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DEVELOPMENT OF RECOMBINANT BACULOVIRUSES FOR INSECT CONTROL

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ABSTRACT

In this review, we provide an overview of the current status of recombinant baculoviruses, describe the development of genetically engineered baculoviruses for use as rapid-action biological insecticides, and provide more detailed information on one particular set of recombinant viruses. The advantages and disadvantages of recombinant baculovirus insecticides, and the importance of risk-assessment studies of these genetically modified organisms, are reviewed. Finally the importance of sensible regulatory strategies to the success and future prospects of this technology is discussed.

PERSPECTIVES AND OVERVIEW

With the advent of recombinant DNA technology and the recent development of rapid-action recombinant baculoviruses, interest in the field potential of these viruses for insect control has increased dramatically. Several major companies and various academic and government laboratories worldwide are currently contributing to this area.

Wild-type baculoviruses are an integral component of the natural biological control of many pest species, and application of wild-type viruses has been very effective for pest management in several cases (31). However, in most cases the wild-type viruses have failed to compete with classical insecticides

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because of several factors, application technology and low field persistence among them. The limitation discussed here is the slow kill by viruses, which not only makes them poorly competitive, but also limits industrial investment in application and formulation technologies for enhanced efficacy. In the past several years, the speed of kill of the viruses has been markedly improved through the use of recombinant DNA technology. These successes have led to industrial investment in related technologies that should make both the wild-type and recombinant viruses more attractive for use in insect pest control.

Several recently published reviews on baculoviruses have covered their biology (1, 3, 39), pathogenesis (99), diversity and molecular biology (7, 59), and application as protein expression vectors (63, 65, 78), as well as the use of wild-type (31, 32) or genetically engineered baculoviruses (8, 42, 67, 79, 103) for insect control. Other articles have focused on specific aspects of recombinant DNA technology and their influence on the efficacy of baculovirus insecticides (10, 74).

INTRODUCTION

Baculovirus Diversity

Baculoviruses are double-stranded DNA viruses that are pathogenic to arthropods—predominantly holometabolous insects. The Baculoviridae contains two genera, as determined by structural properties (80a): the *Nucleopolyhedrovirus* nucleopolyhedroviruses and the *Granulovirus*. The nuclear polyhedrosis viruses (NPV) have virions embedded in a crystalline matrix of the protein polyhedrin. The virions vary in their configuration in that the nucleocapsids may be enveloped singly or in multiples. The occluded viruses are referred to as polyhedra. The granuloviruses (GV) have one, or rarely two, virions embedded in a crystalline matrix of granulin. The nucleocapsid is always enveloped singly.

Efforts to genetically engineer baculoviruses for control of insect pests have centered on the nucleopolyhedroviruses for control of lepidopteran larvae. This review focuses on progress made in genetic engineering of this group for increased use in insect pest control. Systems for engineering these viruses are well developed (57, 63, 78, 86, 95). The technology discussed here can be applied to other viruses or other insect pathogens and commensals as cloning systems are developed.

Baculovirus Life Cycle

Following ingestion of the nucleopolyhedrovirus by the larva, the protective polyhedrin coat dissolves in the midgut (Figure 1). The virions, released from the matrix of the polyhedron, set up sites of primary infection in the cells

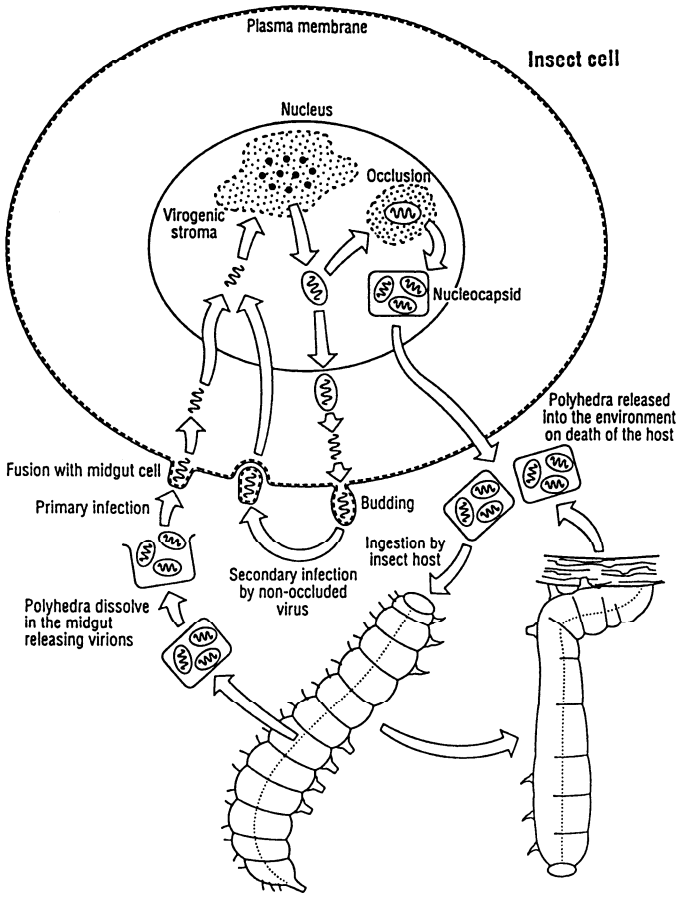

Figure 1 Life cycle of the nucleopolyhedrovirus. Following ingestion of the virus, the polyhedra dissolve in the midgut, releasing nucleocapsids that fuse with the midgut cells. The viral DNA replicates in the nucleus and progeny viruses bud through the cytoplasmic membrane to disseminate the infection. After the initial round of replication, nucleocapsids produced in the nucleus are enclosed within polyhedrin. When the insect dies, the polyhedra are released from the lysed cells. [Courtesy of Intercept Ltd. (8).]

comprising the midgut. Initial rounds of viral replication within the nucleus of the infected cell produce a second viral phenotype, the budded virus or virus particle, which spreads the infection to other tissues. The budded viruses move through the cell membrane and become coated with a viral protein–modified basal plasma membrane. Infection of different larval tissues occurs in a sequential manner, and the virus is hypothesized to use the tracheal system of

the insect as a conduit (30). However, there is some debate about whether this is the primary route of viral transmission within the insect, or whether the hemocytes play this role. At later stages of virus infection, progeny viruses become occluded within the nuclei of the infected cells. Prior to death, larvae infected with some wild-type baculoviruses climb up the plant. They succumb to the virus infection and hang from an elevated position, which facilitates dissemination of the virus as the cadaver decomposes.

Following infection of an insect cell, baculovirus gene expression occurs in a temporally regulated cascade (37). Unlike gene transcription in the three other temporal phases, transcription of the immediate early (IE) genes does not depend on production of other viral proteins because these genes are transcribed by host factors. Their products upregulate the delayed early (DE) genes. Late-gene expression occurs concurrently with the onset of DNA replication, and these genes encode the structural proteins of the virus particles. The very late genes encode proteins involved in the final stages of infection and polyhedron morphogenesis, including the p10 and polyhedrin proteins.

Virus Control of Insect Pests

The potential of baculoviruses for insect pest control was first recognized in the early 1900s (93). Since then, numerous baculoviruses have been used to control, among others, hymenopteran, lepidopteran, and coleopteran pests of crops such as coconuts, cotton, and cabbages, as well as pests of beehives. The control of pests on soybean crops in Brazil and on palm and coconut in the South Pacific by *Anticarsia gemmatalis* NPV and *Oryctes rhinoceros* baculovirus, respectively, is particularly noteworthy (31). The time taken by the virus to kill the host insect from the point of infection ranges from days to weeks, depending on temperature, viral dose, insect age, and the particular host and virus species. During this time, the insect continues to feed. Hence, many of the pests currently controlled by baculoviruses are pests of crops that can sustain some damage without significant economic loss. In addition, baculoviruses have been used successfully when the insects are small and it is possible to apply virus. For example, in Brazil the soybean crops are monitored intensively for larvae for optimal timing of virus application before significant damage has occurred. Genetic engineering of the baculovirus to reduce the time taken by the virus to kill the host insect will not preclude the use of the wild-type viruses in biocontrol but promises to yield viruses more competitive with classical insecticides. This development will significantly increase the potential of baculoviruses for pest control, particularly in row crop agriculture.

Advantages of Recombinant Baculovirus Insecticides

Insect viruses are important components of natural biological control, but this article concentrates on their use as biological insecticides with reduced capac-

ity to recycle in the field. The pressure to find novel means of pest control to reduce reliance on the synthetic chemical insecticides is increasing. The number of compounds currently available to the grower is decreasing as a result of pest resistance (89) and detrimental effects of insecticides on both human health and the environment (19). In this respect, several advantages are associated with the use of baculoviruses as insect-control agents. One of the most important attributes of baculoviruses for pest control is their host specificity. Many baculoviruses infect only a few species, often within the same family. This attribute makes them ideal for incorporation into integrated pest management (IPM) programs because one of the main concepts of IPM is to target pest species that exceed the economic-damage threshold, while leaving the rest of the fauna undisturbed (34). Thus, the whole potential of beneficial organisms can be exploited.

Viral pesticides can be applied using conventional techniques and do not create the problems associated with residues. They do not show cross resistance with chemical compounds. Although insects have shown resistance to baculoviruses in some cases (38), resistance ratios are generally low, and in many cases resistance is unstable in the absence of selection pressure. Apparent negatively correlated resistance to chemical insecticides has even been noted (38a, 72). The use of baculovirus insecticides in IPM programs with other biological-control agents or with classical chemical insecticides may reduce the likelihood of resistance developing to the baculovirus. In some pest-management situations, the recombinant viruses will be used to augment the action of classical insecticides. In fact, some recombinant viruses can synergize and be synergized by classical insecticides, and this complementarity makes the viruses even more attractive as tools for IPM (72).

For the production of baculovirus insecticides, industry is pursuing several strategies, including both in vitro and in vivo technologies. However, wild-type and recombinant viruses can be produced by cottage industries. With carefully produced innocula, local laboratories in developing countries could produce these genetically sophisticated products with minimal technological input.

Disadvantages of Recombinant Baculovirus Insecticides

The enhanced speed of kill of recombinant viruses limits their propensity to recycle in the environment as fewer polyhedra are produced compared with the wild-type virus. Apart from this limitation, the advantages and disadvantages of recombinant and wild-type baculoviruses are the same. In many cases, the traits that are considered advantageous actually limit the utility of the recombinant viruses. For example, the limited host range allows a pest-management specialist to reduce one pest population with precision without disruption of biological-control agents and without the deleterious effects on

nontarget organisms associated with use of classical pesticides and even *Bacillus thuringiensis*. However, this trait also limits the market size for the baculovirus and thus the development costs that can be invested in the technology.

Cloning systems that allow the recombinant technology to be applied to different viruses is lacking even within the Baculoviridae. Hence, the application of genetically engineered baculoviruses for insect control is currently limited to a few pest species. The rate of development of these systems will, one hopes, accelerate. However, of all the limitations facing the use of genetically modified and wild-type viruses, the problems associated with correct application and low persistence are the most serious. The many advantages of the recombinant viruses have attracted industrial investment in research to solve these problems. These technologies will be applicable to wild-type viruses and other biological-control agents intended for augmentative release in pest management, in addition to recombinant viruses. Despite the great potential of recombinant baculoviruses, they do not represent a panacea or even a stand-alone technology for insect control. Rather, they will be used as additional tools for application in IPM programs.

DEVELOPMENT OF RECOMBINANT BACULOVIRUSES

Genetic Engineering of Baculoviruses

The aim of genetic engineering of baculoviruses for use as insecticides is to combine the pathogenicity of the virus with the insecticidal action of a toxin, hormone, or enzyme. Upon infection of the insect larva with the recombinant baculovirus, the foreign protein is expressed. If this protein is toxic to the insect, the insect will die rapidly from this effect, rather than from the viral infection itself. The recombinant approach will probably also be used to improve production, modify host range, and enhance the utility of various insect viruses as biopesticides. However, the goal of the research reviewed here is to reduce the time from infection with the recombinant virus to death of the insect such that feeding damage is below the economic threshold. This goal necessitates an approximate lethal-time ratio (lethal time of test virus divided by lethal time of wild-type virus) (9) of 0.4–0.5 for control of insect pests on many crops. Reduction of the lethal time may also enhance farmer or user acceptance of baculovirus insecticides.

Two major baculovirus-expression systems have been developed for the production of recombinant proteins for research and clinical use. These are based on the nucleopolyhedrovirus derived from the alfalfa looper, *Autographa californica* (AcNPV), and a similar virus from the silkworm, *Bombyx mori* (BmNPV). The sequences of the entire genomes of both AcNPV and BmNPV

have now been determined (2, 69a). Early engineering work was carried out with BmNPV for high levels of protein production in larvae of *B. mori.* As *B. mori* is the only known host for BmNPV, this approach also provided biological containment for the virus (69). Most recent work in developing the virus for insect control has concentrated on AcNPV. A variety of recently developed techniques and transfer vectors greatly facilitate the engineering process (5, 27, 61). Protein-expression systems have also been established in other baculoviruses, such as *Helicoverpa zea* NPV (21) and *Lymantria dispar* NPV (107). This research provides the basis for engineering of these viruses for use as insect pest–control agents in the future.

The circular genome of AcNPV is approximately 134 kilobase pairs (kb) (2). Because of the difficulty of direct manipulation of such a large piece of DNA, engineering of a baculovirus is usually carried out in two steps. First, the foreign gene is incorporated into a baculovirus-transfer vector. Most transfer vectors used are bacterial plasmid, University of California (pUC), derivatives, which encode an origin of replication for propagation in *Escherichia coli* and an ampicillin-resistance gene. The pUC fragment is ligated to a small segment of DNA taken from the viral genome. The foreign gene sequence is incorporated into a cloning site downstream of the promoter selected to drive expression. For the second step, the transfer vector is mixed with DNA from the parental virus. The engineered DNA is incorporated into the virus via homologous recombination events within the nucleus of cultured insect cells. Unlike genetic engineering in plants, which results in a rather random incorporation of new DNA into the genome, the baculovirus system allows the precise insertion of foreign DNA without disruption of other genes. No drug-resistance markers are included in the final clone, which eliminates some of the major objections raised to recombinant organisms (35). Commercial kits and reagents are available for this work, as well as several excellent manuals (57, 86). A number of recently developed alternative approaches for genetic engineering of baculoviruses have been reviewed elsewhere (27).

Early research involved engineering of these viruses for use as protein-expression vectors rather than for insect control (90). The approach involved replacing the gene encoding polyhedrin with the foreign gene of interest. Expression of the foreign gene was driven by the polyhedrin promoter in a polyhedrin-negative virus. Although these viruses can be manipulated successfully in cell culture for production of high levels of foreign protein, they lose any advantage conferred by the polyhedrin coat that protects the virus from inactivation by desiccation and ultraviolet light under field conditions. Replacement of the viral gene encoding the p10 protein (98), which is involved in calyx attachment and nuclear lysis, also resulted in reduced viral fitness. The stability of polyhedra produced by p10-negative viruses is greatly reduced (101).

An alternative approach to replacement of a viral gene with a foreign gene sequence is to duplicate a viral promoter. In this instance, none of the viral genes are lost, and promoters of essential viral genes can be used for expression of foreign proteins. The level and timing of expression of a particular protein by a recombinant baculovirus is determined in part by the promoter chosen to drive transcription of the foreign gene sequence. Currently, the polyhedrin and p10 promoters are used most frequently for expression of recombinant proteins. However, expression under the basic protein promoter was higher in several instances (13, 60a, 91). In the future, the use of early promoters, hybrid promoters, and promoters from other species will increase, particularly with the identification of peptides and proteins that disrupt insect biology at lower expression levels.

Recombinant Baculoviruses Developed for Insect Control

Any gene coding for a protein that disrupts normal larval development or behavior and reduces feeding damage caused by the insect is a candidate for expression by a recombinant baculovirus for insect-control purposes. To establish the suitability of a gene for baculovirus expression, one must consider the mechanism of action and the behavior of the virus after infection to assess whether the gene product is likely to reach its target site. As a general principle, a product that acts on the whole insect systemically is preferable to products that only show toxicity to the infected cells. Although the baculovirus system offers several levels of specificity, agents that exhibit insect-selective toxicity are preferable from a safety standpoint to those that might have a more universal effect. Suitable genes for expression in the baculovirus include insect enzymes and hormones that may disrupt development or homeostasis and insect-selective neurotoxins. The recombinant baculoviruses developed to date have been reviewed more extensively elsewhere (8, 47, 65, 103), but an outline of current developments is presented below.

Recombinant baculoviruses are being developed as biological insecticides for repeated application rather than as biological control agents that establish and recycle in the field. Because the recombinant virus expresses genes for rapid kill of the host, it is subject to strong negative selection pressure and consequently cannot compete with its wild-type counterpart. Should regulators require redundant systems, techniques such as gene deletion can be adopted to further reduce the fitness of the virus. An alternative approach would involve applying preoccluded viruses (POVs), i.e. the nucleocapsids of polyhedrin-negative viruses (106).

Recombinant Baculoviruses Expressing Toxins

Arthropod venoms offer a rich source of insect-selective toxins (50). Indeed, expression of insect-selective toxins in the baculovirus-expression system has

proved to be highly successful for increasing virus efficacy in insect-pest control. The first toxin to be engineered into a baculovirus was the insect-selective toxin derived from the scorpion *Buthus eupeus* (BeIT) (16). However, for unknown reasons, this toxin did not increase the efficacy of the recombinant virus. Two of the toxins produced by the bacterium *Bacillus thuringiensis* (Bt) have been engineered into AcNPV. Of the four classes of Bt isolates, Cryl produces crystals that are toxic to lepidopteran larvae (51). These toxins are thought to generate pores in cell membranes, leading to disruption of osmotic balance and then cell death. Recombinant baculoviruses were constructed to express *B. thuringiensis* subsp. *kurstaki* HD-73 δ-endotoxin (76) and *B. thuringiensis* subsp. *aizawai* 7.21 Cry1A(b) toxin (70). As expected given the mechanism of action of the recombinant proteins, neither of the recombinant viruses constructed showed promise in terms of increased insecticidal efficacy.

Baculoviruses expressing the insect-specific toxin AaIT derived from the North African scorpion *Androctonus australis* are among the most promising recombinant baculoviruses developed to date (69, 73, 94). This toxin acts on the neuronal sodium channel, causing presynaptic excitatory effects. Larvae infected with these recombinant baculoviruses exhibit dorsal arching and increased irritability and cease feeding. Lethal times are reduced by 25–40% compared with those of the wild-type virus, and feeding damage by larvae infected with recombinant virus is reduced by 50% on cabbage compared with damage caused by larvae infected with wild-type viruses (25, 94). The speed of virus kill further increases in the presence of low doses of pyrethroid insecticides (72). Pyrethroid-resistant insects are also killed more quickly by this combination (72). These observations illustrate that the recombinant viruses could be used initially in conjunction with classical insecticides in resistance-management programs.

TxP1, a toxin derived from the straw itch mite, *Pyemotes tritici,* was also expressed in AcNPV (96, 97). The mite uses this toxin, which causes muscle contraction and paralysis, to immobilize insects up to 150,000 times its size. The exact mechanism of action is not known. Recombinant baculoviruses expressing TxP1 had a lethal time 30–40% lower than that of the wild-type virus. These successes with toxins of relatively limited activity on lepidopterous larvae suggest that insect-selective peptides of far greater activity will prove useful with currently used expression systems as well as with systems under the control of earlier promoters. Moreover, the expression of combinations of genes in multiple expression vectors (5), rather than expression of individual genes, should also increase the speed of kill.

A maize mitochondrial protein involved in cytoplasmic male sterility and disease susceptibility has been expressed in AcNPV (60). This protein, URF13, is hydrophobic and binds tightly to the membranes of cells. Injection of larvae of the cabbage looper, *Trichoplusia ni,* with the polyhedrin-negative recom-

binant virus resulted in a 40% decrease in lethal time compared with that of the wild-type virus. URF13 decreased the ability of AcNPV to produce polyhedra when polyhedrin-positive constructs were made. Further research should shed light on the exact mechanism of toxicity of URF13 to the insect host, allowing us to determine whether this protein is suitable for pest-control purposes.

Recombinant Baculoviruses Expressing Insect Hormones

An alternative approach to the expression of insect-selective toxins by a baculovirus is expression of a component of the insect endocrine system (56, 71). The rationale behind this approach is that if a single component is overproduced by the recombinant baculovirus, homeostasis will be disrupted, causing a deleterious effect on the insect. The first recombinant baculovirus to be developed that had enhanced insecticidal activity expressed the diuretic hormone from the tobacco hornworm, *Manduca sexta,* thought to be involved in water balance (64). Larvae of *B. mori* injected with the recombinant BmNPV, which expressed the diuretic hormone, died 20% more quickly than larvae injected with the wild-type virus. This resulted from an alteration in metabolism of larval fluid.

Eclosion hormone, which is involved in ecdysis and shedding of the cuticle during molting, was expressed in AcNPV (28). The recombinant hormone was active, but the efficacy of the recombinant virus was not enhanced relative to the wild-type virus (29). The prothoracicotropic hormone (PTTH) from *B. mori,* which stimulates production of ecdysone, has also been expressed in AcNPV (82). The recombinant viruses produced large amounts of PTTH. No reduction in lethal time was seen upon infection of larvae of the fall armyworm, *Spodoptera frugiperda,* with the recombinant virus, but expression of PTTH markedly inhibited the pathogenicity of AcNPV (82). Other insect hormones have been expressed with little or no success. Considering the complex events involved in homeostasis, the failure of single neurohormones to dramatically enhance the efficacy of recombinant baculovirus insecticides is perhaps not surprising. Once we have a greater understanding of endocrine systems, an improved ability to achieve high-level expression of small peptides, and an understanding of the pharmacokinetics and interactions of neuromodulators, expression of neurohormones may be a fruitful approach in the future.

Ecdysteroid UDP-Glucosyltransferase

The baculovirus AcNPV produces ecdysteroid UDP-glucosyltransferase (egt) (81, 83–85). In vitro studies have shown that this enzyme transfers glucose from UDP-glucose to ecdysteroids involved in molting. Expression of this protein prevents the host insect from molting and effectively keeps the insect

in the feeding stage (85). However, the exact role of egt in vivo has not been demonstrated, and it may have other primary and secondary effects (80b). Deletion of the egt gene from the viral genome results in a 10–20% reduction in lethal time, a 40% reduction in feeding damage, and a 30% reduction in the number of progeny viruses produced. Regulatory agencies view the egt-negative gene-deletion construct with less scrutiny than they do gene-addition constructs, such as those expressing toxins or juvenile hormone esterase. Thus, the egt-negative virus is being used for some early releases to test its effectiveness and thereby pave regulatory pathways for the recombinant baculovirus technology. The egt-negative AcNPV is likely to be the first recombinant baculovirus approved for commercial use as a pesticide (80b). This example demonstrates the utility of gene deletion for improving the speed of kill of a virus. Gene deletion may also prove useful for modification of other traits, such as host range, or for production purposes.

Recombinant Baculoviruses Expressing Juvenile Hormone Esterase

Two epithelial hormones are involved in lepidopteran larval development: 20-hydroxyecdysone, which initiates the molt, and juvenile hormone (JH), which controls the nature of the molt. If the JH titer is high, the molt is isometric to a larger larval stadium. If the JH titer is low, an anisometric molt to the pupa will occur. The reduction in JH titer is a key event in insect development that leads ultimately to termination of feeding and metamorphosis. This decrease in titer occurs through a decrease in biosynthesis caused at least in part by neurohormone(s) and neuromodulators such as allatostatin and allatohibins. Juvenile hormone esterase (JHE) increases as JH titer decreases (40). JHE catalyzes the hydrolysis of the highly stable conjugated methyl ester of the JHs to the corresponding biologically inactive JH acid. JH is intrinsically involved in regulation of gene expression in both larval and adult insects (87).

The agricultural-chemical industry has shown considerable interest in anti-JH agents, because induction of precocious development in a crop pest would reduce feeding damage (92). The expression of JHE in a baculovirus vector is a continuation of this line of research into anti-JH agents.

The coding sequence for JHE from the tobacco budworm, *Heliothis virescens* (46), was expressed in various baculovirus constructs (10). JHE has been expressed in AcNPV under control of the polyhedrin (43, 88), p10 (11, 88), and basic protein (p6.9) promoters (13), as well as a hybrid promoter based on the polyhedrin gene (29). Despite high levels of expression of JHE both in vitro (43) and in vivo (11, 29), the significant improvement of the insecticidal efficacy observed for neonate larvae of *T. ni* (43) was not seen for other stages

or insect species. Compensation for the increased titer of JHE by the regulatory and feedback mechanisms intrinsic to the insect's JH system may explain the limited insecticidal activity of this recombinant virus.

Glycosylation of the molting hormone may reduce or prevent any deleterious effect caused by JHE. Eldridge et al addressed the question of whether the effects of JHE could be masked by the action of egt by expressing JHE in an egt-negative virus (29). In this study, infections of larvae with either egt-positive or egt-negative recombinant viruses expressing JHE did not differ in terms of lethal times, although differences might have appeared upon infection of larvae in earlier stadia.

JHE is an extraordinarily stable protein that does not degrade on incubation in vitro with hemolymph. However, pharmacokinetic analyses showed that the half-life of JHE injected into the hemolymph of larvae of *M. sexta* was only approximately 1 h (53). Further analysis indicated that JHE was being removed by the pericardial cells (15, 54). Electron microscopy of immunogold-labeled sections of these cells showed concentration of the JHE within lysosome-like granules, where it is presumably degraded (15). JHE is removed by a saturable, first-order process, which suggests that the mechanism of uptake may be receptor-mediated endocytosis (53).

To prevent degradation of JHE in the pericardial cells, sequences likely to be involved in the degradation process were altered using site-directed mutagenesis. Specifically, lysine residues at positions 29 and 522 within the peptide sequence of JHE were replaced with arginine residues (100). Baculoviruses were then constructed to express the modified JHEs with the single (AcJHE-29, AcJHE-522), or with both (AcJHE-KK), mutations. Neither the kinetic parameters nor the expression levels of the modified JHEs in vivo or in vitro were significantly different from those of the wild-type JHE. However, infection of *H. virescens* larvae with AcJHE-KK significantly reduced lethal times and feeding damage compared with those observed upon infection with the wild-type, nonengineered virus. Feeding damage was reduced by 50% on lettuce and 36% on cotton leaves. We examined the effects of AcJHE-KK on larvae of *H. virescens* in conjunction with topically applied JH analogues, JHE inhibitors, or anti-JH agents. The lethal time of AcJHE-KK increased when used with JH analogues or JHE inhibitors. These data suggest that catalytic activity is required for this insecticidal effect. When AcJHE-KK was bioassayed in conjunction with anti-JH agents, its lethal time decreased. This result suggests that the insecticidal efficacy of AcJHE-KK results at least in part from an anti-JH activity.

Biochemical analyses of events within the pericardial cell complex after injection of JHE into larvae of *M. sexta* suggest that JHE is bound by a heat-shock protein (Hsp) prior to degradation (BC Bonning, VK Ward, TF Booth, RD Possee & BD Hammock, unpublished data). The Hsp is hypothe-

sized to bind to JHE at a putative lysosome-targeting sequence (17) and to facilitate transport to the lysosome (18). The mutation at position 522 is within the putative lysosome-targeting sequence of JHE. Immunoprecipitation of the wild-type and modified JHEs with anti-Hsp antiserum showed that the modifications significantly reduced binding of the Hsp to JHE. Although the exact mechanism of action of Hsps in chaperoning proteins to the lysosomes is unclear in both this and mammalian systems (49), we propose that the insecticidal activity seen upon infection with AcJHE-KK results from impaired degradation of JHE. Accumulation of JHE-KK within the pericardial cells may reduce the local titer of JH and impair the secretory functions of this organ (33). Variation in efficacy, which sometimes occurs with infection of larvae with AcJHE-KK, may relate to the precise timing of expression of JHE-KK in relation to other physiological events associated with JH action during larval development.

These data suggest that expression of other regulatory proteins and enzymes could be useful in accelerating the kill of recombinant baculoviruses. They also indicate that genetic changes made to enhance the stability or change the distribution of an expressed peptide or protein can be used to improve the performance of recombinant viruses.

Unexpected Results

During the course of research on AcJHE-KK, a simultaneous study to investigate the catalytic mechanism of JHE was under way. To establish the relative contributions of specific amino acids to the catalytic activity of this enzyme, a series of modifications were made to JHE (100). Several of these modifications completely removed the catalytic activity of JHE. For example, the active site Ser201 was replaced to produce JHE-SG, and His446 was replaced to produce JHE-HK. The viruses AcJHE-SG and AcJHE-HK, which express the inactive forms of JHE, were used as control viruses for bioassay of AcJHE-KK. Against expectations, JHE-SG turned out to be highly insecticidal (12). Lethal times were reduced by 20–30% compared with those of the wild-type virus, with a 66% reduction in feeding damage on lettuce and a 55% reduction on cotton. When bioassayed with anti-JH agents, the lethal time of AcJHE-SG was increased, suggesting that the mechanism of action is not related to the degradation or sequestration of JH. About 25% of the larvae infected with AcJHE-SG died during molting, being unable or only partially able to escape from the old exuvium. We propose that the mechanism of action of JHE-SG is related to events at the molt.

A virus was constructed to express a modified form of JHE with mutations at Lys29, Lys522, and Ser201 (MMM van Meer & BC Bonning, unpublished data). This virus, AcJHE-KSK, has comparable efficacy to AcJHE-KK and

AcJHE-SG in terms of feeding damage and lethal times. Similar to results with AcJHE-SG, the lethal time of AcJHE-KSK increased upon application of anti-JH agents during bioassay of the virus. Moreover, the symptoms were similar to those seen upon infection with AcJHE-SG.

The insecticidal efficacies of AcJHE-KK, AcJHE-SG, and AcJHE-KSK do not differ significantly from the efficacy of AcAaIT (12), which is among the best of the toxin-expressing baculoviruses. The recombinant baculoviruses expressing JHE have a possible advantage over toxin-expressing baculoviruses in terms of the public's response to these genetically engineered organisms. In addition, rapid radiochemical (45) and colorimetric assays (75) for JHE activity may greatly facilitate monitoring of the virus under field conditions.

RISK ASSESSMENT OF RECOMBINANT BACULOVIRUS INSECTICIDES

To date, evaluation of the recombinant baculoviruses indicates that they offer great benefit to agriculture with minimal risk. No members of the Baculoviridae infect plants or nonarthropod animals. Selective viruses are most attractive for IPM, but if regulatory barriers are high, industry will be forced to develop viral pesticides that control a broad spectrum of pests. Fortunately, the initial work has focused on viruses that have a relatively narrow host range compared with even highly selective materials such as *B. thuringiensis* and the insect growth regulators. Having developed prototype viruses with enhanced speed of kill, investigators have been conducting extensive tests to ensure that the recombinant viruses do not represent a hazard to nontarget organisms or to the environment (23, 24). The goal of ongoing laboratory-based tests is to ascertain that the genetic manipulation process has not altered the host range of the virus (4, 6). An understanding of the molecular determinants of host range should speed such evaluations (26, 55, 58, 68, 80).

For host-range analyses, large numbers of nontarget organisms are screened for possible infection with the recombinant virus. The potential effects of the expressed protein on predators and parasites of the targeted insect pest are also determined in the laboratory (48). In addition, the genetic stability of the viruses and the likelihood of genetic exchange between viruses infecting the same host insect will be established. Based on these data, field trials have been conducted using various constructs, including AcAaIT (25, 42), and more trials are planned for the near future.

A series of contained field trials carried out with genetically modified baculoviruses in England have addressed questions of virus stability and efficacy (4, 6). In 1989, the Boyce Thompson Institute for Plant Research at Cornell University conducted the first uncontained field trials of genetically engineered baculoviruses (104, 105). The recombinant baculovirus most re-

cently tested for efficacy under field conditions is AcAaIT (25). Data on feeding damage caused by infected larvae of *T. ni* were promising but insufficient to evaluate the commercial potential of the virus.

Because research with recombinant baculoviruses involves recombinant DNA technology, it now requires special scrutiny (77). One hopes that the regulatory authorities will define clear processes for the evaluation and regulation of biological organisms for use in agriculture, using performance-based standards regardless of whether they are generated by classical or recombinant means. Fortunately, the scientists involved in the development of recombinant baculoviruses have themselves set high standards for environmental safety and have designed risk-assessment experiments to test worst-case scenarios. When evaluating the results and the risks and benefits of recombinant-baculovirus technology, we should consider the relative attributes and limitations of competing technologies. Opponents of the technology (20, 102) fail to recognize that all of the rapid-kill viruses are designed as nonrecycling biological pesticides that are at a selective disadvantage compared with the wild-type organisms (41). Thus, there is no evolutionary driver for the virus to replicate in the environment or colonize new hosts. The very nature of the rapid-kill response leads to a dramatic reduction in progeny viruses and a strong negative selection pressure. Thus, these recombinant viruses are more attractive ecologically than organisms provided with traits that give a positive selective advantage.

FUTURE PROSPECTS

New, more potent, insect-selective toxins should be isolated from various organisms within the foreseeable future. Agents with greater activity allow the use of earlier and weaker promoters and may also result in insect death in earlier viral replication cycles (67). Further development of recombinant baculovirus insecticides may proceed along multiple fronts (42, 44). Synergism between insect-selective toxins expressed in the same baculovirus construct represents a new avenue for investigation. Modification of proteins and peptides for enhanced stability in vivo may improve all of the recombinant baculoviruses, especially those that show minimal increases in speed of kill. Apart from the recombinant protein expressed by the virus, formulation components can also be used very effectively to enhance the speed of kill of genetically engineered baculoviruses (22).

Recombinant baculoviruses represent a valuable technology that has great potential for effective integration into pest-management systems. Both the recombinant and wild-type viruses are more environmentally attractive than classical pesticides and may represent a major step toward a more sustainable agriculture. However, the full potential of recombinant baculoviruses will only

be realized if the regulation of their testing and commercial use is rationalized through a risk-based approach (77).

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