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Development and Potential of Genetically Engineered Viral Insecticides

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Introduction

The international market for insecticides is huge. Of the $20 billion annual worldwide market for pesticides, biopesticides make up less than 1%. Baculovirus pesticides account for 0·2% of this (Jutsum, 1988). The relatively small revenues from use of biological control agents has provided little incentive for industrial investment. However, a combination of factors, including increased levels of resistance to synthetic chemical insecticides, public concern over possible health problems associated with chemicals in food, and concern over environmental contamination, have focused attention on alternative means for insect pest control. The advent of recombinant DNA technology provides the potential to improve micro-organisms already in use as insect control agents. The realization that genetically altered organisms can be patented, and used for improved insect control, has provided industry with the incentive needed to justify expenditure on new research and development. Despite the fact that classical chemical pesticides have set a high standard in terms of efficacy, cost and ease of use, early data from use of recombinant DNA techniques indicate that biopesticides are meeting these standards.

For an organism to be amenable to genetic manipulation, the genetics must be well understood, the means must exist to introduce new DNA into the genome of that organism and it must be possible to rear the organism rapidly. Most attention, in terms of enhancement of insecticidal efficacy, has been paid to the bacteria, viruses and fungi, due to their relative simplicity at the genetic level.

Abbreviations: AcNPV, nuclear polyhedrosis virus of *Autographa californica*; BmNPV, nuclear polyhedrosis virus of *Bombyx mori*; Bt, *Bacillus thuringiensis*; BV, budded virus; CAT, chloramphenicol acetyltransferase; egt, ecdysteroid UDP-glucosyltransferase; GV, granulosis virus; IPM, integrated pest management; JH, juvenile hormone; JHE, juvenile hormone esterase; kb, kilobase; NOV, non-occluded virus; NPV, nuclear polyhedrosis virus; OV, occluded virus; SDS, sodium dodecyl sulphate; UV, ultraviolet.

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Baculoviruses are commonly used as powerful expression systems for both prokaryotic and eukaryotic proteins (Luckow and Summers, 1988) and, as a result, the genetic make-up of the viruses has been extensively characterized (Doerfler and Bohm, 1986; Blissard and Rohrmann, 1990). There is now great interest in baculoviruses as genetically engineered insect pest control agents, with the realization that the time taken by the virus to kill its host insect can be hastened by genetic manipulation. Baculoviruses bypass many of the environmental problems associated with chemical pesticide residues, should overcome many resistance mechanisms and may alleviate the extensive damage done by some very serious crop pests. This chapter provides an overview of the techniques used for genetic engineering of viral insecticides and appropriate bioassay techniques; highlights perceived problems associated with use of baculoviruses as insecticidal agents; details research underway to overcome these limitations; and reviews the significant improvements achieved to date. Recent developments in reducing time taken by baculoviruses to kill insect pests, and alteration of the viral host-range by genetic engineering, indicate that recombinant baculoviruses may soon be adopted for insect pest control programmes.

INSECT VIRUSES

Baculoviruses were studied initially in the 1930s for use against forest pests, and were used in North America against the spruce sawfly *Neodiprion sertifer* (Bird and Whalen, 1953) until the advent of chemical pesticides in 1960. Other successful examples of insect pest control by application of baculoviruses include control of the codling moth, *Cydia pomonella*, the gypsy moth, *Lymantria dispar*, the coconut rhinoceros beetle, *Oryctes rhinoceros* in the South Pacific, and soybean pests in Brazil by use of *Anticarsia gemmatalis* NPV (Bedford, 1981; Entwistle and Evans, 1985; Benz, 1986; Hüber, 1986; Moscardi, 1988). As these viruses may take between 4 and 14 days to kill their hosts, they have been used primarily on crops which can sustain a degree of damage to foliage without major economic loss. Insects currently controllable with baculoviruses include pests of cotton, coconuts, bananas and forest pests (*Table 1*). In addition to field and row crop pests, the forestry market in the US is worth $30 million annually and is likely to expand in response to new infestations of the gypsy moth, *Lymantria dispar*, in the Pacific Northwest.

There are seven families of viruses which infect insects: the Baculoviridae, Iridoviridae, Parvaviridae, Polydnaviridae, Picornaviridae, Poxviridae and Reoviridae. The baculoviruses show the greatest potential as biopesticides in the near future. More than 500 species of baculoviruses have been isolated from various insect orders, including the Coleoptera, Hymenoptera, Trichoptera and also the crustacean order, Decapoda (Matthews, 1982; Martignoni and Iwai, 1986). Baculoviruses infect predominantly holometabolous insects and almost all pest species within the Lepidoptera (butterflies and moths) are susceptible to infection by at least one of the baculoviruses. The Lepidoptera include many of the world's most serious pests. It has been estimated that over half of the pesticides used are used to control insects in a

Table 1. Selected insect pests currently controllable with baculoviruses (adapted from Entwistle and Evans, 1985)

Pest	Baculovirus	Crop
Hymenoptera		
Neodiprion sertifer (European pine sawfly)	NPV	Pine
Lepidoptera		
Lymantria dispar (gypsy moth)	NPV	Broadleaved trees
Heliothis sp. (cotton bollworm)	NPV	Cotton, sorghum
Orgyia pseudotsugata (tussock moth)	NPV	Douglas fir
Cydia pomonella (codling moth)	GV	Walnuts, apples
Trichoplusia ni (cabbage looper)	NPV	Brassicas
Coleoptera		
Oryctes rhinoceros (rhinoceros beetle)	BV	Coconuts

single family of Lepidoptera, or other pests released from natural control by this treatment.

The baculoviruses are divided into three subgroups based on their morphology; the nuclear polyhedrosis viruses (NPVs) produce polyhedra containing singly or multiply enveloped nucleocapsids; granulosis viruses (GVs) produce capsule-shaped polyhedra called granules, containing individually occluded, singly enveloped nucleocapsids; and non-occluded viruses (NOVs) have one nucleocapsid per envelope and are not occluded. Baculoviruses are named after the insect species from which they were first isolated, followed by the appropriate baculovirus subgroup name.

Interest in the granulosis viruses (GVs) for genetic manipulation or industrial exploitation has been negligible, partly because they do not grow well in cell culture. However, GVs can be used against a number of pest species, including *Pieris brassicae* on cabbages, and *Plodia interpunctella*, a stored product pest. The high infectivity of some GVs make them very attractive targets if cloning problems can be overcome.

BACULOVIRUS LIFE-CYCLE

The genome of a baculovirus is enclosed within a cylindrical nucleocapsid, 40–60 by 200–400 nm in size, which is enclosed within an occlusion body composed of polyhedrin (Matthews, 1982; Vlak and Rohrmann, 1985). The nucleocapsids within the polyhedrin matrix are referred to as occluded virus (OV). A second viral phenotype, the budded virus (BV) (or non-occluded virus, NOV) is produced when nucleocapsids move to the plasma membrane, lose the nuclear-derived coat in the cytoplasm, and bud through the cytoplasmic membrane into the haemocoel. These BVs disseminate infection among tissues and cells within the insect (*Figure 1*; Keddie, Aponte and Volkman, 1989). Budded viruses are highly infectious to some cultured cells, and to larvae when injected into the haemocoel, but have minimal oral infectivity. It is the occlusion bodies, or polyhedra, which protect the virus in the

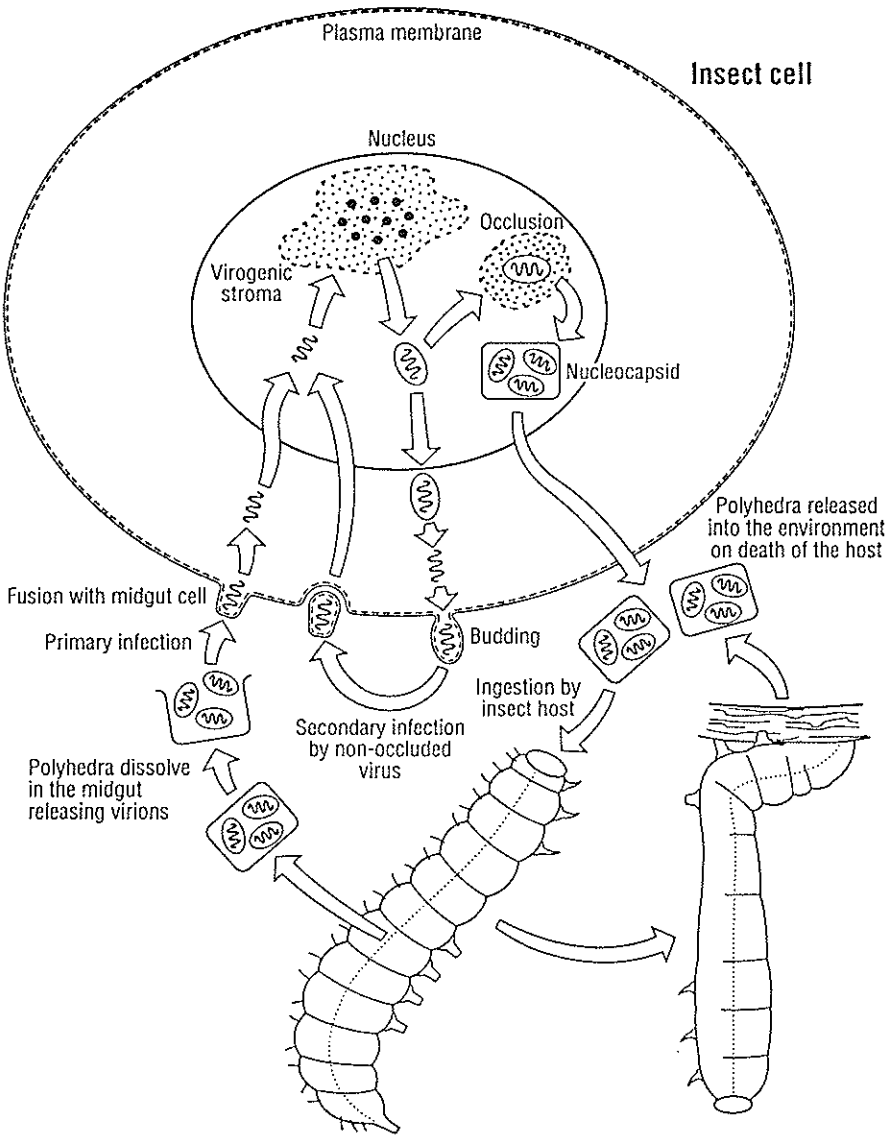

Figure 1. Life-cycle of the nuclear polyhedrosis virus. On ingestion of occluded virus, the polyhedrin coat dissolves in the alkaline midgut of the larva. The nucleocapsids which are released fuse with the midgut cells and the viral DNA moves to the nucleus. Viral DNA replication ensues and progeny nucleocapsids bud through the cytoplasmic membrane and infect other host cells. After the initial round of replication, nucleocapsids produced in the nucleus are enclosed within the protective coat of polyhedrin. When the insect dies, the polyhedra are released from the lysed cells.

environment (Jaques, 1977; Kelly, 1985) and effect transmission of the virus between insects.

On ingestion, the polyhedra move to the midgut where the alkaline environment, and possibly proteolytic action, dissolve the polyhedrin coat, thereby releasing the infectious nucelocapsids. For the granulosis virus, dissolution of the protective granulin coat also releases a factor known as the viral enhancing factor, which degrades the peritrophic membrane, enhancing the likelihood of efficient infection of the midgut cells (Tanada, 1985; Wood and Granados, 1991). The nucleocapsids released from the polyhedra fuse to the midgut epithelial cells, migrate through the cytoplasm, and uncoat in the nucleus (Granados and Lawler, 1981). Viral replication in the nucleus produces progeny nucleocapsids which bud through the plasma membrane. This results in secondary infection of tissues throughout the insect. The virus is spread via haemocytes and tracheal cells to peripheral tissues where secondary infection takes place (Keddie, Aponte and Volkman, 1989; Booth, Bonning and Hammock, 1992). Nucleocapsids may then be enclosed within the polyhedra in the nucleus, or continue to spread infection within the larval host. The polyhedra, which are 1–10 μm in size, account for up to 30% of the dry weight of the infected larvae (Miller, Lingg and Bulla, 1983). There may be 30 or more polyhedra in infected cells, and late instar larvae may produce up to 10^{10} polyhedra before death. The insect may continue to feed for several weeks before death from viral infection, depending on the virus concerned and environmental factors. Death occurs after about 10 rounds of viral replication. The cadaver, which is filled with virus, ruptures easily, releasing millions of polyhedra which are disseminated onto surrounding foliage and soil by the elements. Immediately prior to death, most larvae infected with virus climb up the vegetation and hang by the prolegs from the plant. The mechanism whereby this behaviour is elicited is unknown but the spread of virus from the cadaver is enhanced.

Autographa californica nuclear polyhedrosis virus (AcNPV) has between 100 and 150 genes which are activated in a cascade fashion. Gene expression during the course of baculovirus replication is divided into the immediate early, delayed early, late and very late phases (Kelly and Lescott, 1981). Gene expression in the late phase is mainly concerned with production of nucleocapsid structural proteins (6–20 h post-infection *in vitro*), while production of polyhedra and cell lysis occur in the very late phase (up to 72 h post-infection).

Considerations for use of baculoviruses as insecticides

ADVANTAGES

There are a number of major advantages associated with the use of baculoviruses for pest control. They are non-pathogenic to vertebrates and plants (Summers *et al.*, 1975; Payne, 1982; Miller, Lingg and Bulla, 1983; Miller, 1988), and are unable to penetrate the nuclei of mammalian calls (Carbonell and Miller, 1987). Most baculoviruses are active on a single family or genus (*Table 2*). This restricted host-range is a great advantage as the risks of

deleterious effects on beneficial and non-target organisms are minimal compared to the potential harm caused by the relatively non-selective synthetic chemical insecticides. The lack of any undesirable residues in the environment after use of baculovirus insecticides is of considerable advantage given current concerns over the perceived health and safety problems associated with chemical insecticide use. As these viruses are already present in the environment, their use will not elicit the problems which may arise from the introduction of a new chemical or organism into the field.

Perhaps the most significant advantage for the use of baculovirus insecticides is that they are compatible with other control agents providing greater scope for their use in integrated pest management (IPM) programmes. They are compatible for simultaneous application, and the recombinant viruses may have synergistic effects depending on the mode of action of the foreign protein being expressed.

Of particular interest for insect pest management is *Autographa californica* nuclear polyhedrosis virus (AcNPV), originally isolated from the alfalfa looper. This virus is known to infect 39 species of Lepidoptera (*Table 2*), including those of the major pests *Heliothis, Spodoptera* and *Trichoplusia*. This review will be concerned mainly with the genetic manipulation of AcNPV which has received most attention thus far for development of improved baculovirus insect control agents. However, the techniques pioneered with AcNPV are being applied to a variety of other baculoviruses.

RESISTANCE OF INSECTS TO RECOMBINANT BACULOVIRUSES

Up until now, no resistance to these viruses has been detected on the scale of that encountered for the classical chemical insecticides (Briese, 1986). Baculoviruses have co-evolved with their insect hosts over the millenia and are well adapted to avoid the insect's defence mechanisms to optimize viral production at the expense of the host.

Resistance is 'the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species' (WHO Export Committee on Insecticides, 1957). Developmental, environmental and genetic resistance shown by insects to baculoviruses has been reviewed by Briese (1986). The mechanisms of resistance to baculoviruses have not been studied widely, but a number of differences in susceptibility between populations of the same species have been noted. These susceptibility differences have been caused by inactivation of the virions in the midgut, lack of attachment of virions to midgut cells, inhibition of viral replication in the midgut, discharge of infected midgut cells, or destruction of virions in the haemolymph, in different cases. For example, low pathogenicity of NPV to *Spodoptera frugiperda* involves an unknown mechanism associated with the insect gut (Fuxa and Richter, 1990), and *Pieris brassicae* resistance to GV infection is associated with a post-invasion mechanism (David, 1978).

In all cases of 'resistance' to date, the resistance ratios are fairly low, and insects quickly revert to susceptibility in the absence of exposure to the virus

in question. No cross-resistance from insecticidal baculoviruses to other types of control agents is likely due to the unique mode of viral entry into the host. All of these factors are favourable characteristics in terms of resistance management.

DISADVANTAGES

On the negative side, the self-same specificity listed as an advantage can also act as a disadvantage. Farmers and growers favour insecticidal agents that will deal with all of their pests, as opposed to using a number of different control agents. For example, although AcNPV is highly infectious to *Autographa californica, Estigmene acrea, Heliothis virescens, Spodoptera exigua* and *Trichoplusia ni*, susceptibility of other major cotton or vegetable crop pests such as *Spodoptera littoralis, Heliothis zea, Pectinophora gossypiella* and *Agrotis ipsilon*, is up to 40-fold lower (Payne, 1986). There are many pest situations where the host range of AcNPV will have to be manipulated genetically, other viruses used, or the viruses used with other chemical control strategies.

Table 2. Host-ranges for selected baculoviruses (adapted from Cory and Entwistle, 1990)

Original host insect	Virus	Host-range		
		Families	Genera	Species
Hymenoptera				
Gilpinia hercyniae	NPV	1	1	1
Neodiprion sertifer	NPV	1	1	3
Lepidoptera				
Artogeia rapae	GV	1	1	3
Cydia pomonella	GV	1	3	4
Heliothis armigera	GV	1	3	5
Choristoneura fumiferana	NPV	3	3	3
Helicoverpa zea	NPV	1	1	7
Anticarsia gemmatalis	NPV	2	6	9
Autographa californica	NPV	13	33	39
Euproctis chrysorrhoea	NPV	1	1	1
Mamestra brassicae	NPV	4	26	36
Coleoptera				
Oryctes rhinoceros	NOV	1	2	5

NPV, nuclear polyhedrosis virus; GV, granulosis virus; NOV, non-occluded virus.

Large-scale production of baculoviruses is currently labour intensive; it involves mass rearing and infection of insect larvae, which account for a major part in production costs. Economical cell culture techniques for large-scale baculovirus production have not yet been developed for the agricultural use of baculoviruses (Sheih, 1989). A number of improvements have been and are being made for *in vitro* production systems for recombinant baculoviruses, including enhanced media for virus production and development of novel bioreactors (Shuler *et al.*, 1990). Investment in large-scale cell culture for baculovirus production is very important for the recombinant baculovirus industry; large-scale rearing programmes are not

considered favourably. Cell culture systems are already in place for mass production of proteins expressed by the baculovirus system. It is likely that improvements in culture techniques will result in *in vitro* production of baculoviruses for agriculture becoming an important supplement or alternative to *in vivo* production.

Baculoviruses are best reared in cell culture by batch procedures, rather than by serial passaging by continuous addition of cells and removal of media, which may result in the generation of defective viruses (Van Lier *et al.*, 1990; O'Reilly, Miller and Luckow, 1992).

The fact that baculoviruses are relatively slow at killing their hosts is one of the most important disadvantages associated with baculovirus insecticides, and has restricted their use in the past to crops that can sustain some damage without too much economic loss (*Table 1*; Entwistle and Evans, 1985). Associated with this, and another factor contributing to the limited commercial success of baculoviruses, is that unlike synthetic chemical insecticides, baculoviruses have no contact or systemic action. Pest larvae must consume the baculovirus to become infected, thus good coverage of plants with the virus is required.

The timing of baculovirus application is crucial, and will require a better surveillance system than is currently in operation. Changing surveillance strategy may present some difficulty; the virus must be applied against lower densities of larvae detected in the field than those considered high enough to necessitate application of synthetic chemical insecticides. Also, applications must be carried out against smaller larvae: first instar larvae are often difficult to detect, but they are more susceptible to viral infection than later instars. Spraying of baculoviruses may be required more frequently than application of synthetic chemical insecticides, and precise recommendations for effective application of the viruses will be needed.

OTHER CONSIDERATIONS

The time taken for the virus to kill its host can be reduced by manipulation of the viral genome as illustrated below. The host-range of different viruses may also be extended by classical selection or recombinant techniques, as more is learned of genetic factors influencing the host range (Kondo and Maeda, 1991). In addition, detection and genetic analysis of other viruses, from Diptera for example, may permit application of the same technology currently being applied to baculoviruses to the control of other types of pests, including vectors of disease. In many parts of the world, mosquito-borne disease is rampant due to lack of cost-effective control strategies. About $10 million is currently spent per annum on mosquito control alone. Should a suitable viral agent be isolated, control of mosquitoes by recombinant baculoviruses may provide a novel and much needed alternative means of control. Some of these considerations are discussed in more detail below.

FACTORS AFFECTING HOST RANGE AND POTENTIAL MANIPULATION

Engineering of baculoviruses other than AcNPV and *Bombyx mori* NPV (BmNPV) to target other pest species such as the gypsy moth, celery looper and the spruce budworm is already underway, and provides great potential for exploitation of the baculovirus technology against other insect pest species in the Diptera (flies), Coleoptera (beetles) and other orders.

Two approaches lend themselves to manipulation of viral host range, to extend the number of pest species susceptible to infection. The first involves preparing a cocktail of different viruses with different host-ranges, such that field application will be more effective against a broad range of pest species than application of a single virus type. The second approach involves manipulation of the genome of the virus.

Genetic factors determining host-range have been identified in BmNPV and the host-range manipulated by interspecies genetic exchange of the genomic regions concerned (Kondo and Maeda, 1991). Viral isolates with a wider host-range than either of the parent viruses were generated. These progeny were the result of cross-overs of relatively large areas of the genomes between AcNPV and BmNPV. Extensive analysis would be necessary to ensure that genetic stability, potential genetic exchange between viruses and susceptibility of non-target species had not also inadvertantly been changed by alteration of the host-range in this way.

FORMULATION AND APPLICATION OF BACULOVIRUSES

Optimization of baculovirus formulation presents one of the most challenging aspects of the potential for baculovirus insecticides, but relatively little effort has been invested in this area to date (Hammock and Soderlund, 1986).

Baculoviruses are sensitive to ultraviolet (UV) light (Killick, 1987), and while they may remain stable for years in the soil, they will degrade rapidly if exposed to direct sunlight (Payne, 1982). A variety of adjuvants for formulations are under investigation for optimization of viral shelf-life, suspension of the virus and increased stability under field conditions (Miller, Lingg and Bulla, 1983; Young and Yearian, 1986). An improved formulation can potentially halve the time taken by the virus to kill its host, and may ultimately result in a recombinant viral insecticide that kills in less than 24 h.

Baculoviruses may be applied by air (particularly against forest pests), by a boom-type sprayer for agricultural crops, or by high pressure equipment primarily for fruit and vegetable crops (Entwistle and Evans, 1985). They are generally applied by spray rather than in dust or granule formulations, the wettable powders being the most successful means of application. Adjuvants—surface active agents such as spreaders, stickers and emulsifiers, sunlight screens, buffers and also gustatory stimulants—are added to the tank mixture. The cost of production practices necessitates that the baculoviruses compete with the chemicals for the market. Changes in agricultural technology may be rapid, but generally occur gradually. It is essential that the recombinant viruses fit into current agricultural practice and can be applied

with existing equipment. Certainly some minor changes will be needed such as redefined economic thresholds. However, these changes are also needed as one moves among classical insecticides. Other changes such as more precise monitoring of pest populations will make biologicals far more effective, but they will not be accepted in agriculture until there is a clear and proven economic incentive.

Formulations need to be optimized to maximize speed of kill. With the classical chemical insecticides which act orally rather than topically, formulation and application techniques have proved critical for field efficacy. It is likely that formulation research can significantly improve the effectiveness of baculoviruses and other biologicals.

CONSIDERATIONS FOR USE OF RECOMBINANT BACULOVIRUSES IN THE THIRD WORLD

In many developing countries, insect pest control is restricted due to the relatively high cost of insecticides, and yet these are the countries which have the greatest need for such agents. An insecticide may cost more than 10 times as much in a developing as in a developed country. Most of the 'environmentally soft' chemicals are prohibitively expensive, leading to a reliance in some cases on older organochlorines such as DDT. Food production, disease control, and environmental protection must be considered as global problems.

Considerations for release of recombinant organisms and associated risks are somewhat different in the third, as opposed to first world countries. Potential minor risks associated with baculovirus use, for example, may be far outweighed by the benefits gained in terms of food production from use of this technology in a third world country. There is certainly scope for review of use of such a new technology at the national, rather than the international level (Hammock *et al.*, 1992).

The recombinant baculovirus technology is easily transferrable to the third world. The recombinant technology itself is inexpensive compared to the research and development effort with classical chemicals. Alternatively, isolates of favourable natural or recombinant viruses could be sent by industry, FAO, WHO or other organizations for *in vivo* propagation in developing countries. Baculovirus production by mass rearing and infection of insect larvae is viable on a local, cottage industry scale as manual labour is relatively inexpensive in the third world. Problems of shipment of chemicals which are potentially dangerous to the environment, and the risks associated with transfer of high technology to poorer countries, could thereby be avoided.

The development of recombinant baculoviruses and the potential of these genetically engineered viral insecticides to help alleviate insect pest damage is outlined below.

Production of a recombinant baculovirus

The overall aim for development of a genetically engineered viral insecticide with an enhanced speed of kill is to combine the pathogenicity of the baculovirus with the insecticidal action of a toxin, hormone or enzyme which is active on insects. The principle is to engineer the virus to express the foreign protein, by incorporation of the cDNA coding sequence for the foreign protein into the viral genome. This is facilitated by the relatively simple molecular biology of the baculovirus. The use of baculoviruses for expression of proteins has become a fairly routine technique used in the biological sciences (Miller, 1988; Maeda, 1989a, c; Luckow, 1991). Continued research on the viral genome (particularly for AcNPV and BmNPV) for improved protein production has enhanced our knowledge, facilitating exploitation of the baculovirus for insect control purposes. The entire sequences for the genome of both AcNPV and BmNPV have now been determined by R.D. Possee and S. Maeda, respectively.

Interest in BmNPV stemmed primarily from investment in the silk industry. The BmNPV provides a useful model for insect control; the main advantage for genetic manipulation of BmNPV lies in the fact that huge amounts of foreign protein can be produced using a recombinant virus in the larvae of *Bombyx mori* (Maeda, 1989a, c). AcNPV is itself active against pest species, and has commercial potential for use in the field both as wild-type virus and in its recombinant form.

GENETIC ENGINEERING OF THE BACULOVIRUS GENOME

The genome of AcNPV has a double-stranded, covalently closed circular DNA of 128 kb pairs. Recombinant baculoviruses are constructed in two stages due to the difficulty of manipulating the large genome directly. The foreign gene is incorporated initially into a baculovirus transfer vector. These transfer vectors have characteristics similar to many other plasmids in molecular biology which replicate in *Escherichia coli*.

DEVELOPMENT OF BACULOVIRUS TRANSFER VECTORS FOR PROTEIN EXPRESSION AND THEIR USE FOR DEVELOPMENT OF INSECT CONTROL AGENTS

Most transfer vectors used for construction of recombinant NPVs are plasmid, University of California (pUC) derivatives which usually encode an ampicillin resistance gene and an origin of replication for propagation in *E. coli*. A cloning site is also included for incorporation of the foreign coding sequence downstream of the promoter selected for use.

The polyhedrin protein constitutes about 95% by weight of the occluded virus and 20–50% of the viral protein produced in the very late phase of viral infection. Hence the gene is under the control of a very strong promoter. Since polyhedrin is not necessary for production of non-occluded virus *in vitro*, use of the polyhedrin promoter and disruption of polyhedrin coding

sequences by deletion or fusion has been exploited extensively for protein expression purposes. Genetic engineering of a baculovirus was initially achieved by employing the polyhedrin promoter by deleting part of the coding sequence for polyhedrin in AcNPV (Smith, Fraser and Summers, 1983; Summers and Smith, 1985). The transfer vector was constructed from a fragment of the AcNPV genome at the polyhedrin locus. Alterations (such as insertion of the cDNA sequence encoding a foreign protein) are made within this region. Surrounding the cloning site are sequences homologous to those on either side of the polyhedrin gene in the wild-type virus which are essential for recombination with the viral DNA in the nuclei of insect cells. Genetically engineered viruses are produced by allelic exchange resulting from homologous recombination between the wild-type viral DNA and the sequences around the polyhedrin locus which are included in the transfer vector. Recombinant viruses are identified by the absence of polyhedra in viral plaques produced on a monolayer of cultured insect cells. Human β-interferon was the first foreign protein to be expressed in the baculovirus expression system (Smith, Summers and Fraser, 1983). Since then, baculovirus expression of foreign proteins has become a widely used tool. High yields of protein are produced and correct post-translational modifications (glycosylation, phosphorylation) carried out (Miller, 1988; Maeda, 1989a; Luckow, 1991).

While dispensable for viral replication *in vitro*, the presence of the polyhedrin gene is crucial for the production of AcNPV for insect control purposes. Viruses without polyhedra (non-occluded virus) are not viable for use in the field as insecticidal agents using classical application technology. Non-occluded viruses are very unstable in the field and have extremely low infectivity on ingestion by insect larvae. Clearly, disruption of polyhedron production by genetic manipulation of the baculovirus genome is unsuitable for recombinant insecticidal baculoviruses.

A second approach to production of recombinant baculoviruses was to use the promoter of the p10 protein. Although its precise function is unclear, the p10 protein is thought to be involved in cell lysis, stabilization and assembly of polyhedra by production of the polyhedral membrane (Williams *et al.*, 1989; Van der Wilk, Lent and Vlak, 1987; Vlak *et al.*, 1988; Rohrmann, 1992). The p10 protein is associated with fibrillar structures seen in the nuclei and cytoplasm of infected cells and, like the polyhedrin gene, is dispensable for viral replication *in vitro* (Van der Wilk, Lent and Vlak, 1987). Replacement of the p10 coding sequence by the foreign DNA was carried out to produce a high yield expression vector for protein production (Vlak *et al.*, 1988), and provided the means for producing a recombinant polyhedrin-positive baculovirus with potential for use in the field as a viral insecticide. The viruses lacking the p10 protein had some aberrant polyhedron morphology, and were noted to show some degree of instability.

More recently, engineering of the viral genome for high level expression of the foreign protein has been achieved by duplication of the p10 promoter (Weyer, Knight and Possee, 1990). This process avoids gene deletion from the viral genome altogether and hence bypasses the consequent deleterious

effects on viral infectivity (in the case of deletion of the polyhedrin gene) and stability (in the case of deletion of the p10 gene).

The polyhedrin and p10 promoters employed to drive foreign gene expression are both strong very late viral promoters. The promoter for the basic protein has also been used for foreign gene expression. The basic protein is expressed during the late phase of the viral infection cycle, and is a histone-like binding protein closely associated with the viral DNA within the nucleocapsid. The basic protein promoter was duplicated in the transfer vector at the polyhedrin locus (Hill-Perkins and Possee, 1991). The recombinant virus produced on recombination of the transfer vector DNA with modified viral DNA (Weyer, Knight and Possee, 1990) has the polyhedrin sequence under control of the p10 promoter, and lacks the p10 protein. Use of this system currently provides the means for very high level production of foreign proteins by recombinant baculoviruses, several hours earlier than protein production from the p10 and polyhedrin promoters (Bonning *et al.*, 1993).

There are a wide variety of different plasmid transfer vectors for both AcNPV and BmNPV (Maeda, 1989c; O'Reilly, Miller and Luckow, 1992b). In addition to viral promoters, promoters for non-NPV genes have also been employed, such as the hsp70 promoter from *Drosophila melanogaster* (Vlak *et al.*, 1990). Finally, hybrid promoters derived from the AcNPV polyhedrin promoter have been constructed for high level expression of foreign proteins (Thiem and Miller, 1990). Further development of transfer vectors should provide even greater versatility for construction of recombinant baculovirus insecticides.

For the development of recombinant baculoviruses for use as insecticides, high level, early expression is desirable, although the requirements will vary according to the insecticidal agent being expressed by the recombinant virus. For example, low level, continuous expression of a scorpion toxin may be preferable to high level, late expression according to its mode of action. As genes coding for peptides of higher activity are identified, it will be possible to use earlier and weaker promoters in the development of viral insecticides. Recently, transfer vectors for expression of two genes have become available (Emery and Bishop, 1987; Weyer and Possee, 1991; Wang, Ooi and Miller, 1991), allowing even greater flexibility for production of efficient viral insecticides by genetic manipulation.

CONSTRUCTION OF A RECOMBINANT BACULOVIRUS

A number of new developments have made the introduction of the foreign gene from the transfer vector into the baculovirus a relatively rapid and straightforward process. Once the coding sequence for the foreign protein has been inserted correctly into an AcNPV transfer vector (which is confirmed by restriction analysis of the vector), insect cells such as the *Spodoptera frugiperda* cell line, IPLB-Sf-21 (Vaughn *et al.*, 1977), are co-transfected with plasmid (transfer vector) DNA and with DNA extracted from the appropriate

virus. In the nucleus of cells infected with the mixed DNAs, a recombinant virus is generated by homologous recombination.

Originally, the recombinant virus had to be isolated from non-recombinant virus by a laborious series of plaque assays (Brown and Faulkner, 1977). In this technique, plaques produced by virus infection of a monolayer of insect cells, are screened for the polyhedrin-negative (or polyhedrin-positive) recombinant phenotype. Recombinant virus plaques are isolated and used to infect more cells in a subsequent plaque assay. This is repeated until all plaques have the required recombinant phenotype, i.e. until the recombinant virus is pure, with no background of non-recombinant virus. Purification of recombinant baculoviruses could take several weeks. The advent of polyhedrin-positive systems greatly improved recombinant virus isolation, as it is significantly easier to isolate polyhedrin-positive viruses from a polyhedrin-negative background, rather than vice versa.

A recent advance, in which the viral DNA used for co-transfection is cut with a restriction endonuclease (and hence linearized), has greatly simplified the purification of recombinant baculoviruses. Using this technique, only recombinant viruses will be viable after co-transfection as allelic exchange with the plasmid recircularizes the DNA and only the circular viral DNA is able to replicate (Kitts, Ayres and Possee, 1991). A plaque assay of the medium above the co-transfected cells is carried out 2 days after co-transfection of the cells, to isolate recombinant virus. A single plaque assay is sufficient to isolate the recombinant virus. Provided that plaques are well isolated, further purification of the recombinant virus is unnecessary. Should the viral DNA used in the co-transfection be incompletely cut, a background of viruses lacking the desired gene will be produced. The recombinant viruses, which are polyhedrin-positive, are still distinguishable from the background of polyhedrin-negative virus and can be purified by additional plaque assays.

Lipofectin™ is an efficient agent for transferring the plasmid and linearized viral DNA mixture into the insect cell (Felgner *et al.*, 1987). Lipofectin consists of a cationic lipid that forms liposomes which enclose the DNA and fuse with the plasma membrane of the insect cell, resulting in uptake of the DNA. The DNA and lipofectin are simply mixed for 15 min at room temperature with gentle shaking, before addition to the insect cells. Calcium phosphate precipitation is an alternative but less efficient means of introducing the DNA into the cells. This works by formation of a co-precipitate of DNA and calcium phosphate which is taken up by the cells. Once in the cell, the DNA moves to the cell nucleus where recombination and regeneration take place.

Once a recombinant virus has been isolated and amplified *in vitro*, DNA is extracted and analysed by restriction analysis to confirm that the correct insertion has been made, and that no other recombination or deletion events have taken place. Incorporation of the foreign insert is confirmed by Southern blot analysis, and protein expression is confirmed by sodium dodecyl sulphate (SDS) gel electrophoresis, and/or by assay for the protein itself if appropriate.

Before development of transfer vectors resulting in recombinant viruses which express polyhedrin, polyhedrin-positive viruses could be generated by co-occlusion of the polyhedrin-negative recombinant with polyhedrin-positive wild-type virus (Kuroda *et al.*, 1989; Price *et al.*, 1989). Co-infection of cells with the two virus types results in packaging of some of the polyhedrin-negative recombinant virus in the polyhedra produced by the wild-type virus. This process requires the extra step of co-infection of cells after generation of the recombinant baculovirus. The current transfer vectors, enabling direct construction of recombinant virus expressing polyhedrin, renders co-occlusion somewhat obsolete.

VIRAL AMPLIFICATION *IN VIVO*

Once the correct construct has been generated and protein expression confirmed, the recombinant virus can be amplified by feeding to third instar larvae of, for example, the cabbage looper (*Trichoplusia ni*) or the tobacco budworm (*Heliothis virescens*), for AcNPV. These larvae are harvested and frozen when pale in colour from virus infection (indicating impending death), but before melanization (blackening of the cuticle generally arising from injury). A simple procedure of homogenization and differential centrifugation can then be used to purify the recombinant polyhedra (Bonning *et al.*, 1992a).

Polyhedra are stored in water with 0·02% sodium azide to prevent growth of contaminating micro-organisms at 4°C, and washed thoroughly to remove the azide before counting and use in bioassay. Storage of the virus at 4°C prevents the virus from sticking together, which may occur when the virus is frozen. Unlike the polyhedrin-positive viruses which are relatively stable on storage, the titres of polyhedrin-negative (non-occluded) virus may be reduced 10-fold or more with each freeze-thaw cycle, or on long-term storage at 4°C.

Genetically engineered viral insecticides developed to date

Any gene expressing an agent which perturbs development, reduces feeding, or otherwise disrupts normal insect behaviour resulting in decreased crop damage, is a possible candidate for use in a recombinant baculovirus for insect control purposes. These agents include insect enzymes, hormones and toxins.

RECOMBINANT BACULOVIRUSES EXPRESSING TOXINS

One of the first recombinant baculoviruses for insecticidal use was engineered to express an insect specific toxin (BeIT) from the scorpion *Buthus eupeus* (Carbonell *et al.*, 1988). The coding sequence for the toxin was preceded by the alpha human interferon signal sequence for export of the toxin from virus-infected cells. Expression of the BeIT toxin in the recombinant virus did not enhance efficacy of the virus for pest control purposes, and it was

suggested that insufficient toxin was produced to cause detectable biological activity. This could be for several reasons, including incorrect folding of the toxin, use of an incorrect sequence, or failure of the recombinant baculovirus to produce the threshold of toxin required for biological effect, possibly due to use of an inappropriate signal sequence in the recombinant virus.

A number of different toxins have since been engineered into AcNPV (Maeda and Hammock, 1992), including toxins produced by the bacterium *Bacillus thuringiensis* (Bt). There are four classes of Bt isolates, one of which, Cry1, produces crystals toxic to Lepidopteran larvae (Höfte and Whiteley, 1989). Each toxin appears to have its own toxicity spectrum in insect hosts. Two protoxins from *B. thuringiensis* which are activated by proteolytic cleavage to the toxic form in the insect gut were incorporated into the AcNPV genome by Merryweather *et al.* (1990) and Martens *et al.* (1990). In each case, the active toxin is thought to generate pores in cell membranes, thereby disrupting osmotic balance leading to cell lysis. Merryweather *et al.* (1990) constructed two recombinant viruses incorporating the sequence for the δ endotoxin from *B. thuringiensis* subsp. *kurstaki* HD-73 under control of the p10 or polyhedrin promoter in polyhedrin-positive and -negative constructs, respectively. The lethal dose for the polyhedrin-negative virus could not be ascertained due to feeding inhibition caused by endotoxin contamination of the virus preparation. However, the lethal dose for the polyhedrin-positive construct was not significantly different from that of the non-engineered control virus. Viral products of 130 kDa and 62 kDa were thought to be the protoxin and the active toxin, respectively. The crystal structures characteristically associated with this toxin were not seen on analysis of infected cells by electron microscopy. It was suggested that use of the recombinant viruses expressing the *B. thuringiensis* δ endotoxin may offer an advantage after the initial round of replication, by release of the toxin onto plants after larval death, which would act as a feeding deterrent to other larvae.

Martens *et al.* (1990) incorporated the gene for the Cry 1A(b) toxin of *B. thuringiensis* subspp. *aizawai* 7.21 into AcNPV resulting in a polyhedrin-negative construct. High levels of the Cry1A(b) insecticidal protein were expressed and were visible in cell culture. These crystals inhibited feeding by larvae of the large cabbage white *Pieris brassicae*.

As expression of a Bt toxin by a recombinant virus would only occur within infected cells away from the site of action at the gut, use of Bt toxins in recombinant baculoviruses for insecticidal purposes may be inappropriate.

Two groups (McCutchen *et al.*, 1991; Stewart *et al.*, 1991) introduced an insect-specific toxin from the venom of the North African scorpion *Androctonus australis* Hector into the AcNPV to produce a modified baculovirus control agent. This toxin, AaIT, causes changes in neuronal sodium channel conductance that result in pre-synaptic excitatory effects. Symptoms include cessation of feeding, increased irritability and motility, and dorsal arching, leading to paralysis and eventual death of the insect larva. The toxin, which is 70 amino acids long and has four disulphide bridges, was expressed in AcNPV using the viral gp67 signal peptide sequence (Stewart *et al.*, 1991) and the bombyxin signal sequence (McCutchen *et al.*, 1991). In both cases, feeding

damage caused by recombinant virus-infected larvae was reduced by 50%, and lethal times reduced by about 25% relative to wild-type virus-infected larvae. Despite paralysis of recombinant virus-infected larvae, viral replication continues, producing large numbers of polyhedra. This facilitates the use of viruses expressing this scorpion toxin in the field, as the ability to produce polyhedra *in vivo* is only slightly reduced, despite the rapid immobilization of the insect.

The scorpion toxin AaIT was also expressed with the bombyxin signal sequence, in a recombinant BmNPV (Maeda *et al.*, 1991). Similar effects were seen on infection of larvae of *Bombyx mori* with this recombinant virus; feeding ceased 40 h post-injection of the larvae with non-occluded recombinant virus, and death occurred 60 h post-injection.

A toxin from the straw itch mite *Pyemotes tritici* has also been used for enhancement of insecticidal efficacy of recombinant baculoviruses (Tomalski and Miller, 1991). The mites can debilitate insects up to 150 000 times their size using this toxin, TxP1. Introduction of the cDNA for tox34 into AcNPV reduced time to kill by 30–40% relative to the wild-type virus, paralysing larvae within two days post-injection of the recombinant. The exact mode of action of TxP1 remains to be determined, but it results in muscle contraction and paralysis.

The protoxin expressed by the recombinant virus is 33 kDa in size (291 amino acids). Proteolytic cleavage results in a 27 kDa polypeptide believed to be the active agent. As for the recombinant viruses expressing AaIT, it was found that viral replication was not halted by paralysis of the host larva, although there was a 40% reduction in viral yield relative to the wild-type virus.

Further analysis using recombinant viruses expressing this mite toxin highlighted the importance of the promoter employed to drive expression of the foreign protein (Tomalski and Miller, 1992). A variety of promoters, including a very late promoter, were used: a linker-modified polyhedrin promoter (P_{LSXIV}); an early viral promoter (P_{ETL}); a hybrid promoter derived from both late and very late promoter elements ($P_{cap/polh}$); and a promoter derived from P_{LSXIV} and P_{syn} which is made up of homologous sequences in late and very late promoters (P_{synXIV}) (Tomalski and Miller, 1992). The viral construct using $P_{cap/polh}$ to drive toxin expression resulted in the fastest paralysis (40% decrease in time to kill relative to wild-type virus-infected larvae) and least weight gain (indicating the least feeding damage caused by infected larvae). The timing and strength of the viral promoter, in addition to the potency of the toxin, are important considerations for selection of the optimal promoter for foreign gene expression. For example, expression achieved by the early promoter P_{ETL} was too low to reach the toxin threshold required for larval paralysis. Such weak promoters may still be of use however, for more potent toxins.

As some of these toxins share their target site with other insecticidal agents (the scorpion toxin AaIT with the pyrethroid insecticides, for example), the question of resistance to the agent being expressed by the recombinant viruses arises. The site of action of the toxin AaIT is the sodium channel.

These channels in an insect could be altered by selection from a chemical insecticide and become resistant indirectly to AaIT. If such a resistant insect were infected with a recombinant virus, and the toxin did not paralyse the insect, the insect would eventually succumb to virus infection. Hence, any genetic basis for the resistance mechanism concerned could not be passed on to subsequent generations. Problems from resistance to the foreign toxin or protein being expressed by a recombinant virus are therefore likely to be minimal. Only resistance to the baculovirus itself (as outlined above) could be cause for possible concern.

RECOMBINANT BACULOVIRUSES EXPRESSING INSECT HORMONES

The alternative approach of introducing sequences coding for insect hormones or enzymes involved in the regulation of the insect endocrine system (Keeley and Hayes, 1987; Masler, Kelly and Menn, 1991), has also been adopted for enhancing baculovirus efficacy.

The first recombinant baculovirus with any enhanced insecticidal effect was developed by Maeda (1989). The diuretic hormone from *Manduca sexta* was incorporated into the BmNPV genome (Maeda, 1989b). This hormone (41 amino acids) plays an important part in the fine regulation of water balance. On injection of larvae of *Bombyx mori* with the recombinant virus coding for the hormone (BmDH5), the larval fluid metabolism was altered. Although the diuretic peptide was not detected, these larvae died 20% more quickly than larvae injected with wild-type virus. For this construct, the signal sequence of the cuticle protein (CP2) of *Drosophila melanogaster* was used for export of the virally-produced diuretic hormone.

The cDNA sequence for eclosion hormone, which is associated with ecdysis and brings about shedding of the cuticle at each moult, has been isolated from *M. sexta* (Horodyski, Riddiford and Truman, 1989) and has also been expressed in AcNPV (Eldridge *et al.*, 1991). The action of eclosion hormone is dependent on a complex series of events at a particular stage during the insect life-cycle, and results in a number of physiological and behavioural changes. On injection of the recombinant eclosion hormone into pupae of the fall armyworm, *Spodoptera frugiperda*, eclosion behaviour was initiated. The baculovirus-produced eclosion hormone was also active in *M. sexta*. However, insecticidal efficacy of the virus expressing the hormone was not enhanced in terms of ability to kill, or reduced feeding of infected larvae (Eldridge, O'Reilly and Miller, 1992a). This is perhaps not surprising in view of the timing and complexities associated with the action of eclosion hormone.

IMPLICATIONS OF ECDYSTEROID UDP-GLUCOSYLTRANSFERASE FOR BACULOVIRUS INSECTICIDES

A viral gene encoding ecdysteroid UDP-glucosyltransferase (egt) in AcNPV was reported (O'Reilly and Miller, 1989, 1990, 1991; O'Reilly, Brown and Miller, 1992). Egt transfers glucose from UDP-glucose to the insect moulting

hormones which are ecdysteroids. Expression of egt by the virus keeps the host larva in the feeding state, disrupting normal development and preventing moulting of larvae (O'Reilly and Miller, 1991). The effects of egt were analysed by producing viruses lacking functional coding regions for egt. Fifth instar larvae infected with an egt-positive virus fail to pupate, all signs of pupation (feeding cessation, wandering and spinning) being absent. Egt is a stable 57 kDa polypeptide (506 amino acids) which is secreted into the larval haemolymph. The fact that it is stable may suggest that continued production of egt by the virus is not required during larval infection. The gene is in a hypermutable region of the AcNPV genome, and is readily lost on serial passaging of the virus in cell culture, suggesting a selective disadvantage of the presence of egt under *in vitro* conditions (Kumar and Miller, 1987; O'Reilly and Miller, 1990). The authors also speculate that egt may have been acquired by the virus from an insect host (O'Reilly and Miller, 1990). Larvae infected with egt-negative baculoviruses consume 40% less food and produce 30% fewer progeny than larvae infected with egt-positive viruses, which inevitably gives the egt-positive viruses a considerable selective advantage under field conditions. In addition, larvae infected with egt-positive viruses will not leave the host plant to pupate, thereby enhancing viral spread (O'Reilly and Miller, 1991). Viruses lacking egt show a 10–20% reduction in time to kill relative to the wild-type virus.

DEVELOPMENT OF RECOMBINANT BACULOVIRUS INSECTICIDES EXPRESSING JUVENILE HORMONE ESTERASE

The titre of juvenile hormone (JH) in haemolymph determines the course of larval development (Sehnal, 1985). The larval commitment of tissues is lost in the absence of JH, and release of ecdysone reprogrammes the tissues to become pupal at the moult (Sehnal, 1989). A reduction in JH titre controlled in part by juvenile hormone esterase (JHE) (Hammock, 1985; *see Figure 2*) precedes cessation of feeding before a moult and is necessary for initiation of metamorphosis in the last larval instar.

Anti-juvenile hormone activity has commanded significant interest over the years in the agricultural chemical industry, as induction of precocious development will lead to a reduction in crop damage (Staal, 1986). Expression of JHE in the baculovirus system for insect control purposes is a logical progression of this line of anti-JH research.

The coding sequence for JHE derived from *Heliothis virescens* (Hanzlik *et al.*, 1989) has been introduced into a variety of recombinant baculoviruses for insect control means. JHE has been expressed in different AcNPV constructs under the control of the polyhedrin promoter (Hammock *et al.*, 1990; Roelvink *et al.*, 1992), the p10 promoter (Bonning *et al.*, 1992a; Roelvink *et al.*, 1992), the basic protein promoter (Bonning *et al.*, 1992b), and a hybrid promoter derived from the polyhedrin gene (Eldridge *et al.*, 1992). Although high level expression was obtained from these constructs both *in vitro* (Hammock *et al.*, 1990) and *in vivo* (*Figure 3*; Bonning *et al.*, 1992a; Eldridge *et al.*, 1992), insecticidal efficacy was poor in terms of reduction in feeding,

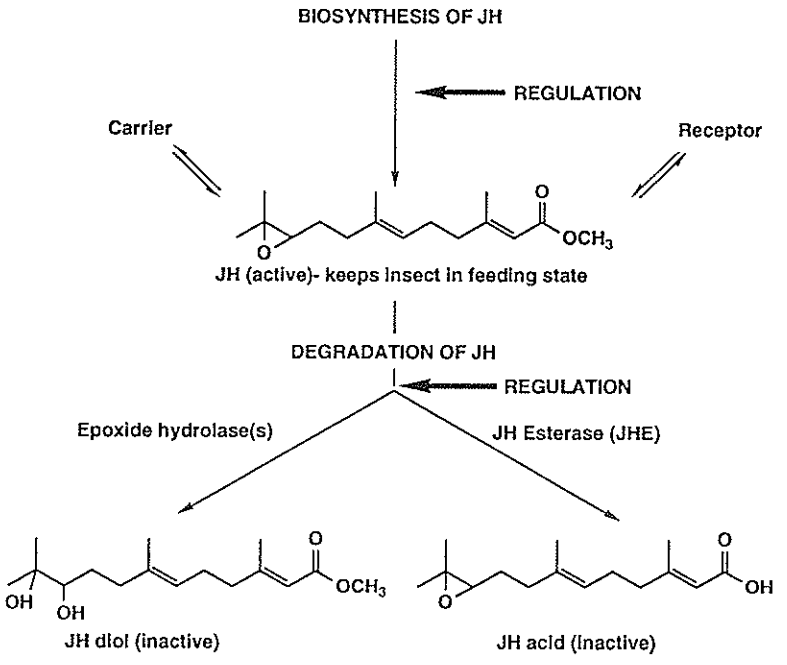

Figure 2. Regulation of the titre of juvenile hormone (JH). JH is produced by the *corpora allata* and exported to the haemolymph. It is degraded by epoxide hydrolases and JHE which hydrolyse JH into the biologically inactive JH acid and JH diol metabolites. JH titres *in vivo* are regulated by both biosynthesis and degradation. These sites of regulation are logical targets for insect control agents.

lethal viral dose, and lethal time of the recombinant viruses. JHE has also been expressed in BmNPV, resulting in very high level expression in larvae of *B. mori* (T.N. Hanzlik, unpublished research). Eldridge *et al.* (1992) also looked at baculovirus expression of JHE in viruses with and without egt, on the premise that glycosylation of the insect moulting hormones could mask the effects initiated by JHE. Fourth and fifth instar larvae of *Trichoplusia ni* were examined, but no differences were found on infection with egt-positive, or egt-negative viruses expressing JHE.

The lepidopteran JHEs examined are very stable *in vitro*. However, pharmacokinetic analysis of the disappearance of JHE injected into *Manduca sexta* larvae showed that the half-life of this protein in insect haemolymph is low; half of the JHE injected had disappeared after about 1 h (Ichinose *et al.*, 1992a). Further analysis showed that JHE is rapidly taken up by the pericardial cells of the insect (Booth, Bonning and Hammock, 1992; Ichinose *et al.*, 1992b), possibly by receptor-mediated endocytosis, where presumably it is degraded.

In order to stabilize the enzyme against proteolytic attack, sequences believed to be involved in the targeting of proteolytic agents (Rogers, Wells and Rechsteiner, 1986; Rechsteiner, 1987; Bachmair, Finlay and Varshavsky, 1986; Bachmair and Varshavsky, 1989) were altered by site-directed

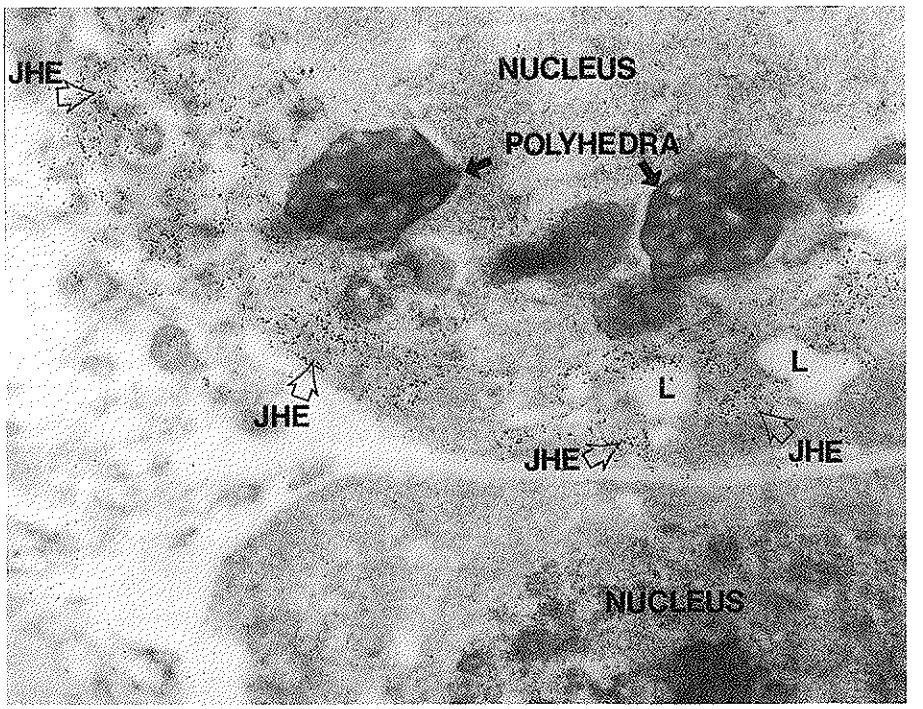

Figure 3. Electron micrograph of section taken from fourth instar larva of *Trichoplusia ni* infected with a recombinant baculovirus expressing JHE (Bonning *et al.*, 1992a). This micrograph shows an infected cell (above) and an uninfected cell (below) in the fat body. The cytoplasm surrounding the nucleus of the infected cell is immunogold labelled for JHE. Two polyhedra are present in the nucleus of the infected cell. L, lipid body. Electron micrograph courtesy of T. Booth.

mutagenesis to produce modified forms of JHE. On expression of these modified JHEs in AcNPV, two highly insecticidal viruses were generated. *Figure 4* compares the bioactivity of the wild-type non-engineered AcNPV with a recombinant virus expressing a scorpion toxin (AcAaIT) (McCutchen *et al.*, 1991) and two recombinant viruses expressing modified JHE (AcJHE1 and AcJHE2) (B.C. Bonning and V.K. Ward, in preparation). These viruses, along with the viruses expressing the mite toxins, are the most effective baculovirus insecticides generated to date by genetic engineering.

JHE AS A REPORTER ENZYME

As shown for baculovirus expression of the mite toxin (Tomalski and Miller, 1992), fundamental research into the characteristics of different viral promoters is essential for enhancement of recombinant baculovirus insecticides. A variety of reporter enzymes have been used for such studies, including β-galactosidase, CAT (chloramphenicol acetyltransferase), and luciferase

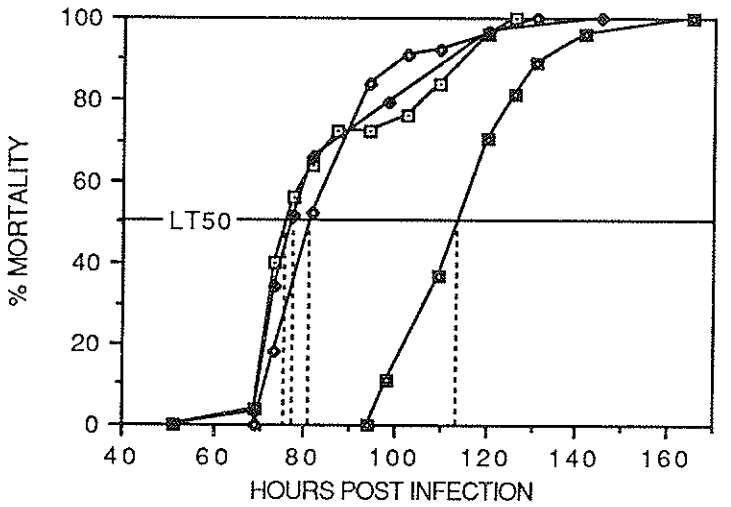

Figure 4. Lethal times (LT) for second instar larvae of *Heliothis virescens* infected with wild-type AcNPV (–■–), a recombinant virus expressing a scorpion toxin (–◆–; McCutchen *et al.*, 1991) and two recombinant viruses expressing modified JHE (–⊡– and –◆–; B.C. Bonning and V.K. Ward, in preparation). These are among the best recombinant baculovirus insecticides developed to date.

(Karp *et al.*, 1992). JHE also proves to be a useful reporter enzyme, with radiochemical (Hammock and Roe, 1985) and colorimetric assays (McCutchen *et al.*, 1992) available. Comparison of JHE expression under control of these promoters highlights differences in protein yield and timing of onset of expression (Bonning *et al.*, 1992, 1993; Roelvink *et al.*, 1992). JHE is a more sensitive reporter enzyme than lacZ, but less sensitive than luciferase (Karp *et al.*, 1992) or CAT, unless the assay technique is modified for enhanced sensitivity (Ward *et al.*, 1992). The fact that JHE is exported from infected cells facilitates the assay of enzyme activity.

Bioassay of genetically engineered baculoviruses

Each new recombinant baculovirus with potential for insecticide use is assessed by bioassay to determine two main parameters: the time taken to kill 50% of the larvae at a given dose (LT_{50}), and the dose of virus taken to kill 50% of the larvae (LD_{50}) (Hughes and Wood, 1986). The time taken to kill is particularly important, being related, but not directly proportional to, the amount of feeding damage sustained. Lethal doses can also potentially be reduced by genetic manipulation.

A variety of techniques have been used in different laboratories for bioassay of recombinant baculoviruses: different insect pest species are tested at different stages and at different temperatures, with different doses of virus; viral doses are administered by a variety of methods including droplet feeding (Hughes, Van Beek and Wood, 1986), by incorporation of the virus into the

diet itself (Eldridge *et al.*, 1992) or injection into the haemocoel (Maeda, 1989b). Incorporation of different doses of virus into the diet and removal of larvae after a specific period of feeding is a less labour-intensive technique than giving specific doses of virus to larvae on small plugs of diet. However, with virus incorporated into the diet, the actual dose of virus ingested is unknown, and variation in feeding between larvae over a 24 h period has not been determined.

As larval susceptibility to viral infection decreases sharply with age, and different insect species vary considerably in susceptibility to the same virus (Briese, 1986), comparison of absolute data (LT_{50} values for example) becomes invalid without reference to data for the wild-type control virus assayed under the same conditions.

LETHAL RATIOS FOR DOSE AND TIME

We propose that bioassay data be presented in the form of lethal ratios for both dose (LRD) and time (LRT), so that data for recombinant viruses are expressed as a function of data acquired for the wild-type control virus bioassayed at the same time under the same conditions. The disparity in bioassay procedures used in the past often makes direct comparison of the LD and LT data hard to interpret.

$$\text{lethal ratio for dose (LRD)} = \frac{LD_{50} \text{ for test virus}}{LD_{50} \text{ for wild-type control}}$$

$$\text{lethal ratio for time (LRT)} = \frac{LT_{50} \text{ for test virus}}{LT_{50} \text{ for wild-type control}}$$

Comparison of absolute data with different assay conditions or from different laboratories can be very misleading. For example, an LT_{50} of 60 h for recombinant virus A and of 80 h for recombinant virus B, suggests that virus A is the most potent. If the LT_{50} values for the wild-type viruses used were 100 and 150 h, respectively, the LRTs are 0·6 for virus A and 0·53 for virus B, indicating that the modification in virus B leads to the greater relative increase in speed to kill, rather than virus A as might be assumed at first glance (Bonning and Hammock, 1992). These ratios, the lethal ratio for time (LRT) or dose (LRD), would then allow direct comparison between data sets from different groups, to assess the relative efficacies of the newly engineered baculoviruses. These ratios are comparable to the resistance ratio used in studies of insecticide resistance.

In addition to the non-engineered wild-type viruses, a recombinant control virus is also often used in bioassays. Data for recombinant controls may indicate any alteration in insecticidal efficacy of the virus caused by the engineering process itself. Genetic manipulation of viral genomes may have deleterious effects on viral fitness, in the same way that genetic change conferring insecticide resistance can affect the fitness of targeted species such

as *Spodoptera exigua* (Brewer and Trimble, 1991), mosquitoes (Curtis, Cook and Wood, 1978) and houseflies (Roush and Plapp, 1982).

Presentation of bioassay data as LRD and LRT at different percentage levels (10, 50 or 90), in addition to actual LD and LT values, will greatly clarify the actual efficacy of new recombinant viruses generated for insect pest control means. There are numerous reasons for carrying out a bioassay. At one extreme, one is interested in mimicking field performance and at another extreme one is asking fundamental questions about the action of the virus or toxin. Different laboratories have devised a series of biological assays, each of which has its advantages in terms of ease of use, accuracy, and other factors. An additional value of bioassays is to compare viral constructs between laboratories. For this application it is important to standardize the technologies used. Suggested bioassay procedures for recombinant baculovirus insecticides are given below.

DETERMINATION OF LETHAL TIME

Infect neonate larvae of *Trichoplusia ni* by droplet feeding (Hughes, Van Beek and Wood, 1986) at 200 polyhedrin inclusion bodies (pibs) μl^{-1}, at least 50 larvae per virus. This technique ensures that all larvae are infected within a 10–15 min period. Use of blue food colouring dye in the virus preparation for the droplet feeding assay provides a means for identifying and transferring only infected larvae to individual diet tubs for subsequent monitoring. Mortality should be scored every 6 or 8 h according to mortality rate. LT_{10}, LT_{50} or LT_{90} values can be determined using the Vistat program (Boyce Thompson Institute, Ithaca, New York, 1990).

DETERMINATION OF LETHAL DOSE

For determination of lethal dose, infect second instar larvae (within a specified weight range according to insect species) by inoculating small diet plugs with appropriate doses of virus (at least five doses per bioassay). Infect 15 larvae per viral dose. After 24 h, only larvae that have completely consumed the diet plug and hence the entire viral dose, are transferred to individual tubs. Mortality of larvae is scored after 8–10 days and LD_{50} values determined using probit analysis (Finney, 1971). For *Heliothis virescens* and other cannibalistic species, first instar larvae can be isolated and maintained with diet in a humid environment until second instar in 96-well microtitre plates.

Bioassays should be repeated two or three times. From a statistical standpoint, multiple replicates of fewer larvae per dose are preferable to one replicate of large numbers of larvae. Most of the same conditions used for bioassay of classical insecticides apply to viruses (Robertson and Preisler, 1992). Replication of bioassays under the same conditions but at different times may produce variable data, but the LRD and LRT values will be similar (Bonning and Hammock, 1992).

A further consideration is that the requirements for the bioassay procedure

may vary according to the foreign gene expressed by the recombinant virus (particularly for insect hormones and enzymes), as well as the host virus and target species. Certain stages in the life-cycle of the insect may be more susceptible to disruption by the expressed agent than others. Hence, bioassays may be appropriate using later instars for the LRT, or neonates for the LRD. For field use, a recombinant virus effective against early instars is preferred to minimize feeding damage caused by the developing larvae.

Development of highly reproducible bioassays and bioassays indicative of field performance are not easy with any material. Comparisons become even more complex with biologicals where a separate, physical analytical method is not available. In addition to the variable of the foreign protein being expressed by the virus, there may also be variation between recombinant viruses in terms of infectivity, size and composition of the polyhedra, and so on. However, the techniques described above ensure rapid, uniform infection with a high dose for determination of the LT_{50} (using the droplet feeding assay of Hughes, Van Beek and Wood, 1986), and accurate infection with specific doses for the LD_{50}.

FEEDING DAMAGE

Despite the emphasis placed on LD_{50} and LT_{50} data for newly engineered viruses, it should be remembered that feeding damage is actually the most important parameter to be considered. The behaviour of an infected larva prior to death is extremely important: an agent such as JHE, when engineered into a baculovirus, may reduce or stop feeding for an extensive period prior to death of the insect (Hammock *et al.*, 1990). In such cases, the LT_{50} will give an underestimate of efficacy of the virus. Feeding damage is not necessarily proportional to LT_{50}; this depends on the foreign protein being expressed by the recombinant baculovirus, which may have antifeedant activity. Assays to quantify feeding damage caused by recombinant virus-infected larvae relative to wild-type virus-infected larvae, are labour intensive, but are recommended for new recombinants with potential for field use.

Risk assessment for genetically engineered baculovirus insecticides

Risk assessment for recombinant baculoviruses needs to be based on a thorough knowledge of basic viral ecology, in particular with respect to host range, persistence, dispersal and potential recombination with other viruses (Tiedje *et al.*, 1989; Cory and Entwistle, 1990). Before field trials can be considered for a recombinant baculovirus, several safety aspects must be rigorously checked in the laboratory to ensure that the virus is stable. First, the precise genetic change in the viral genome must be confirmed by endonuclease restriction or sequencing. The genetic stability of the recombinant baculovirus must then be assessed by repeated passaging through insects in the laboratory, the host-range must be checked to confirm that it has not been altered by the engineering process (Doyle *et al.*, 1990), and the physical stability of the virus must be determined. It is also feasible and advisable to

screen for genetic exchange between engineered viruses and other viruses, or acquisition of host cell DNA sequences by the genetically engineered viruses within the confines of the laboratory.

For field release, it may be advantageous to use recombinants at a selective disadvantage relative to the wild-type virus (with a p10 gene deletion for example; Vlak *et al.*, 1988) so that recombinant viruses are rapidly competed out. These recombinant baculoviruses are being developed as pesticides for repeated application, not as biological control agents which persist in the environment (Hammock, 1992). In addition, the quick-kill recombinant baculoviruses are generally unable to multiply to the same extent as wild-type viruses, which keep the host insect alive for a longer period of time, thus permitting further rounds of viral replication. Larvae infected with wild-type virus dissolve after death, releasing occluded virus into the environment. Recombinant virus-infected larvae die more quickly but often do not dissolve, possibly because the virus does not reach the later stages of infection which overwhelm the host insect. Hence less recombinant virus is produced, and it is also dispersed less efficiently. Deletion of a chitinase gene from the AcNPV genome results in a virus which does not dissolve its larval host at all (R. Hawtin, in preparation). Routine deletion of this gene from recombinant baculoviruses may impair distribution of recombinant viruses in the field.

The phenomenon of impaired dispersal of virus, and overall selective disadvantage relative to the wild-type virus, can be demonstrated in the laboratory by infecting a group of larvae with both wild-type and recombinant virus. On addition of additional larvae (representing successive generations in the field), the wild-type virus (at a selective advantage over the recombinant) will out-compete the recombinant virus after a number of 'generations'. This competing out effect has also been demonstrated *in vitro* using wild-type and genetically manipulated viruses (Huang *et al.*, 1991).

REGULATIONS FOR THE RELEASE OF GENETICALLY MODIFIED ORGANISMS

The regulations for the release of genetically modified organisms are largely undefined, most regulatory authorities being uncertain at the present time of a suitable approach. Generally recombinant organisms are considered favourably in the USA. However, if regulations are brought into effect requiring rigorous and costly testing, the recombinant insecticidal baculovirus technology may be killed. It is essential that the recombinant baculoviruses on the market are able to compete economically with the synthetic chemical insecticides available. Over-extensive testing at the regulatory level may diminish the chances of recombinant baculoviruses becoming a viable alternative means of pest control.

By no means should society fall into the trap of assuming that because a virus (recombinant or wild-type) is 'natural' that *a priori* it is safe. Regulatory requirements must take account of the biology of the virus, the host and the ecosystem involved. The viruses will be less stable and more host-specific than classical insecticides. These factors will result in less environmental

contamination, avoid pest resurgence and delay resistance. Hopefully, this will increase agricultural profitability in both developed and developing countries. Regulatory requirements on classical insecticides have stifled the development of selective compounds ideal for integrated pest management. Hopefully, this error will not occur with biologicals.

The US Environmental Protection Agency (EPA) has encouraged development of pest control means which can be used as alternatives to synthetic chemical insecticides. Increased funding for biotechnology has also been proposed. The EPA, which acts as pesticide quality control manager for the public, promotes the development, registration, marketing and use of safer pesticides. The goal of the EPA is for a greater reliance on integrated pest management (IPM). However, because such 'non-conventional' agents as recombinant baculoviruses differ significantly in their action from the more commonly used insecticides, there are few regulatory precedents for categorization of these new pesticides in terms of associated risks. Registration of more biopesticides in recent years has helped the regulatory process somewhat (Betz, 1986).

Insecticidal products developed by genetic manipulation are subject to regulations for both approval of the final product, and for field release. Most agents are subject to both federal (EPA) and state controls in the USA. This being the case, close association with the regulatory authorities throughout development of such recombinant agents will ensure that research is focused, and the database necessary for field testing and approval is acquired.

PUBLIC ACCEPTANCE OF RECOMBINANT BACULOVIRUS INSECTICIDES

In order to develop a favourable public and regulatory attitude towards recombinant DNA systems, it may be wise to pave the way with approaches that lend themselves to refining risk assessment and which are perceived to be the most innocuous. Hence, release of a recombinant expressing a foreign protein such as juvenile hormone esterase, which is already present in the insect and harmless to man and natural enemies of the target species, ought to be more favourable from a public acceptability standpoint than does release of an agent expressing a toxin of any kind. A carefully planned stepwise programme of introduction of recombinant viruses should be used to test real risks to the ecosystem and allay perceived fears regarding the use of biologicals. Care must be taken to inform the public fully of research on recombinant baculoviruses before their use in the field as insecticidal agents.

FIELD TRIALS OF RECOMBINANT BACULOVIRUS INSECTICIDES

The first field trials on genetically modified viruses were carried out in England in 1986 by the Natural Environment Research Council's Institute of Virology in Oxford (Bishop *et al.*, 1988). These trials, carried out in 1986, 1987 and 1988, were completely contained, and they examined the persistence of various genetically engineered viral constructs under field conditions. Larvae of *Spodoptera exigua* were infected in the laboratory before introduc-

tion at the field test site. The first release was of a genetically marked AcNPV; this virus contained a non-coding piece of DNA. In 1987, the trials involved a crippled virus which did not produce the polyhedrin protective coat, and was also genetically tagged. The third trial involved release of a virus encoding a non-functional gene. Careful attention was paid to publicity and informing the public of events through the media (Bishop, 1986, 1989; Bishop *et al.*, 1988; Cory, 1991).

In 1989 the first uncontained field trials of recombinant baculoviruses were carried out by the Boyce Thompson Institute for Plant Research at Cornell University in the USA. These trials involved release of polyhedrin-negative AcNPV which was co-occluded with wild-type virus (Wood *et al.*, 1990). Laboratory studies showed that the non-occluded virus could not persist due to the unlikely event of co-infection of larvae or larval cells. These trials involved release of larvae of *Trichoplusia ni* into a two-acre cabbage plot, which was then sprayed with polyhedra. These polyhedra contained equal numbers of polyhedrin-positive and -negative virus particles. Monitoring of the site in subsequent years established that loss of the polyhedrin negative virus was relatively rapid.

Potential of genetically engineered viral insecticides

Recombinant DNA technology has provided a significant impetus for the growth of biological control research (Kirshbaum, 1985). Genetically engineered viral insecticides have considerable potential. Through genetic manipulation, the means exist to enhance the speed of kill of baculoviruses, lower the lethal dose and increase the spectrum of target species.

Emphasis for future research should be given to isolating new agents for expression in recombinant baculovirus insecticides. Characterization of factors determining the host-range of the baculoviruses may lead to greater potential for manipulation of the host-range. The majority, if not all, of the factors considered to be disadvantages, such as *in vitro* production, formulation, application, etc., are surmountable; indeed, they are being resolved even now. It will be a very short time before recombinant baculovirus insecticides become a very attractive commercial alternative for insect control.

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