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(54) Title: INSECT DIAGNOSTIC AND CONTROL COMPOSITIONS

(57) Abstract

A diagnostic or control composition is useful to characterize or control insects and comprises a nucleotide sequence coding for juvenile hormone esterase (JHE). The coding sequence may be combined with a promoter sequence regulating the transcription thereof in a recombinant expression vector for use in controlling insects having a juvenile hormone esterase dependency. Preferred embodiments of the invention are recombinant baculoviruses in which a mutated JHE coding sequence provides relatively rapid speed of kill in insects.

## INSECT DIAGNOSTIC AND CONTROL COMPOSITIONS

Field of the Invention

The present invention relates to uses of nucleotide sequences coding for juvenile hormone esterase,
5    and more particularly to recombinant expression vectors including juvenile hormone esterase or mutant coding sequences having uses such as in controlling insects.

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Background of the Invention

The lepidopteran family noctuidae includes some of the most destructive agricultural pests, such as the genera Heliothis, Helicoverpa, Spodoptera and Trichoplusia.
15   For example, included in this family are the tobacco budworm (Heliothis virescens), the cotton leafworm (Alabama argillacea), the spotted cutworm (Amathes c-nigrum), the glassy cutworm (Crymodes devastator), the bronzed cutworm (Nephelodes emmedonia), the fall armyworm (Laphygma
20   frugiperda), the beet armyworm (Spodoptera exigua) and the variegated cutworm (Peridroma saucia). Juvenile hormone esterase is responsible for the stage-specific metabolism of juvenile hormone in such insects.

Juvenile hormone and juvenile hormone esterase
25   have been studied extensively in the Lepidoptera. In the final larval growing stage of these insects, there is a rapid decline in the juvenile hormone titer which initiates the physiological and behavioral events preceding pupation and adult development. This decline in the juvenile
30   hormone titer appears to be regulated by an increase in degradation by juvenile hormone esterase as well as a reduction of biosynthesis. Juvenile hormone esterase activity is very low in the early stadia of larval growth. Even at the peak activity levels in the blood of the final
35   stadium, the concentration of juvenile hormone esterase has

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been estimated at less than 0.1 percent of the total
protein. Yet the enzyme has a high affinity for juvenile
hormone.

5          The initial reduction in juvenile hormone titer
in the last larval stadium initiates a sequence of events
leading to pupation. Powerful and selective chemical
inhibitors of juvenile hormone esterase have been used *in
vivo* to demonstrate the developmental consequences of
blocking the activity of juvenile hormone esterase. For
10         example, a group of the chemical inhibitors of juvenile
hormone esterase are the trifluoromethylketone sulfides, as
described by U.S. Patent 4,562,292, issued December 31,
1985, inventors Hammock et al. Treatment in the final
larval stadium of the tomato hornworm (*Manduca sexta*) and
15         other moth larvae with potent inhibitors can block almost
all of the blood juvenile hormone esterase activity and
cause a delay in the time of metamorphosis, presumably by
allowing juvenile hormone to remain present.

           Classical methods of protein purification have
20         been inefficient for the large-scale purification of
juvenile hormone esterase because the esterase is in
picomole amounts even at its peak levels. More recently,
a purification method for juvenile hormone esterase from
larval blood has been developed. Abdel-Aal and Hammock,
25         *Science*, *233*, pp. 1073-1076 (1986).

           There is a need for genes which when produced in
expression systems will lead to rapid insect death,
disruption of development, and/or cessation of feeding.
Recent efforts have centered on the insect specific toxins
30         from *Bacillus thuringiensis* (Merryweather et al., 1990),
from the scorpions *Buthus eupeus* (Carbonell et al., 1988)
and *Androctonus australis* (Stewart et al., 1991; McCutchen
et al., 1991; Maeda et al., 1991) and from the mite
*Pyemotes tritici* (Tomalski and Miller, 1991).


35         Summary of the Invention
           It is one object of the present invention to
provide a recombinant expression vector that halts feeding

and disrupts the development of insect pests by artificial expression of juvenile hormone esterase at an early stage of development.

One aspect of the present invention comprises a vector with a coding sequence capable of expressing a protein in a host cell expression system. When expressed, the protein degrades, binds with or sequesters juvenile hormone or a juvenile hormone homolog within the host.

A preferred use of the inventive vectors is for expression in insects by infecting susceptible host insect cells with a recombinant baculovirus so that insect development is disrupted. Improved expression of JHE in a baculovirus system (with an improved lethal dose value) has been provided by mutagenesis. This improved expression is important because excessive insect feeding (before the virus acts on the insect pest) can result in unacceptable crop damage. Our improved vectors achieve earlier insect kill.

In another aspect of the present invention a transgenic, non-human organism has a nucleotide sequence introduced therein. The nucleotide sequence preferably is substantially similar to the cDNA illustrated by SEQ ID NOS:1 or 2 or to the gene illustrated by Figure 6. These sequences are effective to alter at least one determinable property of the organism when expressed. A preferred altered property is enhanced degradation of a methyl ester containing compound, such as disruption of juvenile hormone expression.

In another aspect of the present invention, the recombinant expression vectors capable of expression in a host insect or plant cell include coding sequences for a mutant or chimeric protein of a juvenile hormone esterase and a promoter that is heterologous with the coding sequence.

These and other aspects and advantages of the present invention will now be more fully described.

Brief Description of the Drawings

Figure 1 illustrates a restriction map for a cDNA coding for juvenile hormone esterase;

Figure 2 illustrates construction of a plasmid embodiment in accordance with the invention (designated pAcUW21.JHE);

Figure 3 graphically illustrates the expression of wild type and three modified JHEs, which are inventive embodiments, in baculovirus infected Sf cells *in vitro*;

Figure 4 graphically illustrates the expression of JHE in the system used for the Fig. 3 data (baculovirus infected Sf cells *in vitro*), but with three different promoters as additional inventive embodiments;

Figure 5 graphically illustrates the *in vivo* expression of wild type and several modified JHEs post-injection of recombinant baculoviruses into second instar *Manduca sexta*; and

Figure 6 summarizes the JHE gene itself.

Detailed Description of the Preferred Embodiments

The lepidopteran family Noctuidae includes a number of economically destructive agricultural pests. Briefly, two epithelial hormones control metamorphosis in such insects. A steroid hormone, 20-hydroxyecdysone, causes the molt, while a terpenoid hormone, juvenile hormone (JH), determines the nature of the molt. If juvenile hormone titers are high, then the molt is isometric to a larger larval stage. If juvenile hormone titers are low, then anisometric molt to a pupal stage will occur. Thus, the initial reduction in juvenile hormone titer in the last larval stadium is a key event in insect development in that it initiates a sequence of events leading to pupation and early in this sequence of events is the cessation of feeding. This reduction in juvenile hormone titer is accomplished by a reduction of biosynthesis and a tremendous increase in the highly aggressive enzyme known as juvenile hormone esterase (JHE).

Juvenile hormone esterase (JHE) is biochemically
interesting in that it is an extraordinarily efficient
enzyme. The very low $K_m$ 6 x $10^{-8}$ M of juvenile hormone
esterase, its relatively high $k_{CAT}$, and the low molarity of
5      the enzyme even in the last larval stadium indicate that
production of even small amounts of juvenile hormone
esterase will overpower the ability of the corpora allata
to make juvenile hormone (JH). The destruction of the
juvenile hormone will be accelerated by the juvenile
10     hormone hemolymph carrier protein. This carrier protects
juvenile hormone at earlier stages of development, but it
binds juvenile hormone less tightly than the esterase ($K_d$
= 6.1 x $10^{-7}$ M for *H. virescens*), and thus serves to
accelerate degradation of juvenile hormone by juvenile
15     hormone esterase and keeps juvenile hormone out of
lipophilic depots.

Under $V_{max}$ conditions, the juvenile hormone
esterase present in a single fifth stadium noctuid larva
could hydrolyze over 100,000 times as much juvenile hormone
20     each minute as is present in an entire larva at any time
during development. Under physiological conditions,
juvenile hormone esterase acts as an infinitely large sink
capable of extracting juvenile hormone and other
susceptible substrates from lipid depots and carriers by
25     mass action and instantly inactivating it. Thus,
precocious appearance of juvenile hormone esterase will
reduce juvenile hormone titers, typically resulting in
irreversible termination of the feeding stage, attempted
pupation and death of the pest insect.
30     Juvenile hormone esterase is an insect protein
which appears at critical times in the insect's life. It
appears to present no risk to other groups of organisms.
It is nonlethal to an individual cell which allows and
perhaps encourages viral replication; yet the enzyme will
35     fatally disrupt the normal development of the organism.
Because the substrate (juvenile hormone) readily penetrates
membranes, the juvenile hormone esterase need only be
expressed in a few cells to deplete juvenile hormone.

Differential tissue depletion of juvenile hormone is likely
to be even more rapidly fatal to an insect than uniform
depletion.

5          Numerous attempts have been made to purify
juvenile hormone esterases, but purification of the low
abundance enzyme from a small tissue source has proven very
tedious. U.S. Patent 5,098,706, issued March 24, 1992, and
filed concurrently with Serial No. 07/265,507 of which this
is a continuation-in-part, incorporated by reference,
10        exemplifies the administration of an affinity purified
enzyme to insects which results in anti-juvenile hormone
activity.    Such anti-juvenile hormone activity is
effectively lethal, for example in blocking damage by
herbivorous insects.

15          One aspect of the present invention concerns uses
of the coding sequence for juvenile hormone esterase. SEQ
ID NO:1 sets out the coding sequence for one cDNA of JHE,
and SEQ ID NO:2 sets out the coding sequence for another.
These coding sequences are for juvenile hormone esterase
20        from *Heliothis virescens*, although there is homology to
*Helicoverpa zea* (formerly *Heliothis zea*), to *Tricoplusia ni*
and (at lower stringency) hybridization to *Manduca sexta*.
Further, JHE isolated (or derived) from *Heliothis*
*(Helicoverpa) virescens* functions to hydrolyze every known
25        form of JH.    This means that a coding sequence for JHE
derived from *H. virescens* can be used to isolate the gene
or the message from a variety of species.    Figure 6
illustrates the JHE gene for *H. virescens* and gives, among
other things, restriction enzyme sites.

30          The pattern of JHE activity and low message
abundance suggests that expression of the enzyme is tightly
regulated, perhaps at the level of transcription.    In
*Lepidoptera*, the structure and transcriptional control of
developmentally regulated genes has been investigated, but
35        most of these genes are multiple copy and the mRNA is
abundant. By contrast, JHE from *H. virescens* apparently is
encoded by the single gene illustrated by Fig. 6, which
produces a low-abundance message.

JHE Coding Sequence Cloning

            As will also be described in Examples 1 and 2, the
clone containing the sequence of the mRNA transcript of
juvenile hormone esterase from *Heliothis virescens* was
5       isolated from a lambda gt-11 expression library.  To make
the expression library, total RNA was isolated by
homogenizing fat bodies in guanidinium thiocyanate and
centrifugation through cesium chloride.  The fat bodies
were from last instar larvae that had been treated with
10      epofenonane 24 hours previously.  Poly-adenylated RNA was
prepared by one cycle of oligo-dT chromatography from which
cDNA was synthesized and size selected for greater than
1350 base pairs.  The size selected cDNA was suitably
processed and ligated to arms of a lambda-gt11 phage
15      expression vector.  The ligated DNA was then packaged into
phage heads, infected into host cells and plated on a lawn
of host *E. coli.*  The cDNA library was not amplified prior
to screening.

            Screening    was    done    immunochemically    on
20      nitrocellulose filters to which proteins from plated phage
had been bound after induction of protein synthesis.
Clones reacting with antibodies specific for juvenile
hormone esterase were plaque-purified after detection with
immunohistochemical means.  A second round of screening was
25      then conducted upon the isolated clones with hybridization
to a mixture of synthetic nucleotides complementary to the
deduced mRNA sequence possibilities determined from the N-
terminal amino-acid sequence of juvenile hormone esterase.

            The    amino    acid    sequence    was    determined    by
30      automated Edman degradation of the purified protein.  From
this round of screening, three 3,000 basepair clones, 3hv1,
3hv16 and 3hv21 were isolated, subcloned into plasmids and
subjected to restriction analysis.  The later two clones
have their sequences given by SEQ ID NOS. 2 and 1.  Their
35      length matched the 3,000 basepair length of the juvenile
hormone esterase mRNA transcript as determined by Northern
blotting with radiolabeled DNA of clone 3hv21.  An 840
basepair fragment of the 5' coding region of 3hv21 was then

sequenced.  The sequence confirmed the clone to be that
coding for juvenile hormone esterase as the deduced amino-
acid sequence matched 33 of the 35 of the doubly confirmed
amino-acids sequenced at the N-terminus of the purified
5    protein.


Uses of JHE Coding Sequence

        The JHE coding sequence, various mutations and
analogs and restriction fragments thereof have a variety of
uses.  For example, the JHE coding sequence is useful in
10   characterizing insects related to or within the *Heliothis*
group.  Thus, Example 3 describes the utility of the cDNA
as  a  diagnostic  tool.    Once  the  insect  has  been
characterized, in laboratory or field, then appropriate
steps for control may be taken.
15           In addition, the reduction in JH titer in some
insects is accomplished by a combination of reduction in
biosynthesis and an increase in degradation by JHE.  Thus,
the JHE coding sequence can be used in combination with an
agent that reduces the JHE biosynthesis or influences its
20   distribution and kinetics.
             Since  the  juvenile  hormone  esterase  coding
sequence (illustrated by SEQ ID NO:1 and SEQ ID NO:2) has
been  determined,  methods  to  elicit  the  precocious
expression of juvenile hormone esterase for insect control
25   are now possible.
             The  recent  development  of  a  viral  expression
vector from a virus endemic in and selective for noctuid
species permits practical field control systems for the
Boll Worm and other insect pests.  U.S. Patent 4,745,051,
30   issued May 17, 1988, inventors, Smith, et al., incorporated
herein  by  reference  describes  a  method  for  producing  a
recombinant  baculovirus  expression  vector  capable  of
expression of a selected gene in a host insect cell.  The
method exemplified by U.S. Patent 4,745,051 was used to
35   express β-Interferon by infecting susceptible host insect
cells  with  a  recombinant  baculovirus  expression  vector.
Briefly,  baculovirus  DNA  is  cleaved  to  obtain  a  DNA

fragment containing at least a promoter of the baculovirus
gene.  One baculovirus gene is that coding for polyhedrin,
since the polyhedrin protein is one of the most highly
expressed eucaryotid genes known, although other promoter
5      and hybrid promoter sequences may be used.

Although the polyhedrin promoter can be used in
preparing inventive expression vectors, we have found JHE
can be produced even earlier and at still greater levels
under the p10 and basic protein promoters, as illustrated
10     by the data of Fig. 4.  The preferred baculovirus utilized
is *Autographa californica*, although other baculovirus
strains may be suitably utilized.  *Autographa californica*
(AcNPV) is of particular interest as various major pest
species within the genera *Spodoptera*, *Trichoplusia*, and
15     *Heliothis* are susceptible to this virus.

In the present invention, a baculovirus may be
modified by the method described by U.S. Patent 4,745,051,
but by utilizing the juvenile hormone esterase coding
sequence (or a mutant of JHE coding sequence) rather than
20     the $\beta$-Interferon gene.  A recombinant expression vector
thus comprises a JHE coding sequence or a JHE mutant under
the control of a promoter sequence (such as the polyhedrin,
p10 or basic protein promoters), which is heterologous with
the  JHE  coding  sequence  and  which  regulates  the
25     transcription thereof.  Expression of the JHE coding
sequence gene is accomplished by infecting susceptible host
insect  or  plant  cells  so  as  to  produce  precociously
appearing juvenile hormone esterase.  In insect cells the
JHE  appearance,  even  at  low  levels,  disrupts  insect
30     development.  In plant cells the JHE appearance can provide
protection against insect predators.

Aspects  of  the  present  invention  will  be
illustrated  by  a  number  of  examples.   Examples  1-3
primarily describe work obtaining cDNA for JHE and some
35     characterizations for the JHE gene.  Example 4 describes
preparation of improved baculovirus (particularly useful as
insecticidal agents) by forming mutants of the original JHE
nucleotide  sequences,  and  also  discusses  various  other

mutations that can be made. Example 5 describes preparation of inventive vector embodiments with several different promoters. Example 6 describes further characterizations of the JHE gene itself.

5    Preparation of mutants

The degradation of proteins within cells occurs by many pathways, but the best known are the ubiquitin and lysosomal pathways. For degradation by ubiquitin and recognition by many lysosomal enzymes, a protein must
10   either have free lysine (K) residues available on the surface of the protein or appropriate lysine containing sequences respectively. For ubiquitin degradation, appropriate lysines are often found near the N-terminus of the protein. JHE contains a lysine at position 29 and has
15   tryptophan as the N-terminal amino acid, which is a possible susceptible residue for targeting a protein to ubiquitin degradation. In addition, lysine residues occurring in a region rich in proline, glutamate, serine, and threonine are important for both ubiquitin and
20   lysosomal protease recognition. JHE has one such lysine at position 522. These lysines were changed to arginine (R) (K29R and K522R) by site-directed mutagenesis, and then the individual mutants and the combined mutant (K29R + K522R) were tested in the baculovirus system for insecticidal
25   activity.

In addition, an inactive JHE may bind and sequester juvenile hormone rather than degrade it and thus be more effective than active JHE. Thus, natural insect proteins when modified by these methods can have
30   deleterious effects on the insect. A catalytic site mutation of serine 201 to glycine was made (S201G) and tested for insecticidal activity.

The double lysine mutant (sometimes referred to as "KK" and sometimes "29,522" and sometimes as "K29R,
35   K522R") and the catalytic serine mutant (S201G) of recombinant JHE enhance the ability of the AcNPV to kill in both *Heliothis virescens* and *Trichoplusia ni*. The

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insecticidal activity of the catalytically deficient S201G
mutant of JHE is unusual and possibly contradictory to the
current theories of JHE action. The times required for 50%
death of test insects are similar to those required for

5    scorpion toxins that have recently been reported in the
literature, engineered in A.cal NPV, and are a considerably
safer alternative. These viruses can be produced easily in
both tissue culture and in laboratory or industrial insect
colonies, unlike their toxin containing competitors, and

10   represent a minor variation of a protein that occurs
naturally in the insects. As this enzyme occurs in all
insects, the engineering of this JHE into NPVs with
differing host specificity allows a wide spectrum of pests
to be controlled in a rapid and effective manner.

15           In addition to the particular *in vitro* site-
directed mutagenesis technique described by Example 4 to
exemplify the mutation aspect of the invention, there are
numerous techniques for mutagenesis which may be employed
to improve utility. For instance, it is possible to employ

20   other nucleotide-mediated mutagenesis protocols and similar
techniques can be used to create deletions or insertions.
Deletions or insertions may be systematically incorporated,
either by linker-insertion, linker-scan, or nested
deletion. For example, it is possible to randomly insert

25   small linkers into the JHE sequence. Other techniques for
less structured mutagenesis include the use of degenerate
nucleotide pools, misincorporation by DNA polymerases and
chemical mutagenesis. Standard restriction and ligation
techniques can be used to create large deletions or

30   chimeric enzymes. New PCR methods for mutagenesis could
also be used to generate classes of mutations.

             Whether by site-directed mutagenesis techniques
or other mutagenesis protocols, we consider sequences with
significant homology to SEQ ID NO:1 or SEQ ID NO:2 or to

35   the JHE gene itself (illustrated by Fig. 6) to be within
the scope of this invention. By "significant similarity or
homology" is meant that the nucleotides have greater
sequence similarity than by chance alone and that the

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protein coded by the sequence, when expressed in an appropriate host cell expression system, degrades, binds with, or sequesters juvenile hormone or those endogenous proteins commonly recognized as juvenile hormone homologs

5      due to their structural similarity to JH.

Another way of looking at coding sequences that are substantially the same as a juvenile hormone esterase coding sequence are those where the physiological or biological action is as has just been described and the

10     protein expressed by the coding sequence reacts with a methyl ester containing compound. For example, the JHE of *H. virescens* and that of several other species all have high specificity for JH with a high $k_{CAT}/K_M$ ratio ($>10^8$). Juvenile hormone itself is, of course, a methyl ester

15     containing compound (and includes an epoxide functionality), and there are other enzymes of interest where expression of the coding sequence leads to enhanced degradation of JH. For example, epoxide hydrolases are known to act similarly to JHE by hydrolysis of JH to yield

20     biologically inactive diols.

Example 4 illustrates use of mutagenesis in baculovirus expressing JHE as an insecticidal agent, which are improved over recombinant baculoviruses expressing JHE, since several mutants have resulted in improved properties

25     (e.g. reducing the lethal dose significantly in *Heliothis virescens*). For example, a double mutant has resulted in lethal dose improvements so that insects are killed about one day or one and one half days faster than kill rates with the wild type virus. This is significant for crop

30     protection because the plant damage done to crops is a function of time that the crops are vulnerable to the insects.

Example 5 illustrates JHE activity, *in vitro* under the control of several different viral promoters. The

35     expression level of JHE with a basic protein promoter is particularly exciting because greatly increased JHE expression (at levels up to about 5 times higher) have been demonstrated. This is particularly surprising because use

of the basic protein promoter with other coding sequences has indicated lowered expression rather than enhanced expression of the protein coded by the coding sequence. We believe the striking difference in expression levels lies in the fact that JHE is a packaged, or secreted, protein. As a consequence, increased expression levels of secreted or exported proteins should be achievable by combining the basic protein promoter with a nucleotide coding sequence coding for the secreted or exported protein. The sequence and composition of the 19 residues prior to the $NH_2$ terminal Trp of the secreted major form of JHE match well with the consensus for other signal peptides for secretion.

Modified, or mutated, JHEs in accordance with the invention are not limited to just the *H. virescens* insect because the JHE enzyme is capable of hydrolyzing all known JH homologs with similar $k_{CAT}/K_M$ ratios, as well as related methyl esters. This means that JHE and JHE mutants are applicable in many expression systems, and it is clear that JHE can convert a wide variety of methyl esters to the corresponding free acids.

The juvenile hormone esterase shows high selectivity for rapid hydrolysis of methyl and thiomethyl esters. This rapid catalytic activity can be used to yield colorimetric assays using substrates such as octanoic acid thiomethyl ester or methyl $\beta$-(1-pentylthio)propiothiate, methyl $\beta$-(1-pentoxy)propiothioate, and related $\beta$ and $\gamma$ homologs resulting in a catalytic marker. The high stability of juvenile hormone esterase and its rapid turnover of thiomethyl esters also makes it an attractive reporter enzyme. The juvenile hormone esterase expressed in a system such as the baculovirus system can lead to the rapid hydrolysis of a variety of methyl and thiomethyl esters. Methyl esters, thiomethylesters, or methylcarbonates can be removed to activate drugs or pesticides such as clofibric acid methyl ester, bifenox, butoxone methyl ester, dacthal, diclofop-methyl, chlorfenprop-methyl, or esters or carbonates of substituted 2,4-dinitrophenyl uncouplers. The catalytic activity also

can be used to degrade toxic materials containing a methyl ester such as pesticides in environmental samples or drugs or poisons such as warfarin, heroin, or bisacodyl following overdose.

We believe it likely that the biological activity comes from disrupting a fundamental process in insects that is related to turnover of native proteins. Thus, our mutagenesis technique is applicable to the genes of a variety of species and to proteins extending beyond JHE.

Other Uses and Particular Uses of the JHE gene

There are advantages to using the JHE gene itself (as opposed to using a cDNA for JHE) in some systems. For example, use of the gene is believed preferable in transgenic monocots and transgenic (non-human) mammals, and the expression obtained from use of the gene itself can be higher than with cDNA. Although insect control is the utility we presently contemplate as particularly preferred, the JHE gene should also be expressed in plants, mammalian systems, bacteria, fungi, and algae, in addition to insects and viruses.

For example, when the JHE gene is expressed in plants, then the enzyme should act on ester-containing compounds other than insect juvenile hormone to convey herbicide resistance to the plants or may be used in biosynthetic processes to hydrolyze methyl esters selectively. The catalytic activity can be used to remove methyl ester protecting groups in specialty compound synthesis such as methyl esters of retenoic acid or chrysanthemic acid. The catalytic activity on methyl esters can be used to activate or deactivate biologically active materials with a carboxylic acid blocked by a methyl ester.

Examples of deactivation include compounds such as the sulfonyl urea herbicides metsulfuron-methyl, tribenuron-methyl, sulfometuron-methyl, prisisulfuron, bensulfuron-methyl, or the herbicide DME [2-(diphenylmethoxy)acetic acid methyl ester]. The catalytic

activity could be used to hydrolyze methyl esters critical
in the biosynthesis of natural products.  For instance, a
methyl esterase will convert Protoporphyrin IX monomethyl
ester to Protoporphryn IX thus reversing the biosynthesis
5    of the key plant pigment chlorophyll.

Expression of the JHE gene may also protect plants
from invertebrates other than insects.  Expression of the
JHE gene by algae may be useful for control of mosquitos or
other disease vectors.

10    Nucleotide sequences of this invention can be
introduced into non-human organisms to form transgenic
organisms.  Introduction of the sequences may be double or
single stranded form, and can be "sense" or "anti-sense"
(that is, with the sequence reversed with respect to the
15    sequences illustrated such as by SEQ ID NOS:1 or 2 or to
the gene illustrated by Fig. 6).  Shorter fragments, as
small as about 16 sequential bases in length, have various
uses, such as in diagnostic applications, with 16
sequential bases believed to be about the minimum length
20    for hybridization.  The ready availability of JHE from
expression systems of the invention, particularly from
bacteria or viruses, should permit other diagnostic
applications, such as the use of JHE as a reporter enzyme
that can be coupled to antibody.

25                             EXAMPLE 1


Animals

Larvae of *Heliothis virescens* were obtained from
a research facility of Dow Chemical Company located at
Walnut Creek, California.


30    Materials

Radioactively labeled reagents obtained from
Amersham (Arlington Heights, Illinois).  Other chemicals
were of the highest quality available.  Enzymes were
obtained from Promega Biotec (Madison, Wisconsin),
35    Boehringer Mannheim Biochemicals, (Indianapolis, Indiana),

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United States Biochemical Corporation (Cleveland, Ohio) and Sigma (St. Louis, Missouri).

A Bluescript plasmid with a cDNA insert from *H. virescens*, 3hv1 (sequence not shown), 3hv21 (SEQ ID NO:1),

5    and 3hv16 (SEQ ID NO:2), was isolated from the *E. coli* host cells and host chromosomal DNA using the alkaline lysis miniprep procedure described in Maniatis et al., *Molecular Cloning*, Cold Spring Harbor Laboratory (1982). The N-terminal Bam HI restriction fragment from the above

10   preparation was ligated into phage M13 (mp19). Thus, in a 25 μl volume 0.2 μg of Bam HI cut mp19 was added to 1.0 μg of Bluescript plasmid cut with Bam HI. After the addition of ligase the reaction was allowed to proceed 60 minutes at room temperature and overnight at 4°C.

15   Ligated DNA was used to transform competent *E. coli* host cells are described in Rodriquez, *Recombinant DNA Techniques: An Introduction*, Addison-Wesley (1983). Either 0.1 μl, 1 μl or 10 μl of the ligation mixture was added to 200 μl of competent JM101. After incubation on

20   ice the mixture was added to soft agarose at 43-45°C for 2 minutes. The agarose and cells were plated with X-gal and IPTG, and incubated at 37°C. Recombinant transformants were present as white plaques.

Isolated white plaques were picked and grown for

25   6 hours at 37°C in 1xYT medium. Double-stranded DNA was prepared by the method described in Rodriquez, *supra*. Briefly, the host cells were pelleted and resuspended in a sucrose EDTA buffer. RNAse was added and the cells lysed in 1% SDS with 0.2 N NaOH. The host chromosome DNA was

30   pelleted by centrifugation and the supernatant with double-stranded RF M13 DNA removed. From the supernatant double-stranded M13 was precipitated in isopropanol and washed with ethanol. The double-stranded M13 was cut with Bam HI and run on a 0.8% agarose gel with Bam HI cut Bluescript

35   plasmid to ensure the subcloned M13 fragment was the same size as the fragment from the original Bluescript plasmid.

The next step was to conduct a C-test for complementary single-stranded DNA from M13 plaques.

Identification of both orientations of a cloned insert is useful for single-strand sequencing because it is possible to sequence from both ends toward the middle. Essentially, the transformed host cells (JM101) from independent plaques were grown for 4-6 hours in 1xYT medium. Approximately 8 $\mu$l of supernatant from each plaque was removed and supernatant from various combinations of different plaques was mixed with glycerol, salt, and SDS. The mixtures were incubated at 60-70°C for 15 minutes and allowed to cool. A sample of the mixture was placed on a 0.8% agarose gel to detect hybridization by retarded migration in the gel.

Single-stranded DNA from M13 with inserts in opposite orientation was then prepared. To do so, transformed cells were grown for 4-6 hours at 37°C. Single-stranded DNA was extruded into the medium and precipitated with PEG and NaCl. After resuspension protein was removed with phenol chloroform. Several volumes of ethanol was added to the aqueous for precipitation. The pellet was washed in 70% ethanol, dried and resuspended in autoclaved water.

Sequencing of the above single-stranded DNA was based on the chain termination method of Sanger et. al, *PNAS*, 74, 5463 (1977). Bam HI fragments in both orientations in M13 were sequenced. For sequencing reactions, $^{32}$P ATP was used with the reagents and instructions supplied in the sequence kit (United States Biochemical). The sequencing reactions were run on 4% and 6% acrylamide gels. Audioradiographs of the gels were read after overnight exposure of the film to dried gel. The complete Bam HI insert of approximately 840 base pairs was read. This included the putative sequence for the secretion signal peptide and the N-terminal coding sequence which corresponded to the N-terminal amino acid sequence for JHE. The identity of the JHE cDNA insert was initially established in this manner.

EXAMPLE 2

Protein Sequencing

        Juvenile hormone esterase was purified (as described by Abdel-Aal and Hammock, *Science*, *233*, pp. 1073-

5    1076 (1986)) from the hemolymph of last instar larvae of *Heliothis virescens*. The purified preparation was seen to be a single band when analyzed by electrophoresis in the presence of SDS (Laemmli, *Nature*, *227*, pp. 680-685 (1970)) and isoelectric focusing on a polyacrylamide gel having a

10   ph 4.0 to pH 6.5 gradient (Pharmacia, Piscataway, New Jersey). However, when subjected to Edman degradation, two proteins were indicated to be in the preparation. The presence of isoforms of JH esterase in *H. virescens* is consistent with observations of the enzyme in other

15   insects. From the major form was obtained a readable sequence of 35 residues. The signal from the minor form indicated a protein having a two residue extension of Ser-Ala followed by a sequence of five residues identical to the ultimate five residues at the N-terminus of the major

20   form. Amino acid sequencing at the N-terminal of juvenile hormone esterase was done with a Beckman 890M liquid phase sequencer.

Probe Preparation

        Both antibodies against juvenile hormone esterase

25   and nucleotides complementary to the juvenile hormone esterase message were used as probes to detect recombinant clones coding for juvenile hormone esterase. Antisera to juvenile hormone esterase was prepared with New Zealand White female rabbits (Vaitukaitis, *Methods in Enzymology*

30   (Langone and Von Vunakis, eds.) *73*, pp. 46-52, Academic Press, New York (1981)). To reduce background, the antisera was incubated overnight at 4°C with diluted *Heliothis virescens* hemolymph devoid of juvenile hormone esterase activity (the antisera was diluted 1:10 in a

35   solution of 10 mg/ml hemolymph protein in pH 7.4, I=0.2 phosphate buffer containing 0.01% phenyl thiourea). A

final dilution of 1:750 was used for screening. A mixture
of 32 14-mer nucleotides were synthesized using a Syntec
model 1450 synthesizer. Their sequences were complementary
to all the possibilities of the mRNA structure deduced from
the amino acid sequence of the N-terminal. The nucleotides
were purified on a Nensorb 20 nucleic acid purification
cartridge (Dupont Co., Wilmington, Delaware) and end-
labeled with $^{32}$P with T4 polynucleotide kinase and [$^{32}$P]ATP
(>6000 Ci/mmol) by a standard technique (Zeff and
Geliebter, *BRL Focus 9-2*, pp. 1-2 (1987)).

## cDNA Synthesis and Cloning

Total RNA was isolated by homogenizing fat bodies
in guanidinium thiocyanate and centrifugation through
cesium chloride (Turpen and Griffith, *BioTechniques* 4(1),
pp. 11-15 (1986)). The fat bodies were dissected from last
instar larvae that had been treated with epofenonane 24
hours previously to increase the level of juvenile hormone
esterase activity (Hanzlik and Hammock, *J. Biol. Chem.*,
*262*, pp. 13584-13591 (1987)). At the time of treatment,
the larvae weighed 200-300 mg. Polyadenylated RNA was
prepared by one cycle of oligo-dT chromatography (Aviv and
Leder, *Proc. Nat. Acad. Sci. USA*, *69*, pp. 1408-1412 (1972))
using oligo-dT cellulose (Collaborative Research,
Cambridge, Massachusetts). Synthesis of cDNA from 2 $\mu$g of
poly-A RNA was done by the method of Gubler and Hoffman
(Gubler and Hoffman, *Gene*, *25*, pp. 263-269 (1983)) with a
cDNA kit (Amersham) and a variation of its protocol. The
protocol was varied in the priming of the first strand
synthesis wherein 100 ng of random hexamer primers
(Pharmacia) were added 30 minutes after initiation of first
strand synthesis by oligo-dT primers done according to the
protocol. Size selection of the cDNA for >1350 basepairs
was done with gel permeation using a spin column (5 Prime-3
Prime, Paoli, Pennsylvania). The size selected cDNA was
treated with Eco R1 methylase, ligated to Eco R1 linkers
and treated with Eco R1 restriction endonuclease according
to the protocols presented by Huynh et al. (Huynh, Young

and Davis, *DNA Cloning: A Practical Approach* (Glover, D.M.,
ed.), pp. 49-78, IRL Press, Oxford (1984)). The free
linkers were removed by three cycles of ultrafiltration
using Centricon 30 microfiltrators (Amicon, Danvers,
5  Massachusetts). The cDNA was then ligated to arms of a
lambda gt11 expression vector derivative (lambda-ZAP,
Stratagene Cloning Systems, La Jolla, California) according
to the manufacturer's recommendations. The ligated DNA was
then packaged into phage heads using a two extract system
10 (Gigapack Gold, Stratagene Cloning Systems), and plated on
to rec *E. coli* host cells (XL1-Blue, Stratagene Cloning
Systems). Greater than 99% of the clones were recombinant.
The cDNA library was not amplified prior to screening.

Screening
15         Initial screening was done immunochemically on
nitrocellulose filters to which proteins from plated phage
had been bound after induction of protein synthesis.
Clones reacting with antibodies specific for juvenile
hormone esterase were plaque-purified after detection with
20 immunohistochemical means (9) using a kit (Protoblot,
Promega). A second round of screening was then conducted
upon plaque lifts of the isolated clones by hybridization
to the mixture of 14-mer nucleotides. This was done by
incubating the filters with the labeled oligomers at 35°C
25 in 5X SSPE, 3X Denhardt's solution, 100 μg/ml low molecular
weight DNA after prehybridizing in the same solution sans
the oligomers. Washing was done three times at the
hybridizing temperature in a solution consisting of 2X SSC
and 0.1% SDS. Positively reacting clones were then
30 subcloned into the Bluescript SK M13- plasmid (Stratagene
Cloning Systems) by the automatic excision process allowed
by the lambda-ZAP vector.

Sequencing
           Sequencing of the remaining cDNA insert, and to
35 confirm the 840 base pair sequence, was done with the
   dideoxy chain termination method (Sanger, Nicklen and

Coulson, *Proc. Natl. Acad. Sci. USA*, 74, pp. 5463-5467 (1977)) using modified T7 DNA polymerase (Sequenase, United States Biochemical Corp.) and 35S labeled dATP. Templates were generated by using both denature plasmids (Toneguzzo,

5      Glynn, Levi, Mjolsness, and Hayday, *BioTechniques*, 6, pp. 460-469 (1988)) and single stranded DNA from M13 phage (Yanisch-Perron, Vieira, and Messing, *Gene*, 33, pp. 103-119). A rec A- *E. coli* strain (JM109) was used for the amplification of the subclones that were constructed with

10     both restriction fragments and nested deletions (Henikoff, *Gene*, 28, pp. 351-359 (1984)) (kit from Stratagene Cloning Systems). Computer assisted sequence analysis was done with programs written by Pustell (Pustell and Kafatos, *Nucleic Acids Res.*, 12, (1984)).


15     Additional Library and Sequence Analysis

       We decided to construct another cDNA expression library using both random and oligo-dT priming of the first strand. In addition we used cDNA selected for a size greater than 1350 bp for ligation to the vector. Screening

20     40,000 clones of this library produced 25 immunoreactive clones, five of which hybridized to the nucleotide probes. Three of these clones, designated 3hv1, 3hv16 and 3hv21, were subcloned into plasmids and characterized. All three clones contained 3000 bp inserts that had identical

25     restriction patterns when incubated with Eco RI, Xho I and Bam HI. When the clone, 3hv21, was used as a probe on a Northern blot, it hybridized at low stringency to a single band with a 3.0 kb length.

       The amount of screening required to isolate

30     positive clones indicates that the frequency of the JH esterase message during the period of its secretion into the hemolymph during the last instar is relatively low.

       We considered the three clones (3hv1, 3hv21, and 3hv16) to be identical due to their identical length and

35     restriction patterns, although slight differences exist as discussed hereinafter. The sequence of clone 3hv21 is shown by SEQ ID NO:1 (was set out by Fig. 2 of Serial No.

07/725,226 and 07/265,507 of which this is a continuation-
in-part and published in the *Journal of Biological
Chemistry*, *264*:21, pp. 12419-12425 (1989). Figure 1 shows
a restriction map for this clone. The clone was sequenced
100% in both directions. The other clone 3hv16 (SEQ ID
NO:2) was used for baculovirus expression, mutagenesis, and
bioassays.

SEQ ID NO:1 shows the cDNA sequence of JH
esterase, which is a 2989 bp cDNA clone and is nearly a
full length copy of the mRNA transcript. There is a short
19 base sequence prior (5') to the first ATG. The position
and composition of the bases immediately prior to the first
ATG matches the consensus for an insect ribosome binding
site except at position -3 where a G (the second most
frequent base at this site) replaces an A. After the first
ATG, there is a 1714 bp open reading frame followed by an
untranslated 1256 bp region including a 12 base poly(A)
tail. Translation of the open reading frame predicts a 563
residue protein. The sequence and composition of the 19
residues prior to the N-terminal Trp of the secreted major
form of JH esterase match well with the consensus for
signal peptides for secretion. The molecular weight of the
mature protein (*sans* signal peptide) is predicted to be
61,012 Da, which is in agreement with the $M_r$ of 62,000
derived from electrophoresis. The sequences of the
ultimate 35 amino acids derived from Edman degradation of
the major form of JH esterase and that predicted by the
cDNA sequence match except at two sites. The residues Val
10 and Phe 33 predicted by the sequence of clone 3hv21 are
indicted to be Leu and Pro, respectively, on the sequenced
protein (Table 1).

TABLE 1

Amino acid sequence analysis of the N-terminus
of JH esterase from *H. virescens*[1]

| Cycle | Residue(s)[2] | Yield[3] |
|---|---|---|
| 1 | Trp (Ser) | 1120 (230) |
| 2 | Gln (Ala) | 780 (360) |
| 3 | Glu (Trp) | 870 (340) |
| 4 | Thr (Gln) | 100 (310) |
| 5 | Asn (Glu) | 800 (350) |
| 6 | Ser (Thr) | 560 (10) |
| 7 | Arg (Asn) | 400 (390) |
| 8 | Ser | 610 |
| 9 | Val | 740 |
| 10 | Leu | 550 |
| 11 | Ala | 340 |
| 12 | His | 380 |
| 13 | Leu | 580 |
| 14 | Asp | 590 |
| 15 | Ser | 460 |
| 16 | Gly | 360 |
| 17 | Ile | 290 |
| 18 | Ile | 390 |
| 19 | Arg | 290 |
| 20 | Gly | 360 |
| 21 | Val | 300 |
| 22 | Pro | 110 |
| 23 | arg | --- |
| 24 | Ser | 130 |
| 25 | Ala | 250 |
| 26 | Asp | 190 |
| 27 | arg | --- |
| 28 | ile | --- |
| 29 | Lys | 170 |
| 30 | phe | --- |
| 31 | Ala | 130 |
| 32 | ser | --- |
| 33 | Pro | 140 |
| 34 | --- | --- |
| 35 | gly | --- |

[1] Affinity purified JH esterase was subjected to automated Edman degradation on a Beckman model 890M liquid phase sequencer. Derivatized residues were confirmed with two HPLC systems employing reverse phase- and cyano-columns. The amount of protein analyzed was 3 nmol as calculated by Coomasie Blue dye binding.

[2] The initial cycles had strong secondary signals, the identity of which are shown in parenthesis. Residues are capitalized where the identity of the PTH-amino acids were confirmed twice by elution from the two different HPLC

systems and are lower case where the identify was assigned on the basis of elution from only one HPLC system.

³ Yield of secondary PTH-amino acids are shown in parenthesis. Hyphens denote where the yield was not calculated for residues identified on the basis of elution from only one HPLC system.

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In addition, the serine present at the N-terminal of the minor form of JH esterase protein that was sequenced is indicated to be Leu -2 on the cDNA. To answer the question of whether the differences were due to the cloning process or were genuine, we sequenced the 5' region of the two other clones, 3hv1 and 3hv16, which were isolated from the same unamplified library as 3hv21.

We found slight differences among all three clones. The sequence of clone 3hv1 translates identically in the N-terminal region as clone 3hv21, but differs at base 94, which is the last position of a code for serine 6. Clone 3hv16 differs at two bases (50 and 104) from clone 3hv21 in the area coding for the N-terminal, one of which causes a substitution of a phenylalanine for leucine 9 and a leucine for valine 10. The substitution of isoleucine for valine at residue position 10 makes the translation of clone 3hv16 match in 34 of 35 amino acid residues determined from the purified protein.

The previous data indicated at least five slightly different translations of genes for JH esterase, which suggests multiple genes or alleles for JH esterase exist in populations of *H. virescens*. Perhaps contributing to the heterogeneity between the cDNA's and protein sequences is the fact that the protein and RNA were extracted from two different colonies of *H. virescens* and represents natural variation.

There are three consensus poly(A) signal sequences (AATAAA) that start at bases 2299, 2315 and 2951. The presence of three signals for polyadenylation may signify alternative processing of the transcript in the 3' region. Strong evidence from studies of another noctuid moth shows

that there is a constitutively expressed intracellular form of JH esterase throughout its larval stage and thus the larval stage of *H. virescens*. Thus a means of producing an intracellular as well as a secreted form of JH esterase is indicated to exist.

The protein translated from the cDNA clone contains four asparagine residues, Asn 62, Asn 161, Asn 383 and Asn 496, which are candidates for glycosylation. However, preliminary evidence indicates that should this modification be present on the secreted JH esterase from *H. virescens*, mannose and derivatives of glucose are not present. This information indicates that portions of the JHE sequence can be used to make chimeric esterases of altered properties.

Computer analysis of JH esterase sequence

Comparison of the translation of clone 3hv21 to protein sequences in the protein data bank of the National Biomedical Research Foundation and to translations of proteins characterized as esterases, lipases and serine hydrolases in GenBank revealed homologies to five proteins. Identical residue matches (gaps were counted as one substitution regardless of length) of 24.2%, 23.8%, 23.2% and 23.2%, respectively, were noted to human pseudocholine esterase. *Drosophila melanogaster* acetylcholine esterase, electric ray acetylcholine esterase, and *Drosophila melanogaster* esterase -6. In addition, homology to a region situated toward the carboxyl terminal of the large thyroid hormone precursor, bovine thyroglobulin was noted.

EXAMPLE 3

*Heliothis virescens* and *Heliothis zea* are major pest species which are difficult to identify by visual inspection at the larval stage, yet each species is most readily controlled by different insecticides. A molecular probe for *H. virescens* juvenile hormone esterase was used to identify the larval form of one species from the other.

The N-terminal 800 base pair Bam HI fragment from cloned *H. virescens* JHE was labelled with $^{32}$P and hybridized to genomic DNA from *H. virescens* and *H. zea*. The DNA had been cut with the restriction enzyme Xba I, the fragments separated on a 0.8% agarose gel and transferred to a nylon membrane for hybridization with the probe at 65°C. Autoradiographs of the hybridized and washed filter showed that *H. virescens* has at least 6 bands which are approximately 2.9, 3.4, 4.0, 4.3 6.1, 7.6 and 50 kilobases. By contrast, *H. zea* showed none of these bands, but has two diagnostic fragments at 2.0 and 1.5 kilobases. Thus, the JHE probe was useful as a diagnostic tool.

## EXAMPLE 4

Plasmids described below are named with a "p" followed by a code indicating their origin, and after a period the gene that they code for. Viruses are named in a similar fashion with Ac indicating that they were derived from the mNPV (multiple nuclear polyhedrosis virus) of the moth *Autographa californica*.

### Site-directed mutagenesis

The cDNA coding sequence for JHE from clone 3hv16B (SEQ ID NO:2) was selected for mutagenesis. This JHE clone is in the plasmid vector pBluescriptSK+ (Stratagene) and prior to commencement of the mutagenesis reactions, the JHE coding region was removed by digestion with the restriction endonuclease BglII and recloned into the same vector to obtain the reverse orientation of the insert in the vector. This allowed rescue of the appropriate sense strand for site-directed mutagenesis and also enabled the removal of the JHE coding region by both EcoRI and BglII restriction endonucleases.

Rescue of the single strand DNA for mutagenesis was carried out by standard techniques using the mutant M13 bacteriophage M13K07. Briefly, the JHE clone was grown in E.coli strain XL1-blue in 1 ml of LB medium containing

50µg/ml ampicillin. At early log phase, 50µl of culture
was added to 100µl of M13K07 (3x108 pfu/ml) and incubated
at 37°C for 1 hour then 40µl was transferred to 20 ml of
LBpmedia containing 50µg/ml ampicillin and 50µg/ml
5    kanamycin. The culture was incubated overnight at 37°C and
ssDNA purified from the culture supernatant as follows.
The culture was centrifuged at 2,000 g for 10 minutes then
the supernatant was harvested in 16 800µl aliquots in 1.5
ml Eppendorf tubes and 200 µl of 2.5 M NaCl/20% PEG added
10   to each aliquot. After 15 minutes at room temperature, the
samples were centrifuged at 16,000 g for 5 minutes and the
supernatant removed. The samples were the recentrifuged
for 30 seconds and residual supernatant removed. The
pellets were resuspended in 100µl TE (10 mM Tris HCl pH
15   8/0.1 mM EDTA) per 4 tubes and each of the 4 aliquots was
extracted once with buffer saturated phenol and twice with
chloroform then precipitated with ethanol. The purified
ssDNA was resuspended in a final volume of 50µl of TE.

The site-directed mutagenesis reactions were
20   carried out by the method of Kunkel et al. (1985) using
mutant nucleotide sequences complementary to the "rescued"
coding strand obtained from the pBluescript Phagemid. We
selected the sites for mutagenesis as follows. One desired
mutation was lysine (29) to arginine because this is a
25   lysine located near the N-terminus of JHE. Another desired
mutation was lysine (522) to arginine because this is a
lysine located within a potential "PEST" sequence and local
enrichment of Pro, Ser, Glu, and Thr. A third desired
mutation was serine (201) to glycine because conserved
30   catalytic serine motif of Gly X $Ser_{201}$ X Gly.

However, numerous options exist for modification
of the JHE cDNA, or gene, to increase insecticidal activity
or modify the catalytic functions of the enzyme. For
example, it is possible to add or to remove glycosylation
35   sites or to modify the pattern of glycosylation. For many
of the site-directed changes it is possible to add, as well
as remove, regions of the enzyme that confer defined
functions. Other site-directed changes can include

alteration of sites resulting in lability to proteases
(intra- or extracellular), lysosome recognition sites,
tissue (i.e., gut or pericardial) recognition sites, or
additional sites involved with ubiquitination. Sites that
5    affect secretion or subcellular targeting can also be
modified. Endogenous modification of the enzyme, for
example acylation or phosphorylation, can be a goal of
mutagenesis. Larger-scale modifications of the enzyme are
also possible. For instance, C-terminal trucated forms of
10   JHE have already been shown to retain catalytic activity.

Generation of chimeric proteins is another
possibility. One could make chimeric proteins with added
peptides to provide dual catalytic activity, increase
production in an expression system, and/or change
15   pharmacokinetic properties in a target organism. Thus it
may be useful to make a JHE/$\beta$-galactosidase enzyme or a
JHE/acetyl cholinesterase enzyme. Since X-ray analysis of
esterases indicates that they exist in a C-terminal and N-
terminal domain it is straightforward to make chimera of
20   various esterases to alter substrate specificity, kinetic
properties, or pharmacokinetic properties.

The cDNA or gene may be altered to change mRNA
dynamics, rate of transcription, or rate of translation.
As will be further described hereinafter, we have also used
25   several different promoters with the JHE coding sequence.

Of the several separate mutations described to
illustrate this aspect of the invention, one we designated
K29R (lysine 29 mutated to arginine), one as K522R (lysine
522 mutated to arginine), and one as S201G (serine 201
30   mutated to glycine). All mutations were confirmed by
double-stranded sequencing using a sequenase kit (USB).

The K29R, K522R, and S201G JHE mutants were
transferred directly to the baculovirus transfer vector
pAcUW21 (Figure 2) by digestion with BglII, gel
35   purification of the JHE coding region, and cloning into the
BglII site of the baculovirus transfer vector pAcUW21. A
double mutant of JHE (K29R, K522R) was constructed by
removal of the N-terminal half of JHE (containing lysine

29) from the pAcUW21.JHE-K522R construct and replacing this
with the N-terminal half of JHE from the pAcUW21.JHE-K29R
construct. This was done by digesting pAcUW21.JHE-K522R
with BamHI which cuts the clone at position 851 in JHE and

5   also cuts the pAcUW21 vector within the polyhedrin gene.
This region was then replaced with the equivalent region
from pAcUW21.JHE-K29R to regenerate the same vector and
clone but containing the K29R mutation. The correct
orientation of the modified JHE sequence was confirmed in

10  each case by restriction analysis. Transformation, plasmid
preparation and digestions were carried out using standard
techniques (Maniatis et al., 1990). The presence of the
K29R and K522R mutations were confirmed by digestion with
restriction endonucleases. The presence of the S201G

15  mutation was confirmed by sequencing.


Virus Constructs

        The viruses AcUW21.JHE-K29R, AcUW21.JHE.K522R,
AcUW21.JHE-K29R,K522R and AcUW21.JHE-S201G (henceforth
referred to as AcJHE-29, AcJHE-522, AcJHE-29,522 and

20  AcJHE-201 respectively) were made by cotransfection of cell
line IPLB Sf-21 of Spodoptera frugiperda with polyhedron
negative Autographa californica nuclear polyhedrosis virus
(AcNPV) DNA with the respective plasmids with DNA purified
from the polyhedrin negative virus AcRP8 (Matsuura et al.,

25  1987) using Lipofectin Gibco. Homologous recombination
between the recombinant plasmids and the viral DNA resulted
in polyhedrin positive recombinant viruses against a
background of polyhedrin negative non-recombinant virus.
Additional cell lines and types of media can be used to

30  alter the production of JHE in vitro.


Virus Purification

        Recombinant polyhedrin positive viruses were
purified to homogeneity by sequential plaque assays using
standard assay procedures. The expression of JHE by

35  recombinant viruses was confirmed by JHE activity and

SDS-PAGE for the JHE-29,522 mutant and by SDS-PAGE and Western blotting for the JHE-201 mutant.

Propagation of viruses was carried out in Spodoptera frugiperda cells (Sf) IPLB Sf-21 (Vaughn et al., 1977) in ExCell 400 medium (JRH BioSciences) containing 1% Penicillin-Streptomycin (typically containing 100 µg/ml penicillin G and 100 µg/ml streptomycin), at 28°C. Plaque assays were carried out as described by Brown and Faulkner (1977). Purified virus was amplified by larval infection of *Heliothis virescens*. Virus was purified from cadavers by homogenization in double deionized water and differential centrifugation at 4°C. Virus was stored at 4°C in 0.02% sodium azide. Azide was removed by washing before use of virus in bioassays.

The recombinant virus AcUW2(B).JHE (henceforth referred to as AcJHE), engineered to express the unmodified JHE, and the wild type non-engineered virus AcNPV C6 were also used for comparative purposes.

## Baculovirus expression of JHE in vitro

Petri dishes of Sf-21 cells were set up at 1.5 x 106 cells/ml at 28°C, and infected 24 hours later with AcJHE, AcJHE-29, AcJHE-522 or AcJHE-29,522 at 10 pfu/cell. Expression levels of the modified JHEs attained in vitro in Sf cells were monitored at 6 to 12 hour intervals by colorimetric assay for JHE activity (McCutchen, unpublished), for each modified JHE.

Kinetic parameters (Km and Vmax) were determined for JHE-29,522 using tritiated JH III, or $C_6H_{13}OCH_2C(O)SCH_3$ as substrate, to ensure that these were not altered by the modifications of the JHE coding sequence. Kinetic parameters had been determined previously for JHE, JHE-29 and JHE-522. Crude medium containing baculovirus expressed enzyme was used for these studies.

## Bioassay

Second instar larvae of *Trichoplusia ni* or *H. virescens* were infected at various doses of test or control

virus to determine the lethal dose. Diet plugs of uniform
size (made with a Pasteur pipette) were inoculated with
polyhedra (five doses of between 120 to 7 polyhedrin
inclusion bodies). Mid-second instar larvae were allowed
5      to feed on the treated diet plugs in 96 well microtitre
plates for 24 hours, 50 larvae being infected per dose of
virus. After 24 hours, those that had completely consumed
the diet plug and therefore ingested the required dose,
were transferred to individual tubs of diet and maintained
10     at 24°C for *T. ni* and 27°C for *H. virescens*. Controls were
mock infected. AcNPV C6 was used as a reference virus for
comparison with previous bioassay data for other engineered
viruses where the same reference virus was used for
reference (McCutchen et al., 1991; Stewart et al., 1991;
15     Merryweather et al., 1990). Mortality was scored after 9
days. Bioassays were replicated 3 times and lethal doses
calculated using the POLO computer program (Russell et al.,
1977).

Neonate *T. ni* or *H. virescens* were infected using
20     the droplet feeding assay (Hughes et al., 1986) at 2 x 10$^6$
pibs/ml to determine the lethal time for each virus. Fifty
larvae were infected with each virus. Mortality of the
larvae was scored every 4,6 or 8 hours according to the
mortality rate. LT$_{50}$ values were determined using the POLO
25     probit analysis program. Lethal Ratios for Time (LRT) were
determined using AcNPV C6 as the reference strain, for
comparison with other data generated in different
laboratories using different bioassay techniques and
conditions.

30        Second instar larvae of Manduca sexta were
injected with 2 x 10$^4$ pfu (2$\mu$l) of each recombinant virus
(AcJHE, AcJHE-29, AcJHE-522 or AcJHE-29,522) and bled at 24
hour intervals post infection to assess the level of
modified JHE expression in vivo. Five larvae were bled per
35     time point per treatment. Wild type virus (AcNPV C6) was
injected as control. Mock infected controls injected with
media (ExCell 401) were also analyzed. Hemolymph samples
were diluted in PBS containing 100 $\mu$g/ml BSA and stored at

-20°C prior to assay for JHE activity (Hammock and Roe, 1985).

Pharmacokinetics

In order to establish whether the modifications made to the JHE affected the rate of uptake of the enzyme from the hemolymph by the pericardial cells, pharmacokinetic analysis was carried out. Wild type and modified JHEs harvested from cell culture were purified by DEAE anion exchange. JHE (1 nm/min) was injected into second instar M. sexta (30 to 38 mg) and 6 larvae per time point per treatment bled 0.3, 1, 2, 3, and 4 hours post injection. JHE activity in hemolymph samples was determined by radiochemical assay. Half lives were determined by exponential regression analysis, and rate constants of clearance calculated.

Construction of recombinant viruses expressing modified JHE

The specific alterations made in the modified JHE sequences were confirmed by sequencing. The coding sequences were introduced into the baculovirus transfer vector, pAcUW21 (Figure 2), and the correct insertion confirmed by restriction analysis. Sf-21 cells were cotransfected with the appropriate transfer vector and AcRP8 DNA. The recombinant viruses were purified by 4 rounds of plaque purification by screening for polyhedrin positive plaques and JHE activity.

Baculovirus expression in vitro

All three mutated viruses expressing modified JHE (AcJHE-29, AcJHE-522 and AcJHE-29,522) produced active enzyme in cell culture (Figure 3), although expression of JHE-29,522 was lower than expression of JHE, JHE-29 or JHE-522.

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We found that JHE mutants having alterations at sites presumed to be involved with proteolytic degradation by the ubiquitination pathway led to greatly improved

insecticidal efficiency. That is, site-directed mutagenesis significantly improved the speed of kill by baculoviruses containing some of the modified JHEs in both *H. virescens* and *T. ni*. In particular, we found larvae
5    injected at second instar with AcJHE-29, AcJHE-522 or AcJHE-29,522 gave high level expression of JHE from 96 hours post infection (Figure 5). Only low levels of JHE activity (less than 1 nm/min/ml) were detected in hemolymph from uninfected control larvae (injected with medium) and
10   larvae infected with wild type virus or AcJHE.

Lethal doses were determined using the POLO program. For both *H. virescens* and *T. ni*, the LD50 values are comparable to those of the wild type virus AcNPV C6. The LD50 for AcJHE-29,522 is similar to that of the control
15   virus AcJHE.

$LT_{50}$ data (Table 2) show that the virus AcJHE-29,522 has significantly improved $LT_{50}$ of 84 hours in *T. ni* and 80 hours in *H. virescens*, compared to 106 and 114 hours for AcNPV C6 respectively. AcJHE-29 and AcJHE-522
20   give slower kill than AcNPV C6 in *T. ni*, and quicker kill in *H. virescens*.

## TABLE 2

Biological Activity ($LT_{50}$-hours) of
Mutant JHE Expressed in AcNPV

|               | *T. ni* | *H. virescens* |
|---------------|---------|----------------|
| AcNPV C6      | 106     | 114            |
| AcJHE         | 107     | 118            |
| AcJHE-29      | 117     | 106            |
| AcJHE-522     | 120     | 107            |
| AcJHE-29,522  | 84      | 80             |
| AcJHE-201     | 88      | 82             |

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EXAMPLE 5

The basic protein is a 6.9K, arginine-rich
polypeptide, expressed during the late phase of viral
infection (10-20 hours post infection). We used the basic
5    protein promoter in place of the polyhedrin gene in the
plasmid transfer vector pAcMP1 by following a procedure
described by Hill-Perkins and Possee, *Journal of General
Virology*, 71, pp. 971-976 (1991). We ligated the coding
sequence for juvenile hormone esterase from *Heliothis*
10   *virescens* into the pAcMP1 transfer vector at the Bgl II
cloning site, to produce a plasmid we called "pAcMP1JHE."
         Cotransfection of the plasmid pAcMP1JHE with DNA
from the virus AcUW1-PH (by the general procedure described
by Weyer et al., *Journal of General Virology*, 71, pp. 1525-
15   1534 (1990)) resulted in a basic protein-positive,
polyhedrin-positive, p10-negative virus, the polyhedrin
being under control of the p10 promoter at the p10 locus.
JHE is expressed under control of the duplicated basic
protein promoter in this virus (AcMP1JHE).
20          The p10 protein is expressed at high levels during
the very late phase of virus infection. The p10 promoter
has been inserted at the polyhedrin locus in the transfer
vector pAcUW2(B) (Weyer et al., *J. Gen. Virol.*, 71, pp.
1525-1534 (1990)). We inserted the sequence for juvenile
25   hormone esterase at the Bgl II cloning site to produce the
plasmid pAcUW2(B).JHE. Cotransfection of this plasmid with
DNA from AcRP8 produced a polyhedrin-positive, p10-positive
virus, AcUW2(b)JHE, expressing JHE under control of a
duplicated p10 promoter.
30          Turning to Fig. 4, one can see that the just-
described plasmid pAcMP1JHE (that is, with the basic
protein promoter) gave enhanced expression levels of JHE
with respect to levels from the polyhedrin promoter and
even with respect to levels achieved with the p10 promoter.

EXAMPLE 6

Library construction and screening

               DNA was prepared from embryonic tissue derived
        from an outbred laboratory population of *Heliothis*
5       *virescens*.  In order to produce high molecular weight DNA
        care was taken to avoid shearing (Kaiser and Murray 1985),
        and consequently, the size of the purified DNA was larger
        than bacteriophage T4 DNA () 170 kb).  The genomic library
        was constructed as described by Maniatis et al. (1982) and
10      Kaiser and Murray (1985).  Specifically, an optimized MboI
        digestion was used to partially digest the genomic DNA.
        The digested DNA was separated on a sucrose gradient and
        size-selected by pooling fractions corresponding to 10-20
        kb fragments.  Lambda EMBL3 arms (Stratagen) and the size-
15      selected genomic DNA were ligated and then packaged with a
        commercial extract (Gigapak Gold, Stratagene).

               *Escherischia coli* strain LE392 (rec A$^+$) was used
        to plate the library at a density of approximately 30,000
        pfu per 150 x 15 mm petri plate.  Plaques were lifted in
20      duplicate onto nitrocellulose disks, which were prepared
        for hybridization by standard protocols.  To screen the
        library, a 850 bp N-terminal Bam HI fragment from the JHE
        cDNA (Hanzlik et al., 1989) was labelled by random priming
        using hexamers (pdN6, Pharmacia) and $^{32}$P (Amersham).  After
25      hybridization, the last wash was in 0.1 x SSC at 55°C.  The
        washed and dried filters were overlaid with XAR film
        (Kodak) and an intensifying screen for overnight exposure
        at -70°C.  Positive clones, detected as duplicate signals
        on developed film, were isolated by a standard plaque
30      purification protocol.  A phage stock was prepared for each
        clone and used in the process of purifying lambda DNA on
        cesium chloride gradients (Maniatis et al., 1982).

Clone characterization

               A hybridization experiment was conducted to
35      determine how much of the JHE cDNA sequence was present in
        each of the JHE positive genomic clones.  Probes from the

JHE cDNA were hybridized to 5 clones which had been dot-
blotted onto nylon membranes. Hybridization was performed
in 7% sodium dodecyl sulfate, 1 mm EDTA and 0.5 M NaPO$_4$ at
60°C. After hybridization the last wash was in 0.1 x SSC
5       at 65°C.

For further analysis, an 11 kb KpnI fragment
(C11K) from genomic clone C was subcloned into pUC118. To
confirm that clone C11K contained all of the JHE gene, and
to aid restriction mapping of the gene, synthetic
10      nucleotide primers were made to the sequence of the 5' and
3' ends of the full length JHE cDNA clone previously
described in the parent applications and their former
Figure 2. (By "former" Fig. 2, we mean Fig. 2 of Serial
No. 07/725,226 and its predecessor 07/265,507, of which
15      this application is a continuation-in-part. The former
Fig. 2 was found to contain several minor errors in
sequence, which have been corrected in the present SEQ ID
NO:1.) Primer b corresponds to bases 29-46 of the JHE cDNA
sequence and primer f corresponds to bases 2775-2794. A
20      sequence kit (United States Biochemical) was used for dsDNA
sequencing.

A map of clone C11K was generated to verify the
linear correspondence of restriction sites, in relationship
to the JHE cDNA, and for detection of introns within the
25      genomic clone. From the 5' sequencing of C11K an NaeI
restriction site was located upstream of the reading region
and used to restriction map the 5' end of the gene. The 3'
sequence of the genomic clone confirmed the presence of an
XhoI site which was useful for mapping the 3' end of the
30      gene. Using NaeI and XhoI sites as reference points, a map
was constructed by multiple restriction endonuclease
digestions with Bgl II, ClaI, NaeI, NcoI, KpnI, PstI, and
XhoI. From the restriction analysis it was possible to
approximate the position of introns, which were defined by
35      synthesizing nucleotide primers complementary to the cDNA
and sequencing across the intron/exon junctions.

Library construction and screening

        Hybridization of the labelled JHE cDNA BamHI fragment yielded 6 positive clones in a screen of approximately 250,000 primary plaques from the genomic library. Only one of the genomic clones, C, hybridized to each of the cDNA probes and was assumed to contain most, if not all, of the JHE gene. C11K, the 11 kb Kpn fragment, was found to contain the entire JHE gene by sequence homology to the 5' and 3' termini of the JHE cDNA.

        Figure 6 summarizes the JHE gene, which is approximately 11 kb with four introns. Restriction sites used for the mapping of subclone C11K are represented. The exons are represented by solid regions and the introns are represented by stippling. Regions upstream and downstream of the coding region are shown as empty rectangles. The exact location of intron/exon boundaries were determined by sequencing the gene in the regions shown by restriction mapping to have introns. Primers complimentary to JHE cDNA allowed sequencing of most of the intron/exon junctions. To facilitate the sequencing of the junctions at positions 53 and 519, C11K was subcloned into two fragments at the NcoI site. Forward and reverse vector sequencing primers were then used to sequence out to intron junctions on either side of the NcoI site (*). The wedge next to the letter designation of each primer indicates the sequencing orientation from the primer. There is an ordered correspondence between restriction sites in clone C11K and sites in the JHE cDNA, indicating that the clone is representative. Four introns occur in the coding region of the gene and none are found in the 3' untranslated region. A 4.3 kb intron is located in the 19 amino acid leader sequence region, between cDNA bases 52 and 53. The possible significance of a large intron located close to the 5' end of the gene is not clear, but an analogous situation is found in the *Bombyx mori* fibroin gene. The other three introns are relatively small, ranging in size from 154 bp to 204 bp. The consensus sequences (GT-AG),

common to the beginning and end of almost all introns, are
present in the JHE gene.

The foregoing examples illustrate certain
embodiments of the present invention, and are not intended
to limit the scope of the invention, which is defined in
the appended claims.

IT IS CLAIMED:

1.    A vector comprising:
a nucleotide coding sequence corresponding to the cDNA illustrated by SEQ ID NOS:1 or 2, the coding sequence capable of expressing a protein in a host cell expression system, the expressed protein reacting with a methyl ester containing compound.

2.    The vector as in claim 1 wherein the expressed protein degrades, binds with, or sequesters juvenile hormone or a juvenile hormone homolog within the host.

3.    The vector as in claim 2 wherein the expressed protein degrades juvenile hormone or a juvenile hormone homolog with a $k_{CAT}/K_M$ ratio greater than about $10^8$.

4.    The vector as in claim 1 wherein the protein is expressed in a virus infected cell.

5.    The vector as in claim 1 wherein the protein expressed is substantially the same as juvenile hormone esterase but differs therefrom at one or more of the $lysine_{522}$, $lysine_{29}$, and $serine_{201}$ thereof.

6.    The vector as in claim 1 wherein the protein expressed by the coding sequence is a chimeric protein, the chimeric protein including juvenile hormone esterase.

7.    A recombinant expression vector, capable of expression in a host insect or plant cell, comprising:
a juvenile hormone esterase (JHE) coding sequence or a mutated JHE coding sequence; and,
a promoter sequence which is heterologous with the coding sequence and which regulates the transcription thereof.

8.    The expression vector as in claim 7 wherein the coding sequence is a mutant of an insect coding sequence.

9.    The expression vector as in claim 8 wherein the mutant is derived from site-directed mutagenesis.

10.    The expression vector as in claim 8 wherein the JHE is expressed in noctuid insect cell.

11.    The expression vector as in claim 8 wherein the coding sequence is an insert into a baculovirus genome.

12.    The expression vector as in claim 11 wherein the coding sequence is an insert into an *Autographa californica* genome.

13.    The expression vector as in claim 7 wherein the promoter sequence is a polyhedrin promoter, a basic protein promoter, or a p10 promoter.

14.    The expression vector as in claim 7 wherein the promoter sequence is a basic protein promoter.

15.    The expression vector as in claim 14 wherein the coding sequence is a mutant of a juvenile hormone esterase coding sequence.

16.    A composition comprising:
an isolated nucleotide sequence having greater than about 16 sequential bases and being substantially similar to at least a portion of the sequence illustrated by SEQ ID NO:1 or SEQ ID NO:2.

17.    The composition as in claim 16 wherein the nucleotide sequence is double or single stranded.

18.  The composition as in claim 16 wherein the
nucleotide sequence is reversed with respect to SEQ ID NO:1
or SEQ ID NO:2.

19.  A vector, useful for introducing endogenous
5     nucleic acid sequences into an insect organism comprising:
a nucleotide coding sequence capable of over-
expressing a natural protein or a mutant of a natural
protein in an insect organism, the vector disrupting
development of the insect organism when the nucleotide
10    coding sequence is expressed.

20.  The vector as in claim 19 wherein the protein
expressed is juvenile hormone esterase or a mutant thereof.

21.  A transgenic, non-human organism having a
nucleotide sequence introduced into said organism, the
15    nucleotide sequence expressing juvenile hormone esterase or
an epoxide hydrolase capable of degrading insect juvenile
hormone with a $k_{CAT}/K_M$ ratio greater than about $10^8$, the
esterase or hydrolase when expressed being effective to
alter at least one determinable property of the organism.

20        22.  The organism as in claim 21 wherein the
altered property includes disruption of a methyl ester
containing, endogenous compound.

23.  The organism as in claim 21 wherein the
expression of the introduced nucleotide sequence decreases
25    the effective titre of juvenile hormone.

24.  The organism as in claim 21 wherein the
introduced nucleotide sequence is substantially similar to
SEQ ID NO:1, SEQ ID NO:2, or to the gene illustrated by
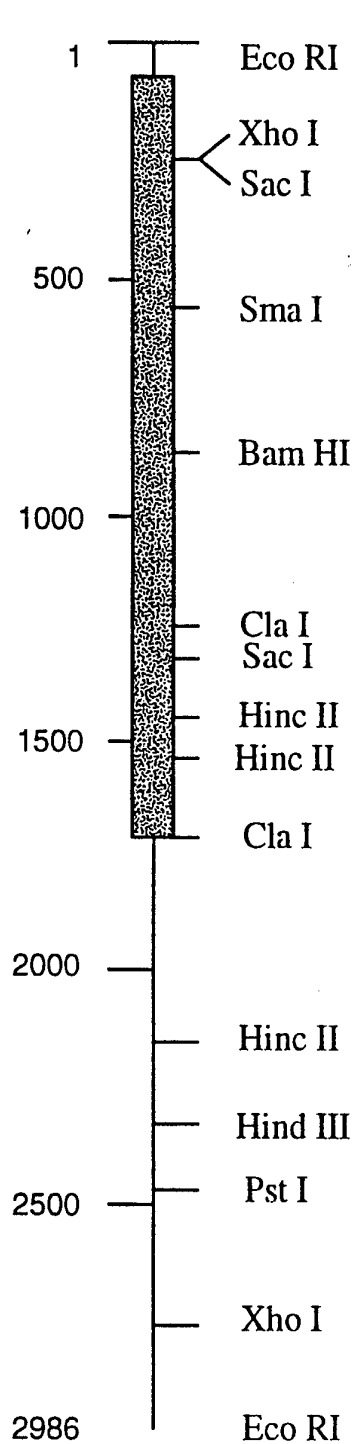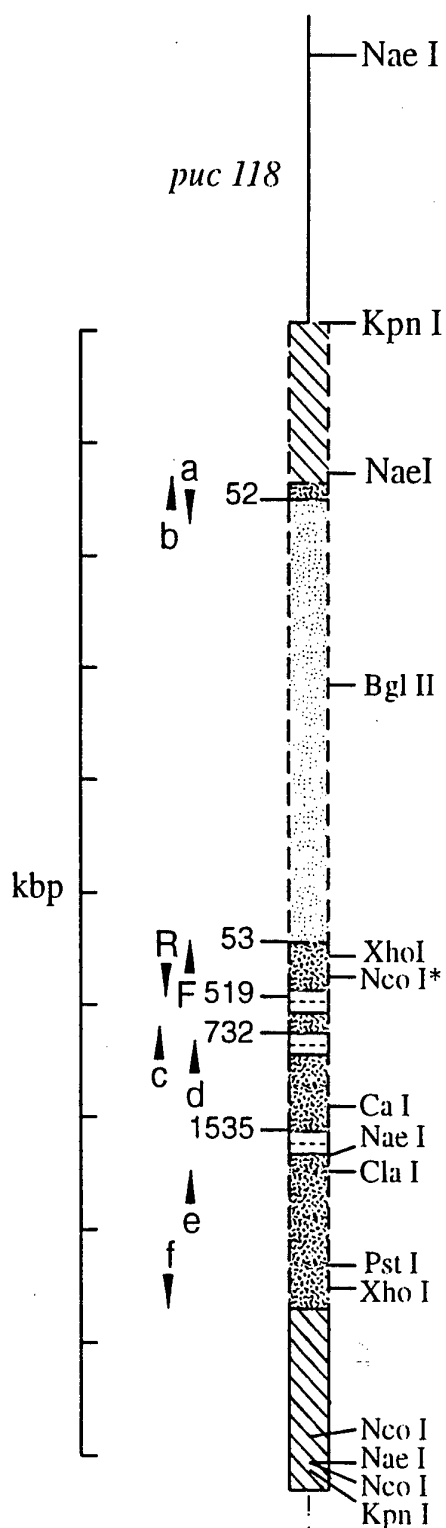Fig. 6.

25.   The organism as in claim 24 wherein the
introduced nucleotide sequence is double or single
stranded.

26.   The organism as in claim 25 wherein the
nucleotide sequence is reversed with respect to SEQ ID
NO:1, SEQ ID NO:2, or to the gene illustrated by Fig. 6.

27.   A method of increasing expression levels of
a protein comprising:
    combining a basic protein promoter with a
nucleotide coding sequence coding for a secreted or
exported protein, the promoter sequence regulating the
transcription of the protein and capable of expressing the
protein in a host cell expression system.

28.   The method as in claim 27 wherein the
secreted or exported protein is juvenile hormone esterase
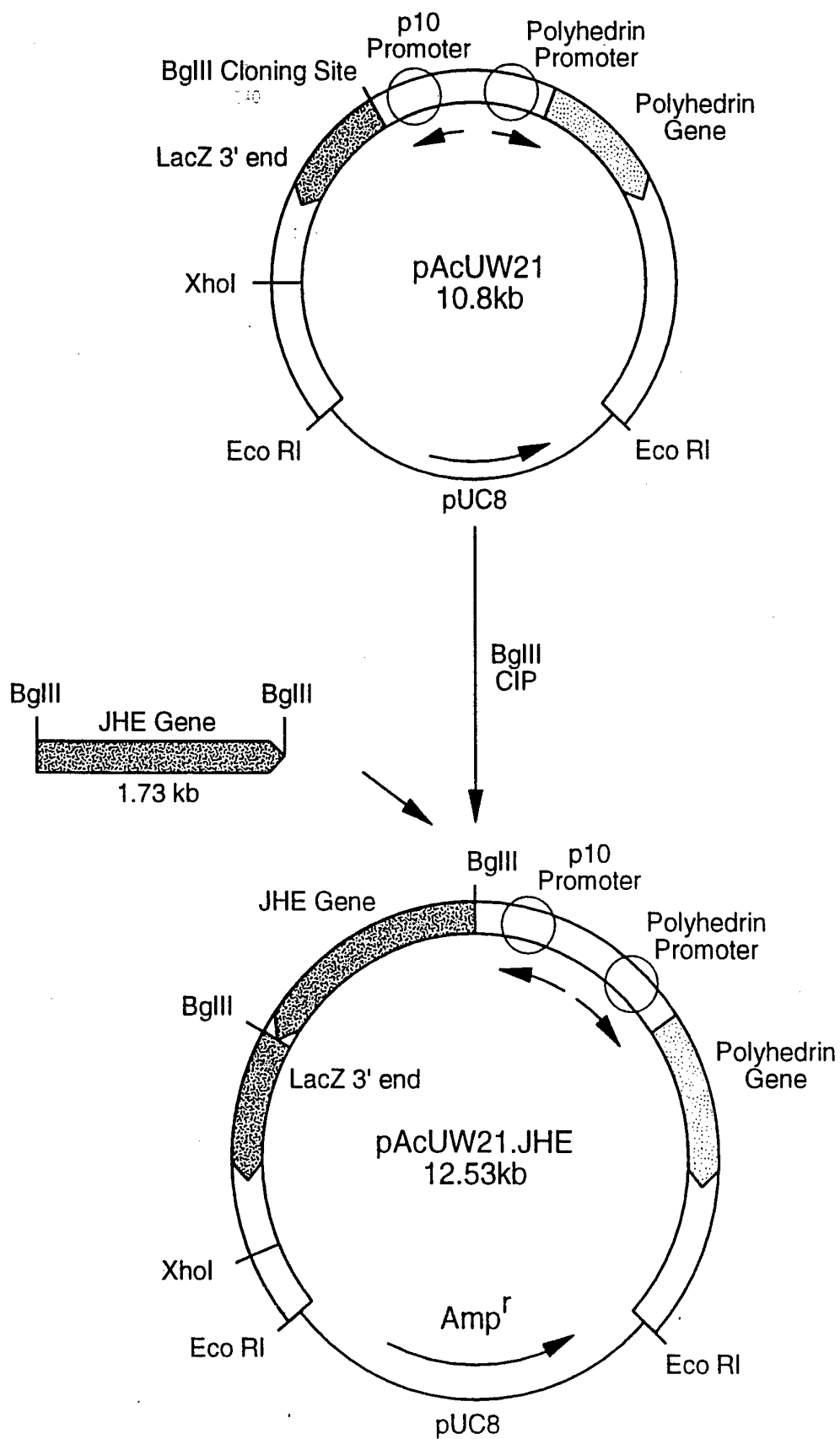or a juvenile hormone esterase mutant.

**FIG._1**
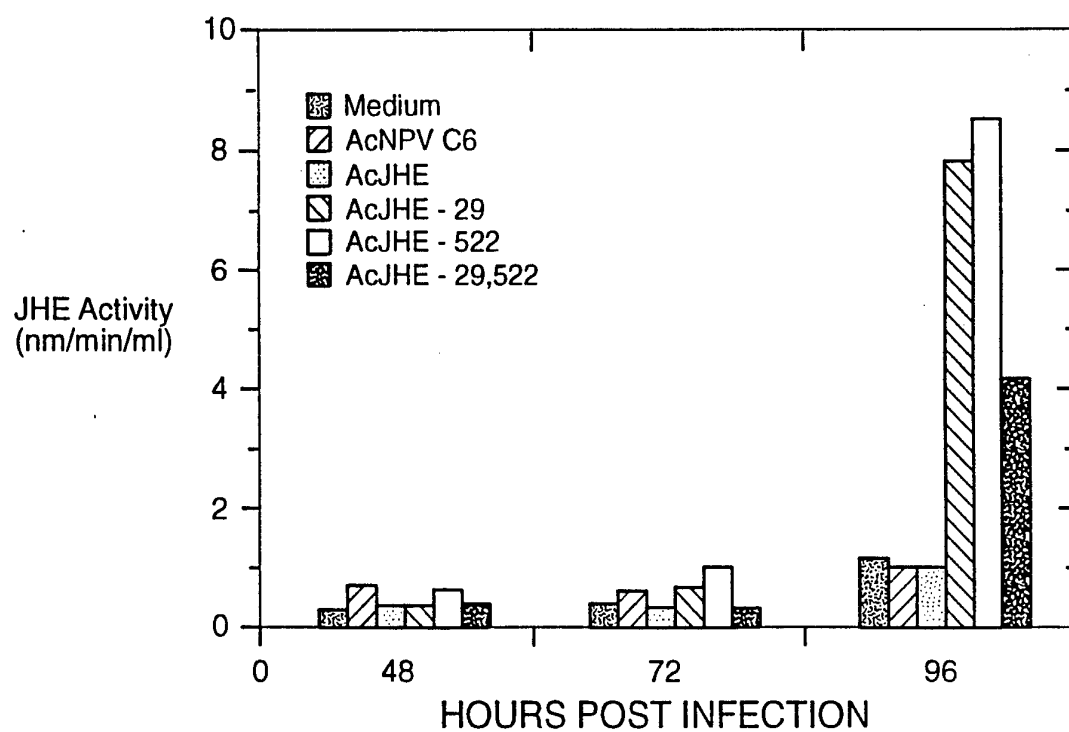
- 1 — Eco RI
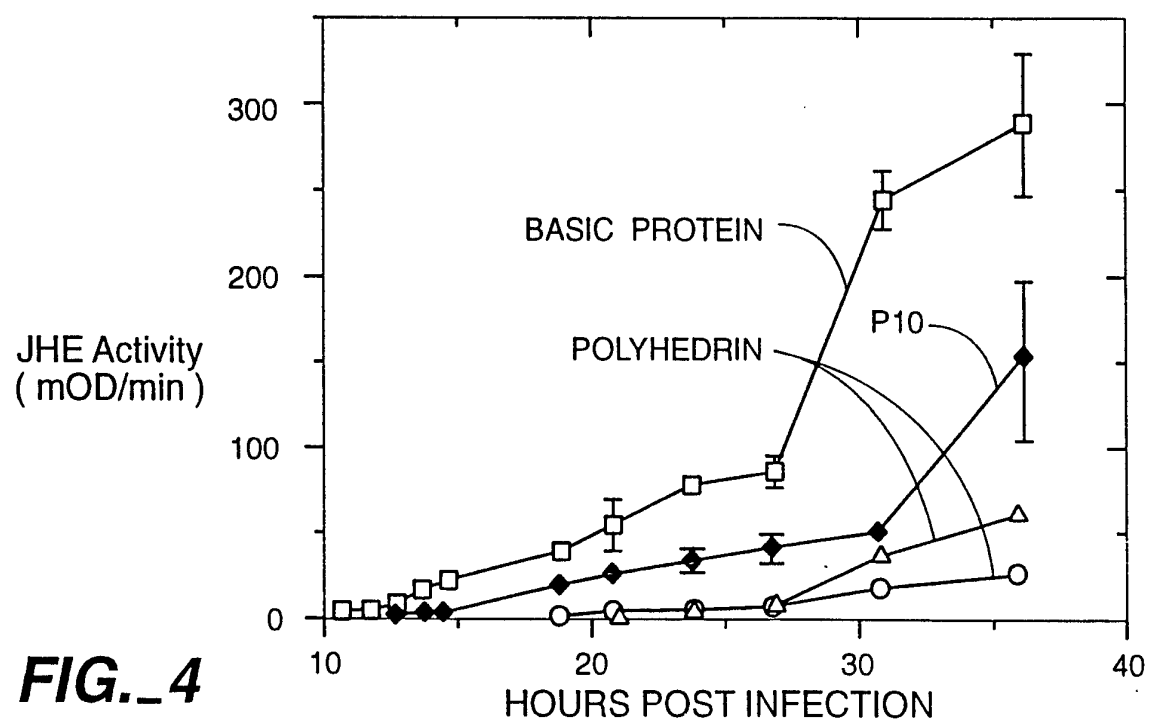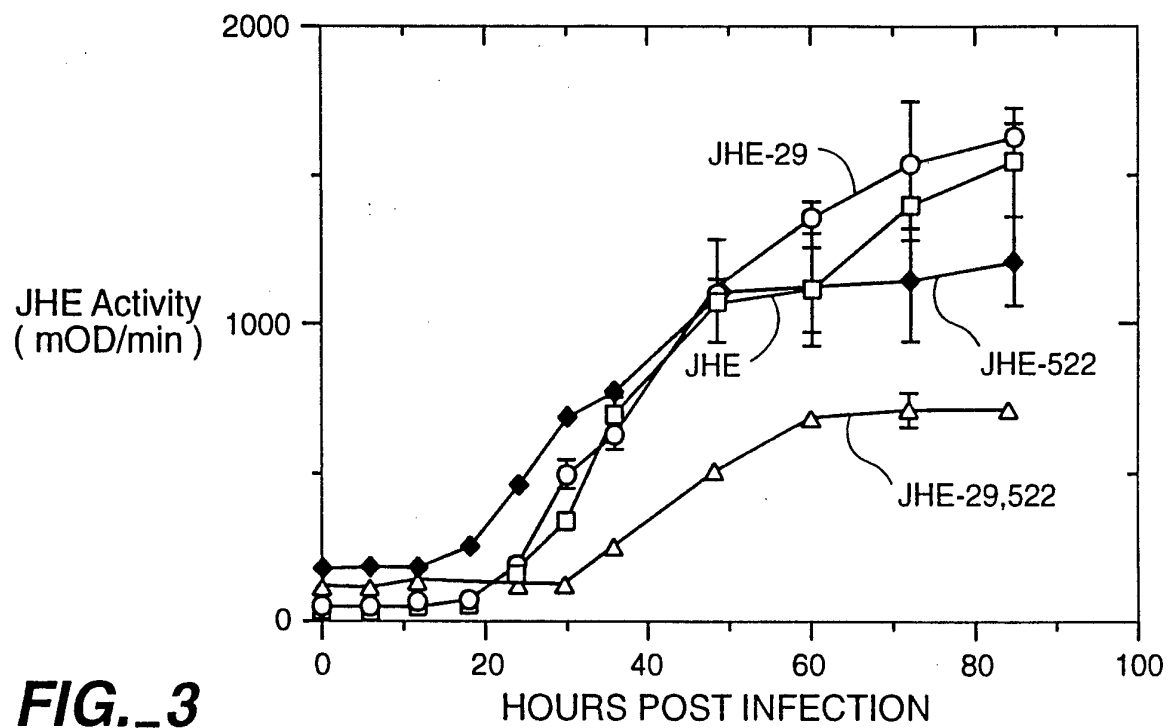- Xho I
- Sac I
- 500 — Sma I
- Bam HI
- 1000
- Cla I
- Sac I
- Hinc II
- 1500 — Hinc II
- Cla I
- 2000
- Hinc II
- Hind III
- Pst I
- 2500
- Xho I
- 2986 — Eco RI

**FIG._6**

- Nae I
- *puc 118*
- Kpn I
- NaeI
- a / b
- 52 — NaeI
- Bgl II
- kbp
- R / F — 53 — XhoI
- 519 — Nco I*
- 732
- c / d
- 1535 — Ca I
- Nae I
- Cla I
- e
- f
- Pst I
- Xho I
- Nco I
- Nae I
- Nco I
- Kpn I

1 / 4

FIG._2

SUBSTITUTE SHEET

FIG._3



FIG._4

**FIG._5**

| A. CLASSIFICATION OF SUBJECT MATTER |
|---|
| IPC(5)    :C12N 5/16, 15/52, 15/85 |
| US CL    :453/69.1, 172.3, 183, 240.2, 320.1; 536/23.2 |
| 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) |
| U.S. :    453/69.1, 172.3, 183, 240.2, 320.1; 536/23.2 |

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 practicable, search terms used)

CAS, BIOSIS, Biotech. Abstr., Medline, APS
search terms: juvenile hormone esterase, seqeunce, vector, plasmid

| C.    DOCUMENTS CONSIDERED TO BE RELEVANT |

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| T | Abstr. Pap. Am. Chem. Soc., 206 Meet., Pt. 1, AGRO122, issued 1993, "Potential of baculo viruses expressing a scorpion toxin and an esterase in agriculture - use of recombinant baculo virus with a Heliothis virescens juvenile-hormone-esterase as an insect biological control agent", see abstract. | 1-28 |

[X] Further documents are listed in the continuation of Box C.          [ ] See patent family annex.

| * | Special categories of cited documents: | "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 |
|---|---|---|---|
| "A" | document defining the general state of the art which is not considered to be part of particular relevance | | |
| "E" | earlier document published on or after the international filing date | "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 |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "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 |
| "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 | "&" | document member of the same patent family |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 October 1993 | NOV 05 1993 |
| Name and mailing address of the ISA/US· Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 | Authorized officer JOHN L. LEGUYADER |
| Facsimile No.   NOT APPLICABLE | Telephone No.    (703) 308-0196 |

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Zool. Sci., Vol. 6, No. 6, issued 1989, Hayakawa, "Juvenile hormone esterase repressive factor in the plasma of parasitized insect larvae", page 1125, only citation available. | 1-28 |
| Y | Zool. Sci., Vol. 7, No. 6, issued 1990, Hayakawa, "Juvenile hormone esterase activity repressive peptide in the parasitized armyworm hemolymph", page 1061, only citation available. | 1-28 |
| T | Int. J. Biochem., Vol. 24, No. 12, issued December 1992, Ward et al., "Analysis of the catalytic mechanism of juvenile hormone esterase by site-directed mutagenesis", pages 1933-1941, see abstract. | 1-28 |
| T | Arch. Insect Biochem. Physiol., Vol. 22, Nos. 3-4, issued 1993, Hammock et al. "Development of recombinant viral insecticides by expression of an insect-specific toxin and insect-specific enzyme in nuclear polyhedrosis viruses", pages 315-344, see abstract. | 1-28 |
| Y | Annals of the New York Academy of Sciences, Vol. 646, issued 1991, Posse et al., "Expression of the proteins with insecticidal activities using baculo virus vectors - recombinant Autographa californica nuclear-polyhedrosis virus vector used for expression of crystal protein and juvenile-hormone-esterase in insect cell culture", pages 234-239, see abstract. | 1-28 |
| Y | Abstr. Pap. Am. Chem. Soc., 202 Meet., Pt. 1, AGRO9, issued 1991, Hammock et al., "Improving the efficacy of baculo virus insecticide by expressing with insect selective proteins", see abstract. | 1-28 |
| Y | Insect Biochem. Mol. Biol., Vol. 22, No. 5, issued 1992, Bonning et al., "Further development of a recombinant baculovirus insecticide expressing the enzyme juvenile hormone esterase from heliothis-virescens", pages 453-458, see abstract. | 1-28 |
| Y | US, A, 5,180,581 (Miller et al.) 19 January 1993, see entire document. | 1-28 |
| X | Nature, Vol. 344, issued 29 March 1990, Hammock et al., "Expresssion and eefects of the juvenile hormone esterase in a baculovirus vector", pages 458-461, see entire article. | 1-28 |
| A | J. Biol. Chem., Vol. 266, issued 5 May 1991, Hayakawa, "Structure of a growth-blocking peptide present in parasitized insect hemolymph", pages 7982-7984, see entire document. | 1-28 |

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X<br>Y | J. Biol. Chem., Vol. 264, issued 25 July 1989, Hanzlik et al., "Isolation and sequencing of cDNA clones coding for juvenile hormone esterase from Heliothis virescens", pages 12419-12425, see entire article. | 1-28<br>1-28 |
| X<br>Y | Applied and Environmental Microbilogy, Vol. 58, No.5, issued May 1992, Eldridge et al., "Insecticidal properties of genetically engineered baculoviruses expressing an insect juvenile hormone esterase gene", pages 1583-1591, see entire article. | 1-28<br>1-28 |
| Y | Biochem. Biophys. Res. Comm., Vol. 185, No. 3, 30 June 1992, Hayakawa, "A putative new juvenile peptide hormone in lepidopteran insects", pages 1141-1147, see entire article. | 1-28 |
| X<br>Y | US, A, 5,098,706 (Hamock et al.) 24 March 1992, see entire document. | 1-28<br>1-28 |
| X | Molecular and Cellualr Biology, Vol. 3, No. 12, issued December 1983, Smith et al., "Production of human beta interferon in insect cells infected with a baculovirus expression vector", pages 2156-2165, see entire article. | 27 |

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