## Genetically Modified Baculoviruses for Pest Insect Control

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In our original comprehensive review article from 2005 and in our update to this article in 2010, we extolled the virtues and cautioned against the limitations of genetically modified (GM) baculoviruses for use in pest insect control. In these articles, we concluded that GM baculoviruses (1) show potency that is comparable to traditional chemical insecticides, (2) pose little or no risk to non-targeted species including humans and the environment, and (3) can make a near immediate positive impact on sustainable pest insect control programs. The scientific literature since our initial comprehensive review continues to support these conclusions. Our enthusiastic support of GM baculoviruses as potent and safe biopesticides that can complement or even synergize traditional chemical insecticides and biocontrol strategies has not changed. The field, however, remains slow to embrace...
our enthusiasm. The high cost of regulatory barriers for GM products coupled with the efficacy and safety of major GM plants as well as new generation insecticides for the control of key noctuid pests, make near-term commercial development of GM baculoviruses unlikely in the United States. In addition, in some countries, there is a general unease with the use of GM products even when there are clear economic advantages to these products. On the other hand, a highly positive trend has been the increased use of natural (i.e., wild type) baculoviruses for pest insect control. The expanded use of natural baculoviruses will likely serve as a positive step in the public acceptance and application of GM baculovirus insecticides. In addition, the use of GM baculoviruses for the production of human therapeutics, first approved in 2007, is another positive step in improving the public’s perception of GM baculoviruses.

Since 2005 several interesting and insightful reviews have been published (Arif, 2005; Beas-Catena et al., 2014; Glare et al., 2012; Inceoglu et al., 2006; Kroemer et al., 2015; Lacey et al., 2015; Moscardi et al., 2011; Szewczyk et al., 2006) that cover the historical aspects of biological control using natural and GM baculoviruses and other microbials as well as the current and future status of biological control. In addition, Gelernter (Gelernter, 2007), Kunimi (Kunimi, 2007), Yang et al. (Yang et al., 2012), and Sun (Sun, 2015) review the current status of the use of baculovirus insecticides and other microbials in Asia. Haase et al. (Haase et al., 2015b) review the current status and future perspectives of baculovirus insecticides in Latin America. Grzywacz et al. (Grzywacz et al., 2014) and Knox et al. (Knox et al., 2015) review the use of baculovirus insecticides in Africa. Moreau and Lucarotti (Moreau and Lucarotti, 2007) review the use of baculoviruses against forest pests in Canada. Summers (Summers, 2006), a pioneer in the development of the baculovirus expression vector system (BEVS) for use in recombinant protein expression, reviews how advances in BEVS played key roles in the development of GM baculovirus biopesticides. The use of BEVS for the production of vaccines and human gene therapy vectors is reviewed by Airenne et al. (Airenne et al., 2013), Haase et al. (Haase et al., 2013), Felberbaum (Felberbaum, 2015), and van Oers et al. (van Oers et al., 2015).

In our original comprehensive review article from 2005 and in our 2010 revision, we characterized genetic modifications of the baculovirus genome into three major approaches (1) insertion of genes encoding hormones and enzymes, (2) insertion of insect-selective toxin genes, and (3) genome modifications. When multiple technologies were combined into a single construct, the genetic modifications resulted in at best a roughly 60% improvement in speed of kill relative to the wild type virus with reductions in feeding damage of roughly 70% (comparison of GM baculovirus- and mock-infected insects). During the past decade, various groups (see below) have continued with single technology and technology-stacked approaches with similar improvements in efficacy. RNA interference (RNAi)- and other RNA-based strategies for control of lepidopteran pest insects have also been proposed and tested (Burand and Hunter, 2013; Katoch et al., 2013; Nandety et al., 2015; Terenius et al., 2011). Although the efficacy of current GM baculovirus constructs is sufficient under many crop protection scenarios, we believe that further improvements (e.g., by improving toxin folding and/or expression, identifying alternative insect-selective toxins, modifying the virus backbone, altering host range, improving formulation, etc.) are possible by sustained efforts from the private and public sectors.

Several groups have continued to genetically modify the baculovirus genome by the insertion of hormone and enzyme genes since our original review in 2005. GM baculovirus constructs designed to express cathepsin B-like proteases (Hong-Lian et al., 2008) and cathepsin L-like (Gramkow et al., 2010; Li et al., 2007, 2008; Sun et al., 2009) proteases have been tested in the laboratory and in multi-year-long field studies. For example, the cathepsin L-like cysteine protease expressing construct HearNPV-cathL of Sun et al. (Sun et al., 2009) is designed to protect cotton against larval cotton bollworm (Helicoverpa armigera Hübner) a species that has developed resistance to chemical and/or Bacillus thuringiensis insecticides. Sun et al. conducted field trials with HearNPV-cathL in 2004 and 2005 in China and found the efficacy of this construct against the cotton bollworm is similar to that of the pyrethroid insecticide β-Cyhalothrin. In the 2005 field trial, they also found that the density of beneficial insects such as the ladybird beetle is similar between GM baculovirus treated and untreated plots, and higher than that found in pyrethroid treated plots. The insecticidal activity of recombinant AcMNPVs expressing the serine protease keratinase or cathepsin L-like protease (ScathL) in larval Spodoptera frugiperda (Gramkow et al., 2010) or CpGV matrix metalloprotease (Ishimwe et al., 2015) or TnGV enhancin (a metalloprotease) (Del Rincon-Castro and Ibarra, 2005) in larval Trichoplusia ni have also been investigated. Of these recombinant AcMNPVs, the median lethal time of the ScathL, keratinase, and enhancin expressing constructs was 66%, 33%, and 17% faster than that of wild type AcMNPV. Lima et al. (Lima et al., 2013) and Wang et al. (Wang et al., 2013) have investigated the roles played by chitinase- and/or cathepsin-encoding genes in the induction of host liquefaction and increased insecticidal activity. Harrison and Bonning (Harrison and Bonning, 2010), Ishimwe et al. (Ishimwe et al., 2015), and Sun (Sun, 2015) review the biological activity and applications of proteases and other enzymes for pest insect control.

In terms of GM baculovirus constructs that carry genes that encode hormones or hormone degrading genes, Wan et al. (Wan et al., 2015) have generated a recombinant AcMNPV that expresses a growth-blocking peptide from Spodoptera exigua and tested the construct in larval S. exigua. They showed that the median lethal time of this virus is approximately 1 day faster than that of wild type AcMNPV. Detvisitsakun et al. (Detvisitsakun et al., 2007) have constructed a recombinant AcMNPV over-expressing a viral fibroblast growth factor that induces accelerated mortality in larval S. frugiperda and Trichoplusia ni. Juvenile hormone esterase (JHE) is an enzyme that hydrolyzes the methyl ester of juvenile hormone (JH). El-Sheikh et al. (El-Sheikh et al., 2011) show that larval Manduca sexta that are infected by a recombinant AcMNPV expressing a stabilized form of JHE causes lower feeding damage to tomato leaf compared to M. sexta infected with wild type AcMNPV or a recombinant AcMNPV expressing wild type JHE.

Since 2005 several groups have continued to genetically modify the baculovirus genome by inserting toxin genes from scorpions (Choi et al., 2008; Jin et al., 2006; Rajendra et al., 2006; Tang et al., 2011; Yuan et al., 2005, 2007), spiders (Ali et al., 2015; Ardlisson-Araujo et al., 2013; Yu et al., 2015), and B. thuringiensis (El-Menofy et al., 2014; Kim et al., 2005). These constructs carry a single toxin gene that is placed at various loci in the baculovirus genome, and the gene is placed downstream of various
types of promoters. In comparison to the older GM baculovirus constructs described below, these recent constructs show comparable improvements in the induction of host cell death and insecticidal efficacy. Shim et al. (Shim et al., 2009, 2013) have developed a “stacked” construct that targets the host at three levels: the gut, nervous system, and systemic baculovirus infection. Their construct showed a reduction in median lethal time of about 50% but perhaps more importantly, the stacked approach may help to delay the onset of resistance. Additionally, serial passage of their unique construct in larval S. exigua results in the rapid loss of the transgene suggesting an additional layer of environmental safety. Jung et al. (Jung et al., 2012) have also taken a stacked approach by generating a recombinant AcMNPV that coexpresses toxins from the spider Araneus ventricosus and B. thuringiensis. They found significant reductions in median lethal dose and median survival time in larval S. exigua and Plutella xylostella that were infected with this construct (in comparison to wild type AcMNPV-infected control larvae). In a related approach, Arrizubieta et al. (Arrizubieta et al., 2015) show improved insecticidal activity in larval cotton bollworm that feed on occlusion bodies that contain a mixture of two genotypic variants of H. armigera single nucleopolyhedrovirus (HeatSNPV). Ortiz and Possani (Ortiz and Possani, 2015) review insect-selective scorpion toxins and discuss barriers to their practical use for pest insect control.

During the past decade, formulation strategies and the relative importance of baculoviruses (both natural and GM) with respect to other microbials and the role of the baculovirus as a delivery system for insecticidal agents have also been reviewed (Hajek et al., 2007; Hynes and Boyetchko, 2006; Lord, 2005; Rosell et al., 2008; Whetstone and Hammock, 2007). Several studies have also investigated optimal and innovative production methodologies of natural and GM baculoviruses in both cultured insect cells (Micheloud et al., 2009; Salem and Maruniak, 2007) and insect larvae (Lasa et al., 2007a; van Beek and Davis, 2007). Goto et al. (Goto et al., 2015) show in a 2-year-long field study that formulating alkaline soluble protein extracts from Xestia c-nigrum granulovirus (XecnGV) capsules with Mamestra brassicae nucleopolyhedrovirus (MabrNPV) results in enhanced MabrNPV infectivity. They suggest that protein extracts from XecnGV capsules are promising additives to baculovirus formulations. The importance of optical brighteners (Ibarguchi et al., 2008; Lasa et al., 2007b) and feeding stimulants (Lasa et al., 2009) on baculovirus formulations have also been investigated. In addition, Licznar et al. (Licznar et al., 2014) and Apaire-Marchais et al. (Apaire-Marchais et al., 2016) show that natural baculoviruses are able to act as synergistic agents that activate calcium-dependent intracellular signaling pathways resulting in the increased sensitivity of insect cells to organophosphate insecticides. Other studies evaluate the use of baculoviruses for the protection of non-major food crops and in situations where traditional chemical insecticides are not acceptable (Gzywacz et al., 2008; Kunimi, 2007; Praet et al., 2006; Sciocco et al., 2009). Studies have also addressed competitive fitness and within-host fitness of GM baculoviruses in which the endogenous egt gene has been deleted (Zwart et al., 2009). Ashour et al. (Ashour et al., 2007) have further investigated the safety of recombinant AcMNPV expressing the insect-selective toxin AaIT.

Numerous studies from the last ten years continue to show that both natural baculoviruses and GM baculoviruses hold clear and substantial benefits for crop protection. Natural and potentially GM baculoviruses are particularly valuable under conditions where traditional chemical insecticides have become ineffective, economically prohibitive or have lost favor with the general public. The advantages of green pesticides, in particular biopesticides that can be produced locally, are the most obvious in developing countries where older and more dangerous pest control materials are often used, and where high costs of both modern pesticides and GM plants are often prohibitive. Since many of the technologies that are used for the production and use of natural and GM baculoviruses are common, increased use of natural baculoviruses may open doors to the use of GM baculoviruses. GM baculoviruses remain a viable alternative to classical chemical pesticides and GM crops that we believe should be utilized when alternative methods of pest insect control are required due to resistance, high cost of traditional insecticides or changes in the regulatory environment and public opinion.

1 Introduction

There are presently more than 20 known groups of insect viruses, which are classified into 19 families (Blissard et al., 2000; Fauquet et al., 2005; Tanada and Kaya, 1993). Among insect pathogenic viruses, members of the family Baculoviridae are the most widely studied and commonly found. Baculoviruses are rod-shaped, enveloped viruses with large, covalently closed, double-stranded (ds) DNA genomes. The Baculoviridae were previously made up of two genera: nucleopolyhedrovirus (NPV) and granulovirus (GV). The NPVs were further divided into two groups (I and II) on the basis of the phylogenetic relationships of twenty distinguishing genes (Herniou et al., 2001). More recently, the Baculoviridae is composed of four genera: alphabaculovirus, betabaculovirus, gammabaculovirus, and deltabaculovirus (Herniou et al., 2012). Baculoviruses produce two types of progeny, the budded virion (BV) and the occlusion-derived virion (ODV), during their life cycle (Granados and Federici, 1986; Miller, 1997). BVs are predominantly produced during an early phase of infection and acquire their envelopes as they bud through the plasma membrane. BVs are responsible for the systemic or cell-to-cell spread of the virus within an infected insect. Continuous cell lines that support high-level production of BVs are available for NPVs, but not for GVs. The availability of these cell lines has been critical for the development of genetically modified NPVs. ODVs are produced during a late phase of infection and are involved in the horizontal or larva-to-larva transmission of the virus. Each ODV is composed of a single nucleocapsid or multiple nucleocapsids surrounded by an envelope. The ODVs are occluded within a protein matrix that helps to protect the embedded ODV(s) from environmental factors. The occlusion body (OB) of the NPV is known as a polyhedron (plural polyhedra) or polyhedral inclusion body, whereas the GV OB is known as a granule. Rohrmann (Rohrmann, 2013) and Ikeda et al. (Ikeda et al., 2015) review the structure and biological properties of baculoviruses are in detail.
Due to their inherent insecticidal activities, natural baculoviruses have been used as safe and effective biopesticides for the protection of field and orchard crops, and forest in the Americas, Europe, and Asia (Black et al., 1997; Copping and Menn, 2000; Haase et al., 2015a; Hunter-Fujita et al., 1998; Lacey et al., 2001, 2015; Moscardi, 1999; Moscardi et al., 2011; Sun, 2015; Vail et al., 1999). Both NPVs and GVs have been successfully registered for use as microbial pesticides by commercial companies and governmental agencies. NPVs of the velvet bean caterpillar *Anticarsia gemmatalis* (AgMNPV) and *Helicoverpa armigera* (HaSNPV) are being used with particular success for the protection of soybean in Brazil (Moscardi, 1999; Moscardi et al., 2011) and cotton in China (Sun, 2015; Sun et al., 2002), respectively. Natural baculoviruses, however, are slower acting and more target specific (i.e., their host specificity is narrow) compared to synthetic chemical pesticides such as the pyrethroids. The general use of natural baculoviruses in developed countries has been limited except against forest pests primarily due to their slow speed of insect killing compared to chemical insecticides, and particularly due to their relatively narrow host specificity, low field stability, and cost of production. As a natural control agent the ‘slow kill’ characteristic allows the virus to replicate to tremendous numbers while allowing its host to feed for several days. Although an attribute in a natural control strategy, this trait is a severe limitation in modern agriculture. This trait and others such as narrow host specificity can, and have been addressed by genetically modifying the baculovirus using recombinant DNA technology.

During the 1980s, the birth of genetically modified (GM) baculoviruses came along with exciting new research in the laboratories of Summers (Summers and Smith, 1987) and Miller (Miller, 1988). They simultaneously exploited a combination of unique characteristics of NPVs to establish the baculovirus expression vector systems (BEVS) that is now in common use for basic research and commercial applications. These characteristics include: (1) the availability of the exceptionally strong polyhedrin gene (*polh*) promoter to drive foreign gene expression, (2) a selection system based upon the visualization of the nonessential (in cultured cells) gene product of the polyhedrin gene, (3) a double-stranded DNA genome that can be easily modified, (4) a rod-shaped capsid that can extend to package additional DNA, and (5) a eukaryotic cell line that supports virus replication at high levels. *Autographa californica* multicapsid NPV (AcMNPV), originally isolated from the alfalfa looper *A. californica* (Vail et al., 1999, 1973), is the baculovirus type species. AcMNPV was used by the Summers and Miller laboratories as the parental baculovirus for BEVS. Another baculovirus, *Bombyx mori* NPV (BmNPV), isolated from the silk moth *B. mori*, was used by Maeda (Maeda, 1989a) as the parental baculovirus in an alternative BEVS that used larvae of *B. mori* for in vivo expression. The methodologies for the construction and use recombinant AcMNPVs and BmNPVs for the expression of heterologous genes have been thoroughly described (Merrington et al., 1999; O’Reilly et al., 1992; Richardson, 1995; Summers and Smith, 1987). These methodologies, with slight modifications, have also been used for the construction of GM baculovirus pesticides.

The studies to date indicate that GM baculoviruses can easily become an integral part of pest insect control, especially in developing countries and for the control of insects that have become resistant to synthetic chemical pesticides (for review see Bonning et al., 2002; Bonning and Hammock, 1996; Hammock et al., 1993; Harrison and Bonning, 2000a; Inceoglu et al., 2001a; McCutchen and Hammock, 1994; Miller, 1995; Wood, 1996). Several innovative and successful approaches have been taken to improve the speed of kill of a baculovirus by genetic modification. These approaches include (1) insertion of a foreign gene into the baculovirus genome whose product alters the physiology of the target host insect or is toxic towards the target host, (2) deletion of an endogenous gene from the baculovirus genome, and (3) incorporation of active toxin into the OV. Combinations of these approaches have also been successful in terms of decreasing the time required to kill the host or more importantly the time required to stop host feeding. Safe and effective protection of the crop from feeding damage should be the goal of a GM baculovirus pesticide. Baculoviruses have been transformed from natural disease agents to efficient pesticides through the above-mentioned discoveries and innovative approaches. Here, we discuss the numerous innovations that have been used to improve the efficacy of baculoviruses for crop protection. Additionally, we will discuss studies that have addressed the safety of natural and GM baculoviruses especially in terms of risk to humans, the environment, and nontarget beneficial insects. As the readers go through the individual sections of this article, they will hopefully find a detailed and comprehensive summary of the filed especially in terms of improving the insecticidal activity of GM baculoviruses. The readers will also find a discussion of the reasons why GM baculoviruses do not receive the interest that they deserve in the concluding remarks. We hope that after reading this review, the reader will be convinced that the currently available GM baculovirus pesticides are effective and safe. Additionally, we hope that the reader will be convinced, as we are convinced, that GM baculoviruses should be used as biological pesticides.

### 2 Insertion of Hormone and Enzyme Genes

#### 2.1 Hormones

Keeley and Hayes (Keeley and Hayes, 1987) were two of the first to suggest the use of an insect neurohormone gene to increase the insecticidal activity of the baculovirus. They wrote “use of an insect baculovirus as an expression vector for neurohormone genes has several advantages as a pest control strategy. (1) Insect viruses are genera- or species-specific so that the virus has a limited host range and would not affect nontarget insects. (2) The natural hormone would be produced at continuous, high levels by the viral expression vector. (3) The combination of natural insect hormones with insect-specific viruses constitutes an ideal insect pest control agent from the environmental standpoint.” Menn and Borkovec (Menn and Borkovec, 1989) further suggested “a neuropeptide gene placed behind a strong non-essential viral promoter is capable of turning an infected cell into a neuropeptide factory within the insect...” Maeda (Maeda, 1989b) pioneered the field by being the first to put these concepts into practice by...
generating a recombinant BmNPV expressing a diuretic hormone gene that disrupted the normal physiology of larvae of the silkworm *B. mori*. Subsequently, at least four biologically active peptide hormones have been expressed using recombinant baculoviruses: eclosion hormone (Eldridge *et al.*, 1991), prothoracicotropic hormone (O’Reilly *et al.*, 1995), pheromone biosynthesis activating neuropeptide (Ma *et al.*, 1998; Vakharia *et al.*, 1995), and neuroparsin (Girardie *et al.*, 2001). Unfortunately, expression of a biologically active hormone by the recombinant baculovirus has only been modestly successful or unsuccessful (Fig. 1) in terms of improving the speed of kill of the baculovirus. In hindsight, this lack of success is not completely unexpected since critical events in the insect’s physiology and life cycle are often protected by sequestration (by physical means or through time) or by overlapping systems. As we learn more about the regulatory mechanisms and timing of the hormonal control systems of insects, we should be able to develop more refined approaches to the use of hormone genes to improve the baculovirus as a pesticide.

2.1.1 Diuretic hormone

Diuretic and anti-diuretic hormones play critical roles in the excretion and retention of water by insects in response to changes in their environment (Coast *et al.*, 2002; Gade, 2004; Holman *et al.*, 1990). The tobacco hornworm *Manduca sexta* encodes a neuropeptide hormone consisting of 41 amino acid residues that stimulates diuresis (Kataoka *et al.*, 1989). Because of the relatively short length of this peptide hormone, Maeda (Maeda, 1989b) was able to generate a synthetic gene encoding the peptide...
and attempted to express this gene in fifth instar larvae of *B. mori* using BmNPV. The synthetic diuretic hormone (DH) gene was designed on the basis of the codon usage of the polyhedrin gene of BmNPV and the gene structure of an amidated peptide from the giant silk moth *Hyalophora cecropia*. The DH gene construct also included a signal sequence for secretion from a cuticle protein (CPII) of *Drosophila melanogaster* (Meigan) (Snyder et al., 1982) and a glycine residue at the C-terminus for amidation. The recombinant BmNPV (BmDH5) carrying the synthetic DH gene killed fifth instar larvae about one day faster than the wild type BmNPV (Maeda, 1989b). This corresponded to a roughly 20% improvement in speed of kill in comparison to the wild type BmNPV. BmDH5 also caused a 30% reduction in hemolymph volume, which was hypothesized to be due to the excretion of water into the Malpighian tubules, and subsequently hindgut and outside (Maeda, 1989b). Biologically active DH, however, was not detected in the circulating hemolymph of these animals (D. A. Schooley, unpublished). The 20% improvement in speed of kill is modest in comparison to more recent GM baculovirus constructs. And, the bioassays were based on injection of BV rather than oral infection with polyhedra (the normal mode of baculovirus infection in the field). Nevertheless, these experiments generated a lot of excitement in the study of genetically modified baculoviruses as pesticides and established some of the groundwork for subsequent GM baculovirus pesticide constructs.

### 2.1.2 Ecdysis hormone

Ecdysis hormones (EH) are neuropeptides that influence several aspects of pupal-adult ecdysis (i.e., eclosion) as well as larval–laryal ecdyses (Holman et al., 1990; Nijhout, 1994). The release of EH from the brain is controlled by a circadian clock within the brain and declining ecdysteroid titers. The overexpression of EH by a recombinant baculovirus has been hypothesized to induce the premature onset of eclosion behavior and molting. A cDNA encoding EH of *M. sexta* has been inserted into recombinant AcMNPVs and shown to express high levels of biologically active and secreted EH (Eldridge et al., 1991, 1992b). Time-mortality bioassays in fifth or sixth instar larvae of *S. frugiperda* injected with vEHEGTD (a recombinant AcMNPV carrying the EH encoding cDNA at the ecdysial UDP-glucosyltransferase (egt) gene locus) showed that the median survival times (ST$_{50}$s) were reduced by approximately 29% (83 vs. 117 h) and 17% (100 vs. 120 h), respectively, in comparison to control larvae injected with wild type AcMNPV. However, in comparison to control larvae injected with vEGETDEL (a control virus in which the egt gene was deleted), the ST$_{50}$s were not significantly different. Similar results were generated by time-mortality bioassays in neonate *S. frugiperda* that were orally inoculated with vEHEGTD, vEGETDEL or AcMNPV. These findings indicated that deletion of the egt gene (rather than expression of EH) was responsible for the reduction in the ST$_{50}$. Eldridge et al. (Eldridge et al., 1992b) thus concluded that EH expression does not provide enhanced biological control properties to AcMNPV. The role of the egt gene in improving speed of kill is discussed later in this article.

### 2.1.3 Prothoracicotropic hormone

Prothoracicotropic hormones (PTTH) or ‘brain hormones’ are neurosecretory polypeptides that stimulate the secretion of ecdysteroids from the prothoracic glands (Holman et al., 1990; Nijhout, 1994). The over expression of PTTH by a recombinant baculovirus has been hypothesized to artificially elevate ecdysteroid levels resulting in the premature induction of molting, which in turn could disrupt insect feeding behavior (Black et al., 1997; Menn and Borkovec, 1989; O’Reilly et al., 1995). O’Reilly et al. (O’Reilly et al., 1995) have constructed a recombinant AcMNPV expressing the mature PTTH of *B. mori* (fused to a secretion signal sequence of sarcotoxin 1A of the flesh fly). This virus expressed PTTH in larvae of *S. frugiperda* resulting in the induction of higher than normal levels of hemolymph ecdysteroids. However, the expressed PTTH produced no observable effects on the development of the *S. frugiperda* and, furthermore, inhibited the pathogenicity of the virus. In the same study, O’Reilly et al. (O’Reilly et al., 1995) expressed the mature PTTH-signal sequence construct using a mutant AcMNPV in which the AcMNPV-encoded egt gene was knocked out. This virus apparently generated an even higher level of peak hemolymph ecdysteroid titer ($1162 \pm 476$ vs. $724 \pm 194$ pg/µL) in comparison to a control virus that expressed PTTH but retained a normal egt gene. This higher ecdysteroid peak, however, was delayed by about 48 h (i.e., the peak occurred at 96 vs. 48 h post infection (p.i.)). These findings suggest that higher levels of hemolymph ecdysteroids are detrimental to the pathogenicity of the virus. In contrast, other studies (as will be discussed later) show that inactivation of the egt gene (putatively resulting in higher ecdysteroid levels) results in improved pathogenicity.

### 2.1.4 Pheromone biosynthesis activating neuropeptide

Pheromone biosynthesis activating neuropeptide (PBAN) is a neurosecretory peptide (33–34 amino acid residues) of the brain that appears to be essential for pheromone biosynthesis and release in some lepidopterans (Holman et al., 1990; Nijhout, 1994). PBAN stimulates pheromone biosynthesis in conjunction with nervous stimuli during the dark phase of the photoperiod. Ma et al. (Ma et al., 1998) expressed a PBAN of the corn earworm *Helicoverpa zea* (Hez-PBAN) fused to the bombyxin (an insect neurohormone) signal sequence for secretion using a recombinant AcMNPV (AcBX-PBAN). AcBX-PBAN expressed biologically active PBAN in cultured cells and larvae of *Trichoplusia ni*. In droplet feeding assays using pre-occluded virus, neonate and third instar larvae of *T. ni* infected with AcBX-PBAN showed ST$_{50}$s that were reduced by 26% (58.3 vs. 79.1 h) and 19% (70.2 vs. 87.0 h), respectively, in comparison to larvae infected with a control virus. Although the mechanism for the improved speed of kill is unknown, Ma et al. (1998) did not observe any morphological differences between larvae that were infected with AcBX-PBAN or the control virus.
2.2 Juvenile Hormone Esterase

V.B. Wigglesworth (Wigglesworth, 1935, 1936) was the first to identify a “juvenile factor” produced by the corpora allata that keeps larval insects in the juvenile state. Subsequently, Röller et al. (Roller et al., 1967) and Meyer et al. (Meyer et al., 1970) showed the chemical structure of “juvenile hormone (JH)”. Six JHs (JH-0, JH-I, JH-II, JH-III, 4-methyl JH-1, and HbJ) have been identified to date all of which are terpenoids derived from farnesenic acid (or its homologs) with an epoxide group at the 10,11 position of one end and a conjugated methyl ester at the other end (Cusson and Palli, 2000; Nijhout, 1994). JH-III appears to be the most common of the JHs being found in all of the insect orders examined to date. In addition to their function as a “juvenile factor” JHs and/or their metabolites are involved in a diverse array of other functionalities including roles in development, metamorphosis, reproduction, diapause, migration, polyphenism, and metabolism (Gilbert et al., 2000; Riddiford, 1994; Truman and Riddiford, 2002). These diverse functionalities clearly indicate that the biosynthesis, transport, sequestration, and degradation of JH and/or its metabolites must be carefully regulated. Conversely, this careful regulation of JH titer opens a window of attack where a recombinant baculovirus expressing an appropriate protein(s) could disrupt the fine balance in JH titer and consequently the insect life cycle. Two pathways for the degradation of JH have been intensively studied in insects (de Kort and Granger, 1996; Hammock, 1985; Roe and Venkatesh, 1990). One involves a soluble esterase, JH esterase (JHE), that hydrolyzes the methyl ester moiety at one end of the JH molecule resulting in a carboxylic acid moiety (Kamita et al., 2003a). The other involves a microsomal epoxide hydrolase, JH epoxide hydrolase (JHEH) that hydrolyzes an epoxide moiety at the other end of the JH molecule to produce a diol.

Hammock et al. (Hammock et al., 1990a) first hypothesized that the natural insecticidal activity of the baculovirus AcMNPV could be improved by expression of a gene encoding HJE. The rationale behind this was that the recombinant AcMNPV would be ingested by early larval instars, and subsequently JHE would be produced at a point in development that is inappropriate for the insect. The observed result was that infected larvae reduced feeding and weight gain, and subsequently died slightly more quickly in comparison to the wild type AcMNPV (Eldridge et al., 1992a; Hammock et al., 1990a, b). Two approaches to improve this technology have included (1) improving the in vivo stability (i.e., reducing removal from the hemolymph and/or degradation) of the JHE enzyme by genetic modification of the JHE gene and (2) increasing or altering the timing of gene expression by using alternative promoters to drive JHE expression. Recombinant baculoviruses expressing JHE have also been used as tools for hypothesis testing with regard to the biological activity of JHE within the insect host (van Meer et al., 2000). JHE proteins from at least five different insect species (H. virescens [Bonning et al., 1992; Hammock et al., 1990a], Choristoneura fumiferana [Feng et al., 1999], B. mori [Hirai et al., 2002], M. sexta [Hinton and Hammock, 2003a], and T. molitor [Hinton and Hammock, 2003b]) have been expressed using recombinant AcMNPVs.

2.2.1 Increase in vivo stability

Studies have shown that recombinant JHE from H. virescens, when injected into larvae of M. sexta, is rapidly recognized and taken up into the pericardial cells from the hemolymph (Booth et al., 1992; Ichinose et al., 1992a,b). This removal of JHE from the hemolymph occurs by a receptor-mediated, endocytotic, saturable mechanism that does not involve passive filtration (Bonning et al., 1997a; Ichinose et al., 1992a,b). The JHE is presumed to be degraded in lysosomes (Booth et al., 1992). At least two putative JHE binding proteins may be involved in transport and/or degradation of JHE in the pericardial cells, including a putative heat shock cognate protein (Hsp) (Bonning et al., 1997a) and P29 (Shanmugavelu et al., 2000, 2001). Receptor-mediated endocytosis of JHE has been demonstrated in early and late larval instars of M. sexta (Ichinose et al., 1992a). Although JHE is normally stable in hemolymph, the half-life of JHE injected into the hemolymph can be as little as 20 min under conditions where endogenous and exogenous proteins including bovine serum albumin, ovalbumin, and hemolymph JH binding protein have half-lives of days (Ichinose et al., 1992a). Thus, the mechanism by which JHE is specifically recognized and removed by the pericardial cells could be a very important target for increasing the half-life of JHE in the hemolymph. There are three ways in which the uptake and degradation of JHE can be disrupted (1) prevention of receptor-mediated uptake by the pericardial cells, (2) disruption of transport to the lysosomes, and (3) prevention of lysosomal degradation.

Bonning et al., (Bonning et al., 1997b) identified two lysine residues that are likely to be on the surface of the JHE protein of H. virescens and potentially involved in uptake or degradation of JHE. These lysine residues are Lys-29 near the N terminus, which is potentially involved in ubiquitin conjugation and Lys-524, which is located within a putative lysosome targeting sequence. A mutated JHE protein (JHE-KK) in which both of these lysine residues were mutated to arginine residues showed decreased efficiency of lysosomal targeting (Bonning et al., 1997b), and binds to the putative JHE binding protein (P29) with significantly less affinity than authentic JHE (Shanmugavelu et al., 2000). However, JHE-KK as well as mutant JHEs in which only one of the lysine residues were mutated to arginine residues showed similar catalytic activities and removal rates of JH from the hemolymph as the authentic JHE. A high-resolution crystal structure should assist with determining the basis of the interaction of JHE and the putative JHE binding protein P29 described by Shanmugavelu et al. (2000). These researchers hypothesize that P29 interacts with JHE of H. virescens and facilitates targeting of JHE to lysosomes within pericardial cells. Other putative JHE binding proteins have been identified in M. sexta, and their possible roles in the degradation of JHE remain to be elucidated (Shanmugavelu et al., 2001). First instar larvae of H. virescens that were infected with a recombinant AcMNPV expressing JHE-KK (AcJHE-KK) under a very late viral promoter died approximately 22% faster (ST50 of 90 vs. 116 h) than control larvae infected with a recombinant AcMNPV expressing the authentic JHE (AcJHE) (Bonning et al., 1999). In similar experiments, first instar T. ni that were infected with AcJHE-KK died approximately 27% faster (ST50 of 83 vs. 113 h) compared to control larvae infected with AcJHE. Kunimi et al. (Kunimi et al., 2003b)
et al., 1996) have analyzed the survival times of third to fifth instar T. ni that were infected with AcJHE-KK. In these older insects, they found that the ST50s of AcJHE-KK-infected third, fourth, and fifth larvae were reduced by roughly 4% (124.8 vs. 130.2 h), 9% (126.6 vs. 138.6 h), and 8% (135.0 vs. 147.0 h), respectively, in comparison to control AcMNPV-infected larvae. In similar bioassays of second to fifth instars of the soybean looper Pseudoplusia includens, AcJHE-KK-infected larvae did not show a reduced survival time in comparison to AcMNPV-infected control larvae (Kunimi et al., 1997). The reasons for these differences in the survival times between species and instars are unknown, however, Bonning et al. (1999) speculated that host- and/or age-specific effects may be involved.

### 2.2.2 Gene silencing and RNAi

AcPR1 is a recombinant AcMNPV that produces biologically active JHE of H. virescens (Hanzlik et al., 1989) under the very late baculoviral p10 gene promoter (Roelvink et al., 1992). AcPR2 is a recombinant AcMNPV in which the same JHE gene is placed under the p10 promoter in the antisense direction. Transcription of this gene generates mRNAs that are antisense to the JHE mRNAs generated by AcPR1. These antisense JHE mRNAs are able to reduce the JHE activity produced by AcPR1 when AcPR1 and AcPR2 are coinfected into Spodoptera frugiperda (Roelvink et al., 1992). By injection of AcPR2 into fifth instar H. virescens, Hajos et al. (Hajos et al., 1999) showed that AcPR2 down regulates JHE activity in more than 95% of the injected larvae. This effect was putatively due to direct RNA-RNA interaction between sense and antisense JHE RNAs. Although 95% of the infected larvae showed a reduction in JHE activity, only 25% of these larvae showed morphogenic alterations (Hajos et al., 1999). These alterations were similar to those induced by the exogenous application of JH (Cymborowski and Zimowska, 1984) or JHE inhibitors (Hammock et al., 1984) to the larvae. On the basis of the effectiveness of this baculovirus-mediated gene silencing approach, Hajos et al. (1999) suggest that the identification of other host gene targets could greatly reduce lethal times or feeding damage of a GM baculovirus pesticide.

RNA interference or RNAi is a sequence-specific mechanism by which a targeted gene is silenced by the introduction of double-stranded RNA (dsRNA) that is homologous to the targeted gene (Denli and Hannon, 2003; Hutvagner and Zamore, 2002). Mechanistically, it is likely that the antisense RNA-mediated gene silencing described by Hajos et al. (1999) involves the RNAi pathway. RNAi has been demonstrated in a diverse range of organisms including plants, fungi, arthropods, and mammals. One biological function of RNAi appears to be as a defense mechanism against RNA viruses in plants and other organisms. Recently, RNAi has been shown to be effective in lepidopteran cell lines (Means et al., 2003; Valdes et al., 2003) and larvae such as the giant silk moth, H. cecropia (Bettencourt et al., 2002) and cabbage looper T. ni (Kramer and Bentley, 2003). Our laboratory has explored the use of RNAi to block the activity and effects of JHE in cultured S. frugiperda cells and larvae of H. virescens. In the larval experiments, a 0.5 kbp dsRNA fragment that corresponds to the 5'-coding sequence of the JHE gene of H. virescens (Hanzlik et al., 1989) was generated. Neonate H. virescens that ingested these dsRNAs within 6 h of hatching showed a statistically significant (two sided Mann-Whitney-Test with an error type I of 99%) increase in weight (in comparison to control larvae) by the second and third days of the fifth larval instar. Additionally, injection of the same 0.5 kbp dsRNAs into larvae of H. virescens on the first day of the fifth instar resulted in a 16 h delay in the median time to pupation in comparison to water-injected or untreated controls (Fig. 2). Treatments were compared with Wilcoxon (Gehan) statistics on a 99% error type I. These comparisons indicated that RNAi against the JHE of H. virescens is effective at prolonging the juvenile state. We are currently in the process of constructing recombinant AcMNPVs carrying JHE gene-derived sequences in a “head-to-head” manner such that the expressed RNAs will form a hairpin loop structure. Such a recombinant AcMNPV may be able to more efficiently induce the RNAi response. Of course, a recombinant virus that is effective at prolonging the juvenile state would be a disaster as a biological insecticide. However, these

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**Fig. 2** Percentage of larvae that pupate over time following treatment with double-stranded RNA, double-distilled water, or untreated. Pupation was scored on the basis of head capsule slippage at 1.5 h or longer intervals.
experiments are important in that they demonstrate the effectiveness of an RNAi approach in the regulation of insect neuroendocrinology and the results should be easily transferred to other significant physiological effectors.

The use of an authentic insect-derived gene to combat the insect is conceptually elegant and potentially safer because one is only trying to express an endogenous gene at an inappropriate time or at higher levels in order to alter the physiology of the insect. From the insect point of view this might be analogous to contracting a serious hereditary genetic disease. In practice, however, overexpression of an insect hormone gene (or a gene involved in hormone metabolism) has not been dramatically effective.

2.3 Proteases

In order to better understand the rationale behind the use of protease encoding genes for improving speed of kill, we will first give a brief review of the midgut barriers faced by the baculovirus. The first barrier that the ODV encounters in the midgut is the fluid within the lumen. The high pH (8 – 11) and endogenous proteases found in the lumen are initially required to release ODVs from the polyhedra or granules. However, digestive enzymes or other factors within the lumen could also lead to the inactivation of the released ODV, if the ODVs do not quickly initiate infection. One such factor is a lipase, Bmlipase-1, isolated from the digestive juice of B. mori that shows antiviral activity against BmNPV (Ponnuvel et al., 2003). The peritrophic membrane (or peritrophic envelope) is the first physical barrier that the ODV faces (Brandt et al., 1978; Richards and Richards, 1977; Tellam, 1996). The peritrophic membrane (PM) is composed of chitin fibrils in a protein-carbohydrate matrix. The specific composition and layout of the PM differs in different lepidopteran species and at different larval instars. The manner in which the ODVs pass this barrier is still not well understood. The second physical barrier that the virus faces is the midgut epithelium. The midgut epithelium is primarily composed of a single layer of columnar cells. The columnar cells show directionality (polarity) in which the luminal border is formed by microvilli (brush border membrane) and the hemocoelic border shows characteristic infolding with large numbers of mitochondria associated with the infolds. Regenerative and endocrine cells are also part of the midgut epithelium. Several pathways have been proposed for how the nucleocapsids pass through the midgut epithelium barrier following the fusion of ODVs with the microvillar membrane (Federici, 1997). One pathway involves entry of the nucleocapsid into the columnar cells, translocation through the cytoplasm, and budding through the hemocoelic border of the same cell without any viral replication. The other pathways involve the generation of progeny nucleocapsids within the columnar cells that can bud (1) through the hemocoelic border, (2) into the tracheal matrix via tracheoblasts or (3) into regenerative cells. Once in the regenerative cells the nucleocapsids may translocate through the cell without replication and through the hemocoelic border or virus replication may occur in these cells as well. Washburn et al. (Washburn et al., 2003) have proposed a ‘hybrid’ pathway in which some nucleocapsids of MNPs uncoat and start replication whereas others pass directly through the columnar cells. The third midgut-associated barrier to systemic infection is the basement membrane (or basal lamina). The basement membrane (BM) is a fibrous matrix composed primarily of glycoproteins, type IV collagen, and laminin that are secreted by the epithelial cells (Ryser, 1998). The BM functions in several roles including structural support of the epithelium or surrounding tissues, filtration, and differentiation (Yurchenco and O’Rear, 1993).

2.3.1 Enhancins

Enhancins are baculovirus-encoded proteins that can enhance the oral infectivity of a heterologous or homologous baculovirus. Enhancin, also known as synergistic factor (SF) or virus enhancing factor (VEF), was first described by Tanada as a lipoprotein of 93–126 kDa that is found within the capsule of the GV of the armyworm Pseudaletia unipuncta (Yamamoto and Tanada, 1978a,b) that enhances the infection of baculoviruses in lepidopteran larvae (Tanada, 1959). The enhancin of the GV of T. ni (TnGV) has been shown to possess malloprotease activity (Lepore et al., 1996; Wang and Granados, 1997). Tanada first suggested that this factor is virus-encoded (Tanada and Hukuhara, 1968) and Hashimoto et al. (Hashimoto et al., 1991) confirmed this by cloning and sequencing of the ref gene from TnGV. Subsequently, enhancin gene homologs have been sequenced from the GVs of Helicoverpa armigera and P. unipuncta (Roelvink et al., 1995) and Xestia c-nigrum (four homologs (Hayakawa et al., 1999)). Enhancin gene homologs have also been identified in two NPVs, Lymantria dispar NPV (two homologs, E1 (Bischoff and Slavicek, 1997) and E2 (Popham et al., 2001)) and Mamestra configurata NPV (Li et al., 2003). Disruption of the E1 enhancin gene homolog of LdMNVP reduces viral potency from 1.4- to 4-fold in comparison to the wild type LdMNVP but does not affect the killing speed of the virus (Bischoff and Slavicek, 1997). Enhancins appear to enhance virus infection by (1) degrading high molecular weight proteins such as mucin (Wang and Granados, 1997) that are found in the PM (Derksen and Granados, 1988; Gallo et al., 1991; Lepore et al., 1996; Wang and Granados, 1997) and/or (2) enhancing fusion of the virus to the midgut epithelium cells (Kozuma and Hukuhara, 1994; Ohba and Tanada, 1984; Wang et al., 1994).

Enhancin genes have been expressed by recombinant AcMNPVs in order to improve the ability of the virus to gain access to the midgut epithelium cells. A recombinant AcMNPV, AcMNPV-enMP2, carrying the enhancin gene of M. configurata NPV under its authentic promoter has been constructed (Li et al., 2003; Popham et al., 2001). In dose-mortality studies, AcMNPV-enMP2 shows a 4.4-fold lower median lethal dose (LD50) (2.8 vs. 12.4 polyhedra/larva) in second instar larvae of T. ni in comparison to the wild type virus (Li et al., 2003). In time-mortality studies, the ST50 of AcMNPV-enMP2-infected larvae was not significantly different from that of wild type AcMNPV-infected larvae when the larvae were inoculated at biologically equivalent doses (i.e., at an LD90 dose). However, when the larvae were inoculated with the same number (i.e., 32 polyhedra/larva) of AcMNPV-enMP2 or wild type
AcMNPV, the AcMNPV-enMP2-inoculated larvae showed an approximately 8% faster ST50 (130 vs. 141 h). Li et al. (2003) suggest that this enhancement in speed of kill may be the result of increasing the number of initial infection foci in the host insect midgut rather than increasing the speed of the spread of the virus infection.

Hayakawa et al. (Hayakawa et al., 2000b) have generated a recombinant AcMNPV (AcEnh26) expressing the enhancin gene from T. ni granulovirus. In bioassays in which third instar larvae of S. exigua were fed a mixture of AcEnh26-infected Sf-9 cells and AcMNPV polyhedra, the LD50 of AcMNPV was 21-fold lower in comparison to feeding with a mixture of control virus-infected Sf-9 cells and AcMNPV (2.69 × 10⁵ vs. 5.75 × 10⁶ polyhedra/larva). In similar experiments, the LD50 of another baculovirus, S. exigua NPV (SeNPV), was 9-fold lower (19.1 vs. 162 polyhedra/larva) when co-infected with AcEnh26-infected Sf-9 cells. In an interesting twist to these experiments, Hayakawa et al. (Hayakawa et al., 2000b) engineered the enhancin gene from T. ni into tobacco plants and used a mixture of AcMNPV and lyophilized transgenic tobacco leaves for bioassay. They found a 10-fold reduction in the LD50 of AcMNPV when the enhancin-expressing tobacco was added to the bioassay. These findings suggest that expression of the enhancin gene in plants (Cao et al., 2002; Granados et al., 2001) or mixing enhancin-expressing plant material in baculovirus pesticide formulations may be an effective method for improving the speed of kill of natural and recombinant baculoviruses.

### 2.3.2 Basement membrane degrading proteases

As mentioned above, the basement membrane is the last midgut-associated barrier that the baculovirus virion must cross prior to the initiation of systemic infection. Harrison and Bonning (Harrison and Bonning, 2001) have constructed recombinant AcMNPVs expressing three different proteases (rat stromelysin-1, human gelatinase A, and cathepsin L from the flesh fly Sarcophaga peregrina) that are known to digest BM proteins. Of these recombinant AcMNPVs, AcMLF9. ScathL expressing cathepsin L under the late baculovirus p6.9 gene promoter generated a 51% faster speed of kill (ST50 of 48 vs. 98 h) in comparison to wild type AcMNPV in neonate larvae of H. virescens. Expression of gelatinase under the p6.9 promoter also improved speed of kill by approximately 12% (ST50 of 86.5 vs. 98 h) in comparison to wild type AcMNPV. On the basis of lethal-concentration bioassays, there were no statistically significant differences in the virulence (i.e., LD50) of these viruses in comparison to the wild type AcMNPV. In bioassays to determine feeding damage, AcMLF9. ScathL-infected larvae consumed approximately 27- and 5-fold less lettuce (29.27 and 5.21 cm², respectively) in comparison to mock- or AcMNPV-infected (1.1 cm²) second instars of H. virescens, respectively. Although the mechanism of this improvement in speed of kill has yet to be determined, Harrison and Bonning (2001) speculated that digestion of BM proteins by the cathepsin L may (1) hasten the spread of infection through the BM, (2) inappropriately activate prophenoloxidase, a key enzyme involved in the formation of melanin during wound healing and the immune response in insects, and/or (3) cause BM damage that results in deregulation of ionic and molecular traffic between the hemocoel and the tissues. Harrison and Bonning (2001) also expressed cathepsin L under the baculovirus early 1el promoter using the recombinant baculovirus AcEI1TV3. ScathL. This virus, however, did not show any improvement in speed of kill. They speculated that expression under the early promoter did not produce sufficient cathepsin L to have an effect on the infected host. The effectiveness of protein expression using early, late, and/or constitutively expressed promoters will be further discussed later.

### 2.4 Other Enzymes and Factors

Chitin is an insoluble structural polysaccharide that is found in the exoskeleton and gut linings of insects (Cohen, 1987). Chitinases are enzymes that can degrade chitin into low molecular weight, soluble and insoluble oligosaccharides (Kramer and Muthukrishnan, 1997). Genes encoding chitinases have been identified in several lepidopteran insects including M. sexta (Gopalakrishnan et al., 1993), B. mori (Daimon et al., 2003; Kim et al., 1998), Hypantria cunea (Kim et al., 1998), S. litura (Shinoda et al., 2001), and C. fumiferanae (Zheng et al., 2002). The genome of B. mori appears to encode multiple, potentially five different, chitinase genes (Daimon et al., 2003). Chitinase and cysteine protease genes are also found in the genomes of NPVs and GVs (Gong et al., 2000; Hawtin et al., 1995; Kang et al., 1998; Ohkawa et al., 1994; Slack et al., 1995). Baculovirus-encoded chitinases and proteases (V-CATH) are thought to be involved in the degradation of the chitinous and proteinaceous components, respectively, of the host cadaver in order to induce liquefaction (Him and Volkman, 2000; O'Reilly, 1997). Hom and Volkman (2000) have suggested another role of the baculovirus-encoded chitinase, namely, proper folding of the nascent V-CATH polypeptides in the endoplasmic reticulum. A recombinant AcMNPV, vAcMNPV·chi, has been generated that expresses the chitinase gene of M. sexta (Gopalakrishnan et al., 1995). When fourth instar S. frugiperda were injected with vAcMNPV·chi, larval death occurred nearly one day earlier than those infected with the wild type virus AcMNPV.

Carbohydrates are the major source of energy that powers insect metabolism and form the material basis of chitin in the cuticle of insects (Cohen, 1987). Thus, the availability of sugars is essential for normal growth and development. Trehalose, a disaccharide composed of two glucose molecules, is the major carbohydrate in the hemolymph of most insects (Friedman, 1978). Sato et al. (Sato et al., 1997) have generated two recombinant AcMNPVs, vTREVL and vERTVL, expressing a trehalase cDNA from the mealworm beetle Tenebrio molitor in the sense and antisense directions, respectively. In Sf-9 cell cultures and larvae of the cabbage armyworm M. brassicae that were infected with vTREVL, biologically active trehalase was secreted into the supernatant and found in the hemolymph, respectively. The survival time of fifth instar larvae of M. brassicae that were infected with vTREVL was longer than control larvae infected with the wild type AcMNPV. Interestingly, the vTREVL-infected larvae showed considerably reduced melting in comparison to the AcMNPV-infected larvae. The survival time of larvae infected with vERTVL was also slightly longer than that of control larvae infected with AcMNPV, but less than that of vTREVL.
URF13 is a mitochondrially-encoded protein of maize that forms pores in the inner mitochondrial membrane (Korth et al., 1991). Korth and Levings (Korth and Levings, 1993) have generated recombinant, occlusion-negative AcMNPVs expressing authentic (BV13T) or mutated (BV13.3940) URF13-encoding genes under the polh promoter. When third or fourth instar larvae of T. ni were injected with BV13T or BV13.3940, 100% died by 60 h post injection. Control larvae that were injected with wild type AcMNPV or a control β-galactosidase expressing AcMNPV lived up to 106 and 100 h p.i., respectively. The LT50 of BV13T or BV13.3940 appeared reduced by about 45% (50 vs. 94 h) in comparison to control virus-infected larvae (estimate based on mortality graph). Although the mechanism of this toxicity was not determined, Korth and Levings (1993) showed that baculovirus-expressed URF13 is localized in the membranes of cells of Sf-9 cultures and larvae of T. ni. They suggested that this membrane localization interferes with normal cellular functions. The URF13-induced cytoxicity, however, appeared to be unrelated to pore formation, because the mutated URF13 expressed by BV13.3940 is unable to form membrane pores (Braun et al., 1989), but the toxicity of BV13.3940 was the same as that of BV13T.

c-MYC is a transcription factor that is involved in various physiological processes including cell growth, proliferation, loss of differentiation, and apoptosis in a variety of organisms (Pelengaris and Khan, 2003). Lee et al. (Lee et al., 1997) have generated a recombinant AcMNPV carrying a 754 bp-long fragment of human c-myc exon II in the antisense direction under the polh promoter. When third or fourth instar S. frugiperda are injected with this virus at an average dose of 3500 plaque forming units (pfu) per mg of body weight, 75% of the larvae stop feeding by three days post infection. This is roughly a two-fold reduction in feeding in comparison to wild type AcMNPV-injected larvae in which about 35% of the larvae stop feeding. This feeding cessation occurs roughly two days before death of the larva. Furthermore, the average survival time of the larvae is reduced by approximately one day. Lee et al. (1997) indicate two important advantages to this antisense strategy. Firstly, this strategy does not require protein synthesis and also avoids any potential problems associated with post-translational modifications or secretion. Secondly, since no foreign protein is produced the likelihood of insect resistance will be reduced.

Sunlight (i.e., UV radiation) is a major factor in the inactivation of baculoviruses in the field (Black et al., 1997; Dougherty et al., 1996; Ignoffo and Garcia, 1992; Ignoffo et al., 1997). In an attempt to reduce UV inactivation, Petrik et al. (Petrik et al., 2003) have generated a recombinant AcMNPV that expresses an algal virus pyrimidine dimer-specific glycosylase, cv-PDG (Furuta et al., 1997), that is involved in the first steps of the repair of UV-damaged DNA. The recombinant AcMNPV (vHSA50L) expresses the cv-PDG encoding A50L gene under the hsp70 promoter of D. melanogaster. Polyhedra of vHSA50L were not more resistant to UV inactivation than the wild type AcMNPV, however, BV of vHSA50L were 3-fold more resistant to UV inactivation. In dose-mortality bioassays in neonate T. ni, the LC50 of vHSA50L and AcMNPV were not significantly different. In neonates of S. frugiperda, however, the LC50 of vHSA50L was reduced by approximately 16-fold (7.1 × 105 vs. 1.15 × 106 polyhedra per ml). Surprisingly, time-mortality bioassays (at an LC50 dose) in vHSA50L-infected neonates of S. frugiperda indicated that the LT50 of vHSA50L was reduced by approximately 41% (73.6 vs. 124.4 h). This reduction in LT50 was even greater (48%, 65.0 vs. 124.4 h) for a construct (vHSA50LORF) that carried A50L excluding the 5′-untranslated region under the hsp70 promoter. In contrast to the neonate S. frugiperda, significant differences were not found in the LT50s of neonate T. ni that were infected with vHSA50L or vHSA50LORF.

3 Insertion of Insect-Selective Toxin Genes

Arthropods that feed on or parasitize other arthropods possess potent venoms that rapidly immobilize their prey. The venoms of arthropods such as scorpions, spiders, and parasitic wasps are composed of a mixture of salts, small molecules, proteins, and peptides (Gordon et al., 1998; Loret and Hammock, 1993; Possani et al., 1999; Zlotkin, 1991; Zlotkin et al., 1985, 1978). Peptide toxins that are specifically active against invertebrates, vertebrates, or both invertebrates and vertebrates have been identified in these venoms. These peptide toxins generally target major ion channels such as the Na+, K+, Ca2+, and Cl− channels although there are several examples of peptides with unusual targets such as the intracellular calcium-activated ryanodine channel (Fajloun et al., 2000). Kinin-like peptides that interfere with physiological events have also been identified in venom (Inceoglu and Hammock, unpublished). Arthropod venoms have been utilized as highly useful sources for the mining of highly potent and selective peptide toxins that selectively paralyze insects. Several of these peptides have been expressed by recombinant baculoviruses for the purpose of improving their speed of kill and insecticidal activity.

3.1 Scorpion Toxins

On average scorpion venom contains from 200 to 500 different peptides. It is conservatively assumed that about 10% of these peptides are insect-selective toxins. Considering that there are roughly 1250 identified species of scorpion, this represents an enormous diversity (25,000 to 62,500) of potent and selective insecticidal peptides for pest control using baculovirus expression. Scorpion toxins are classified on the basis of size and pharmacological target site into two main groups: long and short chain neurotoxins (Loret and Hammock, 1993; Zlotkin et al., 1978). Toxins with selective activity against insects are found in both the long and short chain groups. Long chain neurotoxins are polypeptides of 58–70 amino acid residues, cross-linked by four disulfide bonds, that mainly target voltage-gated sodium channels and to some degree calcium channels (Gordon et al., 1998; Loret and Hammock, 1993; Zlotkin, 1991). Short chain neurotoxins are peptides of 31–37 amino acid residues, cross-linked by three or four disulfide bonds, that mainly target potassium and chloride channels (Loret and Hammock, 1993). The long chain neurotoxins, the
better studied of the two main groups, are further classified into mammal- or insect-selective toxins, with the insect-selective toxins being further classified on the basis of their mode of action into three subgroups: alpha insect toxins, excitatory toxins, and depressant toxins (Gordon et al., 1998; Inceoglu et al., 2001b; Zlotkin et al., 1995). Each of these subgroups target different molecular sites and produce distinct effects upon the voltage-gated sodium channel of the insect (Cestele and Catterall, 2000). In addition, when injected into larvae of the blowfly Sarcophaga falcipulata, toxins in each of these subgroups display unique symptoms. Alpha insect toxins cause contractive paralysis that is delayed and sustained (Eitan et al., 1990). Excitatory toxins, such as AaIT from the North African scorpion Androctonus australis, cause paralysis that is immediate and contractive (Zlotkin, 1991; Zlotkin et al., 1971, 1985). In contrast, depressant toxins, such as LqHT2 from the yellow Israeli scorpion Leirus quinquestriatorius hebraeus, cause transient (until 5 min post injection) contractive paralysis, followed by sustained, flaccid paralysis (Zlotkin, 1991, 1985).

Carbonell et al. (Carbonell et al., 1988) were the first to attempt to express biologically active scorpion toxin, insectotoxin-1 of Buthus eupeus (BeIt), under control of the very late polyhedrin promoter using recombinant AcMNPV constructs (vBeIt-1, vBeIt-2, and vBeIT-3). The vBeIt-1 construct carried the 112 nucleotide-long Belt gene 6 nucleotides downstream of the last nucleotide of the polyhedrin leader. The vBeIt-2 construct carried the Belt gene fused to a 21 amino acid-long signal sequence for secretion of human β-interferon (Ohno and Taniguchi, 1981) 18 nucleotides downstream of the polyhedrin leader. The vBeIt-3 construct expressed Belt as a fusion with the amino-terminal 58 codons of the polyhedrin gene. All three constructs produced similar, high levels of Belt-specific transcripts from the polyhedrin promoter. The vBeIt-1 and vBeIt-2 constructs produced exceptionally low levels of the 4 kDa Belt peptide, whereas the vBeIt-3 construct produced significant amounts of a 13–14 kDa polyhedrin-Belt fusion peptide. Toxic-specific biological activity, however, was not observed by bioassay in larvae of T. ni, Galleria mellonella, and Sarcophaga. Carbonell et al. (1988) speculated that this was the result of (1) toxin instability, (2) a low sensitivity threshold of the toxin bioassay, and/or (3) inability of the polyhedrin-Belt fusion to properly fold.

### 3.1.1 AaIT

The insect-selective neurotoxin AaIT (A. australis insect toxin 1), found in the venom of the scorpion A. australis, was the first scorpion toxin to be expressed by recombinant baculoviruses that showed biological activity (Maeda et al., 1991; McCutchen et al., 1991). AaIT is composed of a single polypeptide chain of 70 amino acid residues cross-linked by four disulfide bonds and is highly specific for the voltage-gated sodium channel of insects (Zlotkin et al., 2000). The toxin induces a neurological response similar to that evoked by the pyrethroid insecticides, but apparently acts at a different site within the sodium channel (see discussion below regarding toxin interactions with chemical pesticides). Maeda et al. (Maeda et al., 1991) constructed a recombinant BmNPV carrying a synthetic AaIT gene (Darbon et al., 1982) that was linked to a signal sequence for secretion of bombyxin and driven by the very late polyhedrin (polh) gene promoter. The recombinant virus, BmAaIT, expressed biologically active AaIT that was secreted into the hemolymph of BmAaIT-infected silkworm larvae. The BmAaIT-infected larvae (second to fifth instar B. mori) displayed symptoms that were consistent with sodium channel binding by AaIT including body tremors, dorsal arching, feeding cessation, and paralysis beginning at 40 h p.i. Death occurred by 60 h p.i. This corresponded to an improvement in speed of kill of approximately 40% in comparison to control larvae infected with the wild type BmNPV.

In related experiments, McCutchen et al. (McCutchen et al., 1991) and Stewart et al. (Stewart et al., 1991) independently generated recombinant AcMNPVs expressing AaIT under the very late baculoviral p10 promoter. The Stewart et al. construct, AcST-3, expressed AaIT as a fusion protein with the signal sequence of the baculoviral GP67 protein. The McCutchen et al. construct, AcAaIT, expressed AaIT as a fusion protein with the bombyxin signal sequence. Bioassays using orally inoculated, second instar larvae of T. ni indicated that the median lethal dose (LD₅₀) of AcST-3 was reduced by about 30% (44 vs. 31 polyhedra per larva) in comparison to the wild type AcMNPV. The median survival time (ST₅₀) of AcST-3-infected larvae (infected with approximately 17.4 polyhedra per larva) was reduced by about 25% (85.8 vs. 113.1 h) in comparison to control larvae infected with AcMNPV. Third instar T. ni that are infected with a dose of AcST-3 that resulted in 100% mortality showed a 50% reduction in feeding damage in comparison to AcMNPV-infected control larvae. The McCutchen et al. construct, AcAaIT, showed similar results by bioassay using second instar larvae of H. virescens. The LD₅₀ of AcAaIT was reduced by about 39% (13.3 vs. 21.9 polyhedra per larva) in comparison to AcMNPV. The ST₅₀ of AcAaIT-infected larvae (infected with 250 polyhedra per larva) was reduced by about 30% (88.0 vs. 125 h) in comparison to control larvae infected with AcMNPV. By droplet feeding assays of neonate H. virescens, the ST₅₀ of AcAaIT-infected larvae was reduced by up to 46% (Inceoglu and Hammock unpublished). McCutchen et al. conducted additional experiments in which third instar larvae of M. sexta (an unnatural host of AcMNPV) were injected with AcAaIT (5 μL containing 1 × 10⁶ pfu). They detected AaIT-specific symptoms as early as 72 h post injection and found a 29% decrease (120 vs. 168 h) in the speed of kill. Additional observations made during this study showed that larvae infected with AcAaIT typically were paralyzed and stopped feeding several hours prior to death. Furthermore, the yields of progeny viruses (polyhedra per cadaver) in AcAaIT-infected larvae (third, fourth or fifth instar T. ni) were only 20–32% of those of control larvae infected with AcMNPV (Fuxa et al., 1998; Kunimi et al., 1996). This suggested that the recombinant virus will be quickly outcompeted in the field by the wild type virus as will be discussed below.

Hoover et al. (Hoover et al., 1995) have further characterized the paralytic effect of AaIT by bioassay using third instar H. virescens that were inoculated with either AcAaIT or AcMNPV. When the AcAaIT- or AcMNPV-infected larvae were placed on greenhouse cultivated cotton plants, they found that the AcAaIT-infected larvae fell off of the plant approximately 5–11 h before death. This ‘knock-off’ effect occurred before the induction of feeding cessation. As a consequence, the amount of leaf area consumed by the AcAaIT-infected larvae was up to 62% and 72% less than that consumed by the AcMNPV- and mock-infected larvae, respectively (Fig. 3). Knock-off effects have also been observed in field trials (on cotton) to assess the efficacy of
recombinant AcMNPV (Cory et al., 1994) or HaSNPV (Sun et al., 2004) expressing AaIT. On the basis of this knock-off effect, Hoover et al. (Hoover et al., 1995) suggested that median survival time is not necessarily predictive of the reduction in the amount of feeding damage that results from the application of a recombinant baculovirus. Cory et al. (Cory et al., 1994) and Hoover et al. (Hoover et al., 1995) also emphasized that the knock-off effect should reduce foliage contamination because unlike AcAaIT-infected larvae that fall off of the plant, wild type virus-infected larvae tend to die on the plant. Consequently, they suggested that the reduction in virus inoculum on the foliage should decrease the spread and recycling of the recombinant virus in comparison to the wild type virus. A discussion of the fitness of a recombinant virus in comparison to the wild type parent is given later in this article.

The AaIT gene has been expressed using other baculovirus vectors, for example, the NPVs of the mint looper Rachiplusia ou (RoMNPV) (Harrison and Bonning, 2000b) and cotton bollworms H. armigera (HzNPV) (Treacy et al., 2000) and H. zea (Tracy et al., 2000; Sun et al., 2004, 2002). RoMNPV is also known as Anagrapha falcifera Kirby MNPV (Harrison and Bonning, 1999). Harrison and Bonning (Harrison and Bonning, 2000b) have generated recombinant RoMNPVs (Ro6.9AaIT) expressing AaIT fused to the bombyxin signal sequence under the very late promoter or other alternative promoter (e.g., early, chimeric, or constitutive) and inserted at the egt gene locus of the virus.

3.1.2 Lqh and Lqq
A series of highly potent insecticidal toxins have been identified and characterized from the venom of yellow Israeli scorpions, L. quinquestriatus hebraeus and L. quinquestriatus quinquestriatus. Both excitatory (e.g., LqqIT1, LqhIT1, and LqhIT5) and depressive (e.g., LqhIT2 and LqqIT2) insect selective toxins have been isolated from these scorpions (Kopeyan et al., 1990; Moskowitz et al., 1998; Zlotkin, 1991; Zlotkin et al., 1993, 1985). Gershburg et al. (Gershburg et al., 1998) have generated recombinant AcMNPVs expressing the excitatory LqhIT1 toxin under the very late p10 (AcLIT1.p10) and early p35 (AcLIT1.p35) gene promoters, and the depressive LqHT2 toxin under the very late polh gene promoter (AcLIT2.pol). These constructs expressed LqHT1 and LqHT2 that were secreted to the outside of the cell under endogenous secretion signals. On the basis of time-mortality bioassays using neonate H. armigera, they found that the median effective times (ET50) for paralysis and/or death of AcLIT1.p10 and AcLIT2.pol were 66 and 59 h. This was an improvement of roughly 24 and 32%, respectively, in comparison to the wild type AcMNPV (ET50 of 87 h).

3.1.3 LqhIT1
In contrast, the LqhIT1 gene is expressed using recombinant AcMNPV (Cory et al., 1994) or RoMNPV (Harrison and Bonning, 2000b) and cotton bollworms H. armigera (HzNPV) (Treacy et al., 2000) and H. zea (Tracy et al., 2000; Sun et al., 2004, 2002). RoMNPV is also known as Anagrapha falcifera Kirby MNPV (Harrison and Bonning, 1999). Harrison and Bonning (Harrison and Bonning, 2000b) have generated recombinant RoMNPVs (Ro6.9AaIT) expressing AaIT fused to the bombyxin signal sequence under the very late promoter or other alternative promoter (e.g., early, chimeric, or constitutive) and inserted at the egt gene locus of the virus.
The ET<sub>50</sub> was also reduced by about 16% (to 73 h) when LqhIT1 was expressed under the early promoter by AcLIT1.p35, although not as dramatically in comparison to AcLIT1.p10. Imai et al. (Imai et al., 2000) have generated a recombinant BmNPV (BmLqhIT2) expressing LqhIT2 fused to a bombyxin signal sequence for secretion (Tomalski and Miller, 1991). The mode of action of TxP-I is unknown, gene as a precursor protein of 291 amino acid residues. The mature protein is secreted from the insect cell following cleavage to tox34 recombinant RoMNPV expressing AaIT under the

recombinant, occlusion-negative AcMNPV (vEV-Tox34) expressing the

recombinant RoMNPVs expressing LqhIT2 under the p6.9 promoter has been generated. The LD<sub>50</sub>s of infected neonate H. zea and H. virescens infected with of Ro6.9LqhIT2 generated ST<sub>50</sub>s that were approximately 41% (107 vs. 181 h), 42% (74.5 vs. 128 h), and 27% (65.5 vs. 89.5 h) faster, respectively, than those generated in control neonates infected with wild type RoMNPV. The ST<sub>50</sub> of Ro6.9LqhIT2-infected neonate H. zea and H. virescens were also significantly lower than control neonates infected with a recombinant RoMNPV expressing the AaIT promoter.

Chejanovsky et al. (Chejanovsky et al., 1995) have generated a recombinant AcMNPV (AcLz22) that expresses the L. quinquestriatus hebraeus-derived alpha toxin, LqhIT (Eitan et al., 1990). The LT<sub>50</sub> of AcLz22 was roughly 35% faster (78 vs. 120 h) than that of the wild type AcMNPV in larvae of H. armigera. Since the LqhIT toxin binds at a different site on the insect sodium channel from that of the excitatory toxins (Cestele and Catterall, 2000; Zlotkin et al., 1978), Chejanovsky et al. (1995) suggested that a baculovirus expressing both alpha and excitatory toxins may yield a synergistic interaction between the toxins. However, they cautioned that Lqh IT toxin lacks absolute selectivity for insects (Eitan et al., 1990), thus a recombinant baculovirus expressing LqhIT is not appropriate as a biological pesticide. The LqhIT toxin, however, should be an effective tool to study the targeting of different types of toxins on the voltage-gated sodium channel. The effectiveness of the use of multiple synergistic toxins is discussed below. Other examples of the expression of insect-selective toxins from L. quinquestriatus hebraeus using alternative promoters, signal sequences for secretion, viral vectors, and/or insertion of the toxin gene at the eg4 gene locus are given later in this article.

### 3.2 Mite Toxins

The insect-predatory straw itch mite *Pyemotes tritici* encodes an insect paralytic neurotoxin TxP-I that induces rapid, muscle-contracting paralysis in larvae of the greater wax moth *Galleria mellonella* (Tomalski et al., 1988, 1989). TxP-I is encoded by the tox34 gene as a precursor protein of 291 amino acid residues. The mature protein is secreted from the insect cell following cleavage of a 39 amino acid-long signal sequence for secretion (Tomalski and Miller, 1991). The mode of action of TxP-I is unknown, however, it is highly toxic (even at a dose of 500 µg/kg) to lepidopteran larvae but not toxic to mice at a dose of 50 mg/kg. A recombinant, occlusion-negative AcMNPV (vEV-Tox34) expressing the tox34 gene under a modified polyhedrin promoter (P<sub>LENV</sub> (Ooi et al., 1989)) was shown to paralyze or kill fifth instar larvae of *T. ni* by two days post injection with 400,000 pfu of BV. In contrast, control larvae injected with BV of wild type AcMNPV never showed symptoms of paralysis (Tomalski and Miller, 1991). Tomalski et al. (Tomalski and Miller, 1992, 1991) have constructed two other occlusion-negative AcMNPVs that express the tox34 gene under early (vETL-Tox34) or hybrid late/very late (vCappolh-Tox34) gene promoters as well as an occlusion-positive AcMNPV (vSp-Tox34) expressing tox34 under a different hybrid late/very late promoter. The activities of these constructs will be discussed later in this article. Lu et al. (Lu et al., 1996) have constructed an occlusion-positive AcMNPV (v6p9tox34) that utilizes the late p6.9 promoter to drive expression of tox34. Use of this late promoter resulted in earlier, by at least 24 h, and higher level of TxP-I expression in comparison to TxP-I expression under a very late promoter. As discussed above, the p6.9 gene promoter is not an early promoter, but is activated earlier and can drive higher levels of expression than very late promoters in tissue culture (Bonning et al., 1994; Hill-Perkins and Possee, 1990). In time-mortality bioassays (at an LC<sub>50</sub> dose), the ET<sub>50</sub> of vp6.9tox34 in neonate larvae of *S. frugiperda* and *T. ni* was reduced by approximately 56% (44.7 vs. 101.3 h) and 58% (41.7 vs. 99.0 h), respectively, in comparison to wild type AcMNPV. In neonate *S. frugiperda* and *T. ni*, the earlier and higher level of TxP-I expression under the late promoter resulted in a 20–30% faster induction of effective paralysis or death in comparison to TxP-I expression under the very late gene promoter.

Burden et al. (Burden et al., 2000) have constructed a slightly different TxP-I encoding gene (tox34.4) by RT-PCR of mRNAs purified from total RNA extracted from *P. tritici* using primers designed to amplify the tox34 open reading of Tomalski and Miller (1991). A recombinant AcMNPV (AcTOX34.4) expressing TxP-I under the p10 promoter has been generated. The LD<sub>50</sub>s of
AcTOX34.4 were not significantly different than those of the wild type AcMNPV in dose-mortality bioassays of second (9.3 polyhedra per larva) and fourth (13.1 polyhedra per larva) instar larvae of T. ni. In time-mortality bioassays, second and fourth instar larvae of T. ni infected with AcTOX34.4 showed a 50–60% reduction (depending on virus dose and instar) in the mean time to death in comparison to control larvae infected with wild type AcMNPV. There was also a dramatic reduction in the yield of progeny virus (number of polyhedra per µg of cadaver) at the time of death. Second and fourth instar larvae of T. ni that were infected with AcTOX34.4 produced roughly 85% and 95% lower yields of polyhedra per unit weight, respectively, in comparison to control larvae infected with AcMNPV. On the basis of pathogen-host model systems that describe how insect viruses may regulate host population density (as will be discussed later in this article), Burden et al. (2000) suggested that the dramatic reduction in yield of AcTOX34.4 will cause it to be outcompeted by the wild type AcMNPV.

### 3.3 Other Toxins

In addition to peptide toxins of scorpion and mite origin, insect-selective and highly potent toxins have been identified from other organisms including spiders, anemones, and bacteria. The spider Agelenopsis aperta and sea anemones Anemonia sulcata and Stichodactyla helianthus possess the insect-selective toxins \( \mu \)-Agα-IV, As II, and Sh I, respectively (Prikhodko et al., 1996). Recombinant AcMNPVs carrying synthetic genes that express \( \mu \)-Agα-IV (vMAg4p + ), As II (vSAt2p + ), or Sh I (vSSh1p + ) under the very late \( \beta\text{-}p\text{X} \) promoter have been constructed (Prikhodko et al., 1996). The As II and Sh I toxins were each expressed as fusion peptides with a signal sequence derived from the flesh fly sarcotoxin IA, whereas \( \mu \)-Agα-IV was expressed as a fusion peptide with the mellitin signal sequence of the honey bee Apis mellifera. In neonate larvae of T. ni, the LC\(_{50}\) of vSSh1p + was slightly lower to that of AcMNPV, but those of vMAg4p + and vSAt2p + were essentially identical. In contrast, in neonate larvae of S. frugiperda the LC\(_{50}\)s of all three viruses were increased by 3.75- to 5.25-fold in comparison to AcMNPV. In time-mortality bioassays, neonate larvae of T. ni and S. frugiperda infected (at an LC\(_{50}\) dose) with any of the recombinant viruses died more quickly than control larvae infected with AcMNPV. In neonate T. ni infected with vMAg4p +, vSAt2p +, and vSSh1p +, the ET\(_{50}\) (median times to effectively paralyze or kill) were reduced by 17% (84.5 vs. 102.0 h), 38% (62.8 vs. 102.0 h), and 36% (65.3 vs. 102.0 h), respectively. In neonate S. frugiperda, the ET\(_{50}\) were reduced by 37% (65.7 vs. 104.3 h), 31% (71.8 vs. 104.3 h), and 35% (67.5 vs. 104.3 h), respectively. Related constructs in which these toxins were expressed under a constitutive promoter or in tandem will be discussed below.

Hughes et al. (Hughes et al., 1997) have generated recombinant AcMNPVs expressing insect-specific toxins DTIX.2 and TaTIX-1 from the spiders Digtaria cantitans and Tegenaria agrestis, respectively. DTIX.2 was expressed by vAcDTIX.2 as a fusion protein with a signal sequence from the AlSP-1 gene of T. ni under the \( \beta\text{-}p\text{h} \) promoter. TaTIX-1 (authentic signal sequence and mature protein) was expressed by vAcTaTIX-1 under the \( \beta\text{-}p\text{h} \) promoter. In time-mortality bioassays, neonate larvae of T. ni, S. exigua, and H. virescens infected (at less than LC\(_{50}\) dose) with vAcDTIX.2 generated ET\(_{50}\)s that were reduced by approximately 9% (62.6 vs. 68.8 h), 9% (71.3 vs. 78.5 h), and 10% (74.1 vs. 81.9 h), respectively, in comparison to control larvae infected with a control virus (Ac-Bb1). Neonate T. ni, S. exigua, and H. virescens infected (at less than LC\(_{50}\) dose) with vAcTaTIX-1 generated ET\(_{50}\)s that were reduced by approximately 20% (35.5 vs. 68.8 h), 18% (64.4 vs. 78.5 h), and 19% (66.1 vs. 81.9 h), respectively, in comparison to control larvae infected with Ac-Bb1. In all cases, larvae infected with the toxin-expressing viruses stopped feeding prior to larvae infected with Ac-Bb1 or wild type AcMNPV. Neonate T. ni, S. exigua, and H. virescens infected with vAcDTIX.2 showed ET\(_{50}\)s (median times to cessation of feeding) that were approximately 33, 28, and 42%, respectively, faster than control larvae infected with Ac-Bb1. Similarly, neonate T. ni, S. exigua, and H. virescens infected with vAcTaTIX-1 showed ET\(_{50}\)s that were approximately 26, 17, and 37%, respectively, faster than control larvae. Interestingly, although the speed of kill of vAcTaTIX-1 was faster, vAcDTIX.2 was able to stop feeding more quickly. Thus, Hughes et al. (Hughes et al., 1997) emphasized that enhanced speed of kill is not necessarily a reliable indicator of the enhanced speed with which feeding is stopped.

The genus Bacillus is composed of gram-positive, endospore forming bacteria. During endospore formation Bacillus thuringiensis (Bt) produces parasporal, proteinaceous, crystalline inclusion bodies that possess insecticidal properties. The biology and genetics of Bt and Bt toxins have been previously reviewed (Aronson and Shai, 2001; Gill et al., 1992; Schnepf et al., 1998). Bt encodes two major classes of lepidopteran-active toxins, cytolytic toxins and \( \delta \)-endotoxins that are encoded by the cyt and cryn genes, respectively. The \( \delta \)-endotoxins of Bt are composed of large (> 120 kDa) protoxin proteins that are solubilized in the alkaline conditions of the insect midgut and processed into the active toxin or toxins by proteases. The active toxins bind to specific receptors found on the insect’s midgut epithelial cells and aggregate to form ion channels. Channel formation leads to disruption of the cells transmembrane potential that leads to osmotic cell lysis. Lethality is believed to be caused by (1) disruption of normal midgut function which results in feeding cessation and starvation of young larvae or (2) Bt septicemia.

Several studies have looked at the effectiveness of the expression of Bt toxin in terms of improving the insecticidal activity of the baculovirus. These studies have investigated the expression of full-length or truncated forms of Bt toxin genes (e.g., crylAb, crylAc, etc.) that are placed under a very late promoter and expressed in AcMNPV (Martens et al., 1990, 1995; Merryweather et al., 1990; Ribeiro and Crook, 1993, 1998) or Hyphantria cunea NPV (Woo et al., 1998). These studies showed that the Bt toxin is highly expressed by the baculovirus and subsequently processed into the biologically active form. However, Bt toxin expression did not increase the virulence of the virus (i.e., the LD\(_{50}\) of the recombinant virus is similar or higher than the LD\(_{50}\) of the wild type virus) nor decrease the ST\(_{50}\) of larvae infected with the recombinant virus. These findings are not completely unexpected considering that the site of action of Bt toxins is the midgut epithelial cell, whereas the baculovirus-expressed Bt protoxin is produced within the cytoplasm of cells within the insect body. Furthermore, the expressed protoxin (1) may not be digested to the active form because
of the lack of appropriate proteases within the cytoplasm, (2) may be poorly secreted, or (3) the mature toxin may be cytotoxic to the cell (Martens et al., 1995). In order to improve toxin secretion, several Bt toxin gene constructs have been generated in which a signal sequence of IHE of H. virescens was fused to the N-terminus of the toxin (Martens et al., 1995). These toxins were translocated across the endoplasmic reticulum (ER) membrane, but they appeared to be retained within one of the ER or Golgi compartments. These findings suggest that the expression of the Bt toxin gene will have little or no effect in improving insecticidal activity once the virus crosses the midgut.

Chang et al. (Chang et al., 2003) have taken a novel and apparently highly successful approach for the delivery of Bt toxin directly to the insect midgut epithelial cells using a recombinant baculovirus that occludes the toxin within its polyhedra. Their strategy was based upon the coexpression of (1) native polyhedrin and (2) a polyhedrin-foreign protein fusion from the same baculovirus (Je et al., 2003). Chang et al. (2003) used this system to coexpress native polyhedrin and a polyhedrin-Cry1Ac-green fluorescent protein (GFP) fusion from the recombinant baculovirus ColorBtrus. ColorBtrus is a recombinant AcMNPV that produces apparently normal polyhedra that occlude Bt toxin and GFP. Although the Cry1Ac toxin was fused at both the N- and C-termini, this fusion protein could be digested by trypsin resulting in an immunoreactive protein that behaved identically to authentic Cry1Ac toxin. They estimated that approximately 10 ng of Cry1Ac was contained in 1.5 × 10^6 ColorBtrus polyhedra. Furthermore, because of GFP incorporation, the ColorBtrus polyhedra fluoresced under UV light. This characteristic should allow the rapid monitoring and detection of ColorBtrus-infected insects in the field (Chang et al., 2003; Chao et al., 1996). In dose-mortality bioassays using second or third instar larvae of the diamondback moth, Plutella xylostella, Chang et al. (2003) showed that the LD_{50} of ColorBtrus was reduced by roughly 100-fold in comparison to wild type AcMNPV (28.3 vs. 2798.3 polyhedra per larva, respectively). By time-mortality bioassays, the ST_{50} of larvae of P. xylostella infected with ColorBtrus was reduced by 63% (33.9 vs. 92.8 h) in comparison to control larvae infected with wild type AcMNPV when the larvae were inoculated at LD_{50} doses (i.e., 80 ColorBtrus or 10,240 AcMNPV polyhedra per larva). Chang et al. (2003) indicated that the ColorBtrus-inoculated larvae displayed symptoms, such as feeding cessation, that were consistent with exposure to Cry1Ac. This suggested that feeding damage to the plant should cease very quickly after ingestion of ColorBtrus. Chang et al. (2003) further suggested that the ‘stacking’ of multiple effectors (i.e., Cry1Ac toxicity and viral pathogenesis) by ColorBtrus should reduce the occurrence of resistance because the few individuals within the population that evolve resistance to the Bt toxin would also have to simultaneously evolve resistance to the baculovirus. Additionally, although the Cry1Ac toxin is highly toxic to a wide range of lepidopteran insects, it is not effective against Spodoptera species such as the beet armyworm S. exigua (Bai et al., 1993). Since the beet armyworm is susceptible to AcMNPV, they suggested that this insect can be used for the efficient in vivo production of ColorBtrus or similar virus.

Besides the toxins mentioned here, new and interesting toxins are frequently being discovered. These novel insect specific toxins can potentially be expressed by baculoviruses. There is a great diversity of available toxins that can be expressed by GM baculoviruses either alone or in combination. We may have not yet reached the limits of improvement of the speed of kill of GM baculoviruses.

3.4 Improvement of Toxin Efficacy by Genetic Modifications

Thus far, our discussion on improving baculovirus pesticides has primarily focused on the expression of an insect-selective toxin gene under late or very late baculoviral promoters. We will now focus on studies that have attempted to improve the efficacy of the toxin-expressing constructs by (1) altering the timing and level of toxin expression, (2) improving folding and secretion efficiency of the toxin, and/or (3) expressing multiple toxins that target different sites within the insect sodium channel. Related to this last point, we will also present studies on synergistic interactions between the expressed toxin and chemical pesticides.

3.4.1 Alteration of the timing and level of expression

In general, the first generation of recombinant baculovirus constructs used a single baculovirus-derived very late (e.g., polh or p10) or late (e.g., p6.9) promoter to drive expression of the toxin gene (or other foreign gene). These promoters generally drive exceptionally high levels of protein expression (i.e., they are very strong promoters), but they require the products of viral early genes for activation. Thus, the very late/late promoters are not activated until a late stage of the baculovirus replication (1u and Milner, 1997). At this late stage of infection, the translational machinery and processing pathways of the cell may be compromised (Jarvis et al., 1990). Thus, a large amount of protein may be produced but not all of the protein will be functionally active due to poor folding or poor post translational modifications and the protein may also be retained within the cell due to poor secretion. Baculovirus early gene promoters on the other hand are recognized by host transcription factors and are activated very soon after the viral DNA is uncoated in the nucleus. At this early stage of infection, the processing and secretory pathways of the cell should be in a less compromised state (in comparison to the late stage of infection). Thus, it is likely that a higher percentage of the expressed proteins will be properly folded, modified, and secreted. Earlier expression may be especially important for the proper folding of neurotoxins because they possess a relatively large number of disulfide bonds (e.g., the 70 amino acid-long AaIT peptide contains four disulfide bonds). Early gene promoters, however, generally drive very low levels of protein expression (i.e., they are weak promoters).

Several groups have attempted to circumvent the potential problems associated with protein expression under very late promoters using (1) early gene promoters, (2) dual or chimeric promoters, and/or (3) constitutively expressed promoters to drive
the expression of a foreign gene. Jarvis et al. (Jarvis et al., 1996) have constructed a recombinant AcMNPV (ie1-AaIT) expressing the AaIT gene under the control of an immediate early gene (ie1) promoter of AcMNPV. In cultured SF-9 cells infected with the ie1-AaIT construct, AaIT was detected as early as 4 h p.i. and continued to accumulate until at least 24 h p.i. At 24 h p.i., the amount of the expressed AaIT peaked and was about equal to that expressed by AcAaIT (AaIT expressed under the very late p10 promoter). In vitro expression assays indicated that there was early and abundant expression of AaIT under the ie1 promoter. These findings suggested that the ie1-AaIT construct should kill infected larvae more quickly than the AcAaIT construct. The results of the insect bioassays using neonate *H. virescens*, however, did not support this. Time-mortality bioassays showed that the ie1-AaIT virus killed neonate *H. virescens* only about 10% faster than the wild type AcMNPV (LT50 of 87.0 vs. 96.5 h). In comparison, AcAaIT killed the neonate *H. virescens* about 22% faster than AcMNPV (LT50 of 75.0 vs. 96.5 h) in parallel experiments. Although Jarvis et al. (1996) were unclear as to the reason(s) for the slower speed of kill of ie1-AaIT in comparison to AcAaIT, they speculated that (1) ie1-AaIT does not produce a threshold dose of AaIT that must be reached before kill rates can be enhanced, (2) duplication of the ie1 promoter usurps limited cellular transcription factors needed by the endogenous ie1 promoter for expression of the IE1 protein, (3) early expression of AaIT in midgut epithelial cells induces these cells to be shed thereby reducing the primary infection in these cells and/or dissemination of the virus into the hemocoel, and (4) the ie1 promoter of AcMNPV has a narrower host range and/or tissue specificity in comparison to the virus as a whole. Interestingly, although ie1-AaIT-infected larvae did not die as quickly as AcAaIT-infected larvae, the relative growth rate of the ie1-AaIT-infected larvae was about 13% lower (0.8 vs. 0.92 mg/larva/day) than that of AcAaIT infected larvae. This suggested that even though they do not die as quickly, the ie1-AaIT-infected larvae may consume less food.

van Beek et al. (van Beek et al., 2003) have compared the efficacy of recombinant AcMNPVs expressing either AaIT or LqhIT2 under various baculoviral early (ie1 or lef3), early/late (39K), or very late (p10) gene promoters in larvae of *H. virescens*, *T. ni*, and *S. exigua*. The larvae were inoculated at both low (approximately LD50) and high (approximately 100 times the LD50) doses of each recombinant virus. By time-to-response bioassays using neonate and third instar *H. virescens* or *T. ni*, and second instar *S. exigua*, they found that the median time to effect (ET50) of the recombinant AcMNPV expressing AaIT under the early hr5/ie1 promoter led in the majority of the cases to a faster response, but a generalized pattern of response in terms of the effect of viral dose and instar was not found. The ‘hr5’ designation indicates that the baculovirus hr5 enhancer sequence was placed immediately upstream of the promoter sequence. By bioassays using third instar larvae of *H. virescens*, they found that there were no significant differences in the ET50 of the recombinant AcMNPVs expressing LqhIT2 under an early (hr5/ie1 or hr5/lef3) or early/late (hr5/39K) gene promoter. Furthermore, regardless of the larval instar or dose applied, when the early hr5/ie1 promoter was used to drive the expression of LqhIT2 or AaIT, the ET50s of the recombinant AcMNPV expressing LqhIT2 were consistently lower (by 8–32 h) in comparison to those of the recombinant AcMNPV expressing AaIT. This was putatively due to differences in the effectiveness of the two toxins.

As described above, Gershburg et al. (1998) have constructed recombinant AcMNPVs expressing the excitatory toxin LqhIT1 or depressant toxin LqhIT2 under either the early p35 gene or very late p10 gene promoters. They found that the recombinant AcMNPV expressing LqhIT1 under the p35 promoter had a slightly slower ET50 (73 vs. 66 h) in comparison to the recombinant AcMNPV expressing LqhIT1 under the p10 promoter. They detected, however, a clear paralytic effect when the p35 promoter was used to express LqhIT1 even when they were unable to detect the LqhIT1 by immunochemical analysis. Thus, they suggested that baculovirus expression of toxin in cells that are adjacent to the target sites of the motor neural tissues overcomes the pharmako-kinetic barriers that the toxin may face when, for example, purified toxin is injected to the insect. Consistent with this hypothesis, Elazar et al. (Elazar et al., 2001) have shown that the concentration of AaIT in the hemolymph of paralyzed larvae (*B. mori*) is approximately 50-fold lower when the paralyzing dose is delivered by a baculovirus (BmAaIT) rather than by the direct injection of purified AaIT. They hypothesized that baculovirus expression of AaIT provides a continuous, local supply of freshly produced toxin (via the tracheal system) to the neuronal receptors, thus providing access to critical target sites that are inaccessible to toxic that is injected. Therefore, a lower level of continuous expression under an early promoter may be sufficient to elicit a paralytic response.

As discussed above, Tomalski et al. (Tomalski and Miller, 1992) have constructed an occlusion-negative recombinant AcMNPV (vETL-Tox34) that expresses the tox34 gene under the baculoviral early P35 promoter. In early fifth instar larvae of *T. ni*, this virus induced much slower paralysis (occurring after 48 h in 95% of larvae) in comparison to control larvae infected with recombinant AcMNPVs (vETL-Tox34, vCappolh-Tox34 or vSp-Tox34) expressing tox34 under a very late or chimeric promoter (all larvae were paralyzed or dead by 48 h). Additionally, the average cumulative weight gain of vETL-Tox34-infected larvae at 24 h post injection was not significantly different to that of control larvae infected with AcMNPV or cell culture medium (mock infection). Lu et al. (Lu et al., 1996) have also constructed a recombinant AcMNPV (vDA26tox34) expressing the tox34 gene under the early Da26 gene promoter. In comparison to vp6.9tox34 (tox34 under a late promoter)- or vSp-tox34 (tox34 under a very late promoter)-infected SF-21 or TN-368 cells (derived from *S. frugiperda* and *T. ni*, respectively), Txp-1 expression was detected at least 24 h earlier (but at dramatically lower levels) in SF-21 and TN-368 cells infected with vDA26tox34. In time-mortality bioassays (at an LC50 dose), the ET50 of vDA26tox34 in neonate larvae of *S. frugiperda* and *T. ni* was reduced by approximately 39% (61.8 vs. 101.3 h) and 28% (71.2 vs. 99.0 h), respectively, in comparison to wild type AcMNPV. However, this was 17.1 and 29.5 h slower, respectively, in comparison to neonate larvae of *S. frugiperda* and *T. ni* that were infected with vp6.9tox34 (ET50s of 44.7 and 41.7 h, respectively). Even though the Txp-1 is expressed significantly earlier under the early promoter, it is apparently not expressed in sufficient quantity, at least initially, to paralyze larvae of *S. frugiperda* or *T. ni*. Popham et al. (Popham et al., 1997) have also expressed the tox34 gene under the early DA26 and late p6.9 promoters (of AcMNPV) by recombinant HzSNPVs (HzEGTDA26tox34 and
HzEGTp6.9tox34, respectively). They found that the ET_{50} of HzEGTDA26tox34 and HzEGTp6.9tox34 in neonate larvae of \textit{H. zea} were reduced by approximately 47\% (35.4 vs. 67.3 h) and 44\% (38.0 vs. 67.3 h), respectively, in comparison to a control virus. In contrast to the results of Lu et al. (Lu et al., 1996), the early promoter was more effective in the case of HzSNPV and neonate \textit{H. zea} suggesting that the effectiveness of a promoter will be virus and insect specific. With toxins of current potency, the use of early (and weak) promoters such as \textit{ie1}, \textit{lef3}, \textit{p35}, \textit{elt} or \textit{DA26} may or may not provide any benefit in terms of improved crop protection. However, if toxins at act at lower concentration (i.e., with much greater potency) are identified, the use of early promoters may offer a great advantage.

Tomalski and Miller (Tomalski and Miller, 1992) have used a chimeric promoter (P_{cap/p35}) comprised of both the late capsid and very late polyhedrin promoter elements to drive the expression of the \textit{tox34} gene in an occlusion-negative recombinant AcMNPV (vCappolh-Tox34). In Sf-21 cell cultures infected with vCappolh-Tox34, TxP-I is detected in the culture medium at 12 h p.i. at levels that are similar to those found in vEV-Tox34 (tox34 under a very late promoter)-infected Sf-21 cells at 24 h p.i. (i.e., high-level expression occurred approximately 12 h earlier). When early fifth instar larvae of \textit{T. ni} were injected with vCappolh-Tox34 approximately 50\% of the insects were paralyzed by 24 h post injection. In comparison, only 10\% or less of control virus (e.g., vEV-Tox34)-infected larvae were paralyzed. Larvae infected with vCappolh-Tox34 also showed a significantly lower cumulative weight gain in comparison to control virus-infected larvae. An occlusion-positive construct (vSp-Tox34) in which the tox34 gene was placed under another hybrid late/very late promoter (P_{Synov} (Wang et al., 1991)) has also been constructed by Tomalski et al. (Tomalski et al., 1991, 1992). The induction of paralysis by vSP-Tox34 in fifth instar \textit{T. ni} was slightly delayed, but all of the larvae were paralyzed or dead by 48 h post injection (as were vCappolh-Tox34-infected larvae) (Tomalski and Miller, 1991, 1992). In time-mortality bioassays (at an LC_{95} dose), the ET_{50} of vSP-Tox34 in neonate larvae of \textit{S. frugiperda} and \textit{T. ni} was reduced by approximately 45\% (55.4 vs. 101.3 h) and 41\% (58.0 vs. 99.0 h), respectively, in comparison to control larvae infected with AcMNPV (Lu et al., 1996; Tomalski and Miller, 1992). Furthermore, the yield (2.1 \times 10^9 vs. 3.5 \times 10^9 polyhedra per larva) of vSP-Tox34 polyhedra from infected last instar larvae of \textit{S. frugiperda} was reduced by approximately 40\% in comparison to AcMNPV-infected control larvae. Tomalski et al. (Tomalski and Miller, 1992) suggested that this reduction in yield will cripple the virus in terms of its ability to compete effectively with the wild type virus in the environment.

Sun et al. (Sun et al., 2004) have generated a recombinant HaSNPV (HaSNPV-AaIT) that expresses the AaIT gene under a chimeric promoter (ph-p69p) consisting of the late p6.9 gene promoter inserted immediately downstream of the very late \textit{polh} gene promoter in the same direction. The AaIT gene cassette was inserted at the egt gene locus of HaSNPV. Detailed dose-mortality bioassays indicated that the \textit{LD}_{50} of HaSNPV-AaIT was unchanged compared to wild type (HaSNPV-WT) or egt gene-deleted (HaSNPV-EGTD) viruses in first to fifth instar larvae of the cotton bollworm \textit{H. armigera}. In time-mortality bioassays, first to fifth instar larvae \textit{H. armigera} infected with HaSNPV-AaIT showed reductions in the \textit{ST}_{50} of 10\% (68.5 vs. 76.5 h), of 20\% (60.5 vs. 75.5 h), 8\% (71.0 vs. 77.5 h), 4\% (104.6 vs. 109.0 h), and 10\% (108.5 vs. 121.0 h), respectively, in comparison to control larvae infected with HaSNPV-EGTD. In comparison to control (first to fifth instar) larvae infected with HaSNPV-WT, the corresponding reductions were 19, 28, 21, and 18\%, respectively. In third to fifth instar \textit{H. armigera} infected with HaSNPV-AaIT, the median times to feeding cessation (\textit{FT}_{50}) were reduced by 25\% (51.5 vs. 68.5 h), 22\% (66.5 vs. 85.0 h), and 12\% (84.5 vs. 96.5 h), respectively, in comparison to control larvae infected with HaSNPV-EGTD. In comparison to control larvae infected with HaSNPV-WT, the corresponding reductions in the \textit{FT}_{50} were 39, 43, and 30\%, respectively. The role of the \textit{egt} deletion in improving speed of kill will be further discussed below. Sun et al. (Sun et al., 2004) did not test constructs in which only the \textit{polh} or p6.9 gene promoter was used to drive expression of the AaIT gene, thus it was not possible to predict if expression under the dual promoter is more effective than expression under a single late or very late promoter.

The \textit{hsp70} promoter of \textit{D. melanogaster} (Snyder et al., 1982) is constitutively active in insect cells (Vlak et al., 1990; Zuidema et al., 1990). Unlike very late or late gene promoters of the baculovirus, the \textit{hsp70} promoter does not require the expression of baculovirus early gene products or the initiation of viral DNA synthesis for its activity. The \textit{hsp70} promoter shows higher activity than early gene promoters, but it is not as active as baculoviral very late or late gene promoters (Morris and Miller, 1992, 1993). McNitt et al. (McNitt et al., 1995) have constructed occlusion-positive, recombinant AcMNPVs expressing AaIT (vHSP70AaIT) and TxP-I (vHSP70tox34) under the \textit{hsp70} promoter. TxP-I was expressed at a significantly higher level under the \textit{hsp70} promoter than under the early \textit{DA26} promoter in both vHSP70tox34-infected Sf-21 and TN-368 cell cultures (Lu et al., 1996; McNitt et al., 1995). Additionally, TxP-I expression was detected as early as 6 and 12 h p.i., respectively, in the culture medium of vHSP70tox34-infected Sf-21 and TN-368 cells. Dose-mortality bioassays using neonate larvae of \textit{S. frugiperda} or \textit{T. ni} indicated that the \textit{LC}_{50} of vHSP70tox34 were unchanged from that of the AcMNPV. Time-mortality bioassays (at an LC_{95} dose) in neonate \textit{S. frugiperda} and \textit{T. ni} showed that the \textit{ET}_{50} of vHSP70tox34 were reduced by approximately 59\% (41.8 vs. 101.3 h) and 46\% (53.8 vs. 99.0 h), respectively, in comparison to control larvae infected with AcMNPV. In comparison to occlusion-positive, recombinant AcMNPVs expressing the \textit{tox34} gene under very late (vSP-tox34) or late (vp6.9tox34) promoters, the \textit{ET}_{50} of \textit{vHSP70tox34} were faster than \textit{vp6.9tox34} (44.7 h) and \textit{vSp-tox34} (55.4 h) in neonate \textit{S. frugiperda}; and faster than \textit{vp6.9tox34} (41.7 h) and slower than \textit{vSp-tox34} (58.5 h) in neonate \textit{T. ni}. This is surprising considering that the overall level of TxP-I secretion under the \textit{hsp70} promoter is substantially lower than that under the p6.9 promoter (Lu et al., 1996). On the basis of these findings Lu et al. (Lu et al., 1996) suggested that expression from a relatively strong constitutive promoter such as \textit{hsp70} is more effective, in some cases, than expression from a very strong late baculovirus promoter. Popham et al. (Popham et al., 1997) have expressed the tox34 gene under the \textit{hsp70} promoter (HzEGThsptox34) at the \textit{egt} gene locus of a recombinant HzSNPV. Time-mortality bioassays (at an LC_{95} dose) in neonate \textit{H. zea} showed that the \textit{ET}_{50} of HzEGThsptox34 was reduced by 34\% (44.1 vs. 67.3 h) in comparison to control larvae infected with an \textit{egt} deletion mutant of HzSNPV (HzEGTdel). In contrast to the results of Lu et al. (Lu et al., 1996), however,
expression of the tox34 gene under the hsp70 promoter was not as effective as expression under the early Da26 (ET50 of 35.4 h) or late P30 (ET50 of 38.0 h) promoters (both of these promoters were of AcMNPV origin).

The hsp70 promoter has also been used to drive the expression of spider and sea anemone toxins. Prikhod’ko et al. (Prikhod’ko et al., 1998) have constructed recombinant AcMNPVs vhsMag4p+, vhsSat2p+, and vhsSh1p+ that express the insect-selective toxins μ-Aga-IV, As II, and Sh I, respectively, under the hsp70 promoter. All of these constructs showed significantly reduced ET50s (at an LD50 dose) in comparison to AcMNPV in neonate larvae of S. frugiperda and T. ni. In neonate S. frugiperda infected with vhsMag4p+, vhsSat2p+, or vhsSh1p+, the ET50s were reduced by approximately 55% (49.0 vs. 110 h), 52% (52.5 vs. 110 h), and 53% (51.9 vs. 110 h), respectively. In neonate T. ni, the ET50s were reduced by 42% (53.6 vs. 99.7 h), 50% (49.0 vs. 99.7 h), and 46% (52.9 vs. 99.7 h), respectively. In both larval species, expression of μ-Aga-IV (mag4 gene), As II (sat2 gene), and Sh I (sh1 gene) under the hsp70 promoter was more effective than expression under the very late P30XV promoter (ET50s of 60.0, 63.6, and 56.8 h, respectively in S. frugiperda, and 84.4, 62.8, and 60.6 h, respectively, in T. ni).

Numerous studies (including several that are not included in our discussion above) have investigated the effectiveness of constitutive and baculoviral promoters (early, late, and very late) used either alone or in combination for the expression of the current generation of toxin genes. These studies, however, do not form a consensus opinion as to which promoter system is the most optimal in terms of improving insecticidal effectiveness of the toxin expressing baculovirus. The toxin gene, parental virus, and host-specific factors all appear to play important roles in determining the effectiveness of a particular promoter system. Given that an early or constitutive promoter does not provide a clear advantage (at least within the context of toxin genes of current potency), the use of a baculoviral late or very late promoter may provide a slight reduction in the risk that a toxin gene could be expressed in a nontarget insect. The argument here is that very late and late gene promoters are inactive in beneficial insects and other organisms (Heinz et al., 1995; McNitt et al., 1995) because the activation of these promoters requires (1) viral DNA replication and (2) late expression factors (LEF) (Lu and Miller, 1997). In AcMNPV there are 18 lef genes that are required for optimal transactivation of expression from late and very late gene promoters. At least seven of the 18 lef genes appear to be involved in DNA replication and at least three lef gene products are part of a virus-induced or virus-encoded RNA polymerase. This RNA polymerase is required for transcription from late and very late genes. Thus, without viral DNA replication and the expression of essential lef genes, transcription from a late or very late gene promoter will not occur. In contrast, early and constitutive gene promoters can potentially be activated in nontarget insect cells (McNitt et al., 1995; Morris and Miller, 1992, 1993). A discussion of the potential risks of associated with gene transfer to nontarget insects and other organisms (e.g., mammals) is given below.

### 3.4.2 Better secretion and folding

In order for a peptide or protein to be secreted to the outside of a eukaryotic cell, it must possess a signal sequence consisting of 15–30 hydrophobic amino acid residues at its amino-terminal. Following the initiation of protein synthesis by ribosomes within the cytoplasm, the newly synthesized signal sequence is bound by a signal recognition particle (SRP). Subsequently, the SRP-signal sequence-ribosome complex binds to a SRP receptor on the cytosolic surface of the rough endoplasmic reticulum (ER) membrane. The signal sequence peptide is then able to cross the ER membrane (and is generally cleaved off within the lumen of the ER) and the remainder of the protein is produced and secreted into the lumen of the ER. Once inside the ER lumen the peptide can undergo appropriate disulfide bond formation and folding. The mature protein then continues to the Golgi apparatus for subsequent release via secretory vesicles to the outside of the cell. In general, once inside the ER lumen, proteins are automatically transported through the Golgi apparatus and secreted (i.e., this is thought to be the default pathway) unless they possess a specific signal that directs them to another location. Thus, the ability of the insect cell SRP to recognize the signal sequence on the baculovirus-expressed protein is a key determinant for its eventual secretion to the outside of the cell. Signal sequences that originate from a variety of organisms (e.g., mammal, plant, yeast, insect, virus, etc.) are recognized by lepidopteran SRPs and can help direct baculovirus-expressed proteins to the ER for subsequent processing and secretion from the cell (O’Reilly et al., 1992). Signal sequences derived from proteins of insect origin (e.g., bombyxin from the silk moth B. mori (Adachi et al., 1989), mellitin from the honey bee A. mellifera (Tessier et al., 1991), and cuticle protein II (CPII) from D. melanogaster (Snyder et al., 1982)) have been shown to be functional when attached to a wide variety of proteins.

In order to improve processing, folding and/or secretion of baculovirus-expressed toxin, van Beek et al. (van Beek et al., 2003) have generated recombinant AcMNPVs expressing LqhIT2 (under the early hr5/ie1 promoter) fused to various secretion signals of insect and non-insect origin. Signal sequences originating from bombyxin, CPII, adipokinetic hormone from M. sexta (L.) (Jaffe et al., 1986); chymotrypsin of Lucilia cuprina (Weidemann) (Casu et al., 1994), AcMNPV gp67 (Whitford et al., 1989), and scorpion neurotoxins BfIT from Hottentotta judaicus (Simon) (Zlotkin et al., 1993), AaIT (Rougis et al., 1989), and LqhIT2 (Zlotkin et al., 1993) were tested. In time-mortality bioassays in third instar larvae of H. virescens (at a dose of polyhedra that was estimated to elicit a response in less than 50% of the larvae), they found that a recombinant AcMNPV expressing LqhIT2 fused to the bombyxin signal sequence elicited the fastest response time (ET50 of 62 h, estimate based on the graph). Recombinant AcMNPVs expressing LqhIT2 fused to the signal sequences of adipokinetic hormone (ET50 of 78 h), chymotrypsin (ET50 of 78 h), LqhIT2 (ET50 of 85 h), and gp67 (ET50 of 72 h) elicited intermediate response times. All of these viruses putatively expressed and secreted active toxin as evidenced by reduced survival times and the occurrence of paralysis. In contrast, recombinant AcMNPV expressing LqhIT2 fused to the signal sequences of CPII, BfIT, and AaIT appeared not to secrete active toxin because insects infected with these constructs exhibited symptoms that is typical of AcMNPV infection. In general they found that the baculoviral gp67 or insect-derived signal sequences (excluding CPII) worked better than the scorpion toxin-derived signal sequences for the secretion of biologically active LqhIT2.
Lu et al. (Lu et al., 1996) have constructed three recombinant AcMNPVs (vSp-BSigtox34, vSp-DCtox34, and vSp-tox21A/tox34) that express TxP-I (under the very late Ppolh promoter) fused to signal sequences originating from sarcotoxin IA of the flesh fly (Matsumoto et al., 1986), CPII, and tox21A (a homolog of tox34) (Tomalski et al., 1993), respectively. The levels of TxP-I secreted into the culture medium of SF-21 cells infected with vSp-DCtox34 or vSp-tox21A/tox34 were similar to control cells infected with vSp-tox34 (TxP-I fused to its own signal sequence). TxP-I secretion was undetectable on the basis of Western blotting in SF-21 cell cultures infected with vSp-BSigtox34. By comparing the levels of TxP-I that were found within the cell (i.e., cell lysate) and in the culture medium (i.e., secreted), it appeared that the CPII secretion signal (vSp-DCtox34) was better than the tox21A signal sequence (vSp-tox21A/tox34) in terms of being able to direct a higher percentage of the expressed TxP-I to the outside of the cell. TxP-I was only detected at very low levels within the cell lysates of SF-21 cells infected with vSp-BSigtox34, thus it is difficult to determine whether the lack of TxP-I in the cell culture medium was the result of poor secretion or poor translation. In dose-mortality bioassays using neonate larvae of T. ni, the LC50s of vSp-BSigtox34 and vSp-DCtox34 were similar to that of AcMNPV, whereas the LC50 of vSp-tox21A/tox34 was roughly double (5.3 × 10^4 vs. 2.2 × 10^4 polyhedra/mL) that of AcMNPV. In time-mortality bioassays (at an LC50 dose) in neonate T. ni, the ET50s of vSp-BSigtox34, vSp-DCtox34, and vSp-tox21A/tox34 were reduced by 26% (70.0 vs. 94.6 h), 47% (49.9 vs. 94.6 h), and 36% (60.8 vs. 94.6 h), respectively, in comparison to control larvae infected with AcMNPV. However, in comparison to neonate T. ni infected with vSp-tox34 (a control virus expressing TxP-I fused to its authentic secretion signal, ET50 of 51.1 h), neonate T. ni infected with viruses expressing TxP-I fused to an alternative signal sequence did not show any improvements in ET50. Additionally, although TxP-I secretion was undetected in SF-21 cells infected with vSp-BSigtox34, neonate T. ni infected with this virus displayed paralysis indicating that the threshold level of toxin required for paralysis is low or that some cells within the larvae may more efficiently produce toxin. On the basis of these studies, Lu et al. (Lu et al., 1996) suggested that the level of toxin secreted into the supernatant is generally diagnostic of how the virus will perform in vivo.

Chaperones found in the cytosol and ER (such as BiP, murine immunoglobulin heavy chain binding protein (Bole et al., 1986)) play key roles in protein folding, transport, and quality control in a wide range of eukaryotic cells including insect cells (Ailor and Betenbaugh, 1999; Ruddon and Bedows, 1997; Trombetta and Parodi, 2003). The coexpression of BiP by a recombinant baculovirus has been shown to reduce aggregation and increase secretion of baculovirus-expressed murine IgG in T. ni-derived cells (Hsu et al., 1994). Tanai et al. (Tanai et al., 2002) have investigated whether coexpression of BiP can improve the secretion of AcAaIT-expressed AaIT (rAaIT) in SF-21 cells. They found that coexpression of BiP increased the amount of soluble rAaIT, however, the amount of active rAaIT that was secreted into the culture medium was not improved. Protein disulfide isomerase (PDI) is another factor within the ER that is known to help protein folding and secretion in insect cells by catalyzing the oxidation, reduction, and isomerization of disulfide bonds in vitro (Hsu et al., 1996; Ruddon and Bedows, 1997). Coexpression of a PDI expressing baculovirus with an AaIT expressing baculovirus was also ineffective in increasing the amount of active AaIT that was secreted into the culture medium (Tanai, Inceoglu, and Hammock, unpublished).

### 3.4.3 Expression of multiple synergistic toxins

At least six distinct receptor sites are found on the voltage-gated sodium channels of mammals and insects that are the molecular targets of a broad range of neurotoxins. Two additional receptors sites are found on the insect sodium channel that are the molecular targets of insect-selective excitatory and depressant scorpion toxins (Cestele and Catterall, 2000). On the basis of binding experiments using radiolabeled toxins, the depressant toxins bind to two noninteracting binding sites (one showing high affinity and the other low affinity) on the insect sodium channel (Cestele and Catterall, 2000; Gordon et al., 1992; Zlotkin et al., 1995). The excitatory toxins bind only to the high affinity receptor (Gordon et al., 1992). Hermann et al. (Hermann et al., 1995) have shown that when excitatory and depressant toxins are simultaneously coinjected into larvae of the blowfly S. falcata or H. virescens, the amount of toxin required to give the same paralytic response is reduced 5–10-fold in comparison to the amount required when only one of the toxins is injected. On the basis of this synergism, they suggested that speed of kill of recombinant baculovirus(es) could be further reduced by (1) the coexpression of two or more recombinant baculoviruses that each express toxin genes with synergistic properties or (2) genetic modification such that two or more synergistic toxin genes are simultaneously expressed.

Regev et al. (Regev et al., 2003) have generated a recombinant AcMNPV (vAclLqIT1-IT2) that expresses both the excitatory LqhIT1 and the depressant LqhIT2 toxins under the very late ppolh promoters, respectively. Time-response bioassays (at an LC50 dose) using neonate H. virescens showed that the ET50 of vAclLqIT1-IT2 was reduced by 41% (46.9 vs. 79.1 h) in comparison to AcMNPV. In comparison, the ET50s of recombinant AcMNPVs expressing only LqhIT1 (vAclLqIT1) or only LqhIT2 (vAclLqIT2) were 57.3 h and 60.0 h, respectively. Thus, expression of both LqhIT1 and LqhIT2 by vAclLqIT1-IT2 resulted in a synergistic effect that reduced the ET50 by 10.4 h (18%) and 13.1 h (22%), respectively. A synergistic effect (15–19%) was also observed following coinfection of neonate H. virescens with a mixture of both vAclLqIT1 and vAclLqIT2 (ET50 of 48.8 h). Time-response bioassays (at an LC50 dose) using neonate H. armigera showed that the ET50 of vAclLqIT1-IT2 was reduced by 24% (69.1 vs. 90.8 h) in comparison to AcMNPV. However, a synergistic effect was not elicited in comparison to neonate H. armigera infected with vAclLqIT1 or vAclLqIT2 alone. Since H. armigera is only a semi-permissive host of AcMNPV, Regev et al. (2003) hypothesized that inefficient oral infection may have diminished any synergistic effect. By intrahemocoelic injection (at an LD95 dose) of third instar larvae of H. armigera, they found that the ET50 of vAclLqIT1-IT2 (72 h) was reduced by 13 h (15%) and 70 h (49%) in comparison to larvae injected with only vAclLqIT1 or vAclLqIT2, respectively. In similar intrahemocoelic injection assays of third instar larvae of S. littoralis (non-permissive by oral infection for AcMNPV), they found that the ET50 of vAclLqIT1-IT2 (54 h) was reduced by 43 h...
Using thoracic neurons of the grasshopper Schistocerca americana Drury, Prikhod’ko et al. (Prikhodko et al., 1998) have shown that the spider and sea anemone toxins, \( \mu \)-Aga-IV and As II, respectively, act at distinct sites on the insect sodium channel and synergistically promote channel opening. These toxins also showed synergism when injected into third instar blowfly Lucilia sericata and fourth instar fall armyworm S. frugiperda. Prikhod’ko et al. (Prikhodko et al., 1998) have generated recombinant AcMNPVs (vMAg4Sat2 and vhsMAg4SAt2) that coexpress both \( \mu \)-Aga-IV and As II toxins under the very late PSynXIV promoter (by vMAg4Sat2) because it takes time to activate this promoter during infection and, once activated, high enough levels of expression of either one of the toxins is sufficient to effectively debilitate the host insect. In the case of coexpression under the hsp70 promoter by vhsMAg4SAt2, they hypothesized that lower ET50 (i.e., a synergistic effect) may not be possible since it takes a minimum period of time (i.e., approximately 50 h) for the virus to initiate both primary and secondary infections within the host. Although synergism was not observed in terms of the ET50 (mean time at which 50% of the test larvae cease to respond to stimulus), synergism was observed in terms of the FT50 (mean time at which 50% of the test larvae cease feeding) of neonate T. ni infected with vhsMAg4SAt2 (at an LC50 dose by droplet feeding). The FT50 of vhsMAg4SAt2-infected neonates was lower than that of vMAg4p+ or vDAhsSAt2, and AcMNPV by approximately 25% (24.3 vs. 32.3 h), 29% (24.3 vs. 34.3 h), and 63% (24.3 vs. 65.8 h). Prikhod’ko et al. (Prikhodko et al., 1998) suggested that 24 h may be the fastest time at which a recombinant baculovirus could stop feeding because of the time (approximately 12 h) that it takes to complete the primary infection cycle within the midgut. Furthermore, they suggested that an FT50 of 24 h is sufficiently rapid to make recombinant baculovirus pesticides competitive with chemical pesticides.

### 3.5 Toxin Interactions With Chemical Pesticides

Pyrethroids, carbamates, and organophosphates are common synthetic chemical pesticides that are used for protection against pest insects worldwide. Pyrethroids currently account for about 20% of the total market for insecticides (Khambay, 2002). Pyrethroids are the most commonly used chemical insecticides for the control of the tobacco budworm H. virescens and cotton bollworm H. zea in North America. Pyrethroids are divided into two groups, type I and type II, on the basis of the absence or presence, respectively, of a cyano (-CN) group in the alpha position of the carboxyl moiety. Both type I and type II pyrethroids target receptors in the sodium channel causing them to remain open and resulting in prolonged sodium influx that causes hyperexcitation of the nervous system of both insects and mammals. Insects, however, are more sensitive to pyrethroid action than mammals due to several factors including the relative concentration of pyrethroid metabolizing enzymes, binding kinetics of the pyrethroid to the receptor, and differential sensitivity of the insect and mammalian sodium channels to pyrethroid action (Soderlund et al., 2002). Although the precise location of the pyrethroid receptor within the insect sodium channel is unknown, the binding site is apparently different from that of peptide toxins produced by spiders, sea anemones, and scorpions (Ghisalbuddin and Soderlund, 1985; Herrmann et al., 1995). The differential binding of pyrethroid and toxin molecules indicate that when they are both present within the sodium channel that they can act independently, synergistically or antagonistically.

McCutchen et al. (McCutchen et al., 1997) have characterized the effect of low concentrations of six chemical pesticides (allethrin, cypermethrin, DDT, endosulfan, methomyl, and profenofos) on the efficacy of wild type and recombinant baculoviruses. They found synergistic interactions when neonate H. virescens were exposed to AcAaIT and low concentrations (LC10-LC20 at 24 h) of the type II pyrethroid (cypermethrin) and a carbamate (methomyl). The interactions of AcAaIT with allethrin, DDT, endosulfan or profenofos were not synergistic but additive. Specifically, neonate H. virescens that were inoculated with 2000 polyhedra per larva (a greater than LC50 dose) of AcAaIT and immediately exposed to an LC10-LC20 dose of cypermethrin or methomyl showed an LT50 of 30 h, a reduction of roughly 58% compared to control larvae infected with the virus alone. Neither synergistic nor antagonistic effects were observed in control experiments in which AcJHE.KK (a recombinant AcMNPV expressing a combination with a low dose of pyrethroid in a pyrethroid-resistant strain (PEG strain) of H. virescens, H. armigera, and S. littoralis.

Pyrethroid-resistant neonate H. virescens that were infected with AcAaIT (at a greater than LC50 dose) died approximately 11% faster (LT50 of 63 vs. 71 h) than pyrethroid-sensitive neonate H. virescens infected with AcAaIT. Furthermore, this decrease in the LT50 was not found in pyrethroid-resistant neonates that were infected with wild type AcMNPV (LT50 of 94 h and 92 h in the resistant and sensitive strains, respectively). McCutchen et al. (McCutchen et al., 1997) thus suggested that the pyrethroid-resistant larvae are more sensitive to the neurotoxin AaIT. They further suggested that the use of AcAaIT or other recombinant baculovirus might provide the means to deter the onset of insecticide resistance in the field and might be used to drive resistant populations toward susceptibility.
In related experiments, Popham et al. (Popham et al., 1998b) tested the effectiveness of the coapplication of the type II pyrethroid deltamethrin and recombinant AcMNPVs expressing μ-Aga-IV, AS II, or Sh I (genes mag4, sat2, and ssh1, respectively) in neonate larvae of *T. ni* and *H. virescens*. The genes encoding these toxins were placed under the very late PsynXIV (vMAg4p +, vSAt2p +, and vSh1p +, respectively) promoters. By diet incorporation bioassays in neonate *T. ni* treated with a low dose (LC50) of deltamethrin and a low dose (LC20) of vMAg4p +, vSAt2p +, or vSh1p +, they found additive effects in terms of larval mortality, but no synergistic effects. By droplet feeding bioassays in neonate *T. ni* treated with a low dose of deltamethrin and a high (LC75 or greater) dose of vMAg4p +, vSAt2p +, or vSh1p +, they found no synergistic effects. Synergistic effects were observed in at least one time response parameter (FT50, ET50, or LT50) of vMAg4p +, vSAt2p +, or vSh1p +, showed a synergistic response in terms of FT50, ET50, and LT50. In similar droplet feeding bioassays in neonate *T. ni* treated with a low dose of deltamethrin and a high dose of vMAg4p +, vSAt2p +, and vSh1p +, they found no synergistic effects. Synergistic effects were observed in at least one time response parameter (FT50, ET50, or LT50) in droplet feeding bioassays in neonate *H. virescens* treated with a low dose of deltamethrin and high dose of a toxin-expressing AcMNPV. In all of the cases where a synergistic effect was observed, the coapplication of a high viral dose was also required. Thus, Popham et al. (Popham et al., 1998b) suggested that (in contrast to the McCutchen et al. (McCutchen et al., 1997) study discussed above) the coapplication of a pyrethroid and a recombinant baculovirus would confer little or no advantage in the field because low doses of the virus would most likely be applied. Furthermore, they suggested that the different outcomes of the McCutchen et al. (McCutchen et al., 1997) study and their study might result from differences in the method of application of the pyrethroid.

A wide variety of venomous and nonvenomous animals, bacteria, and even plants possess peptide toxins that are highly potent and selective for lepidopteran insects and which have the potential to improve the insecticidal activity of recombinant baculoviruses. Rapid methods for the separation, purification, and identification of toxins from venom and other biological matrices have been developed (Nakagawa et al., 1997, 1998). These methods have been successfully used for the identification of a number of insecticidal peptide toxins, for example, AaT5 from *A. australis* (Nakagawa et al., 1997) and ButaIT from the South Indian red scorpion *B. tamulus* (Wudayagiri et al., 2001). ButaIT is a short chain neurotoxin that targets the insect potassium channel, but is particularly interesting because it is highly selective towards the *Heliothine* subfamily but non-toxic against blowfly larvae and other insects. This high level of specificity may provide some unique advantages in comparison to other toxin species. The concept of delivering individual toxins or combinations of toxins via baculoviruses remains a major route to practical application of insect-selective peptide toxins. Whether used alone or in combination, the ever-increasing number of characterized insect-selective toxins, and their compatibility and complementary nature to existing pest control strategies including chemical insecticides provide us with extraordinary tools for “green” pest control.

### 4 Modification of the Baculovirus Genome

#### 4.1 Deletion of the Ecdysteroid UDP-Glucosyltransferase Gene

Ecdysteroids are key molecules in the regulation of physiological events such as molting in insects. More than 60 different ecdysteroids have been isolated from insects and other arthropods, and about 200 phytoecdysteroids from plants (Dinan, 2001; Henrich et al., 1999; Lafont, 2000; Nijhout, 1994). Ecdysone was the first isolated and is the best characterized ecdysteroid. Following its production and secretion from the prothoracic glands, ecdysone is converted into 20-hydroxyecdysone in the hemolymph, epidermis, and fat body. 20-Hydroxyecdysone is the primary active form of the molting hormone in most insects. Ecdysteroids are often bound to specific carrier proteins that putatively help the ecdysteroid to penetrate cell membranes or increase the capacity of the hemolymph for ecdysteroids. Like juvenile hormone, the titer of ecdysteroids in the hemolymph is determined by both their synthesis and their metabolism. During normal insect development, ecdysteroids are transported to the nucleus where they initiate a cellular response. The prevention of ecdysteroid transport results in the interruption of insect growth or abnormal development (or death).

Ecdysteroid UDP-glucosyltransferase is a baculovirus-encoded enzyme that catalyzes the conjugation of sugar molecules to ecdysteroids (O’Reilly, 1995; O’Reilly and Miller, 1989). The conjugation of a hydrophilic sugar molecule to the ecdysteroid prevents it from crossing cellular membranes. Thus, conjugation effectively inactivates ecdysteroid function resulting in the inhibition of molting and pupation. O’Reilly and Miller (O’Reilly and Miller, 1989) were the first to identify a gene, egt, that encodes ecdysteroid UDP-glucosyltransferase in a baculovirus (AcMNPV). Homologs of the AcMNPV egt gene have been identified in approximately 90% (20 NPVs and 8 GVs, Table 1) of the baculovirus genomes that have been searched (Clarke et al., 1996; Tumilasci et al., 2003). Although the egt gene is commonly found in both NPVs and GVs, it is not essential for in vitro (in cultured insect cells) or in vivo (in larval hosts) replication of AcMNPV (O’Reilly and Miller, 1989, 1991). The egt gene has also been shown to be nonessential in the NPVs of *M. brassicae* (Clarke et al., 1996), *L. dispers* (Slavicek et al., 1999), *H. armigera* (Chen et al., 2001), *H. zea* (Treacy et al., 2000), *B. mori* (Kang et al., 2000), and *A. gemmatalis* (Rodrigues et al., 2001) (Table 2). Larvae of *S. frugiperda* or *T. ni* infected with vEGTDEL, an egt deletion mutant of AcMNPV, show earlier mortality and reduced feeding (by about 40%) in comparison to control larvae infected with wild type AcMNPV (Eldridge et al., 1992a; O’Reilly and Miller, 1991; Wilson et al., 2000). Specifically, in time-mortality bioassays (at an LC50 or LC90 dose), the ST50 of vEGTDEL-infected neonate *S. frugiperda* is reduced by approximately 22% (99.7 vs. 127.2 h) in comparison to control larvae infected with AcMNPV. Furthermore, vEGTDEL-infected larvae yield 23% fewer progeny virus (polyhedra) in comparison to AcMNPV-infected larvae. In second or fourth instar
**Table 1** Baculoviruses carrying an ecldysteroid UDP-glucosyltransferase (egt) gene homolog

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus designation</th>
<th>Reference/accession number</th>
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<tbody>
<tr>
<td>Nucleopolyhedrovirus</td>
<td><em>Autographa californica</em> MNPV (AcMNPV)</td>
<td>(O’Reilly and Miller, 1989)</td>
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<td></td>
<td><em>Anticarsia gemmatalis</em> MNPV (AgMNPV)</td>
<td>(Rodrigues et al., 2001)</td>
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<td></td>
<td><em>Amata albiglascia</em> NPV (AalMNPV)</td>
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<td></td>
<td><em>Bombyx mori</em> NPV (BmMNPV)</td>
<td>AF204881</td>
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<td></td>
<td><em>Buzura suppressarina</em> NPV (BusuNPV)</td>
<td>(Gomi et al., 1999)</td>
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<td></td>
<td><em>Choristoneura fumiferana</em> defective MNPV (CfDEFMNPV)</td>
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<td></td>
<td><em>C. fumiferana</em> MNPV (CfMNPV)</td>
<td>(Barrett et al., 1995)</td>
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<td></td>
<td><em>Ecotropis oblique</em> NPV (EobNPV)</td>
<td>AF107100</td>
</tr>
<tr>
<td></td>
<td><em>Epiphyas postvittana</em> MNPV (EppoMNPV)</td>
<td>(Caradoc-Davies et al., 2001)</td>
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<td></td>
<td><em>Heliothis armigera</em> NPV (HearNPV)</td>
<td>(Chen et al., 1997)</td>
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<td></td>
<td><em>H. zea</em> NPV (HzNPV)</td>
<td>(Hu et al., 1997)</td>
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<td></td>
<td><em>Lymantria dispar</em> MNPV (LdMNPV)</td>
<td>(Popham et al., 1997)</td>
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<td></td>
<td><em>Mamestra configurata</em> NPV (MacoNPV)</td>
<td>(Li et al., 2002)</td>
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<tr>
<td></td>
<td><em>M. brassicae</em> MNPV (MbMNPV)</td>
<td>(Clarke et al., 1996)</td>
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<td></td>
<td><em>Orgyia pseudotsugata</em> MNPV (OpMNPV)</td>
<td>(Pearson et al., 1993)</td>
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<td></td>
<td><em>Rachiplusia ou</em> MNPV (RoMNPV)</td>
<td>(Harrison and Bonning, 2003)</td>
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<td></td>
<td><em>Spodoptera exigua</em> MNPV (SeMNPV)</td>
<td>(Ijkel et al., 1999)</td>
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<td><em>S. frugiperda</em> MNPV (SfMNPV)</td>
<td>(Tumilasci et al., 2003)</td>
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<td></td>
<td><em>S. littoralis</em> NPV (SpNPV)</td>
<td>(Barrett et al., 1995)</td>
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<td></td>
<td><em>S. litura</em> NPV (SpltNPV)</td>
<td>(Kikhno et al., 2002)</td>
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**Granulovirus**

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<tr>
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<th>Virus designation</th>
<th>Reference/accession number</th>
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<tr>
<td>Adoxophyes honmai GV (AdhoGV)</td>
<td></td>
<td>(Nakai et al., 2002)</td>
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<tr>
<td><em>A. orana</em> GV (AdorGV)</td>
<td></td>
<td>(Wormleaton and Winstanley, 2001)</td>
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<tr>
<td><em>C. fumiferana</em> GV (CfgV)</td>
<td>AF058690</td>
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<tr>
<td><em>Cydia pomonella</em> GV (CpGV)</td>
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<td>(Luque et al., 2001)</td>
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<tr>
<td><em>Epinotia aporema</em> GV (EpapGV)</td>
<td></td>
<td>(Manzan et al., 2002)</td>
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<tr>
<td><em>Lacanobia oleracea</em> GV (LoGV)</td>
<td></td>
<td>(Smith and Goodale, 1998)</td>
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<tr>
<td><em>Phthorimaea operculella</em> (PophGV)</td>
<td></td>
<td>(Taha et al., 2000)</td>
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<tr>
<td><em>Plutella xylostella</em> GV (PxGV)</td>
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<td>(Hashimoto et al., 2000)</td>
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**Table 2** Genetic modifications at the egt gene locus

<table>
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<th>Parental virus</th>
<th>Virus designation</th>
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<tbody>
<tr>
<td>AcMNPV</td>
<td>VEGTZ</td>
<td>(O’Reilly and Miller, 1989)</td>
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<tr>
<td></td>
<td>vEGTDEL</td>
<td>(O’Reilly and Miller, 1991; Treacy et al., 1997)</td>
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<td></td>
<td>vJHEEGTD</td>
<td>(Eldridge et al., 1992a)</td>
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<tr>
<td></td>
<td>vEGHEGTD</td>
<td>(Eldridge et al., 1992b)</td>
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<tr>
<td></td>
<td>VEGT-PTTHM</td>
<td>(O’Reilly et al., 1995)</td>
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<tr>
<td></td>
<td>AcMNPV-&lt;em&gt; egt&lt;/em&gt;</td>
<td>(Bianchi et al., 2000)</td>
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<tr>
<td></td>
<td>vVP6.9tox34</td>
<td></td>
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<tr>
<td></td>
<td>vVE6.9tox34</td>
<td>(Popham et al., 1997)</td>
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<td>vHSP70tox34</td>
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<td>vVE6.9HSPtox34</td>
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<td>AgMNPV</td>
<td>VEGTDZ</td>
<td>(Rodrigues et al., 2001)</td>
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<td>BmNPV</td>
<td>vAgEGT.1-lacZ</td>
<td>(Pinedo et al., 2003)</td>
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<td>HaSNPV (HearNPV)</td>
<td>BMGTZ</td>
<td>(Kang et al., 2000)</td>
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<td>HaLM2, HaCXW1, HaCXW2</td>
<td>(Chen et al., 2000; Sun et al., 2002)</td>
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<td>HzSNPV</td>
<td>HZGTDel, HZGTp6.9tox34, HZGTsptoxt34, HZGTDA26tox34</td>
<td>(Popham et al., 1997)</td>
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<td>LdMNPV</td>
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<td>(Slavicek et al., 1999)</td>
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<td>MbMNPV</td>
<td>vEGTDZ</td>
<td>(Clarke et al., 1996)</td>
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latter of *T. ni*, vEGTDEL infection reduces the time to death by about 11% in comparison to AcMNPV infection. Virus yield is also lower in fourth instar larvae but not in the second instars. The egt gene, thus, provides a selective advantage to the virus in terms of the production of progeny. Conversely, this suggests that removal of the egt gene generates a virus that is less fit in comparison to the wild type virus. In general, deletion of the egt genes of other NPVs has resulted in improvements in the speed of kill of 15–33% in comparison to the wild type virus (e.g., Chen et al., 2000; Pinedo et al., 2003; Popham et al., 1998a; Slavicek et al., 1999; Treacy et al., 1997, 2000). However, examples in which deletion of the egt gene does not reduce speed of kill are also found. Popham et al. (Popham et al., 1997) found that deletion of the egt gene of HzSNPV had no effect in terms of reducing the ET50 (67.3 vs. 65.4 h) in neonate larvae of *H. zea* in comparison to the wild type virus. Bianchi et al. (Bianchi et al., 2000) found that L350 of AcMNPV-Δegt, an egt gene deletion mutant of AcMNPV, was the same as that of wild type AcMNPV in second and fourth instar larvae of *S. exigua*. Sun et al. (Sun et al., 2004) found that the ST50 of neonate larvae of *H. armigera* infected with HaSNPV-EGTD (egt gene deletion mutant of HaSNPV) was not significantly improved over HaSNPV-infected larvae, whereas second-fifth instar larvae were killed more rapidly. Thus, they suggested that egt gene deletion affects the speed of kill of the virus more strongly in later instars than in earlier instars.

Mechanistically, it is still unclear how deletion of the egt gene improves the speed of kill. In second instar larvae of *S. exigua*, Flipsen et al. (Flipsen et al., 1995) found that an egt gene deletion mutant of AcMNPV caused earlier degradation of the Malpighian tubules in comparison to the wild type AcMNPV. They speculated that this earlier degradation results in the faster speed of kill. Another possibility is that the general level of protein synthesis is elevated in larvae infected with the egt deletion virus (due to relatively higher levels of active ecdysteroids). Consistent with this hypothesis, Kang et al. (Kang et al., 2000) found that virus-encoded and virus-induced proteins are expressed earlier in the infection cycle and at higher levels in larvae of *B. mori* that are infected with BmEGTZ, an egt gene deletion mutant of BmNPV, in comparison to the larvae infected with the wild type BmNPV. Thus, mature progeny virions may be made and released more quickly in larvae infected with BmEGTZ than in larvae infected with BmNPV resulting in a faster systemic infection and death. Kang et al. (2000) have also shown that virus- and certain host-specific proteins are induced in a concentration dependent manner by the injection of purified ecdysteroids into BmEGTZ-infected larvae (24 h prior to injection of ecdysteroids), but not in larvae infected with BmNPV. Furthermore, using a histochemical assay they showed that virus transmission is positively correlated with the amount of ecdysteroids that are injected into a larva (i.e., the higher the amount of ecdysteroids injected, the faster the transmission of the virus). In contrast, O’Reilly et al. (O’Reilly et al., 1995) found that the pathogenicity of a recombinant egt-gene-deleted AcMNPV that expresses biologically active PTH (vEGTPTTHM) is dramatically reduced (LC50 of 280 × 10^7 vs. 3.8 × 10^7 polyhedra per ml) in comparison to vEGTDEL. This effect was not found in a PTH expressing recombinant AcMNPV (vWTPTTHM) in which the egt gene was functional. In vEGTPTTHM-infected larvae of *S. frugiperda*, the titer of ecdysteroids spiked at around 96 h p. i. (1162 ± 476 pg/μL) and quickly dropped at 120 h p. i. (45 ± 12 pg/μL). In contrast, in vEGTDEL-infected larvae, the titer of ecdysteroids increased at 72 h p. i. (519 ± 199 pg/μL) and remained high at 96 h p.i. (282 ± 55 pg/μL) and 120 h p. i. (334 ± 80 pg/μL). Although the peak ecdysteroid titer in vEGTPTTHM-infected larvae appeared to be significantly higher than that found in vEGTDEL-infected larvae, this may not be the case because a higher percentage (54.5 ± 21.0% vs. 32.3 ± 5.1%) of the ecdysteroids in the vEGTPTTHM-infected larvae were in the conjugated form in comparison to vEGTDEL-infected larvae.

An elegant approach to further improve the egt gene deletion construct has been to insert an insect-selective neurotoxin gene cassette into the egt gene locus, thus generating a toxin expressing, egt gene inactivated construct. For example, Chen et al. (Chen et al., 2000) inserted an AaIT-GFP gene cassette (*aait* and green fluorescent protein (*gfp*) genes driven by the *polh* gene promoters of HaSNPV and AcMNPV, respectively) into the egt gene locus of HaSNPV (also known as HearNPV) in order to generate HaCXW2. In time-mortality bioassays, they found that the ST50 of second instar *H. armigera* infected with HaCXW2 was reduced by 32% (63.9 v 94.4 h) and 8% (63.9 vs. 69.5 h) in comparison to control larvae infected with wild type HaSNPV or HaCXW1 (a recombinant HaSNPV carrying *gfp* at its egt gene locus). Sun et al. (Sun et al., 2004, 2002) also showed that HaCXW1 and HaCXW2 were effective pest control agents under field conditions. In their field experiments, second instar *H. armigera* were allowed to feed on HaCXW1- or HaCXW2-treated plots (1.8 × 10^10 polyhedra per 0.015 hectare plot containing 800 cotton plants) for 12 h, then the larvae were transferred to artificial diet and reared at ambient temperature. The ST50 values of the larvae infected with HaCXW1 or HaCXW2 in the field were reduced by 15% (101 vs. 119 h) and 26% (88 vs. 119 h), respectively, in comparison to control larvae that were field-infected with HaSNPV. Treatment of cotton plants in the field with HaCXW1 or HaCXW2 reduced leaf consumption by approximately 50 and 63%, respectively. The results of additional field trials using HaSNPV-AaIT (a recombinant HaSNPV expressing AaIT under a chimeric ph-p69p promoter at the egt gene locus) are discussed below.

From these results it appears that deletion of the egt gene from the baculovirus genome is a useful strategy to improve its speed of kill and consequently its insecticidal activity, but by itself the reduction in speed of kill is too small to be of commercial value. Deletion of the egt gene, however, reduces the number of progeny viruses that are produced in comparison to the wild type virus. Thus, the fitness of the egt gene-deleted virus appears to be reduced in comparison to the wild type virus. Conceptually, removal of an endogenous gene from its genome is safer than the insertion of a heterologous gene because a “new” gene will not be introduced into the environment. In practice, however, we believe that there will be no significant difference in the risk of a recombinant baculovirus pesticide in which an endogenous gene is deleted from the genome or in which a heterologous gene is inserted into the genome. Safety issues and fitness of the virus are further discussed below.
4.2 Removal of Other Nonessential Genes

In a comparative study of seven completely sequenced lepidopteran baculovirus genomes, Hayakawa et al. (Hayakawa et al., 2000a) identified 67 putative genes that were common to all seven. When Herniou et al. (Herniou et al., 2001, 2003) added four additional lepidopteran NPVs and a GV (i.e., a total of twelve completely sequenced lepidopteran baculoviruses) 62 common genes were identified. Considering that the genomes of most baculoviruses encode well over 100 putative genes, these findings suggest that a large number of genes in the genome of lepidopteran baculoviruses may serve auxiliary or host-specific functions or help to improve the fitness of the virus. This hypothesis is consistent with finding that 61 out of the 136 putative genes of BmNPV appear not to be essential for viral replication in BmN cells (i.e., some level of viral replication was detected even when a gene was deleted by replacement with a lacZ gene cassette) (Kamita et al., 2003b and Gomi, Kamita, and Maeda, unpublished). These findings suggest that there are a number of target genes that can be removed from the baculovirus genome in order to decrease fitness, alter host specificity, or simply to obtain a virus that may replicate faster or more efficiently.

Dai et al. (Dai et al., 2000) have identified a mutant of S. exigua MNPV (SeXD1) that is lacking 10.6 kbp of sequence (encoding 14 complete or partial genes including an egt gene homolog) that corresponds to the region between 13.7 and 21.6 map units in the wild type SeMNPV genome. Dose-mortality bioassays in third instar larvae of S. exigua showed that the LD_{50} of SeXD1 and SeMNPV (403 and 124 polyhedra per larva, respectively) were not significantly different. Time-mortality bioassays indicated that the ST_{50} of third instar S. exigua infected with SeXD1 was reduced by 25% (70.2 vs. 93.1 h) in comparison to control larvae infected with SeMNPV. Dai et al. (Dai et al., 2000) did not further examine which gene or genes in this 10.6 kbp region are involved in decreasing the virulence of SeXD1 in comparison to SeMNPV. Considering that deletion of the egt gene from other baculoviruses has been shown to reduce the survival times of larvae infected with these viruses, the egt gene homolog of SeMNPV appears to be a prime candidate as the gene involved in reducing the virulence. However, several of the other genes that were deleted in this region are commonly found in other baculoviruses and may play roles in enhancing infection (e.g., gp37) or dissemination (e.g., v-cath, chIa, and ptp-2) of the virus. Genes that help to enhance virulence or improve dissemination, but are nonessential, are potential targets for deletion in order to reduce the fitness of the virus.

As discussed above, the ST_{50} of larvae that are infected with an egt gene-deleted mutant baculovirus is often reduced in comparison to larvae that are infected with the wild type virus. This reduction in survival time often results in the production of fewer progeny virus (i.e., fewer polyhedra per larva) suggesting that the fitness of the egt gene-deleted virus will also be reduced in comparison to the wild type virus. Two other baculovirus genes that are involved in altering the virulence of the baculovirus are orf603 (Popham et al., 1998a) and Ac23 (Lung et al., 2003) both of AcMNPV origin. The LC_{50} of an AcMNPV variant (V8) in which the orf603 gene is truncated is not significantly different from that of AcMNPV. The AcMNPV V8 variant (as well as other mutants in which the orf603 gene was specifically inactivated) shows an ET_{50} that is reduced by approximately 12% (e.g., 88.5 vs. 100.8 h) in comparison to a revertant virus (i.e., a virus in which the orf603 mutation was repaired). In an attempt to further decrease the speed of kill of the V8 variant of AcMNPV, Popham et al. (Popham et al., 1998a) inserted the tox34 gene under the control of either the p6.9 or hsp70 promoter into the egt gene locus of V8 generating V8EEp6.9tox34 and V8EEHSptoxt34, respectively. In time-mortality bioassays in neonate larvae of S. frugiperda (at an LC_{50} dose), the ET_{50} of V8EEp6.9tox34 and V8EEHSptoxt34 were reduced by approximately 34% (58.2 vs. 88.2 h) and 42% (51.2 vs. 88.2 h), respectively, in comparison to control larvae infected with V8EGTdel, an egt deletion gene from V8. However, in comparison to larvae infected with viruses in which the orf603 gene was not deleted (but expressing the tox34 gene under the p6.9 or hsp70 promoter at the egt gene locus), no differences were found in the ET_{50}. They speculated that the relatively small decrease in speed of kill elicited by deletion of the orf603 gene was masked by expression of the toxin gene.

A low-pH-activated fusion protein called GP64 is found in the envelopes of all group I NPVs, but lacking in group II NPVs. A different low-pH-activated envelope fusion protein called the F protein (Westenberg et al., 2002) has been identified in group II NPVs. F proteins were first identified in LdMNPV (Pearson et al., 2000) and SeMNPV (Ijkel et al., 2000); however, F protein homologs are found in the genomes of all of the sequenced baculoviruses (i.e., both group I and group II NPVs and GVs) (Hayakawa et al., 2000a; Herniou et al., 2001). The F proteins of group II NPVs can functionally substitute for GP64 (Lung et al., 2002), whereas the F proteins of group I NPVs appear not to function in membrane fusion (Pearson et al., 2001). Lung et al. (Lung et al., 2003) found that Ac23, the F protein homolog of AcMNPV, is not essential for AcMNPV replication in Sf-9 or High 5 cells or larvae of T. ni. The growth curve of Ac23null, an Ac23 deletion mutant of AcMNPV, is indistinguishable to those of a repair virus of Ac23null (Ac23null-repair) or wild type AcMNPV. Ac23null and Ac23null-repair also show indistinguishable lethal doses in larvae of T. ni. However, in survival-time bioassays, the survival times of neonate or fourth instar T. ni that are infected with Ac23null are at least 28% (26 h) longer than those of control larvae infected with Ac23null-repair or AcMNPV (i.e., viruses that carry the Ac23 gene). Interestingly, although not quantified, Sf-9 cells infected with Ac23null appear to produce significantly lower yields of polyhedra in comparison to Sf-9 cells infected with the control viruses (G.W. Blissard and O.Y. Lung, personal communications).

Wandering is a behavior that normally occurs only at the end of the larval stage in lepidopteran larvae (Goulson, 1997; Nijhout, 1996). At this stage, holometabolous insects stop feeding, void their gut, and look for a suitable location to pupate. Baculoviruses can artificially induce wandering in infected larvae to increase virus spread (Evans, 1986; Goulson, 1997; Vasconcelos et al., 1996). We have recently found that baculovirus-induced wandering requires a virus-encoded protein tyrosine phosphatase (ptp) gene using a ptp gene-deleted mutant of BmNPV (BmPTPD) (Kamita et al., 2003). Time-mortality bioassays (at an LC_{50} dose) showed that the ST_{50} of BmPTPD- or BmNPV-infected neonate larvae of B. mori (88.1 and 81.7 h, respectively) were not significantly different. BmPTPD-infected neonates, however, did not display any symptoms of virus-induced wandering.
as did the BmNPV-infected neonates. Thus, the *ptp* gene is directly involved in increasing the dissemination of the virus. Removal of this gene may reduce the dispersal (and consequently fitness) of the virus in the field.

### 4.3 Alteration of Host Range

Although baculoviruses have been isolated from more than 400 insect species (Tanada and Kaya, 1993), most baculovirus isolates are permissive only in the original insect host from which they were isolated. Thus, the host range of individual baculoviruses is considered to be narrow. Among baculoviruses, AcMNPV has a relatively wide host range being infectious against more than 30 insect species (Groner, 1986) including a large number of pest insect species. The baculovirus must overcome a number of obstacles both at the organismal and cellular levels in order to replicate and eventually kill the insect host. At the organismal level, the polyhedra must first dissociate and release ODVs that can establish a primary infection. As discussed above, the dissociation of the polyhedra requires high pH and the presence of proteases, whereas the released ODVs must pass through at least three midgut barriers prior to establishing a systemic infection. At the cellular level, the BV must recognize and attach to a receptor on the cell surface, and enter the cell via endocytosis. Subsequently, the nucleocapsid must translocate to a nuclear pore, enter the nucleus, uncoat to release viral DNA, express early genes, initiate viral DNA synthesis, express late genes, and assemble and release of mature virions. The baculovirus is able to express some early genes in a wide variety of insect cells (Miller and Lu, 1997; Thiem, 1997). Significantly, reduced levels of viral DNA synthesis and late gene promoter activity are also observed in some non-permissive insect cell lines. Thus, the host range of a baculovirus may be limited, at least in terms of insect hosts, to its ability to (1) express the entire complement of early genes, (2) synthesize genomic DNA at normal levels, (3) express late/very late genes at normal levels, and/or (4) assemble and release mature virions. Other key factors in the ability of a baculovirus to productively infect a host cell include its ability to inhibit (1) the premature or global cessation of protein synthesis (Thiem, 1997) and (2) virus-induced apoptosis (Clem, 2001; Miller et al., 1998).

The gypsy moth *L. dispar* and the *L. dispar*-derived Ld652Y cell line are nonpermissive hosts of AcMNPV (Chen et al., 1998; Du and Thiem, 1997a; Groner, 1986). AcMNPV infection of Ld652Y cells induces cytotoxicity and a premature cessation of host and viral protein synthesis (Du and Thiem, 1997b; Mazzacano et al., 1999). An *L. dispar* multicapsid NPV (LdMNPV) gene, host range factor 1 (*hrf-1*), has been identified that promotes AcMNPV replication in Ld652Y cells (Du and Thiem, 1997a; Thiem et al., 1996) and gypsy moth larvae (Chen et al., 1998). LdMNPV *hrf-1* does not function as an apoptotic suppressor (Du and Thiem, 1997b), however, in vitro translation assays indicate that the mechanism of translation arrest involves defective or depleted tRNA species (Mazzacano et al., 1999). In neonate larvae of *L. dispar*, the LC₅₀ of a recombinant AcMNPV carrying the *hrf-1* gene of LdMNPV (vAcLdPD) is reduced by greater than 1800-fold (1.2 x 10⁵ vs. 2.2 x 10⁸ polyhedra per mL) in comparison to AcMNPV. The LC₅₀ of vAcLdPD, however, was still 10-fold higher (1.2 x 10⁵ vs. 1.1 x 10⁵ polyhedra per mL) than that of LdMNPV in neonate *L. dispar*. In second instar larvae of *H. zea*, the LC₅₀ of vAcLdPD is 5.8-fold lower (5.49 x 10⁵ vs. 3.17 x 10⁶ polyhedra per mL) than that of AcMNPV. However, the LC₅₀ of AcLdPD was not significantly different to that of AcMNPV in second instar larvae of *P. xylostella* (AcMNPV-resistant insect) and *S. exigua* (AcMNPV-sensitive insect).

Two different types of anti-apoptotic genes, *p35* and *iap*, have been identified in baculoviruses (Bortner and Cidlowski, 2002; Clem, 2001; Clem et al., 1996). The *p35* gene of AcMNPV was first shown by Clem et al. (Clem et al., 1991) to function as an inhibitor of apoptosis in baculovirus-infected SF-21 cells. The P35 protein is an inhibitor of caspases (Bump et al., 1995; Xue and Horvitz, 1995) in a very wide variety of organisms (Bortner and Cidlowski, 2002; Clem, 2001). The first *iap* gene was identified by Crook et al. (Crook et al., 1993) in *Cydia pomonella* GV. Subsequently, *iap* gene homologs have been identified in at least ten other baculoviruses and a wide range of other organisms (Clem, 2001). The wild type AcMNPV induces apoptosis in SL2 cells, a cell line derived from the Egyptian cotton worm *S. littoralis* (Chejanovsky and Gershburg, 1995; Du et al., 1999). The yield of AcMNPV BV in SL2 cells infected with AcMNPV is approximately 2700-fold lower than that of SF-9 cells infected with AcMNPV. A recombinant AcMNPV (vHSP-P35) that over expresses the *p35* gene of AcMNPV under the hsp70 promoter does not induce apoptosis in SL2 cells, however, vHSP-P35 still produces low yields of BV (Gershburg et al., 1997). Lu et al. (Lu et al., 2003) have found that AcMNPV is able to efficiently replicate in SL2 cells and larvae of *S. littoralis*, if it expresses higher levels of IE1 (an essential, multifunction protein that was first identified by its ability to trans-activate baculovirus early genes) relative to IE0 (another protein that is capable of trans-activating baculovirus early genes). The IE0 protein is a longer form (by 54 N-terminal amino acids) of the IE1 product that is the result of transcriptional initiation from the *ie0* transcription initiation site and removal of the *ie0* intron (Friesen, 1997). Lu et al. used two approaches to increase the level of IE1 relative to IE0. Firstly, they generated a mutant virus that expressed lower levels of IE0 by disrupting the *ie0* promoter by insertion of a 519 bp-long DNA fragment or a chloramphenicol acetyltransferase gene. Secondly, they generated a recombinant AcMNPV (vHsp-1) that expressed a second copy of the *ie1* gene under the constitutively expressed hsp70 promoter. Both of these approaches generated genetically modified AcMNPVs with host ranges that extended to *S. littoralis*.

At present, over expression of AcMNPV *ie1* and insertion of LdMNPV *hrf-1* into the AcMNPV genome are the only ways in which the host range of AcMNPV can be extended to include pest insect species. Other AcMNPV genes that are involved in extending host range or dramatically increasing virulence include *p35* (Clem et al., 1991; Clem and Miller, 1993; Griffiths et al., 1999; Hershberger et al., 1992), the DNA helicase gene *p143* (Croizier et al., 1994; Maeda et al., 1993), host cell-specific factor 1 (*hrf-1*) (Lu and Miller, 1995), late expression factor 7 (*lef-7*) (Lu and Miller, 1995), and immediate early gene 2 (*ie-2*) (Prikhodko et al., 1999). The roles of these genes in host range expansion and increase of virulence is discussed in the previous article.
5 Safety of GM Baculoviruses

The safety of a synthetic chemical or biologically based pesticide can never be assured with absolute confidence. Thus, when society considers whether or not a new pesticide is "safe," a better question to ask might be: "What level of risk are we as a society willing to accept for a given benefit?" In the case of chemical pesticides, the general public and a wide array of researchers (scientists, medical doctors, governmental regulators, statisticians, pathologists, ecologists, etc.) have analyzed the actual, potential, and perceived risks that they pose on human health and the environment. In general, all of these stakeholders agree that the benefits of chemical pesticides sufficiently outweigh any short- or long-term risks that they may pose. Society is thus willing (and should be willing) to accept some level of risk for a given benefit (e.g., increased yields of food, fiber, and feed from a given amount of natural resource). There are of course instances in which society has determined that the costs and risks associated with a particular pesticide (e.g., DDT) outweigh the benefits. In terms of the use of a GM baculovirus pesticide, this leads us to the questions of (1) What are the risks associated with the field release of a GM baculovirus? (2) Are these risks worth the benefits? and (3) Are these risks any greater than the risks associated with the use of a chemical insecticide? A number of research studies and essays have addressed the first question above. Miller (Miller, 1995) and Hails (Hails, 2001) suggest that the primary focus of assessing the risks of the field release of a GM baculovirus should include:

1. How the GM baculovirus affects nontarget species.
2. Whether the introduced gene provides a selective advantage to viral replication, survival and/or host range (i.e., is fitness improved).
3. Should the introduced gene transfer to another organism will this event provide a selective advantage to that organism.

5.1 Potential Effects of a GM Baculovirus on Nontarget Species

The potential of natural and recombinant baculoviruses to induce a deleterious effect upon vertebrates has been extensively studied (Black et al., 1997; Burges, 1981). More than two dozen different baculoviruses have been tested in a wide range of vertebrates including ten different mammalian species, birds, and fish. The baculoviruses in these tests were administered by various routes including inhalation, topical application, injection (intravenous, intracerebral, intramuscular, intradermal), and orally. Baculovirus-induced deleterious effects were not observed in any of these tests. Direct and indirect tests on human subjects have also been conducted including oral administration of high (greater than 10⁵ polyhedra) doses of polyhedra of HzSNPV over a period of five days (Heimpel and Buchanan, 1967). The blood of workers that were involved in the production of HzSNPV during a 26 month-long period has also been analyzed for infectious baculovirus, baculoviral antigens or baculoviral antibodies in their blood and none were detected (Black et al., 1997). Furthermore, the ubiquitous baculovirus load from foods in our diet may be quite high (Heimpel et al., 1973; Thomas et al., 1974). For example, Heimpel et al. (1973) found that a square inch (approximately 6.5 cm²) of cabbage taken from a store shelf may contain up to 2 x 10⁶ polyhedra suggesting that consumption of a single serving of cole slaw (16 in² of cabbage) exposes us to up to 3.2 x 10⁷ polyhedra. These studies indicate that we live under constant exposure to baculoviruses with no apparent deleterious effects. The lack of deleterious effects is not completely unexpected since polyhedra are extremely insensitive to the neutral or acidic pH conditions of the vertebrate digestive system. Thus, any polyhedra that are normally ingested while eating cole slaw, for example, pass through the digestive tract and are either excreted intact or should any ODVs be released they are quickly inactivated first by the action of pepsin at low pH and subsequently by the pancreatic proteases at neutral pH (Black et al., 1997; Miller and Lu, 1997).

Beginning in 1995, two studies have shown that baculoviruses can serve as highly efficient vehicles for the transfer of foreign genes into the nuclei of primary cultures of human, mouse, and rat hepatocytes and human hepatoma cell lines (Boyce and Bucher, 1996; Hofmann et al., 1995). Since then, baculoviruses have been shown to be able to transduce a number of mammalian cell types including human neural cells (Sarkis et al., 2000), human fibroblasts (Dwarakanath et al., 2001), human keratinocytes (Condrey et al., 1999), and several established mammalian cell lines (Boyce and Bucher, 1996; Shoji et al., 1997). Non-mammalian, vertebrate cell lines (e.g., fish cells) have also been transduced with a recombinant baculovirus (Leisy et al., 2003; Wagle and Jesuthasan, 2003). At first glance, these studies appear to raise concerns about the risks of baculoviruses that are used for pest control. However, upon careful consideration we believe that they provide further evidence as to the safety of GM baculoviruses. Firstly, these studies show that although the baculovirus can enter the mammalian cell, productive baculovirus infection does not occur even following inoculation at exceptionally high multiplicities of infection (MOI) of up to 1500 pfu per cell using a virus inoculum that was concentrated by ultracentrifugation (Barsoum et al., 1997; Hofmann et al., 1995; Hu et al., 2003; Kost and Condrey, 2002; Shoji et al., 1997). Furthermore, the inoculated cells did not show any visible cytopathic effects, grew normally, and showed normal plating efficiencies. Secondly, in all of these examples the foreign gene is expressed only when placed under a promoter that is specific for mammalian cells (e.g., cytomegalovirus immediate early, Rous sarcoma virus long terminal repeat, simian virus 40, etc.). Foreign gene expression was never observed under a very late baculoviral promoter. Thirdly, although transduction was successful in established cell lines and primary cultures, the direct application of baculoviruses for gene delivery to the liver in vivo is strongly reduced or inhibited because the baculovirus activates the complement (C) system (Hofmann and Strauss, 1998; Sandig et al., 1996). The C system represents a first line of host defense of the innate immune system for the elimination of foreign elements (Liszewski and Atkinson, 1993). Baculoviruses possess a number of characteristics including their inability to (1) replicate in mammalian cells and (2) induce any deleterious effects that make them ideal gene vectors.
delivery vectors for human gene therapy and as a screening systems for proteins and other molecules of human health interest (Lotze and Kost, 2002).

All of the studies to date indicate that there are no known risks associated with human (or other vertebrate) exposure to baculoviruses. Thus, a second focus in our discussion of the safety of recombinant baculoviruses should focus on non-target lepidopteran and other invertebrates such as predatory or beneficial insects. As discussed above and in the previous article, the normal host range of a baculovirus is generally limited to the lepidopteran insect species from which it was originally isolated (Groner, 1986). This assessment of “host range”, however, is potentially problematic because it is often based upon the ability of a baculovirus to elicit virus-specific symptomology or death. Huang et al. (Huang et al., 1997) have tested seven different recombinant NPVs (based on AcMNPV, BmNPV, LdMNPV, or OpMNPV) carrying a reporter gene encoding β-galactosidase, secreted alkaline phosphatase (SEAP), or luciferase under the control of an early (ETL) or very late (polh) promoters. These reporter viruses were tested in 23 different insect species from eight insect orders (Blattodea, Coleoptera, Diptera, Hemiptera, Homoptera, Lepidoptera, Neuroptera, and Orthoptera) and 17 families. The reporter viruses were initially injected into the test insects by hemocoelic injection. Insects that supported virus replication (i.e., on the basis of expression of the reporter protein) by injection were subsequently tested by per os (oral) inoculation with the preoccluded form of the virus. The use of these reporter viruses allowed the detection of both symptomless and pathogenic infections. By the injection experiments, β-galactosidase, SEAP, and luciferase activities were found only in the lepidopteran larvae. Consistent with previous reports, AcMNPV had the widest host range in the ten lepidopteran hosts that were tested, whereas LdMNPV was the most host specific. Host range following per os inoculation was more limited in comparison to inoculation by injection. In fact two species, the monarch butterfly Danaus plexippus and gypsy moth Lymantria dispar, that appeared to be susceptible (to AcMNPV, OpMNPV, and BmNPV) by injection were not susceptible by per os inoculation. Additionally, as pointed out by Cory (Cory and Myers, 2003) the host range of a baculovirus in the field may be considerably narrower due to spatial or temporal differences that are not found in the laboratory.

Other studies have looked at whether natural enemies of lepidopterans such as parasitoids, scavengers, and predators (Boughton et al., 2003; Heinz et al., 1995; Li et al., 1999; McCutchen et al., 1996; McNitt et al., 1995; Smith et al., 2000b) are adversely affected when they prey upon larvae that are infected with recombinant or wild type viruses. The social wasp Polistes metricus is a beneficial insect that preys on lepidopteran larvae (Gould and Jeanne, 1984). McNitt et al. (McNitt et al., 1995) analyzed five developmental parameters (i.e., larval development time, pupal development time, larval mortality, mean nest size, and mean number of adults per nest) in order to assess colony health and individual vigor of P. metricus that fed on lepidopteran larvae infected with recombinant AcMNPVs expressing AaIT or TxP-I. These toxins were expressed under either the constitutive hsp70 or very late polh promoter. There were no significant differences in any of the developmental parameters tested when the P. metricus fed on uninfected or virus-infected (at three days before exposure to P. metricus) fourth or fifth instar Spodoptera exigua. In related experiments, McCutchen et al. (McCutchen et al., 1996) allowed the parasitic wasp Microplitis croceipes to parasitize second instar Heliothis virescens that were subsequently infected with AcAaIT, AcJHE.KK or AcMNPV (at a greater than LC99 dose, 5 × 10⁶ polyhedra per larva) at various times (0, 48, 72, 96 or 120 h) post parasitization. The survival of the parasitoid was less than 4% at 0 and 48 h post parasitization but increased gradually until at 120 h post parasitization when there was no significant difference in the virus- or mock-treated H. virescens. There were no significant differences in terms of time taken for emergence of the adult wasp, ability of the F1 wasps to oviposit, or sex ratios of the F1 wasps. Additionally, there were no significant differences in the time (205–216 vs. 228 h) taken for emergence of the larval parasitoid from virus-infected H. virescens in comparison to mock-infected H. virescens. This was particularly evident in the AcAaIT-infected larvae. McCutchen et al. (1996) suggested that the parasitoid larva responds to the poor condition of its host by prematurely emerging. Additionally, wasps that developed from AcAaIT- or AcJHE.KK-infected larvae were significantly smaller than wasps that developed from mock-infected larvae.

The green lacewing Chrysoperla carnea Stephens, insidious flower bug Orius insidiosus (Say), red imported fire ant Solenopsis invicta Buren, big-eyed bug Geocoris punctipes (Say), congerant lady beetle Hippodamia convergens Guerin-Meneville, and twelve-spotted lady beetle Coleomegilla maculata DeGeer are generalist predators that attack larval lepidopterans such as H. virescens. Heinz et al. (Heinz et al., 1995) have looked at the development times of green lacewings and flower bugs that fed upon near-dead second instar larvae of H. virescens (three per day) that were infected with a greater than LC99 dose (5 × 10⁶ polyhedra per larva) of AcAaIT or AcMNPV. They found no significant differences in the survival percentages of insects that fed upon AcAaIT-, AcMNPV-, or mock-infected H. virescens. There was a significant but short (19.1 vs. 20.6 days) decrease in the larva-to-adult development time in green lacewings that fed upon AcAaIT- or AcMNPV-infected H. virescens in comparison to lacewings that fed upon mock-infected H. virescens. One may speculate that the protein quality in the prey might be reduced due to over expression and agglomeration of the toxin proteins resulting in these subtle differences. Li et al. (Li et al., 1999) have investigated the life history traits (rate of food consumption, travel speed, fecundity, and adult survival) of fire ants, big-eyed bugs, and congerant lady beetles that fed on second instar H. virescens that were infected with one of seven viruses (AcMNPV, HzSNPV, HzSNPV expressing LqhIT2 under the ie1 gene promoter, or AcMNPV expressing LqhIT2 or AaIT under the ie1 or p10 gene promoters) at 24 or 60 h prior to predation by the predator. No significant shifts in the life history characteristics were detected in predators that fed on any of the virus-infected larvae in comparison to predators that fed on healthy larvae.

Boughton et al. (Boughton et al., 2003) have analyzed the adult survival, developmental time, and oviposition rates of green lacewings and twelve-spotted lady beetles that fed upon first or second instar H. virescens that were infected with 100 × LC₅₀ doses of AcMLF9. ScathL (recombinant AcMNPV expressing a basement membrane-degrading protease under the p6.9 gene promoter) or AcMNPV. Unexpectedly, lacewings that fed on the AcMLF9. ScathL-infected H. virescens showed significantly elevated survival rates in comparison to AcMNPV-infected or uninfected H. virescens. Boughton et al. (2003) speculated that the protease expressed by
AcMLF9. ScathL enhanced the efficiency of feeding of the lacewing. No significant differences were found in developmental time, time to onset of oviposition or mean daily egg production of lacewings that fed on virus-infected or uninfected larvae. Most of the twelve-spotted lady beetles that fed exclusively on *H. virescens* (either uninfected or virus-infected) died. There was no evidence that feeding on AcMLF9. ScathL-infected larvae induced any adverse effects in comparison to AcMNPV-infected larvae. Furthermore, neither the lacewing nor lady beetle exhibited any feeding preferences for AcMLF9. ScathL-, AcMNPV, and mock-infected insects. On the basis of these findings, Boughton et al. (2003) concluded that there was no greater risk to insect predators by the use of AcMLF9. ScathL in comparison to the use of AcMNPV as a biological pesticide. Smith et al. (Smith et al., 2000a) have investigated the density and diversity of nontarget predators under field conditions following the application of recombinant baculoviruses (AcMNPV or HzSPNV expressing scorpion toxin LqHT2 under the *ie1* gene promoter) on cotton. They found that predator densities and diversity were similar between recombinant and wild type baculovirus treated plots. In contrast, the chemical pesticide (esfenvalerate) treated plots had consistently lower predator populations.

Taken together, these studies indicate that (1) the amount of baculovirus-expressed toxin or protease that accumulates in the larvae is not sufficient to induce any adverse effects on the predator, (2) the baculovirus is not infectious towards the predatory insects, and (3) the toxin or protease-encoding gene is not expressed in the predatory insect. In some cases there appear to be some costs associated to the beneficial insect that prey upon virus-infected larvae. However, these costs are significantly lower in comparison to the costs associated with the synthetic chemical pesticides. The development of selective recombinant insecticides should augment any IPM program by reducing the impact on non-target species, including beneficial insects. Consequently, the resurgence of primary pests and outbreaks of secondary pests should be minimized.

### 5.2 Fitness of GM Baculoviruses

Fitness is a term that describes the ability of an organism to produce progeny that survive to contribute to the following generation (Cory, 2000). In order to estimate the relative fitness of a recombinant baculovirus in comparison to the wild type baculovirus, five key parameters should be assessed: speed of kill, yield, transmission, dispersal, and persistence. Speed of kill (or time to death), virus yield, and transmission rate can be easily determined by laboratory bioassays (as discussed above) and in some cases under field conditions. Although the relationship between speed of kill (generally quantified in terms of LT50) and virus yield is complex, faster speed of kill generally results in dramatically lower virus yields. This correlation between improved speed of kill and reduced virus yield is found regardless of the parental virus that is genetically modified. For example, the speeds of kill of third, fourth, or fifth instar larvae of *T. ni* that are infected with AcAaIT- or AcHE.KK are reduced by approximately 30% and 8%, respectively, in comparison to control larvae infected with AcMNPV. These faster speeds of kill result in reductions of approximately 80 and 40% in the yields (polyhedra per mg of cadaver) of AcAaIT and AcHE.KK, respectively, in comparison to the yield of AcMNPV (Kunimi et al., 1996). Dramatic reductions of up to 95% in virus yield (polyhedra per μg of cadaver) are found in second and fourth instar larvae of AcTOX34.4-infected *T. ni* in comparison to control AcMNPV-infected larvae (Burden et al., 2000). The corresponding reductions in the mean times to death are 50–60%. A reduction in virus yield is also found by deletion of the egt gene of AgMNPV (Pinedo et al., 2003). The mean lethal times of third instar larvae of *A. gemmatalis* infected with various doses of vAgEGTA-lacZ is reduced by 10–26% in comparison to control larvae infected with the same dose. The yield (polyhedra per gram of cadaver) of vAgEGTA-lacZ is reduced by approximately 50% in comparison to control larvae infected with the wild type AgMNPV. Similar results are found in fifth instar larvae of *S. frugiperda* infected with vEGTDEL which produce 23% fewer polyhedra per insect (the yield of virus per mg of cadaver is not, however, significantly different in comparison to control larvae infected with AcMNPV (O’Reilly and Miller, 1991). O’Reilly et al. (1991) and Ignoffo et al. (Ignoffo et al., 2000) speculated that this correlation between improved speed of kill and reduced virus yield results from the considerably reduced size of recombinant baculovirus-infected larvae at the time of death.

Milks et al. (Milks et al., 2001) have focused on intrahost competition between AcAaIT and AcMNPV or AcAaIT and TnSNPV in larvae of *T. ni* that were synchronously or asynchronously infected. They found no differences in the fitness of the genetically modified or wild type viruses in terms of virus yield. The most important factors in these mixed infections were dose and timing. The virus that was inoculated at the highest dose or the virus that was first inoculated was the one that had the competitive advantage. These findings are not unreasonable when one considers that there are no significant differences in the replication rates of toxin gene carrying and wild type baculoviruses in cell culture.

A key component of the transmission rate of a virus is its pathogenicity or potency (often quantified in terms of the LD50 or LC50). In general, the pathogenicity of a baculovirus is not significantly changed following the insertion of a neurotoxin gene into its genome (Harrison and Bonning, 2000b; McCutchen et al., 1991; Prikhodko et al., 1996; Tomalski and Miller, 1992). Although there are a few exceptions in which relatively small (3-fold or less) increases or decreases have been observed (Chen et al., 2000; Harrison and Bonning, 2000b). Deletion of an endogenous baculovirus gene such as egt also results in no significant (O’Reilly and Miller, 1991; Slavicek et al., 1999) or relatively small (Pinedo et al., 2003) differences in pathogenicity. However, the pathogenicity of the virus can be dramatically increased (i.e., lower LD50 or LC50) by 4- to 100-fold by genetic modifications that improve the ability of the virus to penetrate the midgut (i.e., by the expression of enhancers (Hayakawa et al., 2000b; Popham et al., 2001) or incorporation of Bt toxin into the polyhedra (Chang et al., 2003)). In these cases it is believed that the enhancer or Bt toxin helps the virus to more easily pass through the midgut and establish a more rapid systemic infection. In contrast, however, there were no significant differences in the LC50 values of recombinant AcMNPVs expressing basement membrane degrading proteases (stromelysin-1, gelatinase A, of cathepsin L) under early or late promoters (Harrison and Bonning, 2001).
Another component of the transmission of a virus is the rate at which the virus and host come into contact. This component is obviously more difficult to quantify in the field. As described above, neurotoxin-induced paralysis will cause the insect to fall off of the plant (Cory et al., 1994; Hoover et al., 1995; Sun et al., 2004). This knock-off behavior results in lower levels of foliage contamination, thus the likelihood that a second host insect will come into contact with the recombinant virus (at least during feeding on the plant) will be reduced. In contrast, the wild type virus-infected larvae will most likely die on the plant, thereby increasing the potential for contact between the virus and host. Lee et al. (Lee et al., 2001) have examined this behavioral effect in a greenhouse microcosm. They found that the wild type AcMNPV out-competed recombinant viruses (AcAaIT or AcJHE.SG) for a niche in the greenhouse microcosm. AcMNPV and AcJHE epizootics lasted for eight weeks after the initial release of the virus, whereas the AcAaIT epizootic ended by the fourth week after release. Additionally, AcMNPV polyhedra also increased to greater numbers in the soil in comparison to AcAaIT or AcJHE.SG after eight weeks. A reduction in wandering behavior resulting from the inactivation of the ppi gene may also reduce foliage contamination as discussed above. In both of these examples, the transmission rate of the recombinant baculovirus should be reduced because the likelihood that a host insect and virus coming into contact is reduced.

Dispersal is another parameter that should be assessed in order to determine the fitness of a virus. Abiotic and biotic agents that are known to disperse baculoviruses include rainfall, air currents, predators, parasitoids, scavengers, and grazing mammals (Fuxa, 1991; Fuxa and Richter, 1994). Predatory and scavenging insects have been found to carry (within their digestive tracts) and disperse recombinant virus (in excrement) at rates of up to 125 cm per day over a period of up to 10 days (Lee and Fuxa, 2000). Thus, recombinant virus-induced behavioral changes of the host larvae such as knock-off effects that reduce predation and scavenging will also reduce the dispersal of the virus. Additionally, the ability of the biotic agent itself to disperse the recombinant baculovirus may be reduced. For example, parasitic wasps that develop within larvae of H. virescens that are infected with recombinant baculoviruses are significantly smaller than wasps that develop within wild type virus-infected larvae (McCutchen et al., 1996). Thus, the ability of the smaller wasps to travel, and subsequently disperse the recombinant virus, may be reduced because it has less energy reserves and reduced flight capability in comparison to wasps that developed on wild type virus infected larvae.

Persistence is the final parameter that should be considered when determining the relative fitness of a genetically modified baculovirus. In general inactivation by sunlight (ultraviolet radiation) is the primary route of inactivation of polyhedra in the environment (Black et al., 1997; Ignoffo and Garcia, 1992; Ignoffo et al., 1997). In contrast, soil and foliage are factors that can protect the virus from sunlight and subsequently increase its persistence (Peng et al., 1999). Knock-off or other behavioral changes induced by the recombinant virus may increase their relative concentrations in the soil such that the recombinant’s persistence is increased. In contrast, however, the transmission rate of this recombinant should be reduced because the potential for contact between the virus and host is reduced. The cuticle is another factor that can protect the virus from sunlight such that viral persistence is increased. Fuxa et al. (Fuxa et al., 1998) found that the cadavers of T. ni that are killed by recombinant viruses (AcAaIT, AcJHE.KK or AcJHE.SG) take much longer to disintegrate 4–7 days vs. 1 day) in comparison to AcMNPV-killed T. ni. Another report by Ignoffo and Garcia (Ignoffo and Garcia, 1996) found, however, that there is no significant difference in the time to cell lysis following death by AcAaIT or AcMNPV (lysis took 1.7 vs. 1.5 days, respectively). Clearly, viral fitness is dependent upon a large number of intricately integrated factors. Finally, it is also important to consider as suggested by Hammock (Hammock, 1992) that genetically modified baculoviruses are designed to be biological insecticides and not as biological control agents that will become permanently established. Thus, a recombinant baculovirus should by its very design show reduced fitness in comparison to the wild type.

5.3 Movement of the Introduced Gene to Another Organism

The insecticidal efficacy of natural baculoviruses is dramatically improved by insertion of a foreign gene into the genome or inactivation (deletion) of an endogenous gene from the genome or a combination of both. The insertion strategy generally involves the insertion of an effector gene that encodes a protein that is detrimental to the target insect, alters its life cycle or stops it from feeding. The deletion strategy generally involves the inactivation of an endogenous gene (e.g., egt or orf603) by inserting another gene into its coding sequence. This other gene can be a marker gene such as lacZ or an effector gene as described above. In both cases, the genes are placed under a baculoviral or insect promoter. Several critical points should be kept in mind with these strategies. Firstly, the genes are placed under promoters that are active only in insect cells (and in the case of late/very late baculoviral promoters, these promoters also require the products of baculoviral early genes for activity). Thus, should the effector gene and its promoter somehow jump to the genome of a non-insect cell, the gene will not be expressed. Secondly, the proteins encoded by the effector genes are chosen because they target some critical aspect of the pest insect life cycle or body. The proteins are not biologically active in the non-insects (although it is possible that they may induce an immunological response). Thus, if the effector gene somehow jumps to the genome of a non-insect cell, and if this gene is somehow expressed, detrimental effects will not result.

Genomic variants of baculoviruses are often found in individual field-collected insects (Cherry and Summers, 1985; Hodgson et al., 2001; Maeda et al., 1990; Shapiro et al., 1991), this suggests that recombination and/or transposition events commonly occur between baculovirus genomes. Extensive homology between the donor and recipient DNA molecules and replication of both DNA molecules is required for high frequency recombination to occur (Kamita et al., 2003b). Such conditions may occur when two heterologous viruses (that share some genomic homology) infect the same cell within the same insect. This scenario is the most
likely one in which an effector gene of a GM baculovirus will jump to another organism (i.e., another insect virus). Should the effector gene jump to another virus under these conditions, the fitness of the new recombinant virus will be reduced in comparison to the original GM baculovirus and it too should be rapidly eliminated from the environment. In a second scenario in which the effector gene jumps from the GM baculovirus to the genome of the insect host, the effector gene could cause an adverse effect. However, these effects should be limited to a single individual because once this individual dies the effector gene will also ‘die’. It is also possible that heterologous or random recombination events may also lead to the movement of an effector gene to another organism. The same arguments that were made in regard to the homologous recombination-based movement can be made in this case. However, the likelihood of heterologous recombination is much lower than the likelihood of homologous recombination.

6 Field Testing and Practical Considerations

Laboratory and greenhouse testing has generated a great deal of knowledge about the efficacy, safety, and environmental fate of GM baculovirus pesticides as described above. Mathematical models have also been generated to evaluate the effectiveness and ecological consequences of the release of GM baculoviruses (Dushoff and Dwyer, 2001; Dwyer and Elkinston, 1993; Dwyer et al., 1997). Field testing, however, over both the short- (e.g., single growing season) and long-term (e.g., multiple seasons and years) is still necessary to confirm the findings of laboratory and greenhouse tests and the accuracy of mathematical models. The commercial potential of GM baculoviruses and practical considerations regarding their use can also be determined by field testing. Issues regarding the commercialization of GM baculovirus insecticides including marketing, in vivo and in vitro production, formulation, storage, and public acceptance are discussed in detail by Black et al. (Black et al., 1997).

Some of the earliest field trials of GM baculoviruses (occlusion-negative AcMNPVs carrying junk DNA or lacZ marker gene) were performed in England during the mid to late 1980s (Black et al., 1997; Levidow, 1995). In the United States, the first field trial (a three year study) of a GM baculovirus (a polI gene-deleted AcMNPV that was co-occluded with wild type AcMNPV) was begun in 1989 (Wood et al., 1994). These early field trials were performed primarily to analyze the environmental persistence of the virus. These trials showed that the persistence of occlusion-negative constructs is exceptionally low. The first field trial to test the efficacy of an occlusion-positive, AaIT expressing AcMNPV (AcST-3) was performed in 1993 (Cory et al., 1994). Cory et al. (Cory et al., 1994) found that cabbage plants treated with AcST-3 showed 23–29% lower feeding damage (by third instar larvae of T. ni) in comparison to wild type AcMNPV-treated cabbage plants. This reduction in feeding damage (approximately 50% reduction) was not as large as that found in the laboratory trials (Stewart et al., 1991). The reduction in feeding damage during the field trial was due to the earlier death of AcST-3-infected larvae in comparison to AcMNPV-infected larvae. Cory et al. (Cory et al., 1994) also found that the yield of AcST-3 was 10-fold lower (9.9 x 10^7 vs. 9.6 x 10^6 polyhedra per larva) in comparison to AcMNPV. And, they found that under the high (10^9 polyhedra per ml) dose treatments the majority (62%) of the larvae of AcST-3-treated cabbage were knocked off the plant whereas none were knocked off the AcMNPV-treated cabbage. They speculated that both the behavioral (knock off) and pathological (reduced yield per larva) differences between the recombinant and wild type viruses would have important implications for risk assessment as discussed above.

American Cyanamid Company performed the first field trials in the United States (one in Georgia and the other in Texas) to test the efficacy of an occlusion-positive, egt gene-deleted, AaIT-expressing AcMNPV in 1995 and 1996 (Black et al., 1997). They found that this virus killed target insects faster than wild type AcMNPV or an egt gene-deleted AcMNPV, which resulted in increased control of the target insect. In 1997 and 1998, field trials were conducted by academic scientists and scientists at DuPont Agricultural Products (Smith et al., 2000a) to assess (1) the efficacy of recombinant baculoviruses in protecting cotton, (2) the impact of recombinant virus introduction on predators, and (3) the ability of predators to disperse recombinant baculoviruses. Two occlusion-positive constructs (one based on AcMNPV and the other on HzSNPV) expressing LqhIT2 were tested. Depending upon the timing of the application, they found that the LqhIT2 expressing viruses were able to protect cotton from damage better than the wild type virus (AcMNPV or HzSNPV) and as well as a synthetic chemical pesticide (esfenvalerate). Predator densities and diversity were similar between the recombinant and wild type virus treated plots, whereas plots treated with the chemical pesticide had consistently smaller predator populations. Finally, they found that at two to five days after the initial application of virus, a very small percentage (0.2%) of predators that were caught and evaluated carried DNA from a recombinant virus. In 1998, Trecy et al. (Trecy et al., 2000) (American Cyanamid Company) conducted field trials in the United States (two in Georgia and one in North Carolina) to test the efficacy of two occlusion-positive constructs (AcMNPV- and HzSNPV-based) expressing AaIT in protecting cotton. They found that the recombinant HzSNPV (at 5 or 12 x 10^11 polyhedra per hectare) protected cotton against heliothine complex larvae at slightly better levels than either the recombinant AcMNPV or Bt toxin (Dipel 2X, Abbott Laboratories, at 1121 g (WP) per hectare).

In 2000, Sun et al. (Sun et al., 2004) conducted field trials in People’s Republic of China (in Henan and Hubei provinces) to test the efficacy of HaSNPV-AaIT (an occlusion-positive recombinant HaSNPV expressing AaIT at the egt gene locus) in protecting cotton. Cotton plants plots treated with HaSNPV-AaIT showed significantly less damage to squares, flowers, and bolls in comparison to plots treated with the wild type HaSNPV or HaSNPV-EGTD (HaSNPV with a deletion in its egt gene). In 2001 and 2002 (Hubei province), treatment of cotton plants during the entire growing seasons with HaSNPV-AaIT increased the yield of cotton lint by approximately 18% (1250 vs. 1023 and 1800 vs. 1474 kg per hectare in 2001 and 2002, respectively) in comparison to wild type HaSNPV-treated plots. The yield of cotton lint in plots treated with HaSNPV-AaIT was not significantly different than the yield
1083 and 1994 kg per hectare in 2001 and 2002, respectively) in plots treated with chemical pesticides (ż-Cyhalothrin EC, endosulfan, β-Cypermethrin on different dates).

Numerous field trials have been conducted over the past ten years at geographically distant sites using several types of GM baculovirus constructs and under different cropping situations. These trials show that GM baculoviruses are effective and safe pesticides with efficacy that can be at levels similar to synthetic chemical pesticides or Bt toxin. The field trials also indicate that the recombinant baculovirus will not persist in the environment and have very little, if any, adverse activity against beneficial insects.

7 Concluding Remarks

The majority of people in both developed and developing countries are dependent upon others to produce or otherwise provide the minimum requirements of food, water, and/or shelter that are required for survival. A society must carefully manage and allocate its limited resources in order to provide these minimum requirements for all of its members. Over the past five decades, the use of synthetic chemical pesticides has significantly increased the yields of food, fiber, and feed that producers are able to generate from a given amount of land. Although chemical pesticides have without a doubt improved the efficiency of agricultural output and reduced the incidence of disease by killing disease vectors, chemical pesticides are also a source of environmental pollution and both acute and chronic problems of human health. The inappropriate use of chemical pesticides has also helped to generate pesticide resistant insects. These problems are often intensified in developing countries because of poor governmental regulation and training in the appropriate use of these agents. Furthermore, the high costs of the newest generation of synthetic chemical pesticides are precluding their widespread use in many developing countries. These pressures in the agricultural industry are making biological control agents highly attractive as supplements or replacements for synthetic chemical pesticides in integrated pest management schemes.

While biological pesticides avoid chemical residual problems, many such agents lack sufficient potency and speed of action in the field (at least until recently) to be attractive alternatives. As discussed in this article, a fresh approach to biological insect control involves using the best genes of nature to enhance insecticidal properties of naturally occurring baculoviruses. Our laboratory and numerous others have developed NPVs that have the knock down speed of classical chemical insecticides on the noctuid complex of insect pests while not harming nontarget species. The viruses are applied like classical insecticides, but they present no residue problems, and they are active on insects resistant to classical insecticides. Industry and academic scientists have overcome most of the limitations associated with natural baculovirus pesticides, especially in terms of effective crop protection, through the use of recombinant DNA techniques and other technologies. Furthermore, should baculoviruses appear to offer sufficient commercial potential for investment, many of the remaining limitations (e.g., those that are specific to a particular cropping situation) can be quickly addressed and overcome. The safety of baculoviruses has been thoroughly investigated and there is no evidence that natural or recombinant baculoviruses provide an increased threat to human or environmental health.

A large number of genetically modified baculovirus pesticides have been described in the scientific literature and, undoubtedly, many more have been tested in academic, governmental, and commercial laboratories. With the currently available genes and parental viruses, we believe that the fastest speed of kill (median lethal time) that can be achieved is around 48 h because the virus will most likely have to undergo two replication cycles before the host insect is killed. Feeding cessation (median time to feeding cessation) will occur earlier, perhaps as early as 24–36 h post infection. Feeding cessation at 24 h post infection should be sufficient to make GM baculovirus pesticides competitive with synthetic chemical insecticides in terms of the protection of many types of crops. In terms of a “best performing” GM baculovirus pesticide, AcMNPV offers the widest host range in terms of infectivity against pest insect species. To this parental virus we would insert at the egt gene locus a gene encoding AaIT fused to a bombyxin signal sequence that is expressed under a chimeric late-very late promoter (e.g., P SYNIV). We prefer the AaIT-encoding gene instead of the mite toxin gene (although the mite toxin gene appears to be more insecticidal) because its mode of action is known and the action of AaIT is synergized with pyrethroid insecticides. LqhIT2 is a viable alternative to AaIT. Also, in comparison to the other insect-selective toxins such as those from spiders and sea anemones, AaIT and LqhIT2 have undergone more testing. We prefer expression under a baculovirus late-very late promoter because of the slightly improved selectivity that such a promoter has in comparison to baculovirus early or constitutive promoters. We prefer the bombyxin signal sequence because it has been shown to efficiently secrete a wide variety of proteins in a wide variety of insect cells. Deletion of the egt gene may reduce the fitness of the virus. Other parental viruses such as HzSNPV or RoMNPV are also viable options for pest species that are not highly susceptible to AcMNPV. Additionally, the coexpression of a protease or polyhedrin-Bt toxin fusion protein may help the virus to more quickly penetrate the midgut resulting in a faster systemic infection.

The safety and effectiveness of GM baculoviruses as pest insect controlling agents has been studied for more than 25 years. During this time, thousands of peer-reviewed studies on the basic biology, host range, ecology, efficacy, safety, and applications of natural and recombinant baculovirus have been published. Baculoviruses are very commonly used in hundreds of academic and commercial laboratories as protein expression vectors, as gene transfer vehicles, for surface display, and as a model system to study large DNA viruses. Natural baculoviruses are used as effective pest insect control agents in several regions of the world. In this article, we have primarily reviewed (1) major milestones in the development of recombinant baculoviruses for pest insect control, (2) current strategies to generate recombinant baculoviruses with improved feeding prevention and speed of kill, and (3) potential risks associated with the use of recombinant baculoviruses for pest insect control. A tremendous knowledge base is now available regarding the efficacy and safety of genetically modified baculoviruses for pest insect control. Regardless, there are some that would
argue that there are unseen and unpredictable risks associated with the release of any genetically modified organism into the environment. To this we agree, but we also argue that it is impossible to completely avoid all risk associated with the use of any pesticide. We believe that the benefits afforded by GM baculoviruses far outweigh any unseen or unpredicted risks that they pose, especially in comparison to the potential risks associated with the use of chemical pesticides or genetically modified plants. The current research indicates that GM baculovirus pesticides meet or exceed the high standard of expectation set by synthetic chemical insecticides. GM baculovirus pesticides are safe, effective, and ready for immediate use.

This article shows that we have no doubt succeeded in generating exceptionally safe and efficient biological pesticides, which readily compete with chemical pesticides. The technology has been tested for many years and the results are not completely unsatisfactory, favorable in many cases. However, GM baculoviruses still have limited use in the field. Therefore, we conclude that we should be looking at other reasons why the implementation of an available, safe and effective pest control technology is not moving at a pace it should have moved. Several world-class pesticide manufacturing companies have shown interest in the technology throughout the years. They obtained and licensed critical patents and started their own research programs that positively synergized the efforts of others. On one hand this commercial interest has brought excitement and acceleration to the field, on the other hand the cancellation of their biologicals programs after several years of research have taken a high toll on the commercialization of GM baculoviruses. Were the cancellations based on scientific knowledge on the ineffectiveness or safety of the viruses? We present otherwise. Was profitability an issue? GM baculoviruses now compete with chemical pesticides and examples of profitable industrial manufacturing schemes of non-GM baculoviruses exist. Was public perception an issue? Recombinant organisms have been safely used for many years now and chemical pesticides have more proven side effects than highly specific baculoviruses. Thus, we believe that the lack of the implementation of GM baculoviruses is primarily the result of a psychological effect.

Academic laboratories have already done their part by introducing GM baculovirus technology to the benefit of mankind and by presenting proof on its profitability. However, we in academia may have also shown that it is quite easy to take the technology and use it without regard to the intellectual property rights. This by itself should not be an excuse because legal ways to protect international patent rights exist. To negate the negative psychological effects of seemingly unsuccessful commercialization is, therefore, the utmost problem of the field as we see it. There is also more to do in terms of extending the merits of GM baculoviruses to the end-users. One thing academic researchers might have done more efficiently could be the extension activities. This might have induced the agricultural cooperatives to look at this new alternative earlier on and might have helped them to start production in farm or large scale. The intellectual property rights as they are currently held probably exclude this possibility. So why not publicize all relevant patents to encourage the technology rather than burying it. This is a possibility that all private and public patent holders should consider at this point. The Brazilian experience is exemplary and could be a model for other parts of the world. In that case even though studies were initiated by the government, cooperatives and private companies eventually showed interest in commercial production of non-GM baculoviruses.

Throughout the years, we have attempted many times to come together with the leading scientists in the field to get organized and to initiate private commercial interest. But now is really the time to put all the work done in perspective and either to increase the cooperation between everyone in the field to obtain a positive movement or perhaps to cease our hopes on a group of biopesticides that will never reach the shelves. We believe efforts should be directed towards more practical aspects of the field including increased extension and free access to the technology. A discussion of problems associated with immediate commercialization with the participation of all interested parties in a constructive manner will be critical. Countries where GM baculoviruses are actively used could of course provide wisdom on how to successfully commercialize GM baculoviruses. Data and history from the beginning to the current status presented in this article clearly shows that it would be a great waste to abandon this technology.

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