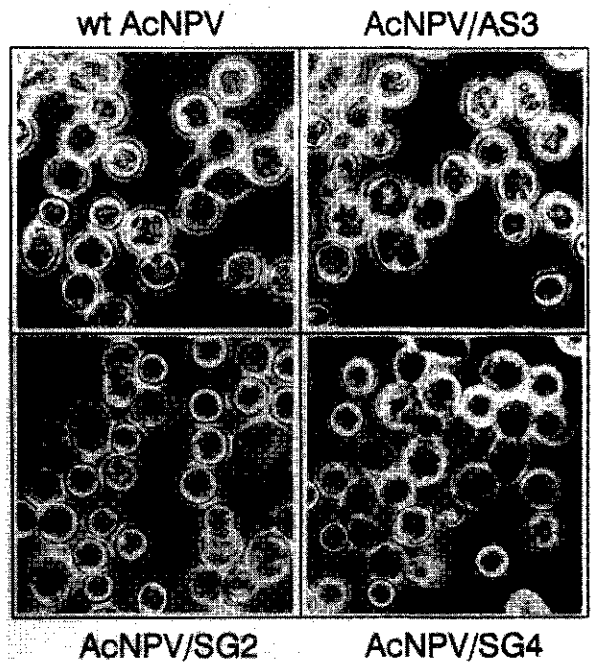

Figure 6.6. Light microscopy of *S. frugiperda* 21 cells infected with *wild-type Ac*NPV, *Ac*NPV/AS3, *Ac*NPV/SG2 and *Ac*NPV/SG4 at 48 h p.i.

The purification of the recombinants *Ac*NPV/SG2 (with signal) and *Ac*NPV/SG4 (without signal) was difficult, which is most likely due to the toxin being intra-cellularly active soon after its production. This resulted in early detachment of cells from the surface,

'swollen' cells late in infection and poor polyhedra formation (Fig. 6.6; compare wt *Ac*NPV and *Ac*NPV/AS3 with *Ac*NPV/SG2 and *Ac*NPV/SG4). In cells infected with these recombinants (Fig. 6.7, lane 4 and 5) the production of both polyhedrin and the recombinant CryIA(b) protein was low. Polyhedrin and *B.t.* toxin were both invisible on a Coomassie brilliant blue-stained gel (Fig. 6.7). However, the presence of CryIA(b) active toxin was observed on immunoblots (Fig. 6.7, lane 4 and 5). No glycosylation and secretion products could be detected in cells and medium after an *Ac*NPV/SG2 infection (not shown). For comparison cells infected with *Ac*NPV/JM1 and *Ac*NPV/JM2 were loaded on the same gel (Fig. 6.7, lane 2 and 3). In these cells the production of polyhedrin is normal and that of the ICPs high.

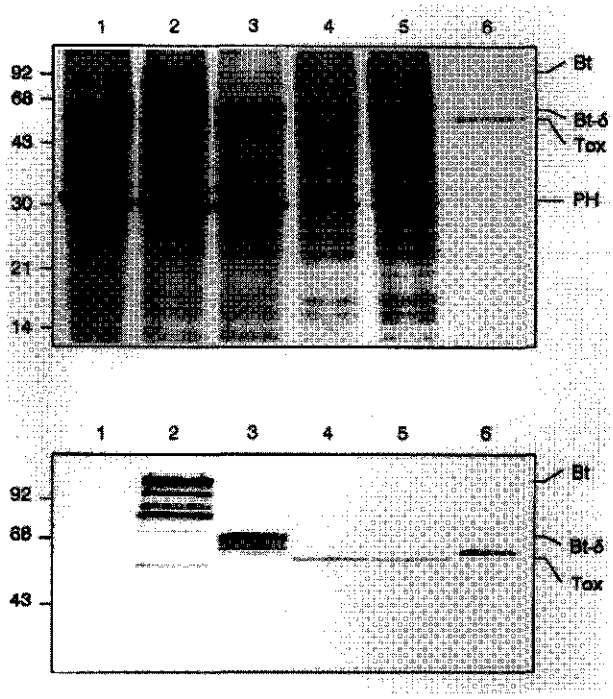

Figure 6.7. SDS/PAGE of 5*10⁴ *S. frugiperda* 21 cells infected with *Ac*NPV/AS3 (1), *Ac*NPV/JM1 (2), *Ac*NPV/JM2 (3) *Ac*NPV/SG4 (4) and *Ac*NPV/SG2 (5) and with a multiplicity of 10 $TCID_{50}$ units per cell, and harvested at 48 h p.i. Lane 6 contains purified "in vitro" released mature CryIA(b) toxin. Top. Coomassie Brilliant blue stained gel; Bottom. Immunoblot using polyclonal CryIA(b) antiserum and peroxidase for detection. Immunoblotting and legend as described in Fig. 6.3.

Biological activity of the different CryIA(b) ICP producing recombinants against insect larvae

The biological activity of the *Ac*NPV recombinants expressing *B.t.* CryIA(b) (pro)toxins constructs was compared with wt *Ac*NPV, the p10-negative recombinant, *Ac*NPV/AS3, and two scorpion toxin gene (*Aa*IT) expressing recombinants. In this assay both the virulence (LD_{50}) and survival time (ST_{50}) for second instar *Spodoptera exigua* larvae was determined. The two scorpion toxin constructs were used for comparison as it was already shown that this toxin is able to enhance the "speed of kill" of *Ac*NPV to *Trichoplusia ni* (McCutchen, *et al.*, 1991). One of these viruses, *Ac*NPV.*Aa*IT, which was used previously, expresses the *Aa*IT gene from a duplicated p10 promoter present in the *Ac*NPV genome in front of the polyhedrin locus (Fig. 6.2). The other recombinant (*Ac*NPV/RH1) expresses the *Aa*IT gene from a p10 promoter in the p10 locus and was constructed using the same p10 parental transfer vector, pAcAS3, which was used for all the cryIA(b) constructs (Fig. 6.2). The recombinants *Ac*NPV/SG2 and *Ac*NPV/SG4, which produce the mature toxin, could not be tested in this assay since they do not produce polyhedra (Fig. 6.6).

The polyhedra used in the bioassay were separated from CryIA(b) protein by a density gradient because the presence of CryIA(b) protein interferes with the bioassay. In the polyhedra suspension derived from the recombinants containing the truncated protoxin, no CryIA(b) protein could be detected on immunoblots, even when large amounts of polyhedra were loaded (data not shown). For the recombinants producing the full-length protoxin we were unable to completely separate the CryIA(b) crystals from polyhedra, since their densities appeared to be almost equal. Therefore, only the recombinant *Ac*NPV/JM2 and *Ac*NPV/FW3 containing the truncated protoxins could be analyzed in bioassay.

The two recombinant viruses, *Ac*NPV/JM2 and *Ac*NPV/FW3, expressing the truncated protoxin constructs did not show any difference in survival time (about 90 hours) and virulence (about 100 polyhedra) as compared to wt *Ac*NPV and *Ac*NPV/AS3. (Table 6.1). The virulence (LD_{50}) of the scorpion toxins-producing recombinants, *Ac*NPV.*Aa*IT and *Ac*NPV/RH1, were also not significantly different from the control viruses (Table 6.1). The survival time of these recombinants, however, was significantly shorter (65 h) than of the other recombinants. There was no significant difference between the two *Aa*IT toxin gene expressing recombinants (Table 6.1).

DISCUSSION

In this paper the potential of the CryIA(b) ICP of *Bacillus thuringiensis* to increase the virulence and the "speed of kill" of the baculovirus *Ac*NPV against insect larvae was investigated. In previous work and in this report it has been shown that biologically active, full-length and truncated protoxins (CryIA(b), CryIA(c), CryIVD) could be produced in high amounts (chapter 2; Merryweather *et al.*, 1990, Ribeiro and Crook, 1993) using the polyhedrin or p10 promoter of *Ac*NPV. In none of these cases, however, the expression of these ICPs constructs resulted in an increase of the virulence (Merryweather *et al.*, 1990; this paper) or in a reduction of the survival time (Ribeiro and Crook, 1993; this paper). Obviously, the (truncated) protoxins produced in all these cases are biologically inactive without proteolytic activation (chapter 2). Therefore, we attempted to secrete these (pro)toxins from insect cells after which activation might occur and we tried to produce the mature toxin to circumvent this activation step.

The signal sequence of the JHE gene was used to translocate the different CryIA(b) protoxins across the ER membrane of infected insect cells. Although at least 20% of the produced CryIA(b) protein was N-linked-glycosylated and, consequently, translocated, almost none of it was secreted (Fig. 6.4 and 6.5). In the medium the glycosylated protoxin is more abundant than the non-glycosylated protoxin (Fig. 6.5). Inside insect cells, however, the opposite is the case (Fig. 6.3 and 6.4) which suggests that the glycosylated protoxins are actively secreted, although to a limited extent. Whether the majority of the CryIA(b) protoxin was retained in the membrane during translocation or in one of the ER or Golgi compartments remains unclear and was not investigated any further.

The majority of the produced CryIA(b) protein was not N-linked glycosylated, which suggests that this part was not translocated at all and remains in the cytoplasm. Secretion and proper glycosylation is often blocked late after infection of baculovirus-infected *S.f.* cells (Jarvis and Summers, 1989) which is possibly due to the shut down of host transcription in the late phase of the baculovirus infection or caused by the exhaustive use of the secretion pathway for virus production. Alternatively, it is possible that the cytoplasmic CryIA(b) protein is produced through initiation of translation at the second AUG, the authentic CryIA(b) translation start codon, of the messenger RNA. This seems unlikely since genes expressed from a baculovirus late promoter usually initiate at the first AUG. (Smith *et al.*,

Table 6.1: Biological activity of the different recombinant viruses against *S. exigua* larvae.

Recombinant	LD_{50}[1]			ST_{50}[2]		
	50	LOWER	UPPER	50	LOWER	UPPER
wt *Ac*NPV	92	61	154	87	86	88
*Ac*NPV/AS3	108	73	163	96	94	99
*Ac*NPV/JM2	71	50	99	90	86	94
*Ac*NPV/FW3	164	95	346	93	91	95
*Ac*NPV.*Aa*IT	66	48	94	63[3]	60	66
*Ac*NPV/RH1	72	51	104	67[3]	65	69

The recombinants were tested on second instar *Spodoptera exigua* larvae (n=48).

[1] The lethal dose is the number of polyhedra ingested to cause 50% mortality.

[2] The survival time is measured in hour post infection.

[3] The survival time of this recombinant is significant shorter than wt *Ac*NPV.

1983b). In addition, the first AUG is in a better context for initiation of translation in eukaryotes (Kozak, 1987). Finally, poor secretion can also be a result of an intrinsic property of the ICP which *in nature* is not a secretory protein.

The efficiency of the *H. virescens* JHE signal sequence *per se* was confirmed by replacing the pseudorabies virus signal sequence of the gX protein in front of a hog cholera E1 gene (Hulst *et al.*, 1993) by the JHE signal. The JHE signal was equally efficient in secreting hog cholera E1 from insect cells (data not shown). A variety of signal sequences from vertebrate and invertebrate origin have been successfully used in baculovirus vectors as signals for secretion (Jarvis *et al.*, 1993), and they function equally well.

Ribeiro and Crook (1993) hypothesized that the production of a mature toxin might result in an effective baculovirus insecticide. However, it was not possible to produce the CryIA(b) mature toxin at high level from the p10 promoter. The occurrence of low virus titres, swollen and detached cells as soon as the p10 promoter becomes active (Roelvink *et al.*, 1992) and the absence of polyhedra suggests that the mature toxin is toxic to insect cells. The cytotoxicity was very similar to what was observed when insects cells were subjected to high concentrations of "in vitro" activated ICPs (Murphy, *et al.*, 1976; Johnson, 1981). When the mature CryIA(b) toxin was produced in *E. coli* a similar cytotoxic effect was observed (chapter 4). Whether specific receptors are involved in these cases remains unclear.

The results presented here as well as in previous reports (Merryweather *et al.*, 1990; chapter 2; Ribeiro and Crook, 1993) now strongly suggest that introduction of *B.t.* ICPs in any form in the genome of a baculovirus is unlikely to result in a recombinant with increased insecticidal activity, since (i) the protoxins produced are inactive and not likely to be activated intracellularly; (ii) the secretion of the *B.t.* protoxins is poor and (iii) production of the mature toxins results in cytotoxicity. Improvement of secretion by removal of the N-linked glycosylation sites present in the CryIA(b) protoxin and subsequent secretion of these protoxin into the midgut might result in a useful recombinant. Alternatively, expression of the mature CryIA(b) toxin from a promoter which is not active in insect cells but is so in larvae might also be effective.

Up till now introduction of mite and scorpion toxins (Tomalski and Miller, 1991, 1992; Stewart, *et al.*, 1991; McCutchen, *et al.*, 1991), a modified JHE (Hammock *et al.*, 1993) or deleting the egt gene from the *Ac*NPV genome have shown to be more promising. In this paper, a recombinant expressing the *Aa*IT toxin gene from the p10 promoter in the p10 locus

was shown to be equally effective (reduction of the survival time by 25 h) as a recombinant expressing the *Aa*IT gene from the p10 promoter located upstream of the polyhedrin gene locus (McCutchen *et al.*, 1991). Hence, the presence of the p10 gene and duplication of the p10 promoter does not influence the effect of these recombinants. The former recombinant has the added advantage that it lacks the p10 gene and hence possibly has some biological containment since nuclear disintegration of cells infected with such a recombinant is hampered (Van Oers *et al.*, 1993).

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CHAPTER SEVEN

SUMMARY AND GENERAL DISCUSSION

I. GENERAL DISCUSSION

Chemical insecticides, which are cheap and act fast, are preferentially used over naturally occurring agents for the control of insect pests both in agriculture and forestry. The occurrence of wide-spread resistance, the damage to the environment and the negative effects on non-target species, generated renewed interest in biological pest control agents. Baculoviruses are most attractive among insect viruses for the control of insect pests, since they are specific for only a few species of predominantly Lepidoptera and Hymenoptera and are harmless for beneficial insects and other organisms including vertebrates (Martignoni, 1984).

Despite these advantages, the commercial use of baculoviruses as insecticides has been hampered for the following reasons: (i) the narrow host range, which makes them inappropriate for broad spectrum use and thus more expensive; (ii) the slow "speed of action", which results in additional crop damage after application (iii) the sensitivity to UV, which results in rapid degradation of infectious virus and (iv) the high dose which is, in some cases, required to incapacitate the target insect. These factors make practical application of baculoviruses expensive if not impossible. For instance, in the Netherlands, baculoviruses (*Spodoptera frugiperda* NPV) were only recently (1993) registered for the control of the beet armyworm. This occurred only because this insect had become resistant against all currently acceptable chemical pesticides. The slow "speed of action" of baculoviruses is the most limiting feature. Suggestions have been made how to improve this negative property by introducing foreign genes in the baculovirus genome (Payne, 1988; Keeley and Hayes, 1987). These proposals mainly focused on baculovirus-driven production of insect-specific toxins (e.g. neurotoxins or *B.t.* toxins) or proteins disturbing the hormonal balance or homeostasis such as hormones (e.g. diuretic, eclosion or prothoracicotrophic hormone) or hormone-modifying enzymes (e.g. juvenile hormone esterase) in infected insects.

In this thesis the potential of the insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis* (*B.t.*) to improve the speed of kill of baculoviruses was investigated. These IPCs were selected because they are (i) highly specific for insects, (ii) very potent and (iii) well-characterized (Höfte and Whiteley, 1989; Gill *et al.*, 1992). The baculovirus *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) was taken as a model virus

because it is the best characterized baculovirus (Ayres *et al.*, 1994) and can be propagated and manipulated *in vitro* (Smith and Summers, 1978; Smith *et al.*, 1983b; Vlak *et al.*, 1990) The CryIA(b) IPC from *B.t. aizawai* was chosen in this study since its host range largely overlaps with that of *Ac*NPV (Höfte *et al.*, 1988; Payne,1986).

II. EXPLOITING INSECTICIDAL CRYSTAL PROTEIN GENES OF *BACILLUS THURINGIENSIS*

ICPs were examined for their potential to improve the insecticidal properties, in particular the "speed of action" of the baculovirus *Ac*NPV. Therefore, it had to be determined first whether sufficient and biologically active IPCs could be produced during a baculovirus infection. The complete cryIA(b) open reading frame (*orf*) was introduced into the genome of *Ac*NPV replacing that of polyhedrin (Chapter 2). In *S.f.* 21 cells infected with this recombinant virus, the cryIA(b) *orf* is thus expressed from the strong late polyhedrin promoter resulting in large amounts of ICP (5% of the total protein) of the correct size (130 kDa) late in the infection (48h pi) (Table 7.1). Most of the expressed protein crystallized into the typical bipyramidal crystals (Bulla *et al.*, 1977) in the cytoplasm of the infected insect cell. These crystals were highly toxic to *P. brassicae* and *H. virescens*, comparable to CryIA(b) proteins produced in *E. coli*, showing that the expressed proteins were fully active. A truncated *orf* coding for the N-terminal 645 amino acids of the protoxin was introduced in a similar way into *Ac*NPV to avoid crystal formation (Chapter 2). This protein, produced in considerable amounts, was also biologically active but, as expected, did not precipitate into crystals, confirming that the C-terminus of the CryIA(b) ICP is required for crystal formation (Höfte and Whiteley, 1989). Both the full-length and the truncated protoxins could be activated *in vitro* into the mature toxin by trypsin and subsequently specifically recognize, at a physiological concentration, putative receptor molecules present on the microvilli of columnar cells using a newly developed histochemical technique (Chapter 3). These results indicate that the *Ac*NPV-expressed ICP is authentic and could in principle carry out its insecticidal action in a baculovirus recombinant.

Additionally literature reports indicated that other functional ICPs (CryIA(b), CryIA(c) and CryIVD) could also be expressed at high level by *Ac*NPV recombinants

from the late polyhedrin or p10 promoter, both in cultured insect cells as well as in larvae (Merryweather, *et al.*, 1990; Pang, *et al.*, 1992, Ribeiro and Crook, 1993; Table 7.1). These results corroborate our observations and show that fully bio-active full-length or truncated protoxins can be produced at high level from either of the two late baculovirus promoters. The expression of these full-length protoxins or truncated protoxins, however, never resulted in improved insecticidal properties of *Ac*NPV (Merryweather, *et al.*, 1990; Ribeiro and Crook, 1993; Chapter 6). Both the effective dose and the median survival time of all these recombinants were similar to that of *wt Ac*NPV (Table 7.1). Protoxins produced are most likely ineffective due to the lack of processing into mature toxins inside insect cells. For the CryIA(c) protoxin, some processing of the protoxin within infected insect cells was reported (Merryweather *et al.*, 1990), but the level was only very low and most likely non-specific.

Trypsin-activated ICPs are toxic when injected in the haemolymph (Lilley *et al.*, 1980; Knowles *et al.*, 1984; Nagamatsu *et al.*, 1984) although to a lesser extent than when ingested orally. Furthermore, toxicity of different ICPs to insect cell lines was reported (Murphy *et al.*, 1976; Johnson, 1981; ibid. 1994; Knowles *et al.*, 1984). This suggests that receptors may also be present on other than midgut cells although receptors present in the midgut have a considerably higher affinity for these toxins or are more abundant. Using this knowledge two approaches may now lead to improved baculovirus insecticides. Either, ICPs could be secreted or a mature toxin could be expressed during a baculovirus infection. (i) In larvae, the expression of protoxins containing a signal peptide might result in the secretion of the ICPs from primary infected midgut and other cells into the haemolymph where activation might occur. The ICPs may also be secreted early in the infection into the midgut lumen since late genes are expressed in columnar cells (Flipsen *et al.*, 1993). In the midgut lumen, the natural target site of these ICPs, proteolytic activation will occur. (ii) Production and subsequent secretion of a mature toxin instead of a protoxin, thereby circumventing the activation process, may result in increased insecticidal properties of *Ac*NPV. Using this approach, receptors facing the haemolymph can effectively bind to ICPs since activation is no longer required. Both approaches were investigated in Chapter 6.

For the introduction of the coding sequences of the mature toxin into the genome of *Ac*NPV first the exact border of the mature toxin had to be determined. Although the

Table 7.1. Insecticidal properties of genetically engineered baculoviruses expressing different ICPs.

Type of gene	Parental Virus	Transfer vector	Promoter	Expression	Activity	ST_{50}[c]	LD_{50}[c]	Ref.
CryIA(c) full-length	*Ac*NPV/(PH)Bt	pAcRP25	PH	++	+	ND	ND	1
CryIA(c) full-length	*Ac*NPV/(PH*)Bt	pAcUW2B	p10	++	+	ND	194%	1
CryIA(c) full-length	*Ac*NPV/Bt73	pAcRP23	PH	++	+	ND	ND	4
CryIA(b) full-length	*Ac*NPV/JM3	pAcDZ1	PH	++	+	ND	ND	2
CryIA(b) a.a. 1 to 646	*Ac*NPV/JM4	pAcDZ1	PH	++	+	ND	ND	2
CryIA(b) full-length	*Ac*NPV/JM1	pAcAS3	p10	++	+	ND	ND	3
CryIA(b) full-length[a]	*Ac*NPV/FW2	pAcAS3	p10	++	+	ND	ND	3
CryIA(b) a.a. 1 to 646	*Ac*NPV/JM2	pAcAS3	p10	++	+	77%	103%	3
CryIA(b) a.a. 1 to 646[a]	*Ac*NPV/FW3	pAcAS3	p10	++	+	178%	107%	3
CryIA(b) a.a. 29 to 607	*Ac*NPV/SG4	pAcAS3	p10	+/-	ND[d]	ND	ND	3
CryIA(b) a.a. 29 to 607[a]	*Ac*NPV/SG2	pAcAS3	p10	+/-	ND[d]	ND	ND	3
CryIA(b) full-length	*Ac*NPV/Btm	pAcRP23	PH	++	+	102%	ND	1
CryIA(b) a.a. 29 to 1155	*Ac*NPV/Bt5	pAcRP23	PH	++	+	98%	ND	1
CryIA(b) a.a. 1 to 730	*Ac*NPV/Bt3	pAcRP23	PH	+	+	99%	ND	1
CryIA(b) a.a. 29 to 730	*Ac*NPV/Bt5/3	pAcRP23	PH	+	+	99%	ND	1
CryIVD[b]	*Ac*NPV/CryIVD	pAcP1	PH	++	+	ND	ND	5
CryIVD[b] a.a. 90 to 643	*Ac*NPV/CryIVD	pAcP1	PH	++	+	ND	ND	5

1 = Merryweather *et al.*, 1991; 2 = chapter 2; 3 = chapter 6; 4 = Ribiero and Crook, 1993; 5 = Pang *et al.*, 1992.
[a] gene preceded by a signal peptide of JHE; [b] gene fused to the polyhedrin gene; [c] Data are presented as suggested by Bonning and Hammock, 1993;
[d] only cytotoxicity to *S.f.* cells observed.

activation process of several ICPs has been studied in detail (Schnepf and Whiteley, 1985; Adang *et al.*, 1985; Höfte *et al.*, 1986; Sanchis *et al.*, 1989; Widner and Whiteley, 1989; Yoshida *et al.*, 1993; Ward and Ellar, 1988) the proteolytic activation at the C-terminus was not completely unravelled. In nature activation of protoxins occurs in the midgut presumably by trypsin-like proteases. One of these enzymes has recently been purified from midgut juice of *Choristoneura fumiferana* (Milne and Kaplan, 1993). The N-terminus of the mature CryIA(b) toxin was determined by amino acid sequencing (Nagamatsu *et al.*, 1984). The N-terminal residue acid Ile29 was located at a potential trypsin-cleavage site (Arg28). At the C-terminus, however, the results were inconclusive. A couple of putative cleavage sites are present: Arg601, Arg619 and Lys621. Arg601 is present as part of an amino acid sequence (IDRIEF) which is highly conserved among ICPs and may represent an actual cleavage site (Höfte *et al.*, 1986; Nakamura *et al.*, 1992). Alternatively, this conserved sequence may be required for the stability of the C-terminus of the mature toxin (Höfte and Whiteley, 1989).

In Chapter 4 it is shown that C-terminal truncated ICPs (e.g. 1-607) in which the conserved IDRIEF sequence was maintained, are protected against trypsin treatment and are still toxic, whereas further truncated derivatives (e.g. 1-601) were not. Thus amino acids beyond Ala607 are not required for toxicity. Proteolytic activation *in vivo* most likely occurs at one of the first available proteolytic cleavage sites C-terminal of Ala607: at Arg619 or Lys621 (Chapter 4; Nakamura *et al.*, 1992). CryIA(b) sequences stretching from Ile29 to Ala607 are, therefore, necessary and sufficient for toxicity. When this peptide was expressed in *E. coli*, it severely inhibited growth of these bacteria suggesting that the mature toxin was cytotoxic to *E. coli* (Chapter 4). This toxicity was most likely not receptor-mediated since a truncated toxin (a.a. 29-429) which lacks the receptor domain, but does contain the 7 α-helices, which are presumed to be involved in pore formation (Li *et al.*, 1991), is also toxic to *E. coli*. These results further indicate that the first 28 amino acids block cytotoxicity in *E. coli*, since a truncated protoxin coding for a.a. residues 1 to 607 is not cytotoxic.

Recombinant baculovirus insecticides should preferably contain an intact polyhedrin gene to facilitate natural infection of larvae and to assay them properly (Entwistle and Evans, 1985; Bishop, 1989). The p10 protein is not essential for a baculovirus infection (Vlak *et al.*, 1988) and the strength of its promoter is comparable to that of the

polyhedrin promoter (Roelvink *et al.*, 1992). Therefore, the p10 locus was chosen for the insertion of ICP gene constructs downstream of the p10 promoter. To facilitate the retrieval of recombinant viruses in the p10 locus, a novel baculovirus vector was developed (Chapter 5). The DNA of this virus could be linearized in the p10 locus by the introduction of a unique restriction site. In a co-transfection with a transfer vector, linearization of viral DNA significantly reduced the number of parental virus, thereby increasing the percentage of recombinants from 1 to 25% and hence reducing the time-consuming plaque-purification to only one round (one week).

For secretion of baculovirus-expressed ICPs from insect cells a secretion signal is required. To achieve this, a baculovirus transfer vector was developed which contains a signal sequence downstream of the p10 promoter. The signal sequence of the juvenile hormone esterase (JHE) gene was chosen because this enzyme itself is efficiently secreted from insect cells, both *in nature* and when expressed via baculovirus vectors (Hammock, *et al.*, 1990). The potential of this secretion signal was demonstrated by using a reporter protein, the hog cholera E1-glycoprotein (Hulst *et al.*, 1993)(Chapter 6).

Finally to investigate the possibilities of ICP to improve baculovirus insecticides a series of different recombinant viruses was produced each expressing a different ICP construct from the p10 promoter: one containing the full-length CryIA(b) protoxin (a.a. 1-1155), another containing a truncated protoxin (a.a. 1-645) and the third containing a mature toxin (a.a. 29-607; based on data of Chapter 4). Three complementary recombinants additionally contained a signal sequence in front of the ICP coding regions. All protoxin constructs were produced in amounts (Chapter 6) comparable to the amounts produced from the polyhedrin promoter. The level of secretion of the CryIA(b) full-length and the truncated protoxins from insect cells however was low (less than 2% of the expressed protein). This could be attributed to intrinsic properties of the CryIA(b) protein since other proteins are efficiently secreted using this signal (chapter 6). Furthermore, secretion is often less efficient in insect cells late after infection (Jarvis and Summers, 1989). Since only 30% of the expressed *B.t.* protein is glycosylated, the poor secretion is presumably the result of a combination of both. Using a similar approach for a truncated CryIA(c) protoxin preceded by the gp67 signal sequence, comparable results were obtained (Merryweather *et al.*, 1991). In nature, ICPs are not secreted and its structure may be inappropriate for this. For instance, two highly hydrophobic areas (a.a.

29 to 80 and 137 to 172) are present near the N-terminus of all ICPs which may serve as a stop transfer signal, retaining the protein in the membrane. Not surprisingly, *Ac*NPV recombinants expressing these protoxins did not have improved insecticidal properties. Since early expression during a baculovirus infection may improve translocation and glycosylation (Jarvis and Summers, 1989), such an approach may result in a higher percentage of glycosylated ICPs but whether this will result in more secreted protein is unlikely.

When the mature toxin was expressed under control of the p10 promoter cytopathic effects were observed as soon as the p10 promoter was transcribed (Chapter 6). This suggests that the mature toxin is also cytotoxic to insect cells in a similar manner as observed in *E. coli* (Chapter 4) which indicates that the mature toxin is able to make pores from the cytosolic side of *S.f.* cells. This cytotoxicity is most likely receptor independent, although receptor sites might also be present in the interior of the cytoplasmic membranes of *S.f.* cells. Due to the cytotoxicity of the mature toxin, these recombinants appearently were unable to produce polyhedra and could thus not be tested for their biological properties *in vivo* in insects. Expression of the mature toxin from a promoter which can be down-regulated *in vitro* may result in a recombinant virus that does produce polyhedra. These polyhedra can then be tested on insects to determine if the expression of the mature toxin results in an baculovirus with improved insecticidal properties.

Thus far, attemps to use ICPs of the bacterium *B. thuringiensis* to improve the insecticidal properties of *Ac*NPV have been unsuccessful (Merryweather, *et al.*, 1990; Pang, *et al.*, 1992, Ribeiro and Crook, 1993; this thesis). When the ICPs are produced as protoxins they are abundantly produced, but unable to operate since activation does not occur inside an infected insect cell. For the activation of the crystals both solubilization in an alkaline environment and digestion by proteolytic enzymes are apparently required. It is unlikely to fulfill these two criteria in infected insect cell unless active ICPs can be produced or the ICPs can be secreted. However, it was impossible to secrete ICPs in significant amounts from infected insect cells. The expression of the mature toxins in insect cells resulted in a cytotoxic effect which severely interfered with the replication of the virus itself. Only a recombinant expressing a mature toxin from a promoter which is

inactive in insect cells but highly expressed in larvae, may result in an interesting recombinant.

In this thesis the research was focussed on improving the "speed of action" of the baculovirus *Ac*NPV exploiting the insecticidal crystal proteins of *Bacillus thuringiensis*. However, as mentioned in the introduction, other insect-specific toxin, hormones or enzymes can be exploited to accelerate the insecticidal effect of baculoviruses. The current status will be shortly reviewed in the next few paragraphs.

III. EXPRESSION OF OTHER INSECTICIDAL TOXIN GENES

Besides the insecticidal toxins produced by *B.t.*, many other insect-specific toxins are known (Zlotkin, 1985). For instance, several groups of predatory arthropods produce neurotoxins to paralyse their prey. These neurotoxins interfere with synaptic transmission in the central nervous system or muscles of insects. As suggested by Zlotkin (1985) insect viruses are perfectly suited to deliver these neurotoxin into the haemolymph of the larva where the toxin can perform its paralytic action. During the course of the study presented in this thesis recombinant viruses were described that express very potent neurotoxins from either the scorpion *Androctonus australis* (*Aa*IT)(Maeda, *et al.*, 1991; Stewart, *et al.*, 1991; McCutchen, *et al.*, 1991) or the mite *Pyemotes tritici* (*Txp*-I)(Tomalski and Miller, 1991; ibid., 1992). These toxins are effectively secreted during *Ac*NPV infection into the haemolymph of infected insects. Recombinants expressing these toxins considerably reduced feeding of infected larvae as compared to *wild-type Ac*NPV (Table 7.2). In an initial report (Carbonell *et al*, 1988), a recombinant *Ac*NPV expressing a similar insect-specific toxin from the scorpion *Buthus eupeus* (BeIt) had no effect at all (Table 7.2). In this case the recombinant toxin may have been unstable, but it might also be possible that not the correct peptide was expressed. In conclusion, these results show that insect-specific neurotoxins can be exploited to improve the insecticidal properties of baculoviruses.

Table 7.2. Overview of genetically engineered baculoviruses.

Type of gene	Recombinant virus	Transfer vector	Promoter	Expression	Activity	ST_{50}[i]	LD_{50}[i]	Larvae assayed	Ref.
DH (hormone)	*Bm*NPV/DH5	pBE284	PH	++	+	86%	ND	*B.mori*	1
BeIT (scorpion toxin)	*Ac*NPV/*Be*IT-1	pEV5	PH	+/-	-	ND	ND		2
BeIT[a]	*Ac*NPV/*Be*IT-2	pEV5	PH	+	-	ND	ND		2
BeIT[a]	*Ac*NPV/*Be*IT-3	pGD-B6874/SalI	PH	++	-	ND	ND		2
JHE	*Ac*NPV/RP23.JHE	pAcRP23	PH	++	+	ND[f]	ND	*T.ni*	3
JHE	*Ac*NPV/UW2B.JHE	pAcUW2B	p10	++	+	102%[g]	463%	*T.ni*	4
JHE (modified)	*Ac*NPV/UW2B.JHE[e]	pAcUW2B	p10	+	+	±75%	ND	*T.ni*	17
*Aa*IT (scorpion toxin)[b]	*Ac*NPV/ST-3	pAcUW2B	p10	+	+	76%[h]	70%	*T.ni*	5
*Aa*IT[c]	*Ac*NPV/UW2B.*Aa*IT	pAcUW2B	p10	+	+	62%[h]	60%	*H.virescens*	6
*Aa*IT[c]	*Ac*NPV/UW2B.*Aa*IT	pAcUW2B	p10	+	+	73%[h]	72%	*S.exigua*	7
*Aa*IT[c]	*Ac*NPV/RH1	pAcAS3	p10	+	+	77%[h]	78%	*S.exigua*	7
*Aa*IT[c]	*Bm*NPV/*Aa*IT	pBK283	PH	+	+	74%[h]	ND	*B.mori*	8
Txp-1 (mite toxin)	*Ac*NPV/EV-Tox34	pEVmXIV	PH(XIV)	+	+	±70%	ND	*T.ni*	9+18
Txp-1	*Ac*NPV/ETL-Tox34	phc-ET-wt	ETL	+	+	±80%	ND	*T.ni*	18
Txp-1	*Ac*NPV/Cappolh-Tox34	pCappolh	Cap+PH	++	+	±60%	ND	*T.ni*	18
Txp-1	*Ac*NPV/Sp-Tox34	pSynXIVVI+X3	Syn(late)+PH	++	+	59%	159%	*T.ni*	18
PTTH[d] (hormone)	*Ac*NPV/PTTHM	pSynXIVVI+X3	Syn(late)+PH			±100%	235%	*S.frugiperda*	10

Table 7.2. continued.

Type of gene	Recombinant virus	Transfer vector	Promoter	Expression	Activity	ST_{50}[i]	LD_{50}[i]	Larvae assayed	Ref.
egt deletion	*Ac*NPV/EGTDEL	pEGTDEL				78%	168%	*S.frugiperda*	11
egt deletion	*Ac*NPV/EGTDEL	pEGTDEL				80%	102%	*T.ni*	12
egt deletion + JHE	*Ac*NPV/JHEEGTD	pSynXIVVI+X3	Late+PH	++	+	76%	107%	*T.ni*	12
egt deletion + *Aa*IT[c]	*Ac*NPV/SO1	pAcAS3	p10	+	+	±70%	100%	*S. exigua*	13
egt deletion + PTTH[d]	*Ac*NPV/EGT-PTTHM	pSynXIVVI+X3	Syn(late)+PH	+	+	±75%	16470%	*S.frugiperda*	10
EH (hormone)	*Ac*NPV/EHDA26Z	pEVmXIV	PH(XIV)	+	+	ND	ND		14
EH	*Ac*NPV/EHEGTZ	pEVmXIV	PH(XIV)	+	+	ND	ND		14
HA5 (hornet allergen)	*Ac*NPV/HA5f17	pEV55	PH	+	+	±95%	ND	*T.ni*	15
URF13	*Ac*NPV/13T	pAcYM1	PH	+	+	±60%	ND	*T.ni*	16

1 = Maeda *et al.*, 1989a; 2 = Carbonell *et al.*, 1988; 3 = Hammock *et al.*, 1990; 4 = Bonning *et al.*, 1992; 5 = Stewart *et al.*, 1991; 6 = McCutchen *et al.*, 1991; 7 = Chapter 6; 8 = Maeda *et al.*, 1991; 9 = Tomalski and Miller, 1991; 10 = O'Reilly *et al.*, submitted; 11 = O'Reilly and Miller, 1991; 12 = Eldridge *et al.*, 1992; 13 = Maus and Martens, unpublished; 14 = Eldridge *et al.*, 1991; 15 Tomalski *et al.*, 1993; 16 = Korth and Levings, 1993; 17 = Hammock *et al.*, 1993; 18 = Tomalski and Miller, 1992; DH = diuretic hormone of *Maduca sexta*; *Be*IT = *Buthus eupeus* scorpion toxin; JHE = Juvenile hormone esterase of *Heliothis virescens*; *Aa*IT = *Androctonus australis* scorpion toxin; *Txp*-1 = *Pyemotes tritici* mite toxin (Tox35); PTTH = *Bombix mori* prothoracicotropic hormone; *egt* = ecdysteroid UDP-glucosyl transferase; EH = Eclosion hormone of *Manduca sexta*; HA5 = *Dolichovespula maculata* hornet antigen 5; Maize mitochondrial protein URF13. [a] gene preceded by a signal peptide of human interferon α, [b] gp67, [c] the neuropeptide bombixin or [d] the sarcotoxin 1A of *S. peregrina*; [e] gene fused to the polyhedrin gene; [f] reduction of feeding witnessed; [g] no reduction of feeding observed; [h] paralysis occurred 10 hours before death; [i] Data are presented as suggested by Bonning and Hammock, 1993.

IV. EXPRESSION OF GENES THAT INTERFERE WITH LARVAL METABOLISM

Most activities of the larvae affecting homeostasis or development, such as water balance, feeding behavior, molting etc., are regulated by hormones. Over-expression of these hormones in larvae might disturb the balance, causing early feeding arrest. Thus far, only few hormones or enzymes tuning hormone levels have been exploited. A recombinant *Bombyx mori* NPV (*Bm*NPV) over-expressing the diuretic hormone (which regulates the water balance), reduced the survival time (LT_{50}) of infected larvae by 20% (Maeda, 1989a; Table 7.2). This was the first evidence showing that over expression of insect hormones had the potential of improving baculovirus insecticidal properties.

Another study involved the baculovirus-driven expression of the juvenile hormone esterase (JHE). This enzyme regulates the level of juvenile hormone (JH) in the haemolymph and is normally produced prior to a molt to decrease the levels of JH in the haemolymph. A decrease in JH titer in the haemolymph and an increase of ecdysone levels are the signs for the larvae to start molting (Koolman, 1990). Molting is usually accompanied by a feeding arrest of the larvae until the end of the molt. Although, the JHE enzyme was highly expressed both in insect cells and in larvae, the application of this recombinant did not result in an early feeding arrest of infected larvae (Bonning *et al*, 1992; Table 7.2).

The genome of *Ac*NPV contains an enzyme, the ecdysteroid UDP glucosyl transferase (*egt*) (O'Reilly and Miller, 1989), which prevents larvae from molting by inactivating ecdysone. This enzyme, therefore, extends the larval stages resulting in a higher yield of progeny virus and presumably interferes with the action of enzymes such as JHE. Deletion of the *egt* gene from the *Ac*NPV genome indeed led to shorter (normal) larval stages and resulted, surprisingly, in reduced median survival time (30%) of infected larvae (O'Reilly & Miller, 1991; Table 7.2). The mechanism, however, remains unclear.

The inactivation of ecdysone masks the possible action of JHE, since ecdysone is required to initiate a molt. Overexpressing JHE in an *egt* minus virus may, therefore, result in a premature feeding arrest. This, however, appeared not to be the case (Eldridge *et al*, 1992; Table 7.2). The authors suggest that the action of JHE is counteracted by the production of new JH, which is carefully controlled by releasing hormones, allatostatin

and allatotropin, and by the rapid removal of JHE enzyme from the haemolymph by pericardial cells (Booth *et al.*,1994) to stabilize the JH concentration there. It seems as if the effect of proteins that interfere with homeostasis, such as JH and DH, is automatically balanced by the action of feed-back mechanisms that restore homeostasis. So, when these type of genes are introduced they need to be modified to circumvent the natural quest for homeostasis as demonstrated by Hammock *et al.* (1993) for JHE. This modified JHE gene, made more stable by removing the ubiquitination sites in the enzyme which are required for efficient removal of the enzyme from the haemolymph, was introduced into *Ac*NPV. When this recombinant virus was tested on larvae the survival time of these larvae was significantly reduced (Table 7.2). Expression of this modified JHE gene in a virus lacking the *egt* gene may result in a more effective virus.

Recently, a recombinant virus was produced which expresses the prothoracicotropic hormone (PTTH), another hormone involved in the onset of molting (O'Reilly *et al.*, 1994, submitted). In nature, PTTH is produced by neuro-secretory cells in the brain of the larva where it stimulates the prothoracic gland to secrete ecdysone into the haemolymph. In the fat body and the Malpighian tubules, ecdysone is subsequently metabolized to 20-hydroxyecdysone, the bio-active form. 20-Hydroxyecdysone recognizes receptors which are present in the nucleus of target cells. Receptor recognition results in the expression of genes regulating the molt (for review Palli *et al.*, 1994). Expression of the *Bombyx mori* PTTH in the genome *Ac*NPV and in an *Ac*NPV *egt* deletion mutant did not result in improved insecticidal properties (Table 7.2). However, it remains to be determined whether the *Bombyx mori* PTTH is bio-active in *S. frugiperda* larvae which were used in this experiment. Surprisingly, the dose required to kill *S. frugiperda* larvae was increased about a hundred times for the PTTH-expressing *egt* minus recombinant only (Table 7.2). This suggests that the expressed PTTH is indeed bio-active and secondly that ecdysone severely affects a baculovirus infection. In conclusion, genes which interfere with the larval metabolism have proven successful for the engineering of baculovirus insecticides. Other hormones, such as allatostatin which down-regulates JH expression or the eclosion hormone which initiates the shedding of the cuticula, may appear fruitful in the near future. Alternatively, the expression of other JH modifying enzymes (e.g. JH epoxide hydrolase) may also prove successful.

V. OTHER FACTORS THAT MIGHT IMPROVE THE "SPEED OF ACTION"

Besides the type of foreign gene introduced, three other factors influence the effect of the introduced genes. (i) the level of expression. This should be high enough to produce a dose which is effective. The level of expression of a certain protein is determined by the strength of the promoter, the stability and the translatability of the messenger RNA (e.g. non-translated leader, Kozak rules, codon usage and removal sequences that de-stabilize mRNA, poly-adenylation signals) and the stability of the protein. For baculovirus proteins expressed from a very-late promoter it has been shown that an intact 5' non-translated leader is a prerequisite for high level of expression (Possee and Howard, 1987; Rankin *et al.*, 1988). In transgenic plants expressing ICPs of *Bacillus thuringiensis* it has been shown that removal of poly-adenylation signals and sequences involved in degradation of mRNA stabilize the messenger and increase the production of the foreign gene about 100 times (Perlak *et al.*, 1990; Honée, 1992). (ii) the timing of expression. The foreign protein needs to be produced as soon as possible after application of the virus, preferably already in the midgut. This makes the choice of a suitable promoter very important. Thus far, most of the introduced genes have been expressed from the strong late polyhedrin or p10 promoters resulting in high levels of expression but late after the primary infection. In larvae, it still takes about 60 h before the foreign protein is detected into the haemolymph when expressed from the late p10 promoter (Bonning *et al*, 1992). Using strong host (midgut) promoters, the *Drosophila melanogaster* heat shock promoter (hsp), baculovirus early (*etl*) or late (p6.9; CAP) promoters alone or chimaeric one's (CAP-PH; SYN-PH) might result in an earlier response of the insect to a baculovirus infection (Zuidema *et al.*, 1990; Morris and Miller, 1992; Thiem and Miller, 1990; Hill-Perkins and Possee, 1990; Wang *et al.*, 1991). The disadvantage of exploiting some of the early or late promoter elements is their relatively low transcriptional activity as compared to the very late promoters (Bonning *et al.*, 1994), but for very potent proteins this approach might be considered. Until now only for the expression of mite toxin, *Txp*-I, different kinds of promoters were tested and compared (Tomalski & Miller, 1992). Unfortunately, these promoters were not compared in polyhedrin-positive viruses on larvae. Caution is required when host, heat-shock or early baculovirus promoters are used, as they are also recognized in non-host insects

which may be undesirable in terms of biosafety (Morris and Miller, 1992). (iii) Intrinsic properties of the expressed protein. The amino acid sequence of the foreign protein can be modified, e.g. to obtain more potent, secretable or more suitable proteins as has been shown for the JHE (Hammock *et al.*, 1993). Also for *Aa*IT, which seems very instable, improvement might be achieved by stabilizing the protein itself.

VI. CONCLUDING REMARKS AND FUTURE PROSPECTS

Improvement of baculovirus insecticides via the introduction of *B.t.* ICPs was investigated. Although the expression of the ICPs in general was high, it was impossible to target these to the haemolymph or the midgut lumen, the target sites for these toxins. Exploiting the mature toxin still leaves a few options to improve baculoviruses using ICPs but this remains to be determined. Tailoring for appropriate delivery as has been suggested in the second paragraph of the discussion may need some further investigation. The specificity of *B.t.* toxins for only target insects remains an attractive property of these proteins for improvement of baculovirus insecticides.

At least four genes (*egt* deletion, JHE, *Aa*IT and *Txp*-I) increased the speed of kill of baculoviruses significantly. The median survival time of larvae with the most potent recombinant virus was reduced by 30% compared to the *wt Ac*NPV. More importantly, the damage caused to plants was reduced by 45%. Besides these four successfull approaches, the potency of other genes, such as poneratoxin, philanthotoxin, bradykinins, JH epoxide hydrolase, eclosion hormone or allatostatin can be investigated. And, as has been suggested in a previous paragraph, the effect of recombinants expressing insect specific hormones, toxins or enzymes may be further optimized by promoter choice and stabilizing the messenger or the protein, potentially resulting in more suitable recombinants. Besides improvement of the insecticidal properties of baculoviruses, the expression of toxins, hormones and enzymes may also unravel the mode of action of these important protein *in vivo*. Especially for JHE, PTTH, EH, allatostatin, allatotropin and DH, baculoviruses may prove valuable in the near future.

Thus far, most engineering efforts have been focussed on the baculovirus *Ac*NPV, but in the near future other baculoviruses, e.g. *Spodoptera exigua* NPV, *Cydia pomonella* Granulosis Virus, *Orgyia pseudotsugata* NPV, *Choristoneura fumiferana* NPV,

Lymamtria dispar NPV and *Heliothus zea* NPV having different host-ranges will be exploited. For a number of these viruses no cell culture systems are yet available making genetic engineering of these viruses difficult. Recombinant of these viruses may be produced in yeast as described by (Patel *et al.*, 1990) or by *in vivo* recombination in larvae (Croizier *et al.*, 1994).

Despite the positive outlook of some of the presently available engineered baculovirus recombinants, definite proof of their usefullness should come from the application of these viruses in the field. Recently, the first field trials were performed with a recombinant virus, *Ac*NPV/ST-3 (See table 7.2), expressing the scorpion toxin *Aa*IT (Cory *et al.*, 1994). Under field conditions it was shown that this recombinant virus reduced damage by 25% compared to *wild-type* virus demonstrating that engineered baculoviruses can be superior to the *wild-type* under field conditions. Important prerequisite for the use of engineered baculoviruses for pest control is a thorough investigation of the risks associated with the release of these recombinant viruses in the environment. Finally, care must be undertaken when applying these improved insecticides since inappropriate use of both *wild-type* and engineered baculoviruses may result in the development of resistance.

REFERENCES

Adams, J.R., R.H. Goodwin and Th.A. Wilcox. 1977. Electron micriscopic investigation on invasion and replication of insect baculoviruses *in vivo* and *in vitro*. Biol. Cellulaire **28**:261-268.

Adang, M.J., M.J. Staver, T.A. Rocheleau, J. Leighton, R.F. Barker and D.V. Thompson. 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. Gene **36**:289-300.

Aronson, A.I., E.S. Han, W. Mcgaughey and D. Johnson. 1991. The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects. Appl. Environm. Microbiol. **57**:981-986.

Ayres, M.D., S.C. Howard, J. Kuzio, M. Lopez-Ferber and R.D. Possee. 1994. The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology **202**:586-605.

Barjac, de H. and E. Frachon. 1990. Classification of *Bacillus thuringiensis* strains. Entomophaga **35**:233-240.

Becker, D. and D. Knebel-Mörsdorf. 1993. Sequence and temporal appearance of the early transcribed baculovirus gene HE65. J. Virol. **67**:5867-5872.

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. **7**:1513-1523.

Bishop, D.H.L. 1989. Genetic engineered viral insecticides. A progress report. 1986-1989. Pestic. Sci. **27**:173-189.

Blissard, G.W. and G.F. Rohrmann. 1990. Baculovirus diversity and molecular biology. Ann. Rev. Entomol. **35**:127-155.

Blissard, G.W. and J.R. Wenz. 1992. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. J. Virol. **66**:6829-6835.

Bohmfalk, G.T. 1986. Practical factors influencing the utilization of baculoviruses as pesticides. *In* The biology of baculoviruses. Vol. II. Granados, R.R. and B.A. Federici, Eds., CRC press, Boca Raton, Fl., p.223.

Bonning, B.C. and B.D. Hammock. 1993. Lethal ratios: an optimized strategy for presentation of bioassay data generated from genetically engineered baculoviruses. J. Invertebr. Pathol. **62**:196-197.

Bonning, B.C., M. Hirst, R.D. Possee and B.D. Hammock. 1992. Further development of a recombinant baculovirus insecticide expressing the enzyme juvenile hormone esterase from *Heliothis virescens*. Insect Biochem. Molec. Biol. **22**:453-458.

Bonning, B.C., P.W. Roelvink, J.M. Vlak, R.D. Possee and B.D. Hammock. 1994. Superior expression of juvenile hormone esterase and beta-galactosidase from the basic protein of *Autographa californica* nuclear polyhedrosis virus compared to the p10 protein and polyhedrin promoters. J. Gen. Virol. **75**:1551-1556.

Booth, T.F., B.C. Bonning and B.D. Hammock. 1992. Localization of juvenile hormone esterase during development in normal and in recombinant baculovirus infected *Trichoplusia ni* Larvae. Tissue Cell **24**:267-273.

Bravo, A., K. Hendrickx, S. Jansens and M. Peferoen. 1992a. Immunocytochemical analysis of specific binding of *Bacillus thuringiensis* insecticidal crystal proteins to Lepidopteran and Coleopteran midgut membranes. J. Invertebr. Pathol. **60**:247-253.

Bravo, A., S. Jansens and M. Peferoen. 1992b. Immunocytochemical localization of *Bacillus thuringiensis* insecticidal crystal proteins in intoxicated insects. J. Invertebr. Pathol. **60**:237-246.

Briese, D.T. 1986. Insect resistance to baculoviruses. *In* The biology of baculoviruses. Vol. II. Granados, R.R. and B.A. Federici, Eds., CRC press, Boca Raton, Fl., p.223.

Bulla, L.A. Jr., K.J. Kramer and L.I. Davidson. 1977. Characterization of the entomocidal parasporal crystal of *Bacillus thuringiensis*. J. Bacteriol. **130**:375-383.

Caballero, P., D. Zuidema, C. Santiago-Alvarez and J.M. Vlak. 1992. Biochemical and biological characterization of four isolates of *Spodoptera exigua* nuclear polyhedrosis virus. Biocontr. Science and Technol. **2**:145-157.

Carbonell, L.F., M.R. Hodge, M.D. Tomalski and L.K. Miller. 1988. Synthesis of a gene coding for an insect-specific scorpion neurotoxin and attemps to express it using baculovirus vectors. Gene **73**:409-418.

Carroll, J., J. Li and D.J. Ellar. 1989. Proteolytic processing of a coleopteran-specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. Biochem. J. **261**:99-105.

Carson, D.D., L.A. Guarino and M.D. Summers. 1988. Functional mapping of an *Ac*NPV immediately early gene which augments the IE-1 trans-activated 39K gene. Virology **162**:444-451.

Carson R. 1965. Silent spring. Harmondsworth, Penguin, 317pp.

Charlton, C.A. and L.E. Volkman. 1993. Penetration of *Autographa californica* nuclear polyhedrosis virus nucleocapsids into IPLB Sf 21 cells induces actin cable formation. Virology **197**:245-254

Clem, R.J., M. Flechheimer and L.K. Miller. 1991. Characterization of apoptosis by a baculovirus gene during infection of insect cells. Science **254**:1388-1390.

Cory, J.S., M.L. Hirst, T. Williams, R.S. Hails, D. Goulson, B.M. Green, T.M. Carthy, R.D. Possee, P.J. Cayley and D.H. Bishop. 1994. Field trial of a genetically improved baculovirus insecticide. Nature **370**:138-140.

Crawford, A.M. and L.K. Miller. 1988. Characterization of an early gene accelerating expression of late genes of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J. Virol. **62**:2773-2781.

Croizier, G., L. Croizier, O. Argaud and D. Poudevigne. 1994. Extension of the *Autographa californica* nuclear polyhedrosis virus host range by interspecific replacement of a short DNA sequence in the p143 helicase gene. Proc. Natl. Acad. Sci. USA **91**:48-52.

Cunningham J.C. 1988. Baculoviruses - their status compared to *Bacillus thuringiensis* as microbial insecticides. Outlook on Agriculture **17**:10-17.

Dai, S.M. and S.S. Gill. 1993. *In vitro* and *in vivo* proteolysis of the *Bacillus thuringiensis* subsp. *israelensis* CryIVD protein by *Culex quinquefasciatus* larval midgut proteases. Insect. Biochem. Molec. Biol. **23**:273-283.

Denolf, P., S. Jansens, M. Peferoen, D. Degheele and J. Van Rie. 1993a. 2 Different *Bacillus thuringiensis* delta-endotoxin receptors in the midgut brush border membrane of the European corn borer, *Ostrinia nubilalis* (Hubner) (Lepidoptera, Pyralidae). Appl. Environm. Microbiol. **59**:1828-1837.

Denolf, P., S. Jansens, S. Vanhoudt, M. Peferoen, D. Degheele and J. Van Rie. 1993b. Biotinylation of *Bacillus thuringiensis* insecticidal crystal proteins. Appl. Environm. Microbiol. **59**:1821-1827.

Donovan, W.P., C.C. Dankocsik and M.P. Gilbert. 1988. Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **170**:4732-4738.

Eldridge, R., F.M. Horodyski, D.B. Morton, D.R. O'Reilly, J.W. Truman, L.M. Riddiford and L.K. Miller. 1992. Expression of an eclosion hormone gene in insect cells using baculovirus vectors. Insect Biochem. **21**:341-351.

Eldridge, R., D.R. O'Reilly, B.D. Hammock and L.K. Miller. 1992. Insecticdal properties of genetically engeneered baculoviruses expressing an insect juvenile hormone esterase gene. Appl. Environm. Microbiol. **58**:1583-1591.

Emery, V.C. and D.H.L. Bishop. 1987. The development of multiple expression vectors for high level synthesis of eukaryotic proteins: Expression of LCMV-N and *Ac*NPV polyhedrin protein by recombinant baculovirus. Protein Engin. 1:359-366.

English, L.H., T.L. Readdy and A.E. Bastian. 1991. Delta-endotoxin-induced leakage of Rb-86+-K+ and H_2O from phospholipid vesicles is catalyzed by reconstituted midgut membrane. Insect Biochem. **21**:177-184.

Entwistle, P.F. and H.F. Evans. 1985. Viral control. *In* G.A. Kerkut and L.I. Gilbert (Eds.), Comprehensive insect physiology, biochemistry and pharmacology **13**:347-412.

Feitelson, J.S., J. Payne and L. Kim. 1992. *Bacillus thuringiensis*: Insects and beyond. Biotechnology **10**:271-275.

Ferré, J, M.D. Real, J. Van Rie, S. Jansens and M. Peferoen. 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. Proc. Natl. Acad. Sci. USA **88**:5119-5123.

Finney, D.J. 1971. Probit analysis. Cambridge University Press Cambridge.

Flipsen, J.T.M., J.W.M. Van Lent, R.W. Goldbach and J.M. Vlak. 1993. Expression of polyhedrin and p10 in the midgut of *Ac*NPV-infected *Spodoptera frugiperda* larvae: an immunoelectron microscopical investigation. J. Invertebr. Pathol. **61**:17-23.

Friesen, P.D. and L.K. Miller. 1986. The regulation of baculovirus gene expression. Curr. Top. Microbiol. Immunol. **131**:31-49.

Fuchs, L.Y., M.S. Woods and R.F. Weaver. 1983. Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: a novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. J. Virol. **41**:641-646.

Ge, A.Z., N.I. Shivarova and D.H. Dean. 1989. Location of the *Bombix mori* specificity domain on a *Bacillus thuringiensis* δ-endotoxin protein. Proc. Natl. Acad. Sci USA **86**:4037-4041.

Gill, S.S., E.A. Cowles and P.V. Pietrantonio. 1992. The mode of action of Bacillus thuringiensis endotoxins. Ann. Rev. Entomol. **37**:615-636.

Glaser, R.W. and J.W. Chapman. 1913. The wilt disease of gypsy moth caterpillars. J. Econ. Entomol. **6**:479.

Granados, R.R. and B.A. Federici (Eds.). 1986. The biology of baculoviruses. Vol. I and II. CRC press, Boca Raton, Florida.

Granados, R.R. and K.A. Lawler. 1981. *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. Virology **108**:297-308.

Guarino, L.A. and M.D. Summers. 1986. Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene. J. Virol. **57**:563-571.

Guarino, L.A. and M.D. Summers. 1988. Functional mapping of *Autographa californica* nuclear polyhedrosis virus genes required for late gene expression. J. Virol. **62**:463-471.

Haider, M.Z. and D.J. Ellar. 1989. Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxin: interaction with phospholipid vesicles. Biochem. Biophys. Acta **978**:216-222.

Haider, M.Z., B.H. Knowles and D.J. Ellar. 1986. Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal delta-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. Eur. J. Biochem. **156**:531-540.

Hammock, B.D., B.C. Bonning, R.D. Possee, T.N. Hanzlik and S. Maeda. 1990. Expression and effects of the juvenile hormone esterase in a baculovirus vector. Nature **344**:458-461.

Hammock, B.D., B.F. McCutchen, J. Beetham, P.V. Choudary, E. Fowler, R. Ichinose, V.K. Ward, J.M. Vickers, B.C. Bonning, L.G. Harshman, D. Grant, T. Uematsu and S. Maeda. 1993. Development of recombinant viral insecticides by expression of an insect-specific toxin and an insect-specific enzyme in nuclear polyhedrosis viruses. Arch. Insect Biochem. Physiol. **22**:315-344.

Hanzlik, T.N., Y.A.I. Abdel-Aal, L.G. Harshman and B.D. Hammock. 1989. Isolation and sequencing of cDNA clones coding for juvenile hormone esterase from *Heliothis virescens*. J. Biol. Chem. **264**:12419-12425.

Harrap, K.A. 1970. Cell infection by a nuclear polyhedrosis virus. Virology **42**:311-318.

Hawtin, R., K. Arnold, L.H. Chappel, M.D. Ayres, P.A. Kitts, G.W. Gooday and R.D. Possee. 1992. Identification of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus. Presentation at the 11th ASV-meeting, Ithaca, NY, USA.

Hill-Perkins, M.S. and R.D. Possee. 1990. A baculovirus expression vector from the basic protein promoter of *Autographa californica* nuclear polyhedrosis virus. J. Gen. Virol. **71**:971-976.

Hink, F. 1970. Established insect cell line from the cabbage looper, *Trichoplusia ni*. Nature **226**:466-467.

Hofmann, C., H. Vanderbruggen, H. Höfte, J. Van Rie, S. Jansens and H. Van Mellaert. 1988. Specificity of *Bacillus thuringiensis* delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. USA **85**:7844-7848.

Höfte, H., H. de Greve, J. Seurinck, S. Jansens, J. Mahillon, C. Ampe, J. Vandekerckhove, H. Vanderbruggen, M. van Montagu and M. Zabeau. 1986. Structural and functional analysis of a cloned delta endotoxin of *Bacillus thuringiensis berliner* 1715. Eur. J. Biochem. **161**:273-280.

Höfte, H., J. van Rie, S. Jansens, A. van Houtven, H. Vanderbruggen and M. Vaeck. 1988. Monoclonal antibody analysis and insecticidal spectrum of three types of lepidopteran-specific insecticidal crystal proteins of *Bacillus thuringiensis*. Appl. Environm. Microbiol. **54**:2010-2017.

Höfte, H. and H.R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53**:242-255.

Honée, G. 1992. The specificity of *Bacillus thuringiensis* crystal proteins. Thesis. Wageningen. 127p.

Honée, G. and B. Visser. 1993. The mode of action of *Bacillus thuringiensis* crystal proteins. Entomol. Exp. Appl. **69**:145-155.

Honée, G., W. Vriezen and B. Visser. 1990. A translation fusion product of two different insecticidal crystal protein genes of *Bacillus thuringiensis* exhibits an enlarged insecticidal spectrum. Appl. Environm. Microbiol. **56**:823-825.

Horton H.M. and J.P. Burand. 1993. Saturable attachment sites for Polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. J. Virol. **67**:1860-1868.

Hulst, M.M., D.F. Westra, G. Wensvoort and R.J.M. Moorman. 1993. Glycoprotein E1 of Hog Cholera virus expressed in insect cells protects swine from Hog Cholera. J. Virol. **67**:5435-5442.

Ishiwata, S. 1901. On a kind of severe flacherie (sotto disease) (I.) Dainihon Sanshi Keiho **9**:1-5.

Jarvis, D.L. and M.D. Summers. 1989. Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. Mol. Cell. Biol. **9**:214-223.

Jarvis, D.L., M.D. Summers, A. Garcia and D.A. Bohlmeyer. 1993. Influence of different signal peptides and prosequences on expression and secretion of human plasminogen activator in the baculovirus system. J. Biol. Chem. **268**:16754-16762.

Johnson, D.E. 1981. Toxicity of *Bacillus thuringiensis* entomocidal protein toward cultured insect tissue. J. Invertebr. Pathol. **38**:94-101.

Johnson, D.E. 1994. Cellular toxicities and membrane binding characteristics of insecticidal crystal proteins from *Bacillus thuringiensis* toward cultured insect cells. J. Invertebr. Pathol. **63**:123-129.

Keddie, B.A., G.W. Aponte and L.E. Volkman. 1989. The pathway of the infection of *Autographa californica* Nuclear Polyhedrosis Virus in a insect host. Science **243**:1728-1730.

Keeley, L.L. and T.K. Hayes. 1987. Speculations on biotechnology applications for insect neuroendocrine research. Insect Biochem. **17**:639-651.

King, L.A. and R.D. Possee. 1992. The baculovirus expression system. Chapman and Hall, London, pp. 229.

Kitts, P.A., M.D. Ayres and R.D. Possee. 1990. Linearization of baculovirus DNA enhances the recovery of recombinant baculovirus expression vectors. Nucl. Acid Res. **18**:5667-5672.

Kitts, P.A. and R.D. Possee. 1993. A method for producing recombinant baculovirus expression vectors at high frequency. Biotechniques **14**:810.

Knebel-Mörsdorf, D., A. Kremer and F. Jahnel. 1993. Baculovirus gene ME53, which contains a putative Zinc finger domain, is one of the major early-transcribed genes. J. Virol. **67**:753-758.

Knowles, B.H. and D.J. Ellar. 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. Biochim. Biophys. Acta **924**:509-518.

Knowles, B.H., W.E. Thomas and D.J. Ellar. 1984. Lectin-like binding of *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action. FEBS letters **168**:197-202.

Kogan, P.H. and G.W. Blissard. 1994. A baculovirus early promoter is activated by host transcription factor binding to CACGTG and GATA elements. J. Virol. **68**:813-822.

Kool, M., C.H. Ahrens, R.W. Goldbach, G.F. Rohrmann and J.M. Vlak. 1994. Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. Proc. Natl. Acad. Sci. submitted.

Koolman, J. 1990. Ecdysteroids. Zoological science **7**:563-580.

Korth K.L. and Ch.S. Levings. 1993. Baculovirus expression of the maize mitochondrial protein URF13 confers insecticidal activity in cell cultures and larvae. Proc. Natl. Acad. Sci. USA **90**:3388-3392.

Kovacs, G.R., J. Choi, L.A. Guarino and M.D. Summers. 1992. Functional dissection of the *Autographa californica* nuclear polyhedrosis virus immediate-early-1 transcriptional regulatory protein. J. Virol. **66**:7429-7437.

Kovacs, G.R, L.A. Guarino and M.D. Summers. 1991. Novel regulatory properties of the IE1 and IE0 trans-activators encoded by the baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus. J. Virol. **65**:5281-5288.

Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNA. Nucl. Acids Res. **15**:8125-8132.

Krappa, R., A. Behn-Krappa, F. Jahnal, W. Doerfler and D. Knebel-Mörsdorf. 1992. Differential factor binding at the promoter of early baculovirus gene PE38 during viral infection: GATA motif is recognized by an insect protein. J. Virol. **66**:3493-3503.

Krappa, R. and D. Knebel-Mörsdorf. 1991. Identification of the very early transcribed baculovirus gene PE-38. J. Virol. **65**:805-812.

Krieg, A. 1986. *Bacillus thuringiensis*, ein mikrobielles insectzid: Grundlagen und anwendung. *In* Acta Phytomedica **10**, 191 pp.

Kunkel, T.A. 1985. Rapid and efficient site-directed mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. **82**:488-492.

Kuroda, K., A. Gröner, K. Frese, D. Drenckhahn, Ch. Hauser, R. Rott, W. Doerfler and H.D. Klenk. 1989. Synthesis of biological active influenza virus hemagglutinin in insect larvae. J. Virol. **63**:1677-1685.

Kuzio, J. and P. Faulkner. 1991. Identification of a cysteine proteinase encoded by the *Autographa californica* nuclear polyhedrosis virus. Sequence in EMBL/Genebank Acc. No. M67451.

Kuzio, J., R. Jaques and P. Faulkner. 1989. Identification of P74, a gene essential for virulence of baculovirus occlusion bodies. Virology **173**:759-763.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685.

Li, Y.H., A.L. Passarelli and L.K. Miller. 1993. Identification, sequence, and transcriptional mapping of lef-3, a baculovirus gene involved in late and very late gene expression. J. Virol. **67**:5260-5268.

Li, J., J. Carroll and D.J. Ellar. 1991. Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. Nature **353**:815-821.

Lilley, M., R.N. Ruffell and H.J. Somerville. 1980. Purification of the insecticidal toxin in crystals of *Bacillus thuringiensis*. J. Gen. Microbiol. **118**:1-11.

Lu, A. and E.B. Carstens. 1992. Nucleotide sequence and transcriptional analysis of the p80 gene of *Autographa californica* Nuclear Polyhedrosis Virus: a homologue of the *Orgyia pseudotsugata* Nuclear Polyhedrosis Virus capsid-associated gene. Virology **190**:201-209.

Lu, A. and E.B. Carstens. 1993. Immediate-early baculovirus genes transactivate the p143 gene promoter of *Autographa californica* Nuclear Polyhedrosis Virus. Virology **195**:710-718.

Luckow, V.L. and M.D. Summers. 1988. Trends in the development of baculovirus expression vectors. Bio/Technol. **6**:47-55.

Maeda, S. 1989a. Increased insecticidal effect by a recombinant baculovirus carrying a synthetic diuretic hormone gene. Biochem. Biophys. Res. Commun. **165**:1177-1183.

Maeda, S. 1989b. Expression of foreign genes in insects using baculovirus vectors. Ann. Rev. Entomol. **34**:351-372.

Maeda, S., S.L. Volrath, T.N. Hanzlik, S.A. Harper, K. Majima, D.W. Maddox, B.D. Hammock and E. Fowler. 1991. Insecticidal effects of an insect-specific neurotoxin expressed by a recombinant baculovirus. Virology **184**:777-780.

Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Martignoni, M.E. 1984. Baculovirus: an attractive alternative, p.51-67. *In* W. Y. Garner and J. Harvey (eds.), Chemicals and biological controls in agriculture and forestry, American Chemical Society, Washington, D.C.

McCutchen, B.F., P.V. Choudary, R. Crenshaw, D.W. Maddox, S.G. Kamita, N. Palekar, S. Volrath, E. Fowler, B.D. Hammock and S. Maeda. 1991. Development of a recombinant baculovirus expressing an insect selective neurotoxin: potential for pest control. Biotechnol. **9**:848-852.

Mcgaugy W.H. and M.E. Whalon. 1990. Managing insect resistance to *Bacillus thuringiensis* toxins. Science **258**:1451-1455.

Merryweather, A.T., U. Weyer, M.P.G. Harris, M. Hirst, T. Booth and R.D. Possee. 1990. Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin. J. Gen. Virol. **71**:1535-1544.

Merryweather, A.T., U. Weyer, M.P.G. Harris, M. Hirst, T. Booth and R.D. Possee. 1991. Genetically engineered baculovirus insecticides containing a *Bacillus thuringiensis* delta endotoxin. First international conference on *Bacillus thuringiensis*. Oxford, UK, July 1991.

Miller, L.K. 1989. Insect baculoviruses: Powerful gene expression vectors. BioEssays **11**:91-95.

Miller, L.K. 1988. Baculoviruses as gene expression vectors. Ann. Rev. Microbiol. **42**:177-199.

Milne, R. and H. Kaplan. 1993. Purification and characterization of a trypsin-like digestive enzyme from spruce budworm (*Choristoneura fumiferana*) responsible for the activation of δ-endotoxin from *Bacillus thuringiensis*. Insect Biochem. Molec. Biol. **23**:663-673.

Morris, T.D. and L.K. Miller. 1992. Promoter influence on baculovirus-mediated gene expression in permissive and non-permissive insect cell lines. J. Virol. **66**:7397-7405.

Murphy, D.W., S.S. Sohi and P.G. Fast. 1976. *Bacillus thuringiensis* enzyme-digested delta endotoxin: Effect on cultured insect cells. Science **194**:954-956.

Nagamatsu, Y., Y. Itai, C. Hatanaka, G. Funatsu and K. Hayashi. 1984. A toxic fragment from the entomocidal crystal protein of *Bacillus thuringiensis*. Agric. Biol. Chem. **48**:611-619.

Nakamura, K., R.M. Murai-Nishioka, M. Shimizu, K. Oshie, K. Mikitani, K. Oeda and H. Ohkawa. 1992. Insecticidal activity and processing in larval gut juices of genetically engineered 130-kDa proteins of *Bacillus thuringiensis* subsp. *aizawai*. Biosci. Biotech. Biochem. **56**:1-7.

Oddou, P., H. Hartmann and M. Geiser. 1991. Identification and characterization of Heliothis virescens midgut membrane proteins binding *Bacillus thuringiensis* delta-endotoxins. Eur. J. Biochem. **202**:673-680.

Oddou, P., H. Hartmann, F. Radecke and M. Geiser. 1993. Immunologically unrelated *Heliothis* Ssp. and *Spodoptera* Ssp. midgut membrane-proteins bind *Bacillus thuringiensis* CryIA(b) delta-endotoxin. Eur. J. Biochem. **212**:145-150.

Oeda, K., K. Inouye, Y. Ibuchi, K. Oschie, M. Shimizu, K. Nakamura, R. Nishioka, Y. Takada and H. Ohkawa. 1989. Formation of crystals of the insecticidal proteins of *Bacillus thuringiensis* subsp. *aizawai* IPL7 in *Escherichia coli*. J. Bacteriol. **171**:3568-3571.

Ogiwara, K., L.S. Indrasith, S. Asano and H. Hori. 1992. Processing of δ-endotoxin from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and HD-73 by gut juices of various insect larvae. J. Invertebr. Pathol. **60**:121-126.

Oker-Blom, C., C. Jansson, M. Karp, C. Lindqvist, J.M. Savola and K. Akerman. 1993. Functional analysis of the human α2c-C4 adrenergic receptor in insect cells expressed by a luciferase based expression vector. Biochem. Biophys. Acta. **1176**:269-275.

O'reilly D.R., T.J. Kelly, E.P. Masler, B.S. Thyagaraja, R.M. Robson and L.K. Miller. Overexpression of *Bombix mori* prothoracicotropic hormone in insect cells and larvae using baculovirus vectors. Submitted.

O'reilly D.R. and L.K. Miller. 1989. A baculovirus gene blocks insect molting by producing acdysteroid UDP-glycosyl transferase. Science **245**:1110-1112.

O'reilly D.R. and L.K. Miller. 1991. Improvement of a baculovirus pesticide by deletion of the *egt* deletion. Bio/technol. **9**:1086-1089.

Palli, S.R., K. Touhara, J.P. Charles, B.C. Bonning, J.K. Atkinson, S.C. Trowell, K. Hiruma, W.G. Goodman, T. Kyriakides, G.D. Prestwich, B.D. Hammock and L.M. Riddiford. 1994. A nuclear protein from larvae of *Manduca sexta*: a putative receptor for the metamorpic action of juvenile hormone. Proc. Natl. Acad. Sci. USA **91**:6191-6195.

Pang, Y., R. Frutos and B.A. Federici. 1992. Synthesis and toxicity of full-Length and truncated bacterial CryIVD mosquitocidal proteins expressed in lepidopteran cells using a baculovirus vector. J. Gen. Virol. **73**:89-101.

Passarelli, A.L. and L.K. Miller. 1993a. Three baculovirus genes involved in late and very late gene expression: IE-1, IE-N and lef-2. J. Virol. **67**:2149-2158.

Passarelli, A.L. and L.K. Miller. 1993b. Identification and characterization of lef-1, a baculovirus gene involved in late and very late gene expression. J. Virol. **67**:3481-3488.

Passarelli, A.L. and L.K. Miller. 1993c. Identification of genes encoding late expression factors located between 56.0 and 65.4 map units of the *Autographa californica* nuclear polyhedrosis virus genome. Virology **197**:704-714.

Passarelli, A.L., J.W. Todd and L.K. Miller. 1994. A baculovirus gene involved in transient late gene expression encodes a 102 kDa polypeptide with a motif conserved in RNA polymerases. Sequence in EMBL/Genebank Acc. No. U04879.

Patel, G., K. Nasmyth and N. Jones. 1992. A new method for the isolation of recombinant baculovirus. Nucl. Acids Res. **20**:97-104

Payne, C.C. 1988. Pathogens for the control of insects: where next? Philosophical transactions of the Royal Society of London. **B 318**:225-248.

Payne, C.C. 1986. Insect pathogenic viruses as pest control agents. Fortschr. Zool. **32**:183-200.

Pearson, M.N., R.L. Quant-Russell, G.F. Rohrmann and G.S. Beaudreau. 1988. P39, a major baculovirus structural protein: immunocytochemical characterization and genetic localization. Virology **167**:407-413.

Perlak, F.J., R.L. Fuchs, D.A. Dean, S.L. McPherson and D.A. Fischhoff. 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. USA **88**: 3324-3328.

Possee, R.D. and S.C. Howard. 1987. Analysis of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. Nucl. Acids Res. **15**:10233-10248.

Possee, R.D., Sun, T.P., Howard, S.C., Ayres, M.D., Hill-Perkins, M. and K.L. Gearing. 1991. Nucleotide sequence of the *Autographa californica* nuclear polyhedrosis virus 9.4 kbp *Eco*RI-I and *Eco*RI-R (polyhedrin gene) region. Virology **185**:229-241.

Price, P.M., C.F. Reichelderfer, B.E. Johansson, E.D. Kilbourne and G. Acs. 1989. Complementation of recombinant baculoviruses by coinfection with wild-type virus facilitates production in insect larvae of antigenic proteins of hepatitis B virus and influenza. Proc. Natl. Acad. Sci USA **86**:1435-1456

Rankin, C., B.G. Ooi and L.K. Millar. 1988. Eight base pairs encompassing the transcriptional start point are the major determinants for baculovirus polyhedrin gene expression. Gene **70**:39-49.

Ravoahangimalala, O., J.F. Charles and J. Schoeller-Raccaud. 1993. Immunological localization of *Bacillus thuringiensis* serovar *israelensis* toxins in midgut cells of intoxicated *Anopheles gambiae* larvae (Diptera: Culicidae). Res. Microbiol. **144**:271-278

Ribeiro, B.M. and N.E. Crook. 1993. Expression of full-length and truncated forms of crystal protein genes from *Bacillus thuringiensis* subsp. *kurstaki* in a baculovirus and pathogenicity of the recombinant viruses. J. Invertebr. Pathol. **62**:121-130.

Rodems, S.M. and F.D. Friesen. 1993. The *hr5* transcriptional enhancer stimulates early expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication. J. Virol. **67**: 5776-5785.

Roelvink, P.W., M.M.M. van Meer, C.A.D. de Kort, R.D. Possee, B.D. Hammock and J.M. Vlak. 1992. Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedron and p10 genes. J. Gen. Virol. **73**:1481-1489.

Rohrmann, G. 1986. Polyhedrin structure. J. Gen. Virol. **67**:1499-1513.

Ryerse, J.S., J.R. Beck Jr. and P.B. Lavrik. 1990. Light microscope immuno localization of *Bacillus thuringiensis kurstaki* delta-endotoxin in the midgut and Malpighian tubes of the tobacco budworm, *Heliothis virescens*. J. Invertebr. Pathol. **56**:86-90.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory,Cold Spring Harbor, N.Y..

Sanchis, V., D. Lereclus, G. Menou, J. Chaufaux, S. Guo and M.M. Lecadet. 1989. Nucleotide sequence and analysis of the N- terminal coding region of the *Spodoptera*-active delta-endotoxin gene of *Bacillus thuringiensis aizawai* 7.29. Mol. Microbiol. **3**:229-238.

Schesser, J.H., K.J. Kramer and L.A. Bulla Jr. 1977. Bioassay for homogeneous parasporal crystal of *Bacillus thuringiensis* using the tobacco hornworm, *Manduca sexta*. Appl. Environm. Microbiol. **33**:878-880.

Schnepf, H.E. and H.R. Whiteley. 1985. Delineation of a toxin-encoding segment of a *Bacillus thuringiensis* crystal protein gene. J. Biol. Chem. **260**:6273-6280.

Shivakumar, A.G., G.J. Gundling, T.A. Benson, D. Casuto, M.F. Miller and B.B. Spear. 1986. Vegetative expression of the delta-endotoxin genes of *Bacillus thuringiensis* subsp. *kurstaki* in *Bacillus subtilus*. J. Bacteriol. **166**:194-204.

Slatin, S.L., C.K. Abrams and L.H. English. 1990. Delta-endotoxins form cation-selective channels in planar lipid bilayers. Biochem. Biophys. Res. Commun. **169**:765-772.

Smith, G.E. and M.D. Summers. 1978. Analysis of baculovirus genomes with restriction endonucleases. Virology **98**:517-527.

Smith, G.E., M.J. Fraser and M. D. Summers. 1983a. Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: Deletion mutants within the polyhedrin gene. J. Virol. **46**:584-593.

Smith, G.E., M.D. Summers and M.J. Fraser. 1983b. Production of human beta interferon in insect cells infected with a baculovirus expression vector. Molec. Cell. Biol. **3**:2156-2165.

Smith, G.E., J.M. Vlak and M.D. Summers. 1983c. Physical analysis of *Autographa californica* nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular weight protein. J. Virol. **42**:15-225.

Smits, P.H. 1987. Nuclear polyhedrosis virus as biological control agent of *Spodoptera exigua*. Thesis. Wageningen. The Netherlands. 127p.

Smits, P.H. and J.M. Vlak. 1988. Selection of nuclear polyhedrosis viruses as biological control agents of *Spodoptera exigua* (Lepidoptera: Noctuidae). Entomophaga **33**:299-308.

Steinhaus, E.A. 1956. Microbial control: The emergence of an idea. J. Agric. Sci **26**:107-160.

Stewart, L.M.D., M. Hirst, M. Lopez-Ferber, A.T. Merryweather, P.J. Caylay and R.D. Possee. 1991. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. Nature **352**:85-88.

Summers, M.D. and G.E. Smith. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures, p.1555, Texas Agricultural Experiment Station, Bulletin.

Thiem, S.M. and L.K. Miller. 1989. A baculovirus gene with a novel transcription pattern encodes a polypeptide with a zinc finger and a leucine zipper. J. Virol. **63**:4489-4497.

Tomalski, M.D., T.P. King and L.K. Miller. 1993. Expression of hornet genes encoding venom allergen antigen 5 in insects. Arch. Insect Biochem. Physiol. **22**:303-313.

Tomalski, M.D. and L.K. Miller. 1991. Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. Nature 352:82-85.

Tomalski, M.D. and L.K. Miller. 1992. Expression of a paralytic neurotoxin gene to improve insect baculoviruses as biopesticides. Bio/Technology **10**:545-549.

Van Lent, J.W.M., J.T.M. Groenen, E.C. Klinge-Roode, G.F. Rohrmann, D. Zuidema and J.M. Vlak. 1990. Localization of the 36-kDa polyhedron envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. Arch. Virol. **111**:103-114.

Van Oers, M.M., J.T.M. Flipsen, C.B.E.M. Reusken, E.L. Sliwinsky, R.W. Goldbach and J.M. Vlak. 1993. Functional domains of the p10 protein of *Autographa californica* nuclear polyhedrosis virus. J. Gen. Virol. **74**:563-574.

Van Oers M.M. 1994. Functional analysis of the baculovirus 10 kilodalton protein. Thesis. Wageningen. 143p.

Van Rie, J., S. Jansens, H. Höfte, D. Degheele and H. van Mellaert. 1989. Specificity of *Bacillus thuringiensis* delta-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. Eur. J. Biochem. **186**:239-247.

Van Rie J., S. Jansens, H. Höfte, D. Degheele and H. van Mellaert. 1990a. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Appl. Environm. Microbiol. **56**:1378-1385.

Van Rie, J., W.M. McGaughey, D.E. Johnson, B.D. Barnett and H. van Mellaert. 1990b. Mechanism of insect resistance to the microbial insecticide *Bacillus-thuringiensis*. Science **247**:72-74.

Van der Wilk, F., J.W.M. van Lent and J.M. Vlak. 1987. Immunogold detection of polyhedrin, p10 and virion antigens in *Autographa californica* nuclear polyhedrosis virus-infected *S. frugiperda* cells. J. Gen. Virol. **68**:2615-2623.

Vaughn, J.L., R.H. Goodwin, G.J. Tompkins and P. McCawley. 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro **13**:213-217.

Vialard, J.E. and C.D. Richardson. 1993. The 1,629-nucleotide open reading frame located downstream of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene encodes a nucleocapsid associated phosphoprotein. J. Virol. **67**:5859-5866.

Visser, B., T. van der Salm, W. van den Brink and G. Folkers. 1988. Genes from *Bacillus thuringienses entomocidus* 60.5 coding for insect specific crystal proteins. Mol. Gen. Genet. **212**:219-224.

Vlak, J.M. 1979. The proteins of nonoccluded *Autographa californica* nuclear polyhedrosis virus produced in an established cell line of *Spodoptera frugiperda*. J. Invertebr. Pathol. **34**:110-118.

Vlak, J.M. 1993. Genetic engineering of baculoviruses for insect control. *In*: "Molecular approaches to pure and applied entomology" (M.J. Whitten and J.G. Oakeshott, Eds.). pp. 90-127. Springer-Report, New York.

Vlak, J.M., A. Schouten, M. Usmany, G.J. Belsham, E.C. Klinge-Roode, A.J. Maule, J.W.M. van Lent and D. Zuidema. 1990. Expression of cauliflower mosaic virus gene I using a baculovirus vector based upon the p10 gene using a novel selection method. Virology **179**:312-320.

Vlak, J.M., F.A. Klinkenberg, K.J.M. Zaal, M. Usmany, E.C. Klinge-Roode, J.B.F. Geervliet, J. Roosien and J.W.M. van Lent. 1988. Functional studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10-ß-galactosidase fusion gene. J. Gen. Virol. **69**:65-776.

Volkman, L.E. and P.A. Goldsmith. 1985. Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: Inhibition of entry by adsorptive endocytosis. Virology **143**:185-195.

Volkman, L.E. and M.D. Summers. 1977. *Autographa californica* nuclear polyhedrosis virus: comparative infectivity of occluded, alkali liberated and non-occluded forms. J. Invertebr. Pathol. **30**:102-103.

Walters, F.S., S.L. Slatin, C.A. Kulesza and L.H. English. 1993. Ion channel activity of N-terminal fragments from CryIA(c) delta-endotoxin. Biochem. Biophys. Res. Commun. **196**:921-926.

Wang, X., B.G. Ooi and L.K. Miller. 1991. Baculovirus vectors for multiple gene expression and for occluded virus production. Gene **100**:131-137.

Ward, E.S. and D.J. Ellar. 1988 Cloning and expression of two homologous genes of *Bacillus thuringiensis* subsp. *israelensis* which encode 130-kilodalton mosquitocidal proteins. J. Bacteriol. **170**:727-735.

Weyer, U., S. Knight and R.D. Possee. 1990. Analysis of very late gene-expression by *Autographa-californica* nuclear polyhedrosis-virus and the further development of multiple expression vectors. J. Gen. Virol. **71**:1525-1534.

Whitford, M. and P. Faulkner. 1992. Nucleotide sequence and transcriptional analysis of a gene encoding gp41, a structural glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J. Virol. **66**:4763-4768.

Whitt, M.A. and J.S. Manning. 1988. A phosphorylated 34-kDa protein and a subpopulation of polyhedrin are thiol linked to the carbohydrate layer surrounding a baculovirus occlusion body. Virology **163**:33-42.

Widner, W.R. and H.R. Whiteley. 1989. Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. J. Bacteriol. **171**:965-974.

Wilson, M.E., T.H. Mainprize, P.D. Friezen and L.K. Miller. 1987. Location, transcription, and sequence of a baculovirus gene encoding a small arginine-rich polypeptide. J. Virol. **61**:661-666.

Wilson, M.E. and Consigly R.A. 1985. Characterization of a protein kinase activity associated with purified capsids of the granulosis virus infection *Plodia interpunctella*. Virology **143**:526-535.

Wood, H.A. 1991. Development of genetically enhanced baculovirus pesticides. *In* "Biotechnology for biological control of pests and vectors" (K. Maramorosh, ed.) Academic Press, New York, pp. 69-76.

Yoshida, K., Y. Fujita, E. Mukai, K. Sen, M. Himeno, H. Sakai and T. Komano. 1993. Importance of the central region of 130-kDa insecticidal proteins of *Bacillus thuringiensis* var *israelensis* for their activity *in vivo* and *in vitro*. Biosci. Biotech. Biochem. **57**:584-590.

Zanotto, P.M.A., B.D. Kessing and J.E. Maruniak. 1993. Phylogenetic interrelationships among baculoviruses: Evolutionary rates and host associations. J. Invertebr. Pathol. **62**:147-164.

Zlotkin, E. 1985. Toxins derived from arthropod venoms specifically affecting insects. *In* Comprehensive Insect Physiology. Vol. 10. Kerkut G.A. and L.I. Gilbert, Eds., Pergamon press, Oxford, p.499-546.

Zuidema, D., E.C. Klinge-Roode, J.W.M. Van Lent and J.M. Vlak. 1989. Construction and analysis of an Autographa californica nuclear polyhedrosis virus mutant lacking the polyhedral envelope. Virology **173**:98-108.

Zuidema, D., A. Schouten, M. Usmany, A.J. Maule, G.J. Belsham, J. Roosien, E.C. Klinge-Roode, J.W.M. Van Lent and J.M. Vlak. 1990. Expression of cauliflower mosaic virus gene I in insect cells using a novel polyhedrin-based baculovirus expression vector. J. Gen. Virol, **71**:2201-2209.

SAMENVATTING

Een belangrijk deel van de schade in land-, tuin- en bosbouw wordt veroorzaakt door vraat van insekten. Om deze schade te beperken zijn er de afgelopen decennia op grote schaal chemische bestrijdingsmiddelen ingezet. Het grootschalige gebruik van deze insekticiden resulteerde echter in selectie van resistente insekten, waardoor vervolgens meer van deze insekticiden moest worden toegediend. Het gebruik van chemische bestrijdingsmiddelen in het algemeen leidde tot een onaanvaardbare belasting van het milieu, hetgeen de overheid heeft genoopt het gebruik van deze middelen sterk te beperken en het onderzoek naar milieuvriendelijke alternatieve beheersmaatregelen sterk te stimuleren. Eén van de alternatieven is het gebruik van virussen die insekten kunnen doden.

Baculovirussen zijn specifiek voor geleedpotigen, waartoe ondermeer insekten behoren. Zij vormen potentieel een veilig en biologisch-verantwoord alternatief. Deze virussen hebben echter een aantal belangrijke nadelen. Vanwege hun specificiteit kunnen ze maar tegen een beperkt aantal insekten worden ingezet. Dit is een belangrijk economisch nadeel. Met een specifiek baculovirus kunnen slechts één of enkele insektenplagen bestreden worden en dit garandeert slechts een kleine omzet. Desondanks is recentelijk een baculovirus, onder de naam Spod-X^R, met succes op de Nederlandse markt gebracht om de Floridamot, die resistent is tegen vrijwel alle toegelaten chemisch insekticiden, in de bloementeelt te bestrijden. Een tweede belangrijke belemmering voor een effectief gebruik van baculovirussen als insekten-bestrijdingsmiddel, is het feit dat baculovirussen zich relatief langzaam vermenigvuldigen in het insekt. Het duurt daarom minstens nog een week eer de aangetaste insekten ophouden met eten als gevolg van de virusinfectie. In deze periode kan het gewas nog zeer veel (vraat)schade oplopen. Doel van het in dit proefschrift beschreven onderzoek was te onderzoeken of de effectiviteit van baculovirussen kon worden verhoogd door ze zodanig genetisch te modificeren dat ze tijdens hun infectie snel-werkende, insekt-specifieke toxines produceren.

Bij de aanvang van het onderzoek leken de insekt-specifieke kristaleiwitten, die geproduceerd worden door de bodembacterie *Bacillus thuringiensis* tijdens sporulatie,

veelbelovend omdat ze zeer toxisch zijn voor insektelarven, snel werken en biologisch afbreekbaar zijn. Daarnaast overlapt de gastheerreeks gevoelig voor deze toxische kristaleiwitten grotendeels met die van baculovirussen en is hun werkingsmechanisme reeds uitgebreid onderzocht. Bovendien hebben deze toxines geen effect op andere levensvormen, zoals de mens, hogere dieren en planten.

Allereerst werd onderzocht of het prototype van de baculovirussen, het kernpolyedervirus van de spanrups *Autographa californica* (*Ac*NPV), in staat zou zijn tijdens infectie deze *B. thuringiensis* toxines in voldoende mate en in een biologisch actieve vorm te produceren (Hoofdstuk 2). In insektecelcultures bleek, dat tijdens een infectie met een recombinant-*Ac*NPV, waarin het cryIA(b) toxine-gen achter de late polyhedrine-promoter van het virus was geplaatst, grote hoeveelheden biologisch actief protoxine geproduceerd konden worden. Deze protoxines bleken in insektecellen uit te kristalliseren in de voor deze eiwitten karakteristieke bi-piramidale vorm. Daarnaast konden deze kristallen in een alkalisch milieu opgelost worden en waren de hieruit vrijgekomen protoxines (130 kDa) met een trypsine te activeren tot actief toxine ($\pm$ 60 kDa). Deze toxines bleken vervolgens specifiek te binden aan microvilli van middendarmcellen van gevoelige insekten. Deze toetsen toonden aan dat het door *Ac*NPV geproduceerde protoxine op zichzelf biologisch actief is (Hoofdstukken 2 en 3), maar dat het virus na toevoeging van dit protoxine-gen geen grotere effectiviteit (snellere doding) ten opzichte van insektelarven tentoonspreid.

Kristaleiwitten van *B. thuringiensis* zijn toxisch voor larven ongeacht of ze oraal danwel via injectie in de hemocoel worden toegediend aan het insekt. In de hemocoel is alleen het trypsine-geactiveerde toxine werkzaam, kennelijk omdat daar de activatie tot toxine niet kan plaatsvinden. Willen kristaleiwitten dus in staat zijn de effectiviteit van baculovirussen te verhogen dan zullen deze eiwitten tijdens de virusinfectie uitgescheiden dienen te worden in het lumen van de middendarm of in de hemocoel. Bij secretie in de hemocoel zal om bovengenoemde reden het actieve toxine tot expressie gebracht moeten worden. Beide opties zijn verder onderzocht.

Om het actieve toxine tot expressie te kunnen brengen werd bepaald welk deel van het protoxine overeenkomt met het actieve toxine. Het N-terminale aminozuur van het actieve toxine was reeds bekend (isoleucine 29 van het protoxine); de C-terminus echter nog niet precies. Met behulp van plaatsgerichte mutagenese werd bepaald dat een polypeptide dat de aminozuurresiduen 29 tot en met 607 van het protoxine bevat, zonder trypsine-activatie

werkzaam is (Hoofdstuk 4). Verrassend genoeg bleek dit polypeptide ook toxisch te zijn voor *E. coli* cellen die, voor zover bekend, geen receptoren voor *B. thuringiensis* toxines bezitten. Zelfs wanneer het domein van het toxine dat bindt aan de receptor in middendarmcellen uit dit toxine werd gedeleteerd, bleek dit ingekorte eiwit toxisch voor *E. coli* te zijn, hetgeen suggereert dat in het geval van *E. coli* geen receptor nodig is om poriën in membranen te introduceren.

Om de diverse *B. thuringiensis*-toxineconstructen in insektecellen en -larven te kunnen testen werden deze geïnserteerd in het *Ac*NPV-genoom onder controle van de promoter van het p10 gen. Dit gen is niet essentieel voor de infectie van larven, maar speelt een rol bij de verspreiding van het virus in het milieu. Daarnaast garandeert de p10 promoter (evenals de polyhedrine promoter) hoge expressie laat in infectie. Omdat het genoom van een baculovirus relatief groot is (130 kilobaseparen), is het alleen mogelijk een nieuw gen aan het virusgenoom toe te voegen via homologe recombinatie van een transfervector waarin het nieuwe gen geflankeerd wordt door virale sequenties en het virus DNA. Homologe recombinatie treedt van nature slechts met een lage frequentie op (± 0.5%). Om recombinante baculovirussen op efficiëntere wijze te kunnen produceren werd eerst een recombinant-baculovirus geconstrueerd, waarbij het p10 gen vervangen werd door een unieke knipplaats voor een restrictie-enzym. Hierdoor kon het virale DNA voorafgaand aan een co-transfectie gelineariseerd worden. Lineair viraal DNA is niet infectieus doordat er geen replicatie van het genoom kan plaatsvinden. Pas na recombinatie met een transfervector ontstaat weer een circulair viraal genoom dat wel repliceert. Door gebruikmaking van dit principe kon het percentage recombinante virussen na een co-transfectie verhoogd worden tot 20 à 30%, waardoor de isolatie van de recombinante virussen aanmerkelijk werd versneld (Hoofdstuk 5).

Om te bepalen of de toevoeging van *B. thuringiensis*-kristaleiwitten de effectiviteit van baculovirusinfectie bij insekten kan verhogen, is een aantal verschillende recombinante virussen gemaakt (Hoofdstuk 6). Eén van deze recombinanten bracht het volledige protoxine tot expressie, een tweede een verkort protoxine waardoor het eiwit niet meer uitkristalliseert en een derde het actieve toxine uit Hoofdstuk 4. Een drietal gelijksoortige recombinanten werd geproduceerd, waarvan de genoemde polypeptides waren voorzien van een signaalpeptide, om uitscheiding ervan uit cellen mogelijk te maken. Tijdens de virusinfectie bleek dat de protoxines in alle gevallen in grote hoeveelheden geproduceerd werden.

Daarnaast bleek dat er nagenoeg geen kristaleiwitten aangetroffen werden in het medium van cellen die geïnfecteerd waren met recombinanten waarvan de protoxines voorzien waren van een signaalpeptide. Dit betekent dat er geen noemenswaardige uitscheiding had plaatsgevonden. Het feit dat dit kristaleiwit moeilijk te secreteren is, wordt waarschijnlijk veroorzaakt door de intrinsieke eigenschappen van het eiwit, want het gebruikte signaalpeptide bleek voor andere eiwitten uitstekend te functioneren.

Recombinanten, die een actief toxine tot expressie brengen, waren moeilijk te verkrijgen. Het actieve toxine is zeer waarschijnlijk cytotoxisch voor insektecellen. Deze recombinanten produceerden tijdens de infectie ook geen polyeders en waren daarnaast zeer instabiel. Polyeders zijn noodzakelijk voor een goede biologische analyse (biotoets) van recombinante virussen. De onmogelijkheid om polyeders te isoleren van deze recombinant leidde ertoe dat de laatstgenoemde recombinanten niet verder biologisch getoetst konden worden. Van de overige recombinanten werd uiteindelijk de biologische activiteit voor larven van de Floridamot (*Spodoptera exigua*) bepaald. Zowel de mediaan letale dosis (LD_{50}) als de mediaan letale tijd (LT_{50}) van deze geteste recombinanten bleek niet significant te verschillen van die van het *wild type* virus. Derhalve kon geconcludeerd worden dat *B. thuringiensis*-protoxines, alhoewel op zichzelf biologisch actief, niet in staat zijn de dodingssnelheid van deze baculovirussen te verhogen. De reden hiervoor is dat de in insektecellen geproduceerde protoxines vermoedelijk biologisch inactief zijn tijdens de infectie, omdat er geen activatie intracellulair kan plaatsvinden. Bovendien bleek dat het niet mogelijk was om deze protoxines uit te scheiden naar een plaats waar deze activatie wel zou kunnen optreden, zoals bijvoorbeeld in de middendarm, zodat de recombinanten, waarvan het protoxine voorzien was van een signaalpeptide, qua biologische activiteit niet van het *wild type* virus verschilden.

Het in dit proefschrift beschreven onderzoek geeft aan dat insekt-specifieke toxines van *B. thuringiensis* op dit moment (nog) niet geschikt zijn om de effectiviteit van baculovirussen bij de bestrijding van insekten te verhogen. Verbetering van de secretie van het protoxine of regulering van de expressie van het actieve toxine door gebruik te maken van een induceerbare promoter zou mogelijk in de toekomst kunnen resulteren in bruikbare recombinante virussen op basis van het toxine van *B. thuringiensis*.

ACCOUNT

Martens, J.W.M., G. Honée, D. Zuidema, J.W.M. Van Lent, B. Visser and J.M. Vlak. 1990. Insecticidal activity of a bacterial Crystal protein expressed by a recombinant baculovirus in insect cells. Appl. Environ. Microbiol. **56**:2764-2770.

Martens, J.W.M., G. Honée, D. Zuidema, J.W.M. Van Lent, B. Visser and J.M. Vlak. 1991. Insecticidal activity of a *Bacillus thuringiensis* toxin expressed by baculovirus recombinants. Proceedings Third European Meeting "Microbial Control Of Pests". p. 185-190.

Martens, J.W.M., Bosch, D., Visser, B. and J.M. Vlak. Mapping and characterization of the entomocidal domain of the CryIA(b) protoxin. Submitted to Molecular and General Genetics.

Martens, J.W.M., M.M. Van Oers, J.M. Vlak and P.J. Oudshoorn. Efficient recovery and screening of baculovirus p10-based recombinants. Journal of Virological Methods. In press.

Martens, J.W.M., M. Knoester, F. Weijts, A.J.A. Groffen, Z. Hu, D. Bosch, B. Visser and J.M. Vlak. Characterization of baculovirus insecticides expressing tailored *Bacillus thuringiensis* CryIA(b) insecticidal protein constructs. Submitted to the Journal of Invertebrate Pathology.

Martens, J.W.M., J.T.M. Flipsen, A.J.A. Groffen, J.M. Vlak and D. Bosch. Rapid method for detecting specific binding of insecticidal crystal proteins of *Bacillus thuringiensis* to midgut sections. In prepation.

CURRICULUM VITAE

Op zondag 16 augustus 1964 ben ik, John Martens, in het brabantse Rijsbergen geboren. In 1982 behaalde ik het VWO diploma aan de Scholengemeenschap (KSE) te Ettenleur en besloot ik in datzelfde jaar biologie te gaan studeren aan de toenmalige Landbouwhogeschool. Na een afstudeervak "Moleculaire Biologie" o.l.v dr. Cees Waalwijk op het voormalige Ital instituut en een stage "Moleculaire Carcinogenese" bij dr. Harry van Steeg en dr. Coen van Kreijl op het Rijks Instituut voor Volksgezondheid en Milieuhygiëne behaalde ik in november 1988 het felbegeerde ingenieurs diploma. Van maart 1989 tot maart 1993 was ik werkzaam als Onderzoeker in Opleiding (OIO), gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), bij de vakgroep Virologie van de Landbouwuniversiteit onder leiding van dr. Just Vlak en prof. Rob Goldbach, alwaar ik het in dit proefschrift beschreven onderzoek heb uitgevoerd. Dit onderzoek werd in nauwe samenwerking uitgevoerd met dr. Bert Visser en dr. Dirk Bosch van het Centrum voor Plantveredeling en Rassen Onderzoek (CPRO) te Wageningen. Na een korte periode (6 maanden) als part-time toegevoegd onderzoeker te zijn geweest eveneens aan de vakgroep Virologie en een iets lagere periode (6 maanden) om dit proefschrift af te ronden ben ik sinds half april 1994 voor de periode van 1 jaar werkzaam als Post-doc bij de vakgroep Endocrinologie en Voortplanting van de Erasmus universiteit te Rotterdam bij prof. Frank de Jong met als hoofddoel de receptor van het hormoon inhibine te karakterizeren cq. te kloneren.