BIOLOGICAL INSECT CONTROL AGENTS EXPRESSING INSECT-SPECIFIC MITE TOXIN GENES, METHODS AND COMPOSITIONS

TECHNICAL FIELD OF THE INVENTION

The present invention relates to methods and compositions for improved biological control of insect pests. More particularly, the present invention relates to the efficient expression of insect-specific toxins coding sequences in baculoviruses for use as biological insect control agents.

BACKGROUND OF THE INVENTION

Interest in the biological control of insect pests has arisen as a result of disadvantages of conventional chemical pesticides. Chemical pesticides generally affect beneficial as well as nonbeneficial species, and insect pests tend to acquire resistance to such chemicals. Furthermore, chemical residues pose environmental hazards and possible health concerns. Biological control presents an alternative means of pest control which can reduce dependence on chemical pesticides.

Baculoviruses are a large group of evolutionarily related viruses which infect only arthropods [Miller, L.K. (1981) in *Genetic Engineering in the Plant Sciences*, N. Panopoulous, (ed.), Praeger Publ., New York, pp. 203-224; Carstens, (1980) *Trends in Biochemical Science* 52:107-110; Harrap and Payne (1979) in *Advances in Virus Research*, Vol. 25, Lawfer et al. (eds.), Academic Press, New York, pp. 273-355, Granados, R. R. and Federici, B. A. eds. (1986) *The Biology of Baculoviruses*, Vol. 1, *Biological Properties and Molecular Biology*, CRC Press Inc., Boca Raton, FL). Some baculoviruses only infect insects

which are pests of commercially important agricultural and forestry crops. Other baculoviruses are known which specifically infect other insect pests, e.g., mosquitoes and fleas. Such baculoviruses are potentially valuable as biological control agents. A potential advantage of baculoviruses as biological pesticides is their host specificity. Because individual baculovirus strains usually only infect one or a few species of insects, they pose little or no risk to man or the environment, and can be used without adversely affecting beneficial insect species.

Baculovirus subgroups include nuclear polyhedrosis viruses, now called nucleopolyhedroviruses (NPVs) and granulosis viruses, now called granuloviruses (GV). In the occluded forms of baculoviruses, the virions (enveloped nucleocapsids) are embedded in a crystalline protein matrix. This structure, referred to as an occlusion body, is the form found extraorganismally in nature, and it is generally responsible for spreading the infection between insects. The characteristic feature of the NPVs is that many virions are embedded in each occlusion body, which is relatively large (up to 5 micrometers). Occlusion bodies of SNPVs (single nucleopolyhedrosis viruses) are smaller and contain a single virion with multiple nucleocapsids each. Multiple nucleopolyedrosis viruses (MNPVs) have multiple nucleocapsids per virion and multiple virions per occlusion body. Granulosis viruses (GVs) have a single viron with one nucleocapsid per occulusion body. The crystalline protein matrix of the occlusion bodies of these forms is primarily composed of a single 25 to 33 kDa polypeptide which is known as polyhedrin or granulin. Gröner et al. in *The Biology of Baculoviruses*, Vol. 1, supra, which is incorporated by reference herein, in Chap. 9, Tables 2 and 7 provides an extensive list of NPV hosts and GV hosts.

In nature, infection is initiated when an insect ingests food contaminated with baculovirus particles, typically in the form of occlusion bodies. The occlusion bodies dissociate under the alkaline conditions of the insect midgut, releasing the virions which then invade epithelial cells lining the gut. Pre-occlusion bodies are also infective (WO 97/08297, published 3/6/97). Within a host cell, the baculovirus migrates to the nucleus where replication takes place. Initially, specific viral proteins are produced within the infected cell via the transcription and translation of so-called "early genes." Among other functions, these proteins are required for the replication of the viral DNA, which begins 4 to 6 hours after the virus enters the cell. Viral DNA replication proceeds up to about 24 hours post-infection (pi). From about 8 to 24 hours pi, infected cells express "late genes" at high levels. These include components of the

nucleocapsid which surround the viral DNA during the formation of progeny virus particles. Production of progeny virus particles begins around 12 hours pi. Initially, progeny virus migrate to the cell membrane where they acquire an envelope as they bud out from the surface of the cell. The nonoccluded virus particles can then infect other cells within the insect. Polyhedrin synthesis begins approximately 18 hours after infection and increases to very high levels by 24 to 48 hours pi. At about 24 hrs pi, there is a decrease in the rate of nonoccluded virus production, and most progeny virus particles are then embedded in occlusion bodies. Occlusion body formation continues until the cell dies or lyses. Some baculoviruses infect virtually every tissue in the host insect so that at the end of the infection process, the entire insect is liquified, releasing extremely large numbers of occlusion bodies which can then spread the infection to other insects. [Reviewed in *The Biology of Baculoviruses*, Vol. I and II, Granados and Federici (eds.), CRC Press, Boca Raton, FL, 1986].

Baculoviruses which are derivatives of AcMNPV and are useful as expression vectors have been described in U.S. Patent No. 5,244,805 (Miller, issued September 14, 1993); Rankin et al. (1988) *Gene* 70:39-49; Ooi et al. (1989) *J. Mol. Biol.* 210:721-736, Thiem and Miller (1990) *Gene* 91:87-95. Particularly strong late and very late promoters include the modified polyhedrin promoter LSXIV, the hybrid Cap/Polh promoter and the synthetic promoter Syn. However, there is a need for baculoviruses which cause insects to cease feeding earlier than prior art baculoviruses so that crop damage is minimized.

Baculoviruses with improved insecticidal properties have been described. For example, AcMNPV in which the *egt* (ecdysone glucosyl transferase) gene has been inactivated causes earlier cessation of feeding and earlier larvae death as compared to larvae infected with wild-type AcMNPV [See, e.g., U.S. Patent No. 5,352,451 (Miller et al., issued October 4, 1994].

Pyemotes tritici, the straw-itch mite, is one of thirteen known species of mites in the genus Pyemotes, all of which are predatory and which possess venoms causing mild to extreme toxicity in target insects. The thirteen known species can be divided into two morphological groups which also differ in host preference, methods of dispersal and toxicity to their target prey, and in the effects of their toxins on insects and man. The *scolyti* and *ventricosus* groups are summarized in Table 1. Most members of the *ventricosus* group have extremely insect-toxic venoms. The mite venoms do not appear to be specific for particular insects, since the venoms are toxic to a wide

variety of insect host and nonhost species. However, the *P. tritici* toxins do not appear to be toxic to mammals.

Insect-specific toxins in the venom of *P. tritici*, have been purified and characterized [Tomalski et al. (1988) *Toxicon* 26:127-132; Tomalski et al. (1989) *Toxicon* 27:1151-1167]. These toxins are produced in female mites and injected into insect prey as components of the venom, resulting in paralysis of the prey, which allows the feeding female mite to become fully gravid, thus ensuring adequate nutrients for reproduction. The toxin designated TxP-I has been purified to apparent homogeneity; it has an apparent molecular weight of 27,000, as determined by SDS-polyacrylamide gel electrophoresis. Two other components were resolved which exhibit molecular weights of 28,000 and 29,000; these two components comprise TxP-II. Based on peptide mapping and immunoblot experiments, it was concluded that the protein components of TxP-I and TxP-II are isoproteins [Tomalski et al. (1989) supra]. DNA sequences encoding *P. tritici* toxin proteins have been isolated and characterized, and expressed in AcMNPV. See, e.g., U.S. Patent No. 5,266,317, which is incorporated by reference herein in its entirety.

Insect-specific neurotoxins have also been found in the venoms of other arthropods including, but not limited to, scorpions, wasps and spiders [Zlotkin (1985) in *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, I. Kerkut and L.I. Gilbert (eds.) Pergamon Press, Oxford, U.K., pp. 499-546]. Several insect-specific toxins (and corresponding coding sequences) from scorpions and other insect predators have also been described [See, e.g., EP 505 207 (published September 23, 1992, Cayley et al.); Maeda et al. (1991) *Virology* 184:777-780; McCutchen et al. (1991) *Bio/Technology* 9:848-852; Stewart et al. (1991) *Nature* 352:85-88]. Merryweather et al. (1990) *J. Gen. Virology* 71:1535-1544 reported the construction of baculovirus containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin expressed the control of the polyhedrin promoter.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-1E presents nucleotide and amino acid sequences of signal sequences tested with the itch mite toxin coding sequences. The signal sequences shown correspond to *tox34* (Fig. 1A; nucleotides 12-56 of SEQ ID NO:1), sarcotoxin IA gene of *S. peregrina* (Fig. 1B; SEQ ID NO:10), the cuticle gene of *D. melanogaster* (Fig. 1C; SEQ ID NO:12), *tox21A* from *P. tritici* (Fig. 1D; nucleotides 119-208 of SEQ ID NO:3) and a modified *tox34* signal sequence (Fig 1E; SEQ ID NO:14). The amino acid sequence of each signal peptide is highlighted by a shaded box

below its corresponding nucleotide sequence. The mature N-terminus of *tox34* is indicated by the amino acid residues in an open box. In the case of the sarcotoxin IA gene signal (Fig. 1B) a glycine residue was introduced onto the mature end of Tox34. Half arrows indicate the location and direction of PCR primers used to generate *tox34* with the *tox21A* signal sequence (Fig. 1D). A shaded box in the nucleotide sequence of A shows the location of a complementary TAAG sequence which was mutated at the base pair marked by an asterisk in Fig. 1E.

Figure 2A-2C presents schematic diagrams showing the polyhedrin gene region of baculovirus recombinants expressing *tox34* with alternate signal sequences (Fig. 2A); under control of different promoters (Fig. 2B); and with a modified native *tox34* signal sequence (Fig 2C). The name of each virus is shown on the left. All recombinant viruses contain the toxin gene inserted into the AcMNPV genome upstream of and in the opposite orientation to the polyhedrin gene (polh) and its promoter (PP). The signal sequence (labeled above) and promoter used to drive toxin expression (labeled below with arrow) are shown for each recombinant.

Figure 3 illustrates the effect of different insect signal sequences on expression and secretion of Tox34 in SF-21 cells. Cell lysates (lanes 1 to 6) or supernatants (lanes 7 to 12) from SF-21 cells infected with the indicated viruses were harvested at 48 h post infection (p.i.) and the proteins were separated by SDS-PAGE and visualized by immunoblotting.

Figures 4A-4B illustrate the effects of different promoters on Tox34 expression and secretion in TN-368 cells. Cell lysates (Fig. 4A) or supernatants (Fig. 4B) from TN-368 cells infected with the indicated viruses were harvested at the indicated times post infection. Proteins were separated by SDS-PAGE, blotted onto a membrane and probed with antibody directed against Tox34. Tox34 and its precursor (pTox34) form are indicated on the right.

Figure 5 provides a comparison of the secreted levels of Tox34 from vDA26tox34- or vHSP70tox34-infected SF-21 or TN-368 cells. SF-21 (lanes 1 to 10) or TN-368 (lanes 11 to 20) cells were infected with vDA26tox34 or vHSP70tox34, and supernatants were collected at the indicated times p.i. Proteins in the supernatant fractions were concentrated, separated by SDS-PAGE and blotted onto a membrane. Tox34 was detected using an antibody specific for purified Tox34.

Figs. 6A-6B illustrates the effects of the complementary TAAG sequence in the native *tox34* signal sequence on expression and

secretion of the Tox34 protein. Proteins in cell lysates (Fig. 6A) or supernatants (Fig. 6B) from TN-368 cells infected with the indicated viruses at 18, 24 and 48 h p.i. were separated by SDS-PAGE, transferred onto membranes, and probed with anti-Tox34 antibody.

Figure 7 is a diagram showing the plasmid transfer vectors used to construct recombinant HzSNPV viruses. Plasmid pHzEGT contains a complete *egt* gene while all others contain deletions and/or insertions in the *egt* gene. Viral sequences were inserted at the EcoRI site of pBluescript KS on the left while the junction at the right is a fusion of a viral *SalI* site and the vector XhoI site. Restriction sites indicated with the viral DNA inserts are *SalI* (S), *Bsu*36I (*Bsu*) and *Sse8387I* (*Sse*). Promoters (hsp70, DA26 or p6.9) are shown by shaded or dark boxes while the foreign gene insert (GUS or *tox34*) are indicated by open boxes.

Figure 8 shows the results for an assay of EGT activity in the culture supernatants removed from infected Hz cells. Cells were either mock infected or infected with HzSNPV or AcNPV. UDP-glucose (Glc) or UDP-galactose (Gal) were used as substrates. The products of the reactions were separated from the substrates by thin layer chromatography. The position of the [^{3}H] ecdysone substrate is indicated (E) on the right as are the positions of the ecdysone-glucose (E-Glc) or ecdysone-galactose (E-Gal) products.

Figure 9A is a diagram of the HindIII restriction map of HzSNPV indicating the position of the IE-1 and *polh* genes [Cowan et al. (1994) *J. Gen. Virol.* 75:3211-3218]. Figure 9B provides the nucleotide sequence of the 5' end of a 2.1 kB *Cla*I fragment of *Hind*III-C which contains the *Bsu*36I site(SEQ ID NO:22).

Figures 10A-10B provide the nucleotide sequence of HzSNPV *egt* gene and the deduced amino acid sequence. The predicted translation start and stop codons and a potential polyadenylation site are in bold type. The *SalI* sites within the EGT coding sequences are indicated by double underlining. See also SEQ ID NOS: 23 AND 24.

Figure 11A is a phylogenetic tree of baculovirus ecdysteroid glycosyl transferases for which sequence information is available. The single most parsimonious tree with length of 1538 and a consistency index of 0.81 was constructed using the Branch and Bound Search program of Paup 3.1 [Swofford, D.L. (1993) Phylogenetic analysis using parsimony. Version 3.1, computer program distributed by the Illinois Natural History Survey, Champaign, IL]. Numbers above the lines are the number of changes between the node and virus, while the underlined

numbers below the lines indicate the frequency of that cluster after bootstrap analysis with 100 replicates. Figure 11B presents aligned amino acid sequences of baculovirus ecdysteroid UDP glucosyl transferases. The EGT sequences analyzed include those of AcMNPV [O'Reilly and Miller (1990) *J. Virol.* 64:1321-1328]; *Buzura suppressaria* NPV, BsSNPV [Hu et al. (1997) *Virus Res.* 47:91-97]; *Bombyx mori* NPV, BmNPV [Genbank Accession No. L33180]; *Choristoneura fumiferana* NPV, CfMNPV and its associated defective virus, CfDEF [Barrett et al. (1995) *J. Gen. Virol.* 76:2447-2456]; *Lymantria dispar* NPV, LdMNPV [Riegel et al. (1994) *J. Gen. Virol.* 75:829-838]; *Mamestra brassicae* NPV, MbMNPV [Clarke et al. (1996) *J. Gen. Virol.* 77:2865-2871]; *Orgyia pseudotsugata* NPV, OpMNPV [Ahrens et al. (1997) *Virology* 229:381-399; Genbank Accession No. U75930]; *S. littoralis* NPV, SlMNPV [Faktor et al. (1995) *Virus Genes* 11:47-52] and *Lacanobia oleracea* GV, LoGV [Genbank Accession No. Y08294].

SUMMARY OF THE INVENTION

It is an object of the present invention to provide baculoviruses which have been genetically engineered to contain and express insect-specific toxin genes under the control of promoters which allow the expression of the toxin coding sequence such that there is sufficient expression of the toxin sequence at a time earlier than enabled by prior art viruses, with the result that infected insects stop feeding and die sooner than with infection by prior art viruses. As specifically exemplified, the toxin genes expressed are those from insect-parasitic mites such as those of the genus *Pyemotes*, particularly those from *ventricosus* group of the *Pyemotes*. In a specific embodiment the insect-specific paralytic neurotoxin coding sequence is Tox34 derived from *Pyemotes tritici*; this coding sequence is provided in SEQ ID NO:1; a second specific embodiment of an insect paralytic neurotoxin coding sequence is termed Tox21a herein (SEQ ID NO:3; amino acid sequence, SEQ ID NO:4), also from *Pyemotes tritici*. It will be understood in the art that other insect-specific paralytic neurotoxin coding sequences from mites can be isolated and identified by nucleotide sequence homology, as determined in hybridization experiments [See, e.g., Hames and Higgins (1985) *Nucleic Acid Hybridization*, IRL Press, Washington, DC] employing sequence information provided herein. Insect-specific paralytic toxin coding sequences from insect-predacious mites which have at least 70% nucleotide sequence homology to the coding sequence in SEQ ID NO:1 and which encode toxins with substantially similar biology activity in insects are within the scope of the present invention.

As disclosed herein, the recombinant HzSNPV in which the itch mite toxin coding sequence is expressed under the regulatory control of the AcMNPV 6.9K promoter or a heat shock promoter, preferably from an insect heat shock gene or gene family such as *hsp70*, *hsp83*, *hsp22* or *hsp23*. A preferred heat shock promoter is the *Drosophila melanogaster hsp70* promoter. The baculoviruses in which an insect-specific toxin gene is expressed under the control of a promoter such as the *D. melanogaster hsp70* promoter or the AcMNPV 6.9K promoter (or a DA26 promoter) are improved over prior art baculoviruses as insect control agents. These recombinant HzSNPV derivatives cause insect paralysis and death sooner than those constructs in which a mite toxin coding sequence is expressed under the control of very late promoters such as the polyhedrin promoter.

Genetically engineered baculoviruses for insect control other than the exemplified AcMNPV and HzSNPV can be produced using the teachings of the present Specification taken with what is well known to the art. Toxins other than those encoded by the *tox34* and *tox21a* coding sequences can be inserted under the regulatory control of a heat shock promoter, preferably the *hsp70* promoter or *6.9K* or a DA26 promoter promoter as disclosed herein to produce killing properties when compared with baculoviruses in which the toxin coding sequences are inserted under the regulatory control of late or very late promoters.

The invention also includes a recombinant DNA molecule comprising a coding sequence for an insect-specific paralytic neurotoxin wherein said encoded insect-specific paralytic neurotoxin is expressed under the regulatory control of a heat shock promoter, preferably from an animal, more preferably from an insect, especially the *Drosophila hsp70* promoter or the AcMNPV *6.9K* promoter. A DA26 promoter can also be used. In particular, the *hsp70* promoter is highly expressed in insect cells from a wide variety of species. Thus, genetically engineered baculoviruses expressing an insect-specific toxin under the regulatory control of this promoter are surprisingly improved in the time at which paralysis occurs and in terms of the insect species in which such a genetically engineered baculovirus is effective as an insect control agent. Particularly preferred embodiments of such a genetically modified baculovirus are those AcMNPV and HzSNPV derivatives which are occluded; preoccluded viruses are also useful in insect toxic compositions and methods for control of insect pests using same. For any example of a nonoccluded nuclear polyhedrosis or granulosis virus derivative, the skilled artisan understands how to construct an analogous occluded virus without the expense of undue experimentation.

Since there is significant homology among some genes of different baculoviruses, the skilled artisan will also understand how to insert the toxin gene, fused to an appropriate promoter, into the genomes of other baculoviruses in similar nonessential locations.

Accordingly, the invention includes a baculovirus which has been genetically modified to contain and express a gene encoding an insect-specific toxin, preferably a paralytic neurotoxin, under the regulatory control of a promoter which allows strong gene expression at a time relatively early after infection, especially as compared with other promoters such as that of the polyhedrin gene.

As specifically exemplified, the invention also provides a baculovirus which has been genetically modified to contain and express a coding sequence for an insect-specific paralytic neurotoxin of a mite of the genus *Pyemotes*, specifically from a mite of the species *Pyemotes tritici*. A specifically exemplified toxin coding sequence has a nucleotide sequence as given in SEQ ID NO:1 from an aspartate encoded at about nucleotide 120 to a cysteine encoded at about nucleotide 873. An alternative coding sequence for an insect-specific neurotoxin of an insect-predacious mite is that as shown in SEQ ID NO:3, from an aspartate encoded at about nucleotide 120 to a cysteine encoded at about nucleotide 873. Also within the scope of the present invention are mite toxin coding sequences having at least about 70% nucleotide sequence identity to the exemplified coding sequences as provided in SEQ ID NO:1 and SEQ ID NO:3.

As exemplified, the baculovirus derivative is an NPV baculovirus, specifically, an AcMNPV derivative or an HzSNPV derivative.

Another object of the invention is an insect-toxic composition comprising an insect-toxic amount of a baculovirus, genetically engineered to express an insect-specific paralytic neurotoxin at a level that results in a toxic effect on a targeted insect, and an agriculturally or otherwise environmentally acceptable carrier. Such compositions can be employed for protection of plants from insect pests. Preferred control agents are those which express an insect-specific paralytic neurotoxin gene from an insect-parasitic mite, and particularly those mites of the genus *Pyemotes*. It is preferred that the baculovirus particles are present in the occluded or preocculuded form. As specifically exemplified, the baculovirus derivative is an AcMNPV derivative or an HzSNPV derivative, and the recombinant baculovirus expresses an insect-specific toxin at an insect-toxic or insect-paralyzing level at a time sooner than that enabled by prior art viruses.

It is a further object of the invention to provide a method for the biological control of an insect pest comprising the step of applying an insect-toxic composition which contains an insect-toxic amount of a baculovirus which has been genetically engineered to express an insect-selective toxin gene such as an insect-specific paralytic neurotoxin gene from an insect-parasitic mite in an effective amount at a time earlier than enabled by prior art viruses. Such an insect-toxic composition is applied in the vicinity of a targeted insect, an insect habitat or to an area, plant or environment that is to be protected from the insect pest. The amount of said baculovirus derivative in said composition and the level of expression of said toxin coding sequence the baculovirus are such that said composition produces a toxic effect in a targeted insect, resulting in a reduction or, more preferably, a cessation of feeding. Preferred baculovirus derivatives include AcMNPV derivatives and HzSNPV derivatives. The occluded forms of genetically altered nuclear polyhedrosis viruses are most useful in the present invention. The skilled artisan understands that the genetically altered virus expressing the insect toxin may itself be capable of occlusion or that occlusion may be achieved by other means, e.g., by coinfection with an occlusion-positive virus. Useful promoters for toxin coding sequence expression include the heat shock promoters, preferably those from the animal kingdom, more preferably from an insect, and desirably from the *hsp70*, *hsp83*, *hsp22* and *hsp23* gene families, e.g., a *D. melanogaster hsp70* promoter. Particularly preferred for use in the control of insect-specific toxin gene expression are those heat shock promoters which are relatively strongly constitutively expressed. However, a number of heat shock promoter sequences are well known and available to the art. Preferably, the insect toxin coding sequence is expressed under the regulatory control of the *Drosophila melanogaster hsp70* promoter or the AcMNPV *6.9K* promoter. The invention includes a method for the control of insect pests comprising the step of applying an insect-toxic amount of the insecticidal composition of the present invention to a habitat of said insect pests, for example, to plants.

Similarly, it is an object to provide baculoviruses which are genetically altered to express an insect-specific paralytic neurotoxin coding sequence, which are effective against insect pests other than those which attack or are harmful to plants. Such an agent can be incorporated into insect-toxic, insect-paralytic, or insecticidal compositions along with environmentally acceptable carriers as understood in the art, and can be used in a method to control a target insect pest susceptible to the particular baculovirus employed. For example, there are baculoviruses known to specifically infect each of mosquitoes and fleas. See, Beard et al. (1989) *J. Invertebrate Path.*

54:128-131 and Federici (1980) *Virology* 100:1-9. The target insect guides the ordinary skilled artisan in the selection of the particular baculovirus modified to express paralytic toxin.

Especially preferred in the recombinant baculoviruses, insecticidal compositions and methods of the present invention are those baculoviruses in which an insect-specific neurotoxin coding sequence is expressed and in which an ecdysteroid UDP-glycosyl transferase gene has been inactivated.

DETAILED DESCRIPTION OF THE INVENTION

A biological insect control agent is an agent effective in the control of insect pests. As used herein, insect control agents include baculoviruses which have been genetically modified to express an insect-specific toxin, preferably an insect-specific paralytic neurotoxin, in a way that leads to a cessation of feeding, insect paralysis or insect death at a time sooner than prior art baculoviruses due to the use of promoters which promote higher levels of toxin gene expression and at a time earlier than with previously described baculoviruses.

Control can refer to limitation of feeding behavior or to killing of an insect pest. A biological insect control agent of the present invention has an insect-toxic effect that is attributable at least in part to the expression of an insect-specific toxin coding sequence. An insect-toxic effect relates to any adverse effect on a targeted insect and is observable as paralysis and/or killing of that insect or as a change in normal behavior of the targeted insect such as in feeding behavior, righting response or other stereotypic behaviors. This toxic effect occurs due to early and efficient expression of such a toxin coding sequence.

Insect-predacious mites are those mites which feed on insects. Many of such mites inject venom into the insect hosts on which they feed. Such venom contains insect-specific paralytic neurotoxins to immobilize the host insects. Mites expressing insect-specific paralytic toxin genes include those within the *ventricosus* group including *P. anobii*, *P. beckeri*, *P. emerginatus*, *P. schwerdtfegeri*, *P. tuberculatus*, *P. tritici*, *P. ventricosus* and *P. zwoelferi*.

An insect-specific paralytic neurotoxin is a polypeptide which causes paralysis of a sensitive insect larva or adult, but has no significant toxic effect on other organisms. The paralytic effect may initially be observed as an effect on mobility or other behaviors of

the insect, including feeding behavior. Insect-specific neurotoxins are those which adversely affect insects, and have negligible effects on higher animals, especially mammals. The insect-specific paralytic neurotoxin of this invention is specifically exemplified by Tox34 and Tox21a, and/or the TxP-I and TxP-II proteins produced by *P. tritici*. The deduced amino acid sequences for two representative insect-specific paralytic proteins are presented in SEQ ID NO:2 and SEQ ID NO:4. A toxin that is functionally equivalent to the neurotoxins of this invention effects a similar muscle contractile paralysis in insects as is caused by Tox34 and Tox21a. It is well known in the biological arts that certain amino acid substitutions can be made in protein sequences without affecting the function of the protein. Generally, conservative amino acid substitutions or substitutions of similar amino acids are tolerated without affecting protein function. Similar amino acids can be those that are similar in size and/or charge properties, for example, aspartate and glutamate and isoleucine and valine are both pairs of similar amino acids. Similarity between amino acid pairs has been assessed in the art in a number of ways. For example, Dayhoff et al. (1978) in *Atlas of Protein Sequence and Structure*, Vol. 5, Suppl. 3, Chapter 22, pages 345-352, incorporated by reference herein, provides frequency tables for amino acid substitutions which can be employed as a measure of amino acid similarity. Dayhoff et al.'s frequency tables are based on comparisons of amino acid sequences for proteins having the same function from a variety of evolutionarily different sources.

Additional functional equivalents of insect-specific paralytic neurotoxins as defined herein include polypeptides with portions of amino acid sequences with substantial identity to Tox34 or Tox21a or polypeptides which themselves are a portion of a full length TxP-I protein or which have the amino acid sequence of a Tox34 or Tox21a protein into which an insertion has been made, and which retain the biological activity, in these examples, contractile muscle paralysis.

Insect-specific paralytic neurotoxin genes may be found in insect-predacious mites, including but not limited to those listed in Table 1, particularly those within the *ventricosus* group, or in other insect parasites or predators. Genes homologous to the *tox34* and *tox21a* genes of the present invention may be identified in mites or other sources by nucleic acid hybridization to sequences disclosed in the present invention or by cross-reaction of toxin molecules with antibody specific for the toxins of the present invention or by any other means known to the art, including the use of PCR technology carried out using oligonucleotides corresponding to conserved or unambiguous regions of the toxin gene(s) exemplified herein. In

principle, any insect-specific paralytic neurotoxin gene may be identified and that gene expressed in a baculovirus vector. Biological activity of the expressed protein can be readily determined and similarly, the efficacy of such a genetically modified vector can be assessed using the teachings of the present invention in combination with techniques well known to the art.

Other known insect-specific toxins include those from scorpions and spiders [see, e.g., Bougis et al. (1988) in *Proc. World Congress on Animal Natural Toxins*, pp. 94-101; EP 417,906; and EP 507,207]. Functional equivalents of published coding sequences and recombinant baculoviruses can be generated by the skilled artisan using techniques and information well-known to the art, e.g., in a manner similar to that described hereinabove for the mite toxins.

A recombinant DNA molecule, as used herein, does not occur in nature, and it is one which has been produced either by natural processes using known methods and directed by man to produce a desired result or artificially produced from parts derived from heterologous sources, which parts may be naturally occurring or chemically synthesized molecules, and wherein those parts have been joined by ligation or other means known to the art.

Genetically modified to contain and express an insect-specific toxin gene, such as an insect-specific paralytic neurotoxin gene, means that nucleotide sequences encoding such a protein and directing its synthesis are introduced into a baculovirus genome so that the modified baculoviruses can produce that neurotoxin protein. Any means known to the art may be used to insert the expressible neurotoxin gene into a particular baculovirus.

In the present invention, promoter and/or promoter-associated sequences direct gene expression, i.e., control transcription and translation of a nucleotide sequence encoding an insect-specific toxin in the infected target insect. Particularly preferred promoters are heat shock promoters such as those from the *hsp70*, *hsp83*, *hsp22* and *hsp23* gene families, especially the *Drosophila melanogaster hsp70* promoter, and the AcMNPV (or other baculovirus) *6.9K* promoter. Alternatively a baculovirus DA26 promoter can be used.

It will be understood that the goals of a skilled artisan will determine the choice of particular regulatory sequences and/or promoters. For example, with baculovirus promoters, if high levels of expression are required, then an especially strong promoter, expressed at a very early time after infection and in a wide variety of insect

cells, is appropriate. This is consistent with the goal of limiting the feeding of an insect larva to the shortest possible time (or to extend the effective host range of the insect virus).

An NPV baculovirus isolated from *Autographa californica* (Lepidoptera: Noctuidae), specifically AcMNPV, is exemplified in the present disclosure. The terms AcMNPV and AcNPV are synonymous. The infectivity of most NPVs is reported to be restricted to members of the genus, family or order of the original host. AcMNPV baculoviruses replicate in several families of Lepidoptera, but their infectivity is reported to be limited to that order. A second specifically exemplified baculovirus modified to achieve improved efficacy as an insecticide, is HzSNPV, which was isolated from the cotton bollworm, *Helicoverpa zea*. HzSNPV infects and kills most species of *Helicoverpa (Heliothis)*. In the mid 1970s, HzSNPV was registered and commercially produced as a pesticide (Elcar™) by Sandoz Corp. to control infestations of the cotton bollworm [Ignoffo, C.M. (1981) Living Microbial insecticides. In: *Essays in Applied Microbiology* (eds. J.R. Norris and M.H. Richmond) John Wiley & Sons, New York, pp. 2-31]. This product, however, did not compete successfully with the newly introduced pyrethroid pesticides. The poor field performance of Elcar™ was due, in part, to the slow rate that the virus killed the insect pest compared to contact chemical pesticides; the delay between virus application and insect death can result in significant crop damage [reviewed by Miller, L.K. (1995) *J. Invertebr. Pathol.* 65:211-216].

The art understands how to insert an expressible gene into a viral genome at a site which does not interfere with viral replicative functions. Similarly, the skilled artisan can select a promoter with desired strength and temporal expression to drive the expression of an insect-specific toxin gene in a desired baculovirus vector. The target insect dictates the virus selected, and the particular virus to be engineered will guide the skilled artisan in the selection of an appropriate promoter.

A number of promoters have been used to control the expression heterologous coding sequences in recombinant baculovirus systems. The three classes of viral promoters for viruses such as AcMNPV are early, late and very late promoters [see, e.g., Morris and Miller (1992) *J. Virol.* 66:7397-7405]. Early promoters include the ETL promoter of AcMNPV, which has been described in U.S. Patent No. 5,266,317, the DA26 promoter and the IE0, IE1 and IEN promoters [O'Reilly et al. (1990) *J. Gen. Virol.* 71:1029-1037; Carson et al. (1991) *J. Virol.* 65:945-951; Kovacs et al. (1991) *J. Virol.* 65:5281-5288. Late AcMNPV promoters include 6.9K, the capsid (*vp39*) promoter [Hill-Perkins and Possee

(1990) *J. Gen. Virol.* 71:971-976; Thiem and Miller (1989) *J. Virol.* 63:2008-2018]. Very late promoters include the polyhedrin and a synthetic promoter [U.S. Patent No. 5,244,805, L. Miller, issued 1993] and the modified polyhedrin promoter LSXIV [Ooi et al. (1989) *J. Molec. Biol.* 210:721-736; U.S. Patent No. 5,244,805 (L. Miller, issued 1993]. Very late baculovirus promoters, as exemplified in AcMNPV, include the polyhedrin and p10 promoters [Kelly and Lescott (1981) *Microbiologica* 4:35-57; Miller, L.K. (1988) *Ann. Rev. Microbiol.* 42:172-199; Bonning et al. (1994) *J. Gen. Microbiol.* 75:1551-1556]. See also Thiem and Miller (1990) *Gene* 91:87-94 for further discussion of late and very late gene expression. See also *The Baculoviruses*, ed., L.K. Miller, Plenum Press, New York, 1997.

A heat shock promoter with a significant level of constitutive expression of a downstream, operably linked coding sequence is exemplified by *hsp70* promoters, in particular, the *D. melanogaster hsp70* promoter [See, e.g., Toerek and Karch (1980) *Nucl. Acids Res.* 8:3105-3123]. This promoter has been used for the expression of heterologous coding sequences in recombinant baculovirus vectors with varying degrees of success [Morris and Miller (1992) supra].

For a general discussion of heat shock genes, their promoters and heterologous expression driven by them, see, e.g., Nover, L. (1987) *Enzym. Microb. Technol.* 9:130-144; Amin et al. (1988) *Mol. Cell. Biol.* 8:3761-3760, and references cited in said references, all of which are incorporated by reference herein in their entireties. It is a general property of *hsp70*, in Drosophila and in other organisms that there is a relatively high constitutive level of downstream gene expression. *hsp70* promoter and promoter-associated sequences from a variety of insect, animal, plant and yeast sources are well known in the art.

In the context of the present application, a recombinant DNA molecule is produced via human intervention, and it contains nucleotide sequences which in nature are not covalently joined or associated. Chemical synthesis or *in vitro* enzyme ligation can effect the joining, or recombination can be accomplished where the input sequences are introduced into a single cell in the laboratory and predicted resulting progeny are analyzed and purified.

As used herein, an insect control agent is a composition or the active ingredient of a composition which has an adverse affect on insect pests. Feeding by insects is reduced in response to the genetically engineered baculoviruses of the present invention as a result of the expressed toxin, and death of the insect follows. An insect control agent of this invention preferably is an insect virus

genetically engineered to express a heterologous gene encoding an insect-specific toxin. Specific examples of such toxin proteins include, but are not limited to, Tox34 and Tox21a, with the amino acid sequences disclosed in SEQ ID NO:2 and SEQ ID NO:4, respectively, as well as known scorpion and spider toxins.

To determine if HzSNPV possessed and expressed an *egt* gene, the presence of EGT activity in supernatants from uninfected and infected HzUNDK cells was determined by enzymatic assays using [^{3}H] ecdysone and UDP-glucose or UDP- galactose as substrates. Using UDP-glucose as a substrate, the supernatants from ACMNPV- and HzSNPV-infected cells converted ecdysone to a product of altered polarity which was previously identified as an ecdysone-glucose conjugate (Fig. 8). This EGT activity was not observed in uninfected HzUNDK cells, indicating that HzSNPV induced an EGT activity during infection and possessed an *egt* gene. When UDP-galactose was used as a substrate, the EGT activity in AcMNPV-infected cell extracts was able to form an ecdysone-galactose conjugate, but HzSNPV-infected cells were unable to use UDP-galactose as a substrate (Fig. 8), indicating a difference in the substrate specificities of the AcMNPV and HzSNPV EGTs [O'Reilly et al. (1992) *Insect Biochem. Molec. Biol.* 22:313-320].

Restriction digests of the Elcar isolate of HzSNPV were identical to those isolates previously described bv Knell and Summers (1984) *J. Gen. Virol.* 65:445-450 and Cowan et al. (1994) *J. Gen. Virol.* 75:3211-3218. A set of overlapping cosmids representing the entire HzSNPV genome was isolated and characterized. The basic physical map of the HzSNPV genome was confirmed and further refined (Fig. 9A). The genes in the polyhedrin gene (*polh*) region, e.g. the IE1 gene, are known to be in reverse order to that of the conventional AcMNPV map [Cowan et al. (1994) supra]. Using the ACMNPV *egt* gene as a hybridization probe on Southern blots failed to give a strong hybridization signal under the conditions used. As an alternate approach to mapping the *egt* gene, cosmid plasmid clones corresponding to regions flanking the *polh* gene were screened by PCR using degenerate oligonucleotide primers which were constructed based on consensus sequences from conserved regions of the *egt* gene. PCR amplication using an HzSNPV cosmid which spanned *Hind*III-J through -E fragments and a plasmid clone containing the *Hind*III-C fragment as templates generated a PCR product of the size expected for the *egt* gene. One of the PCR products was cloned and sequenced and found to contain a portion of the *egt* gene. The sequences generated were used to prepare oligonucleotide primers which allowed the sequencing of the entire HzSNPV *egt* gene. The *egt* gene was located entirely within the *Eco*RI Q fragment (Fig. 9A) and is transcribed from the same strand as the *polh* and IE1 genes. The DNA

sequence of HzSNPV *egt is* presented in Fig. 10A-10B; see also SEQ ID NOs: 23 and 24. The percent sequence identity and similarity of HzSNPV EGT with other known EGTs are presented in Table 7, and Fig. 11 presents a phylogenetic tree based on amino acid sequence comparisons of several baculoviral *egt* genes.

The HzSNPV genome was found to have no *Sse*8387I sites and a single *Bsu*36I site which was located within the *Hind*III-C fragment (Fig. 9A). The sequence surrounding this restriction site was determined (Fig. 9B). The region contained no open reading frames (ORFS) of 50 codons or more and exhibited no homology with other known viral genes by computer analysis. This site was eliminated from the virus genome by restriction digestion, filling-in the 3 bp cohesive ends, and religation. The infectivity and virulence of the resulting virus lacking the *Bsu*36I site, HzSNPV(*Bsu*36I'), was determined by measuring the LC_{50}s and ET_{50}s, respectively, in *H. zea* neonates. Duplicate LC_{50} assays (Tables 6 and 7) showed that the elimination of the *Bsu*36I site had little or no effect on the infectivity of the virus, and duplicate ET_{50} assays (Tables 8 and 9) showed that the virulence of the modified virus was also essentially unaltered. The *Bsu*36I' virus was then used for the construction of all subsequent virus constructions since the ability to insert genes into the viral genome by direct cloning offers a rapid and useful means of constructing viral recombinants [Ernst et al. (1994) *Nucl. Acids Res.* 22:2855-2856; Lu and Miller (1996) *Biotechniques* 21:63-68] and insertion of *Bsu*36I and *Sse*8387I sites into the *egt* region of the *Bsu*36I' virus simplifies direct cloning into this region.

The next step in genetically engineering HzSNPV was to insert a marker gene, the *E. coli* GUS gene, into the *egt* gene of HzSNPV and screen for viruses which produced a blue color in the presence of the GUS indicator, X-Gluc (see Example 4). This virus was then used as a parental virus to construct additional HzSNPV recombinants using the presence of white plaques as a screen for double-crossover recombinants in allelic replacement.

An *egt* deletion virus, three *tox34*-expressing recombinant viruses and an *egt* deletion virus containing *Bsu*36I and *Sse*8387I sites within the *egt* locus were constructed by allelic replacement using the appropriate transfer plasmids (see Fig. 7). Each of the *tox34*-expressing recombinants contained a different promoter: the *D. melanogaster* hsp70 promoter, the early AcMNPV DA26 (ORF 16) viral promoter or the late AcMNPV p6.9 gene (ORF 100) promoter. The infectivities and virulence of these toxin-expressing viruses were compared to HzSNPV, HzSNPV (*Bsu*36I') and *egt* deletion mutant (HzEGTdel)

virus in *H. zea* neonates (Tables 8-11). The LC_{50}s were found to be similar, if not identical, for all the viruses tested. All viruses containing *tox34* had significantly lower ET_{50}s, indicating that all three promoters used were functional in *H. zea* larvae and that toxin gene expression reduced the ET_{50}s substantially. The recombinant expressing *tox34* under the control of the viral DA26 promoter exhibited a lower ET_{50} than the recombinant containing the *tox34* gene under the control of the *Drosophila* hsp70 promoter in this species. The AcMNPV p6.9 promoter also consistently performed better than the hsp70 promoter and was similar to, although possibly slightly less effective than, the AcMNPV DA26 promoter. The vEGTDA26*tox34* virus decreased the ET_{50} to less than 40 h, 40% less than wt HzSNPV. The ET_{50}s for HzEGTdel was not lower than those for HzSNPV wt and HzSNPV (*Bsu36I'*).

The HzSNPV *egt* gene is located between 93.1 and 94.5 m.u. of the HzSNPV map and is predicted to encode a 515 amino acid polypeptide with less than 50% sequence identity with other known baculovirus EGTs. Like other baculovirus *egt* gene products [O'Reilly et al. (1992) *Insect Biochem. Molec. Biol.* 22:313-320], it is predicted to have a cleavable signal sequence at its amino-terminus and lacks a C-terminal transmembrane domain. The HzSNPV EGT has the seven amino acids which are found to be absolutely conserved among all EGTs and UDP-glycosyltransferases [O'Reilly, D.R. (1995) *Insect. Biochem. Molec. Biol.* 25:541-550]. Domain II of EGTs [O'Reilly (1995) supra] from amino acid residues 254-267, is the most conserved region among all baculovirus EGTs including the HzSNPV EGT. In contrast to the AcMNPV EGT [O'Reilly et al. (1992) *Insect Biochem. Molec. Biol.* 22:313-320], the HzSNPV EGT is unable to use UDP-galactose as a substrate. The site for UDP-sugar binding is predicted to lie within domains III and IV of the EGT polypeptides (O'Reilly (1995) supra]. HzSNPV EGT shares the most sequence identity to the MbNPV EGT (70%) and the most similarity to SlNPV (Table 7; see also Fig. 11A for phylogenetic tree).

Although deletion of the AcMNPV *egt* gene reduced the LT_{50} of this virus in two different species [O'Reilly and Miller (1991) *BioTechnology* 9:1086-1089], a similar reduction in the rate of mortality was not observed for the HzSNPV *egt* deletion mutant in *H. zea* neonate larvae. Because wild-type HzSNPV acts more quickly than wild-type AcNPV in its respective host, the effect of *egt* (e.g. 15% reduction in LT_{50}) appears too subtle to be observed in this species or in this larval instar. Expression of *egt* is known to block the molting of the host insect [O'Reilly and Miller (1991) supra] and also prevents the degeneration of the malpighian tubules during the infection process [Flipsen et al. (1995) *J. Virol.* 69:4529-4532].

Promoter-dependent effects on *tox34* expression and larval paralysis have been previously reported in AcMNPV [Tomalski and Miller (1992) *BioTechnology* 10:545-549; Lu et al. (1996) *BioTechniques* 21:63-68]. Both the *Drosophila* hsp70 and viral p6.9 promoters were considerably more effective than the *polh* promoter in reducing the ET_{50} of AcMNPV in both *T. ni* and *S. frugiperda*, and the relative effectiveness of the p6.9 and hsp70 promoters was species-dependent [Lu et al. (1996) *Biol. Control.* 7:320-332]. The DA26 promoter, however, was less effective in reducing the ET_{50} of AcMNPV in these two species than the *polh*, p6.9 or hsp70 promoters. We compared the effects of placing *tox34* under the control of *Drosophila* hsp70, AcMNPV DA26, or AcMNPV p6.9 promoters within the context of the HzSNPV genome in *H. zea* neonates and found that all toxin-expressing recombinant viruses had a reduced effective time to paralysis/mortality relative to wt HzSNPV. The most effective viral promoter was AcMNPV DA26 although it was only slightly more effective than the AcMNPV p6.9 promoter. The *D. melanogaster* hsp70 promoter was somewhat less effective under these conditions than either the hsp70 or DA26 promoters. Both early and late AcMNPV promoters were effective in the context of the HzSNPV genome. Without wishing to be bound by theory, it is predicted that the equivalent HzSNPV promoters are as or more effective than the AcMNPV promoters.

We have successfully improved the properties of HzSNPV as a pesticide through genetic engineering technology to provide the first recombinant HzSNPV. With an ET_{50} of less than 40 hrs, the HzDA26tox34 recombinant is the fastest acting baculovirus reported to date. Genetically engineered HzSNPV derivatives are also useful as gene expression vectors.

The mature form of the Tox34 protein secreted from insect cells infected with recombinant AcMNPV expressing *tox34* [Tomalski and Miller (1991) *Nature* 352:82-85] is thought to be the same form as that produced by mites. TxP-I is synthesized as a precursor protein of 291 amino acids; the first 39 amino acids are a signal sequence absent from the mature secreted product. Since mature Tox34 is a secreted protein that must interact with the secretory pathway of infected insect cells, we investigated the influence of different signal peptides on expression and secretion of Tox34. Secretion of some heterologous proteins from baculovirus-infected cells appears to be affected by the nature of the signal sequence [Tessier et al. (1991) *Gene* 98:177-183; O'Reilly et al. (1995) *Insect Biochem. Mol. Biol.* 25:475-485].

Three different signal sequences derived from the sarcotoxin IA gene of *Sarcophaga peregrina* (flesh fly), the cuticle gene of *D.*

melanogaster, and a homolog of *tox34* (*tox21A*) were substituted for the native *tox34* signal sequence (see Figs. 1A-1E) and placed under control of the modified polyhedrin promoter P_{synXIV} in AcMNPV (Fig. 2A-2C). The levels of Tox34 expressed in recombinant virus-infected SF-21 cells and secreted into the tissue culture media at 48 h pi were compared by Western blot analysis using polyclonal antibody specific for Tox34 (Fig. 3). Intracellular levels of toxin produced at 48 h p.i. varied (Fig. 3, lanes 1-6). Expression from vSp-BSigtox34 produced less intracellular toxin protein (Fig. 3, compare lanes 2 and 3) while vSp-DCtox34 and vSp-tox34 infected-cells produced similar amounts of intracellular toxin (Fig. 3, compare lanes 2 and 4). In contrast, intracellular levels of toxin in vSp-tox21A/tox34 infected cells were approximately 3-fold higher than in vSP-tox34 infected cells (Fig. 3, lanes 2 and 5). No Tox34 was detected in wild-type AcMNPV infected cells (Fig. 3, lane 1). The 15-fold reduction of intracellular toxin in vSp-tox21A infected cells relative to vSp-tox34 (Fig. 3, lane 6) probably reflects inefficient recognition of Tox21A by the Tox34 antibody. Toxin produced in and secreted from cells infected with vSp-BSigtox34 and vSp-DCtox34 appear to be about 5 kDa smaller than Tox34. Without wishing to be bound by any particular theory, this is believed to represent differences in the site or efficiency of cleavage governed by the alternate signal sequences. The N-terminal sequence of TxP-I is known [Tomalski et al. (1989) *Toxicon* 27:1151-1167]; the N-terminal sequence of mature recombinant Tox34 has not be empirically determined.

The heterogeneity in sizes of intracellular Tox34 in vSp-tox21A/tox34 infected cells has been observed previously [Tomalski and Miller (1991) *Nature* 352:82-85; Tomalski and Miller (1992) *Bio/Technology* 10:545-549] and probably represents precursors of the mature Tox34 protein. The ratio of the precursor to the mature forms of Tox34 increases with time p.i. [Tomalski and Miller (1992) supra], likely reflecting the fact that the cell's secretory pathway becomes functionally compromised during the later phases of infection [Jarvis et al. (1993) *J. Biol. Chem.* 268:16754-16762]. Because there are 3-fold higher levels of toxin expressed in cells infected with vSp-tox21A/tox34 than with vSp-tox34, the presence of these heterogenous products suggests that the former is expressed at higher levels than the latter and/or that the *tox21A* signal sequence is not efficiently processed. Both scenarios can lead to an accumulation of unprocessed forms of Tox34 in vSp-tox21A/tox34 infected cells.

None of the changes to the signal sequence of *tox34* ultimately increased the amount of secreted toxin. The amount of Tox34 secreted by vSp-tox34, vSp-DCtox34, and vSp-tox21/tox34 infected cells at 48 h p.i. was similar (Fig. 3, lanes 8, 10, 11) while Tox34 was undetectable

in the supernatant fractions of vSp-BSigtox34 and vSp-tox21A infected cells (Fig. 3, lanes 9 and 12). Again, and without wishing to be bound by any particular theory, the apparent absence of Tox21A is believed to be due to poor reactivity with the Tox34 antibody. The low levels of secreted toxin from vSp-BSigtox34 infected cells is believed due to translational or post-translational problems since the levels of the toxin transcripts appear to be similar. The additional glycine residue added to the N-terminal sequence of the mature toxin may be responsible for this effect.

Neonate *T. ni* larvae were infected *per os* with occluded virus from each recombinant virus to evaluate the effect of changing the signal sequence of *tox34* on the LC_{50} (concentration of occluded virus required to kill 50% of the test insects) and the ET_{50} (time required to paralyze or kill 50% of the test insects) of each recombinant virus (Table 2).

No significant differences in the LC_{50} for the different viruses were observed; thus, the expression of *tox34* does not compromise ability to initiate infection relative to wild-type. In all cases, *per os* infection of *T.ni* larvae by *tox34* expressing viruses resulted in paralysis. vSp-tox34 and vSp-DCtox34 infected larvae were paralyzed approximately 50% faster than wild-type AcMNPV, vSp-tox21A and vSp-tox21A/tox34 about 35% faster than wild-type, and vSp-BSigtox34 about 25% faster. The ET_{50} of vSp-tox34 is in close agreement with previously published results [Tomalski and Miller (1992) supra]; however, the ET_{50} of vSp-tox21A is approximately 15 h longer than what was reported for the same construct by Tomalski et al. (1993) *Toxicon* 31:319-326.

The ET_{50} values for vSp-tox34 and vSp-DCtox34 correlate well with the levels of toxin detected in the supernatants of infected cells; however, the ET_{50} of vSp-tox21A/tox34 was longer than vSp-tox34 and vSp-DCtox34 even though similar levels of toxin were secreted. This suggests that not all of the secreted toxin from vSp-tox21A/Tox34 infected cells is biologically active. Although no toxin was found in the supernatant from vSp-BSigtox34 infected cells, neonate *T. ni* larvae infected *per os* with vSp-BSigtox34 exhibited paralysis, indicating that the threshold level of toxin required for paralysis of *T. ni* neonates is low or that some insect tissues are more efficient in producing active toxin from this construct. Fourth instar *T. ni* injected hemocoelically with 2.0×10^5 pfu of vSp-BSigtox34 exhibited paralysis after 48 h. In general, the level of toxin secreted into culture supernatant was predictive of virus performance *in vivo*.

S1 nuclease analysis was performed to determine whether the low level of toxin produced by vSp-BSigtox34 infected cells was at the level of transcription or translation. A 648 bp or a 668 bp *Hin*dIII-*Nde*I probe, uniquely end-labeled at the *Nde*I site, was used in S1 nuclease protection assays with total RNA isolated from vSp-BSigtox34 or vSp-tox34 infected cells at 0, 6, 12, 24 and 36 h p.i. Protected probe corresponding to transcriptional initiation within the TAAG motif of the P_{synXIV} promoter was observed as early as 12 h p.i., and continued to increase through 36 h p.i., with RNA prepared from both vSp-BSigtox34- and vSp-tox34-infected cells. The amount of protected probe at each time point was similar between the two viruses, indicating that the low level of toxin produced in vSp-BSigtox34 infected cells was not due to reduced transcription.

Interestingly, another transcriptional start site was mapped to a TAAG sequence on the complementary strand within the native *tox34* signal sequence which was not present in RNA isolated from vSp-BSigtox34 infected cells. Primer extension analysis confirmed that this additional start site corresponded to the reverse TAAG motif. Similar interference from duplex RNA in S1 nuclease and primer extension analysis has been described [Ooi and Miller (1991) *J. Gen. Virol.* 72:527-534; Lu and Carstens (1992) *Virology* 190:201-209]. This result had potential relevance to *tox34* expression since the formation of duplex RNA at the 5' end of *tox34* mRNA can inhibit its translation, thereby decreasing Tox34 levels.

vSp-tox34 infection of neonate *T. ni* larvae (*tox34* expressed under the transcriptional control of the hybrid very late promoter, P_{synSIV}) resulted in paralysis of larvae about 45% faster than wild-type. Therefore, it was of interest to evaluate the effect of promoters for potentially earlier expression of *tox34* in infected cells as a means to further decrease the ET_{50} of *tox34*-expressing recombinants, four recombinant viruses expressing *tox34* under control of the *D. melanogaster* HSP70 promoter (vHSP70tox34), the early AcMNPV DA26 gene promoter (vDA26tox34), the late AcMNPV 6.9K DNA binding protein gene promoter (vp6.9tox34), and the very late P_{synXIV} promoter (vSp-tox34) were constructed (see Fig. 2A and the Examples). vETL-tox34, in which the *P. tritici* Tox34 toxin coding sequence is expressed under the control of the early ETL promoter [Tomalski and Miller (1992) *Bio/Technology* 10:545-549] was not as desirable as the vp6.9tox34 or the vHSP70tox34 as an insect control agent.

Analyses of time courses of *tox34* expression in TN-368 cells infected with these four viruses and wild-type AcMNPV illustrated the relative levels and timing of *tox34* expression in these cells. Western

blot analysis using antibody against Tox34 detected Tox34 in the supernatants and in cell lysates of virus-infected cells at 0, 6, 12, 24 and 48 h p.i. (Figs. 4A-4B). No Tox34 was detected in wild-type AcMNPV-infected cells throughout the time course of infection in either the cell lysate or supernatant fractions (Fig. 4A and 4B, lanes 1 to 5). Surprisingly, Tox34 was not found in the lysates from vHSP70tox34-infected cells, and it was first observed in the supernatant at 24 h p.i., with increased levels found by 48 h p.i. (Figure 4B, lanes 9 and 10). This lack of accumulation in the cellular fraction suggests that Tox34 is efficiently secreted from the infected cell. The levels of Tox34 in vDA26tox34-infected cells were too low to detect in either intracellular or extracellular fractions (Figs. 4A and 4B, lanes 11 to 15). vp6.9tox34-infected cells showed the highest expression of Tox34 of all promoters tested, and substantial levels of Tox34 were detected in both fractions at both 24 and 48 h p.i. (Figs. 4A and 4B, lanes 19 and 20). Expression of Tox34 in vp6.9tox34-infected cells was at least 24 h earlier than *tox34* under very late promoter control, where Tox34 was not detected until 48 h p.i. (Figs. 1A and 4B, compare lanes 19 and 24, and lanes 20 and 25). The two immunoreactive species found in vp6.9tox34- and vSp-tox34-infected cell lysates represent mature Tox34 and its unprocessed, precursor form.

The response of neonate *S. frugiperda* (Table 3) or *T. ni* larvae (Table 4) to infection by virus expression of *tox34* under the control of different promoters was studied to determine whether the time to paralysis was affected by the promoter and whether there were species-specific differences. In both species the LC_{50}s of all four recombinants were similar to the LC_{50} of wild-type AcMNPV. The higher LC_{50} values for wild-type AcMNPV in *S. frugiperda* (about 200 fold) relative to *T. ni* neonates have been previously documented [Clem et al. (1994) *J. Virol.* 75:1551-1556]. In *T. ni* larvae, vp6.9tox34 performed best, reducing time to paralysis by about 60% compared to wild-type, and vp6.9tox34 outperformed vSp-tox34 by 20%. A similar ET_{50} was found for vp6.9tox34 in *S. frugiperda* larvae, but in this case vHSP70tox34 performed as well, if not better. An additional species-specific difference in ET_{50} was found with vDA26tox34, which was more effective in *S. frugiperda* larvae than *T. ni* larvae by about 10 h. These results indicate that there are differences in the relative strengths of these two "early" promoters in the two insect species.

To determine if a promoter-specific effect was detectable in cell culture, time course studies of *tox34* expression in vHSP70tox34- and vDA26tox34-infected SF-21 and TN-368 cells were performed (Fig. 5). Because levels of Tox34 secreted from vHSP70tox34 and vDA26tox34 were low to undetectable (Figs. 4A and 4B), proteins in the supernatant

fraction were precipitated with 20% trichloroacetic acid (TCA) prior to separating the proteins by SDS-PAGE and detecting Tox34 by immunoblot analysis. Comparison of the relative levels of Tox34 secreted by each cell line revealed that the HSP70 promoter was significantly stronger than the DA26 gene promoter in both cell lines (Fig. 5, compare lanes 1-5 with lanes 11-15, and lanes 6-10 with lanes 16-20). A comparison of each promoter in the two cell lines showed that detectable levels of Tox34 from both the DA26 and HSP70 promoters appear at least 24 h earlier in SF-21 than in TN-368 cells. Longer exposures of this blot detected Tox34 as early as 6 and 12 h p.i. in the supernatants of vHSP70-tox34-infected SF-21 and TN-368 cells, respectively. This analysis of the level and timing of *tox34* expression with vHSP70tox34 and vDA26tox34 in the two cell lines at the protein level corresponds well with the relative time to paralysis in neonate bioassays (Tables 4 and 5).

S1 nuclease analysis revealed that a transcriptional start site was located on the complementary strand within the native *tox34* signal sequence. Transcripts initiating from this site could potentially form duplex RNA with *tox34* transcripts in infected cells to interfere with the translation of *tox34*.

Oligonucleotide site-directed mutagenesis was performed on pSp-tox34 and pSp-p6.9tox34 to change the CTTAA motif to a CTTGA motif (See Fig. 1E) within the *tox34* signal coding sequence without altering the amino acid sequence of the signal peptide. The effect of this altered signal peptide coding sequence was tested using recombinant virus expressing the modified *tox34* gene (*tox34m*) under 6.9K (vp6.9tox34m) or P_{synXIV} (vSp-tox34m) promoter control. Cell lysates and supernatants were examined for Tox34 or Tox34m expression from 18 to 48 h p.i. (Figs. 6A and 6B). In vSp-tox34- and vSp-tox34m-infected cells, Tox34 was detected in both fractions at 48 h p.i. Expression of Tox34 or Tox34m from the 6.9K promoter was observed at 18 h p.i. The relative levels of intracellular Tox34 were approximately 2 to 3 fold higher in cells infected with vp6.9tox34m than with vp6.9tox34, indicating that removal of the reverse TAAG motif increased the intracellular levels of Tox34 protein. This difference was not observed in vSp-tox34 and vSp-tox34m infected cells. Without wishing to be bound by theory, it is believed that the complementary TAAG motif acts as a late rather than a very late promoter during infection, and consequently, RNA initiating from this promoter primarily effects translation from *tox34* expressed from the late 6.9K promoter. No differences in the extracellular levels of Tox34 were observed between vp6.9tox34 and vp6.9tox34m infected cells (Fig. 5B). It is possible that the level of Tox34 produced in vp6.9tox34-infected cells has overwhelmed the cell's secretory pathway

so that even higher levels of precursor Tox34 produced in vp6.9tox34m infected cells do not affect the level of secreted Tox34.

vp6.9tox34m and vSp-tox34m were also used in *per os* infection of neonate *S. frugiperda* and *T. ni* larvae to determine whether there were any differences in the LC_{50} and ET_{50} of these recombinant viruses. No differences were observed in the LC_{50}s of each virus in either species relative to wild-type AcMNPV (Table 6). The ET_{50} of vp6.9tox34m was moderately longer than vp6.9tox34 by 4.5 and 5.6 h in *S. frugiperda* and *T. ni* larvae, respectively, and was just outside the limits of standard error. vSp-tox34m performed better than vSp-tox34 in both species. Its effect seemed to be greater in *S. frugiperda* larvae, decreasing the time to paralysis by about 7 h.

None of the heterologous signal sequences tested increased the levels of secreted Tox34 in cell culture, and none improved the time to paralysis of *T. ni* infected with these viral recombinants. Promoter choice, however, did significantly influence the timing and level of secreted toxin in cell culture, and surprisingly, certain promoters dramatically improved the properties of the virus as a biopesticide.

The major effect of changing the signal sequence was to affect the intracellular levels of Tox34. The levels of toxin in vSp-tox21A/tox34 infected cells accumulated to a higher level than in vSp-tox34 infected cells at 48 h p.i., suggesting that the *tox21A* signal sequence was less efficient at directing the secretion of Tox34 than the native *tox34* signal sequence. Very low amounts of toxin were detected in vSp-BSigtox34 infected cells even though similar levels of toxin transcripts were found in vSp-BSigtox34 and vSp-tox34 infected cells. The stability of Tox34 may be affected as a result of the change in the signal sequence or the N-terminal residue of the mature polypeptide. This signal sequence has been used successfully to promote the secretion of active prothoracicotropic hormone using the baculovirus expression system [O'Reilly et al. (1995) supra]. The addition of an extra glycine residue to the amino terminus of the mature Tox34 may have destabilized rather than stabilized Tox34.

Modification of the native *tox34* signal sequence to remove the complementary TAAG motif did not increase the level of secreted Tox34 in cell culture, but it did significantly increase the levels of intracellular Tox34 and its precursors. This suggested that while removal of the TAAG sequence increased expression of Tox34 in the cell, processing and transport through the secretory pathway was the limiting step in obtaining increased extracellular toxin levels. A decrease in the ET_{50} of vSp-tox34m relative to vSp-tox34 in *S. frugiperda* larvae was

observed, suggesting that there was some *in vivo* effect of altering this reverse TAAG motif. These results suggest that it is advantageous to use this modified signal for those promoters (e.g. HSP70) which are expressed earlier and continue to be expressed late in infection but produce less toxin than the threshold level required for paralysis.

All of the toxin-expressing recombinant viruses (regardless of promoter) reduced the effective time to paralysis/mortality relative to wild-type AcMNPV. The most effective viral promoter in the two species tested was the late 6.9K DNA binding protein gene promoter of AcMNPV. *Tox34* under 6.9K promoter control was expressed both earlier (at least 24 h) and at greater levels than *tox34* under control of a hybrid promoter composed of both late and very late promoter elements [Tomalski and Miller (1992) supra]. Superior expression mediated by the *6.9K* promoter over either the *p10* or polyhedrin gene promoters has been previously reported with respect to the expression of juvenile hormone esterase and ß-galactosidase [Bonning et al. (1994) *J. Gen Virol.* 75:1551-1556]. The earlier synthesis and secretion of Tox34 in vp6.9tox34 infected SF-21 cells reflects the performance of the recombinant virus *in vivo*.

Tox34 under control of the early *DA26* promoter was the least effective in both species, a result also found with another early promoter (ETL) of AcMNPV [Tomalski and Miller (1992) supra]. The results indicate that although *tox34* is expressed earlier in these cells, it is not initially expressed at the threshold level needed to paralyze larvae. The *hsp70* promoter has been shown to be a relatively strong promoter when compared to early viral promoters [Morris and Miller (1992) *J. Virol.* 66:7397-7405] and this promoter was found to drive higher levels of *tox34* expression than the *DA26* promoter in both *T. ni* and *S. frugiperda*. Surprisingly, *tox34* expressed under the control of the *hsp70* promoter resulted in the shortest ET_{50} in *S. frugiperda* larvae, even through overall levels of Tox34 secreted under the *hsp70* promoter were substantially lower than *tox34* expressed under *6.9K* promoter control. This indicates that *tox34* expression from a strong constitutive promoter can, in at least some cases, be more effective than expression from a strong late viral promoter. The differences observed for ET_{50} values for vHSP70tox34 and vDA26tox34 in the two species suggests that the effectiveness of a particular promoter is host-dependent.

As discussed above, there is a longfelt need in the art for biological pesticides which are especially selective for target insect pests. Baculoviruses are being considered to fill this need, but most baculoviruses require from four to fourteen days to kill their insect

hosts, and during this time the insects continue to feed and effect significant damage to crops and other vegetation. Genetic modifications of baculovirus genomes have resulted in certain improvements of baculoviruses as insect control agents by reducing the time to mortality in infected insects. The expression of insect predacious mite toxins which are specific for insects by recombinant baculoviruses has led to reduction of feeding time in infected insects; the present invention provides further improvement in such baculoviruses. Changes made to the signal sequence of the insect-specific toxin did not improve the level of secreted mite toxin, and in fact, the specific changes to the signal sequence tested resulted in an increase in the time to paralysis of infected insects. Surprisingly, however, promoter choice was a key factor in improving the time of expression, increasing the levels of toxin protein, and reducing the time to paralysis in a host-dependent manner. Without wishing to be bound by theory, it is proposed that the most effective promoter for driving toxin expression in most insect hosts is either an AcMNPV *6.9K* promoter or a heat shock promoter, in particular, the *Drosophila hsp70* promoter.

Insecticidal compositions suitable for applications to plants to control insect pests comprise an agriculturally suitable carrier and an insect control agent. Application of an insecticidal composition of this invention can protect plants from insect pests by reducing feeding by and killing of susceptible insects.

The skilled artisan knows how to choose an insect control agent, e.g., an insect virus, which is suitable for the control of a particular insect pest. It will be understood by those skilled in the art that the insect pests can be exposed to the insect control agent of the present invention by conventional methods including ingestion, inhalation or direct contact of the insect control agent.

A primary use of the genetically engineered baculoviruses of the present invention will be as components of agricultural compositions for applying to plants, plant environments or distributed in baits to effect the biological control of insect pests. It will also be possible to use the insect control agents of the present invention in the control of other insect pests with the appropriate choice of the particular organism genetically modified to express an insect-specific paralytic neurotoxin. For example, there are baculoviruses known to specifically infect each of mosquitoes, beetles and fleas, besides the common Lepidopterans. The target insect guides the skilled artisan in the selection of the insect control agent expressing the paralytic toxin, and the particular agent constrains the selection of an

appropriate promoter sequence. Many variations of preparing such agriculturally suitable and/or environmentally acceptable compositions for insect control are known in the art.

The concentration of the genetically engineered baculovirus required to produce insecticidally effective compositions for the control of an insect pest depends on the type of organism and neurotoxin used and the formulation of the composition. The insecticidally effective concentration of the insect control agent within the composition can readily be determined experimentally, as understood by the skilled artisan. For example, the insecticidally effective concentration of a virus can be readily determined using bioassay techniques known to the art.

Agricultural compositions for control of insect pests of plants must be suitable for agricultural use and dispersal in fields. Similarly, compositions for the control of other insect pests must be environmentally acceptable. Generally, components of the composition must be non-phytotoxic and not detrimental to the integrity of the occluded virus. Foliar applications must not damage or injure plant leaves. In addition to appropriate solid or, more preferably, liquid carriers, agricultural compositions may include sticking and adhesive agents, emulsifying and wetting agents, but no components which deter insect feeding or any viral functions. It may also be desirable to add components which protect the insect control agent from UV inactivation or components which serve as adjuvants to increase the potency and/or virulence of an entomopathogen. Agricultural compositions for insect pest control may also include agents which stimulate insect feeding.

Reviews describing methods of application of biological insect control agents and methods and compositions agricultural application are available. See, for example, Couch and Ignoffo (1981) in *Microbial Control of Pests and Plant Disease* 1970-1980, Burges (ed.), chapter 34, pp. 621-634; Corke and Rishbeth, ibid, chapter 39, pp. 717-732; Brockwell (1980) in *Methods for Evaluating Nitrogen Fixation*, Bergersen (ed.) pp. 417-488; Burton (1982) in *Biological Nitrogen Fixation Technology for Tropical Agriculture*, Graham and Harris (eds.) pp. 105-114; and Roughley (1982) ibid, pp. 115-127; *The Biology of Baculoviruses*, Vol. II, supra, and references cited in the above. Wettable powder compositions incorporating baculoviruses for use in insect control are described in EP 697,170 (Ahmed, published Feb. 21, 1996) incorporated by reference herein.

Monoclonal or polyclonal antibodies, preferably monoclonal, specifically reacting with a toxin protein encoded by a particular

coding sequence identified using the present methods may be made by methods known in the art. See, e.g., Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY and Goding (1986) *Monoclonal Antibodies: Principles and Practice*, 2d ed., Academic Press, New York.

Standard techniques for cloning, DNA isolation, amplification and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described in O'Reilly et al. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, W.H. Freeman, New York, NY; Sambrook et al. (1989) *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press, Plainview, New York; Maniatis et al. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Plainview, New York; Wu (ed.) (1993) *Meth. Enzymol.* 218, Part I; Wu (ed.) (1979) *Meth Enzymol.* 68; Wu et al. (eds.) (1983) *Meth. Enzymol.* 100 and 101; Grossman and Moldave (eds.) *Meth. Enzymol.* 65; Miller (ed.) (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose (1981) *Principles of Gene Manipulation*, University of California Press, Berkeley; Schleif and Wensink (1982) *Practical Methods in Molecular Biology*; Glover (ed.) (1985) *DNA Cloning* Vol. I and II, IRL Press, Oxford, UK; Hames and Higgins (eds.) (1985) *Nucleic Acid Hybridization*, IRL Press, Oxford, UK; and Setlow and Hollaender (1979) *Genetic Engineering: Principles and Methods*, Vols. 1-4, Plenum Press, New York. Abbreviations and nomenclature, where employed, are deemed standard in the field and commonly used in professional journals such as those cited herein. Each reference cited in the present application is incorporated by reference herein in its entirety.

This invention is illustrated by the following examples, which are not to be construed in any way as imposing limitations on the scope thereof. It is understood that resort can be made to various other embodiments, modifications, alternatives and equivalents of the procedures materials and techniques specifically described which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

THE EXAMPLES

Example 1. Insect Cells and Viruses

All AcMNPV viruses are originally derived from AcMNPV L-1 [Lee and Miller (1978) *J. Virol.* 27:754], and are plaque-purified and propagated in *Spodoptera frugiperda* IPLB-SF-21 cells (Sf cells, *Spodoptera frugiperda* [Vaughn et al. (1977) *In Vitro* 13:213-217] or TN-368 cells (Tn) *Trichoplusia ni* cells [Hink, W.F. (1970) *Nature* 226:466-467] in TC-100 medium (GIBCO, Grand Island, New York) supplemented with 10% fetal bovine serum and 0.26% tryptose broth at 27°C as described previously [O'Reilly et al. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, W.H. Freeman, New York, NY]. AcMNPV Viruses were titered using IPLB-SF-21 cells and the standard plaque assay as described by O'Reilly et al. (1992) supra. AcMNPV L1 served as the wild-type virus for comparative purposes.

Recombinant viruses vSptox34 and vHSP70tox34 contain the *tox34* coding sequence expressed under the regulatory control of the synthetic hybrid viral promoter P_{synXIV} [Wang et al. (1991) *Gene* 100:131-137] and the *Drosophila melanogaster* hsp70, respectively [Tomalski and Miller (1992) *Bio/Technology* 10545-549; McNitt et al. (1995) *Bio. Control* 5:267-278]. vHSP70tox34 was constructed starting with pEVptox34 digested with EcoRI and BglII to release a *tox34* fragment (see U.S. Patent No. 5,266,317) and plasmid pHSP70PLV1+CAT [Morris and Miller (1992) *J. Virol.* 66:7397-7405] which was digested with EcoRI and BglII to produce a vector fragment and a *cat* fragment, which is discarded. The vector fragment contains the *D. melanogaster hsp70* promoter with sequence from about -500 to +231 [see Toeroek and Karch (1980) *Nucl. Acids Res.* 8:3105-3123]. Insertion of the Tox34 coding sequence in the proper orientation was confirmed by restriction endonuclease analysis (digests with XbaI, NdeI, and a double digest with EcoRI and BglII). The resulting transfer plasmid was cotransfected into SF21 cells with DNA of the virus vSynV1-gal, an AcMNPV derivative containing *lac ZI* in place of *polh*. Recombinant viruses were identified by their white, occulusion-positive phenotype on Xgal plates, and their genotype was confirmed by restriction endonuclease analysis. Recombinant virus vSp-tox21A expresses the *tox21A* coding sequence, a homolog of the *tox34* coding sequence also isolated by cDNA cloning from *Pyemotes tritici* insect-predacious mites, under the regulatory control of the P_{synXIV}.

Hz 105/UND-K cells (HzUNDK), clonally isolated from IPLB-Hz 107S [Corsaro et al., (1989) *J. Virol Methods* 5:283-292], were provided by Dr. Malcolm Fraser (University of Notre Dame, Notre Dame, IN) and were maintained at 27°C in TCIOO medium supplemented with 10% fetal bovine serum and 0.26% tryptose broth, as previously described [O'Reilly et al. (1992) *Baculovirus Expression Vectors: A. Laboratory Manual*, W. H.

Freeman, new York, NY]. A plaque-purified isolate of the Elcar™ strain of HzSNPV, provided by Dr. William Rice (USDA-ARS Rice Research Center, Crawley, LA), served as the parental wild type (wt) virus and was propagated in HzUNDK cells. HzSNPV viruses are readily available in the art.

Example 2: EGT Assays

HzUNDK cells (2 x 10^6 per 60 mm dish) were infected with wt HzSNPV or AcMNPV at a multiplicity of infection of 10. After 48 h post-infection the infected cell culture supernatant was collected and transferase activity was assayed using 100 µl of supernatant with [^{3}H] ecdysone (Dupont, NEN Research Products) and UDP-galactose or UDP-glucose serving as the substrate [O'Reilly et al. (1992) *Insect Biochem. Molec. Biol.* 22:313-320]. Ecdysone was separated from ecdysone-sugar conjugates by thin layer chromatography on silica gel plates, and the radiolabel was detected by autoradiography.

Example 3: Identification and Sequencing of the HzSNPV *egt* Gene

A cosmid library of the HzSNPV genome was constructed using SuperCos (Stratagene, LaJolla, CA) as the vector. Selected cosmid and plasmid clones containing HzSNPV genomic fragments were screened by polymerase chain reaction (PCR) using two degenerate primers designed from two highly conserved regions of known UDP-glucosyltransfereases. The sequence (where I is inosine, Y is C or T, S is G or C, K is G or T, M is A or C, W is A or T, H is A or C or T, B is G or T or C, V is G or C or A, and N is all four nucleotides) of the primers included a 5' terminal *BamHI* or *EcoRI* site respectively: 5' GC GGA TCC AIY GTG SWG TWY NTK GGM GG 3' (SEQ ID NO: 16) [corresponding to SVQYLGG (SEQ ID NO: 17) in the AcMNPV EGT sequence] and 5' GC GAA TTC GGM ABV MHC ACC AKN GG 3' (SEQ ID NO: 18) [originally intended to correspond to PMVCLP (SEQ ID NO: 19) in many EGT sequences].

PCR amplification using an HzSNPV cosmid, which spanned the *Hind*III J through E fragments, and a plasmid clone containing the HindIII-C fragment as templates generated a PCR product of the size expected for the *egt* gene. The sequence of the PCR product confirmed that it was derived from the HzSNPV *egt* gene. The entire gene was then sequenced in both directions with the aid of synthetic oligonucleotide primers which provided sufficient overlap between contiguous sequences for confident alignments and unambiguous sequence information.

Nucleotide sequences of known *egts* available in GenBank were aligned using the Pileup and Boxshade programs. (See Fig. 11B-11C) Amino acid sequences were compared using the Bestfit analysis (Genetics

Computer Group, University of Wisconsin, Madison, WI). Phylogenetic relationships based on the sequence comparison are shown diagrammatically in Fig. 11A.

Example 4: Construction of HzSNPV Transfer Vectors and Recombinant HzSNPV Viruses

The *egt* gene was found to be located entirely within the *Eco*RI Q fragment. This fragment was cloned from HzSNPV into the EcoRl site of Bluescript II KS+ (Stratagene) and a 600 bp *Sal*I/EcoRI fragment downstream of *egt* was removed to produce pHzEGT (Fig. 7). pHzEGT was digested with *Sal*I which removed an internal segment of *egt* and the ends filled in by Klenow. The vector fragment was then ligated to a blunt-ended fragment containing the *E. coli* β-glucuronidase gene (GUS), under the control of the *D. melanogaster* hsp70 promoter, and *Bsu*36I and *Sse*8387I sites on either side of GUS to form pEGThspGUS. Additionally it was ligated to a DA26 promoted-, p6.9 promoted-, or a *D. melanogaster* HSP70 promoted-*tox34* gene. The DA26 and p6.9 promoters were derived from AcMNPV [Lu et al. (1995) *Biol Control.* 7:320-332]. A plasmid with a deletion in *egt* was made from *Sal*I-digested pHzEGT by ligation of the vector fragment. The resulting plasmid, pEGTdel, was used to generate a plasmid, pEGTlinker, with unique *Bsu*36*I* and *Sse*8387I sites within *egt* by digesting the plasmid DNA with *Sal*I and ligating it to an oligonucleotide with *Sal*I cohesive ends and unique *Bsu*361 and *Sse*8387I sites (Fig. 7). The oligonucleotide was constructed by annealing the following primers together (5' T CGA CCT CAG GGC AGC TTA AGG CCT GCA GG 3' (SEQ ID NO: 20) and 5' TCG ACC TGC AGG CCT TAA GCT GCC CTG AGG 3') (SEQ ID NO: 21).

HzSNPV was found to have a unique *Bsu*36I site which was located within the *Hind*III-C fragment. A 2.1 kb *Cla*I fragment containing the *Bsu*36I site was cloned from *Hind*III-C and the region surrounding the site was sequenced. The site was eliminated from the virus by digesting viral DNA with *Bsu*36I, filling in with Klenow polymerase and religating the DNA. The DNA was then digested with *Bsu*36I again and transfected into HzUNDK cells. Viruses emerging from these transfections were plaque-purified, amplified and tested for the loss of the *Bsu*36I site. Selected viruses lacking the *Bsu*36I site were then tested for their infectivity (LC_{50}) and virulence (LT_{50}) in *H.zea* neonates.

The *Bsu*36I$^-$ virus, HzSNPV(*Bsu*36I$^-$), was allelically recombined with pEGThspGUS to create an EGT deleted virus that yielded blue plaques in HzUNDK cells in the presence of X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide). Using this virus, HzSNPV(*Bsu*36I$^-$)-EGThspGUS or more simply HzEGThspGUS, the following

recombinant viruses were generated by allelic replacement [O'Reilly et al. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, W. H. Freeman, New York, NY] using transfer plasmids HzEGTdel, HzEGTDA26*tox34*, HzEGTp6.9*tox34*, and HzEGTHSP*tox34* (Fig. 7). To enhance recombination, viral DNA was linearized with *Bsu*36I before transfection of viral and plasmid DNA. Virus recombinants were screened for a white, occlusion positive, plaque phenotype. Viruses were further plaque purified and then amplified. Viral DNA was analyzed by restriction endonuclease analysis to confirm allelic replacement.

Example 5: Insect Bioassays with HzSNPV and its Derivatives

The LC_{50} and ET_{50} (mean time to effectively paralyze 50% of test larvae) of viruses were determined using neonate *H. zea*. Bioassays were conducted as droplet feeding assays according to the protocols developed by Hughes et al. (1986) *J. Invertebr. Pathol.* 48:189-192. Neonates were fed known concentrations of viral occlusion bodies (PIBs) suspended in 5% sucrose and 1 mg/ml FD&C blue #1 dye (Hilton Davis, Cincinnati, OH) by placing them in the center of a 60 mm plastic petri dish and providing them with 0.5 µl droplets of the PIB suspension pipetted on the bottom near the edge of the dish. Larvae that had ingested the PIBs within 30 mins, as determined by their blue color, were transferred to fresh diet (described as *S. frugiperda* diet in O'Reilly et al. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, W. H. Freeman, New York, NY] and monitored approximately every six hours. Five virus concentrations with 30 insects per dose were tested for each virus. ET_{50}s were determined by the Vistat 2.1 program [Hughes, P.R. (1990) *ViStat: Statistical package for the analysis of baculovirus bioassay data*, Boyce Thompson Institute, Cornell University, Ithaca, NY] and LC_{50}s were determined using Polo-PC [Robertson and Prieler (1992) Polo-PC. In "*Pesticide Bioassays with Arthropods.*" CRC Press, Boca Raton, FL].

Example 6: Construction of recombinant viruses with alternate signal sequences

All recombinant viruses were constructed by allelic replacement using previously described methods [O'Reilly et al. (1992) supra]. Transfer plasmids were cotransfected into SF-21 cells with vSynVI-gal [Wang et al. (1991) supra] and recombinant viruses were selected based on a white occlusion-positive plaque phenotype. Each recombinant virus was verified using appropriate restriction endonuclease digestion analysis. All recombinant viruses contain *tox34* inserted upstream of and in the opposite direction to the polyhedrin gene.

The virus, vSp-BSigtox34, containing *tox34* fused in frame to the sarcotoxin IA gene signal sequence from the flesh fly *Sarcophaga peregrina* [O'Reilly et al. (1995) supra] was constructed as follows: Two oligonucleotide primers, tox34up and tox34down, corresponding to nucleotides 118 to 138 and complementary to nucleotides 862 to 876, respectively, of *tox34* (SEQ ID NO:3) [Tomalski and Miller (1991) *Nature* 352:82-85] were used in a polymerase chain reaction (PCR) to amplify a 777 base pair fragment containing *tox34* without its native signal sequence. The primer tox34up was designed to add an extra glycine residue to the N-terminus of the mature *tox34* gene product (see Fig. 1A and 1B): N-terminal glycine residues reportedly stabilize recombinant gene products [Bachmair et al. (1986) *Science* 34:179-186]. The PCR-amplified product was digested with *Hin*dIII and *Sma*I (recognition sites incorporated into the primers) and inserted in frame into plasmid pBSig [O'Reilly et al. (1995) supra] between the *Eco*RI site, blunt-ended with Mung Bean nuclease, and the *Hin*dIII site. The resulting construct, pBSigtox34, contains *tox34* fused with the sarcotoxin IA signal sequence. The junction between the signal sequence and *tox34* was confirmed by sequence analysis (Fig. 2B). In addition, the entire *tox34* PCR product was sequenced to ensure that no mutations were introduced during PCR. The transfer vector, pSp-BSigtox34, was constructed by digesting pBSigtox34 with *Bam*HI, filling in the ends with the large fragment of DNA polymerase I (Klenow) followed by digestion with *Bgl*II. A fragment containing BSig*tox34* was gel-purified and inserted into pSp-tox34 between a blunt-ended *Eco*RI site and the *Bgl*II site placing BSig*tox34* under control of the P_{synXIV} promoter (Figure 2A, vSp-BSigtox34).

Recombinant virus vSp-DCtox34 (Fig. 2A), containing *tox34* fused with the *Drosophila* cuticle gene signal sequence (Fig. 1C) [Snyder et al. (1982) *Cell* 29:1027-1040] was constructed by digesting the plasmid pBSIGtox34SmaI with *Bam*HI and *Sma*I and inserting a 24 base pair oligonucleotide containing *Esp*3I and *Bsp*MI sites. The plasmid pBSigtox34SmaI is a derivative of pBSigtox34 containing a *Sma*I site at the junction between the sarcotoxin IA signal sequence and *tox34*. The resulting plasmid, pEBtox34, contains two unique restriction sites (*Esp*3I and *Bsp*MI) upstream of the mature *tox34* sequence in place of the sarcotoxin IA signal sequence. The oligonucleotide was designed so that digestion with *Esp*3I and *Bsp*MI and subsequent filling in of the ends with Klenow polymerase generates blunt ends into which any signal sequence can be inserted in-frame with *tox34* in an optimal baculovirus late/very late AUG context [O'Reilly et al. (1992) supra]. Two complementary oligonucleotides containing the *Drosophila* cuticle signal sequence with an *Esp*3I site were annealed, digested with *Esp*3I, blunt-ended with Klenow, and inserted into the *Esp*3I and *Bsp*MI sites of

pEBtox34 after the ends were filled in with Klenow. The resulting construct, pEBDCtox34, contains *tox34* fused in-frame with the *Drosophila* cuticle signal sequence (Fig. 1C). The correct junction between the cuticle signal sequence and *tox34* was verified by sequence analysis. The *Drosophila* cuticle signal sequence-*tox34* gene fusion (DCtox34) was transferred into pSp-tox34 by digesting pEBDCtox34 with *Bam*HI, filling in the ends with Klenow and digesting with *Bgl*II. The fragment containing DCtox34 was then cloned into pSp-tox34 digested with *Eco*RI, blunt ended, and digested with *Bgl*II, thus placing DCtox34 under control of the P_{synXIV} promoter (Fig. 2A, vSp-DCtox34).

Recombinant virus vSp-tox21A/tox34 (Fig. 2A) containing a hybrid toxin gene composed of the mature *tox34* gene fused with the *tox21A* signal sequence [Tomalski et al. (1993) supra] was constructed using the technique of gene splicing by overlap extension [Horton et al. (1989) *Gene* 77:61-68]. First, the *tox21A* signal sequence was amplified with primers "a" (SEQ ID NO:5) and "b" (SEQ ID NO:6) (Fig. 1D) using pBS-tox21A [Tomalski et al. (1993) supra] as a template. These primers correspond to the first 24 nucleotides of the *tox21A* signal sequence and are complementary to nucleotides 94 to 120 of *tox34*, respectively. The mature *tox34* gene was PCR amplified using two primers, one complementary to primer "b" (Fig. 1A, primer "c", SEQ ID NO:7), and the second one complementary to nucleotides 862 to 876 [Tomalski and Miller (1991) supra] at the 3' end of *tox34* that was previously used to amplify *tox34* in vSp-BSigtox34 (tox34down, see above). The PCR products from these two independent amplifications were combined and further amplified using primer "a" and tox34down, which resulted in a single fragment containing *tox34* with the tox21A signal sequence (tox21A/tox34). Tox21A/tox34 was digested with *Eco*RI and *Bgl*II (recognition sites incorporated into primers "a" and tox34up) and cloned into the corresponding sites in pSp-tox34 (see Fig. 2A).

Example 7: Construction of recombinant AcMNPV viruses with tox34 under control of different cellular and viral promoters

vp6.9tox34 and vDA26tox34 (Fig. 2B) were generated using allelic replacement as described previously [O'Reilly et al. (1992) supra] using the transfer plasmids described below. p6.9tox34 was constructed by cloning a 933 bp *Eco*RI fragment containing *tox34* from pSp-tox34 into p6.9hc between a *Bgl*II site and a *Kpn*I site blunt-ended with T4 DNA polymerase. p6.9hc is a reporter plasmid containing the chloramphenicol acetyltransferase gene (*cat*) under control of the late *6.9K* core DNA binding protein gene promoter of AcMNPV [Todd et al. (1996) *J. Virol.* 70:2307-2317]. The transfer vector, pSp-p6.9tox34, was constructed by cloning a 1.1 kb *Eco*RI/*Eco*RV fragment from p6.9tox34 containing *tox34* under *6.9K* promoter [Wilson et al. (1987) *J. Virol.*

61:661-666] control into pSp-tox34 digested with *Eco*RI and *Eco*RV (see Fig. 2B).

vDA26tox34 was constructed using two oligonucleotide primers corresponding to nucleotides -283 to -264 and complementary to -22 to -1 of the AUG of the early *DA26* gene mRNA of AcMNPV [O'Reilly et al. (1990) *J. Gen. Virol.* 71:1029-1037]. The 290 bp PCR product was digested with *Eco*RV and *Bgl*II (recognition sites incorporated into the primers) and cloned between the *Eco*RV and *Bgl*II sites of pCAPCAT [Thiem and Miller (1990) supra] reported plasmid containing *cat* under control of the late capsid protein gene promoter of AcMNPV [Thiem and Miller (1989) *J. Virol.* 63:4489-4497]. The resulting construct, pDA26CAT, contains the *DA26* gene promoter in place of capsid protein gene promoter. A 933 bp fragment containing *tox34* was removed from pSp-tox34 by digestion with *Eco*RI, blunt-ended with Klenow, and cloned into pDA26CAT digested with *Bgl*II and *Kpn*I and treated with T4 DNA polymerase. This construct was digested with *Eco*RI and *Eco*RV, and a 1.2 kb fragment containing *tox34* under *DA26* promoter control was inserted into the corresponding sites of pSp-p6.9tox34 resulting in the transfer vector pSp-DA26tox34 (see Fig. 2B)

Example 8: Construction of recombinant viruses without a reverse TAAG sequence in the native tox34 signal sequence

Viruses vp6.9tox34m and vSp-tox34m (Fig. 2C), with a mutated reverse TAAG sequence in the native *tox34* signal sequence (Fig. 1E), were generated by site-directed mutagenesis of transfer plasmids pSp-p6.9tox34 and pSp-tox34 using the Transformer Kit (Clontech, Palo Alto, California) according to the manufacturer's protocol. Two primers, a selection primer 5'-GGG TCG ACA CAG CTG CAG CTC-3' (SEQ ID NO:8) which eliminates a *Bgl*II site in both parent plasmids, and a mutagenic primer 5'-GCC ATT ATC AAT CAA GGA AAT AT-3' (base change is underlined; SEQ ID NO:9), complementary to nucleotides 104 to 126 of *tox34* [Tomalski and Miller (1991) supra], which eliminates the reverse TAAG sequence were used with the kit. Transfer plasmids, pSp-p6.9tox34m and pSp-tox34m, were sequenced to verify that the base change was present in the *tox34* signal sequence.

Example 9: Time course of tox34 expression in insect cells

SF-21 or TN-368 cells (1.0 x 10^6 cells per 35 mm plate) were infected with virus at a multiplicity of infection of 10 plaque forming units (pfu) per cell. At various times post-infection (p.i.) the tissue culture media were collected, and the cells were lysed in 2X electrophoresis sample buffer [O'Reilly et al. (1992) supra]. Proteins from cell lysates and extracellular fluids were analyzed by SDS-PAGE on

10% polyacrylamide gels, transblotted onto Millipore Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Massachusetts), and probed with a polyclonal antibody specific for Tox34 [McNitt et al. (1995) supra; Tomalski et al. (1989) supra]. Tox34 was visualized using the ECL Western blotting detection kit (Amersham Corp., Arlington Heights, Illinois) and quantitated using a Molecular Dynamics densitometer 300A (Sunnyvale, California).

Example 10: RNA isolation and S1 nuclease analysis

Total RNA was isolated from vSp-BSigtox34 and vSp-tox34 infected SF-21 cells at various times p.i. by the guanidinium isothiocyanate method [Chirgwin et al. (1979) *Biochemistry* 24:5294-5299]. S1 nuclease analysis of *tox34* transcripts in vSp-BSigtox34 or vSp-tox34 infected cells was performed using 25 μg of total RNA and either a 648 bp or a 668 bp *Nde*I-*Hin*dIII fragment uniquely end-labeled at the *Nde*I site. DNA-RNA hybridization was performed with 80% formamide-40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)-0.4 M NaCl-1 mM EDTA at 30°C overnight. S1 nuclease reactions were carried out as described in Sambrook et al. (1989) supra.

Example 11: Larval bioassays for AcMNPV and Recombinants

Polyhedral inclusion bodies (PIBs) were prepared by infecting fifth instar *Spodoptera frugiperda* larvae with 2.0×10^5 pfu of budded AcMNPV from each of the recombinant viruses. PIBs were isolated as previously described [Eldridge et al. (1992) *Biol. Control* 2:104-110]. The LC_{50} (concentration of occluded viruses required to kill 50% of the test larvae) and the ET_{50} (mean time to effectively paralyze or kill 50% of the test larvae) of toxin-expressing viruses and wild-type AcMNPV were determined using neonate *Trichoplusia ni* or *S. frugiperda* as previously described [Tomalski and Miller (1992) supra]. Six virus concentrations with 60 insects per dose were used to test each virus. LC_{50} and ET_{50} data were analyzed by Probit analysis [Daum, R.J. (1970) *Nature* 226:466-467] and ViStat 2.1 analysis [Hughes (1990) *ViStat: Statistical package for the analysis of baculovirus bioassay data*, Boyce Thompson Institute, Cornell University, Ithaca, NY], respectively. All bioassays were performed at least twice and the results presented represent an average of the results.

Example 12: Recombinant AcMNPV Virus Construction

pEV-Tox34 was constructed by inserting the EcoRI fragment containing the Tox34 coding sequence into EcoRI-cut pEVmodXIV, which supplied the powerful LSXIV promoter and sequences flanking the polyhedrin gene of AcMNPV. DNA of wild-type AcMNPV and pEV-Tox34 were cotransfected into insect cells as described in Miller et al. (1986) supra, and a recombinant virus was isolated and designated vEV-Tox34

after selection on the basis of its occlusion-negative phenotype and screening for the proper allelic replacement events by restriction endonuclease analysis and Southern hybridization.

Expression of the Tox34 gene in vEV-Tox34-infected insect cells was tested as follows. Sf21 cells were separately infected with AcMNPV and vEV-Tox34 as described in Lee et al. (1978) supra; Miller et al. (1986) supra, and the cell culture fluids from control (uninfected), AcMNPV and vEV-Tox34-infected cells were collected after 48 hrs of infection. Larvae of the wax moth *Galleria mellonella* were each injected with 5 microliter aliquots of culture fluids. Insect larvae injected with the culture fluid from vEV-Tox34-infected cells were paralyzed within 2 minutes whereas the insect larvae injected with fluid from wild-type AcMNPV-infected cells showed no paralytic response over an extended time period (several days). Paralyzed larvae were visually immobile, they lacked the righting response (the ability to turn themselves upright after turned onto their dorsal sides) and they failed to spin silk to line their burrows (a stereotypic behavior of wax moth larvae). Control larvae exhibited movement, the righting response and silk-spinning behavior. These results indicated that a neuroparalytic toxin was produced in the VEV-Tox34-infected cells, but not in cells infected with wild-type AcMNPV, via expression of the Tox34 cDNA coding sequence and that this toxin was secreted into the extracellular medium. The Tox34 gene product effects contractile muscle paralysis in the challenged insect larvae.

To test the ability of a baculovirus carrying the Tox34 gene to control insect larval feeding behavior during infection, insects were infected with vEV-Tox34 by injecting purified budded virus into the hemolymph of test larvae. *T. ni* larvae in about early fourth instar were injected with TC-100 medium (mock-infected) or medium containing budded virus particles from cell cultures infected with either wild-type AcMNPV or vEV-Tox34 (4×10^5 plaque-forming units of virus per larva). Control larvae included those larvae injected with culture medium or with wild-type AcMNPV. Insects injected with VEV-Tox34 were paralyzed (immobilized and lacked righting response) by 36 hr after injection.

In the above-described virus construction, the Tox34 coding sequence is expressed under the regulatory control of the very late LSXIV baculovirus promoter [see also U.S. Patent No. 5,244,805 (Miller) issued September 19, 1993] which is not expressed until about 18 hrs pi in cells infected at a high multiplicity of infection (moi; i.e. 10 viruses/cell) or until 24-30 hrs pi in cells infected at a moi of 1.

Thus, it was not unexpected that the paralytic effects of baculovirus-mediated Tox34 expression were not observed until about 36 hrs pi.

Transplacement plasmid phc-ETL-Tox34 was constructed with the Tox34 coding sequence expressed under the regulatory control of the ETL promoter of AcMNPV [described in Crawford et al. (1988) *J. Virol.* 62:2773-2778, incorporated by reference herein]. The Tox34-containing EcoRI fragment was inserted into the EcoRI site of phc-dET, which was derived from phcwt [Rankin et al. (1988) supra] by replacing the polyhedrin promoter between the EcoRV site and the BglII site with the ETL promoter sequences extending from -6 (relative to the ETL translational initiation ATG at +1,+2,+3) to approximately 300 bp upstream of the ETL coding sequences. The transplacement plasmid and wild-type AcMNPV were cotransfected and appropriate nonoccluded recombinants were isolated and characterized.

Table 1. Toxicity and a Partial List of Host Preferences for Species of Mites in the Genus Pyemotes

	TOXICITY		
	INSECTS	HUMANS	HOSTS
ventricosus group			
anobii	extreme	(?)	Curculionidae Scolytidae Buprestidae Anobiidae
beckeri	extreme	(?)	Lyctidae Scolytidae
emarginatus	mild	mild	Cecidomyiidae
schwerdtfegeri	extreme	mild	Anobiidae Buprestidae
tritici	extreme	extreme	Cucujidae Curculionidae Kalotermitidae Vespidae
tuberculatus	(?)	(?)	Anobiidae
ventricosus	extreme	extreme	Apoidea Chalcidoidea
zwoelferi	extreme	extreme	Cecidomyiidae
scolyti group			
dimorphus	mild	none	Scolytidae
dryas	mild	none	Scolytidae
giganticus	mild	none	Scolytidae
parviscolyti	mild	none	Scolytidae
scolyti	mild	none	Scolytidae

Modified from Cross and Moser (1975) Ann. Entomol. Soc. Am. 68:723-732

Table 2. Response of neonate *Trichoplusia ni* to infection by wt AcMNPV or recombinant baculoviruses expressing toxins with alternate signal sequences.

Dose response[1]				
		95% fiducial limits		
virus	LC_{50} (PIBs/ml)	upper	lower	slope
wt AcMNPV	2.2×10^4	2.6×10^4	1.8×10^4	2.25
1vSp-tox34	2.0×10^4	2.3×10^4	1.6×10^4	2.13
vSp-tox21A	3.5×10^4	4.1×10^4	3.0×10^4	2.53
vSp-BSigtox34	1.6×10^4	2.8×10^4	0.8×10^4	2.08
vSp-tox21A/tox34	5.3×10^4	6.5×10^4	4.3×10^4	1.95
vSp-DCtox34	2.6×10^4	3.1×10^4	2.2×10^4	2.34

Time response[2]		
virus	ET_{50} ± s.e. (h)	slope
wt AcMNPV	94.6 ± 1.6	14.8
vSp-tox34	51.1 ± 0.9	13.2
vSp-tox21A	62.1 ± 0.9	18.8
vSp-BSigtox34	70.0 ± 2.6	6.4
vSp-tox21A/tox34	60.8 ± 2.0	8.1
vSp-DCtox34	49.9 ± 1.0	12.5

[1] determined by probit analysis
[2] determined by ViStat 2.1 analysis at LC_{95} dose

Table 3. Response of neonate *Spodoptera frugiperda* larvae to oral infection by wt AcMNPV and recombinants expressing *tox34* under control of alternate promoters.

Dose response[1]				
		95% fiducial limits		
virus	LC_{50} (PIBs/ml)	upper	lower	slope
wt AcMNPV	1.3×10^6	1.7×10^6	1.0×10^6	1.43
vHSP70tox34	1.6×10^6	1.8×10^6	1.0×10^6	1.49
vDA26tox34	1.1×10^6	1.4×10^6	0.8×10^6	1.42
vp6.9tox34	0.6×10^6	0.9×10^6	0.3×10^6	1.23
vSp-tox34	0.8×10^6	1.0×10^6	0.5×10^6	1.31

Time response[2]		
virus	ET_{50} ± s.e. (h)	slope
wt AcMNPV	101.3 ± 2.5	9.9
vHSP70tox34	41.8 ± 2.5	8.2
vDA26tox34	61.8 ± 2.0	7.2
vp6.9tox34	44.7 ± 1.7	7.4
vSp-tox34	55.4 ± 2.0	7.0

[1] determined by probit analysis
[2] determined by ViStat 2.1 analysis at LC_{95} dose

Table 4. Response of neonate *Trichoplusia ni* larvae to oral infection by wt AcMNPV and recombinants expressing *tox34* under control of alternate promoters

Dose response[1]				
		95% fiducial limits		
virus	LC_{50} (PIBs/ml)	upper	lower	slope
wt AcMNPV	5.4×10^3	7.2×10^3	3.5×10^3	1.80
vHSP70tox34	4.5×10^3	9.5×10^3	1.0×10^3	1.90
vDA26tox34	7.0×10^3	8.9×10^3	5.3×10^3	2.10
vp6.9tox34	2.0×10^3	3.5×10^3	0.6×10^3	1.58
vSp-tox34	1.6×10^3	2.8×10^3	0.5×10^3	1.58

Time response[2]		
virus	ET_{50} ± s.e. (h)	slope
wt AcMNPV	99.0 ± 2.0	13.9
vHSP70tox34	53.8 ± 1.0	14.3
vDA26tox34	71.2 ± 1.1	15.9
vp6.9tox34	41.7 ± 1.4	12.3
vSp-tox34	58.5 ± 1.4	12.7

[1] determined by probit analysis
[2] determined by ViStat analysis at LC_{95} dose

Table 5. Response of neonate *S. frugiperda* larvae to oral infection by wt AcMNPV or recombinant virus expressing *tox34* with or without a reverse TAAG sequence in the *tox34* signal sequence.

Dose response[1]				
		95% fiducial limits		
virus	LC_{50} (PIBs/ml)	upper	lower	slope
wt AcMNPV	3.6×10^5	4.7×10^5	2.5×10^5	1.45
vp6.9tox34	1.5×10^5	2.2×10^5	0.9×10^5	1.30
vp6.9tox34m	3.6×10^5	4.7×10^5	2.6×10^5	1.53
vSp-tox34	2.8×10^5	7.1×10^5	1.5×10^5	1.34
vSp-tox34m	2.5×10^5	3.4×10^5	1.7×10^5	1.45

Time response[2]		
virus	ET_{50} ± s.e. (h)	slope
wt AcMNPV	103.0 ± 2.1	11.5
vp6.9tox34	43.9 ± 1.6	8.9
vp6.9tox34m	48.4 ± 1.8	6.9
vSp-tox34	54.9 ± 1.5	8.5
vSp-tox34m	47.9 ± 1.1	11.3

[1] determined by probit analysis
[2] determined by ViStat 2.1 analysis at LC_{95} dose

Table 6. Response of neonate *T. ni* larvae to oral infection by wt AcMNPV or recombinant virus expressing *tox34* with or without a reverse TAAG sequence in the *tox34* signal sequence.

Dose response[1]

virus	LC_{50} (PIBs/ml)	95% fiducial limits upper	95% fiducial limits lower	slope
wt AcMNPV	9.2×10^3	4.6×10^4	1.4×10^4	1.37
vp6.9tox34	3.6×10^3	6.0×10^3	1.1×10^3	1.64
vp6.9tox34m	1.1×10^4	1.5×10^4	7.7×10^3	1.40
vSp-tox34	6.5×10^3	9.0×10^3	4.1×10^3	2.01
vSp-tox34m	6.3×10^3	8.1×10^3	4.4×10^3	2.46

Time response[2]

virus	ET_{50} ± s.e. (h)	slope
wt AcMNPV	100.5 ± 2.3	11.5
vp6.9tox34	41.5 ± 1.6	8.2
vp6.9tox34m	46.7 ± 1.5	8.8
vSp-tox34	59.2 ± 1.5	12.7
vSp-tox34m	55.9 ± 1.2	14.8

[1] determined by probit analysis
[2] determined by ViStat 2.1 analysis at LC_{95} dose

Table 7. Length, percent similarity and identity of known EGT amino acid sequences to HzSNPV EGT

Virus	Length	Similarity	Identity
MbNPV	528	70.0	49.8
LdNPV	488	65.9	49.6
SlNPV	515	70.8	49.1
AcNPV	506	67.6	46.2
BmNPV	507	67.6	46.2
CfNPV	491	61.4	42.4
CfDEF	493	63.9	45.2
OpNPV*	293	62.2	44.1
LoGV	460	65.1	43.4

* Partial sequence of 293 amino acids

Table 8. Dose-mortality response of neonate *Heliocoverpa zea* larvae infected *per os* with wild-type HzSNPV and various recombinants. Bioassay #1.

Virus	LC50 (PIB/ml)	95% Fiducial Limit lower	upper	Slope	Hetero-geneity
HzSNPV Elkar	1.1×10^3	0.6×10^2	2.7×10^3	1.2 ± 0.3	0.6
HzSNPV (BSU361-)	1.7×10^3	1.1×10^3	2.5×10^3	2.1 ± 0.4	1.0
HzEGTdel	2.2×10^3	0.6×10^3	4.4×10^3	1.6 ± 0.3	1.0
HzEGTp6.9tox34	0.8×10^3	0.2×10^3	1.6×10^3	1.1 ± 0.2	0.6
HzEGThsptox34	1.2×10^3	0.4×10^3	2.1×10^3	1.2 ± 0.2	0.9
HzEGTDA26tox34	0.7×10^3	0.9×10^3	1.3×10^3	1.1 ± 0.3	0.3

Table 9. Dose-mortality response of neonate *Heliocoverpa zea* larvae infected *per os* with wild-type HzSNPV and various recombinants. Bioassay #2.

Virus	LC50 (PIB/ml)	95% Fiducial Limit lower	upper	Slope	Hetero-geneity
HzSNPV Elkar	1.4×10^3	0.9×10^3	1.9×10^3	2.2 ± 0.5	0.04
HzSNPV (BSU361-)	1.3×10^3	0.6×10^3	2.0×10^3	1.3 ± 0.4	0.9
HzEGTdel	1.7×10^3	0.8×10^3	2.7×10^3	1.2 ± 0.2	0.6
HzEGTp6.9tox34	1.6×10^3	1.1×10^3	2.5×10^3	1.7 ± 0.3	0.6
HzEGThsptox34	2.3×10^3	1.2×10^3	5.0×10^3	1.9 ± 0.3	1.2
HzEGTDA26tox34	2.1×10^3	1.1×10^3	4.9×10^3	1.9 ± 0.3	1.1

Table 10. Time-mortality response of neonate *Heliocoverpa zea* larvae infected *per os* with wild-type HzSNPV and various recombinant viruses. Bioassay #1.

Virus	LC50 ET50 ± SE	LC50 Slope ± SE	LC95 ET50 ± SE	LC95 Slope ± SE
HzSNPV Elkar	64.2 ± 2.8	8.9 ± 1.9	63.4 ± 1.8	12.0 ± 2.0
HzSNPV (BSU361-)	61.1 ± 2.7	10.3 ± 2.4	64.0 ± 1.3	16.0 ± 2.8
HzEGTdel	72.4 ± 5.2	7.1 ± 1.9	62.4 ± 1.6	13.2 ± 2.4
HzEGTp6.9tox34	43.9 ± 1.2	19.1 ± 5.1	40.5 ± 1.0	13.3 ± 2.2
HzEGThsptox34	49.5 ± 1.2	19.2 ± 4.9	46.4 ± 0.7	23.5 ± 4.6
HzEGTDA26tox34	39.3 ± 1.5	11.5 ± 2.5	36.8 ± 0.7	18.2 ± 3.2

Table 11. Time-mortality response of neonate *Heliocoverpa zea* larvae infected *per os* with wild-type HzSNPV and various recombinant viruses. Bioassay #2.

Virus	LC50 ET50 ± SE	LC50 Slope ± SE	LC95 ET50 ± SE	LC95 Slope ± SE
HzSNPV Elkar	64.3 ± 2.8	8.9 ± 1.7	65.4 ± 2.2	12.0 ± 2.0
HzSNPV (BSU361-)	62.2 ± 2.3	11.6 ± 2.5	58.3 ± 1.9	10.0 ± 1.6
HzEGTdel	67.3 ± 3.8	9.6 ± 2.6	67.3 ± 2.3	9.5 ± 1.5
HzEGTp6.9tox34	41.6 ± 1.2	14.5 ± 3.0	38.0 ± 0.7	18.3 ± 3.1
HzEGThsptox34	44.0 ± 0.9	20.0 ± 3.9	44.1 ± 0.7	20.6 ± 3.6
HzEGTDA26tox34	39.1 ± 1.5	12.0 ± 2.8	35.4 ± 0.6	19.0 ± 3.9

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Georgia Research Foundation, Inc.
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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 924 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 12..887

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 129..884

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CTTATTAATT A ATG AAA ATT TGT ACA TTT TTT ATT CCT TTA TTC AAA ATG      50
             Met Lys Ile Cys Thr Phe Phe Ile Pro Leu Phe Lys Met
             -39             -35                 -30

AAC TTG TTT TTT TTA TTT ATT ATT CCA ACA ATT TTA GCA GTT AAA CCT      98
Asn Leu Phe Phe Leu Phe Ile Ile Pro Thr Ile Leu Ala Val Lys Pro
    -25                 -20                 -15

TTT AGG TCT TTT AAT AAT ATT TCC TTA ATT GAT AAT GGC AAT GTC GAA     146
Phe Arg Ser Phe Asn Asn Ile Ser Leu Ile Asp Asn Gly Asn Val Glu
-10                 -5                  1                   5

TCT GTA AGA GCA GTA GTT ATT GAT TAT TGT GAT ATT AGA CAT CCA AAT     194
Ser Val Arg Ala Val Val Ile Asp Tyr Cys Asp Ile Arg His Pro Asn
            10                  15                  20

AAT TTA TGT AAA AAA CAT TTT GAA ATC GAT TCA TAT TGG AAT GAT GAT     242
Asn Leu Cys Lys Lys His Phe Glu Ile Asp Ser Tyr Trp Asn Asp Asp
        25                  30                  35

ACG GAT TGT TTT ACA AAT ATT GGA TGC AAA GTA TAT GGA GGA TTT GAT     290
Thr Asp Cys Phe Thr Asn Ile Gly Cys Lys Val Tyr Gly Gly Phe Asp
    40                  45                  50

ATT ATT GGT GGT CAT ACC CCT AAA GTT GGA ACT GTA TGT AGA CTT AAA     338
Ile Ile Gly Gly His Thr Pro Lys Val Gly Thr Val Cys Arg Leu Lys
55                  60                  65                  70

AAA GGA GAA AAT AAA TTT GGA TAT TGT AAT TCA AAG GGA AAT TGC GTT     386
Lys Gly Glu Asn Lys Phe Gly Tyr Cys Asn Ser Lys Gly Asn Cys Val
                75                  80                  85

GAA AGA GAT TTT AAA GAA AGT TTT GGA ATA TCT ATA AAA ATA AAA GGA     434
Glu Arg Asp Phe Lys Glu Ser Phe Gly Ile Ser Ile Lys Ile Lys Gly
            90                  95                  100

ATT TCT AAT AAA GGA GAT GAT GAA CCA GCA TGT CCA CAA TAT AAA AAT     482
Ile Ser Asn Lys Gly Asp Asp Glu Pro Ala Cys Pro Gln Tyr Lys Asn
        105                 110                 115

ACT TGG ATT AAT TAT GGG AAA TGT AAT GAA CCT TAT TAT TGT GGA ACA     530
Thr Trp Ile Asn Tyr Gly Lys Cys Asn Glu Pro Tyr Tyr Cys Gly Thr
    120                 125                 130

AAT CAT GGA TTA TTT TAT GCA AAC AAA AGA AAA CTC GAT TAC TTT CCC     578
Asn His Gly Leu Phe Tyr Ala Asn Lys Arg Lys Leu Asp Tyr Phe Pro
135                 140                 145                 150

ACA GAC GGT GAA AAA TGT AAT TCA AAT AAT ATA CCA TAT GCT GTT TGT     626
Thr Asp Gly Glu Lys Cys Asn Ser Asn Asn Ile Pro Tyr Ala Val Cys
                155                 160                 165

TAT TTA GGA AGA TGT CAT ACA ACA GGT GGT TTT TTT AGT GAA TTT GGA     674
Tyr Leu Gly Arg Cys His Thr Thr Gly Gly Phe Phe Ser Glu Phe Gly
            170                 175                 180

ACT ATT GTT AAA AAT GTC GAA ATC GTA ACT TTA TCA GAT GGA AAG AAC     722
Thr Ile Val Lys Asn Val Glu Ile Val Thr Leu Ser Asp Gly Lys Asn
        185                 190                 195
```

```
AGT TCT AGA AGA GGA AAA CAT AAA AAT TTA CCT ACT TCT AAA GTA TTT          770
Ser Ser Arg Arg Gly Lys His Lys Asn Leu Pro Thr Ser Lys Val Phe
    200                 205                 210

GAT AGT TAT AGT ATA TAT GAT ATT GAT CCT AAA AAT TGG AAA ATT GAA          818
Asp Ser Tyr Ser Ile Tyr Asp Ile Asp Pro Lys Asn Trp Lys Ile Glu
215                 220                 225                 230

GAT GAT GAT AAA GAT GTT ACT GTT CAT GAA AAT ACA TTA GAT CCA AAA          866
Asp Asp Asp Lys Asp Val Thr Val His Glu Asn Thr Leu Asp Pro Lys
                235                 240                 245

AGT GAT TCA AGA CTG TGT TAA ATTTTTAAAA ATTTGATTTT TTTAAATAAA             917
Ser Asp Ser Arg Leu Cys  *
            250

TGTCAAT                                                                 924
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 292 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Lys Ile Cys Thr Phe Phe Ile Pro Leu Phe Lys Met Asn Leu Phe
-39                 -35                 -30                 -25

Phe Leu Phe Ile Ile Pro Thr Ile Leu Ala Val Lys Pro Phe Arg Ser
                -20                 -15                 -10

Phe Asn Asn Ile Ser Leu Ile Asp Asn Gly Asn Val Glu Ser Val Arg
            -5                  1                   5

Ala Val Val Ile Asp Tyr Cys Asp Ile Arg His Pro Asn Asn Leu Cys
 10                  15                  20                  25

Lys Lys His Phe Glu Ile Asp Ser Tyr Trp Asn Asp Asp Thr Asp Cys
                30                  35                  40

Phe Thr Asn Ile Gly Cys Lys Val Tyr Gly Gly Phe Asp Ile Ile Gly
            45                  50                  55

Gly His Thr Pro Lys Val Gly Thr Val Cys Arg Leu Lys Lys Gly Glu
        60                  65                  70

Asn Lys Phe Gly Tyr Cys Asn Ser Lys Gly Asn Cys Val Glu Arg Asp
    75                  80                  85

Phe Lys Glu Ser Phe Gly Ile Ser Ile Lys Ile Lys Gly Ile Ser Asn
 90                  95                  100                 105

Lys Gly Asp Asp Glu Pro Ala Cys Pro Gln Tyr Lys Asn Thr Trp Ile
                110                 115                 120

Asn Tyr Gly Lys Cys Asn Glu Pro Tyr Tyr Cys Gly Thr Asn His Gly
            125                 130                 135

Leu Phe Tyr Ala Asn Lys Arg Lys Leu Asp Tyr Phe Pro Thr Asp Gly
        140                 145                 150
```

```
Glu Lys Cys Asn Ser Asn Asn Ile Pro Tyr Ala Val Cys Tyr Leu Gly
    155                 160                 165

Arg Cys His Thr Thr Gly Gly Phe Phe Ser Glu Phe Gly Thr Ile Val
170                 175                 180                 185

Lys Asn Val Glu Ile Val Thr Leu Ser Asp Gly Lys Asn Ser Ser Arg
                190                 195                 200

Arg Gly Lys His Lys Asn Leu Pro Thr Ser Lys Val Phe Asp Ser Tyr
            205                 210                 215

Ser Ile Tyr Asp Ile Asp Pro Lys Asn Trp Lys Ile Glu Asp Asp Asp
        220                 225                 230

Lys Asp Val Thr Val His Glu Asn Thr Leu Asp Pro Lys Ser Asp Ser
    235                 240                 245

Arg Leu Cys  *
250
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1241 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 119..988

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
GAATTCCAAC AACAGTGCCT TTGGGCGGCC GCACTGGTCT TAACTTTTTT CTCTTTTTTA      60

GCAGCGGCCA TGATAATTTA ATAATCTTAT TTACAAATTT TTTATTATTT TATTCAGA       118

ATG AAC TTG TAT TTT TTA TTT TTT ATT TCA ACT ATT TTA GCA GCT AAA       166
Met Asn Leu Tyr Phe Leu Phe Phe Ile Ser Thr Ile Leu Ala Ala Lys
  1               5                  10                  15

CCT TTC AAT TCT TTT AAT AAA ACT TCA TTA ATT GAT GAA GGA GTT GAC       214
Pro Phe Asn Ser Phe Asn Lys Thr Ser Leu Ile Asp Glu Gly Val Asp
            20                  25                  30

AAC GAT GAC GAT ATT GTC TCT AAA AGA GCA GTA GTT ATT GAT TAT TGT       262
Asn Asp Asp Asp Ile Val Ser Lys Arg Ala Val Val Ile Asp Tyr Cys
        35                  40                  45

GAT ACT AGA CAT CCA AAT AAT TTA TGT AAA AAA TAT TTT GAA ATC GAT       310
Asp Thr Arg His Pro Asn Asn Leu Cys Lys Lys Tyr Phe Glu Ile Asp
    50                  55                  60

TCA TAT TGG AAT GAT GAT ACG GAT TGT TTT ACA AAT ATT GGA TGC AAA       358
Ser Tyr Trp Asn Asp Asp Thr Asp Cys Phe Thr Asn Ile Gly Cys Lys
65                  70                  75                  80
```

```
GTA TAT GGA GGA TTT GAT ATT ATT GGT GGT AAA GCT CCT AAA ATT GGA          406
Val Tyr Gly Gly Phe Asp Ile Ile Gly Gly Lys Ala Pro Lys Ile Gly
                 85                  90                  95

ACT GTA TGT AGA CTT AAA AAA GGA AAA AAT AAA TTT GGA TAT TGT AAT          454
Thr Val Cys Arg Leu Lys Lys Gly Lys Asn Lys Phe Gly Tyr Cys Asn
            100                 105                 110

TCA AAA GGA AAT TGC GTT GAA AGA GAT TTT ATT GAA AGT TTT GGA GTA          502
Ser Lys Gly Asn Cys Val Glu Arg Asp Phe Ile Glu Ser Phe Gly Val
        115                 120                 125

TCT ATA AAA ATA AAA GGA ATT TCT CAT AGA GGA GAT GAT GAA CCA GCA          550
Ser Ile Lys Ile Lys Gly Ile Ser His Arg Gly Asp Asp Glu Pro Ala
    130                 135                 140

TGT CCA CTT TAT GAA AAT ACT TGG ATT AAT TAT GGA AAA TGT AAT GAA          598
Cys Pro Leu Tyr Glu Asn Thr Trp Ile Asn Tyr Gly Lys Cys Asn Glu
145                 150                 155                 160

CCT TAT CAT TGT GGA ACA AAT TAT GGG TTA TTT TAT GCA AAC AAA AGA          646
Pro Tyr His Cys Gly Thr Asn Tyr Gly Leu Phe Tyr Ala Asn Lys Arg
                165                 170                 175

AAA CTC AAT TAC TTT CCT GAT AAC GGT CAA AAA TGT AAT TCA AAA TAT          694
Lys Leu Asn Tyr Phe Pro Asp Asn Gly Gln Lys Cys Asn Ser Lys Tyr
            180                 185                 190

GAA ATA TAC GGT GTA TGT TAT TTA GGA CGC TGT CAT GGA ACA GGA AAT          742
Glu Ile Tyr Gly Val Cys Tyr Leu Gly Arg Cys His Gly Thr Gly Asn
        195                 200                 205

TTT TCA AAT GGT GAA GTT TTT AGT GAA TTT GGA ACT ATT TTT AAA GAT          790
Phe Ser Asn Gly Glu Val Phe Ser Glu Phe Gly Thr Ile Phe Lys Asp
    210                 215                 220

GTC GAA ATT GTA ACT TTA TCA GAT GGA AAG AAC AGT TCT AAA AGA GGA          838
Val Glu Ile Val Thr Leu Ser Asp Gly Lys Asn Ser Ser Lys Arg Gly
225                 230                 235                 240

AAA CAT AAA AAT TTA CAT GGT TCT AAA GTA TTT GAT AGT AAT GGT ATA          886
Lys His Lys Asn Leu His Gly Ser Lys Val Phe Asp Ser Asn Gly Ile
                245                 250                 255

TAT GAT ATT GAT CCT AAA AAT TGG AAA ATT GAA GAT GAT GAT AAA GAT          934
Tyr Asp Ile Asp Pro Lys Asn Trp Lys Ile Glu Asp Asp Asp Lys Asp
            260                 265                 270

ATT ACT GTT CAT GAA AAT GCT GGA GAT CCA AAA AGT GAT TCA AGA CGT          982
Ile Thr Val His Glu Asn Ala Gly Asp Pro Lys Ser Asp Ser Arg Arg
        275                 280                 285

TGT TAA ATTTTTAAAT ATTTGATTTT TTTTAAATAA ATATAAATCT ATATATTTAA          1038
Cys  *
    290

TAATATAATT TCTTTTAATT TTTAAATTAG TAAAATTTCG ATAATTTTAC TTAATTTTTT      1098

AAATTTACTA AATTGACTAA TTTTATTAAG AAGTAACTTC TAAAAAATTT GATTTTTTTT      1158

AAAACAAATA ATTATAAATA TTTTTTAATT AAATAAATTT AATAATTACA AGATAAAAAA      1218

AAAAAAAAAA AAAAAAGGAA TTC                                              1241
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 290 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Asn Leu Tyr Phe Leu Phe Phe Ile Ser Thr Ile Leu Ala Ala Lys
 1               5                  10                  15

Pro Phe Asn Ser Phe Asn Lys Thr Ser Leu Ile Asp Glu Gly Val Asp
            20                  25                  30

Asn Asp Asp Asp Ile Val Ser Lys Arg Ala Val Val Ile Asp Tyr Cys
        35                  40                  45

Asp Thr Arg His Pro Asn Asn Leu Cys Lys Lys Tyr Phe Glu Ile Asp
    50                  55                  60

Ser Tyr Trp Asn Asp Asp Thr Asp Cys Phe Thr Asn Ile Gly Cys Lys
65                  70                  75                  80

Val Tyr Gly Gly Phe Asp Ile Ile Gly Gly Lys Ala Pro Lys Ile Gly
                85                  90                  95

Thr Val Cys Arg Leu Lys Lys Gly Lys Asn Lys Phe Gly Tyr Cys Asn
            100                 105                 110

Ser Lys Gly Asn Cys Val Glu Arg Asp Phe Ile Glu Ser Phe Gly Val
        115                 120                 125

Ser Ile Lys Ile Lys Gly Ile Ser His Arg Gly Asp Asp Glu Pro Ala
    130                 135                 140

Cys Pro Leu Tyr Glu Asn Thr Trp Ile Asn Tyr Gly Lys Cys Asn Glu
145                 150                 155                 160

Pro Tyr His Cys Gly Thr Asn Tyr Gly Leu Phe Tyr Ala Asn Lys Arg
                165                 170                 175

Lys Leu Asn Tyr Phe Pro Asp Asn Gly Gln Lys Cys Asn Ser Lys Tyr
            180                 185                 190

Glu Ile Tyr Gly Val Cys Tyr Leu Gly Arg Cys His Gly Thr Gly Asn
        195                 200                 205

Phe Ser Asn Gly Glu Val Phe Ser Glu Phe Gly Thr Ile Phe Lys Asp
    210                 215                 220

Val Glu Ile Val Thr Leu Ser Asp Gly Lys Asn Ser Ser Lys Arg Gly
225                 230                 235                 240

Lys His Lys Asn Leu His Gly Ser Lys Val Phe Asp Ser Asn Gly Ile
                245                 250                 255

Tyr Asp Ile Asp Pro Lys Asn Trp Lys Ile Glu Asp Asp Asp Lys Asp
            260                 265                 270

Ile Thr Val His Glu Asn Ala Gly Asp Pro Lys Ser Asp Ser Arg Arg
        275                 280                 285

Cys *
    290
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGAACTTGT ATTTTTTATT TTTT 24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATCAATTAAT GAAGTTTTAT TAAAAGA 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCTTTTAATA ATATTTCCTT AATTGAT 27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonculeotide primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGTCGACAC AGCTGCAGCT C 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide primer"

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCATTATCA ATCAAGGAAA TAT 23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Coding sequence of Sarcotoxin IA signal sequence and start of toxin protein"

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..81

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..69

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ATG AAC TTC CAA AAC ATA TTC ATA TTC GTG GCG TTA ATA TTG GCG GTG      48
Met Asn Phe Gln Asn Ile Phe Ile Phe Val Ala Leu Ile Leu Ala Val
 1               5                  10                  15

TTC GCG GGA CAA TCT CAG GCG GGG GAT AAT GGC                        81
Phe Ala Gly Gln Ser Gln Ala Gly Asp Asn Gly
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Asn Phe Gln Asn Ile Phe Ile Phe Val Ala Leu Ile Leu Ala Val
 1               5                  10                  15

Phe Ala Gly Gln Ser Gln Ala Gly Asp Asn Gly
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Coding sequence for signal sequence and beginning of Drosophila melanogaster cuticle protein"

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..57

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..48

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATG TTC AAG TTT GTC ATG ATC TGC GCA GTT TTG GGC CTG GCG GTG GCC      48
Met Phe Lys Phe Val Met Ile Cys Ala Val Leu Gly Leu Ala Val Ala
 1               5                  10                  15

GAT AAT GGC                                                        57
Asp Asn Gly
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Phe Lys Phe Val Met Ile Cys Ala Val Leu Gly Leu Ala Val Ala
 1               5                  10                  15

Asp Asn Gly
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Coding sequence for modified tox34 signal sequence and start of mature Tox34 protein"

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..126

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..112

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
ATG AAA ATT TGT ACA TTT TTT ATT CTT TTA TTC AAA ATG AAC TTG TTT        48
Met Lys Ile Cys Thr Phe Phe Ile Leu Leu Phe Lys Met Asn Leu Phe
  1               5                  10                  15

TTT TTA TTT ATT ATT CCA ACA ATT TTA GCA GTT AAA CCT TTT AGG TCT        96
Phe Leu Phe Ile Ile Pro Thr Ile Leu Ala Val Lys Pro Phe Arg Ser
            20                  25                  30

TTT AAT AAT ATT TCC TTG ATT GAT AAT GGC                               126
Phe Asn Asn Ile Ser Leu Ile Asp Asn Gly
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Lys Ile Cys Thr Phe Phe Ile Leu Leu Phe Lys Met Asn Leu Phe
  1               5                  10                  15

Phe Leu Phe Ile Ile Pro Thr Ile Leu Ala Val Lys Pro Phe Arg Ser
            20                  25                  30

Phe Asn Asn Ile Ser Leu Ile Asp Asn Gly
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide."

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..28
(D) OTHER INFORMATION: /note= "At position 10, N is inosine; at position 21, N is A, G, T or C."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCGGATCCAN YGTGSWGTWY NTKGGMGG 28

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser Val Gln Tyr Leu Gly Gly
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide."

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /note= "At position 23, N is A, T, G or C."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCGAATTCGG MABVMHCACC AKNGG 25

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Pro Met Val Cys Leu Pro
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide."

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TCGACCTCAG GGCAGCTTAA GGCCTGCAGG 30

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide."

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCGACCTGCA GGCCTTAAGC TGCCCTGAGG 30

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 395 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
CGATAACAAC TTTAAAGTAA CCATATTATG GAACACTTGA CCGCACACCC AAATAGAATG      60

ACAAAGAATG TTTTCATCGT TTCGTCGCCC ACACAATTCA AACATAACGT TATCTTTAAA     120

GATAACAAAT GATGACATAT ATTAAATTAT GGTGCAATAT ACATGACACA AACAACTTAC     180

GTCATCGTAA CCTTGAATTA AAATGTAAAA ACAATTTGTG ATATCGTTAA TTCTAGGAAG     240

TTGGGCACAA ACAACTTACG TCATCGTAAC CTTAGGTCAA ATCGTTAATT CTAGGAAGTT     300

GTGCACAAAC AACTTACGTC ATACATGTTA TTAATCATTT GCGGTGCAAT CGTCATCGGA     360

TCAAACGATT TCGGTTAAAT TTCGACACTG GTGTG                                395
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2025 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 320..1867

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
TCGACAGAGT TATATTATTT ATTAATGTGT TGCTGTTGTT GCGTATGACA TCATAGGTAT      60

TAGTACTGTC ATTGCTAAAT ATAGACATGA AATAATTATC TTCGTTGAGG TTATCACGAA     120

CACTAGTAGT CATCGTGACG ATAGATATCT GTAATACACA CATCAAAGTA AACATGTTTA     180

CTTAAACAGT AACTGAATAA TAATTTCAAC ATACGCACGC CACTATAAGA TGCAGCATCC     240

CGTCCGTTGG TCATCTTTCG ATAAACGCTC TGACCCATAA ACGGACGTGC GCTAATTTTT     300

TTTTATTGCT AAATTCAAA ATG TAC AAA CAA ATA ATA ACT ATG TTA TTG TTG      352
                     Met Tyr Lys Gln Ile Ile Thr Met Leu Leu Leu
                       1               5                  10

GTG TTG TTT CTG TCG GTT CTG GAT GGA GCG CGT ATC CTG TGC GTT TTT      400
Val Leu Phe Leu Ser Val Leu Asp Gly Ala Arg Ile Leu Cys Val Phe
            15                  20                  25

CCT GTT CCT TCG TAC AGT CAT CAT GCA GTG TTC GAA GCT TAC ACC AAT      448
Pro Val Pro Ser Tyr Ser His His Ala Val Phe Glu Ala Tyr Thr Asn
        30                  35                  40

GCT CTA GCG TCG CGT GGC CAT ACA ATA GTC AGA ATT ACA CCG TTT CCC      496
Ala Leu Ala Ser Arg Gly His Thr Ile Val Arg Ile Thr Pro Phe Pro
    45                  50                  55
```

```
ACT AAG AAA AAC GAT TCA TCC AAC GTG ACA GAT GTC GAC GTT AGC TTG          544
Thr Lys Lys Asn Asp Ser Ser Asn Val Thr Asp Val Asp Val Ser Leu
 60                  65                  70                  75

TCG AAA GAT TAT TTT AAA AGT CTT GTG GAC CGA TCT AGA CTG TTC AAG          592
Ser Lys Asp Tyr Phe Lys Ser Leu Val Asp Arg Ser Arg Leu Phe Lys
                 80                  85                  90

AAA CGA GGC GTT ATT TCG GAA ACG TCC AGC GTG ACC GCT CGC AAT TAC          640
Lys Arg Gly Val Ile Ser Glu Thr Ser Ser Val Thr Ala Arg Asn Tyr
             95                 100                 105

ATC AGT CTA GTA CAC ATG TTG ATT GAT CAA TTC TCT GTG GAG AGT GTA          688
Ile Ser Leu Val His Met Leu Ile Asp Gln Phe Ser Val Glu Ser Val
        110                 115                 120

CGA CAA TTG ATC GAA TCC AAC AAT GTT TTC GAT TTG TTG GTG ACC GAA          736
Arg Gln Leu Ile Glu Ser Asn Asn Val Phe Asp Leu Leu Val Thr Glu
    125                 130                 135

GCC TTT CTA GAT TAT CCT CTG GTG TTT TCG CAT TTG TTT GGC GAT GTG          784
Ala Phe Leu Asp Tyr Pro Leu Val Phe Ser His Leu Phe Gly Asp Val
140                 145                 150                 155

CCT GTC ATA CAA ATT TCG TCG GGT CAC GCT TTG GCC GAA AAT TTT GAG          832
Pro Val Ile Gln Ile Ser Ser Gly His Ala Leu Ala Glu Asn Phe Glu
                160                 165                 170

ACA ATG GGA GCC GTG AGC CGA CAT CCC ATT TAC TAT CCA AAT TTG TGG          880
Thr Met Gly Ala Val Ser Arg His Pro Ile Tyr Tyr Pro Asn Leu Trp
            175                 180                 185

CGC AAC AAA TTT CAA AAT TTA AAC GTT TGG GAG ATA ATA ACG GAA ATC          928
Arg Asn Lys Phe Gln Asn Leu Asn Val Trp Glu Ile Ile Thr Glu Ile
        190                 195                 200

TAT ACA GAA CTG GTG CTG TAC TTG GAA TTT GCT CGT TTA GCC GAC GAA          976
Tyr Thr Glu Leu Val Leu Tyr Leu Glu Phe Ala Arg Leu Ala Asp Glu
    205                 210                 215

CAA ACT AAA ATG CTT CGC CAT CAA TTC GGA CCA AAC ACG CCC AGC GTG         1024
Gln Thr Lys Met Leu Arg His Gln Phe Gly Pro Asn Thr Pro Ser Val
220                 225                 230                 235

GAA GAA CTG CGA CAA CGC GTT CAA TTA TTG TTT GTG AAT ACG CAT CCG         1072
Glu Glu Leu Arg Gln Arg Val Gln Leu Leu Phe Val Asn Thr His Pro
                240                 245                 250

CTG TTT GAT AAT AAC AGA CCA GTA CCG CCG AGT GTA CAA TAT TTG GGA         1120
Leu Phe Asp Asn Asn Arg Pro Val Pro Pro Ser Val Gln Tyr Leu Gly
            255                 260                 265

AGT CTA CAT CTT GAT CGA AAC AAT GAT GTC GAC GAA CAG CAA ACG ATG         1168
Ser Leu His Leu Asp Arg Asn Asn Asp Val Asp Glu Gln Gln Thr Met
        270                 275                 280

GAC TAT AAT TTG ATG CAA TTT TTA AAT AAT TCT ACA AAC GGT GTG GTG         1216
Asp Tyr Asn Leu Met Gln Phe Leu Asn Asn Ser Thr Asn Gly Val Val
    285                 290                 295

TAC GTG AGC TTC GGT ACG TCT ATA CGA GTT TCA GAC ATG GAC GAC GAA         1264
Tyr Val Ser Phe Gly Thr Ser Ile Arg Val Ser Asp Met Asp Asp Glu
300                 305                 310-----             315

TTT CTG TTT GAA TTT ATA ACA GCT TTC AAG CAA TTA CCC TAT AAT ATA         1312
Phe Leu Phe Glu Phe Ile Thr Ala Phe Lys Gln Leu Pro Tyr Asn Ile
                320                 325                 330
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TTG TGG AAG ACC GAT GGA ATG CCC ATG GAA CAC GTA CTG CCT AAA AAT      1360
Leu Trp Lys Thr Asp Gly Met Pro Met Glu His Val Leu Pro Lys Asn
            335                 340                 345

GTG TTG ACA CAA ACT TGG CTG CCG CAA CAC CAT GTA TTG AAA CAC AGC      1408
Val Leu Thr Gln Thr Trp Leu Pro Gln His His Val Leu Lys His Ser
        350                 355                 360

AAT GTA GTT GCT TTT GTT ACT CAA GGC GGA ATG CAG TCA ACG GAC GAA      1456
Asn Val Val Ala Phe Val Thr Gln Gly Gly Met Gln Ser Thr Asp Glu
    365                 370                 375

GCC ATC GAC GCT TGT GTA CCA CTA ATC GGA ATC CCG TTT ATG GGC GAC      1504
Ala Ile Asp Ala Cys Val Pro Leu Ile Gly Ile Pro Phe Met Gly Asp
380                 385                 390                 395

CAA GCA TAC AAT ACC AAT AAA TAC GAA GAA CTC GGA ATC GGA CGC AAC      1552
Gln Ala Tyr Asn Thr Asn Lys Tyr Glu Glu Leu Gly Ile Gly Arg Asn
                400                 405                 410

CTC GAT CCC GTA ACG CTC ACA AGT CAT ATT TTG GTG TCT GCC GTT TTA      1600
Leu Asp Pro Val Thr Leu Thr Ser His Ile Leu Val Ser Ala Val Leu
            415                 420                 425

GAT GTG ACC GTC AAC AAC AAG AGT CGC TAC ACA GAT AAT ATT AAA GCA      1648
Asp Val Thr Val Asn Asn Lys Ser Arg Tyr Thr Asp Asn Ile Lys Ala
        430                 435                 440

TTG AAT CGT TCC ACT AAT TAT CGA ACA CGG AAA CCT ATG GAA AAG GCC      1696
Leu Asn Arg Ser Thr Asn Tyr Arg Thr Arg Lys Pro Met Glu Lys Ala
    445                 450                 455

ATC TGG TAC ACA GAA CAT GTA ATT GAT AAT GGT AAA AAT CCC ATT TTA      1744
Ile Trp Tyr Thr Glu His Val Ile Asp Asn Gly Lys Asn Pro Ile Leu
460                 465                 470                 475

AAA ACG AAG GCC GCC AAC GTA TCG TAT AGC AAA TAT TAT ATG AGT GAT      1792
Lys Thr Lys Ala Ala Asn Val Ser Tyr Ser Lys Tyr Tyr Met Ser Asp
                480                 485                 490

ATC ATC GTT CCT GTT ATA ACG TTT TTG GTA ATG ACT CAT TTG GGT CAG      1840
Ile Ile Val Pro Val Ile Thr Phe Leu Val Met Thr His Leu Gly Gln
            495                 500                 505

GCT ATT CGG CGG TTG GTT GTT ATT TAA TACTGTATGA CAATGTACAC            1887
Ala Ile Arg Arg Leu Val Val Ile  *
        510                 515

ATGTGTTAAT AAAAAAGGCA TTACTAATAT TTAGATTGTT TCAAATTATT TACGCATGAC    1947

TACCCGTCTC CTATTGCGCA GCTACGCTAG CTTTAAATAC AGCCGATGGC GTAGTAAAGT    2007

TCATTTAAAT ATCTAAAT                                                  2025
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(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 516 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

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Met Tyr Lys Gln Ile Ile Thr Met Leu Leu Leu Val Leu Phe Leu Ser
 1               5                  10                  15

Val Leu Asp Gly Ala Arg Ile Leu Cys Val Phe Pro Val Pro Ser Tyr
            20                  25                  30

Ser His His Ala Val Phe Glu Ala Tyr Thr Asn Ala Leu Ala Ser Arg
        35                  40                  45

Gly His Thr Ile Val Arg Ile Thr Pro Phe Pro Thr Lys Lys Asn Asp
    50                  55                  60

Ser Ser Asn Val Thr Asp Val Asp Val Ser Leu Ser Lys Asp Tyr Phe
65                  70                  75                  80

Lys Ser Leu Val Asp Arg Ser Arg Leu Phe Lys Lys Arg Gly Val Ile
                85                  90                  95

Ser Glu Thr Ser Ser Val Thr Ala Arg Asn Tyr Ile Ser Leu Val His
            100                 105                 110

Met Leu Ile Asp Gln Phe Ser Val Glu Ser Val Arg Gln Leu Ile Glu
        115                 120                 125

Ser Asn Asn Val Phe Asp Leu Leu Val Thr Glu Ala Phe Leu Asp Tyr
    130                 135                 140

Pro Leu Val Phe Ser His Leu Phe Gly Asp Val Pro Val Ile Gln Ile
145                 150                 155                 160

Ser Ser Gly His Ala Leu Ala Glu Asn Phe Glu Thr Met Gly Ala Val
                165                 170                 175

Ser Arg His Pro Ile Tyr Tyr Pro Asn Leu Trp Arg Asn Lys Phe Gln
            180                 185                 190

Asn Leu Asn Val Trp Glu Ile Ile Thr Glu Ile Tyr Thr Glu Leu Val
        195                 200                 205

Leu Tyr Leu Glu Phe Ala Arg Leu Ala Asp Glu Gln Thr Lys Met Leu
    210                 215                 220

Arg His Gln Phe Gly Pro Asn Thr Pro Ser Val Glu Glu Leu Arg Gln
225                 230                 235                 240

Arg Val Gln Leu Leu Phe Val Asn Thr His Pro Leu Phe Asp Asn Asn
                245                 250                 255

Arg Pro Val Pro Pro Ser Val Gln Tyr Leu Gly Ser Leu His Leu Asp
            260                 265                 270

Arg Asn Asn Asp Val Asp Glu Gln Gln Thr Met Asp Tyr Asn Leu Met
        275                 280                 285

Gln Phe Leu Asn Asn Ser Thr Asn Gly Val Val Tyr Val Ser Phe Gly
    290                 295                 300

Thr Ser Ile Arg Val Ser Asp Met Asp Asp Glu Phe Leu Phe Glu Phe
305                 310                 315                 320

Ile Thr Ala Phe Lys Gln Leu Pro Tyr Asn Ile Leu Trp Lys Thr Asp
                325                 330                 335

Gly Met Pro Met Glu His Val Leu Pro Lys Asn Val Leu Thr Gln Thr
            340                 345                 350
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Trp Leu Pro Gln His His Val Leu Lys His Ser Asn Val Val Ala Phe
        355                 360                 365

Val Thr Gln Gly Gly Met Gln Ser Thr Asp Glu Ala Ile Asp Ala Cys
    370                 375                 380

Val Pro Leu Ile Gly Ile Pro Phe Met Gly Asp Gln Ala Tyr Asn Thr
385                 390                 395                 400

Asn Lys Tyr Glu Glu Leu Gly Ile Gly Arg Asn Leu Asp Pro Val Thr
                405                 410                 415

Leu Thr Ser His Ile Leu Val Ser Ala Val Leu Asp Val Thr Val Asn
            420                 425                 430

Asn Lys Ser Arg Tyr Thr Asp Asn Ile Lys Ala Leu Asn Arg Ser Thr
        435                 440                 445

Asn Tyr Arg Thr Arg Lys Pro Met Glu Lys Ala Ile Trp Tyr Thr Glu
    450                 455                 460

His Val Ile Asp Asn Gly Lys Asn Pro Ile Leu Lys Thr Lys Ala Ala
465                 470                 475                 480

Asn Val Ser Tyr Ser Lys Tyr Tyr Met Ser Asp Ile Ile Val Pro Val
                485                 490                 495

Ile Thr Phe Leu Val Met Thr His Leu Gly Gln Ala Ile Arg Arg Leu
            500                 505                 510

Val Val Ile  *
        515
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WE CLAIM:

1. A recombinant DNA molecule comprising a promoter selected from the group consisting of a baculovirus 6.9K promoter, a baculovirus DA26 promoter and a heat shock gene promoter, and a coding sequence for an insect-specific toxin, said coding sequence being operably linked to and expressed under the regulatory control of said promoter.

2. The recombinant DNA molecule of claim 1 wherein said coding sequence encodes an insect-specific neurotoxin.

3. The recombinant DNA molecule of claim 2 wherein said coding sequence encodes an insect-specific paralytic neurotoxin from a mite of the genus *Pyemotes*.

4. The recombinant DNA molecule of claim 3 wherein said coding sequence is as given in SEQ ID NO:1, nucleotides 120 to 873, or a sequence having at least about 70% nucleotide sequence homology thereto, and encoding a protein having insect-specific neurotoxin activity.

5. The recombinant DNA molecule of claim 4 wherein the encoded insect-specific paralytic neurotoxin comprises an amino acid as shown in SEQ ID NO:4.

6. The recombinant DNA molecule of claim 4 wherein the encoded insect-specific paralytic neurotoxin comprises an amino acid sequence as shown in SEQ ID NO:2, from an aspartate encoded at about nucleotide 120 to a cysteine encoded at about nucleotide 873 in SEQ ID NO:1.

7. A baculovirus genetically engineered to contain a promoter selected from the group consisting of baculovirus 6.9K, baculovirus DA26 promoter and a heat shock gene promoter and a coding sequence for an insect-specific toxin, said coding sequence being expressed under the regulatory control of said promoter.

8. The baculovirus of claim 7 wherein said coding sequence encodes an insect-specific paralytic neurotoxin of a mite of the genus *Pyemotes*.

9. The baculovirus of claim 8 wherein the said coding sequence is as given in SEQ ID NO:1, nucleotides 120 to 873, or a sequence having at least about 70% nucleotide sequence homology thereto, and encoding a protein having insect-specific neurotoxin activity.

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10. The baculovirus of claim 9 wherein the encoded insect-specific paralytic neurotoxin comprises an amino acid sequence as shown in SEQ ID NO:2.

11. The baculovirus of claim 10 wherein the encoded insect-specific paralytic neurotoxin comprises an amino acid sequence as shown in SEQ ID NO:4.

12. The baculovirus of claim 7 wherein said baculovirus is a nucleopolyhedrovirus.

13. The baculovirus of any of claims 7 to 12 wherein said nucleopolyhedrovirus is *Autographa californica* Nuclear Polyhedrosis Virus (AcMNPV)or *Helicoverpa zea* Nucleopolyhedrosd Virus (HzSNPV).

14. The baculovirus of any of claims 7 to 12 wherein said heat shock promoter is a *Drosophila melanogaster hsp70* promoter.

15. The baculovirus of claim 14 which is vHSP70tox34.

16. The baculovirus of claim 13 which is v6.9-Tox34 or vDA26-Tox34.

17. The baculovirus of claim 13 which is vHzDA26tox34, vHSP70tox34, vHzEGTHSP70tox34, vHz6.9tox34, and vHzEGT6.9tox34, vHzHSP70tox34 and vHzEGTDA26tox34.

18. The baculovirus of any of claims 7 to 17 in which a gene encoding an ecdysteroid UDP-glucosyl transferase has been inactivated.

19. An insect toxic composition comprising an amount of a baculovirus of any of claims 7 to 18 effective for causing a toxic effect on a target insect, and further comprising an agriculturally acceptable carrier.

20. A method for the control of insect pests comprising the step of applying the insect-toxic composition of claim 19 to a habitat of said insect pests.

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