The present invention provides, inter alia, a polynucleotide comprising a region encoding at least two of the insecticidal toxin subunits selected from those comprised in SEQ ID Nos. 2, 4, 6, 8, and 10 and that encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the said spliced RNA being capable of hybridising with the extension products of the primers depicted in SEQ ID Nos. 20 and 21, or SEQ ID Nos. 22 and 23, or SEQ ID Nos. 24 and 25 using the sequence depicted in SEQ ID No. 1 as a template, with the proviso that the polynucleotide does not encode only the combination of the subunits comprised in SEQ ID Nos. 2 and 8. The invention also provides an insect pathogen, particularly a recombinant baculovirus comprising the said polynucleotide, and various modifications thereof.
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**BIOLOGICAL INSECT CONTROL AGENT**

The present invention relates, *inter alia*, to the biological control of insect pests, to genetically engineered insect pathogens capable of exerting such control, and to the polynucleotide sequences (and vectors containing them) engineered into such pathogens which enable them to exert such control.

The venoms of many social wasps have been extensively studied and are known to contain a potent array of biologically active amines, pain-producing neuropeptides, allergens and neurotoxins. Much less understood are the venoms of solitary wasps, especially those which lead a parasitic lifestyle. Many solitary parasitic wasps prey upon insects and more than 250 species have been observed to paralyse their host. Many of these species are in the family *Braconidae*. The majority of braconid wasps are primary parasites. Adults lay their eggs almost exclusively in or on other insects and, after hatching, the wasp larvae feed upon their host. One braconid species that has attracted attention is *Bracon hebetor* (*Bracon hebetor* = Microbracon = Habrobracon). *Bracon hebetor* (*B. hebetor*) is a small (3 mm) parasite of Lepidopteran larvae which have a cryptic, or cocooning, lifestyle. Adult female wasps deposit eggs on the outside of host larvae while simultaneously injecting a paralysing venom. Within minutes, the host larvae become uncoordinated and eventually suffer complete paralysis. Although not directly fatal, this paralysis is permanent and immobilises the insect until the wasp larvae emerge to feed upon their host. The venom of *B. hebetor* possesses an extremely potent paralysing activity. In larvae of the greater waxmoth, *Galleria mellonella* (*G. mellonella*), it has been estimated that complete and permanent paralysis occurs at levels of 1 part venom to 200,000,000 parts host haemolymph. Furthermore, the venom shows selective toxicity towards insects and between insect orders. Spider, crayfish, frog, rat and guinea-pig neuromuscular preparations all appear to be insensitive to the venom.

The paralysing component of *B. hebetor* venom is thought to act by presynaptically blocking excitatory glutamatergic transmission at neuromuscular junctions, possibly by inhibiting the release of synaptic vesicles.

Venoms from many arthropods that prey on insects have been found to contain toxins which selectively act on insects. However, since the published information on proteinaceous toxins from *B. hebetor* suggests that multiple toxins may exist it was first necessary to purify and characterise a toxin that had high neurotoxic activity to Lepidopteran larvae. We purified
and characterised two neurotoxic proteins, which for ease of reference have been designated bracon toxin 1 and 2 (hereinafter BrhTX-1, and BrhTX-2). *Inter alia*, the present invention provides polynucleotide sequences which encode combinations of the subunits comprised by BrhTX-1.

According to the present invention there is provided a polynucleotide comprising a region encoding at least two of the insecticidal toxin subunits selected from those comprised in SEQ ID Nos. 2, 4, 6, 8, and 10 and that encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the said spliced RNA being capable of hybridising with the extension products of the primers depicted in SEQ ID Nos 20 and 21, or SEQ ID Nos 22 and 23, or SEQ ID Nos 24 and 25 using the sequence depicted in SEQ ID No. 1 as a template, with the *proviso* that the polynucleotide does not encode only the combination of the subunits comprised in SEQ ID Nos. 2 and 8.

"SEQ ID No. 2" includes a protein which is identical to that depicted in SEQ ID No. 2 with the *proviso* that the Proline residue at position 93 in the amino acid sequence is replaced by Leucine.

By "capable of hybridising" is meant hybridisation with the sequence depicted in SEQ ID No. 1 following incubation of the extension products with the SEQ ID No. 1 sequence at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS.

In a preferred embodiment of the polynucleotide the said subunits are the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No. 6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10. At least one of the said proteins may optionally comprise a heterologous N-terminal extension in the form of a signal or secretory peptide.

The invention also provides a polynucleotide comprising a region encoding at least one of the insecticidal toxin subunits selected from those comprised in SEQ ID Nos. 2, 4, 6, 8 and 10 and that encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the said spliced RNA being capable of hybridising with the extension products of the primers depicted in SEQ ID Nos 20 and 21, or SEQ ID Nos 22 and 23, or SEQ ID Nos 24 and 25, using the sequence depicted in SEQ ID No. 1 as a template, wherein the region has
been modified in that mRNA instability motifs and/or fortuitous splice regions are removed, or insect-pest preferred codons are used so that expression of the thus modified polynucleotide in the said insect yields substantially similar protein having a substantially similar activity/function to that obtained by expression of the unmodified polynucleotide in the organism in which the protein encoding regions of the unmodified polynucleotide are endogenous.

It is preferred that the modified region encodes subunits which are the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No. 6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10. At least one of the said proteins may optionally comprise a heterologous N-terminal extension in the form of a signal or secretory peptide. The insect pest may be Lepidopteran, and the said organism may be an Hymenopteran of the superfamily Ichneumonoidea, in particular a wasp of the family Braconidae. The skilled man is aware that the family Braconidae includes the genera Apanteles, Microbracon and Stenobracon.

At least one of the protein encoding sequences in the region may be under expression control of a viral promoter, or insect strong promoter, which is not down regulated or otherwise silenced when the polynucleotide is introduced into the cells of an insect which is or becomes infected by an insect virus. Suitable promoters are known to the skilled man and include, for example, the baculovirus p10 promoter and the polyhedrin promoter.

In one embodiment of the invention, the said region of the polynucleotide preferably encodes not less than two and not more than four of the toxin subunits selected from the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No. 6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10. In a further embodiment, the region likewise encodes not less than two and not more than three of the toxin subunits, and in a still further embodiment of the invention the region encodes two of the toxin subunits selected from the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No. 6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10, with the proviso that the region does not encode only the combination of the toxin subunits comprised in SEQ ID Nos. 2 and 8.

In a particularly preferred embodiment, the polynucleotide comprises a region encoding the three proteins represented by amino acids 22 to 182 in SEQ ID No. 4 or amino
acids 22 to 184 in SEQ ID No. 10; amino acids 32 to 176 in SEQ ID No. 6 and amino acids 23 to 275 in SEQ ID No. 8.

Where the region has been modified - as indicated above - by the provision of insect pest preferred codons, or removal of mRNA instability motifs or splice regions the region may encode only one of the toxin subunits selected from the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No. 6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10.

The invention also provides a nucleotide sequence, encoding an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 50 and 55°C in single strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with single strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID Nos. 1, 3, 5, 7, 9, or 19. “SEQ ID No. 1” includes a polynucleotide which is identical to that depicted in SEQ ID No. 1, with the proviso that the triplet CCA at positions 289-291 is replaced with the triplet CCA. It is preferred that the nucleotide sequence, encodes an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 55 and 60°C in single strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.5 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID Nos. 1, 3, 5, 7, 9, or 19. It is more preferred that the nucleotide sequence, encodes an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID Nos. 1, 3, 5, 7, 9, or 19.

The invention also provides a nucleotide sequence, encoding an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 50 and 60°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence represented by nucleotides 61 to 486 in SEQ ID No. 1; nucleotides 285 to 766 in SEQ ID No. 3; nucleotides 147 to 584 in SEQ ID No. 5;
nucleotides 161 to 219 in SEQ ID No. 7; or nucleotides 85 to 573 in SEQ ID No. 9. It is more preferred that the nucleotide sequence encodes an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence represented by nucleotides 61 to 486 in SEQ ID No. 1; nucleotides 285 to 766 in SEQ ID No. 3; nucleotides 147 to 584 in SEQ ID No. 5; nucleotides 161 to 219 in SEQ ID No. 7; or nucleotides 85 to 573 in SEQ ID No. 9.

It will be appreciated that the said nucleotide sequence can constitute a whole or part of the said region of the polynucleotide.

The invention still further provides a cell transformed with the polynucleotide or complementary sequence of the invention, as well as an organism regenerated from the transformed cell.

The invention still further provides an insect pathogen, or other biological control agent, comprising the polynucleotide or complementary sequence of the invention. Included within the term biological control agent are viral, prokaryotic or eukaryotic organisms which when brought into association with an insect are capable of infecting the insect and/or interfering with the normal biochemical, physiological or electrophysiological processes and ultimately leading to the death of the insect. Suitable biological control agents within the scope of the invention include those based on bacterial, viral and fungal pathogens of insects. Bacterial pathogens include for example Bacillus species such as B. thuringiensis, B. cereus and the like. Fungal pathogens of insects include for example Beauvaria species such as B. bassiana.

Viruses are particularly preferred. Suitable insect viral pathogens include baculoviruses, entomopoxviruses, reoviruses, iridoviruses, paroviruses, rhabdoviruses, picornaviruses and picorna-like viruses, nodaviruses, ascoviruses and retroviruses, with baculoviruses being preferred. Baculoviruses can be classified as a nuclear polyhedrosis virus (NPV) (includes NPVs with singly enveloped nucleocapsids (SNPV)) and NPVs with multiply enveloped nucleocapsids (MNPV)), granulosis virus (GV), and non-occluded baculovirus. Examples of baculoviruses include: Autographa californica MNPV (AcMNPV), Anagraphe falcifera MNPV (AfMNPV), Bombyx mori MNPV (BmMNPV), Anticarsia gemmatalis MNPV (AgMNPV), Cydia pomonella GV (CpGV), Helicoverpa armigera SNPV (HaSNPV),
Helicoverpa zea SNPV (HzSNPV), Lycantra dispar MNPV (LdMNPV), Choristoneura fumiferana MNPV (CfMNPV), Mamestra brassaic MNPV (MbMNPV), Neodiprion sertifer SNPV (NsSMNPV), Orgyia pseudotsugata MNPV (OpMNPV), Pieris rapae GV (PrGV) and Spodoptera exigua MNPV (SeMNPV).

The recombinant baculoviruses may be mixed or otherwise treated with known fluorescent brighteners, in particular those of the stilbene disulphonic acid group. Such brighteners may enhance the intrinsic activity of baculoviruses, and provide enhanced levels of protection of the virus against ultra-violet light, which helps to retain the activity of the virus during exposure to uv light.

Alternatively the biological control agent can be a genetically modified plant endophyte in which the genome has been altered to incorporate the polynucleotide or complementary nucleotide sequence of the present invention. When such an endophyte is brought into association with a plant the toxin subunits encoded by the introduced DNA may be produced by the endophyte within the plant and exert toxic effects on insects feeding on or dwelling within the plant.

In a further variation the biological control agent can be a plant itself, particularly a crop plant being grown for food or fibre products, in which the plant genome has been modified by incorporation into it of the polynucleotide or complementary nucleotide sequence of the invention. Accordingly, plant tissue may be transformed, by means (electroporation, microprojectile mediated transformation, Agrobacterium mediated transformation, protoplast transformation, etc.) known to the skilled man, with the polynucleotide or complementary sequence and regenerated, again by known means, into intact fertile whole plants.

The invention still further provides insecticidal compositions comprising the following combinations of proteins:

(i) the proteins represented by amino acids 17 to 158 in SEQ ID No. 2 and 22 to 182 in SEQ ID No. 4;
(ii) the proteins represented by amino acids 17 to 158 in SEQ ID No. 2 and 32 to 176 in SEQ ID No. 6;
(iii) the proteins represented by amino acids 22 to 182 in SEQ ID No. 4 and 32 to 176 in SEQ ID No. 6;
(iv) the proteins represented by amino acids 22 to 182 in SEQ ID No. 4 and 23 to 275 in SEQ ID No. 8;
(v) the proteins represented by amino acids 32 to 176 in SEQ ID No. 6 and 23 to 275 in SEQ ID No. 8;
(vi) the proteins represented by amino acids 17 to 158 in SEQ ID No. 2, amino acids 32 to 176 in SEQ ID No. 6, and amino acids 23 to 275 in SEQ ID No. 8;
(vii) the proteins represented by amino acids 17 to 158 in SEQ ID No. 2, amino acids 22 to 182 in SEQ ID No. 4, and amino acids 32 to 176 in SEQ ID No. 6;
(viii) the proteins represented by amino acids 17 to 158 in SEQ ID No. 2, amino acids 22 to 182 in SEQ ID No. 4, and amino acids 23 to 275 in SEQ ID No. 8;
(ix) the proteins represented by amino acids 22 to 182 in SEQ ID No. 4, amino acids 32 to 176 in SEQ ID No. 6, and amino acids 23 to 275 in SEQ ID No. 8;
(x) the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4, amino acids 32 to 176 in SEQ ID No. 6, and amino acids 23 to 275 in SEQ ID No. 8.

Each of the insecticidal compositions given above may be augmented by - or at least one of the proteins in the composition replaced by - protein represented by amino acids 22 to 184 in SEQ ID No. 10, and/or that encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the said spliced RNA being capable of hybridising with the extension products of the primers depicted in SEQ ID Nos 20 and 21, or SEQ ID Nos 22 and 23, or SEQ ID Nos 24 and 25, using the sequence depicted in SEQ ID No. 1 as a template.

The invention still further provides a method of controlling insects, comprising exposing them or their habitat to one or more of the following: (i) cells transformed with the polynucleotide or complementary sequence of the invention; (ii) organisms regenerated from the transformed cells; (iii) recombinant insect pathogens comprising the polynucleotide or sequence of the invention; and (iv) the insecticidal composition disclosed in the two immediately preceding paragraphs.

The invention still further provides the use of the inventive polynucleotide or sequence in the preparation of a recombinant insect-pathogen, particularly a baculovirus for the biological control of insect pests.
The invention still further provides a polynucleotide comprising a region encoding the insecticidal toxin subunit encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the said spliced RNA being capable of hybridising with the extension products of the primers depicted in SEQ ID Nos 20 and 21, or SEQ ID Nos 22 and 23, or SEQ ID Nos 24 and 25 using the sequence depicted in SEQ ID No. 1 as a template, the translation product of the polynucleotide and recombinant insect-enteropathogenic viruses comprising the said polynucleotide or a nucleotide encoding the spliced variant thereof.

The invention will be further apparent from the following description taken in conjunction with the associated Figures and Sequence listings.

Figure 1 is a composite sequence of the genomic region encoding the BrhTX-1(a) cDNA, putative exons are indicated in bold and translation of the putative message is shown below the coding sequence.

Figure 2 is an alignment of homologous regions of the genomic clones pBH(a)λ3.4-Pst.2 and pBH(a)λ3.5-Pst.5, the former contains the sequences that encode BrhTX-1(a) (see Figure 1).

Figure 3 is a plasmid map of pMMS1.

Figure 4 is a plasmid map of pAcUW21.

SEQ ID No. 1 shows a first nucleotide sequence comprising a region encoding a first insecticidal toxin subunit (BrhTX-1a) having a molecular weight of about 17kDa. SEQ ID No. 2 shows the translational product of the ORF identified in SEQ ID No. 1. Amino acids 1-16 of the sequence shown in SEQ ID No. 2 constitute a putative leader or signal sequence. Amino acids 17 to 158 of the sequence constitute the mature toxin-subunit.

SEQ ID No. 3 shows a second nucleotide sequence comprising a region encoding a second insecticidal toxin subunit (BrhTX-1b) having a molecular weight of about 18kDa.

SEQ ID No. 4 shows the translational product of the ORF identified in SEQ ID No. 3. Amino acids 1 to 21 of the sequence shown in SEQ ID No. 4 constitute a putative leader or signal sequence. Amino acids 22 to 182 of the sequence constitute the mature toxin-subunit.

SEQ ID No. 5 shows a third nucleotide sequence comprising a region encoding a third insecticidal toxin subunit (BrhTX-1c) having a molecular weight of about 21kDa as judged by SDS electrophoresis.
SEQ ID No. 6 shows the translational product of the ORF identified in SEQ ID No. 5, together with a putative signal peptide.

SEQ ID No.7 shows a fourth nucleotide sequence comprising a region encoding a fourth insecticidal toxin subunit ((BrhTX-1d) having a molecular weight of about 32kDa. SEQ ID No. 8 shows the translational product of the ORF identified in SEQ ID No. 7. Amino acids 1 to 22 of the sequence shown in SEQ ID No. 8 constitute a putative leader or signal sequence. Amino acids 23 to 275 of the sequence constitute the mature toxin-subunit.

SEQ ID No. 9 shows a fifth nucleotide sequence - substantially similar to that depicted in SEQ ID No. 3 - comprising a region encoding a fifth insecticidal toxin subunit ((BrhTX-1e) having a molecular weight of about 18kDa. SEQ ID No. 10 shows the translational product of the ORF identified in SEQ ID No. 9. Amino acids 1 to 21 of the sequence shown in SEQ ID No. 10 constitute a putative leader or signal sequence. Amino acids 22 to 184 of the sequence constitute the mature toxin-subunit.

SEQ ID No. 11 shows a genomic sequence which comprises part of a spliced RNA that is related to the cDNA comprised by pBrhTX-1(a)1.1, and which encodes a toxin subunit which is substantially similar to the protein depicted in SEQ ID No. 2.

SEQ ID No. 12 shows the sequence of pBrhTX-1(a)GBsp1 and pBrhTX-1(a)GBsp3; SEQ ID No. 13 shows the sequence of pBrhTX-1(a)GBsp2 and pBrhTX-1(a)GBsp4; SEQ ID No. 14 shows the sequence of the 700bp Spe I fragment isolated from the λ(a)G-2.1.1 genomic clone.

SEQ ID Nos. 15-18 and 20 - 44 show the sequences of the various PCR primers.

SEQ ID No. 19 shows a contiguous genomic sequence which encodes part of a spliced RNA that is related to the cDNA comprised by pBrhTX-1(a)1.1, and which encodes a protein which is substantially similar to the protein depicted in SEQ ID No. 2

SEQ ID No. 45 discloses a putative toxin-encoding-sequence derived from the genomic sequence depicted in SEQ ID No. 19 and SEQ ID No. 46 discloses the translation product of the nucleotide sequence depicted in SEQ ID. No. 45.

International Patent Application No. PCT/GB95/02720 (Publication No. WO 96/16171) describes the isolation and characterisation of BrhTX-1 and BrhTX-2. The application also describes, inter alia, the sequence analysis of BrhTX-1 and the cloning of the sequences encoding the subunits of this toxin. These descriptions are incorporated by
reference into the present application. Unless specifically indicated to the contrary, all amino acid and nucleotide sequences which are depicted in, or are referred to in the following Examples have already been published in International Patent Application No. PCT/GB95/02720 (Publication No. WO 96/16171). Accordingly, such known sequences are not included in the sequence listing appended to this application.

Example 1 - Identification of a BrhTX-1(a) coding sequence in addition to that disclosed in WO96/16171

Northern Analysis of 17kDa cDNA Sequence

Batches of mRNA for Northern analysis are isolated from approximately 300 female Bracon hebetor wasps using a “QuickPrep” Total RNA Extraction Kit (Pharmacia, Uppsala, Sweden). Integrity and estimates of concentration of isolated RNA from each preparation are made by electrophoresis through MOPS/formaldehyde, 1% agarose gels. Blots for hybridisation are made with a total of 20 female equivalents per lane from 1% agarose gels containing MOPS/formaldehyde. Transfers to hybridisation membrane are made using 20-2x SSC gradients and hybridisations are carried out in buffers containing 50% formamide at 42°C.

\(^{32}\)P-labelled probes are synthesised by PCR from short regions at the 5’ end in the middle and at the 3’ end of the BrhTX-1 cDNA. For all probes the cDNA clone, BrhTX-1(a)1.2 digested with Not I is used as template. Primers for the 5’ end are BH(a)F5 and BH(a)R5 (SEQ ID Nos 20, 21), middle BH(a)F3 and BH(a)R6 (SEQ ID Nos 22, 23) and for the 3’ end BH(a)F6 and BH(a)R4 (SEQ ID Nos 24, 25)

Reaction conditions for generation of \(^{32}\)P-labelled probes using the above primers are as follows:

- 5 minutes at 95°C
- 30 seconds at 95°C, 30 seconds at 45°C, 1 minutes at 72°C (5 cycles); then
- 30 seconds at 95°C, 30 seconds at 50°C, 1 minute 72°C (30 cycles) then,
- 5 minutes at 72°C

Blots are then hybridised with one of the 3 probes (5’, middle or 3’) as detailed above.

Results from this analysis are shown in Table 1:
The minimum size of the message (as deduced from the cDNA clone) is about 560 bases - with all of the 3’ untranslated accounted for i.e. the clone contains the polyA tail. It therefore seems likely that the 706 base band identified above corresponds to the message encoding BrhTX-1(a). The presence of the 1694 base band however suggests that another RNA species is produced that contains sequences related to regions of the BrhTX-1(a)1.1 cDNA clone.

Genomic Southern Analysis

Genomic DNA is prepared as described for the Examples in WO-99/16171, and 5-10mg aliquots are digested with the restriction enzymes Aat II, Acc I, Apa I, Bam HI, Bcl I, Bsp HI, Cla I, Eco RI, Eco RV, Hind III, Kpn I, Mbo II, Nco I, Nde I, Not I, Pst I, Sac I, Sac II, Sal I, Sma I, Spe I, Xba I and Xho I. Fragments are then separated by electrophoresis through 0.7% agarose gels in TAE buffer and the DNA blotted onto nitrocellulose membranes (Nitro-pure, supported nitrocellulose, MSI, MA, USA) in a 2-20x SSC gradient and bonded to the membrane by baking at 80°C for 2 hours. 32P-labelled probes are synthesised by PCR using the primers BH(a)F2 and BH(a)R4 (SEQ ID Nos 26, 25) and pBrhTX-1(a)1.2 plasmid DNA as target DNA under the following conditions:

5 minutes at 95°C
30 seconds at 95°C, 30 seconds at 45°C, 1 minute at 72°C (5 cycles); then
30 seconds at 95°C, 30 seconds at 50°C, 1 minute 72°C (30 cycles) then,
5 minutes at 72°C

Pre-hybridisation and hybridisation are performed as previously described.

The majority of restriction digests reveal a single hybridising fragment of over 10Kb. Eight enzymes however, generate fragments of under 10 Kbp. In some instances more than one

<table>
<thead>
<tr>
<th>Probe</th>
<th>Major Hybridising Band</th>
<th>Minor Hybridising Band</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’</td>
<td>706b and 1694b</td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>706b</td>
<td>1694b</td>
</tr>
<tr>
<td>3’</td>
<td>1694b</td>
<td>706b</td>
</tr>
</tbody>
</table>
hybridising fragment is observed - with one of the fragments hybridising more strongly than
the other. The sizes and relative hybridisation intensity of the fragments from these digests are
summarised below.

<table>
<thead>
<tr>
<th>restriction enzyme</th>
<th>major size</th>
<th>minor size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xba I</td>
<td>5.7 Kbp</td>
<td>2.5 Kbp</td>
</tr>
<tr>
<td>Spe I</td>
<td>&gt;16 Kbp</td>
<td>0.7 Kbp</td>
</tr>
<tr>
<td>Nde I</td>
<td>7.0 Kbp</td>
<td>0.9 Kbp</td>
</tr>
<tr>
<td>Bsp HI</td>
<td>3.8 Kbp</td>
<td>1.2 Kbp</td>
</tr>
<tr>
<td>Mbo II</td>
<td>1.1 Kbp</td>
<td></td>
</tr>
<tr>
<td>Pst I</td>
<td>3.4 Kbp</td>
<td></td>
</tr>
<tr>
<td>Bcl I</td>
<td>1.4 Kbp</td>
<td></td>
</tr>
<tr>
<td>Acc I</td>
<td>2.9 Kbp and</td>
<td>1.5 Kbp</td>
</tr>
</tbody>
</table>

Initial analysis of the above data suggests that there is a single BrhTX-1(a) gene in the
Bracon genome, and that the gene contains an Acc I, Xba I, Spe I, Nde I and Bsp HI site.
However, further Southern analysis using the restriction enzymes Pst I, Xho I and Nde I and
the three probes designed to be homologous to regions at 5’ end, middle and 3’ end of
BrhTX-1(a) cDNA gives the results presented in Table 2. In contrast to the results obtained
using a probe generated from the cDNA using the primers BH(a)F2 and BH(a)R4 (SEQ ID
Nos 26, 25) the results using the much smaller region-specific probes indicates that there is a
second sequence in the Bracon hebetor genome with sequence similarity to that of the
BrhTX-1(a) cDNA. This latter observation is in agreement with the Northern hybridisation
results that indicate a second mRNA with sequence homology to the pBrhTX-1(a) cDNA
clone.
Table 2. Results of Southern Analysis Performed on 17Kda cDNA using Region-Specific Probes.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Enzyme</th>
<th>Major Hybridising Band</th>
<th>Minor Hybridising Band</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'</td>
<td>Pst I</td>
<td>3.7Kb</td>
<td>4.7Kb</td>
</tr>
<tr>
<td></td>
<td>Xho I</td>
<td>&gt;23Kb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nde I</td>
<td>6.1 Kb</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>Pst I</td>
<td>3.7Kb</td>
<td>4.7Kb</td>
</tr>
<tr>
<td></td>
<td>Xho I</td>
<td>&gt;23Kb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nde I</td>
<td>6.1Kb and 0.9Kb</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'</td>
<td>Pst I</td>
<td>3.7Kb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xho I</td>
<td>&gt;23Kb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nde I</td>
<td>0.9Kb</td>
<td></td>
</tr>
</tbody>
</table>

Generation of a Genomic Library from *Braccon hebetor*

Genomic DNA was prepared from female wasps as previously described and subjected to partial digestion with *Sau*3A I. The digested DNA is then ligated into λGEM-11 Xho I half site arms under conditions specified by the supplier (Promega, Madison, WI). After packaging the phage are plated into *E. coli* Strain LE392. Subsequent titration and amplification of the library was carried out using this strain.

Isolation and Sub-cloning of Genomic Sequences

The λGEM-11 genomic library is plated and plaque lifts carried out as previously described for cDNA libraries. ³²P-labelled primers are synthesised by PCR as described above using the BH(a)F2 and BH(a)R4 primers (SEQ ID Nos 26, 25). Pre-hybridisation and hybridisation of filters from plaque-lifts are performed under the conditions described for genomic Southern analysis. The primary screen identifies over a hundred plaques that hybridise strongly to the probes. Six of these are selected and taken through a further three rounds of plaque purification. DNA is then purified from the phage as previously described and restriction digests performed to estimate the size of the inserts. These insert sizes and designation of phage are shown below:
Approx insert size

$\lambda B\text{H}(a)G$-2.1.1  21.0 Kbp

$\lambda B\text{H}(a)G$-3.1.1  19.1 Kbp

$\lambda B\text{H}(a)G$-4.1.1  15.0 Kbp

$\lambda B\text{H}(a)G$-5.1.1  18.9 Kbp

$\lambda B\text{H}(a)G$-6.1.1  19.3 Kbp

$\lambda B\text{H}(a)G$-6.2.1  20.5 Kbp

DNA is isolated from these phage and digested with the enzymes Eco RI, Spe I and Xho I and subjected to Southern blot analyses using the same probe and conditions as outlined above for their isolation. This analyses demonstrate that the clones all share a number of identical bands and are thus related to each other. Because of the close relationship between the clones further analyses are therefore concentrated upon one of the phage namely, $\lambda B\text{H}(a)G$-2.1.1

Southern analysis of the genomic $\lambda B\text{H}(a)G$-2.1.1 which includes the restriction enzymes Bsp HI Spe I and Pst I reveals fragments of 400bp, 700bp and 3.7kbp and 4.7kbp respectively that hybridise strongly to the 344bp probe generated by PCR from the cDNA clone, pBrhTX-1(a)1.2 (WO-99/16171) and the primers BH(a)F2 and BH(a)R4 (SEQ ID 26, 25). Consequently, these fragments are isolated after electrophoresis on 0.8% agarose gels and cloned into the Nco I and Spe I sites of pGEM5z(f)+ (Promega) and pBluescript SK- for the Bsp HI and Spe I fragments respectively and into the Pst I site of pGEM for Pst I fragments.

**Sequencing of the Lambda Genomic Clone and Subclones**

Sub-cloned Bsp HI fragments clones (pBrhTX-1(a)GBsp1, pBrhTX-1(a)GBsp2, pBrhTX-1(a)GBsp3, pBrhTX-1(a)GBsp4) and Spe I fragments (pBrhTX-1(a)GSpE1, pBrhTX-1(a)GSpE2, pBrhTX-1(a)GSpE3, pBrhTX-1(a)GSpE4, pBrhTX-1(a)GSpE5, pBrhTX-1(a)GSpE6) are sequenced using the ABI Prism Dye terminator cycle sequencing kit with AmpliTaq DNA Polymerase, FS and analysed using the ABI 373 Automated Sequencer.

Two distinct sequences are obtained from the four Bsp HI sub-clones analysed. pBrhTX-1(a)GBsp1 and pBrhTX-1(a)GBsp3 are identical (SEQ ID No 12) while pBrhTX-1(a)GBsp2 and pBrhTX-1(a)GBsp4 are also identical to each other (SEQ ID No 13).
However, an alignment of these two sequences reveals that although similar they are not identical. Analysis of the six \textit{Spe} I subclones reveals a single sequence (SEQ ID No 14).

Initial sequence analysis is also carried out by direct sequencing from \textit{\lambda}BH(a)G-2.1.1 using the primers BH(a)F2, BH(a)F3, BH(a)F6, BH(a)R6, BH(a)R4, BH(a)R7, BH(a)R8, (SEQ ID Nos 26, 22, 24, 23, 25, 27, 28) and the ABI Prism dye-terminator cycle sequencing kit with AmpliTaq DNA Polymerase, FS. Samples from cycle sequencing reactions are analysed using the ABI 373 Automated Sequencer. No sequence is obtained from the primers BH(a)F2 and BH(a)F3 and the sequencing signal from BH(a)R7 gives dual peaks at some bases. These dual peaks can in most instances be resolved by reference to the graphic summary of the sequencing analysis. Using these sequence data an initial sequence is obtained spanning the whole of the region containing all of the putative cDNA sequence represented by the pBrhTX-1(a)1.2.

Alignment of this initial sequence is then carried out with the pBrhTX-1(a)GBsp1/3 sequence which showed a high degree of homology. In contrast alignments performed with the pBrhTX-1(a)GBsp2/4 sequence shows considerable differences from the putative genomic sequence (including two deletions/insertions of 2 and 3 bp). As all of these fragments are isolated and cloned from a single \textit{\lambda} genomic clone, the implication from the above data is that there is more than one sequence in that \textit{\lambda} clone with relatively high homology to the 5' end of the BrhTX-1(a) cDNA.

The two Pst I sub-clones (termed pBH(a)\textit{\lambda}3.5-Pst.2 and pBH(a)\textit{\lambda}3.5-Pst.5) are sequenced using the primers BH(a)F2, BH(a)F3, BH(a)F6, BH(a)R6, BH(a)R4, BH(a)R7, BH(a)R8 (SEQ ID Nos 26, 22, 24, 23, 25, 27,28) and the ABI Prism dye-terminator cycle sequencing kit with AmpliTaq DNA Polymerase, FS. A number of \textit{Hinc} II, \textit{Xba I} and \textit{Acc I} fragments are also subcloned from the pBH(a)\textit{\lambda}3.5-Pst.2 and pBH(a)\textit{\lambda}3.5-Pst.5 subclones and sequenced using the automated dye-terminator method described above.

From all of the sequence data available a consensus of the genomic region encoding the mRNA from which the BrhTX-1(a) mRNA is derived (SEQ ID 19). This sequence is shown in Figure 1 with the putative intron/exon structure and the translation of the deduced mRNA. This sequence is contained completely within the pBH(a)\textit{\lambda}3.5-Pst.2 subclone.

Sequence analysis of the pBH(a)\textit{\lambda}3.5-Pst.5 subclone and the subclones derived from it produce a single contiguous sequence that contains all of the pBrhTX-1(a)GBsp2/4 sequence
(SEQ ID 13) and the 700bp Spe I subclones (SEQ ID 14) derived from \( \lambda BH(a) \)G-2.1.1. This contiguous sequence is shown in SEQ ID 19. An alignment of this sequence with the genomic sequence that encodes BrhTX-1(a) is shown in Figure 2.

While the genomic sequence that encodes BrhTX-1(a) and the sequence (SEQ ID 19) that shows homology to it, are both located in the 21.0 kbp fragment of genomic DNA contained in \( \lambda BH(a) \)G-2.1.1, the arrangement of the sequences relative to each other is not known. To determine this four primers are designed as shown below and in SEQ ID Nos 29, 30, 31, 32.

- BH(a)PST2R 5'-GTA ACC AGC TAA GCA TAA CG- 3'
- BH(a)PST2L 5'-GTT ATA CAC AGA GGA TCA GGG AG-3'
- BH(a)PST5R 5'-AAT ACA GTC CCT ATA TAC CC-3'
- BH(a)PST5L 5'-GGG ATG GGA ATA ATG ATG TCA-3'

These primers are designed from sequences at the termini of the two Pst I subclones (BH(a)\( \lambda \)3.5-Pst.2 and pBH(a)\( \lambda \)3.5-Pst.5) with their 5'-3' orientation being away from the middle of those clones. All of the pairwise combinations of these primers are then used in PCR experiments under the following conditions using \( \lambda BH(a) \)G-2.1.1 DNA as a target.

- 5 minutes at 95\(^\circ\)C
- 30 seconds at 95\(^\circ\)C, 30 seconds at 45\(^\circ\)C, 1 minutes at 72\(^\circ\)C (5 cycles); then
- 30 seconds at 95\(^\circ\)C, 30 seconds at 50\(^\circ\)C, 1 minute 72\(^\circ\)C (30 cycles) then,
- 5 minutes at 72\(^\circ\)C

Only the BH(a)PST2L and BH(a)PST5R combination produces an amplicon i.e. a fragment of 305 bp. This indicates that the two fragments are virtually adjacent to each other within the \( \lambda BH(a) \)G-2.1.1 clone. Sequencing with the primers BH(a)PST2L and BH(a)PST5R directly from the \( \lambda BH(a) \)G-2.1.1 DNA confirms that the Pst I fragments are adjacent in the genomic clone. In relation to the putative transcriptional orientation of the sequence encoding BrhTX-1(a), the homologous sequence is located in the fragment situated upstream.

**Example 2 - Cloning of subunit (b) of BrhTX-1**

This is performed as described in WO96/16171. Briefly, from the N-terminal sequence given in Sequence ID No. 2 of WO96/16171, two multi-species primers are designed and are indicated below:

- BH(b)A 5'-AC(TCA) TTG TT(TC) AC(TCA) GA(TC) CG(TC) AA-3'
BH(b)B 5’-GG (ATG)CC (AG)AA (AGT)GT (TC)TT (AG)TC-3’

These primers are used in a PCR experiment to generate a PCR product from cDNA synthesised from female mRNA as previously described. The PCR reaction conditions for a 0.2 µl Taq DNA Polymerase/50 µl reaction are as follows:

5 minutes at 95°C; then
30 seconds at 95°C; 1.5 minutes at 50°C; 1 minute at 72°C (5 cycles); then
30 seconds at 95°C; 1.5 minutes at 55°C; 1 minute at 72°C (30 cycles); then
5 minutes at 72°C.

This PCR product are of approximately 54 bp, as estimated by PAGE in 15% gels in TBE buffer. Amplification products are visualised in the gels by staining with ethidium bromide. The fragment is then cloned into the EcoRV site of the commercially available plasmid pBluescript SK- that has been tailed with a thymine residue (T-tailing) using Taq DNA Polymerase. The cloned PCR product is sequenced using the following dye primers which are available from Applied Biosystems Inc:

Universal M13-20 dye-primer: 5’-CAG GAA ACA GCT ATG ACC-3’
M13 reverse dye-primer: 5’-TGT AAA ACG ACG GCC AGT-3’

Sequencing is carried out using an ABI 370 A DNA analysis system (Applied Biosystems). The sequence for the PCR product is given below.

5’-ACC TTG TTT ACA GAC CGC AAG TGG TGT GGA CGT GCC GAT AAG ACT TTC GGC CC-3’

(ii) Screening of library and Isolation of Clones

Library plating is performed as described in WO96/16171. Plaque lifts are performed in duplicate onto Nitropure nitrocellulose membranes, 137 mm 0.45 micron, (Microb Separations, Westboro, MA, USA). In the primary screen 3.6x10^5 plaques per filter (4 filters in total) are screened, in secondary and tertiary screens 100-250 plaques per filter are screened.

32P-labelled probes are made by PCR. PCR is carried out in the presence of 32P-dATP using the oligonucleotides BH(b)A and BH(b)B shown in Sequence ID Nos. 38 and 39 of WO96/16171, and the cloned PCR product carrying the sequence shown in Sequence ID No. 40 (WO96/16171) as the target.

The following are the PCR conditions:
95°C for 5 minutes prior to the addition of Taq polymerase; then
95°C for 30 seconds, 45°C for 1.5 minutes and 72°C for 1 minute (5 cycles); then
95°C for 30 seconds, 50°C for 1.30 minutes and 72°C for 1 minute (30 cycles); then
72°C for 5 minutes (1 cycle).

Filters are probed with the \(^{32}P\)-labelled probe described above under the same pre-
hybridising and hybridising conditions as described for the Genomic Southern Blot of subunit
BrhTX-1(a). Ten plaques that hybridise to this probe are initially identified. Three of these
are purified through second and third round screens to homogeneity. Plaque purified phage
are picked into 1ml of SM buffer plus 10 \(\mu\)l of chloroform.

Characterisation of Clones

The plaque-purified phage are screened for the presence and size of a cDNA insert by
PCR analysis using the oligonucleotides \(\lambda\)gt 11 forward and \(\lambda\)gt 11 reverse. Lambda DNA
for PCR is prepared from purified stocks by boiling 10 \(\mu\)l of the stock for 5 minutes. Two \(\mu\)l
of this DNA is then used in PCR experiments. The amplified fragments are sized by
electrophoresis through 0.8% agarose gels in TAE buffer and sized against markers of known
size. Of the three purified phage, an insert of approximately 500 bp is found in one phage,
which phage is designated \(\lambda\)BrhTX-1 (b)1.

Subcloning and Sequencing of Clones

Phage DNA is purified by isopycnic centrifugation in CsCl as described in Sambrook

The cDNA insert from \(\lambda\)BrhTX-1 (b)1 is removed by digestion with \(Not\) I and ligated
into pBluescript SK- that is digested with \(Not\) I and phosphatase treated. The insert of the
cloned is sequenced using the ABI dye-primer sequencing kit (as described above). The
sequence of the plasmid clone, pBrhTX-1(b)1, insert is given in Sequence ID No. 41 of
WO96/16171. The amino acids 27-44 match those of the N-terminal sequence shown in
Sequence ID No. 2 of WO96/16171, but the very short apparent size of the ORF encoded by
the sequence in Sequence ID No. 41 suggests that the clone is severely truncated. Two
primers to the ORF at the 5' end of the clone, namely BH(b)C and BH(b)D as shown in
Sequence ID Nos. 42 and 43 respectively of WO96/16171 are designed. Using these primers
and pBrhTX-1(b)1.1 as a target a \(^{32}P\)-labelled probe by PCR is generated under the following
conditions:
5 minutes at 95°C;
30 seconds at 95°C; 1.5 minutes at 45°C; 1 minute at 72°C (5 cycles); then
30 seconds at 95°C; 1.5 minutes at 50°C; 1 minute at 72°C (5 cycles); then
5 minutes at 72°C.

The library is then re-screened using the conditions described above. Six strongly
hybridising plaques are purified through to homogeneity in three rounds of plaque
purification. cDNA inserts are removed from the phage by digestion with *Not* I and ligated
into pBluescript SK- that has been digested with *Not* I and phosphatase treated. The sizes of
the inserts are estimated by digestion with *Not* I and separation of restriction fragments
through 0.8% agarose gels in TAE buffer.

The following cDNA insert sizes are estimated:

λBrhTX-1(b)2 - about 500 bp; and λBrhTX-1 (b)3, λBrhTX-1(b)4, λBrhTX-1(b)5 and
λBrhTX-1 (b)6 all at about 1200 bp.

The plasmid designated pBrhTX-1(b)6 is sequenced using the ABI 370 A DNA
analysis system and the previously described dye primers. The sequencing is completed using
the ABİ dye-terminator system in conjunction with the BH(b)C, BH(b)D primers and two
additional primers as shown below.

BH(b)E    GTTGTCATACACCCCTG
BH(b)F    AGAACGAGATGTTATTGTAT

The nucleotide sequence obtained is shown in SEQ ID No. 3 Translation of the
nucleotide sequence is shown in Sequence ID No. 4, and gives a protein of either 182 or 165
amino acids depending upon the initiation codon used. The alternative proteins have
hydrophobic leader sequences of either 21 or 4 amino acids respectively, giving a mature
peptide of 161 amino acids.

**Analysis of Clones**

**Peptide Digestion/Sequencing** - As in WO96/16171, the alignment of the sequences obtained
against the deduced amino acid translations is shown below:

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>MIKPGETYGDVTK</th>
<th>EWWHDNA</th>
<th>LLPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA Sequence</td>
<td>MIKPGETYGDVTK</td>
<td>EWWHDNA</td>
<td>LLPR</td>
</tr>
<tr>
<td>Peptide Sequence</td>
<td>PHTVYDKHESLQ</td>
<td>DVHDNAGTLPR</td>
<td></td>
</tr>
<tr>
<td>Peptide Sequence</td>
<td>PHTVYDKHESLY</td>
<td>WVHDNA</td>
<td>LLPQ</td>
</tr>
</tbody>
</table>
It is not unusual for the terminal amino acids to be subject to mis-sequencing; however, one of the peptide sequences sequenced on two separate occasions contains a characteristic GT amino acid pair. These GT pair is missing in the ORF predicted for the sequenced clone. It can therefore be deduced that the clone pBrhTX-1(b)6 contains a sequencing artefact or that the cDNA from which it is generated contains an error, i.e. a six bp deletion generated during synthesis/cloning. The region containing the apparent "GT" pair/deletion is recloned by PCR. Primers BH(b)E and BH(b)F are used in the PCR experiments with cDNA as the target, to generate an amplification product. cDNA is synthesised from female B. hebetor mRNA as previously described for the construction of the cDNA library.

The amplification products are then cloned into the EcoRV site of pBluescript that are T-tailed as previously described. These clones are then sequenced and all are found to contain the same sequence as pBrhTX-1(b)6 flanking the apparent GT anomaly, but have an additional six bp corresponding to the codons for G and T, i.e. GGAACT.

From these data it appears that the GT anomaly arises during the cDNA synthesis/cloning of the original λ clone. The consensus sequence for the pBrhTX-1(b) cDNA and the putative ORF are shown in SEQ ID No. 9.

Genomic Southern Blot - Southern blots of male and female B. hebetor genomic DNA digested with Pst I, EcoRV, Acc I and Xho I are generated as previously described in WO96/16171. A 32P-labelled probe is generated by PCR from the 5' region of the pBrhTX-1(b)6 clone using the primers BH(b)C and BH(b)D under the following conditions:

- 5 minutes at 95°C;
- 30 seconds at 95°C; 1.5 minutes at 45°C; 1 minute at 72°C (5 cycles); then
- 30 seconds at 95°C; 1.5 minutes at 50°C; 1 minute at 72°C (5 cycles); then
- 5 minutes at 72°C.

Hybridisations is carried out with the probe as previously described. Results are identical for male and female DNA. In the Pst I digest two hybridising bands are observed of 13.0 kbp and 4.1 kbp. This result is consistent with the presence of a Pst I site in the probe hybridisation region of the cDNA sequence. The EcoRV, Acc I and Xho I digests produce single hybridising bands of 3.3 kbp, 13.0 kbp and 7.0 kbp respectively.

In a second experiment, 32P-labelled probes are made from the 3' region of the pBrhTX-1(b)5 clone using the primers BH(b)E and BH(b)F under the following conditions:
5 minutes at 95°C;
30 seconds at 95°C; 1.5 minutes at 45°C; 1 minute at 72°C (5 cycles); then
30 seconds at 95°C; 1.5 minutes at 50°C; 1 minute at 72°C (5 cycles); then
5 minutes at 72°C.

These probes hybridise to Southern blots prepared as previously described using male
and female genomic DNA that has been digested with Bcl I, Nde I, Bgl II and Pst I. Results
are identical for male and female DNA with the Bcl I and Bgl II digests single hybridising
bands of 1.7 kbp and 9.1 kbp are generated. Although these results do not fully agree with the
presence of both these sites within the probe hybridising region, i.e. one would normally
expect to see two hybridising bands, both sites are relatively close to the ends of the probe
region and the fragment containing the smaller portion of the hybridising region can evade
detection with the large probes that are used in this experiment. In the Nde I and Pst I digests,
single hybridising bands of 2.4 and 22.2 kbp are detected.

Recovery of an alternative form of the BrhTX-1(b) subunit

The aim of recovering an alternative form of the BrhTX-1(b) subunit is to obtain a
cDNA without the long 5' untranslated end and encoding the GT amino acid pair which
peptide sequencing suggests might be present in some BrhTX-1(b) proteins but which is not
encoded by clone pBrhTX-1(b)6. Such results might be explained if there are polymorphic
alleles of the BrhTX-1(b) gene, alternatively, the two forms of BrhTX-1(b) may be encoded
by mRNA molecules generated by differential processing of the primary gene transcript.

The strategy chosen to access DNA sequences encoding the candidate alternative
form of BrhTX-1(b) is to use PCR to obtain products from the Bracon genomic and cDNA
libraries. The PCR products can then be cloned and sequenced to check if the correct editing
had taken place and whether the GT amino acids are encoded.

The PCR primers (KED(b)F and KED(b)R - see below) are designed for this purpose
so that just the coding sequence of the gene is obtained, i.e. no 5' nor 3' untranslated regions.
Suitable restriction sites for cloning and an optimised Kozak sequence are included in the
primers.

KED(b)F

5' - TTTAGATCTCGCGCCGCCACCATGTCATACTATGTAAAT - 3'

BglII NotI Kozak Start
KED(b)R

5' - CCGGAATTCGCGGCGCTATTATTCAGTTGGAATCTAAAG -3'

EcoRI  NotI  Stop  Stop

The PCRs are performed using 0.5μl (approximately 5x10⁴ pfus) of genomic or cDNA library as template, 100pmol of each primer, 16μl Ultrapure dNTPs (Pharmacia) [1.25mM of each], 10μl 10x buffer [500mM KCl, 100mM Tris, pH8.3, 15mM MgCl₂, 0.1% (w/v) gelatin] and 0.8μl (4 units) Taq DNA polymerase (Perkin/Elmer Cetus). The reactions are carried out in a Hybaid OmniGene programmable dri-block and subjected to the following temperature regime:

5 minutes at 95 °C;
30 seconds at 95 °C;
30 seconds at 47 °C;
1.2 minutes at 72 °C;
35 cycles followed by a final incubation period of 7 minutes at 72 °C.

No products are obtained from the genomic library. However a product of the expected size is obtained from the cDNA library. This product is digested with BgIII and EcoRI. It is then cloned into BgIII and EcoRI-digested, phosphatase-treated pMMS1. (pMMS1 is a derivative of pUC19, the polylinker of which has been modified so that it contains BgIII and EcoRI restriction endonuclease sites. pMMS1 still confers the ability to metabolise X-gal. A map of pMMS1 is shown in Figure 3.

The PCR product is sequenced using a Sequenase kit (USB, Cleveland, Ohio) and primers shown below:

BH(b)F1:  5' - CGATGGTATCGTAACAT -3'
BH(b)F2:  5' - CGAATAAAGGATTTTCAA -3'
BH(b)F3:  5' - CACCCCATACTGTATATG -3'
BH(b)R1:  5' - CCAGCTCTTGGCGGAGAA -3'
BH(b)R2:  5' - ATGCAATATTTCCACAAC -3'
BH(b)R3  5' - TTCTGCAGCAATCACCAAA -3'

The sequencing data shows that the PCR product encodes a BrhTX1-(b) subunit including the GT amino acid pair.

Example 3 - Production of recombinant polyhedrin positive AcMNPV derivatives
expressing the BrhTX-1(a) subunit of the toxin from *B. hebetor*

Subcloning the cDNA into pAcUW21  pAcUW21 is a commercially available baculovirus transfer vector (R&D Systems Europe Ltd., Abingdon, Oxon, UK) which can be conveniently used to generate recombinant polyhedrin positive (pol*) AcMNPV derivatives. It contains the polyhedrin gene under the control of the natural polyhedrin promoter whilst foreign genes can be inserted (in either EcoRI or Bgl II sites) so that they can be transcribed from the p10 promoter. A plasmid map of pAcUW21 is shown in Figure 4.

The cDNA encoding the BrhTX-1(a) subunit is excised from clone pBrhTX-1(a)1.1 by EcoRI digestion. The released insert is then cloned into EcoRI digested, phosphatase-treated pAcUW21 with T4 DNA ligase in appropriate buffers (Sambrook 1989). Ligation progeny are recovered by transformation of *E.coli* DH5α cells under standard conditions (Sambrook 1989). Transformants are selected on LB agar plates containing 100μg/ml ampicillin. Transformant colonies are grown overnight in L-broth containing 100μg/ml ampicillin at 37°C and candidate pAcUW21/BrhTX-1(a) recombinant plasmids are recovered using a Wizard minipreparation kit (Promega Corporation, Madison, WI). These plasmids are then subjected to sequence analysis across the insert/vector junctions using internal primers BH(a)F4 and BH(a)R4 (shown below and in SEQ ID No. 15 and 25 respectively) and using a Sequenase (USB, Cleveland, Ohio) kit.

\[ BH(a)F4: \quad 5'-TTATATGAAGTTCTTAGA-3' \]

\[ BH(a)R4: \quad 5'-TTAAATGATTCCAACGC-3' \]

Two transformants, designated pACBH(a).1 and pACBH(a).2, have inserts in the correct orientation and are selected for recombinant baculovirus construction. A caesium chloride plasmid preparation is used for this purpose.

**Cotransfection of Spodoptera frugiperda (Sf21) insect cells** Cotransfection is carried-out using the BacPAK6 kit (Clontech Laboratories, Palo Alto, CA, USA). Cotransfection of Sf21 insect cells with *Bsu*36 I-digested BacPAK6 DNA and each transfer vector containing the BrhTX-1(a) subunit is carried according to the manufacturer’s recommendations.

**Isolation of recombinant AcMNPV** A plaque assay is performed using standard methods (King, L.A. and Possee, R.D. (1992) *The Baculovirus Expression System - A Laboratory Guide*. Chapman and Hall, London.) with cotransfection supernatant at dilutions of $10^1$, $10^2$, $10^3$ as well as neat supernatant. Individual polyhedrin positive plaques, which fail to stain
blue when exposed to the chromogenic substrate X-Gal, are picked into sterile 1.5ml microcentrifuge tubes containing 0.5ml TC100/10% FCS medium.

Mini-amplification of the recombinant viruses  Mini-amplifications are carried-out using standard methods (King and Possee, 1992). Tissue culture flat flasks (25cm²) (Corning, New York, USA) are seeded with 1x10⁶ Sf21 cells and incubated overnight at 28°C to form a monolayer. Next day they are infected with 0.25ml of the virus stock obtained from the isolated plug. The flasks are then incubated at 28°C for 6 days. The supernatants are then collected and cell debris and polyhedrin inclusion bodies (hereinafter referred to as PIBs) pelleted by centrifugation in Falcon 2097 tubes at 3000rpm in a Sorvall RT6000B bench top centrifuge. Supernatants are stored at 4°C. Pellets are resuspended in 4ml sterile distilled water. PIBs are released by sonication in an MSE Soniprep 150, 20 seconds on, 20 seconds off for 2 cycles. The number of PIBs per ml is ascertained using a counting chamber (Weber Scientific International Ltd.) and a Nikon Labophot light microscope. PIB solutions are diluted to 1x10⁷ PIB/ml to be used in biological assessment of the virus activity against first instar Heliothis virescens larvae.

Example 4 - Production of recombinant polyhedrin positive AcMNPV derivatives expressing the BrhTX-1(b) subunit of the toxin from B. hebetor

Subcloning the BrhTX-1(b) cDNA into pAcUW21  The cDNA encoding the Brh-TX-1(b) subunit is excised from clone pBrhTX-1(b)6 by NsiI digestion. The insert is blunt-ended by filling in using the Klenow fragment of DNA polymerase using standard methods (Sambrook 1989). The insert is then cloned into EcoRI-digested, Klenow polymerase blunt-ended, phosphatase-treated pAcUW21 with T4 DNA ligase in appropriate conditions for blunt end ligations (Sambrook 1989). Ligation progeny are recovered by transformation of E.coli DH5α cells under standard conditions (Sambrook 1989). Transformants are selected on LB agar plates containing 100μg/ml ampicillin. Transformant colonies are grown overnight in L-broth containing 100μg/ml ampicillin at 37°C. Candidate pAcUW21/BrhTX-1(b) recombinant plasmids are recovered using a Wizard minipreparation kit (Promega Corporation, Madison, WI). These plasmids are then subjected to sequence analysis across the vector/insert junctions using PACF1 and PACR1 primers (see below and SEQ ID Nos. 17 and 18) and a Sequenase kit (USB, Cleveland, Ohio). One transformant, designated pACBH(b).1, with the insert in the
correct orientation is selected for recombinant baculovirus construction. A caesium chloride plasmid preparation is used for this purpose.

\[
\begin{align*}
\text{PACF1:} & \quad 5' - \text{TTCCTTACGC} \text{GAAATACG} - 3' \\
\text{PACR1:} & \quad 5' - \text{GAATTATTA} \text{CAAATCAT} - 3'
\end{align*}
\]

Co-transfection of S/21 cells, isolation and mini-amplification of recombinant AcMNPV is as described in Example 3.

**Example 5 - Production of recombinant polyhedrin positive AcMNPV derivatives expressing an alternative form of the BrhTX-1(b) subunit of the toxin from *B. hebetor***

Subcloning the cDNA into pAcUW21 The insert encoding the edited BrhTX-1(b) subunit is as described in Example X is excised from pMMSed18 by *Bgl*II and *EcoRI* digestion. This insert is then cloned into *Bgl*III, *EcoRI*-digested, phosphatase-treated pAcUW21 with T4 DNA ligase under appropriate conditions and ligation progeny are recovered by transformation of *E. coli* DH5α cells under standard conditions (Sambrook 1989). Transformants are selected on LB agar plates containing 100µg/ml ampicillin.

Transformant colonies are grown overnight in L-broth containing 100µg/ml ampicillin at 37°C. Candidate pAcUW21/BrhTX-1(b) recombinant plasmids are recovered using a Wizard minipreparation kit (Promega Corporation, Madison, WI). These plasmids are then subjected to sequence analysis across the vector/insert junctions performed using PACF1 and PACR1 primers shown in Example 3 and a Sequenase kit (USB, Cleveland, Ohio). One transformant, designated pACBHE(b).2, with the insert is selected for recombinant baculovirus construction. A caesium chloride plasmid preparation is used for this purpose. Cotransfection of S/21 cells, isolation and mini-amplification of recombinant AcMNPV is described in Example 3.

**Example 6 - Production of recombinant polyhedrin positive AcMNPV derivatives expressing the BrhTX-1(c) subunit of the toxin from *B. hebetor***

Subcloning the cDNA into pAcUW21 The cDNA encoding the BrhTX-1(c) subunit is excised from clone \(\lambda\text{BrhTX-1(c)5}\) by *NotI* digestion. The insert is blunt-ended by filling in using the Klenow fragment of DNA polymerase using standard methods (Sambrook 1989). It is then cloned into *EcoRI*-digested, Klenow polymerase blunt-ended, phosphatase-treated pAcUW21 with T4 DNA ligase under appropriate conditions for blunt end ligations (Sambrook 1989). Ligation progeny are recovered by transformation of *E. coli* DH5α cells
under standard conditions (Sambrook 1989). Transformants are selected on LB agar plates containing 100μg/ml ampicillin. Transformant colonies are grown overnight in L-broth containing 100μg/ml ampicillin at 37°C. Candidate pAcUW21/BrhTX-1(c) recombinant plasmids are recovered using a Wizard minipreparation kit (Promega Corporation, Madison, WI). These plasmids are then subjected to sequence analysis across the vector/insert junctions using PACF1 and PACR1 primers and a Sequenase kit (USB, Cleveland, Ohio). One transformant, designated pACBH(c).1, with the insert in the correct orientation to be expressed from the vector p10 promoter is selected for recombinant baculovirus construction. A caesium chloride plasmid preparation is used for this purpose. Cotransfection of Sf21 cells and isolation and mini-amplification of recombinant AcMNPV is as described in Example 3.

Example 7 - Production of recombinant polyhedrin positive AcMNPV derivatives expressing the BrhTX 1(d) subunit of the toxin from B. hebetor

Subcloning the cDNA into pAcUW21 The cDNA encoding the BrhTX-1(d) subunit is excised from clone pBrhTX-1(d).1.2 by EcoRI digestion. The released insert is cloned into EcoRI-digested, phosphatase treated pAcUW21 with T4 DNA ligase in appropriate buffer conditions (Sambrook 1989). Ligation progeny are recovered by transformation of E.coli DH5α cells under standard conditions (Sambrook 1989). Transformants are selected on LB agar plates containing 100μg/ml ampicillin. Transformant colonies are grown overnight in L-broth containing 100μg/ml ampicillin at 37°C. Candidate pAcUW21/BrhTX-1(d) recombinant plasmids are recovered using a Wizard minipreparation DNA purification kit (Promega Corporation, Madison, WI). These plasmids are then subjected to sequence analysis across the insert/vector junctions performed using vector specific primers PACF1 and PACR1 and using a Sequenase kit (USB, Cleveland, Ohio). One transformant, pACBH(d).1 with the insert in the correct orientation is selected for recombinant baculovirus construction. A caesium chloride plasmid preparation is used for this purpose. Cotransfection of Sf21 cells and isolation and mini-amplification of recombinant AcMNPV is as described in Example 3.

Example 8 - Physical characterisation of recombinant AcMNPV by Southern Blot Analysis

These analyses are undertaken to check that the physical environment and structure of the recombinant baculoviruses is the same as that of the relevant transfer vector.

Preparation of recombinant AcMNPV DNA 1x10^6 Sf21 cells are used to seed 35mm Nunc Petri dishes (Gibco BRL, Paisley, Scotland). The plates are incubated at room temperature
for 15 minutes to allow cells to attach and form a monolayer. The media is then removed and 1ml (approximately 1x10^7 Non-occluded virions [NOVs]) of mini-amplification supernatant is added. The plates are left 1 hour at room temperature for infection to occur. The inoculum is then removed and 1.4ml TC100/10%FCS medium is added. The plates are incubated at 28°C overnight. Next morning the infected cell monolayers are harvested by scraping them off the plate, transferring to a 1.5ml microcentrifuge tube and centrifugation at 4000rpm for 2 minutes. The supernatant is then removed and the cell pellet resuspended in 250μl TE. Next 250μl lysis buffer (50mM Tris HCl [pH8], 5% β-mercaptoethanol, 0.4% w/v SDS, 10mM EDTA) is added. The solution is mixed gently to obtain a viscous cell lysate. 12.5μl proteinase K (10mg/ml in TE, predigested at 37°C for 30minutes) and 2.5μl RNase A (10mg/ml in H2O) are added and the tube incubated at 37°C for 30minutes. The solution is extracted twice with 500μl (50:50) TE-saturated phenol/chloroform:isoamyl alcohol [24:1] and once with 500μl chloroform:isoamyl alcohol [24:1]. Infected cell DNA is precipitated by adding 50μl 3M Na acetate and 2 volumes absolute ethanol. Precipitation is for 5 minutes at room temperature and the DNA pelleted by centrifugation at 14 000rpm for 15 minutes. The DNA pellet is washed twice in 70% ethanol, air-dried for 5 minutes at room temperature and resuspended in 100μl TE, pH8 overnight at 4°C.

Digestion of the baculovirus infected cell DNA and separation by agarose gel electrophoresis

15μl of each infected cell DNA preparation is digested using EcoRI (EcoRI and BgIII for BrhTX-1(c) and edited BrhTX-1(b) subunits). The digests are run on a 0.8%, 1 xTBE agarose gel and the DNA transferred to nylon membranes (Hybond-N, Amersham, UK) using standard "Southern Blot" methods (Sambrook 1989).

Hybridisation of the subunit cDNA probe to the recombinant baculovirus DNA

Preparation of the probe The subunit cDNA is excised from the appropriate vector using EcoRI for BrhTX-1(a), BrhTX-1(d) subunits, NsiI for BrhTX-1(b) and EcoRI/BgIII for BrhTX-1(c) and edited BrhTX-1(b) subunits. 25ng of the excised subunit DNA is denatured (by boiling for 10min) and then labelled by random priming with α-32P dCTP (Amersham International, Amersham, UK) and Klenow polymerase (NBL Gene Sciences Ltd., Cramlington, Northumberland, UK) using standard methods (Sambrook 1989). This probe is denatured by incubation in a boiling water bath immediately before use.
Hybridisation The membrane is UV cross-linked in a Stratalinker (Stratagene, USA) and pre-
hybridised at 65°C for 2 hours in 5 x SSPE, 5 x Denhardt’s Reagent, 0.5% SDS and 200µg/ml
salmon sperm DNA in a Techne hybridisation oven. Hybridisation is carried-out in 5 x SSPE,
5 x Denhardt’s reagent, 0.5% SDS plus labelled probe at 65°C for 16 hours. Membranes are
washed in 3 x SSC (20 x SSC: 3.0M NaCl, 0.3M trisodium citrate); 0.1%SDS at 65°C for 4 x
15 minutes and then exposed to Kodak X-AR film at -80°C with intensifying screens. If there
is a high background, another series of washes are performed at 0.1 x SSC; 0.1% SDS, 65°C
4 x 15 minutes and the films re-exposed to film.

Example 9 - Construction of recombinant AcMNPV derivatives capable of co-expression of
BrhTX-1 subunits.

This assembly, which is intended to provide for efficient, co-ordinated, high level
expression of combinations of the various toxin subunits (BrhTX-1(a), BrhTX-1(b), BrhTX-
1(c) and BrhTX-1(d)), can be achieved by use of AcMNPV transfer vectors which have been
constructed to allow introduction of three or four heterologous genes downstream of
independent polyhedrin or p10 very late promoters. Such transfer vectors can be custom built,
in which case they could be designed so that they also carry an intact functional polyhedrin
gene and can therefore be used to generate polyhedrin positive (pol+) progeny by in vivo
recombination with one of several convenient intermediate AcMNPV viral vector DNA
preparations, including: Baculogold™ (Pharmingen), BacPAK6™ (Clontech) or AcRP23.lacZ
(Pharmingen).

Alternatively, commercially available co-expression transfer vectors can be used. In the
case that three subunits are selected, pAcAB3 (Pharmingen) may be used. This can
accommodate up to three heterologous genes under the transcriptional control of either two
p10 promoters or one polyhedrin promoter. In addition, pAcAB4 (Pharmingen)) is also
available. This expression vector can accommodate up to four heterologous genes under the
transcriptional control of two p10 promoters and two polyhedrin promoters. However, if this
transfer vector is used to construct recombinant AcMNPV derivatives by recombination with
conventional intermediate AcMNPV viral vectors, such as Baculogold™, BacPAK6™ or
AcRP23.lacZ, only polyhedrin negative AcMNPV derivatives are made. These can be
bioassayed by injection into suitable lepidopteran hosts e.g. 3rd/4th instar H. virescens.
However in a preferred embodiment they are used to construct polyhedrin positive (Pol+)
AcMNPV derivatives directly by in vivo recombination with Bsu36 I linearised AcUW1-PH DNA (Weyer et al.(1990) J.Gen.Virol. 71 1525-1534) since this AcMNPV derivative has a functional polyhedrin gene in place of its non-essential p10 gene and has a replaceable lacZ gene within the region homologous and hence exchangeable with the above mentioned transfer vectors (see also PCT/GB95/00677). Pol+ recombinant baculoviruses can be readily employed in a variety of bioassays, including that described in Example 10 and model plant protection studies, when delivered per os.

The skilled worker will appreciate that the above options provide various permutations for construction of recombinant baculovirus derivatives capable of co-expression of the various subunits of the toxin.

Example 9(a) Construction of recombinant AcMNPV derivatives capable of co-expression of four subunits of BrhTX-1

Concerning the combination of BrhTX-1(a), BrhTX-1(b), BrhTX-1(c) and BrhTX-1(d) one may initially insert the BrhTX-1(d) gene, isolated from pBrhTX-1(d)1.2 as an EcoRI fragment and blunt ended by a fill-in reaction with Klenow DNA polymerase performed under standard conditions (Sambrook 1989), into a similarly filled BamHI site in the transfer vector pAcAB4. DNA sequence analysis is then used with likely recombinant plasmids to select those with an intact BrhTX-1(d) gene correctly orientated for transcription from one of the two polyhedrin promoters in pAcAB4. Next an insert encoding the BrhTX-1(c) gene, isolated from pBrhTX-1(c)5 as a NotI fragment and blunt ended by a fill-in reaction with Klenow DNA polymerase, is introduced into a similarly filled SpeI site in an authenticated preparation of the intermediate pAcAB4/BrhTX-1(d) transfer vector. DNA sequence analysis is then again used to select those recombinant plasmids with an intact BrhTX-1(c) gene correctly orientated for functional transcription from one of the two p10 promoters carried by pAcAB4. One such authenticated pAcAB4/BrhTX-1(c)/BrhTX-1(d) recombinant transfer vector preparation is selected for further work and subject to BglII digestion to linearise it downstream of the other p10 promoter and blunt ended by Klenow fill in reaction. NotI digestion is then used to isolate a BrhTX-1(b) gene from pBrhTX-1(b)6. This fragment is again blunt ended by Klenow fill-in before ligation with the BglII pAcAB4/BrhTX-1(c)/BrhTX-1(d) preparation under appropriate conditions to promote blunt end fragment joining. Again recombinant transfer vectors containing the correctly orientated BrhTX-1(b)
gene are recognised by DNA sequence analysis. Finally, the selected \( \text{pAcAB4/BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d)} \) recombinant transfer vector is subject to \( \text{StuI} \) digestion to linearise it just downstream of the remaining polyhedrin promoter. \( \text{EcoRI} \) digestion is then used to isolate a \( \text{BrhTX-1(a)} \) gene from a suitable \( \text{pUC19/BrhTX-1(a)} \) recombinant. This fragment is again blunt ended by Klenow fill-in before ligation with the \( \text{StuI} \) \( \text{pAcAB4/BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d)} \) preparation under appropriate conditions to promote blunt end fragment joining. The target recombinant transfer vectors containing the correctly orientated \( \text{BrhTX-1(a)} \) gene are then recognised by DNA sequence analysis. An ethidium bromide/CsCl purified preparation of the selected \( \text{pAcAB4/BrhTX-1(a)/BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d)} \) recombinant transfer vector is then prepared for use in assembly of recombinant pol+ AcMNPV derivatives by co-tranfection of \( \text{S/21} \) cells with \( \text{Bsu361} \) linearised AcUW1-PH DNA as described in Example 3.

Alternatively, initially four independent linkers are inserted into the \( \text{EcoRI/HindIII} \) sites of \( \text{pAlter-1 vector (Promega)} \) to assemble four independent intermediate cloning vectors to facilitate cloning of the four subunits into \( \text{pAcAB4} \). Then an insert encoding the \( \text{BrhTX-1(d)} \) gene, isolated from \( \text{pBrhTX-1(d) 1.2} \) as an \( \text{EcoRI} \) fragment, is introduced into an \( \text{EcoRI} \) site in one of the \( \text{pAlter-1 intermediate cloning vectors which carries a pair of Spe9 sites flanking the} \text{EcoRI site. DNA sequence analysis is then used with likely recombinant plasmids to select those with an intact} \text{BrhTX-1(d)} \) gene. \( \text{SpeI} \) digestion is then utilised to isolate the \( \text{BrhTX-1(d)} \) gene from the \( \text{pAlter-1 intermediate vector. This fragment is subsequently introduced into an XbaI digested pAcAB4. DNA sequence analysis is then performed on recombinant plasmids to select those with an intact} \text{BrhTX-1(d) gene correctly orientated for transcription from one of the two polyhedrin promoters in pAcAB4. An insert encoding the} \text{BrhTX-1(b) gene, isolated from pBrhTX-1(b) 6 as a NotI fragment, is then introduced into a NotI site in one of the pAlter-1 intermediate cloning vectors which carries a pair of} \text{BglIII sites flanking the NotI site. Again, DNA sequence analysis is used to select recombinant vectors with an intact} \text{BrhTX-1(b) gene. This} \text{BrhTX-1(b) gene is then released from the pAlter-1 vector with the BglIII restriction enzyme to clone it into the BamHI site in an authenticated preparation of the intermediate pAcAB4/BrhTX-1(d) transfer vector. DNA sequence analysis is then again performed on the product in order to select those recombinant plasmids with an intact} \text{BrhTX-1(b) gene correctly orientated for functional transcription.
from the remaining polyhedrin promoter in pAcAB4. *EcoRI* digestion is then used to isolate a BrhTX-1(a) gene from pUC19/BrhTX-1(a), to clone it into an *EcoRI* site in one of the pAlter-1 intermediate cloning vectors which carries a pair of flanking Xba1 sites flanking the *EcoR1* site. Once again, recombinant vectors containing the intact BrhTX-1(a) gene are recognised by DNA sequence analysis. One authenticated pAcAB4/BrhTX-1(d)/BrhTX-1(b) recombinant transfer vector preparation is selected for further work and subject to *SpeI* digestion to linearise it downstream of one of the p10 promoters. *XbaI* digestion is then used to release the BrhTX-1(a) gene from pAlter-1 before ligation with the *SpeI* digested pAcAB4/BrhTX-1(d)/BrhTX-1(b) preparation. Recombinant transfer vectors containing the correctly orientated BrhTX-1(a) gene are recognised by DNA sequence analysis. Finally, *NotI* digestion is used to isolate a BrhTX-1(c) gene from a suitable pBrhTX-1(c)5 recombinant and introduce it into the last pAlter-1 cloning vector which carries a pair of BamH1 sites flanking the *EcoR1* site. Recombinant progeny of this cloning step are again analysed by sequencing, to isolate a plasmid with an intact BrhTX-1(c) gene. The selected pAcAB4/BrhTX-1(d)/BrhTX-1(b)/BrhTX-1(a) recombinant transfer vector is then subjected to *BglIII* digestion to linearise it just downstream of the remaining p10 promoter. Finally, the BrhTX-1(c) gene, isolated from pAlter-1 by *BamHI* digestion, is ligated to the *BglIII* pAcAB4/BrhTX-1(d)/BrhTX-1(b)/BrhTX-1(a) preparation. The target recombinant transfer vectors containing the correctly orientated BrhTX-1(c) gene are then recognised by DNA sequence analysis. An ethidium bromide/CsCl purified preparation of the selected pAcAB4/BrhTX-1(a)/BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d) recombinant transfer vector is then prepared for use in assembly of recombinant pol+ AcMNPV derivatives by co-transfection of Sf21 cells with Bsu36 I linearised AcUW1-PH DNA.

The skilled man will appreciate that adapters may be used to introduce an *EcoRI* or a *NotI* digested subunit into pAcAB4. Initially the BrhTX-1(d) gene, isolated from pBrhTX-1(d)1.2 may be inserted as an *EcoRI* fragment, into an *XbaI* digested pAcAB4 vector using an *XbaI-EcoRI* adapter. DNA sequence analysis is then used with likely recombinant plasmids to select those with an intact BrhTX-1(d) gene correctly orientated from transcription from one of the two polyhedrin promoters in pAcAB4. Next an insert encoding the BrhTX-1(b) gene, isolated from pBrhTX-1(b)6 as a *NotI* fragment, is introduced into the *BamHI* site of an authenticated preparation of the intermediate pAcAB4/BrhTX-1(d) transfer vector utilising a
BamHI/BglII-NotI adapter. DNA sequence analysis is then again used to select those recombinant plasmids with an intact BrhTX-1(b) gene correctly orientated for functional transcription from the second polyhedrin promoter carried by pAcAB4. One such authenticated pAcAB4/BrhTX-1(d)/BrhTX-1(b) recombinant transfer vector preparation is selected for further work and subject to SpeI digestion to linearise it downstream of one of the two p10 promoters. EcoRI digestion is then used to isolate a BrhTX-1(a) gene from pUC19/BrhTX-1(a). An SpeI-EcoRI adapter is again utilised to allow ligation of the BrhTX-1(a) gene with the SpeI pAcAB4/BrhTX-1(d)/BrhTX-1(b) preparation under appropriate conditions. Again recombinant transfer vectors containing the correctly orientated BrhTX-1(a) gene are recognised by DNA sequence analysis. Finally, the selected pAcAB4/BrhTX-1(d)/BrhTX-1(b)/BrhTX-1(a) recombinant transfer vector is subjected to BglII digestion to linearise it just downstream of the remaining p10 promoter. NotI digestion is then used to isolate a BrhTX-1(c) gene from a suitable pBrhTX-1(c)5 recombinant. A BamHI/BglII-NotI adapter is again utilised to allow ligation of the BrhTX-1(c) gene with the BglII pAcAB4/BrhTX-1(d)/BrhTX-1(b)/BrhTX-1(a) preparation under appropriate conditions.

The target recombinant transfer vectors containing the correctly orientated BrhTX-1(c) gene are then recognised by DNA sequence analysis and are purified as indicated above.

Alternatively, the two methods described above can also be combined to produce a pAcAB4/BrhTX-1(a)/BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d) recombinant transfer vector.

Example 9(b) Construction of recombinant AcMNPV derivatives capable of co-expression of three subunits of BrhTX-1

Concerning the production of recombinant baculovirus derivatives capable of providing for co-expression of BrhTX-1(a), BrhTX-1(c) and BrhTX-1(d) a pACAB4/BrhTX-1(c)/BrhTX-1(d) recombinant transfer vector is selected as indicated above, and subjected to Stu I digestion to linearise it downstream of the other polyhedrin promoter. Eco RI digestion is then used to isolate a BrhTX-1(a) gene from a suitable pUC19/BrhTX-1(a) recombinant plasmid. This fragment is again blunt ended by Klenow fill-in before ligation with the Stu I pAcAB4/BrhTX-1(c)/BrhTX-1(d) preparation under appropriate conditions to promote blunt end fragment joining. Again recombinant transfer vectors containing the correctly orientated BrhTX-1(a) gene are recognised by DNA sequence analysis. An ethidium bromide/CsCl purified preparation of the selected pAcAB4/BrhTX-1(a)/BrhTX-1(c)/BrhTX-1(d)
recombinant transfer vector is then prepared for use in assembly of recombinant pol+
AcMNPV derivatives by co-transfection of Sf21 cells with Bsu36 I linearised AcUW1-PH
dNA as described above for the four subunit assembly.

In the case that the recombinant baculoviruses should comprise genes encoding
BrhTX-1(a), BrhTX-1(b) and BrhTX-1(d), a pACAB4/BrhTX-1(d) recombinant transfer
vector is constructed as indicated above for either the three or four subunit assembly. Next an
insert encoding the BrhTX-1(b) gene, isolated from pBrhTX-1(b)6 as a NotI fragment and
blunt ended by a fill-in reaction with Klenow DNA polymerase, is introduced into a similarly
filled BglII site in an authenticated preparation of the intermediate pACAB4/BrhTX-1(d)
transfer vector. DNA sequence analysis is then used to select those recombinant plasmids
with an intact BrhTX-1(b) gene correctly orientated for functional transcription from one of
the two p10 promoters carried by pACAB4. One such authenticated pACAB4/BrhTX-
1(b)/BrhTX-1(d) recombinant transfer vector preparation is selected for further work and
subject to StuI digestion to linearise it downstream of the other polyhedrin promoter. Eco RI
digestion is then used to isolate a BrhTX-1(a) gene from a suitable pUC19/BrhTX-1(a)
recombinant plasmid. This fragment is again blunt ended by Klenow fill-in before ligation
with the StuI pACAB4/BrhTX-1(b)/BrhTX-1(d) preparation under appropriate conditions to
promote blunt end fragment joining. Again recombinant transfer vectors containing the
correctly orientated BrhTX-1(a) gene are recognised by DNA sequence analysis. An ethidium
bromide/CsCl purified preparation of the selected pACAB4/BrhTX-1(a)/BrhTX-1(b)/BrhTX-
1(d) recombinant transfer vector is then prepared for use in assembly of recombinant pol+
AcMNPV derivatives by co-transfection of Sf21 cells with Bsu36 I linearised AcUW1-PH
dNA.

In the case that the recombinant baculoviruses comprise genes encoding the BrhTX-
1(b), BrhTX-1(c) and BrhTX-1(d) subunits, an authenticated pAcAB4/BrhTX-1(c)/BrhTX-
1(d) recombinant transfer vector preparation is produced as indicated above and subjected to
BglII digestion to linearise it downstream of the other p10 promoter. NorI digestion is then
used to isolate a BrhTX-1(b) gene from pBrhTX-1(b)6. This fragment is again blunt ended
by Klenow fill-in before ligation with the BglII pACAB4/BrhTX-1(c)/BrhTX-1(d)
preparation under appropriate conditions to promote blunt end fragment joining. Again
recombinant transfer vectors containing the correctly orientated BrhTX-1(b) gene are
recognised by DNA sequence analysis. An ethidium bromide/CsCl purified preparation of the selected pAcAB4/BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d) recombinant transfer vector is then again prepared for use in assembly of recombinant pol+ AcMNPV derivatives by co-transfection of Sf21 cells with Bsu36 I linearised AcUW1-PH DNA.

Concerning the construction of recombinant baculovirus derivatives capable of co-expression of BrhTX-1(a), BrhTX-1(b) and BrhTX-1(c), one may initially insert the BrhTX-1(c) gene, isolated from pBrhTX-1(c)5 as a Not I fragment and blunt ended by a fill-in reaction with Klenow DNA polymerase performed under standard conditions (Sambrook 1989), into a similarly filled Spe I site in the transfer vector pAcAB4. DNA sequence analysis is then used with likely recombinant plasmids to select those with an intact BrhTX-1(c) gene correctly orientated for transcription from one of the two p10 promoters in pAcAB4. Next an insert encoding the BrhTX-1(b) gene, isolated from pBrhTX-1(b)6 as a Not I fragment and blunt ended by a fill in reaction with Klenow DNA polymerase, is introduced into a similarly filled Bgl II site in an authenticated preparation of the intermediate pAcAB4/BrhTX-1(c) transfer vector. DNA sequence analysis is then used to select those recombinant plasmids with an intact BrhTX-1(b) gene correctly orientated for functional transcription from the other p10 promoter carried by pAcAB4. One such authenticated pAcAB4/BrhTX-1(b)/BrhTX-1(c) recombinant transfer vector preparation is selected for further work and subject to Stu I digestion to linearise it downstream of one of the polyhedrin promoters. Eco RI digestion is then used to isolate a BrhTX-1(a) gene from a suitable pUC19/BrhTX-1(a) recombinant plasmid. This fragment is again blunt ended by Klenow fill in before ligation with the Stu I pAcAB4/BrhTX-1(b)/BrhTX-1(c) preparation under appropriate conditions to promote blunt end fragment joining. Again recombinant transfer vectors containing the correctly orientated BrhTX-1(a) gene are recognised by DNA sequence analysis. An ethidium bromide/CsCl purified preparation of the selected pAcAB4/BrhTX-1(a)/BrhTX-1(b)/BrhTX-1(c) recombinant transfer vector is then prepared for use in assembly of recombinant pol+ AcMNPV derivatives by co-transfection of Sf21 cells with Bsu36 I linearised AcUW1-PH DNA.

Example 9(c) Construction of recombinant AcMNPV derivatives capable of co-expression of two subunits of BrhTX-1
In addition to the AcMNPV transfer vectors used in the production recombinant baculoviruses comprising genes providing for expression of three or four toxin subunits, suitable commercially available co-expression transfer vectors can be used for the production of viruses comprising genes encoding only two subunits. One suitable example, pAcUW51 (Pharmingen), can accommodate up to two heterologous genes under the transcriptional control of one p10 promoter or one polyhedrin promoter.

Recombinant baculovirus derivatives capable of co-expression of BrhTX-1(a) and BrhTX-1(b) can be made by a method which involves the insertion, into an EcoRI digested phosphatase-treated pAcUW51 plasmid, of a BrhTX-1(a) gene isolated from a suitable pUC19/BrhTX-1(a) recombinant plasmid as an EcoRI fragment. DNA sequence analysis is then used with likely recombinant plasmids to select those with an intact BrhTX-1(a) gene correctly orientated for transcription from the p10 promoter. An insert encoding the BrhTX-1(b) gene is isolated from pBrhTX-1(b)6 as a NotI fragment and blunt-ended by a fill-in reaction with Klenow DNA polymerase. This is introduced into a similarly filled Bam HI site in an authenticated preparation of the intermediate pAcUW51/BrhTX-1(a) transfer vector. DNA sequence analysis is then used with likely recombinant plasmids to select those with an intact BrhTX-1(b) gene correctly orientated for transcription from the polyhedrin promoter. One such authenticated pAcUW51/BrhTX-1(a)/BrhTX-1(b) recombinant transfer vector preparation is selected for further work. A caesium chloride purified preparation of the selected pAcUW51/BrhTX-1(a)/BrhTX-1(b) recombinant transfer vector is prepared for use in assembly of recombinant pol+ AcMNPV derivatives by co-transfection of Sf21 cells with Bsu36 I linearised AcUW1-PH DNA as described in PCT/GB95/00677. Pol+ AcMNPV derivatives capable of co-expressing BrhTX-1(a) and BrhTX-1(b) are then isolated by the method described above in Example 3.

This method can be repeated for the production of recombinant baculoviruses comprising sequences encoding other binary combinations of subunits, for example, (i) BrhTX-1(a) and BrhTX-1(c); (ii) BrhTX-1(b) and BrhTX-1(c); (iii) BrhTX-1(b) and BrhTX-1(d); (iv) BrhTX-1(c) and BrhTX-1(d).

Whatever the combination of subunit encoding sequences the recombinant baculoviruses comprise, Pol+ AcMNPV derivatives capable of co-expressing the various combinations of sub-units are isolated by:
- 36 -

- Plaque purification of the co-transfection supernatants on Sf21 monolayers by selecting for pol+/lacZ- plaques by standard procedures (King & Possee 1992).

- Restriction digestion/Southern blot analysis of mini-amplifications of candidate recombinant viral DNA to confirm purity and the appropriate diagnostic restriction pattern in the vicinity of the introduced combinations of genes, for example, (i) BrhTX-1(a)/BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d); (ii) BrhTX-1(a)/BrhTX-1(c)/BrhTX-1(d); (iii) BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d); (iv) BrhTX-1(a)/BrhTX-1(b)/BrhTX-1(c); (v) In vitro protein expression studies to demonstrate that the viruses have the capacity to express immunologically detectable BrhTX-1(a), BrhTX-1(b), BrhTX-1(c) and BrhTX-1(d).

The selected AcMNPV/BrhTX-1(a)/BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d); AcMNPV/BrhTX-1(a)/BrhTX-1(c)/BrhTX-1(d); AcMNPV/BrhTX-1(b)/BrhTX-1(c)/BrhTX-1(d); AcMNPV/BrhTX-1(a)/BrhTX-1(b)/BrhTX-1(c) etc. recombinant baculovirus isolates are then subject to diet based bio-assays on first instar H. virescens larvae as described in Example 10.

Example 10 - Diet surface dosing assay for evaluation of viruses vs. Heliothis virescens larvae

Petri dishes (Falcon model 1006, 50 x 9mm, tight fit lid) are filled with a thin layer of a freshly made, pinto bean based, lepidopteran diet (velvetbean caterpillar diet - Green et al 1976 Louisiana State University) sufficient to evenly cover the floor of the dish and labelled as appropriate for the test treatment list (one to two dishes per treatment). Twenty 1½” clear ‘minipots’ (Ashwood Plastics, London - cat. no. 4161) per virus treatment, are similarly prepared.

The virus stock solution concentrations are measured by using a counting chamber (Weber Scientific International Ltd.) and diluted to a concentration of 1x10^6 PIB/ml using deionised water. In Primary tests two tenfold serial dilutions are made to give a dose range, for each virus tested, of 1x10^6, 1x10^5 and 1x10^4 PIB/ml. In follow up tests on viruses of interest the dose range consists of five rates in four-fold serial dilution i.e. 1x10^6, 2.5x10^5, 6.3x10^4, 1.6x10^4, 3.9x10^3 PIB/ml.

Using a sterile syringe, 0.1ml of the appropriate treatment solution is deposited on the surface of the diet in each of the pre-labelled Falcon dishes. The solution is spread evenly over
the surface of the diet using a sterile inoculating loop. Control dishes are treated in the same way with an equivalent volume of deionised water. The dishes are left to dry thoroughly prior to infestation.

Approximately 30 first instar *Heliothis virescens* larvae of uniform size are transferred to the surface of the diet in each dish using a fine artists paint brush. The dishes are tightly sealed and stored in trays in a holding room at 27°C and 60% relative humidity.

After 24 hours on the treated diet surface, twenty larvae per treatment are individually transferred to the prepared minipots of clean diet using a fine artists paint brush (one larva per minipot). Each minipot is labelled with the appropriate treatment and replicate number. The minipots are held under the same conditions as the treated Falcon dishes (see above) for the duration of the test.

Assessments of mortality and symptomology are made at three, four, and seven days after exposure to the treated diet. The numbers of dead and affected larvae are recorded and used for calculating percentage mortality/affected.

**AcMNPV/BrhTX-1(c) recombinants**

Table 3 summarises data generated comparing the BrhTX-1(c) clones with wild type and Tox 34#4 AcMNPV. The three compare three clones over a comprehensive dose range. Whilst the insecticidal activity - at 3DAT - appears to be inconsistent between tests, the three selected clones all show good intrinsic viral activity compared to the wild type.
<table>
<thead>
<tr>
<th></th>
<th>Rate (PIB/ml)</th>
<th>3 DAT</th>
<th>4 DAT</th>
<th>7 DAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AcNPV wild type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 x 10^6</td>
<td>0</td>
<td>65</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2.5 x 10^4</td>
<td>-</td>
<td>40</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>6.3 x 10^4</td>
<td>-</td>
<td>5</td>
<td>20</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>3.9 x 10^3</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td><strong>AcNPV pAc Tox34#4</strong></td>
<td>1.0 x 10^6</td>
<td>70(30)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^5</td>
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<td>75</td>
<td>80</td>
</tr>
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</tr>
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<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3.9 x 10^3</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
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<td>95</td>
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<td>2.5 x 10^3</td>
<td>-</td>
<td>35(15)</td>
<td>75</td>
</tr>
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<td>65</td>
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<td></td>
<td>1.6 x 10^4</td>
<td>-</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>3.9 x 10^3</td>
<td>-</td>
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<td>0</td>
</tr>
<tr>
<td><strong>Bracon 21#m</strong></td>
<td>1.0 x 10^6</td>
<td>10</td>
<td>45(10)</td>
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</tr>
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<td></td>
<td>2.5 x 10^3</td>
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<td>95</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6.3 x 10^4</td>
<td>0(5)</td>
<td>50</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>1.6 x 10^4</td>
<td>-</td>
<td>25</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>3.9 x 10^3</td>
<td>-</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td><strong>Bracon 21#p</strong></td>
<td>1.0 x 10^6</td>
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<td>70(20)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^5</td>
<td>-</td>
<td>75(10)</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>6.3 x 10^4</td>
<td>-</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
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<td>-</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>3.9 x 10^3</td>
<td>-</td>
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<td>5</td>
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<tr>
<td><strong>Control 1</strong></td>
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<td>0</td>
<td>10</td>
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<tr>
<td><strong>Control 2</strong></td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>10</td>
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</tbody>
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Table 3

**AcMNPV/BrhTX-1(b) recombinants**

Table 4 summarises data generated in a diet surface dosing assays comparing the edited BrhTX-1(b) (SEQ ID No. 9) clones with wild type and Tox 34#4 AcMNPV.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Rate (PIB/ml)</th>
<th>% Mortality (% Affected)</th>
<th>3 DAT</th>
<th>4 DAT</th>
<th>7 DAT</th>
</tr>
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<tbody>
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<td>AcNPV wild type (Ac9)</td>
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<td>95</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^5</td>
<td>10</td>
<td>42</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^4</td>
<td>-</td>
<td>15</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>AcNPV pAc Tox34#4</td>
<td>1.0 x 10^6</td>
<td>95</td>
<td>95</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^5</td>
<td>5(5)</td>
<td>15</td>
<td>15</td>
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</tr>
<tr>
<td></td>
<td>1.0 x 10^4</td>
<td>5</td>
<td>5</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Bracon 18#a</td>
<td>1.0 x 10^6</td>
<td>5</td>
<td>11</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^5</td>
<td>-</td>
<td>-</td>
<td>40</td>
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</tr>
<tr>
<td></td>
<td>1.0 x 10^4</td>
<td>-</td>
<td>-</td>
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<td>10</td>
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<tr>
<td>Bracon 18#c</td>
<td>1.0 x 10^6</td>
<td>10</td>
<td>58</td>
<td>100</td>
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</tr>
<tr>
<td></td>
<td>1.0 x 10^5</td>
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<td>15(10)</td>
<td>60</td>
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</tr>
<tr>
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<tr>
<td>Bracon 18#d</td>
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</tr>
<tr>
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<td>1.0 x 10^4</td>
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<td></td>
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<tr>
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<td>95(5)</td>
<td>100</td>
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</tr>
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<td>30(5)</td>
<td>50</td>
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<tr>
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</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td></td>
</tr>
</tbody>
</table>

Table 4 shows the insecticidal activity of recombinant baculovirus based on he

5 AcUW1-PN/pAcAB4 system comprising genes encoding the BrhTX-1(a) (17KDa -SEQ ID No. 2), BrhTX-1(b) (18kDa - SEQ ID No. 4), Brh-TX-1(c) (21kDa -SEQ ID No. 6) or BrhTX-1(d) (32kDa - SEQ ID No. 8) toxin subunits.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Rate (PIB/ml)</th>
<th>% Mortality (% Affected)</th>
<th>3 DAT</th>
<th>4 DAT</th>
<th>5 DAT</th>
<th>7 DAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcNPV wild type</td>
<td>1.0 x 10^6</td>
<td>0^5(5)</td>
<td>40</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>(Ac9)</td>
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<td>20</td>
<td>80</td>
<td>95</td>
<td></td>
</tr>
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<tr>
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<td>AcNPV</td>
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</tr>
<tr>
<td></td>
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<td>20^10(10)</td>
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<tr>
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<td></td>
</tr>
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</tr>
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<td>0</td>
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<td>95</td>
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</tr>
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<td>1.0 x 10^5</td>
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<td>15^15(15)</td>
<td>65^30(30)</td>
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<td>30^40(40)</td>
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</tr>
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<td>55^30(30)</td>
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<td></td>
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</tr>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>1.0 x 10^5</td>
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<td>5</td>
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<td>0</td>
<td>5</td>
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</tr>
</tbody>
</table>

Table 5

Table 6 shows the insecticidal activity of recombinant baculovirus comprising various combinations of the genes encoding the Brh TX-1(a) (17kDa - SEQ ID No. 2), BrhTX-1(b)
(18kDa - SEQ ID No. 10), BrhTX-1(c) (21kDa - SEQ ID No. 6) or BrhTX-1(d) (32kDa -
SEQ ID No. 8) toxin subunits.

<table>
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<tr>
<th>Virus</th>
<th>Rate (PIB/ml)</th>
<th>3 DAT</th>
<th>4 DAT</th>
<th>5 DAT</th>
<th>7 DAT</th>
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<tr>
<td>AcNPV wild type (Ac9)</td>
<td>1.0 x 10^6</td>
<td>0</td>
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<td>75</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^5</td>
<td>-</td>
<td>5</td>
<td>60</td>
<td>85</td>
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<tr>
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<td>1.0 x 10^4</td>
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<td>-</td>
<td>15</td>
<td>30</td>
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<td>AcUW1-PH/pVL1392</td>
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<td>50(5)</td>
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<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^6</td>
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<td>15(5)</td>
<td>85</td>
<td>95</td>
</tr>
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<td>10</td>
<td>15</td>
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<tr>
<td>AcNPV pAc Tox34#4</td>
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<td>5(10)</td>
<td>65</td>
<td>75</td>
<td>75</td>
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<tr>
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<td>10</td>
<td>10</td>
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<td>1.0 x 10^4</td>
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<td>-</td>
<td>-</td>
<td>0</td>
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<tr>
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<td>5(5)</td>
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<td>95(25)</td>
<td>100</td>
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<tr>
<td></td>
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<td>0</td>
<td>60(25)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^5</td>
<td>-</td>
<td>-</td>
<td>15(10)</td>
<td>50</td>
</tr>
<tr>
<td>Bracon BH4#50 Seq ID Nos 6 and 8</td>
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<td>0</td>
<td>11(10)</td>
<td>58(25)</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^6</td>
<td>-</td>
<td>5</td>
<td>20</td>
<td>55</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
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<td>95(25)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^6</td>
<td>-</td>
<td>5</td>
<td>60(25)</td>
<td>95</td>
</tr>
<tr>
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<td>-</td>
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<td>10(15)</td>
<td>75(25)</td>
<td>100</td>
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<tr>
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<td>80(10)</td>
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<td>-</td>
<td>35(5)</td>
<td>55</td>
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<td>30(5)</td>
<td>75</td>
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<td>25</td>
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<td>1.0 x 10^6</td>
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<td>75(10)</td>
<td>100</td>
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<td>60</td>
</tr>
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<td>0</td>
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<td>40(20)</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^6</td>
<td>-</td>
<td>-</td>
<td>15(10)</td>
<td>70</td>
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<tr>
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<td>-</td>
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<td>20</td>
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<td>10(15)</td>
<td>95</td>
<td>100</td>
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<td>1.0 x 10^6</td>
<td>-</td>
<td>10(5)</td>
<td>35(10)</td>
<td>85</td>
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<td>-</td>
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<td>30</td>
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<tr>
<td>Control 2</td>
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</tr>
</tbody>
</table>

Table 6

Whilst the present invention has been particularly described with reference to the production of recombinant baculoviruses comprising nucleotide sequences depicted in various of SEQ ID Nos. 1, 3, 5, 7 and 9, the skilled man will appreciate that the baculoviruses may be engineered
to contain other sequences which are also part of the present invention. For example, the baculoviruses may comprise a polynucleotide comprising a region encoding a protein encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the said spliced RNA being capable of hybridising with the extension products of the primers depicted in SEQ ID Nos 20 and 21, or SEQ ID Nos 22 and 23, or SEQ ID Nos 24 and 25 using the sequence depicted in SEQ ID No. 1 as a template. SEQ ID No. 45 discloses a toxin encoding sequence derived from the genomic clone depicted in SEQ ID No. 19 and SEQ ID No. 46 discloses the translation product of the nucleotide sequence depicted in SEQ ID No. 45. The present invention thus includes Baculoviruses which are engineered to comprise the SEQ ID No. 45 sequence, alone or in combination with one or more of the other toxin encoding sequences disclosed herein.
(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ZENECO Ltd
(B) STREET: 15 Stanhope Gate
(C) CITY: London
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): W1Y 6LN

(A) NAME: Commonwealth Scientific and Industrial Research Organisation
(B) STREET: 407 Royal Parade
(C) CITY: Parkville
(D) STATE: Victoria
(E) COUNTRY: Australia
(F) POSTAL CODE (ZIP): 3052

(ii) TITLE OF INVENTION: Biological Insect Control Agent

(iii) NUMBER OF SEQUENCES: 46

(iv) COMPUTER READABLE FORM:
(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 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 564 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) ORIGINAL SOURCE:
(A) ORGANISM: Braccon Hebetor

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 13..487

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

```plaintext
TTTGATATACATGGAAATTATGATATCTCATTATTTATCTCTTATTACCA
1
Met Lys Phe Leu Tyr Leu Ile Leu Leu Leu Ala
5
10
GGAGATACATGTCACCGGAGACATCTGAGAAGAAATTAT
15
Gly Val Val Ser Phe Asn Pro Glu Thr His Arg Glu Cys Lys Asn Tyr
20
25
```

48
96
TGC GCC AAA GAG CAC GGC GAG GAA TAT CGT ACG TGG TCT TTC CGT TAC
Cys Ala Lys Glu His Gly Glu Glu Tyr Arg Thr Trp Ser Phe Arg Tyr
                                      144
                                      30
                                      40
GAA CTT GGT GAT ATT TTT AAA TGG GTT TGC ACT CAC GGA AAG AAT CTT
Glu Leu Gly Asp Ile Phe Lys Cys Val Cys Thr His Gly Lys Asn Leu
                                      192
                                      45
                                      50
                                      55
                                      60
ATG GGA AGC GAG AAT TAT GGT AAG TGT AGA GAA GCA TGT ATT CAA AAT
Met Gly Ser Glu Asn Tyr Gly Lys Cys Arg Glu Ala Cys Ile Gln Asn
                                      240
                                      70
                                      75
CAT GGA GCG GGA GCC TTT AAA TAT GCC TTT CCC ATA TAC AGC GAA GTA
His Gly Ala Gly Gly Phe Lys Tyr Ala Phe Pro Ile Tyr Ser Glu Val
                                      288
                                      80
                                      85
                                      90
CCA GCA TCA TGG GCA TGC ATA TGC ACT CAG GAG AAA AAT AAG ACA TTT
Pro Ala Ser Trp Ala Cys Ile Cys Thr Gln Glu Lys Asn Thr Phe
                                      336
                                      95
                                      100
                                      105
TGT ATA CAT GCT TGC GAA ATT CAT CAC AAG GCC CCA CCT AAG AAT
Cys Ile His Ala Cys Ser Glu Ile His His Lys Ala Pro Pro Lys Asn
                                      384
                                      110
                                      115
                                      120
CCC ATA GTC ATG AAA AAT GGA CAA TGC TAC TAC CAA GAT CAC AGG GTT
Pro Ile Val Met Lys Asn Gly Gln Cys Tyr Tyr Gln Asp His Arg Gly
                                      432
                                      125
                                      130
                                      135
                                      140
GTG GAC AGG TAT TGT GAA GTT TAT AGG TTC TTA GAT GCG TGG GAA
Val Asp Arg Tyr Cys Glu Val Tyr Met Lys Phe Leu Asp Ala Leu Glu
                                      480
                                      145
                                      150
                                      155
TCA ATT T AAACAAATGATC AAATCATGT TATCAATGAA GAAGAATCAA TGAATTAATA
Ser Ile
                                      537
                                      35
                                      564
ATAATTATCA AAAATCAAAA AAAAAAAA

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 158 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

50 Met Lys Phe Leu Tyr Leu Ile Leu Leu Leu Ile Ala Gly Val Val Ser
                                      1
                                      5
                                      10
                                      15
Phe Asn Pro Glu Thr His Arg Glu Cys Lys Asn Tyr Cys Ala Lys Glu
                                      20
                                      25
                                      30
His Gly Glu Tyr Arg Thr Trp Ser Phe Arg Tyr Glu Leu Gly Asp
                                      35
                                      40
                                      45
Ile Phe Lys Cys Val Cys Thr His Gly Lys Asn Leu Met Gly Ser Glu
                                      60
                                      50
                                      55
                                      60
Asn Tyr Gly Lys Cys Arg Glu Ala Cys Ile Gln Asn His Gly Ala Gly
                                      65
                                      70
                                      75
                                      80
Gly Phe Lys Tyr Ala Phe Pro Ile Tyr Ser Glu Val Pro Ala Ser Trp
  85
  90
Ala Cys Ile Cys Thr Glu Glu Lys Asn Lys Thr Phe Cys Ile His Ala
  100
  105
Cys Ser Glu Ile His His Lys Ala Pro Pro Lys Asn Pro Ile Val Met
  115
  120
  125
Lys Asn Gly Glu Cys Tyr Tyr Glu Asp His Arg Gly Val Asp Arg Tyr
  130
  135
  140
Cys Glu Val Tyr Met Lys Phe Leu Asp Ala Leu Glu Ser Ile
  145
  150
  155

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 1197 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
  (A) ORGANISM: Bracon Hebetor

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

CACGCCTACT TAGATAATTT CTCAATTCTT TGCAAAATTAT GAAAATAAGTG CAAGAGATGT
40
GTATGACACC TCAATCTGAG TTTGTCATTAT ATTCGGAGG AGATAAATAAG GAAGTCTCTG
  120
TGTACAAAG AAAAATCTCT CATATAAATC TGGCATTTTTT CGGTGAGAGA GAAAAAAGAAA
  180
CCCTGAAAAA CTGAGTAAGG CAATAATTTT NCCTCATAAAC AATGTCATACT ATATGTAATAA
  240
TAATTTTTGG TGGCTACTG AGTGGGACAT CGATGATGAC GTCAAACATT TTTACAGACC
  300
GAAGATGCTGG TGGAGTGTTT GATAAGACTT TTGCTCTCTTAC ACGTGGCTA GAAAGGAGGGT
  360
TTGGTGATTG TGCAGAGAAT CATGACAGCT GGGCCGCGAT GATTAACCA GGAGAGACTT
  420
ATGGAAGATGT TACGAATATA GAATTTTCAA ATATGAGGAA ATGCCAGATGT GACTATGCAT
  480
TTTTTCAATGC TCTTGACCGCT TCAATGGTA AAAGAAAAA TGTTGTGGAA ATATGCAATT
  540
TTGGCATGTC ATCAACACCC TTTACTTCTA TGAAGATGCT CGGTGCTAAA ATATCACCCC
  600
ATACTGTATA TGATAAACAA CAACTCACTT ATCAACTTAT ACTACACAAA GATAATTTTA
  660
AGGAGTGCGGT GCATGATAAT GCTCTTCTCCC CGCAAGAGCT GGGGATTAAA ATGAGGGCATG
  720
TGTGGGAGAC ACTGATGGCA TGGATGACTT TTAGATTTCC AACTGAATAA TAAATATCC
  780
AAATACAGAT ATCCCTTTCG TAAAATGTCG TAAAACATGAT TGTTTAGATG AATGGTAAAT
TAATGAAAAG ATGTGATGAA AATGTCTGAA GTAACCTNNNG GATNNAGCAT ATAATATATA
ATATTTGCCCT TATNGATAA ACTCTTACCN TTAANAAAGG AAAAAAGGAG AGGNGTAGGA
GGAGGATTAG GATATTTTAC AAAAATATTA AACAATTAGA TCTTCTGTAAT
ATGATGATG CATGATTTAA ATACAAATAAC ATCTCGTCTC CATAGTACAA TGAAAAAGAA
CATAACAGTA TGCAACAAAA TAATGACGGT AAATATCTAT GTATGTATGT AGAGAGAGA
AAATAAAAAAT AGTTAGACAG GTCCAAAAA AAAAAAAAAAAAAAAAA

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 182 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bracon Hebetor

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

```
Met Ser Ile Ile Cys Lys Ile Ile Leu Leu Val Leu Leu Ser Trp Thr
  1   5       10      15
Ser Met Val Ser Ser Thr Leu Phe Thr Asp Arg Lys Trp Cys Gly Arg
  20    25   30
Ala Asp Lys Thr Phe Gly Pro Ser Arg Ser Leu Gly Gly Gly Val Gly
  35   40    45
Asp Cys Cys Arg Ser His Asp Ser Cys Gly Arg Met Ile Lys Pro Gly
  50   55    60
Glu Thr Tyr Gly Asp Val Thr Asn Gly Phe Ser Asn Ile Trp Glu
  65    70   75     80
Cys Arg Cys Asp Tyr Ala Phe Phe Gln Cys Leu Gln Arg Ser Asn Gly
  85    90
Lys Met Lys Asn Val Val Glu Ile Leu His Phe Asp Val Val Asn Thr
  100   105   110
Pro Cys Tyr Phe Met Lys Asp Gly Arg Ala Lys Ile Ser Pro His Thr
  115   120   125
Val Tyr Asp Lys His Glu Ser Leu Tyr Gln Leu Ile Leu His Lys Asp
  130   135   140
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Asn Phe Lys Glu Trp Val His Asp Asn Ala Leu Leu Pro Gln Glu Leu  
145 150 155 160  
Gly Ile Lys Asp Glu His Val Trp Glu Thr Leu Met Ala Trp Met Asp  
165 170 175  
Phe Arg Phe Pro Thr Glu  
180  

(2) INFORMATION FOR SEQ ID NO: 5:  

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 669 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: unknown  

(ii) MOLECULE TYPE: cDNA  

(iii) HYPOTHETICAL: NO  

(iv) ANTI-SENSE: NO  

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Bracon Hebetor  

(ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 147..581  

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

```
35 AAAAGACTAA AAATAAGAAA AAAAAACATA GAAGAATGTT TACAATAATT TATAATTTTTA  
60  
AACTTTTACATT TCTATTAGTT CCGTGCCTGGA GTTTTCTCAAC CTACGCTGGG TATGGTGAAT  
120  
ATAATCGGTC CATTACTAAG CGACAG ATG GAC GAT GGT GAG ACG TGC GAA AGG  
173  
       Met Asp Asp Gly Glu Thr Cys Glu Arg  
       160 165  

TGT TTG AAT CCA CTC GAA TTA GTA AAT GAC GCT GTA GAC TCG TGC ATT  
45  
Cys Leu Asn Pro Leu Glu Leu Val Asn Asp Ala Val Asp Ser Cys Ile  
       170 175 180  

GAA GCT CAT GAG GAA TGT GAG GAA TTC ATT GAA GCC GGG ATG GAA ATG  
50  
Glu Ala His Glu Glu Cys Glu Glu Phe Ile Glu Gly Gly Met Glu Met  
       185 190 195  

CTT CAT GTA CAC AAT CCA GGA AAC TTC CCA GTC TCC AAA TGT GTA TGC  
55  
Leu His Val His Asn Pro Gly Asn Phe Arg Val Ser Lys Cys Val Cys  
       200 205 210 215  

GAC ATT GGC CTC AAG GAG TGC CTC ACT ACT CAT CTC GAA ATG AGT TTC  
60  
Asp Ile Ala Leu Lys Glu Cys Leu Thr Thr His Pro Glu Met Ser Phe  
       220 225 230  

AAA TTT GCT AAA GCA CTC TTT TTT GAT TTG CTT GCT CCA CCC TGT TTT  
65  
Lys Phe Val Lys Ala Leu Phe Phe Asp Leu Leu Ala Pro Pro Cys Phe  
       235 240 245  

GAT CAG ATT GCT GAT TGG GGT AAG AAA AAA TTG AAA AAT AAG CAG GCA  
70  
```

461
Asp Gln Ile Ala Asp Trp Gly Lys Lys Leu Lys Asn Lys Gln Ala
250 255 260

TTT TCA CTG CAT GAT TTA CAA TCA GCT GCC CAC GCG CTC TGG CAA ACA 509
Phe Ser Leu His Asp Leu Gln Ser Ala Ala His Ala Leu Trp Gln Thr
265 270 275

CTC TAT GAC GCT GTC AAG GGC ATA GCT CAG GAT GTC GGA CAT GCT GCA 557
Leu Tyr Asp Ala Val Lys Gly Ile Ala Gln Asp Val Gly His Ala Ala
280 285 290 295

CAT TCT TTT GAA AAA ATG TTA CAG TAA CAG GTA AAC ATG AAA AAA GGTCCATGAT 611
His Ser Phe Glu Lys Met Leu Gln
300

AGTAGAATAC AGTATGTT GTATAATAAA ATAAATATT CAGAATGATA AAAAAAA 669

20 (2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 176 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Trp Gly Leu Ser Phe Leu Leu Val Pro Cys Trp Ser Phe Ser Thr
1 5 10 15

Tyr Ala Gly Cys Gly Gly Tyr Asn Arg Ser Ile Thr Lys Arg Gln Met
20 25 30

Asp Asp Gly Glu Thr Cys Glu Arg Cys Leu Asn Pro Leu Glu Leu Val
35 40 45

Asn Asp Ala Val Asp Ser Cys Ile Glu Ala His Glu Glu Cys Glu Glu
50 55 60

Phe Ile Glu Gly Gly Met Glu Met Leu His Val His Asn Pro Gly Asn
65 70 75 80

Phe Arg Val Ser Lys Cys Val Cys Asp Ile Ala Leu Lys Glu Cys Leu
85 90 95

Thr Thr His Pro Glu Met Ser Phe Lys Phe Val Lys Ala Leu Phe Phe
100 105 110

Asp Leu Leu Ala Pro Pro Cys Phe Asp Gln Ile Ala Asp Trp Gly Lys
115 120 125

Lys Lys Leu Asn Lys Gln Ala Phe Ser Leu His Asp Leu Gln Ser
130 135 140

Ala Ala His Ala Leu Trp Gln Thr Leu Tyr Asp Ala Val Lys Gly Ile
145 150 155 160

Ala Gln Asp Val Gly His Ala Ala His Ser Phe Glu Lys Met Leu Gln
165 170 175
(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1057 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) ORIGINAL SOURCE:
(A) ORGANISM: Bracon Hebetor

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 95..919

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

25 CGCGGCCGCT GTTGATATAT AACAATTAT TAAAAATTTTC AAGTGAAAG AAAAACTATC

TTTTTTTTTTT TTTTTTTTT TTTCTAATT TAAA ATG CAT TTC TCC GCC TCC

Met His Phe Phe Ala Ser

150

30 ATC CTG GTA TGC TTC TTA CTG GGC AAG GCA ATT CAT GAT GTG GAA GGA

Ile Leu Val Cys Phe Leu Leu Leu Gly Lys Ala Ile His Asp Val Gly Gly

155 160 165

35 ATA ATA AAT GGT CAT GAT GCT ACT GAG GGA CAA TTT CCC CAT ATG GCT

Ile Ile Asn Gly His Asp Ala Thr Glu Gly Gln Phe Pro His Met Ala

170 175 180

40 TAT TTA CAA GCA TCA GCT GGA AAG TGT TCT TAT GGA TGT GGC GGT GCT

Tyr Leu Gln Ala Ser Ala Gly Lys Cys Ser Tyr Val Cys Gly Ala

185 190 195

45 CTT CTA ACT AAA AAA CAT ATT ATG ACA GCT GCT CAT TGT GTA GCA ATG

Leu Leu Thr Lys Lys His Ile Met Thr Ala Ala His Cys Val Ala Met

200 205 210 215

50 CAC AGA ACG GGA AAT ATT AAA GTA GCC CTT GGT GTT ACG CAT TTT CAT

His Arg Thr Gly Asn Ile Lys Val Ala Leu Gly Val Thr Asp Phe His

220 225 230

55 AAT AAG CCA TCA ATG CAA CAA AGA AAG GTT GAA CAT ATA AAA GTC CAT

Asn Lys Pro Ser Met Glu Glu Arg Lys Val Glu His Ile Lys Val His

235 240 245

60 TCT GAG TAC AAA GGA GGA AGG CGT AAG TCA TTA AAA AAT TGG TAT CGC

Ser Glu Tyr Lys Gly Gly Arg Arg Lys Ser Leu Lys Asn Trp Tyr Arg

250 255 260

TCC ATA CAT CGT ACA TTG ACA GGA CCG TCT GGG GAT AAA GAA TAC AAT

Ser Ile His Arg Thr Phe Thr Pro Ser Gly Asp Lys Gly Tyr Asn

265 270 275

GAT ATT GCT ATT ACA AGC TTG AGC CAG GAA GTA ACA CTA GGA CCA GTA

544
Asp Ile Ala Ile Ile Thr Leu Ser Gln Glu Val Thr Leu Gly Pro Val
280  285  290  295

GTA AAG ACT ATT AAT TTA CCC CCA AAG AGC TAT CGG CCT TCT TTT GAT
5 Val Lys Thr Ile Asn Leu Pro Pro Lys Ser Tyr Arg Leu Pro Phe Asp
300  305  310

CAA GAT GCT AGA TGG TCG GGC TTT GGC CGA ACA GTC ATT GTC AAA GAA
10 Gln Asp Ala Arg Leu Ser Gly Phe Gly Arg Thr Val Ile Val Lys Glu
320  325

AAT GAT CCA ATT CCT CCA CCC ACT ACA CAT TTA CAA TGG CTA GAT ATG
15 Asn Asp Pro Ile Pro Pro Pro Thr Thr His Leu Gln Trp Leu Asp Met
330  335  340

AAG GTT CTT CAT TCA CGA GAT GCT ATT GTC ACT GAT AGT GAA TTT CTC
20 Lys Val Leu His Ser Arg Asp Ala Ile Val Thr Asp Ser Glu Phe Leu
345  350  355

GCT GAT AAA GAA TAT GGT GAT GGA ACT TGG TCT AAT GCA GCT AAG GGA
25 Ala Asp Lys Glu Tyr Asp Gly Thr Gln Trp Ser Asn Ala Ala Lys Gly
360  365  370  375

GAC ACG GGT AGT CCC TTA GTC AAG GAT AAT CAA GTA ATT GCC GTA GCC
30 Asp Ser Gly Ser Pro Leu Val Lys Asp Asn Gln Val Ile Gly Val Ala
380  385  390

GTT TCT GTG AGT GAT GAA GAA CAT ACT ACA CGC TTT CAA ATA GTC ACT
35 Val Ser Val Ser Asp Glu Glu His Thr Thr Arg Phe Gln Ile Val Thr
400  405

TAT TAT TTG GAT TGG ATC AAG AAA TAT GCC GAA CTT GCG TAAAAGAAT
40 Tyr Tyr Leu Asp Trp Ile Lys Tyr Ala Glu Leu Ala
410  415  420

AAAGAGCAA ATTGCTCAGA TGTTGAATAT ACACATTTCC AATAAGCTCA GAAAAATCG
45

ATTATATGT AATAAAAAA TTAAGAATTG TTTTCTCTCT TTTACAGAA GAATTTGGCG
50

CGTGAAATT

(2) INFORMATION FOR SEQ ID NO: 8:
45

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 275 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met His Phe Phe Ala Ser Ile Leu Val Ser Phe Leu Leu Gly Lys Ala
1  5 10  15

Ile His Asp Val Glu Gly Ile Ile Asn Gly His Asp Ala Thr Glu Gly
20  25  30

Gln Phe Pro His Met Ala Tyr Leu Gln Ala Ser Ala Gly Lys Cys Ser
35  40  45

Tyr Val Cys Gly Gly Ala Leu Leu Thr Lys Lys His Ile Met Thr Ala
50  55  60

1057
Ala His Cys Val Ala Met His Arg Thr Gly Asn Ile Lys Val Ala Leu
65
70
75
80
Gly Val Thr Asp Phe His Asn Lys Pro Ser Met Gln Gln Arg Lys Val
85
90
95
Glu His Ile Lys Val His Ser Glu Tyr Lys Gly Gly Arg Arg Lys Ser
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Leu Lys Asn Trp Tyr Arg Ser Ile His Arg Thr Phe Thr Gly Pro Ser
115
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125
Gly Asp Lys Glu Tyr Asn Asp Ile Ala Ile Ile Thr Leu Ser Gln Glu
130
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140
Val Thr Leu Gly Pro Val Val Lys Thr Ile Asn Leu Pro Pro Lys Ser
145
150
155
160
Tyr Arg Leu Pro Phe Asp Gln Asp Ala Arg Leu Ser Gly Phe Gly Arg
165
170
175
Thr Val Ile Val Lys Glu Asn Asp Pro Ile Pro Pro Pro Thr Thr His
180
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Leu Gln Trp Leu Asp Met Lys Val Leu His Ser Arg Asp Ala Ile Val
195
200
205
Thr Asp Ser Glu Phe Leu Ala Asp Lys Glu Tyr Gly Asp Gly Thr Trp
210
215
220
Ser Asn Ala Ala Lys Gly Asp Ser Gly Ser Pro Leu Val Lys Asn
225
230
235
240
Gln Val Ile Gly Val Ala Val Ser Val Ser Asp Glu Glu His Thr Thr
245
250
255
Arg Phe Gln Ile Val Thr Tyr Tyr Leu Asp Trp Ile Lys Lys Tyr Ala
260
265
270
Glu Leu Ala
275

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 594 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bracon Hebetor

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 22..573

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

```
5  AGATCTGCGG CGCGGCCACC A ATG TCA ATC ATA TGT AAA ATA ATC TTG TTG
   Met Ser Ile Ile Cys Lys Ile Ile Leu Leu
   280  285

10 GTG CTA CTG AGT TGG ACA TCG ATG GTA TCG TCA ACA TTA TTT ACA GAC
    Val Leu Leu Ser Trp Thr Ser Met Val Ser Ser Thr Leu Phe Thr Asp
    290  295  300

15 CGA AAG TGG TGT GGA CGT GCC GAT AAG ACT TTT GGT CCT TCA CGG TCG
    Arg Lys Trp Cys Gly Arg Ala Asp Lys Thr Phe Gly Pro Ser Arg Ser
    305  310  315

20 CTA GGA GGA GGT GGT GAT TGC TGC AGA AGT CAT GAC AGC TGT GGC
    Leu Gly Gly Val Gly Asp Cys Cys Arg Ser His Asp Ser Cys Gly
    320  325  330

25 CGC ATG ATT AAA CCA GGA GAG ACT TAT GGA GAT GTT ACG AAT AAA GGA
    Arg Met Ile Lys Pro Gly Thr Tyr Gly Asp Val Thr Asn Lys Gly
    335  340  345

30 TTT TCA AAT ATT TGG GAA TGC CGA TGT GAC TAT GCA TTT TTT CAA TGT
    Phe Ser Asn Ile Trp Glu Cys Arg Cys Asp Tyr Ala Phe Phe Gln Cys
    350  355  360  365

35 CTT CAG CGT TCC AAT GGT AAA ATG AAA AAT GTT GAG GAA ATA TGT CAT
    Leu Gln Arg Ser Asn Gly Lys Met Lys Asn Val Val Ile Leu His
    370  375  380

40 TTT GAC GTT GTC AAT ACA CCC TGT TAC TTC ATG AAA GAT GGC CGT GCT
    Phe Asp Val Val Asn Thr Pro Cys Tyr Phe Met Lys Asp Gly Arg Ala
    385  390  395

45 AAA ATA TCA CCC CAT ACT GTA TAT GAT AAA CAC GAA TCA CTC TAT CAA
    Lys Ile Ser Pro His Thr Val Tyr Asp His Glu Ser Leu Tyr Gln
    400  405  410

50 CTT ATA CTA CAC AAA GAT AAT TTT AAG GAG TGG GTG CAT GAT AAT GCT
    Leu Ile Leu His Asp Asn Phe Lys Glu Trp Val His Asp Asn Ala
    415  420  425

55 GGA ACT CTC CTT CGG CGA GAG CTG GGA ATT AAA GCT GAG CAT GTG TGG
    Gly Thr Leu Leu Pro Arg Glu Leu Gly Ile Lys Ala Glu His Val Trp
    430  435  440  445

60 GAG ACA CTG ATG GCA TGG ATG GAC TTT AGA TTT CCA ACT GAA
    Glu Thr Leu Met Ala Trp Met Asp Phe Arg Phe Pro Thr Glu
    450  455

TAATGAGCGG CGCGAATTC C

594
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(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 184 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

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Asp Lys Thr Phe Gly Pro Ser Arg Ser Leu Gly Gly Gly Val Gly

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Asp Cys Cys Arg Ser His Asp Ser Cys Gly Arg Met Ile Lys Pro Gly

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Lys Met Lys Asn Val Val Glu Ile Leu His Phe Asp Val Val Asn Thr

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Pro Cys Tyr Phe Met Lys Asp Gly Arg Ala Lys Ile Ser Pro His Thr

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Asn Phe Lys Glu Trp Val His Asp Asn Ala Gly Thr Leu Leu Pro Arg

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Met Asp Phe Arg Phe Pro Thr Glu

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 1326 base pairs
   (B) TYPE: nucleic acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: Bracon Hecetor

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

TGGATAAAA TCATGAAATT TTTATATCTA ATACTCCTTT TAATCGCAGG AGTAGTATCA

TTCAATCCGG AGACACGTAAT GAAATXGGA AAATTTTTTT AATTAATCATTCTATAAT
CAATTCAGTT GTTCACAAATTTTTTGTTT CATTTTTTATT AAAATTATAC CAATAATGAT 180
AATAAAAATT TATTTTCGCC TTTAAATTGA TAAAAATTAG ATCGGGAAATG TAAGAATTAT 240
TGCGCCAAAG AGCAGCGGCA GGAATATCGT AGCTGCGCTT TCCGGTACGAG AACTAGGTGAT 300
ATTTTTTAAAT GTTTTTGGAC GTAAGTAACA AAATTTTCAT TACTAATATA CTTCCTTTAT 360
TATTCCATGAT TAAATCAAGA TTTATTAATT AATGAATTAT GTGTGACTTC ATTATGTAAG 420
TCACGGAAAG AATCTTATGG GAAGCGAGAA TTAAGTGAAG TATTATCTCA AAAATTTTAG 480
TTTTGGTTTA ATAAATATTAA TTTTTCTTTA AATTATCCGT CTTCTGGCC AGTGAAGTGT 540
AGAGAGAGCT GTATTCCAAA TGTGGAGGCG GGGGCTTTTA AATATGCTTT TCCCATATAC 600
AGCGAAATTC TAGCATCATG GGCATGCAAT TGCACTATAGT "AACATTAGA TGTAATTATT 660
ATTATTTCTA TTTAAATCCA ATATAATTAT AATATAATCG TATAATGTTT TTAGTCAGGA 720
GAAAAATAAG ACATTTTGTA TACATGCTTG CTCAGAAATT CATCACAAGG CCCACCTAAA 780
GAATCCCATG GTTATGAATA ATGGGAATAATG CTACTACCAA GATCACAAGG GTGTGACAG 840
GTATTTGGAA GTTTATATAG ACGTCTTCTGA TCGCTTGAA TCAATTTAAC AATGATCAA 900
TTTTCACGTAT CAATGAGAGA AGGATTATGA ATCAAATATA AATAACCAA AAATCATGAT 960
TTTTTTTACA ATATTTAAAA AAAAGGCTAC ATTTGATTTT TAACTGTTCG ATATAATACC 1020
CCCTTCTCCAA TTCAACTACT CCTCTCATCT CAATTAGAAA CAACCTAAAA ACTAATGAT 1080
TCCAATACCC GAAACGAAAC AATTTACATA GCATAGAAG AGCTTAATTG CCTAAAAATT 1140
AATGCATAAT TAGGGAGAG CACTAATAA AGGGGAGGG GCTCCGGTTCC AAAAAATCAT 1200
TTTGTATATA TACTTTTTA AGAATGACTT TCTTTCTGCT ATCCCATTTTC TTTTTCTCCT 1260
TCCCCACTC GGAATATCT CTTGCGTATA TATATATAGC TACGTTAGAT CCTGGCAACT 1320
TGGAAC 1326

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 358 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bracon Hebetor

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
CATGGAAATT TTTATATCTA ATACTCCCTT TAATTGCAGG AGTAGTATCA TTCAATCCGG
AGACACGTAA GTAAATTGGA AAATTTTTTT AATTAATCA ATTCTATAAT CAATTTTATT 120
GTTCACTTAT TTGGTTTTTT CATTCTTTAT AAAATTGAC CAATAATGAT AATAAAATT 180
TATTTTTCGC TTTAAATTTA TAAATTTTAG ATCTGGAATG TAAGAATTAT TGGGCCAAAAG 240
AGACGCAGGA GGAATACCGT ACCTGTTCTT TCCGTACGA ACTTGTTGAT ATTTTTAATAT 300
GTGTTTGGAC GTCAGTAAACA AAATTTTCAT TACTAATATA TTCTTTTTATT ATTCATGG 358

(2) INFORMATION FOR SEQ ID NO: 13:
(I) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 358 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bracon Hebetor

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:
CATGGAATTT TTTATATCTA ATACCCCCCTT TAATTGCAGG AGTAGCATCA TTCAATGCCG 60
CCATAGCTAA GTAAATTGGA TTTTTTTTTTTTTTTTTTTTTAAATTTTAT TTAATTCTATA ATTAAATTATA 120
TTGTTCACTT TTTTTTTTTC ATTTTTATTA AAATATTACC AAATAGTGA ATATAAAATT 180
ATTTTTTCGGT TTTAAATTTT AAAAAATTTAGT ATCTGGAATG TAAGAATTAT TGGGCCAAAAG 240
GCACGCGGAG GGAATAGCTA CATGTTCTTT TCCGGTGGA ATGGGGTGT CTTTTAATG 300
TATTGCAGG TAAGTACAAAAATTTTTCAT GACTACATTC TTCTTTTTTTTTTATGG 358

(2) INFORMATION FOR SEQ ID NO: 14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 737 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bracon Hebetor

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:
ACTAGTATTC TTTTCTTGTT TTTTCTGATT AATCAAGAT TTATATATTAT AATGAATATG 60
TGTGTTGATT TGATTATAA GATCCGAAAA CCACCTATG AGAACAGAGA ATATAGGTTA 120
GATATCTACTT AGAATTATTA CTTTTGTTTT ATTAATTCA ATTTTCTTTA CTTTTTAA 180
CTCTATATTTC TCTCTCTCTCT CTCTTCCTCCATA ATCAAGATATTGCTAGACAA AAATGATTTC 240
AACAGCATTAG AGCCGGAGGC TTAAAAATAG CTCGAGCAT ATACAGTGAA GTACCAGCTT 300
CAGGGCATTG CATGTCACG TAGTTAACAT TAGATGCTAT TATTATATAA ATATTTTTAA 360
ATCAATAATT TTAATAAAC AATTGTATGA TGTTTTTTAGT CAGGGAAAAA ATAGACATA 420
TTGTATCATG CCGTGCAGTA AAGTTGAGAAA AAGGCCCCA CCTATGAATCCCATAGTGAT 480
GAAGAATGGA GAATGCTACT ATCAAGATCA CAGGGTGGT GAGGGCTGCTG GGGAGTTGGA 540
AATGAATACG ATGGATGAA TAAATTCACAA ATTTATCAAA TTTTCGTTATT CAAATGAGGA 600
AGAATAATAA ATCAATAATA ATATAAACA ATCAATAACAT TTGTACAAAA TCATTTAAAA 660
AAAAAACGCT ACATCGGTAT TTTAAGCTCT CGAGATACTA ATGCTTTTGGT GAGGGAGAT 720
TTAAGGCTTG TACTAGT 737

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TTATATGAAG TTCTTTAGA 18

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTAAATTGAT TCCAACGC 18

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTCCTTACCG GAAATACG

5

(ii) MOLECULE TYPE: nucleic acid

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

GAATTATTAT CAAATCAT

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(iii) MOLECULE TYPE: DNA (genomic)

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

TCTAGAACAA TGATATAGGA CCGTTGTTAG GTAAAAAGCA GTGCCAATTG TCTATAGTC

TAGCCGCTAC AAGGCCCTA AAACACGTA AAGACAAAAA AGCTATAGCG GTGATAATA

GAGTGCCGTT AGAGGGGCGG TGTAACCAATT TGCAAAATTAT GGTTATCCATC ATTCGTCATA

TAACATACA AAATCTAAA ATATCTAAA ACTCCTCCAA ATAACCTAAA AAAAAAAA

AAAAAAGATTC TGAAGCTGTT GTAAGAGATA TGGAGGAGCT GTGACTGGTC ATGGAGTTGT

ACAGTAATAG GCTTACGTTG ATTTTCAC ACACTAGTAA TAAATATGGA GATAATAAA

AAATACCTGT GATTCTTAGT CTATTTAAA AAAATTTTCA TTGGATATAC CGGAGTACAA

GAGCTATATA AAGATGAGTA AGGGTTAGCA TAAGTATAAT TCAATTTCTT TCAAGAGAG

TTCAGATAAA TCATGAAAT TTTATATCTA ATACCCCTTT TAATGCAAG AGTAGCATCA

TTCAATGCAG CCATACGTA AAAAAATGGA TTTTTTTTTT TTTAAAAATT CAATTCCTA

ATTCAATTTT ATTGGTACAAT TTTTTTTTTT TATTTTTATT AAAATTAC ACAATAGAT

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720
AATATAAATT TATTTTTCGC TTTAAAATTGA TAAGAATTGAT 780
TGGCCCAAG AGCACGGGCA GAAATGGTCT ACATGGTCTT TTGTTACGA ACTGGGTGAT 840
ACGTTCATAT GTATTTCGAC GTAAGTACA AAAAAATCAT TACTAGTATT CTTCTTCAT 900
TTTTCATGAT TAAATCAAGA TTTAATTAAT AATGAATTAT GTGTGGTGAT TGATTATAT 960
AGTATCGGAA ACGGCCCTCAT GAGAACAGAG AATAAGTGTA AGTATCTACT TAGAATTAT 1020
AGTTTTGGTT TAATAATTCT AATTTTTCTT ACTTTTAAAT ACTCTATTAT TCTCTCTCTC 1080
TCTCTCTCC CAAATCAAGATA AGTGAGACCA AAAAAATGATT CAACAGCATG GAGCGGGAGG 1140
CTTTAAATAT GCTTCAGCA TATACAGTGA AGTACCAGCT TCATGGGCAT GCATGGCAC 1200
GTATGTAACAC TTGATGCTTA TTATTAT TTTTTTTTTA AATCAATAAT TTAAAATAAATA 1260
CAATGTATG ATGTTTCTAG TCAGGTGAAA AATAAGACAT ATGTATACACA TGCTTGCATG 1320
AAAGTTGAAAG AAAAGCCCC ACCTATGAAT CCCATAGTGA TGGAAAAATGG AGAATGCTAC 1380
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ATATATAAAC AATCAATAAC TTTGTATTTA AATCATTTAAA AAAAAAGGCC TACATTTGGAT 1560
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CGAAACAGCC AGTTCCACCTT TTGAGAAGCA CCTCTATACA CATACATGTT TGCGGCAAATA 1680
TGTTGAAATT TATGGCGCAA ATTTTTTTTT ACATCCCTCA TTAAATGCCT AAGCCGTACA 1740
AGTTTTAATT GACACACTGTT TGAAGGCGGA ACTTTAATAA TGATGGAAAG AAAGGAAAGA 1800
AATGTTTGGAA A 1811

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: DNA

(ii) MOLECULE TYPE: nucleic acid

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

ATGAAAATTT TATATCTAAT 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GTCTCCGGAT TGAATGATA

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CATATACAGC GAAGTACC

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CATAACTATG GGATCTTAG G

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

AAATGGACA ATGCTACTAC C

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

TTAAATGTAT TCCAAGC

(2) INFORMATION FOR SEQ ID NO: 26:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

AATGTGTTTG CACTCAG

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CCTCCCGCT CCATGATTIT G

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CCTCGCGGTG CTCTTTGGC

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GTAACCAGC TAAGCATAAC G

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GTATAACG AGAGGATCA GGGAG
(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

AATACAGTCC CTATATAACCC

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: DNA
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: nucleic acid
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GGGATGGGAA TAATGATGTC A

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GACTCTGGGA GCCCG

(2) INFORMATION FOR SEQ ID NO: 34:

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

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

TTGACACCAG ACACACTGGT AATG

(2) INFORMATION FOR SEQ ID NO: 35:

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

(ii) MOLECULE TYPE: DNA

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GCTGATGTGC TGCAAGGCGA TTAAG

(2) INFORMATION FOR SEQ ID NO: 36:

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

(ii) MOLECULE TYPE: DNA

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

TTCACAGAGG AAACAGCTAT GAC

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

TCGTCAAGTG AAGAATTA

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

TTATATGAAG TTCTTPQA

(2) INFORMATION FOR SEQ ID NO: 39:
(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 18 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

GCGCAATTA TTCTTCAC

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 18 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

TAACTATGGG ATCTTAG

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 18 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

ATATTTAAG CCTCCGC

(2) INFORMATION FOR SEQ ID NO: 42:

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

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

TTYAAYCCNG ARACNCAYNG NGA
(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 20 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

GCNAAGACG ASGNGARAG

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 59 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

TTCAACCCNG ARACNGACNG NGARNNAAR AACNCAANNNG CNAARGARCA TGGNGARAG

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 456 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: Bracon Hebetor

(ix) FEATURE:
   (A) NAME/KEY: CDS
   (B) LOCATION: 1..456

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

ATG AAA TTT TTA TAT CTA ATA CCC CTT TTA ATT GCA GGA GTA GCA TCA
Met Lys Phe Leu Tyr Leu Ile Pro Leu Leu Ile Ala Gly Val Ala Ser
1      5     10     15
TTC AAT GCG GCC ATA GAT CGT GAA TGT AAG AAT TTT TGC GCC AAA GAG
  Phe Asn Ala Ala Ile Asp Arg Glu Cys Lys Asn Phe Cys Ala Lys Glu
  20 25 30

CAC GGC GAG GAA TAT GCT ACA TGG TCT TCT CGT TAC GAA CTG GGT GAT
  His Gly Glu Tyr Ala Thr Trp Ser Phe Arg Tyr Glu Leu Gly Asp
  35 40 45

ACG TTT AAA TGT ATT TGC ATC GGA AAG GCC CTC ATG AGA ACA GAG AAT
  Thr Phe Lys Cys Ile Cys Ile Gly Asn Gly Leu Met Arg Thr Glu Asn
  50 55 60

AAT GAT AAG TGT AGA CAA AAA TGT ATT CAA CAG CAT GGA GCG GCA GGC
  Asn Asp Lys Cys Arg Gln Lys Cys Ile Gln Gln His Gly Ala Gly Gly
  65 70 75 80

TTT AAA TAT GCC TCC AGC ATA TAC ATG GAA GTA CCA CCT GTCA TGG GCA
  Phe Lys Tyr Ala Phe Ser Ile Tyr Ser Glu Val Pro Ala Ser Thr Ala
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TGC ATG TGC CAG GTG AAA AAT AAG ACA TAT TGT ATA CAT GCT TGG ATG
  Cys Met Cys Glu Val Lys Asn Lys Thr Tyr Cys Ile His Ala Cys Met
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AAA GTT GAA GAA AAG GCC CCA CCT ATG AAT CCC ATA GTG ATG AAA AAT
  Lys Val Glu Glu Lys Ala Pro Pro Met Asn Pro Ile Val Met Lys Asn
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GGA GAA TGC TAC TAT CAA GAT CAC AGG GGT GTT GAG AGG TCG TGT GAA
  Gly Glu Cys Tyr Tyr Glu Asp His Arg Gly Val Glu Arg Ser Cys Glu
  130 135 140

TTG GAA ATG AAT AGC ATT GAT
  Leu Glu Met Asn Ser Ile Asp
  145 150

(2) INFORMATION FOR SEQ ID NO: 46:

  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 152 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: protein

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

Met Lys Phe Leu Tyr Leu Ile Pro Leu Leu Ile Ala Gly Val Ala Ser
  1  5 10  15

Phe Asn Ala Ala Ile Asp Arg Glu Cys Lys Asn Phe Cys Ala Lys Glu
  20 25 30

His Gly Glu Gly Tyr Ala Thr Trp Ser Phe Arg Tyr Glu Leu Gly Asp
  35 40 45

Thr Phe Lys Cys Ile Cys Ile Gly Asn Gly Leu Met Arg Thr Glu Asn
  50 55 60

Asn Asp Lys Cys Arg Gln Lys Cys Ile Gln Gln His Gly Ala Gly Gly
  65 70 75 80
Phe Lys Tyr Ala Phe Ser Ile Tyr Ser Glu Val Pro Ala Ser Trp Ala
   85         90         95
Cys Met Cys Gln Val Lys Asn Lys Thr Tyr Cys Ile His Ala Cys Met
   100        105        110
Lys Val Glu Glu Lys Ala Pro Pro Met Asn Pro Ile Val Met Lys Asn
   115        120        125
Gly Glu Cys Tyr Tyr Gln Asp His Arg Gly Val Glu Arg Ser Cys Glu
   130        135        140
Leu Glu Met Asn Ser Ile Asp
  145        150
CLAIMS

1. A polynucleotide comprising a region encoding at least two of the insecticidal toxin subunits selected from those comprised in SEQ ID Nos. 2, 4, 6, 8, and 10 and that encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the said spliced RNA being capable of hybridising with the extension products of the primers depicted in SEQ ID Nos 20 and 21, or SEQ ID Nos 22 and 23, or SEQ ID Nos 24 and 25 using the sequence depicted in SEQ ID No. 1 as a template, with the proviso that the polynucleotide does not encode only the combination of the subunits comprised in SEQ ID Nos. 2 and 8.

2. A polynucleotide according to claim 1, wherein the said subunits are the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No. 6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10.

3. A polynucleotide according to claim 2 wherein at least one of the said proteins comprises a heterologous N-terminal extension in the form of a signal or secretory peptide.

4. A polynucleotide comprising a region encoding at least one of the insecticidal toxin subunits selected from those comprised in SEQ ID Nos. 2, 4, 6, 8, and 10 and that encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the said spliced RNA being capable of hybridising with the extension products of the primers depicted in SEQ ID Nos 20 and 21, or SEQ ID Nos 22 and 23, or SEQ ID Nos 24 and 25 using the sequence depicted in SEQ ID No. 1 as a template, wherein the region has been modified in that mRNA instability motifs and/or fortuitous splice regions are removed, or insect-pest preferred codons are used so that expression of the thus modified polynucleotide in the said insect yields substantially similar protein having a substantially similar activity/function to that obtained by expression of the unmodified polynucleotide in the organism in which the protein encoding regions of the unmodified polynucleotide are endogenous.
5. A polynucleotide according to claim 4, wherein the said subunits are the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No. 6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10.

6. A polynucleotide according to claim 5, wherein at least one of the said proteins comprises a heterologous N-terminal extension in the form of a signal or secretory peptide.

7. A polynucleotide according to any one of claims 4-6, wherein the insect pest is Lepidopteran, and the said organism is an Hymenopteran of the superfamily Ichneumonoidea, in particular a wasp of the family Braconidae.

8. A polynucleotide according to any of the preceding claims wherein at least one of the protein encoding sequences in the region is under expression control of a viral promoter, or insect strong promoter, which is not down regulated or otherwise silenced when the polynucleotide is introduced into the cells of an insect which is or becomes infected by an insect virus.

9. A polynucleotide according to the preceding claim, wherein the promoter is selected from the group consisting of the baculovirus p10 promoter and the polyhedrin promoter.

10. A polynucleotide according to any preceding claim, wherein the region encodes not less than two and not more than four of the toxin subunits selected from the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No. 6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10.
11. A polynucleotide according to any preceding claim, wherein the region encodes not less than two and not more than three of the toxin subunits selected from the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No.6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10.

12. A polynucleotide according to any preceding claim, wherein the region encodes two of the toxin subunits selected from the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No.6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10.

13. A polynucleotide according to any one of claims 1 to 9, wherein the region encodes the three proteins represented by amino acids 22 to 182 in SEQ ID No. 4 or amino acids 22 to 184 in SEQ ID No. 10; amino acids 32 to 176 in SEQ ID No.6 and amino acids 23 to 275 in SEQ ID No. 8.

14. A polynucleotide according to any one of claims 4-7, wherein the said modified region encodes one of the toxin subunits selected from the proteins represented by amino acids 17 to 158 in SEQ ID No. 2; amino acids 22 to 182 in SEQ ID No. 4; amino acids 32 to 176 in SEQ ID No.6; amino acids 23 to 275 in SEQ ID No. 8; and amino acids 22 to 184 in SEQ ID No. 10.

15. A nucleotide sequence, encoding an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 50 and 55°C in single strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with single strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID Nos. 1, 3, 5, 7, 9, or 19.
16. A nucleotide sequence, encoding an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 55 and 60°C in single strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.5 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID Nos. 1, 3, 5, 7, 9, or 19.

17. A nucleotide sequence, encoding an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID Nos. 1, 3, 5, 7, 9, or 19.

18. A nucleotide sequence, encoding an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 50 and 60°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence represented by nucleotides 61 to 486 in SEQ ID No. 1; nucleotides 285 to 766 in SEQ ID No. 3; nucleotides 147 to 584 in SEQ ID No. 5; nucleotides 161 to 219 in SEQ ID No. 7; or nucleotides 85 to 573 in SEQ ID No. 9.

19. A nucleotide sequence, encoding an insecticidal toxin sub-unit, which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence represented by nucleotides 61 to 486 in SEQ ID No. 1; nucleotides 285 to 766 in SEQ ID No. 3; nucleotides 147 to 584 in SEQ ID No. 5; nucleotides 161 to 219 in SEQ ID No. 7; or nucleotides 85 to 573 in SEQ ID No. 9.

20. A polynucleotide according to any one of claims 1-13, wherein the said region comprises the nucleotide sequence of any one of claims 15 to 19.
21. A cell transformed with the polynucleotide of any one of claims 1-13 or 20, or the nucleotide sequence of any one of claims 14 to 19.

22. An organism regenerated from the cell of the preceding claim.

23. An insect pathogen comprising the polynucleotide of any one of claims 1-13 or 20, or the nucleotide sequence of any one of claims 14 to 19.

24. A pathogen according to the preceding claim, in the form of an insect virus.

25. A pathogen according to claim 23, in the form of a recombinant baculovirus such as HaSNPV, AcMNPV or AFMNPV, a fungus or a Bacillus bacterium.

26. An insect virus according to either of claims 24 or 25, in combination with a fluorescent brightener particularly those comprising a stilbene diphosphonic acid group, other uv-stabiliser and/or an anti-oxidant.

27. An insecticidal composition comprising the following combinations of proteins:

   (i) the proteins represented by amino acids 17 to 158 in SEQ ID No.2 and 22 to 182 in SEQ ID No. 4;
   (ii) the proteins represented by amino acids 17 to 158 in SEQ ID No.2 and 32 to 176 in SEQ ID No. 6;
   (iii) the proteins represented by amino acids 22 to 182 in SEQ ID No. 4 and 32 to 176 in SEQ ID No. 6;
   (iv) the proteins represented by amino acids 22 to 182 in SEQ ID No.4 and 23 to 275 in SEQ ID No. 8;
   (v) the proteins represented by amino acids 1 to 145 in SEQ ID No. 4 and 23 to 275 in SEQ ID No. 8;
   (vi) the proteins represented by amino acids 17 to 158 in SEQ ID No.2, amino acids 32 to 176 in SEQ ID No. 6, and amino acids 23 to 275 in SEQ ID No. 8;
(vii) the proteins represented by amino acids 17 to 158 in SEQ ID No.2, amino acids 22 to 182 in SEQ ID No.4, and amino acids 32 to 176 in SEQ ID No.6;
(viii) the proteins represented by amino acids 17 to 158 in SEQ ID No.2, amino acids 22 to 182 in SEQ ID No.4, and amino acids 23 to 275 in SEQ ID No.8;
(ix) the proteins represented by amino acids 22 to 182 in SEQ ID No.4, amino acids 32 to 176 in SEQ ID No.6, and amino acids 23 to 275 in SEQ ID No.8;
(x) the proteins represented by amino acids 17 to 158 in SEQ ID No.2; amino acids 22 to 182 in SEQ ID No.4, amino acids 32 to 176 in SEQ ID No.6, and amino acids 23 to 275 in SEQ ID No.8.

28. A composition according to the preceding claim, wherein the composition is augmented by - or at least one of the proteins in a composition is replaced by - the toxin subunit represented by amino acids 22 to 184 of SEQ ID No. 10 or that encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the said spliced RNA being capable of hybridising with the extension products of the primers depicted in SEQ ID Nos 20 and 21, or SEQ ID Nos 22 and 23, or SEQ ID Nos 24 and 25, using the sequence depicted in SEQ ID No. 1 as a template.

29. A method of controlling insects, comprising exposing them or their habitat to the cell of claim 21, organism of claim 22, pathogen of claims 23-26 or composition of claims 27 or 28.

30. Use of the polynucleotide of any one of claims 1-13 and 20 or the nucleotide sequence of any one of claims 14 - 19, in the preparation of a recombinant baculovirus for the biological control of insect pests.

31. Use of the cell of claim 21, organism of claim 22, pathogen of claims 23-26 or composition of claims 27 or 28 to control insect pests.

32. A polynucleotide comprising a region encoding the insecticidal toxin subunit encoded by the spliced RNA derived from the genomic clone depicted in SEQ ID No. 19, the
said spliced RNA being capable of hybridising with the extension products of the
primers depicted in SEQ ID Nos 20 and 21, or SEQ ID Nos 22 and 23, or SEQ ID
Nos 24 and 25 using the sequence depicted in SEQ ID No. 1 as a template, the
translation product of the polynucleotide and recombinant entomopathogenic viruses
comprising the said polynucleotide or a nucleotide comprising the spliced variant
thereof.
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FIGURE 3

Multiple cloning site

NdeI (183)
EcoO 109 I (2574)
Ast II (2517)
SaoI (2501)
Xmn I (2291)
Sea I (2177)

lac I

M13mp

Hae II (1050)
Hae II (650) *
All III (805)
pBR322 on

pMMS1
2686bp

pBR322

AMF

* site not unique

CtR101 (1779)

SUBSTITUTE SHEET (RULE 26)
**FIGURE 4**

**pAcUW21**

9267 bp
unique sites underlined

Amp<sup>R</sup>

F1 Ori

**p10 promoter**

**polyhedrin promoter**

**polyhedrin gene**

- **AlwN1 (8493)**
- **Gsu1 (7929)**
- **PvuII (9085)**
- **Sph1 (230)**
- **BstX1 (1248)**
- **PvuII (2542)**
- **EcoR1 (2548)**
- **BglII (2554)**
- **BamH1 (3079)**
- **Kpn1 (3539)**
- **SnaB1 (3721)**

**PvuII (6245)**

**Eag1 (5920)**

**PvuII (5667)**
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 C07K14/435 A01N63/02

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS/searchED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>A</td>
<td>WO 95 26410 A (ZENECA LTD) 5 October 1995 cited in the application see the whole document</td>
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**Further documents are listed in the continuation of box C.**

**Patent family members are listed in annex.**

* Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document but published on or after the international filing date
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  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed
  - "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  - "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  - "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  - "&" document member of the same patent family

**Date of the actual completion of the international search**

8 September 1997

**Date of mailing of the international search report**

16.09.97

Name and mailing address of the ISA

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NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo ml,
Fac. (+31-70) 340-3016

Authorized officer

De Kok, A
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