MOLECULAR CLONING AND PHYSICAL MAPPING OF BERTHA ARMYWORM, *Mamestra configurata*, NUCLEAR POLYHEDROSIS VIRUS GENOME AND PRELIMINARY STUDY OF GEOGRAPHIC ISOLATES

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SUMMARY OF DISSERTATION

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ABSTRACT

Bertha armyworm, *Mamestra configurata* (Lepidoptera: Noctuidae), is an important pest of cruciferous oilseed crops in western Canada. A nuclear polyhedrosis virus, MacoNPV, isolated from *M. configurata*, has demonstrated as high as 95% infection in field populations of bertha armyworm. MacoNPV isolates from different geographic areas differ in terms of their virulence to bertha armyworm. Restriction endonuclease (REN) fragment analyses show that all of the geographic isolates are closely related viruses but with some distinct REN pattern differences. Most of these geographic isolates are heterogenous mixtures of genotypes. The thesis describes the cloning and physical mapping of the 156.9 kbp genome of the MacoNPV-90/2 geographic isolate, including 112 restriction sites for six common REN, *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sma*I and *Xho*I. Twenty plaque purified strains of MacoNPV were isolated in a cultured *Mamestra brassicae* (Mbr) insect cell line. The *Eco*RI restriction patterns of these pick plaque (pp) strains fell into 10 different categories. In order to investigate the differences among these pp strains, and between these strains and the parental geographic isolates in terms of REN patterns, virulence to insect hosts, and their growth rates in insect cell lines, some of these isolates were selected for bioassays in bertha armyworm larvae and in the Mbr cell line.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. Introduction

Insects, together with diseases and weeds, are the three major groups of crop pests. Damage caused by insect pests is tremendous. In the United States alone, losses in potential production of crop and other cultivated plants caused by insect pests have a value of about $30 billion annually, despite the application of more than 100,000 tonnes of insecticides (Gillott, 1995). Chemical pesticides have played, and continue to play, important roles in the control of these pests. However, the massive use of chemical insecticides has caused some major problems. The first is the development of resistance of insects and mites to one or more of the chemical insecticides, which has made the search for suitable synthetic insecticides increasingly difficult. The second is the non-specificity of action of these chemicals, which destroy beneficial as well as pest species. Disruption of populations of beneficial species causes the pests to rebound with great force, so that additional insecticidal treatments are required, because the pest species typically can recover from insecticide application more rapidly than their natural enemies. The third is the potential hazard, both direct and indirect, of many of the synthetic insecticides to humans, livestock, and wildlife (Gillott, 1995). These problems have greatly restricted the broad use of chemical control. Biological control, which was first used long before the use of chemical pesticides and has obvious advantages over chemical pesticides

due to its high specificity and limited environmental problems, was thus postulated to be an alternative to chemical control or an adjunct in the development of an integrated pest management strategy.

Biological control includes the regulation of pest populations by the introduction, establishment, and multiplication and spread of natural enemies (parasitic and predatory insects or other beneficial arthropods, and pathogens) (reviewed by Rosen, 1985; Entwistle and Evan, 1985). Depending on the nature of the control agent and the pest, the concept of biological control includes four strategies: (1) natural control in which the agent is an endemic species and the pest is either endemic or introduced; (2) augmentative control in which populations of an endemic control agent are increased, either by cultural means in the field or by mass-rearing and release; (3) classical biological control where an exotic control agent is imported to control an introduced, coevolved pest; and (4) neoclassical biological control in which the agent is an exotic species brought in to control a native pest (Gillott, 1995).

Microbial control, the use of microorganisms (viruses, bacteria, protozoa, fungi, and nematodes) as control agents, is an important part of biological control. The strategies for using microorganisms to regulate pest populations include: (1) introduction, which means to introduce pathogens into the pest's ecosystem. This approach applies to the situation where there is no pathogen (e.g., for some exotic pests) in the pest's

ecosystem. The pathogens may exert permanent regulation on the pest populations or there may be no recycling of pathogen when introduced into the pest's ecosystem, depending on the relationship between the pathogen and the host insects; (2) augmentation, to place additional amounts of a naturally occurring pathogen into the ecosystem to increase disease prevalence. This approach is useful when natural epizootics are asynchronous with pest outbreaks or when the incidence of disease is too low to be economically valuable; and (3) conservation, to adjust the ecosystem so to provide conditions for the maximum development of insect pathogens. For example, irrigation may increase the humidity and may trigger epizootics of fungal disease in pest populations.

Bertha armyworm, *Mamestra configurata* Walker (Lepidoptera: Noctuidae), is an important oilseed crop pest in western Canada. Several potential microbial control agents, including various commercial *Bacillus thuringiensis* (Bt) products and formulations of Bt delta-endotoxin, have been tested for the control of bertha armyworm. Of these agents, a naturally occurring insect virus, which was originally isolated from bertha armyworm, has been shown to have great potential for microbial control of bertha armyworm. The present work is on the preliminary characterization of this virus. This work will aid in the development of this virus as a potential control agent for bertha armyworm.

2. Literature Review

2.1. Bertha Armyworm

2.1.1. Occurrence, Host Range and Damage to Crops

Bertha armyworm is a widely distributed polyphagous species. This insect has been reported from California, New Mexico, Arizona, Colorado, to British Columbia and the Prairie provinces of Canada. This species was cited in economic entomological literature as early as 1928 (King, 1928). Flax, sweet clover, and alfalfa were the major plants attacked by bertha armyworm before 1960, but now the widely grown oilseed, canola (*Brassica napus* and *B. campestris*), is its major host species (Jones and Heming, 1979; Turnock, 1988). Diet quality is an important factor affecting the growth, survival, and fecundity of bertha armyworm (Bailey, 1976; Turnock, 1985). Some components of the host species, such as the glucosinolate profile and sinalbin (p-hydroxybenzyl glucosino-late) in mustard seedlings, significantly affect the feeding and growth of bertha armyworm larvae (McCloskey and Isman, 1993; Bodnaryk, 1991).

In the Canadian prairies, bertha armyworm is univoltine. Moths start emerging in early June. The females deposit eggs in clusters on leaves of a host plant. Larvae feed on these leaves and then chew into the flower buds, seed pods, bolls or fruit. There are six larval instars. Larvae pupate in the soil between August and September, and winter is spent in the pupal instar (Jones and Heming, 1979; Turnock, 1988). Soil temperature

apparently affects the spatial distribution of bertha armyworm outbreaks. For example, bertha armyworm pupae did not survive a Manitoba winter in snow-free field plots, but 55% survived in plots with 5-10 cm snow cover. However, winter soil temperature did not by itself determine initiation or termination of bertha armyworm outbreaks on canola (Lamb et al., 1985).

The last two instars cause the most damage (Bracken & Bucher, 1977). Bracken (1987) showed that the losses caused by bertha armyworm are two-fold, comprising yield loss and reduction of seed quality. Damaged pods had greater proportions of green and broken seeds, which caused lower seed grade, and these defects were evident in seed from plots with 21% or more damaged pods. The author therefore suggested that a control decision be made before 20% of the pods are damaged.

2.1.2. Control

It is suggested that control measures be put in place when the density of bertha armyworm reaches 20 larvae/m^2 of ground surface (the currently recommended economic threshold) (Bracken and Bucher, 1984; Jones and Johnson, 1994). In outbreak years, chemical insecticides, including p,p'-dichlorodiphenyl-trichloroethane (DDT), malathion, methomyl, chlordimeform, and permethrin, have been used for bertha armyworm control (Lee et al., 1972; Bodnaryk, 1976, 1977, 1982; Wylie and Bucher, 1977). However, because of the well known side-effects of chemical pesticides, efforts have been made to use natural enemies to control this pest. These include the use of native parasitoids

(Arthur and Mason, 1985; Arthur and Powell, 1989, 1990), the investigation of a foreign parasitoid (Arthur and Mason, 1986), and the use of microbial pathogens (Morris, 1986; Bucher and Turnock, 1983; Turnock, 1988).

Naturally occurring insect parasites [e.g. *Banchus flavescens* Cress., and *Athrycia cinerea* (Coq.)] and pathogens, including entomophthoran fungi, microsporidia and baculoviruses, have been isolated from bertha armyworm populations across western Canada (Wylie and Bucher, 1977; Bucher and Turnock, 1983; Erlandson, 1990). Pathogenic microorganisms play important roles in maintaining bertha armyworm populations at relatively low levels. In areas or in years when insect populations are high, diseases are major mortality factors (Wylie and Bucher, 1977). Pathogenic microorganisms are therefore considered attractive agents for biological control of insect pests.

Various commercial Bt (Dipel 132® and Thuricide 48 VL®) products and formulations of Bt delta-endotoxin have been bioassayed against 3rd- to 6th-instar larvae of the bertha armyworm. However, Bt is not particularly effective for bertha armyworm, compared to other insect species against which Bt is currently used (Morris, 1986; Morris, 1988; Trottier et al., 1988).

In natural populations of bertha armyworm, the infection with a baculovirus can occur at epidemic levels, implying the importance for the further study of this virus as a biological control agent.

2.2. Baculoviruses

Baculoviruses (Baculoviridae) are a diverse group of insect viruses. They are generally considered safe for use as biological control agents, because their host range is restricted to invertebrates and in most cases to insect hosts. Moreover, they have no structural similarity to any of the mammalian or plant virus groups (Blissard and Rohrmann, 1990). Baculoviruses have good potential as biological insecticides (reviewed by Entwistle and Evans, 1985; Podgwaite, 1985; Huber, 1986). Historically, studies emphasized the potential of using baculoviruses in biological control from the perspective of their pathogenic effects. However, in recent years, many studies have been conducted on the possibility of using baculoviruses as expression vectors for the production of heterologous proteins (King and Possee, 1992; Shuler et al., 1995). In this section the following topics will be introduced: the structure, classification, host range and life cycle of baculoviruses, the use of baculoviruses in biological control, the methods used to characterize baculovirus genomic structure, and a review of genetic engineering approaches to increase the efficiency of baculoviruses in biological control.

2.2.1. Structure and Classification

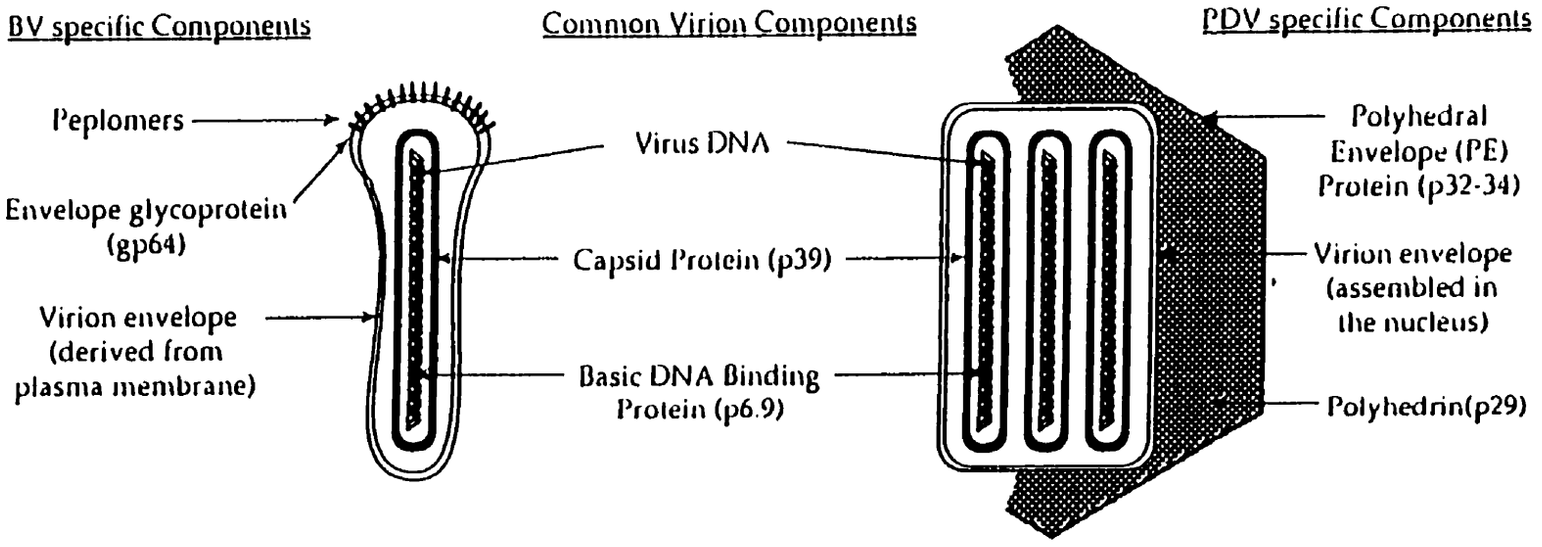
Baculoviruses have large (80-200 kbp), covalently closed circular dsDNA genomes (King and Possee, 1992; Arif, 1986; Rohrmann, 1992). Typically, the virus particles (called virions)

are occluded within a crystalline protein matrix, forming the typical occlusion body (OB). Figure 2.1 shows the overall structure of a baculovirus. The viral genomic DNA is associated with a highly basic (arginine-rich) protein of 6.5-7.9 kDa (Rohrmann, 1992; King and Possee, 1992) called basic protein or DNA-binding protein, which compresses the viral DNA together. The DNA-protein complex is packaged by a 39 kDa capsid protein, forming the characteristic large, rod-shaped (baculo = rod) nucleocapsid (NC). One or more NC are enveloped within a typical bilayer membrane, forming the virion. While the length of the NC ranges from 200-400nm, depending on the size of the genome, the width is typically about 36nm (King and Possee, 1992; Fraser, 1986). The virions are packaged within OB consisting largely of a single crystallized protein of 30 kDa.

Historically baculoviridae were divided into three subgroups of viral types (genera) based on morphological characteristics (Francki et al., 1991). These genera are placed into two subfamilies based on the presence (Eubaculovirinae) or absence (Nudibaculovirinae) of OB. Figure 2.2 shows the typical structure of the three genera: 1) *nuclear polyhedrosis virus* (NPV) with the characteristic feature that several virions are occluded in an OB. This will be discussed in detail below; 2) *granulosis virus* (GV) (type species: *Plodia interpunctella* [Pi] GV), with virions containing single NC packaged one per OB; and 3) *non-occluded baculovirus* (NOB) (type species: *Heliothis zea* [Hz] NOB). This group consists of virions which are not packaged into OB at any stage of their life cycle.

Figure 2.1. Baculovirus structural components. The two baculovirus virion phenotypes are shown diagramatically with shared and phenotype-specific components indicated. The extracellular virus (ECV) phenotype is represented by a virion with a single nucleocapsid, and the polyhedra derived virus (PDV) phenotype is represented by a diagram of an MNPV. Adapted from Blissard and Rohrmann (1990).

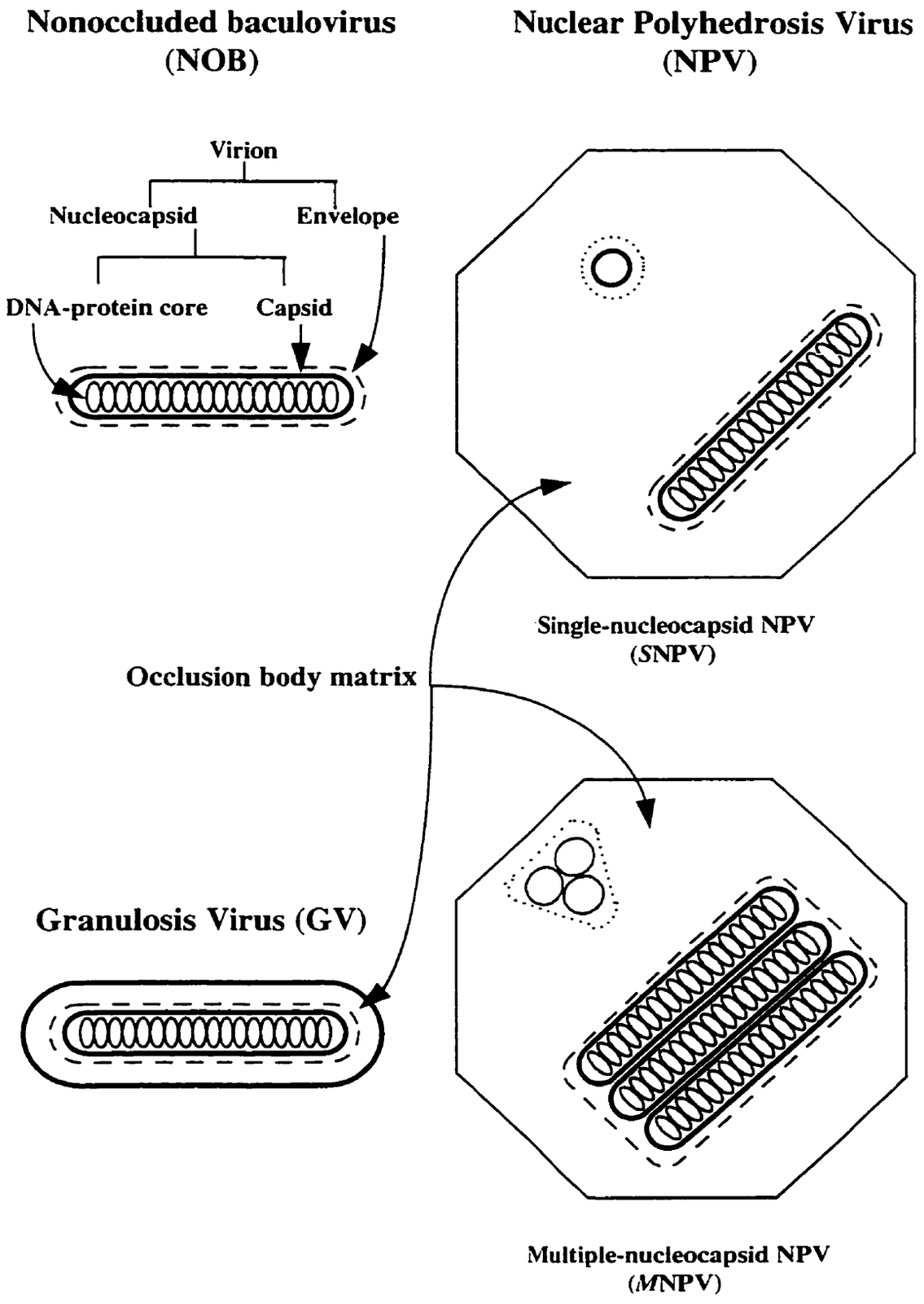
Baculovirus Phenotypes



Budded Virus (BV)

Polyhedra Derived Virus (PDV)

Figure 2.2. Schematic representation of the Baculoviridae. The SNPVs and MNPVs contain numerous virions per OB; MNPV virions may contain as many as 17 nucleocapsids per envelope. Not drawn to scale. Adapted from Bilimoria (1986).


Nonoccluded baculovirus
(NOB)
Nuclear Polyhedrosis Virus
(NPV)
Virion
Nucleocapsid
Envelope
DNA-protein core
Capsid
Single-nucleocapsid NPV
(SNPV)
Occlusion body matrix
Granulosis Virus (GV)
Multiple-nucleocapsid NPV
(MNPV)

In the case of NPV, the large OB are polyhedral and develop within the cell nucleus. It is for these characteristics that this group of viruses is named (Federici, 1986). The OB, called polyhedra, may be cuboidal, tetrahedral, dodecahedral, or irregular. Their size ranges from 0.5 to 15 µm (Federici, 1986), though most OB are between 0.6 and 2.5 µm in diameter (Summers, 1977; Blissard and Rohrmann, 1990; Adams and McClintock, 1991). Although in diseased larvae polyhedra vary in size, depending on their stage of development, in any one nucleus most polyhedra will be of approximately the same size and at about the same stage of maturation (Federici, 1986). NPV are subdivided into two morphological subgroups (subgenera) according to the extent of the aggregation of their NC: the single-nucleocapsid NPV (SNPV) (type species: *Bombyx mori* [Bm] SNPV) in which only a single NC is found per envelope; and the multi-nucleocapsid NPV (MNPV) (type species: *Autographa californica* [Ac] MNPV) in which several NC are packaged per envelope (Figure 2.2).

Although, as indicated previously, baculoviruses are traditionally named after the host insect from which they were first isolated and the subgroup to which they belong, it is apparent that this method does not reflect the genetic characteristics of the virus, particularly if a virus has more than one host or if more than one virus is isolated from the same host species. Based on a comparison of OB protein serological and sequence data from 10 occluded baculoviruses, Rohrmann (1986) arranged occluded baculoviruses into four subgroups which did not

always correspond to their morphological classification. By alignment of polyhedrin amino-acid sequences as well as DNA sequences of polyhedrin genes of NPV isolated from different lepidopteran species, and comparison of baculovirus phylogeny with Lepidoptera phylogeny, Zanotto et al. (1993) suggested that the common designation of baculoviruses based on their hosts is not adequate for baculovirus classification as it does not reflect the genetic relatedness of the viruses. Based on polyhedrin gene sequences, the authors placed lepidopteran NPV into two groups, each of which included some MNPV and some SNPV. They suggested that baculovirus taxonomy take into consideration a concept of species based on relevant viral characteristics rather than merely the first documented host and/or viral morphotype.

In the Sixth Report of the International Committee on Taxonomy of Viruses (Volkman et al., 1995), the classification and nomenclature of baculoviruses were changed. The family Baculoviridae now includes only two genera: *Nucleopolyhedrovirus* which is equivalent to the previous *Nuclear polyhedrosis virus*, and *Granulovirus* which is equivalent to the previous *Granulosis virus*. However, the so-called *non-occluded baculovirus* is not included. The regulation of nucleocapsid packaging are unknown and for some species packaging arrangements may be variable. Therefore, in the most recent revision of baculovirus nomenclature, the designation of S/M NPV was only retained for species where variability has not been reported (e.g., AcMNPV and

BmNPV) and for distinct viruses that would otherwise have identical designations under the current nomenclature (e.g., *Trichoplusia ni* [Tn] SNPV and MNPV) (Volkman et al., 1995). Moreover, most baculoviruses are now designated using first two letters each from the genus and species of host insect. For example, NPV isolate from bertha armyworm, *Mamestra configurata*, is designated MacoNPV. This paper will follow the designation by the International Committee on Taxonomy of Viruses (Volkman et al., 1995), no matter how the viruses have been designated in the references.

2.2.2. Host Range and Tissue Tropism

More than 520 NPV have been identified in insects from at least 8 orders, although the majority are from Lepidoptera (Beard *et al.*, 1989). Generally, NPV are quite species-specific in terms of infection, and cross-transmission is usually restricted to host genera within the same family. However, some NPV have wider host ranges. For example, AcMNPV can infect at least 32 species in 12 families (Granados and Williams, 1986). Though NPV which infect insects from different orders have also been reported (Al-Fazairy and Hassan, 1988; Bensimon, 1987), this needs to be confirmed.

Some NPV, such as those of sawflies, infect only the gut epithelium, although most are polyorganotrophic. OB have been observed in the hypodermis, hemocytes, fat body, tracheal matrix,

muscle cells, nerve ganglia, Malpighian tubules, testes, and pericardial cells of diseased insects (Evans and Entwistle, 1987; Granados and Williams, 1986).

In general, GV are more species-specific and are limited to Lepidoptera. GV also exhibit limited tissue tropism. For example, *Spodoptera frugiperda* (Spfr) GV replicates only in the fat body cells of *S. frugiperda* larvae, and *Trichoplusia ni* (Tn) GV infects only the midgut and fat body (Granados and Williams, 1986).

2.2.3. Infection Pathways and Life Cycle

Outside their hosts, baculoviruses exist in the form of OB. The most common route of infection is *per os* (Granados and Williams, 1986). Other routes of infection, such as transovum transmission (Etzel and Falcon, 1976) or introduction by parasites (Beegle and Oatman, 1975; Irabagon and Brooks, 1974; Levin et al., 1979), are possible, but are considered less important due to their relatively low frequency of occurrence (Granados and Williams, 1986; Faulkner, 1981). Occluded viruses become infectious on ingestion of OB by a host larva. In the infection process, they have a unique bi-phasic replication cycle. After OB ingestion, the virions are released from the OB in the high pH environment of the insect midgut. In NPV, virions associated with polyhedra are called *polyhedra derived virus* (PDV). In Lepidoptera, the highly alkaline digestive juice, pH

9.5-11.5, and enzymatic degradation by digestive proteases play an important role in the dissolution of OB. Because the foregut and hindgut are lined with cuticle, and thus serve as physical barriers to infection, the midgut epithelial cells are the primary sites for virus attachment and entry (Adams and McClintock, 1991; Granados, 1980; Granados and Williams, 1986; Mazzone, 1985).

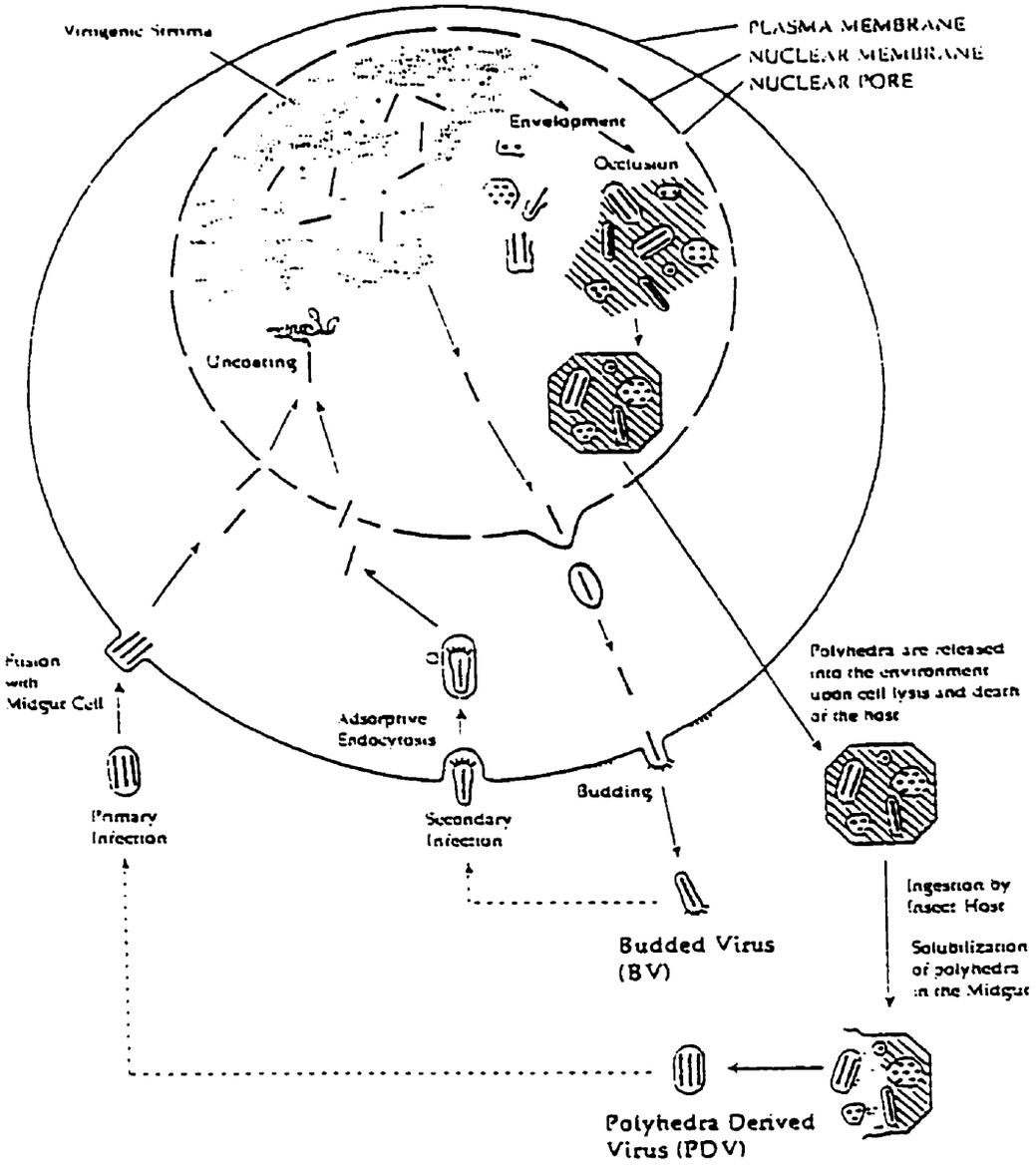
When the virions are released, the first barrier they must overcome is the peritrophic membrane, a thin acellular structure composed of chitin, proteins, various mucopolysaccharides and hyaluronic acid-like compounds (Lysenko, 1981; Richards and Richards, 1977). The peritrophic membrane acts as a mechanical barrier to protect the gut epithelial cells from mechanical injury and infection by pathogens (Orihel, 1975; Brandt *et al.*, 1978). It is now generally accepted that baculovirus infection causes physical/chemical changes in the structure of the peritrophic membrane, resulting in increased peritrophic membrane fragility (Derksen and Granados, 1988; Granados and Corsaro, 1990). After the PDV has negotiated the peritrophic membrane, its lipoprotein envelope directly fuses with the plasma membrane of the midgut epithelial cells. It has been suggested that in the lepidopteran midgut, columnar epithelial cells are the only cell type involved in the infection process (Granados, 1980; Granados and Williams, 1986). However, initial infection of *T. ni* midgut by AcMNPV occurred in both columnar epithelial and regenerative cells has also been reported (Keddie et al., 1989).

Although fusion can occur throughout the pH range of 4 to 11, the optimum condition for PDV fusion to microvilli is the alkaline pH normally found in lepidopteran larval midgut (Horton and Burand, 1993). After enveloped nucleocapsids (NC) fuse with the microvilli membrane, the naked NC enter the cell cytoplasm and uncoat at a nucleus pore. The viral genome is released into the nucleus, followed by gene expression and viral DNA replication. The nucleus enlarges and the progeny NC develop within and around an area with condensed material referred to as virogenic stroma. The hypertrophy of nuclei is the first and most characteristic evidence of viral infection of the midgut cells (Adams and McClintock, 1991; Granados and Williams, 1986). Some of progeny NC are released into the cytoplasm by means of vesicles originating from the nuclear envelope. These NC bud through the plasma membrane, and acquire a host-derived envelope with virus encoded proteins on the envelope. These viruses are called *extracellular virus* (ECV), also referred to as *budded virus* (BV) or *nonoccluded virus* (NOV). They pass through the connective tissue (basal lamina) between the midgut and hemocoel, into the hemocoel, and initiate secondary infection of other insect tissues, establishing systemic infections (Adams and McClinton, 1991; Evans and Entwistle, 1987; Granados and Williams, 1986). The replication cycle in secondary tissues is similar to that in midgut cells except that late in infection virus are occluded in the nucleus (Figure 2.3). The OB are made up primarily of virus-encoded products. Progeny NC destined to be

occluded are enveloped within membranes that are synthesized *de novo* within the infected nucleus and are later occluded within polyhedra (PDV). Unlike ECV, the PDV in the highly stable OB serves as the vehicle for the transmission of "packages" of infectious virus from one host to another by protecting the virions in various environmental conditions (Summers, 1977; Payne and Mertens, 1983; Adams and McClintock, 1991).

Previously hemocytes had been thought to be a major conduit for spreading virus throughout the host once the virus has passed through basal lamina. However, the insect tracheal system is now considered to be a major route in the systemic spread of baculoviruses in the host (Engelhard et al., 1994). In many NPV, occlusion of virions does not occur in midgut cells (Blissard and Rohrmann, 1990). However, Flipsen et al. (1993) reported that polyhedra are detected in both columnar and regenerative cells of the midgut epithelium. The polyhedra in the regenerative cells are of normal size and contain virus particles, and those in the columnar cells are relatively small and do not contain virus particles. Although OB are typically observed at 24 hr post inoculation in cell culture infection (or 2-3 days in infected insect tissues), they are released into the environment only upon the death of the host and the lysis of the host cells. They serve as inoculum for other insect hosts thus completing the life cycle of baculoviruses (Blissard and Rohrmann, 1990). Figure 2.3 shows the life cycle of baculoviruses.

Figure 2.3. Typical cellular infection cycle of the NPV. Polyhedra are ingested by a susceptible insect and solubilized by the high pH of the insect midgut. Virions of the polyhedra derived virus (PDV) phenotype are released and enter midgut epithelial cells by fusion with microvilli. Nucleocapsids are transported to the nucleus where uncoating of the viral DNA occurs, followed by gene expression and viral DNA replication. Progeny nucleocapsids are observed assembling within and around a dense virogenic stroma. Some progeny nucleocapsids are budded through the nuclear membrane and transported to the plasma membrane but apparently lose the nuclear-derived envelope in the hemocoel acquiring the budded virus (BV)-specific envelope that contains the virus-encoded envelope glycoprotein (gp64). These virions (of the BV phenotype) appear to be specialized for secondary infection of other host cells. A second group of progeny nucleocapsids become enveloped within the nucleus by a de novo assembled envelope. These virions are subsequently occluded within polyhedrin protein that crystallizes around them. Maturation of the polyhedra includes the addition of a polyhedral envelope around the periphery of the forming occlusion bodies. Upon insect death and cell lysis, the polyhedra are released into the environment. Adapted from Blissard and Rohrmann (1990).


Virogenic Stroma
PLASMA MEMBRANE
NUCLEAR MEMBRANE
NUCLEAR PORE
Envelopment
Occlusion
Uncoating
Fusion with Midgut Cell
Adsorptive Endocytosis
Budding
Polyhedra are released into the environment upon cell lysis and death of the host
Primary Infection
Secondary Infection
Budded Virus (BV)
Ingestion by Insect Host
Solubilization of polyhedra in the Midgut
Polyhedra Derived Virus (PDV)

Although the two phenotypes, PDV and ECV, are superficially similar in structure, it is generally accepted that they differ in virion morphology, protein composition, tissue specificity, and mode of viral entry into the host cells, as shown in Table 2.1.

Braunagel and Summers (1994) have done a detailed comparison between AcMNPV ECV and PDV, including viral envelope and NC structural proteins, lipid and fatty acid profiles. They reported different protein profiles in preparations of ECV and PDV. ECV is enriched for proteins at 67, 45, and 35 kDa, while proteins at 89, 70, 60, 50, and 25 kDa are enriched in PDV. Two proteins, PDV-E66 and PDV-E43, are identified as being specific for the PDV envelope. There are more N-glycosylated proteins in ECV than in PDV, with ECV-specific glycoproteins found at 137, 128, 89, 45, and 40 kDa. PDV glycoproteins are 70, 53, 49, 42, 40, and 31 kDa. Most differences occur in the envelope portion, though obvious differences in the composition of nucleocapsids have also been observed. For example, the 31 kDa glycoprotein is only present in the PDV nucleocapsid. Most phosphoproteins of both ECV and PDV are predominantly found in the viral envelopes. The predominant phosphoprotein of ECV is 85 kDa, whereas PDV major phosphoprotein is 36 kDa. The major phospholipid of ECV is phosphatidylserine (50%), while those of PDV are phosphatidylcholine and phosphatidylethanolamine (39 and 30%, respectively). Comparison of the phospholipid composition of PDV, produced in the nucleus of infected cells, with that of uninfected *S. frugiperda* (Sf9)

Table 2.1. Comparison between the components of extracellular virus (ECV) and polyhedra derived virus (PDV).

Feature	ECV	OB+PDV
Virion morphology:		
Nucleocapsid(s)/virion	one	one or more
viral envelope	loose-fitting	tightly-fitting
Source of virion envelope	derived from plasma membrane	assembled in the nucleus de novo
Protein composition:		
viral-encoded gp64	+	-
polyhedral envelope protein	-	+
polyhedrin	-	+
Tissue specificity in insect host:	cells and tissues within hemocoel	columnar epithelial cells in insect midgut
Infectiousness to cell culture:	highly infectious	less infectious
Mode of viral entry:	absorptive endocytosis	fusion of viral envelope with microvilli

nuclei demonstrated significant differences between these two membrane systems, supporting the theory of the *de novo* synthesis of PDV membranes in the nucleus (Braunagel and Summers 1994).

2.2.4. Polyhedrin and p10 Gene

Baculoviruses have large dsDNA genomes with the potential to encode more than 100 genes (Ayres et al., 1994). These genes are expressed in a regulated fashion and in an ordered cascade in infected insect cells. Based on the time course in which they are transcribed, baculovirus genes are divided into four temporally ordered tiers designated immediate early, delayed early, late, and very late (Blissard and Rohrmann, 1990; King and Possee, 1992). Polyhedrin and p10 genes are two of the most extensively studied baculovirus genes. Both genes have very strong promoters and are hyperexpressed very late in infection, with their gene products accounting for 50% of the total protein in infected cells (King and Possee, 1992; Shuler et al., 1995). These two genes are reviewed below:

2.2.4.1. Polyhedrin

OB protein is essential for the stabilization of viral particles in the environment and, therefore, for keeping the virus infectious. OB protein, referred to as polyhedrin in NPV, is a protein of about 245 amino acids with a molecular weight of approximately 30 kDa. It is the major component of the

baculovirus OB forming a protective crystalline matrix around the occluded virus. The overall structure resists solubilization except under strongly alkaline conditions such as occur in the host insect's midgut (Rohrmann, 1992). Because the polyhedrin genes are highly conserved in baculoviruses, Vlak and Smith (1982) proposed that the viral DNA restriction endonuclease (REN) fragment containing the polyhedrin gene be used as the zero point of the physical map.

Although it is not essential for viral replication in cell culture, the OB protein gene is the most conserved baculovirus gene. There is over 80% homology among lepidopteran baculovirus polyhedrins, about 50% homology between lepidopteran baculovirus polyhedrins and granulins and about 40% homology between lepidopteran baculovirus polyhedrins and hymenopteran baculovirus polyhedrins (Rohrmann, 1986). Because the polyhedrin genes are highly conserved in baculoviruses, Vlak and Smith (1982) proposed that the viral DNA REN fragment containing the polyhedrin gene be used as the zero point of the physical map. The 5' flanking sequences of polyhedrin genes from different baculoviruses share a highly conuserved 12-nt consensus sequence of A/TATAAGNANTT/AT surrounding the TAAG core, followed by an AT-rich sequence consisting of from 75 to 89% A+T (Rohrmann, 1986). The sequences between the TAAG core and ATG start codon are considered to contribute to the very late burst expression of polyhedrin, and are therefore called "burst" sequences (Morris and Miller, 1994).

The efficiency of polyhedrin nuclear localization is tightly

coupled to its rate of biosynthesis. An examination of the biosynthesis, localization, and assembly of polyhedrin at different times after infection by Jarvis et al. (1992) showed that nuclear localization of polyhedrin becomes more efficient as the occlusion phase of infection progresses. They also found that compared with wild-type virus, an AcMNPV few-polyhedra (FP) mutant, which produces smaller numbers of viral occlusions containing few or no virions, produced polyhedrin more slowly and localized it to the nucleus less efficiently at the beginning of the occlusion phase of infection (24 h pi). It has been revealed that the expression of a viral 25K protein gene, which is called FP locus protein gene and is inactivated in the FP mutant, is directly or indirectly associated with an enhancement of polyhedrin biosynthesis and nuclear localization at the beginning of the occlusion phase (Beames and Summers, 1988; Kool and Vlak, 1993). This enhancement effect was considered to be necessary to ensure the normal assembly of viral occlusions (Jarvis et al., 1992).

2.2.4.2. p10

The p10 protein forms extensive fibrillar structures (Wilk et al., 1987; Williams et al., 1989; Russell et al., 1991; Cheley et al., 1992) which are commonly found in the nucleus and cytoplasm of infected cells. p10 protein is also commonly found associated with polyhedra (Russell et al., 1987) and is thought to have an important role in the formation of the polyhedron envelope (Wilk

et al., 1987; Rohrmann, 1992). Polyhedra produced by p10 mutant viruses have irregular, pitted surfaces that differ from that of wild-type polyhedra and are less stable (Williams et al., 1989). Despite its obvious importance in the formation of polyhedron envelope and the stabilization of baculovirus OB, the p10 gene is much less conserved than the polyhedrin gene and many other open reading frames (ORF) that have been sequenced (Rohrmann, 1992; Zuidema et al., 1993).

The p10 protein may also play an important role in nuclear disintegration. Absence of the p10 protein prevents the lysis of host cells and the release of polyhedra from cells infected with p10(-) mutant (Williams et al., 1989). Experiments using a recombinant AcMNPV with the p10 coding sequence replaced by *Spodoptera exigua* (Se) MNPV p10 sequence indicated that p10 itself is not enough for cell lysis. Instead, interaction of p10 with at least one virus-specific factor is required (Oers et al., 1994). Like polyhedrin gene, p10 gene is dispensable for baculovirus replication in cultured insect cell lines.

2.2.5. Strategies for Physical Mapping of Baculovirus

Physical mapping is a preliminary step in the characterization of the structure of viral genome and description of the location of REN sites on the genome. It is essential for more detailed studies on the viral genome, such as gene mapping, transcription analysis, origin of replication analysis, and functional mapping.

Restriction endonucleases are basic tools for physical mapping. They are endo-deoxyribonucleases that digest double-stranded DNA after recognizing specific nucleotides sequences by cleaving two phosphodiester bonds, one within each strand of the duplex DNA. There are three types of REN classified according to their gene and protein structure, cofactor dependence, and specificity of binding and cleavage. Only type II REN are absolutely site-specific and hydrolyze specific phosphodiester bonds within or in close proximity to their recognition sequences. The recognition sequences typically are four (tetranucleotide target) or six (hexanucleotide target) nucleotides in length with a twofold axis of symmetry (palindromic recognition sequence). Type II REN consistently produce specific size fragments for a specific DNA sequence, and are by far the most useful REN in molecular biology and recombinant DNA techniques. Many type II REN (e.g. *Eco*RI) generate DNA fragments with protruding 5' cohesive ends; some others (e.g. *Pst*I) generate fragments with protruding 3' cohesive ends; whereas still others (e.g. *Sma*I) cleave DNA at the axis of symmetry to produce blunt-ended fragments (Maniatis et al., 1982). The recognition sequences and cleavage sites for the six REN which are used for DNA restriction pattern analysis and physical map construction in the present study are shown in Table 2.2.

The frequency of restriction sites for a specific REN depends on the length of its recognition sequence and the base composition (C+G%) of target DNA. Assuming that nucleotides are distributed

randomly along DNA, a tetranucleotide target will occur on average once every 4^4 (i.e. 256), and a hexanucleotide target will occur once in 4^6 (i.e. 4096) nucleotides (Maniatis et al., 1982).

Because type II REN cut target DNA at specific site and produce specific size fragments, the fragment pattern of an REN digest upon gel electrophoresis is specific for particular virus isolates and can be used to distinguish between virus species and strains.

Many strategies have been used to construct physical maps of viral genomes, including the following:

2.2.5.1. Double Digestion

The double digestion method utilizes reciprocal double digestion of isolated or cloned restriction fragments with a second REN in order to determine the number and location of sites for the second REN within the initial REN fragment. In this manner, data can be obtained on the location of REN sites and size of fragments generated in various regions of the genome. For example, the presence or absence of smaller *Hin*dIII REN fragments within a large *Eco*RI fragment can be determined by the double digestion of the isolated or cloned *Eco*RI fragment with *Eco*RI+*Hin*dIII. Double digest analysis can also be used to identify the overlapping region from two REN fragments generated by different REN (e.g., *Eco*RI and *Hin*dIII).

Table 2.2. Recognition sequences and cleavage sites for REN used in the present study.

Enzyme	Recognition sequences and cleavage sites	Cohesive ends	
*Bam*HI	5′ G↓G-A-T-C-C 3′ 3′ C-C-T-A-G↑G 5′	5′ G 3′ 3′ C-C-T-A-G 5′	5′ G-A-T-C-C 3′ 3′ G 5′
*Eco*RI	5′ G↓A-A-T-T-C 3′ 3′ C-T-T-A-A↑G 5′	5′ G 3′ 3′ C-T-T-A-A 5′	5′ A-A-T-T-C 3′ 3′ G 5′
*Hind*III	5′ A↓A-G-C-T-T 3′ 3′ T-T-C-G-A↑A 5′	5′ A 3′ 3′ T-T-C-G-A 5′	5′ A-G-C-T-T 3′ 3′ A 5′
*Pst*I	5′ C-T-G-C-A↓G 3′ 3′ G↑A-C-G-T-C 5′	5′ C-T-G-C-A 3′ 3′ G 5′	5′ G 3′ 3′ A-C-G-T-C 5′
*Sma*I	5′ C-C-C↓G-G-G 3′ 3′ G-G-G↑C-C-C 5′	5′ C-C-C 3′ 3′ G-G-G 5′	5′ G-G-G 3′ 3′ C-C-C 5′
*Xho*I	5′ C↓T-C-G-A-G 3′ 3′ G-A-G-C-T↑C 5′	5′ C 3′ 3′ G-A-G-C-T 5′	5′ T-C-G-A-G 3′ 3′ C 5′

↓ ↑ : cleavage site

2.2.5.2. Southern Blot Hybridization

This method is a more direct method for mapping REN fragments and is based on the fact that REN fragments located in the same region on the viral genome will have homologous DNA sequences which can be detected by hybridization analysis.

2.2.5.3. Cross-blot Hybridization

Cross-blot hybridization is a modified Southern blot hybridization technique which allows the determination of the sequence homology between many fragments simultaneously. Unlabelled "cold" REN-digested DNA is separated by electrophoresis on a preparative (single well) agarose gel and Southern blotted onto a nitrocellulose membrane. The DNA fragments are fixed on the membrane by baking at 80°C. A "hot" REN digest, *in vitro* end-labelled usually with ^{32}P dCTP with DNA polymerase I is also electrophoresed on a preparative agarose gel. The above membrane with immobilized DNA is placed on top of the radioactive gel at right angle to the original gel such that each cold band bound to the nitrocellulose membrane interacts with each "hot" band in the gel. The "hot" REN fragments are then transferred by Southern blot to the unlabelled DNA bands on the nitrocellulose sheet under hybridization conditions. After the membrane is washed, it is wrapped and exposed to X-ray film to detect radioactive signals from the hybridized "hot" bands (Loh et al., 1981; Possee and Kelly, 1988).

Based on the double digestion and hybridization results,

viral REN fragments can be pieced together, and a physical map of the viral genome can be constructed. Figure 2.4 shows a circular physical map of AcMNPV genome with the *Eco*RI restriction sites and the positions of some ORF.

2.2.5.4. The Conventional Way of Linearizing Physical Maps

NPV have circular, dsDNA genomes. A number of baculovirus genomic physical maps have been constructed, for example, AcMNPV (Miller and Dawes, 1979; Summers et al., 1980, Ayres et al., 1994), *Rachiplusia ou* (Ro) MNPV (Summers et al., 1980), *S. frugiperda* (Sf) MNPV (Loh et al., 1981), SeMNPV (Brown et al., 1984), *Mamestra brassicae* (Mb) MNPV (Wiegers and Vlak, 1984; Possee and Kelly, 1988), *Panolis flammea* (Pafl) NPV (Possee and Kelly, 1988; Weitzman et al., 1992), *Anticarsia gemmatalis* (Ag) MNPV (Johnson and Maruniak, 1989), and BmNPV (Maeda and Majima, 1990). As noted above, the polyhedrin gene is highly conserved among NPV (Rohrmann, 1986). Vlak and Smith (1982) suggested that the smallest REN fragment containing the polyhedrin gene be used as the start site (zero point) of the linearized physical map of NPV genomes. The zero point of AcMNPV is positioned between *Eco*RI-I and *Eco*RI-B (Figure 2.4a).

2.2.5.5. The Size Range of Genomes

Baculoviruses have genomes that range from 80 kbp to 160 kbp. The size of the total viral genome is estimated by summation

Figure 2.4. A circular physical map of AcMNPV genome. *Eco*RI fragments are indicated and map units (0-100) are labelled on the inside of the circle. Relative locations and orientations of some ORF are indicated as solid arrows around the circle. Abbreviations used are the following: *basic* DNA *bp* (p6.9), basic DNA binding protein (6.9 kd); *bv efp* (gp64), budded virus envelope fusion protein (64 kd); *capsid* (p39), major capsid protein (39 kd); *cath*, cathepsin; *cg30*, *Hin*dIII-C/*Eco*RI-G 30 kd protein; *da16*, *Hin*dIII-D/*Eco*RI-A 16 kd protein; *da26*, *Hin*dIII-D/*Eco*RI-A 26 kd protein; *da41*, *Hin*dIII-D/*Eco*RI-A 41 kd protein; DNA pol, DNA polymerase; *egt*, ecdysterioid UDP-glucosylttransferase; *ets*, *Hin*dIII-E-*Eco*RI-T-small; *fp1* (25 kd), few polyhedra locus protein (25 kd); *gp37*, glycoprotein 37 kd (*slp*, spheroidin-like protein); HR, homologous repeat; *p35*, suppressor of apoptosis; *ie0*, immediate early gene 0; *ie1*, immediate early gene 1; *ie-n (ie2)*, immediate early gene 2; *lef-1*, late expression factor 1; *lef-2*, late expression factor 2; *me53*, major early 53 kd; *orf1629*, 1629 nt ORF; *orf603*, 603 nt ORF; *pcna (etl)*, proliferating cell nuclear antigen (*Hin*dIII-E-*Eco*RI-T-large); *pe38*, *Pst*I-*Eco*RI 38 kd; *pep (pp34)*, polyhedral envelope protein (phosphoprotein 34 kd); *pk*, Protein kinase; *polyhedrin*, Major occlusion protein; *pp31 (39k)*, phosphoprotein 31 kd (originally named 39 kd protein); *ptp*, protein tyrosin/serine phosphatase; *sod*, superoxide dismutase; *ubi*, ubiquitin. Adapted from Volkman et al. (1995).

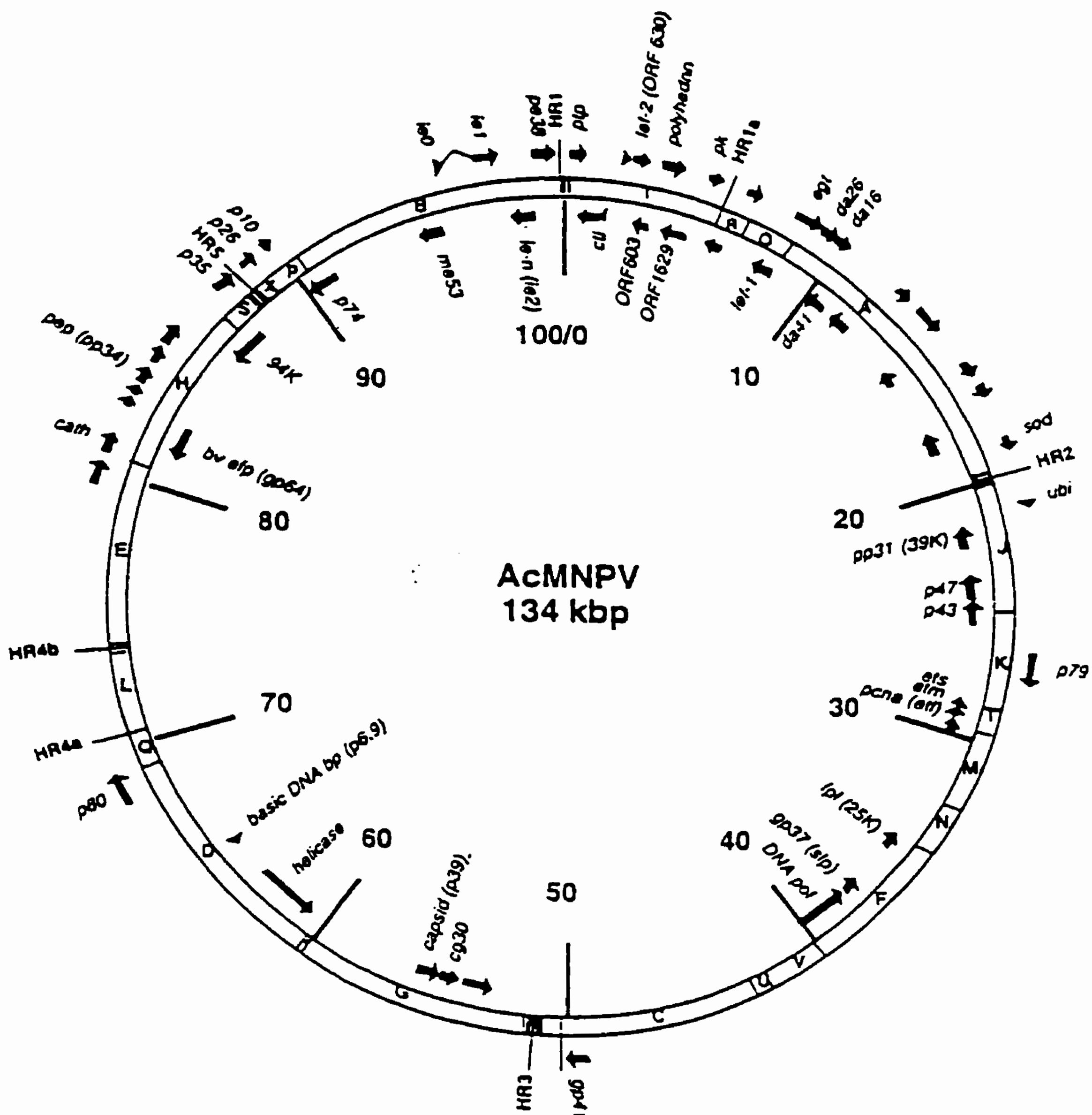
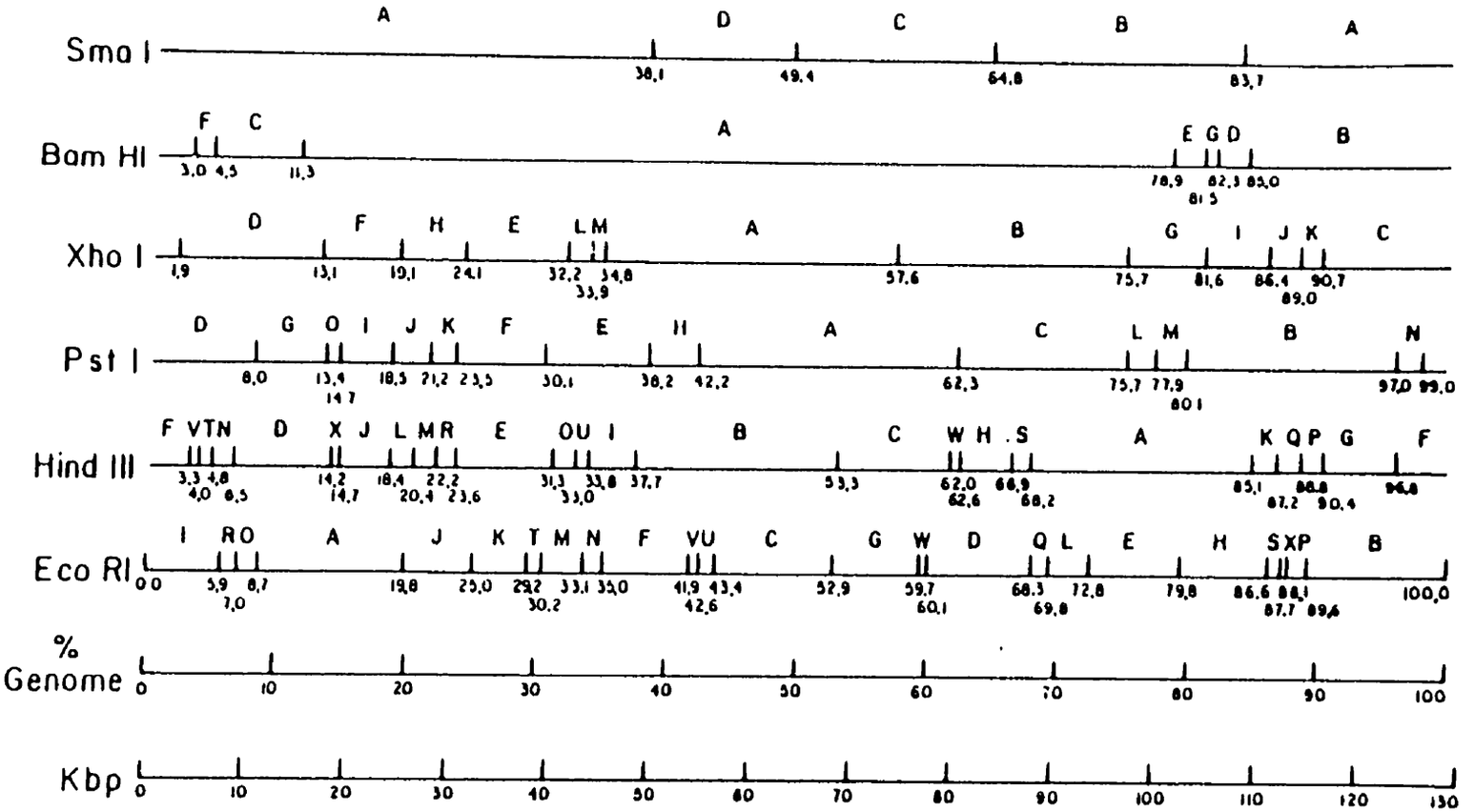

AcMNPV
134 kbp
100/0
10
20
30
40
50
60
70
80
90
HR1
HR1a
HR2
HR3
HR4a
HR4b
HR5
polyhedron
lef-2 (ORF 630)
ORF603
ORF1629
lef-1
me53
p74
94K
bv efp (gp64)
cath
pep (pp34)
p35
p26
p10
sod
ubi
pp31 (39K)
p47
p43
p79
pcna (etl)
lef-1
gp37 (slp)
DNA pol
helicase
basic DNA bp (p6.9)
capsid (p39)
cg30
gp41
p80
ie1
ie0
pp38

Figure 2.4a. Physical map of AcMNPV HR3 DNA for the enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sma*I, and *Xho*I. The map was linearized at the junction of the *Eco*RI-I and -B fragments. Each cleavage site is indicated with a horizontal line and is labelled as a percentage of the genome. Comigrating restriction fragments are designated as a single letter in alphabetical order starting from 0.0% (e.g., double bands *Hin*dIII-NO were shown as *Hin*d-N and -O on the physical map). Adapted from Cochran et al. (1982).


Sma I
A D C B A
38.1 49.4 64.8 83.7
Bam HI
F C A E G D B
3.0 4.5 11.3 78.9 82.3 85.0 81.5
Xho I
D F H E L M A B G I J K C
1.9 13.1 19.1 24.1 32.2 34.8 33.9 57.6 75.7 81.6 86.4 90.7 89.0
Pst I
D G O I J K F E H A C L M B N
8.0 13.4 14.7 18.5 21.2 23.5 30.1 38.2 42.2 62.3 75.7 77.9 80.1 97.0 99.0
Hind III
F VTN D X J L M R E OU I B C W H . S A K Q P G F
3.3 4.8 4.0 6.5 14.2 14.7 18.4 20.4 22.2 23.6 31.3 33.8 33.0 37.7 53.3 62.0 62.6 66.9 68.2 85.1 87.2 88.8 90.4 96.8
Eco RI
I RO A J K T M N F VU C G W D Q L E H SXP B
0.0 5.9 7.0 8.7 19.8 25.0 29.2 30.2 33.1 35.0 41.9 42.6 43.4 52.9 59.7 60.1 68.3 69.8 72.8 79.8 86.6 87.7 88.1 89.6 100.0
% Genome
0 10 20 30 40 50 60 70 80 90 100
Kbp
0 10 20 30 40 50 60 70 80 90 100 110 120 130

of the mean sizes of REN fragments generated by each of different REN enzymes used in physical mapping studies (Wiegers and Vlak, 1984; Crook et al., 1985; Crawford, et al., 1985; Possee and Kelly, 1988; Johnson and Maruniak, 1989; Jehle et al., 1992). The genome sizes of AcMNPV isolates range from 128 kbp to 134 kbp (Cochran et al., 1982; Ayres et al., 1994). The complete nucleotide sequence of one of the AcMNPV isolates, containing 133,894 base pairs, has been determined (Ayres et al., 1994). The physical maps of virus genomes are often useful in determining regions of difference among strains of virus.

2.2.5.6. Gene Mapping

The position of specific gene(s) on the viral genome are determined based on the fact that the viral specific polyadenylated RNA transcribed in infected cells shares nucleotide sequence homology with viral genomic DNA. Polyadenylated RNAs from infected insect cells are selected on oligo(dT)-Sepharose columns. They are separated on agarose gels and transferred on nitrocellulose filter (Northern blot). Viral-specific polyadenylated RNA are visualized by hybridization to viral genomic DNA or cloned viral DNA fragment probes. A cDNA library can be synthesized from a polyadenylated RNA preparation and screened by hybridization with total viral genomic DNA or the cloned viral DNA fragment probes. These cDNA could be used with various expression vectors to produce the quantities of the gene products.

The order of genes along different baculovirus genomes is often quite similar (Leisy et al., 1984; Arif et al., 1985; Possee and Kelly, 1988; Jehle et al., 1992). This feature of baculoviruses is referred to as co-linear organization of the genome. It is a useful concept in detailed comparison between viral species. Figure 2.5 is an example showing the co-linear organization of AcMNPV and *Orgyia pseudotsugata* (Op) MNPV based on cross-hybridization studies.

2.2.6. Genetic Variation among Natural Populations

Among natural populations of baculoviruses, isolates with slight differences in genomic structure have often been reported (Wiegers and Vlak, 1984; Crook et al., 1985; Cherry and Summers, 1985; Brown et al., 1985; Smith and Crook, 1988; Erlandson, 1990; Shapiro et al., 1991). These isolates may also differ in terms of virulence to host insects (Williams and Payne, 1984; Erlandson, 1990). Sometimes one viral isolate may contain a heterogeneous mixture of genotypic variants, indicated by the presence of submolar DNA fragments in the REN restriction patterns (Erlandson, 1990; Weitzman et al., 1992).

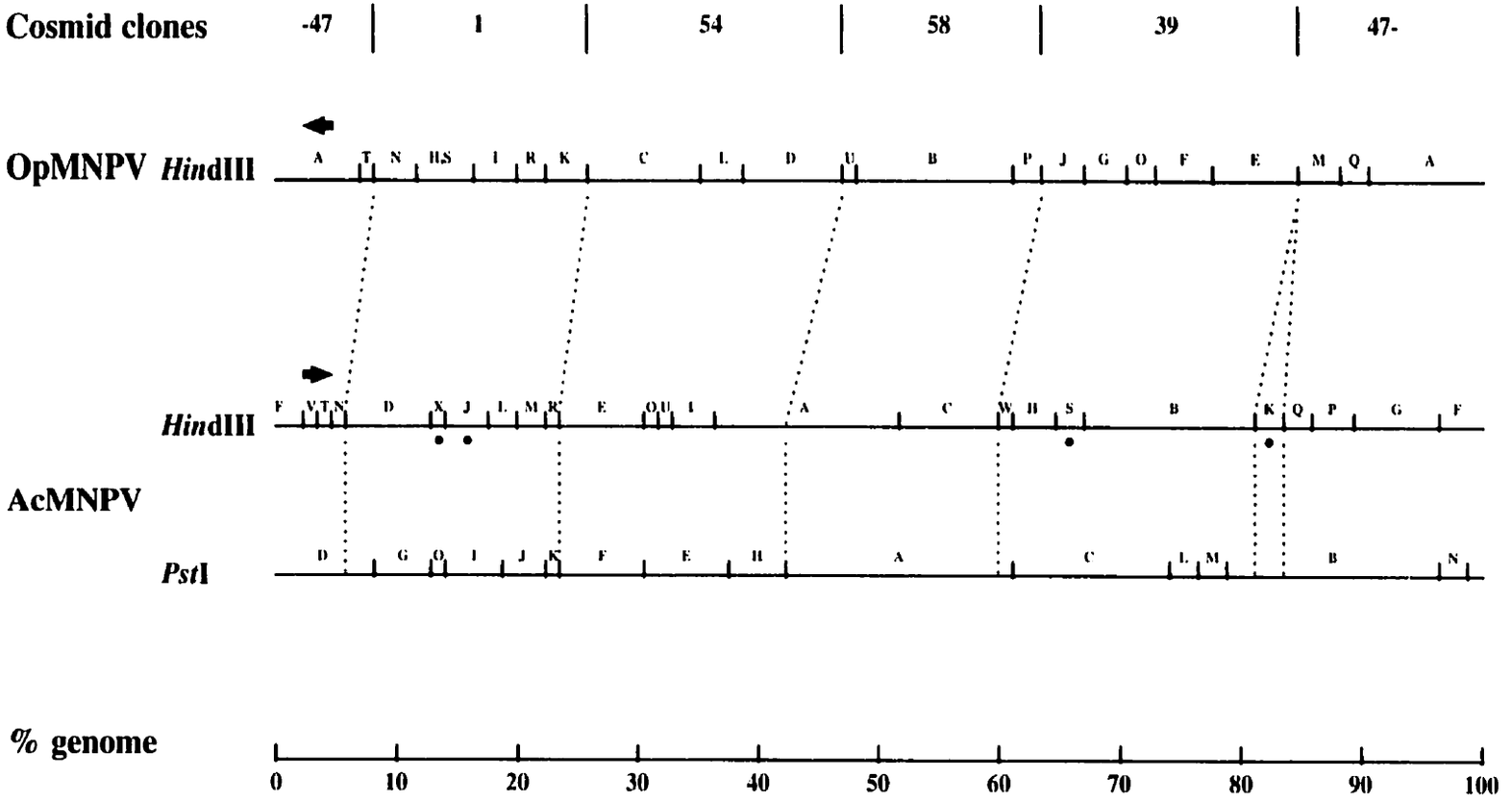


Figure 2.5. Alignment of the OpMNPV map with the cross-hybridizing region of AcMNPV. The arrows above the restriction maps indicate the positions and directions of transcription of the polyhedrin gene for each virus. Dashed lines connecting the two maps indicate the regions cross-hybridizing with each of the OpMNPV *Hin*dIII fragment cosmid clone. Dots indicate AcMNPV fragments not hybridized. Modified from Leisy et al 1984.

2.2.7. Use of Baculoviruses in Insect Biological Control

Baculoviruses have good potential as biological control agents (reviewed by Entwistle and Evans, 1985). The following advantages of viral insecticides make them attractive alternatives to chemical pesticides for insect pest control:

1. Unlike chemical insecticides, which have produced problems due to non-target toxicity, most baculoviruses infect only a limited number of insect species. For example, the host range of GV is confined to Lepidoptera; the host range of NPV is also limited to closely related insect species, most often to several species within the Lepidoptera, Coleoptera, Hymenoptera, and Diptera.

2. Baculoviruses replicate in host insects, thus leading to biological magnification with additional inoculum being produced. This extends the time frame of their impact.

3. There is less potential for resistant insect populations to develop and no cross resistance with chemical insecticides has been noted.

4. Baculoviruses do not produce residue problems, which have always been associated with chemical insecticides.

5. Conventional application technologies for chemical insecticides, such as ultra low volume spray and bait formulation can be used for viral insecticide application. Also, production of insect viruses can be done with low energy input technology, especially when high production, alternative host insects are

available. This approach may be especially useful in developing countries.

However, there are some significant disadvantages which hamper the large-scale development and application of baculoviruses as biological control agents, for example, the difficulties of large-scale production and storage of baculoviruses. As well, because of the host specificity and the fact that baculoviruses are only useful against feeding stages of the insect pests, the market potential of any particular baculovirus product is limited. However, the major drawback to the large-scale use of baculoviruses is the slowness of their effect, which causes a lag period of 1 to 4 weeks between the application of baculoviruses and their effect. This lag allows the larvae to feed longer and thereby damage the host plant. This lag is unacceptable in the protection of many crops, especially where cosmetic damage seriously reduces the value of a crop (e.g., fruit), in comparison with the rapid knock-down effect of chemical insecticides.

It is speculated that by engineering genes encoding insect-specific hormones, enzymes or toxins into baculoviruses, a recombinant virus capable of killing the infected host in a shorter time could be developed (Hawtin et al., 1992). Because the polyhedrin and p10 genes are dispensable for virus replication in insect cell culture, efforts have been made to produce recombinant baculoviruses by inserting foreign genes into the coding regions of these two very late genes under the control of their promoters

(reviewed by Possee et al., 1993; Bonning and Hammock, 1996). In the case of the polyhedrin promoter, polyhedrin-minus recombinant baculoviruses have been co-occluded with wild-type virus to ensure their *per os* infectivity. Experiments demonstrate that the persistence of a polyhedrin-minus virus in a cycling virus population is limited by the co-occlusion process (Wood et al., 1994). The biological stability of the polyhedrin-minus recombinant virus has been investigated in field studies. The results are a rapid decline in the percentage of polyhedrin-minus recombinant viruses in the population of occluded viruses from field-infected host larvae, indicating that the genetically crippled virus will rapidly degrade in the environment after killing its host (Bishop and Possee, 1990; Wood et al., 1994).

Metabolic and developmental processes within the insect, such as water balance and moulting, are regulated hormonally. Thus, the overexpression of genes for insect hormones or enzymes by baculoviruses might disrupt these processes. For example, a synthetic gene based on *Manduca sexta* diuretic hormone, together with a signal peptide coding sequence from *Drosophila melanogaster* cuticle protein, was introduced into the BmNPV genome in place of the polyhedrin gene-coding region. This recombinant virus produces the diuretic hormone in virus-infected cells. A 30% reduction in hemolymph volume has been reported in larvae infected with the recombinant virus and mortality occurs 1 day earlier than for larvae infected with wild-type virus (Maeda, 1989).

Juvenile hormone is important in insect metamorphosis. Its

level at its sites of action is regulated in part by juvenile hormone esterase, which hydrolyses the chemically stable, conjugated methyl ester to yield juvenile hormone acid (Hammock, 1985). Thus, overproduction of juvenile hormone esterase is expected to inhibit the routine metabolism of insects and result in the abolition of feeding and premature pupation (Hammock et al., 1990). When a recombinant AcMNPV, with the *H. virescens* juvenile hormone esterase gene inserted into the polyhedrin gene coding region, is fed to *T. ni* larvae, first-instar larvae but not older larvae, showed reduction in feeding (Hammock et al., 1990). The relatively low level of juvenile hormone esterase produced in later larval stages are thought to be responsible for the failure of the recombinant baculovirus expressing juvenile hormone esterase to significantly alter feeding behaviour and mortality in host insects compared to wild-type virus (Possee et al., 1993).

Insect eclosion hormone is a neuropeptide which has a number of physiological and behavioral functions related to molting. A *Manduca sexta* cDNA encoding an eclosion hormone has been inserted into the AcMNPV genome under the control of a strong, modified polyhedrin promoter. Biologically active eclosion hormone, effective in both homologous and heterologous insect species, has been synthesized and secreted at high levels in recombinant-virus infected cells (Eldridge et al., 1991, 1992). However, for unclear reasons, expression of the eclosion hormone gene during infection of late instar *S. frugiperda* has no effect on the ability of AcMNPV to limit insect feeding or hasten death (Eldridge et al.,

1992).

The above examples indicate that although the chances of an insect becoming resistant to its own gene products are small, it is difficult to produce a recombinant virus that overcomes the tightly regulated processes of the insect host.

The expression of insect-specific toxin genes, including the Bt δ-endotoxin gene, by baculoviruses has been an attractive approach to enhance baculovirus efficiency. The δ-endotoxin is active against a wide range of insect species (Huber and Luthy, 1981). The toxin is produced as a 130 kDa protoxin which is cleaved to a 62 kDa active toxin in the midgut upon ingestion by an insect host. The presence of active toxin in the midgut causes an immediate reduction in feeding, and it is thought that the toxin generates pores in the midgut epithelial cell membrane, leading to disruption of osmotic control and eventually to cell lysis (reviewed by Luthy and Ebersold, 1981). When the complete δ-endotoxin gene is introduced into AcMNPV in place of the polyhedrin gene, it is expressed at a high level without interference with AcMNPV production in infected *S. frugiperda* cells. The recombinant virus expressed toxin protein is found in large crystals with an ultrastructure similar to that in sporulated Bt cells. Recombinant virus infected-cell extracts inhibited feeding of the large cabbage white butterfly, *Pieris brassicae* (L.), indicating the virus expressed an authentic δ-endotoxin protein (Martens et al., 1990).

The endotoxin coding sequence has also been inserted into the

AcMNPV genome under the control of the p10 promoter to produce polyhedrin-positive virus (Merryweather et al., 1990). However, the AcMNPV recombinant has no increased insecticidal effectiveness against *T. ni* larvae. It is suggested that the protoxin produced by the recombinant virus in insect hosts is not processed into active toxin (Merryweather et al., 1990).

While insect-specific toxins which act in the gut appear to be poor prospects for increasing the toxicity of baculoviruses, the use of insect-specific neurotoxins, including a mite neurotoxin (Tomalski and Miller, 1991) and a scorpion neurotoxin (Stewart et al., 1991), seems to be a more attractive proposition. Females of the mite *Pyemotes tritici* inject an extremely potent venom into their insect prey which causes muscle-contraction and paralysis. The active component in the venom, TxP-I, is effective against a broad range of insect species but not mice. The neurotoxin gene (Tox-34) has been cloned from a cDNA expression library produced with mite mRNA and the nt sequence determined. The predicted sequence of TxP-I has a hydrophobic domain potentially useful as signal for secretion into the venom apparatus. Insect cells infected with a recombinant virus expressing the toxin gene secrete three polypeptides related to TxP-I. *T. ni* larvae infected with the recombinant virus responded more rapidly to infection than those infected with wild-type virus. Fifth-instar *T. ni* larvae injected with a lethal dose of budded recombinant virus were paralysed within two days of infection, whereas larvae injected with the same dose of wild-type

virus never showed paralysis. The induced paralysis of the larvae prevents the larvae from feeding but does not prevent the replication of the recombinant virus. On oral infection with a low dose of occluded virus, neonate larvae infected with recombinant virus succumbed to paralysis and/or death at a faster rate than those infected with wild-type virus (Tomalski and Miller, 1991).

The North African scorpion, *Androctonus australis* Hector, produces an insect-specific neurotoxin (Zlotkin et al., 1971) in its venom. The toxin causes specific modifications to the Na+ conductance of neurons, producing a presynaptic excitatory effect that leads to paralysis and death (Walther et al., 1976; Teitelbaum et al., 1979). A synthetic *A. australis* insect toxin (AaHIT) gene with a copy of the AcMNPV gp67 signal peptide sequence (Whitford et al., 1989) incorporated at its 5' end to facilitate secretion of the neurotoxin by insect cells has been introduced into AcMNPV under the control of a p10 promoter to produce the polyhedrin-positive recombinant virus (Stewart et al., 1991). The virus-directed intracellular expression of AaHIT was detected as early as 18 h pi.; maximal expression occurred at 36 h pi, and declined at 48 h pi. Expression of the neurotoxin by the virus caused a reduction in both the median survival time (ST_{50}, decreased 24%) and the median lethal dose (LD_{50}, decreased 30%) in *T. ni* larvae. Third-instar *T. ni* larvae infected with recombinant virus caused 50% less damage to cabbage leaves than individuals infected with wild-type AcMNPV. In a field trial, many of the larvae infected with an AcMNPV recombinant virus expressing AaHIT

fell off the plant due to virus-induced paralysis. Larvae infected with the recombinant virus died earlier than those infected with wild-type virus (Cory et al., 1994), indicating that genetic modification can improve the efficacy of baculoviruses.

Some other insect-specific toxin genes are under investigation. The cDNA encoding hornet antigen 5, which is a major allergen found in the venom of the baldfaced hornet, *Vespula maculata* (L), has been cloned into AcMNPV. Insect cells infected with the recombinant virus produce and secrete a novel protein which has an electrophoretic mobility similar to that of authentic mature hornet antigen 5 (HA5) and reacts specifically with a polyclonal antiserum raised to HA5. Although HA5 gene products are not acutely toxic when injected into insect larvae, infection of fifth-instar larvae with the recombinants resulted in premature melanization of the larvae and lower weight gain than larvae infected with wild-type virus. The HA5 gene product is therefore expected to have an indirect, possibly cytotoxic or biochemical, effect on insects (Tomalski et al., 1993). This gene may be useful in further genetic modification of baculoviruses.

About 50 peptidic toxins purified from venom of the primitive hunting spider, *Plectreurys tristis* (Simon), have been reported to be paralytic and/or lethal when injected into insect pests such as larvae of *H. virescens*, *S. exigua*, and *M. sexta*. Amino acid sequences for some of the insecticidal peptides have been determined. These toxins (referred as plectoxins after and genus name of spider) are expected to be useful in insect control after

delivery by recombinant baculoviruses (Quistad and Skinner, 1994).

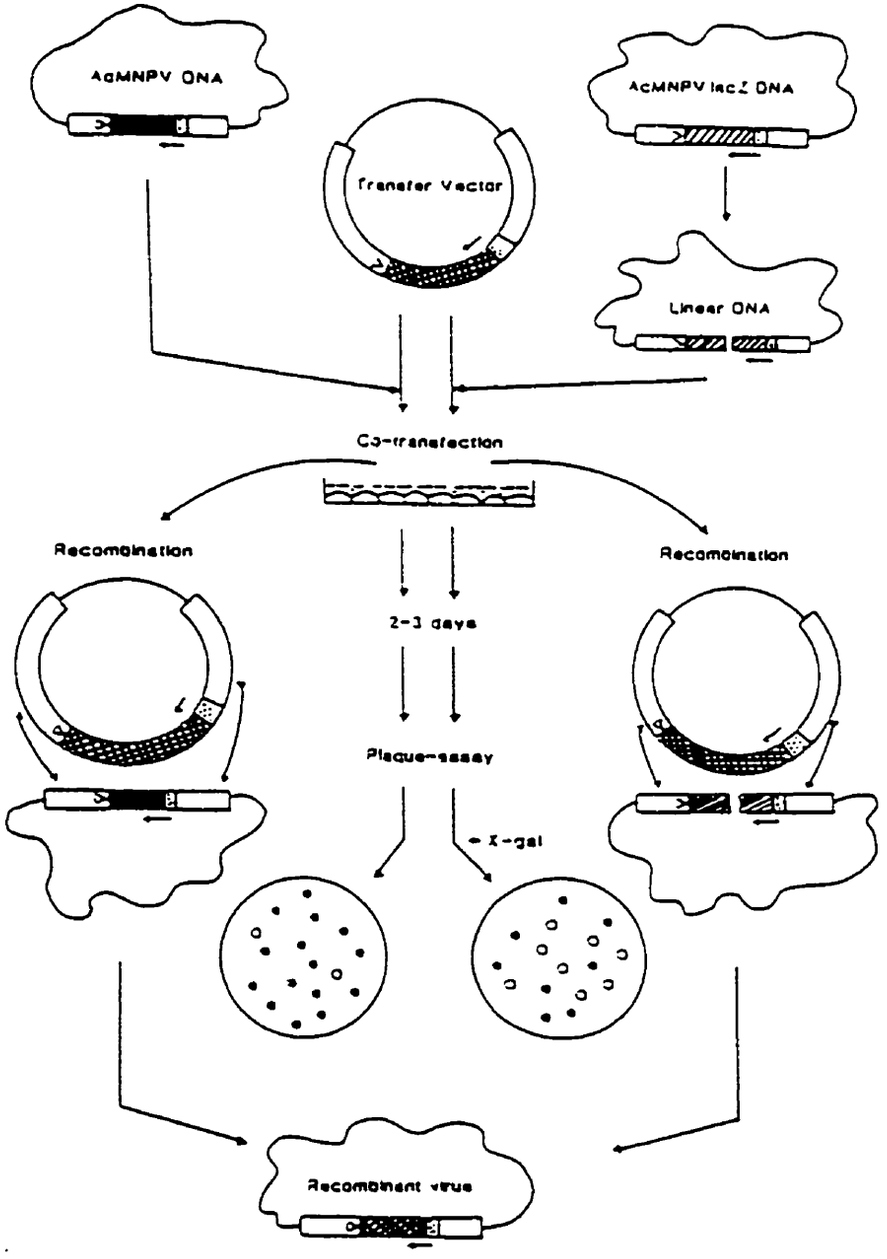
2.2.8. Use of Recombinant Baculoviruses for Foreign Gene Expression

Recombinant baculoviruses have been used as expression vectors for production of heterologous proteins in insect cell lines. Because of its large size, the baculovirus genome is difficult to directly manipulate. Usually, the development of recombinant baculovirus is carried out in two steps. First, the foreign gene is incorporated into a baculovirus transfer vector. Portions of virus genome spanning the gene of interest for promoter utilization (e.g., polyhedrin or p10 gene) are inserted into bacterial plasmids and propagated in *E. coli* cells. Part or all of the viral gene coding region is removed using REN or exonucleases, leaving the promoter intact. Foreign gene coding sequences with compatible restriction ends are ligated into the recombinant bacterial plasmid under the control of viral promoter. This plasmid is called the transfer vector. Second, the engineered DNA is incorporated into the virus via homologous recombination with the parental virus as shown in Figure 2.6. In this way, a recombinant virus with the foreign gene under the control of the chosen promoter is produced.

Insect cell lines have some advantages over mammalian cell lines. Most typically used insect cell lines are continuous cell lines that are subject to contact inhibition and can be cultured

in either anchorage-dependent or anchorage-independent modes, and have characteristics somewhat intermediate between normal and transformed mammalian cell lines (Shuler, 1995). For the expression of eukaryotic genes, expression systems from baculovirus-infected insect cell cultures have obvious advantages over mammalian cell line and bacterial cell line systems. Since baculoviruses infect eukaryotic cells, post-translational processing of foreign gene products can be facilitated (which bacterial gene products cannot), although insect cells do show some differences in the pattern of protein glycosylation (posttranslational modification) compared to that in vertebrate cells (King and Possee, 1992; Shuler et al., 1995; Luckow, 1995). As baculoviruses have a large genome, they are able to accommodate large pieces of foreign DNA without affecting their normal replication and DNA packaging. Also, because baculoviruses do not infect vertebrates, it is safe to use these promoters for vertebrate gene product amplification. Insect cells are amenable to large-scale volume production in fermentation systems; thus, baculovirus expression systems can be scaled up for large-volume production (King and Possee, 1992; Shuler et al., 1995).

Figure 2.6. Schematic representation of the production of a recombinant baculovirus. The plasmid transfer vector contains the polyhedrin promoter (stippled box) and transcription termination signals (open arrow head) flanked by AcMNPV sequences (open box). A foreign gene (Cross-hatched box) is inserted under the control of the polyhedrin promoter and then mixed with infectious AcMNPV DNA (or linearized AcMNPV DNA, e.g. AcMNPV *lacZ*, a recombinant AcMNPV encoding *lacZ* gene). Insect cells are co-transfected with the transfer vector and virus DNA with either calcium phosphate co-precipitation, electroporation or lipofection. In the course of the resulting infection, the sequences flanking the foreign gene recombine with the homologous sequences in the virus genome (homologous recombination) and thus replaces the polyhedrin gene (solid box) or *lacZ* gene (hatched box) with the foreign gene coding sequences. The progeny virus are screened for polyhedrin- or *lacZ*-negative viruses by plaque-assay or by dot-blot hybridization followed by plaque-assay. Recombinant viruses are identified by the production of polyhedrin- or *lacZ*-negative plaques (open circles) compared with the production of polyhedrin- or *lacZ*-positive plaques (solid circles) by the parental virus. Adapted from King and Possee (1992).


AcMNPV DNA
Transfer Vector
AcMNPV lacZ DNA
Linear DNA
Co-transfection
Recombination
Recombination
2-3 days
Plaque-assay
+ X-gal
Recombinant virus

As mentioned before, both polyhedrin and p10 gene are dispensable for virus replication in cultured cell lines and both genes have very strong promoters. Foreign genes have been inserted into the baculovirus genome under the control of either the polyhedrin or p10 gene promoters. In addition, both genes are expressed very late in infection. Thus, if a cytotoxic protein is to be expressed, it will be synthesized late in infection and not affect the normal replication of recombinant virus. An additional advantage is that the replacement of the polyhedrin gene with a foreign gene allows for relatively easy visible selection of polyhedrin-minus recombinants. Moreover, when a polyhedrin-minus virus is used as the infectious virus DNA and is co-transfected with a transfer vector containing a functional polyhedrin gene, it becomes quite easy to pick out polyhedrin-positive recombinant viruses from the background of polyhedrin-minus non-recombinants (King and Possee, 1992). The *E. coli* β-galactosidase gene, which confers a blue phenotype on the recombinant virus when expressed in insect cells in the presence of X-gal, has also been used for recombinant virus selection (King and Possee, 1992; Luckow, 1995).

Although the production levels of the baculovirus genes in the early and late categories do not match those of the very late polyhedrin and p10 genes, they are still potentially useful in the development of expression vectors, especially in cases when the early expression of foreign gene is required. However, because most of these gene products are essential for virus replication, they cannot be replaced directly in the manner of the polyhedrin

and the p10 genes. Usually, it is necessary to duplicate the promoters of these genes at alternative, non-essential locations in the virus genome (King and Possee, 1992; Bonning and Hammock, 1996). Higher expression under the control of basic protein gene (a late gene) promoter has also been reported (Bonning et al., 1994).

2.2.9. Baculoviruses Occurring in Bertha Armyworm

A MNPV (MacoNPV) was isolated from *M. configurata* in different geographic areas of western Canada (Erlandson, 1990). The MacoNPV isolates from different geographic areas (geographic isolates) differ in their virulence to the host insect. Preliminary studies showed that the LD_{50} to neonate bertha armyworm ranged from 11.9 to 73.5 viral OB per larva (Erlandson, unpublished data), indicating the virus has good potential as a biocontrol agent for bertha armyworm. The fact that the isolates have varied virulence to their host may provide an opportunity for the selection of great virulent strains from a broad geographic area. Preliminary studies also showed that all but one of the viral geographic isolates are heterogeneous mixtures of genotypes (Erlandson, personal communication), which theoretically makes it possible to derive several genotypic variants of the virus.

Though in natural populations of bertha armyworm, the level of larval mortality due to NPV infection typically runs <40%, MacoNPV infections can occur at epidemic levels. Infection prevalence as high as 95% has been recorded in late season

populations of bertha armyworm (Erlandson, 1990). Because bertha armyworm infected with MacoNPV always die before pupation, this high level of infection means that MacoNPV can play an important role in reducing natural host populations.

The above features of MacoNPV indicate the importance of further study of this virus, including the detailed characterization of the viral genomic structure, comparison between MacoNPV geographic isolates and between MacoNPV and other baculoviruses, the investigation of the relationship between viral genomic structure and viral infectivity and/or virulence to its insect hosts, the study of viral gene expression, and the selection of highly virulent viral strains for biological control agents by genetic engineering.

3. Objectives

Bertha armyworm is an important insect pest for oilseed crops in western Canada. Our long-term goal is to discover or to generate highly efficient biological control agents for this insect pest. As part of this goal, the primary objective of the present study is to describe the physical structure of the MacoNPV genome, that is, to characterize the REN restriction patterns of the viral genome, to map the locations of REN restriction sites on the viral genome, and finally to construct a physical map. The physical map of the viral genome will be useful in the comparison of MacoNPV with other baculoviruses and in the comparison of MacoNPV geographic isolates. A secondary objective of the study is to purify some homogenous genotypic strains from the geographic isolates through pick plaque purification, and preliminary characterization of these viral strains in terms of REN patterns and their virulences to insect host and their growth in cultured insect cells. These data will be useful for further study to discover the possible relationships between viral genomic structure and infectivity and/or virulence.

4. REN Analysis, Molecular Cloning and Physical Mapping of MacoNPV Genomic DNA

Physical mapping of the DNA genome of MacoNPV provides a preliminary description of the physical structure of the virus genome in terms of the position of REN cleavage sites on the genome.

One of the basic techniques used to manipulate the viral DNA REN fragments is the process of molecular cloning of the REN fragments in bacterial plasmids. To do this, viral DNA and the selected plasmid vector DNA were cleaved with (an) appropriate REN, the viral DNA fragment(s) were ligated with the linearized plasmid DNA. Suitable bacterial host cells were transformed with the plasmids (recombinant and non-recombinant) to propagate the plasmids, and the transformed host cells were screened for the presence or absence of recombinant plasmids.

Southern blot hybridization was used to determine the relative position of REN fragments on the viral DNA genome. This technique is based on the DNA base pair complementarity of an *in vitro* labelled single-stranded DNA (ssDNA) probe to the denatured ssDNA fragments on Southern blot membranes. The REN DNA fragments, which showed clear hybridization with each other when either cloned or excised REN fragments are *in vitro* labelled and used as probes to Southern blot membranes, were considered to share common nucleotide sequences and therefore map to the same region of the MacoNPV genome. All the Southern blot hybridization

results described in the following sections were pieced together based on this principle.

4.1. Material and Methods

4.1.1. Insects

Cultures of bertha armyworm were kindly provided by Agriculture and Agri-Food Canada, Saskatoon Research Centre. Leaf discs with egg masses were placed in inverted 30 ml diet cups and neonate larvae were isolated within 12-24 hr of hatching for use in bioassays. Later instar larvae for virus production were obtained from the culture colony and set up at 5 larvae/cup. Larvae were maintained on a semi-synthetic diet (Bucher and Bracken, 1976) at 21°C, 60% RH, and a 20:4 L:D photoperiod.

4.1.2. Source of Virus

The five MacoNPV geographic isolates used in the present study, designated MacoNPV-86/1, -90/1, -90/2, -90/3, and -90/4, were kindly provided by Dr. Martin Erlandson, Agriculture and Agri-Food Canada, Saskatoon Research Centre. The five geographic isolates were collected from Carrot River (Saskatchewan, 53°22'N, 103°19'W), Lamont (Alberta, 53°50'N, 112°38'W), Wilkie (Saskatchewan, 52°30'N, 108°41'W), Vermillion (Alberta, 53°22'N, 110°51'W) and Lamont (Alberta, 53°50'N, 112°38'W), respectively. MacoNPV-90/1 and -90/4 were isolated from different fields near Lamont, Alberta. All isolates except MacoNPV-90/2 contained REN fragment bands present at sub-molar ratios when analyzed by

agarose gel electrophoresis and these were considered to be heterogeneous mixtures of genotypes (Erlandson, personal communication). The geographic isolate MacoNPV-90/2 was therefore chosen for physical mapping of the viral genome because the results would be more easily interpreted in the absence of submolar bands.

4.1.3. Virus Purification and Viral DNA Preparation

MacoNPV virus stocks were produced by infection of 4th-instar bertha armyworm larvae by contamination of the diet surface with 1.4×10^4 OB/cm^2. Virus production and OB isolation, virion purification, and viral DNA extraction essentially followed the methods described by Erlandson (1990). Briefly, infected larvae were homogenized in 5-10x their volume of distilled water using a polytron tissue homogenizer. After filtration through cheesecloth, the homogenate was pelleted by low-speed centrifugation (1,400x g). The pellet containing MacoNPV OB and tissue debris was resuspended in 0.5% sodium dodecyl sulfate (SDS) and incubated at 37°C for 2-6 hr. The suspension was again filtered through four layers of cheesecloth and the OB pelleted by low-speed centrifugation. Finally, the OB suspension was purified by isopycnic centrifugation on a 40 to 66% w/w sucrose density gradient with a Beckman SW28 rotor at 89,500x g for 90 min. The OB band was collected with a Pasteur pipet, washed in distilled water and centrifuged at 1,400x g for 15 min. The concentration of viral OB was determined by counting

the OB under a microscope with a hemocytometer.

The OB pellet was resuspended in distilled water at approximately $1x10^8$ OB/ml, and the virions were released from the OB or polyhedra by incubation in alkaline OB dissolution buffer (0.1M Na_2CO_3, 0.17M NaCl, 0.001M NaEDTA, pH 10.8) and purified by centrifugation on a 40 to 66% w/w sucrose density gradient with a Beckman SW28 rotor at 89,500x g for 90 min. The virion bands were collected from the gradient to a fresh tube using a pasteur pipette and pelleted by centrifugation. Virions were resuspended in TE buffer, pH 7.2. Virus DNA was extracted from virion suspensions by the addition of 1/10 volume of 10% SDS and proteinase K to a final concentration of 50µg/ml and incubated at 37°C for 4 hr. The preparation was extracted four times with an equal volume of TE-saturated phenol:chloroform:iso-amyl alcohol (25:24:1) and once with an equal volume of chloroform/iso-amyl alcohol. The viral DNA in the aqueous phase was then dialysed against TE, pH 7.5, for 72 hr, with three changes of buffer.

4.1.4. REN Digestion of Viral and Plasmid DNA and Agarose Gel Electrophoresis of DNA Fragments

Six REN, *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sma*I and *Xho*I (GIBCO-BRL), were used to digest the viral genome. Virus DNA was digested with REN in a core buffer system from the supplier (REact3 for *Bam*HI and *Eco*RI; REact2 for *Hin*dIII, *Pst*I, and *Xho*I; and REact4 for *Sma*I) by incubation at 37°C for 2 to 3 hr. Plasmid DNA was digested in a similar fashion, but for a shorter

digestion period.

Digested viral DNA fragments were electrophoresed on 0.8%, 0.6%, or 0.4% agarose gels at 50 V for 15 to 22 hr to separate the fragments. Gels were stained with ethidium bromide and photographed on a Foto/PrepI (Fotodyne) transilluminator using Polaroid type 57 or type 55 positive/negative film. The viral genome has few restriction sites for *Bam*HI (8) and *Sma*I (3) and the fragments from these two enzyme digests did not separate well upon agarose gel electrophoresis. Therefore, the genome was double-digested with these two enzymes to improve separation of the fragments. To do this, genomic DNA was digested with *Sma*I for 2 hr using REact4; then, REact3 and *Bam*HI were added and the reaction continued for a further 2 hr.

4.1.5. Molecular Weight Estimates for MacoNPV REN Fragments

Lambda DNA/*Hin*dIII fragments and GIBCO-BRL high-molecular weight DNA standards were used as size markers for agarose gel electrophoresis. Film negatives of photographed gels were scanned on a densitometer (Pharmacia LKB), and the size of viral DNA fragments was estimated using GelScan XL software (Pharmacia LKB). The densitometer detects and quantifies the absorbance of light by the image of the DNA REN fragment bands on the film negatives, converts the information regarding the position and density of each band into digital information which is then analyzed by the GelScan XL software. Each band is displayed as a peak by the GelScan XL software. The area of the peak represents

the relative amount of DNA in the band, and the position of the peak represents the migration distance of the band. The GelScan XL software computes the size of DNA REN fragments by comparing the migration distance of the viral DNA REN fragments with that of the DNA size standards.

4.1.6. Molecular Cloning of MacoNPV Genomic Fragments

Viral genomic DNA was digested with various REN and the fragments cloned into plasmid pUC18 or pBluescript SK+, which was digested with the appropriate REN, using standard techniques (Maniatis *et al.*, 1982). Briefly, the REN-digested viral and plasmid DNA was diluted in distilled water, 1/5 volume of 5X ligation buffer and an appropriate amount of T4 DNA ligase were added to the DNA mixture. This reaction mixture was incubated at 15-18°C for 18 hr. The ligation reaction was diluted 5-10 times with sterile TE, pH 7.2. A 20µl aliquot of competent DH5α *E. coli* (strain DH5α from GIBCO-BRL) cells was added to 1-5µl of diluted DNA. The cell suspension was incubated on ice for 30 min. The cells were heat shocked at 42°C for 45 sec, rapidly cooled in ice for 2 min. Finally, 380µl of LB broth were added to the transformed *E. coli* DH5α cells and incubated at 37°C for 1 hr to express the ampicillin-resistant gene on the plasmid vector. The transformed cells were spread on LB agar plates containing 100µg/ml ampicillin and 50µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) for screening. Positive selection for transformed bacterial colonies was based

on the presence of the ampicillin resistance gene present on the plasmid vector whose expression is required for growth on medium containing ampicillin. Additionally, the blue/white selection of colonies was based on the expression of β-galactosidase. The plasmid vectors used contain bacterial lac promoter/*lac*Z α-peptide and a multiple cloning site in this region. The *E. coli* cells transformed with non-recombinant plasmid express lacZ α-peptide which is able to cleave X-gal producing blue color. However, when a DNA fragment is cloned into the multiple cloning site, the lacZ α-peptide coding region is interrupted. Thus, the cells transformed with recombinant plasmids give rise to white colonies, while those transformed with non-recombinant plasmids give rise to blue colonies. The plasmid pUC18 was used for the cloning of *Eco*RI, *Hin*dIII, *Pst*I and *Bam*HI+*Sma*I viral DNA fragments, and the plasmid pBluescript for the cloning of viral DNA fragments with *Xho*I site(s). Specific viral REN fragments not generated by shotgun cloning (random cloning of viral DNA fragments) were excised from agarose gels, purified using GeneClean glass milk (Bio 101), and ligated with the appropriately digested plasmid DNA.

4.1.7. *In Vitro* Labelling of DNA Probes by Nick Translation

Viral genomic DNA and DNA fragment clones were labelled *in vitro* with biotin by nick translation (BRL-BioNick Translation Kit) and used as probes in Southern blot hybridizations. The BRL-BioNick Translation Kit contains both DNase I and *E. coli* DNA

polymerase I. The DNase I produces randomly distributed nicks in DNA at low enzyme concentrations in the presence of Mg^{2+}. The DNA polymerase I has both 5'→3'-exonucleolytic and 5'→3'-polymerase functions. It synthesizes DNA complementary to the intact strand in a 5'→3' direction using the 3'-OH termini of the nick as a primer. Its 5'→3'-exonucleolytic activity simultaneously removes nucleotide in the direction of synthesis. The polymerase activity sequentially replaces nucleotides removed by exonucleolytic activity with Biotin-labelled deoxyribonucleoside triphosphates.

Most of the cloned viral-fragment probes were made from miniprep DNA. However, for the 22 kbp *Hin*dIII-A fragment, which we have been unable to clone, a labelled probe was made by excising the DNA band from an agarose gel, purifying the DNA by centrifugation through siliconized glass wool, and then nick translating the isolated DNA.

4.1.8. Southern Blot Hybridization

Southern blot hybridization was done following the PhotoGene™ (GIBCO-BRL) Nucleic Acid Detection System methods with minor modifications. Viral DNA was digested with various REN, and the digest mixture was electrophoresed on 0.6% agarose gels. The DNA immobilized in gels was denatured by incubating in 20 gel volumes of 1.5 M NaCl, 0.5 M NaOH (pH 10.8) twice at room temperature, 20-30 min each. After being neutralized in 20 gel volume of 1.0 M Tris-HCl, 1.5 M NaCl (pH 7.5) twice, 20 min each, the denatured ssDNA fragments in the gel were blotted onto nylon

membranes (PhotoGene™ membrane, GIBCO-BRL) by a wet blot method overnight with 10X SSC as transfer media (Maniatis et al., 1982). The membranes were briefly washed in 5X SSC for 5 min at room temperature, placed on a piece of clean filter paper and air dried for 30 min. The membranes were wrapped in the filter paper and baked for 3 hr in an oven pre-heated at 80°C. The Southern blot membranes were prehybridized overnight at 42°C in 50% formamide (or 25% formamide for low-stringency hybridization), 6x SSPE, 5x Denhardt's solution, 1% (w/v) SDS, and 200mg/ml denatured salmon sperm DNA. The nick-translated DNA probes were hybridized to virus DNA on Southern blot membranes overnight at 42°C in 50% formamide (or 25% formamide for low-stringency hybridization), 10% (w/v) dextran sulfate, 6x SSPE, 5x Denhardt's solution, 1% (w/v) SDS, and 200mg/ml denatured salmon sperm DNA. After hybridization, the probes were removed and the Southern blot membranes were incubated in initial (2x SSC, 0.1% SDS) and final (2x SSC) wash buffers at room temperature for 5 min. For high-stringency hybridization, one intermediate wash was performed in 0.1x SSC, 0.5% SDS at 50°C for 30 min. For low-stringency hybridizations, two intermediate washes were done in 6x SSC, 0.1% SDS at 50°C for 15 min each. Blotto (5% Carnation skim milk in Dulbecco's PBS) was used for blocking at 37°C for 1-3 hr. The biotin-labelled DNA probes were detected on hybridized membranes using a PhotoGene Detection System (GIBCO-BRL). Briefly, a streptavidin-alkaline phosphatase conjugate (SA-AP) was used to specifically bind to the biotin

group on the probe DNA hybridizing to the immobilized ssDNA on the nylon membrane. The membrane was then incubated with a detection reagent (substrate for alkaline phosphatase) which luminesced when dephosphorylated. The luminescence was detected using X-AR or BioMax X-ray films (Kodak).

4.1.9. Construction of the Physical Map of the Viral Genome

The zero point for the physical map of MacoNPV was determined by hybridizing the AcMNPV *Hin*dIII-V fragment clone, containing the middle and 3' portion of the AcMNPV polyhedrin gene, with Southern blots of MacoNPV genomic DNA at low stringency (25% formamide). The AcMNPV *Hin*dIII-V fragment cloned in the plasmid polink26 was kindly provided by Dr. Tom Roberts (Veterinary Infectious Diseases Organization, Saskatoon, Saskatchewan). The conventional practice of defining an REN fragment containing the polyhedrin gene as the zero point of the REN map (Vlak and Smith, 1982) was followed.

The relative order of the restriction fragments was determined from the Southern blot hybridization results and double digests of cloned viral REN fragments. The restriction sites in terms of base pairs from the zero point were aligned and graphically depicted using Clone-Manager ver.4 (Scientific and Educational Software) to produce a physical map of the viral genome.

4.2. Results

4.2.1. REN Fragment Patterns of MacoNPV Isolate 90/2 DNA, and Size Estimates for REN Fragments of Viral Genome

Figure 4.1 shows the fragment patterns observed when MacoNPV-90/2 was subject to REN digestion. As proposed by Vlak and Smith (1982), REN fragments were designated alphabetically starting with A for the largest fragment in each digest, lower-case letters were used following upper-case letters, and comigrating REN fragments were given multiple-letter designations. For example, there are five sets of comigrating fragments in the *Eco*RI pattern (*Eco*RI-DE, -IJ, -NO, -PQ, and -UV), four in the *Hin*dIII restriction pattern (*Hin*dIII-EF, -OP, -ST and -bc), four in the *Pst*I pattern (*Pst*I-BC, -DE, -MN, and -VW), and two in the *Xho*I pattern (*Xho*I-JK and -LM) (Figure 4.1, lane 1, 2, 3, and 5), respectively.

At least 123 REN restriction fragments were detectable, including 26, 32, 24, 11, 19, 8, and 3 fragments for *Eco*RI, *Hin*dIII, *Pst*I, *Bam*HI+*Sma*I (produced by 8 *Bam*HI restriction sites and 3 *Sma*I restriction sites), *Xho*I, *Bam*HI, and *Sma*I, respectively (Figure 4.1).

Densitometric scans were used to estimate the size of viral DNA fragments. Figure 4.2 shows a densitometric scan of a gel lane containing the MacoNPV *Hin*dIII fragment pattern with the numbered peaks representing the respective REN fragments. Table 4.1 summarizes the size estimates for MacoNPV REN fragments. The final size estimate for each fragment is the mean of 13 separate

Figure 4.1. Restriction endonuclease (REN) fragment patterns of MacoNPV isolate 90/2 DNA. MacoNPV-90/2 virus DNA was purified and digested with six REN: *Eco*RI (lane 1), *Hin*dIII (lane 2), *Pst*I (lane 3), *Xho*I (lane 5), *Bam*HI (lane 6) and *Sma*I (lane 7) (GIBCO-BRL) or double digested with *Bam*HI plus *Sma*I to improve separation of the fragments (lane 4). Lambda *Hin*dIII fragments (λ) were used as size standards.

Digested viral DNA was electrophoresed on 0.6% agarose gels at 50 V for 17 hr to separate the fragments. Gels were stained with ethidium bromide and photographed on a Foto/PrepI (Fotodyne) transilluminator using Polaroid type 55 or type 57 positive/ negative films.

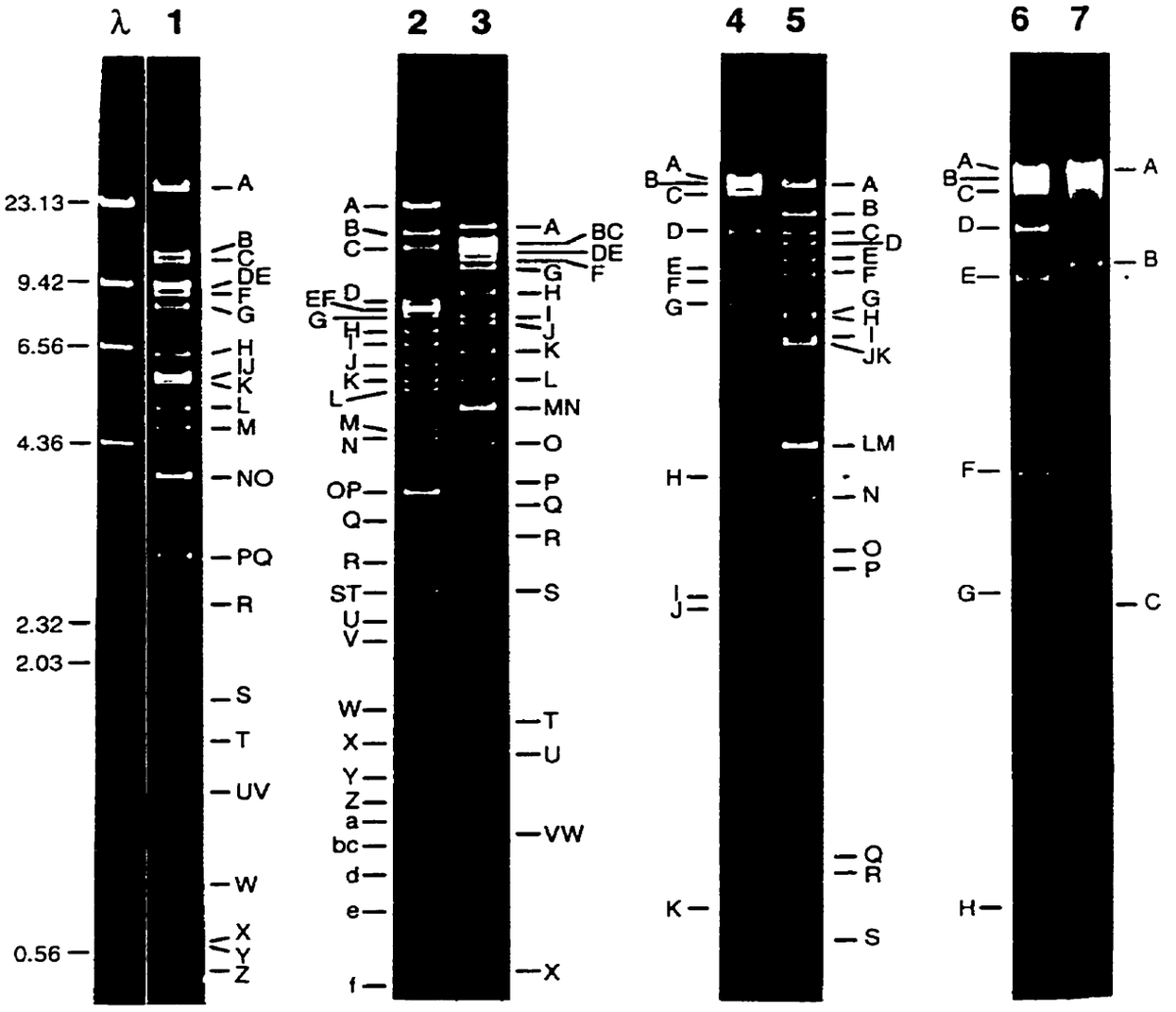

λ
1
2
3
4
5
6
7
23.13
9.42
6.56
4.36
2.32
2.03
0.56
A
B
C
DE
F
G
H
IJ
K
L
M
NO
PQ
R
S
T
UV
W
X
Y
Z
EF
OP
ST
U
V
BC
MN
bc
d
e
f
JK
LM
N
O
P
Q

Figure 4.2. A densitometer scan of agarose gel representing MacoNPV/*Hin*dIII bands analyzed by the GelScan XL software. Each band is displayed as a peak by the GelScan XL software. The area of the peak represents the relative amount of DNA in the band, and the position of the peak represents the migration distance of the band. The size of DNA REN fragments was determined by comparing the migration distance of the viral DNA REN fragments with that of the DNA size standards.

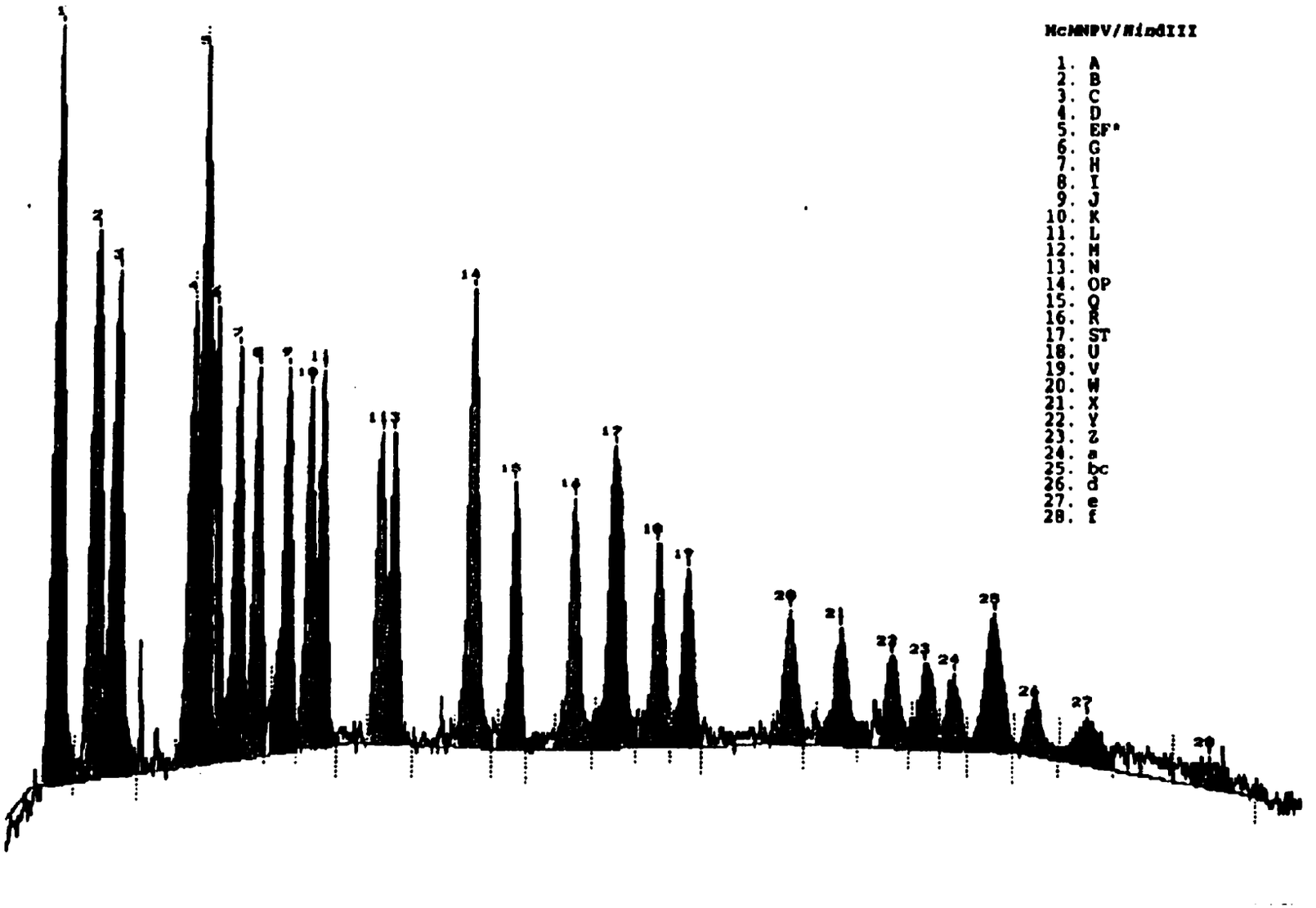

HcMNPV/HindIII
1. A
2. B
3. C
4. D
5. EF*
6. G
7. H
8. I
9. J
10. K
11. L
12. M
13. N
14. OP
15. Q
16. R
17. ST
18. U
19. V
20. W
21. X
22. Y
23. Z
24. a
25. bc
26. d
27. e
28. f

Table 4.1. Size of restriction endonuclease fragments (kbp) of MacoNPV 90/2.

Fragment	*Hin*dIII	*Eco*RI	*Pst*I	*Xho*I	*Bam*HI+*Sma*I
A	22.00	38.20	15.53	36.15	45.40
B	16.05*	12.20	13.25	18.35	35.40
C	13.66*	11.40	13.10	14.18	27.27
D	8.45*	9.55*	12.10*	12.78	14.20
E	8.11*	9.55	12.10*	10.88*	10.41
F	8.08*	9.02	11.20	9.86	9.30
G	7.73*	8.39	10.86	7.64*	8.14*
H	7.08*	6.42*	8.77	7.47	3.78
I	6.59*	5.84*	7.67*	6.66*	2.48
J	6.00*	5.84	7.34*	6.48*	2.38
K	5.61*	5.70*	6.32	6.48*	0.68
L	5.41*	5.10	5.59	4.20*	
M	4.57*	4.69*	4.98	4.20	
N	4.40*	3.92*	4.98	3.49*	
O	3.64*	3.92*	4.30	2.89*	
P	3.64*	2.96*	3.73	2.71*	
Q	3.29*	2.96*	3.45	0.84*	
R	2.85*	2.51	3.11*	0.76*	
S	2.58*	1.71	2.57*	0.55*	
T	2.58*	1.42	1.53*		
U	2.33*	1.11*	1.30*		
V	2.17*	1.11*	0.92*		
W	1.61*	0.76*	0.92*		
X	1.37*	0.59*	0.51*		
Y	1.17*	0.55*			
Z	1.06*	0.47*			
a	0.97*				
b	0.87*				
c	0.87*				
d	0.76*				
e	0.65*				
f	0.47*				
TOTAL	156.62	155.89	156.13	156.57	159.44

* Restriction endonuclease fragment has been cloned.

electrophoretic runs. The mean size estimates for the MacoNPV-90/2 DNA genome was determined by summation of the sizes of REN fragments generated by each of the REN enzyme, and these ranged from 155.9 to 159.4 kbp, depending on the REN used.

4.2.2. Molecular Cloning of MacoNPV Genomic Fragments

More than 70 viral REN fragments were cloned into plasmid pUC18 or pBluescript, including 15 from the *Eco*RI digest, all but 1 (*Hin*dIII-A) of the 32 fragments from the *Hin*dIII digest, 11 from the *Pst*I digest, 12 from the *Xho*I digest and 1 from the *Bam*HI+*Sma*I double digest (indicated by * in Table 4.1). Most fragments are represented by two or more clones. The identification of the REN fragment clones was initially made by digestion with the respective REN and electrophoretic analysis on agarose gels using total MacoNPV-90/2 genomic DNA digests for comparison. At least one of the clones for each REN fragment was used in Southern blot hybridization analysis to confirm their identity. Some clones, for example *Hin*dIII-EF, were subcloned for further studies. The cloned viral REN DNA fragments cover approximately 99% of the viral genome.

Some of the co-migrating REN fragment clones could not be separated or identified by size upon agarose gel electrophoresis. These REN fragment clones were identified by double digestion or by Southern blot hybridization analysis on the basis of differential hybridization patterns. For example, a number of *Pst*I restriction fragment clones containing insert fragments of

the same size as *Pst*I-DE were derived by shotgun cloning. When these clones were double digested with *Hin*dIII and *Pst*I and separated by agarose gel electrophoresis with MacoNPV genomic DNA *Hin*dIII fragments as markers, two restriction patterns were revealed: clones pMc-Pst9 and pMc-Pst3.2 contain fragments comigrating with *Hin*dIII-I and -Y, while clones pMc-Pst1, pMc-Pst21, and pMc-Pst23 contain fragments comigrating with *Hin*dIII-M, -V, -W, and -bc (Figure 4.3), indicating that both types of clones are authentic viral DNA representing clones of both of the *Pst*I-DE fragments. Another example is an *Eco*RI cloned fragment of the size of *Eco*RI-DE (pMc-Eco3) which was confirmed as an authentic viral DNA fragment when the fragment clone was double digested with *Eco*RI+*Hin*dIII and separated by agarose gel electrophoresis with MacoNPV genomic DNA *Hin*dIII fragments as markers. This fragment clone contained REN fragments comigrating with *Hin*dIII-R, -W, -bc, and -e (Figure 4.4).

When double digestion was insufficient for separating co-migrating fragment clones, cross hybridization between two clones was performed. For example, GelScan XL analysis (data not shown) showed that *Pst*I-V and -W are comigrating REN fragments. A number of *Pst*I fragment clones of this size were obtained by shotgun cloning, and miniprep DNA from some of these clones were used as probes in Southern blot hybridization analysis. Southern blot hybridization results showed that both pMc-Pst4 and -Pst22 fragment clones hybridized to the *Pst*I-VW fragments in MacoNPV-90/2 DNA digests, indicating that both are authentic viral

Figure 4.3. Double-digest analysis of MacoNPV *Pst*I-DE fragment clones. Two MacoNPV-*Pst*I fragment clones digested with *Pst*I (lane 2, pMC-Pst-1; lane 3, pMcPst-9) or with *Pst*I+*Hin*dIII (lane 5, pMC-Pst-1; lane 6, pMc-Pst-9) were electrophoresed on 0.6% agarose gel, with MacoNPV-90/2 genomic DNA digested with *Pst*I (lane 1) and *Hin*dIII (lane 4) as standards (negative image). The two *Pst*I REN fragment inserts co-migrated with *Pst*I-DE (lane 2 and 3). Clone pMc-Pst1 double digested with *Pst*I and *Hin*dIII contain fragments comigrating with *Hin*dIII-M, -V, -W, and -bc (lane 5), while clone pMc-Pst9 double digested with *Pst*I and *Hin*dIII contain fragments comigrating with *Hin*dIII-I and -Y (lane 6), indicating that both were authentic viral DNA clones representing both *Pst*I-DE fragments. The plasmid band is indicated with an arrow.

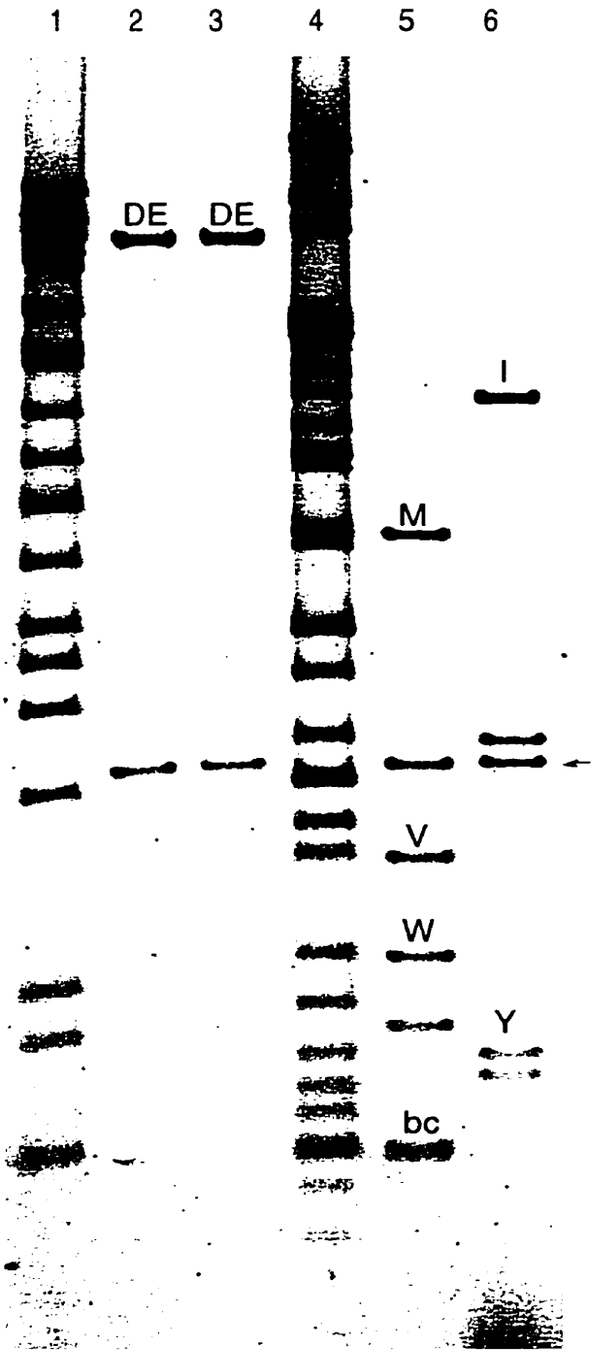
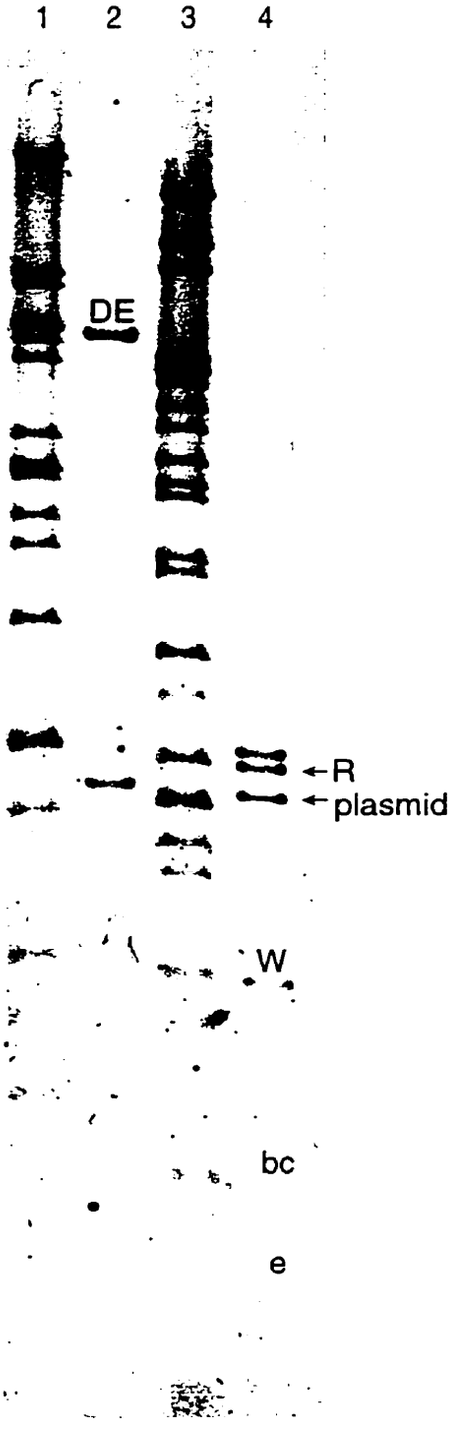

1
2
3
4
5
6
DE
DE
I
M
V
W
Y
bc

Figure 4.4. Double digest analysis of an MacoNPV EcoRI-DE fragment clone. An MacoNPV-*Eco*RI fragment clone (pMc-Eco3) digested with *Eco*RI (lane 2) or *Eco*RI+*Hin*dIII (lane 4) was electrophoresed on 0.6% agarose gel, using MacoNPV-90/2 genomic DNA digested with *Eco*RI (lane 1) and *Hin*dIII (lane 3) as standards (negative image). pMc-Eco3 was of the size of *Eco*RI-DE (lane 2) and contained REN fragments comigrating with *Hin*dIII-R, -W, -bc, and -e (lane 4).


1
2
3
4
DE
R
plasmid
W
bc
e

*Pst*I-VW fragment clones. Also, both of these cloned fragments hybridized to *Bam*HI-A, *Eco*RI-DE, *Hin*dIII-G, *Sma*I-B, *Xho*I-LM and *Bam*HI+*Sma*I-E. Those fragment clones, which were indistinguishable from each other based on the hybridization patterns with MacoNPV genomic DNA blots, were identified by Southern blot hybridization of one cloned fragment with another. In the above case, the pMc-Pst4 and -Pst22 did not hybridize with each other (Figure 4.5), indicating that pMc-Pst4 and -Pst22 represent distinct *Pst*I-VW fragment clones. The clones of the comigrating viral REN fragment *Eco*RI-UV were identified and confirmed in the same way.

Very small viral REN DNA fragments stained very faintly on agarose gels. Some fragments (<0.7 kbp) were cloned and identified as follows. There are three *Eco*RI fragments of about 0.5 kbp, as indicated by the densitometric scan of an *Eco*RI digest pattern of the viral genome (Figure 4.6). DNA separating in this region of an agarose gel was excised from the gel, purified with GeneClean (GIBCO BRL) and cloned into a pUC18 plasmid. Numerous white, recombinant colonies were generated. Mini-prep DNA from 30 of these colonies (including: pMc-Eco7, -Eco8, -Eco29, -Eco30, and -Eco32-57) were digested with *Eco*RI, separated by agarose gel electrophoresis with *Eco*RI digested MacoNPV-90/2 genomic DNA fragments as standards, and blotted onto nylon membranes. Mini-prep DNA from three of these clones (pMc-Eco7, -Eco8 and -Eco53) were used as probes to screen the plasmid clones. These clone probes did not hybridize to each other in

Figure 4.5. Discrimination of MacoNPV *Pst*I-VW fragment clones. A series of MacoNPV *Pst*I fragment clones pMc-Pst4 (P4), -Pst7 (P7), etc, were digested with *Pst*I, separated on 0.6% agarose gels, and blotted on nylon membranes (PhotoGene™, GIBCO-BRL). pMc-Pst4 and -Pst22 fragment clones, both authentic MacoNPV PstI-VW fragments, were used as hybridization probes on panel A and B, respectively.

The pMc-Pst4 fragment probe hybridized to the insert band of pMc-Pst4 and -Pst7 (lane 1 and 2, panel A), indicating these two clones represent the same MacoNPV *Pst*I-VW fragment. pMc-Pst4 probe did not hybridize to the insert band of pMc-Pst22; and fragment probe pMc-Pst22 did not hybridize to the insert band of pMc-Pst4 (lane 1, panel B), indicating that pMc-Pst4 and pMc-Pst22 are distinct *Pst*I-VW fragment clones. The pMc-Pst22 fragment probe also hybridized to the insert band in pMc-Pst40, -Pst41, -Pst42, and -Pst43 (lanes 5-7, panel B), indicating these fragment clones represent the same viral fragment as pMc-Pst22. A band of plasmid origin (pUC18) was indicated by an arrow.

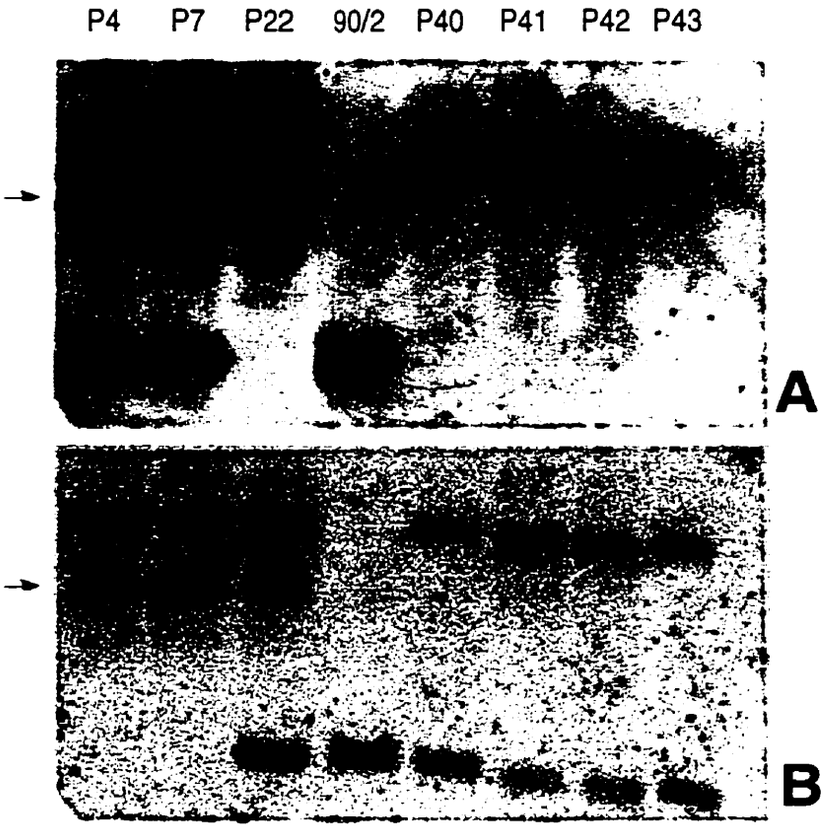
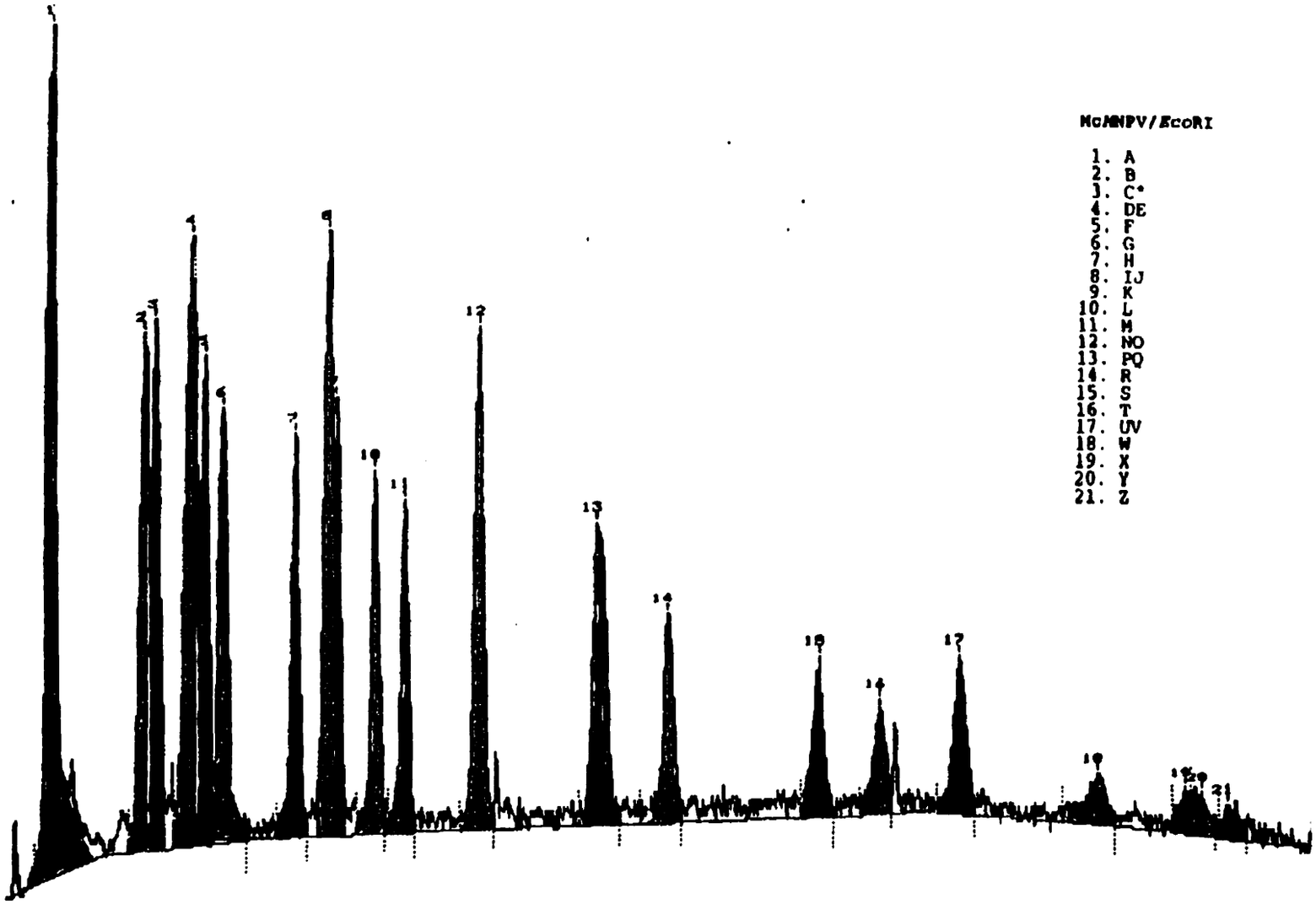

P4
P7
P22
90/2
P40
P41
P42
P43
A
B

Figure 4.6. A densitometer scan of agarose gel representing MacoNPV/*Eco*RI fragments analyzed by the GelScan XL software.


HcMNPV/EcoRI
1. A
2. B
3. C
4. DE
5. F
6. G
7. H
8. IJ
9. K
10. L
11. M
12. NO
13. PQ
14. R
15. S
16. T
17. UV
18. W
19. X
20. Y
21. Z

terms of the insert bands but they all hybridized to MacoNPV-90/2 genomic DNA fragments of an appropriate size (Figure 4.7), indicating that three different viral fragments had been cloned. Based on Southern blot hybridization data, three subsets of clones could be identified each representing one of the *Eco*RI-X, -Y, -Z fragments.

4.2.3. Southern Blot Hybridization and Physical Mapping

4.2.3.1. Hybridization Results and the Alignment of Restriction Endonuclease Fragments

The REN fragment map of the MacoNPV DNA genome was pieced together based on the concept that fragments from different REN digests sharing common nucleotide sequences will map to the same region of the viral genome. For example, the cloned MacoNPV *Hin*dIII-O fragment hybridized to *Eco*RI-B, -M, -T; *Hin*dIII-OP; *Pst*I-G; *Xho*I-B, -G; and *Bam*HI+*Sma*I-A (which equivalent to *Bam*HI-A and *Sma*I-A) (Figure 4.8). These fragments were eventually mapped to the same region on the viral genome (Figure 4.9). The results of similar hybridization experiments using biotin-labelled MacoNPV REN cloned fragments as probes to Southern blots of MacoNPV-90/2 DNA digested with various restriction endonuclease enzymes are shown in Tables 4.2 and 4.3).

The hybridization data generated using the *Hin*dIII fragment clones and *Hin*dIII-A DNA eluted from a preparative gel were used to align the REN fragments (Table 4.2). For example, clone pMcHind-EF (clone 157) fragment hybridized to *Xho*I-JK, -LM and to a

Figure 4.7. Southern blot analysis of subsets of MacoNPV *Eco*RI-XYZ fragment clones. MacoNPV-90/2 genomic DNA and MacoNPV *Eco*RI fragment clones digested with *Eco*RI were separated on 0.6% agarose gel and blotted on nylon membranes (PhotoGene™, GIBCO-BRL). The authentic genomic insert fragments were identified by comparison to the same blot probed with biotin-labelled total MacoNPV DNA.

Three fragment clones, pMc-Eco7 (panel A), pMc-Eco8 (panel B), and pMc-Eco53 (panel C), were used as probes in Southern blot hybridization. Each of these probes hybridized to MacoNPV-90/2 EcoRI-XYZ fragment region. pMc-Eco7 probe hybridized to pMc-Eco43, -47, -49 to -52, -54, and -55 (panel A); pMc-Eco8 probe hybridized to pMc-Eco56 and -57 (panel B); and pMc-Eco53 probe hybridized to pMc-Eco44 to -46, -48, -52, and -53 (panel C). pMc-Eco52 hybridized to both pMc-Eco7 (panel A) and pMc-Eco53 (panel C), indicating it may contain double insert fragments.

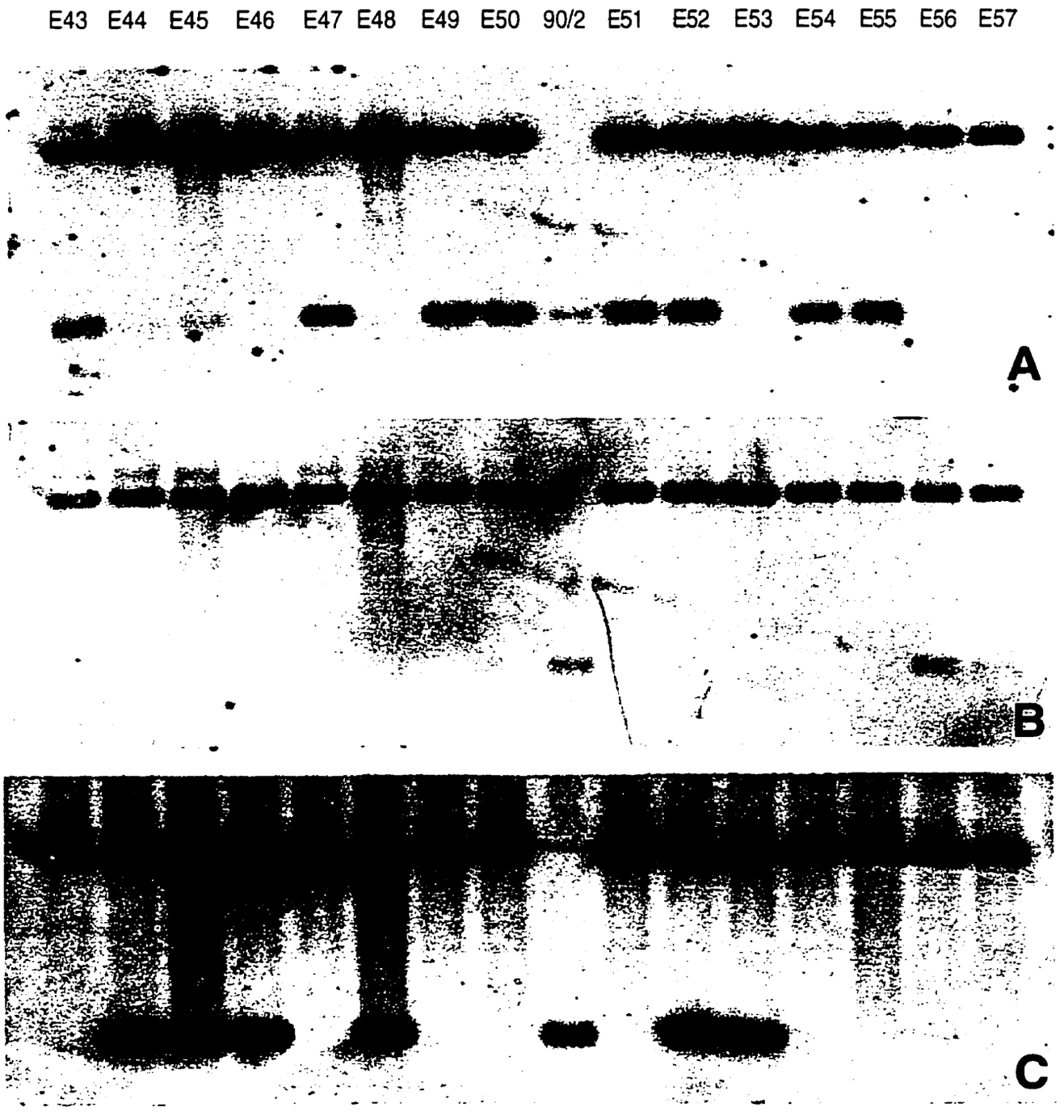

E43 E44 E45 E46 E47 E48 E49 E50 90/2 E51 E52 E53 E54 E55 E56 E57
A
B
C

Figure 4.8. Southern blots of REN digested MacoNPV DNA probed with *in vitro* labelled cloned viral fragments. MacoNPV genomic DNA was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), *Pst*I (lane 3), *Bam*HI+*Sma*I (lane 4), and *Xho*I (lane 5), electrophoresed on 0.6% gels, and the separated DNA fragments blotted onto nylon membranes (PhotoGene™, GIBCO-BRL). The membranes were prehybridized overnight at 42°C in prehybridization solution. Nick-translated DNAs, labelled with biotin, were used as probes (Panel A, total genomic DNA; Panel B, pMc-Hind-O fragment cloned in pUC18 plasmid vector) to the immobilized DNA on the nylon membrane by hybridizing overnight at 42°C in hybridization solution. The membranes were washed at the appropriate temperature and salt concentration to remove non-specifically bound DNA probe. The biotin-labelled DNA probes were detected on hybridized membranes using a PhotoGene Detection System (GIBCO-BRL). The fragments hybridizing to MacoNPV clone probes were identified by comparison with the same blot probed with biotin-labelled total MacoNPV DNA.

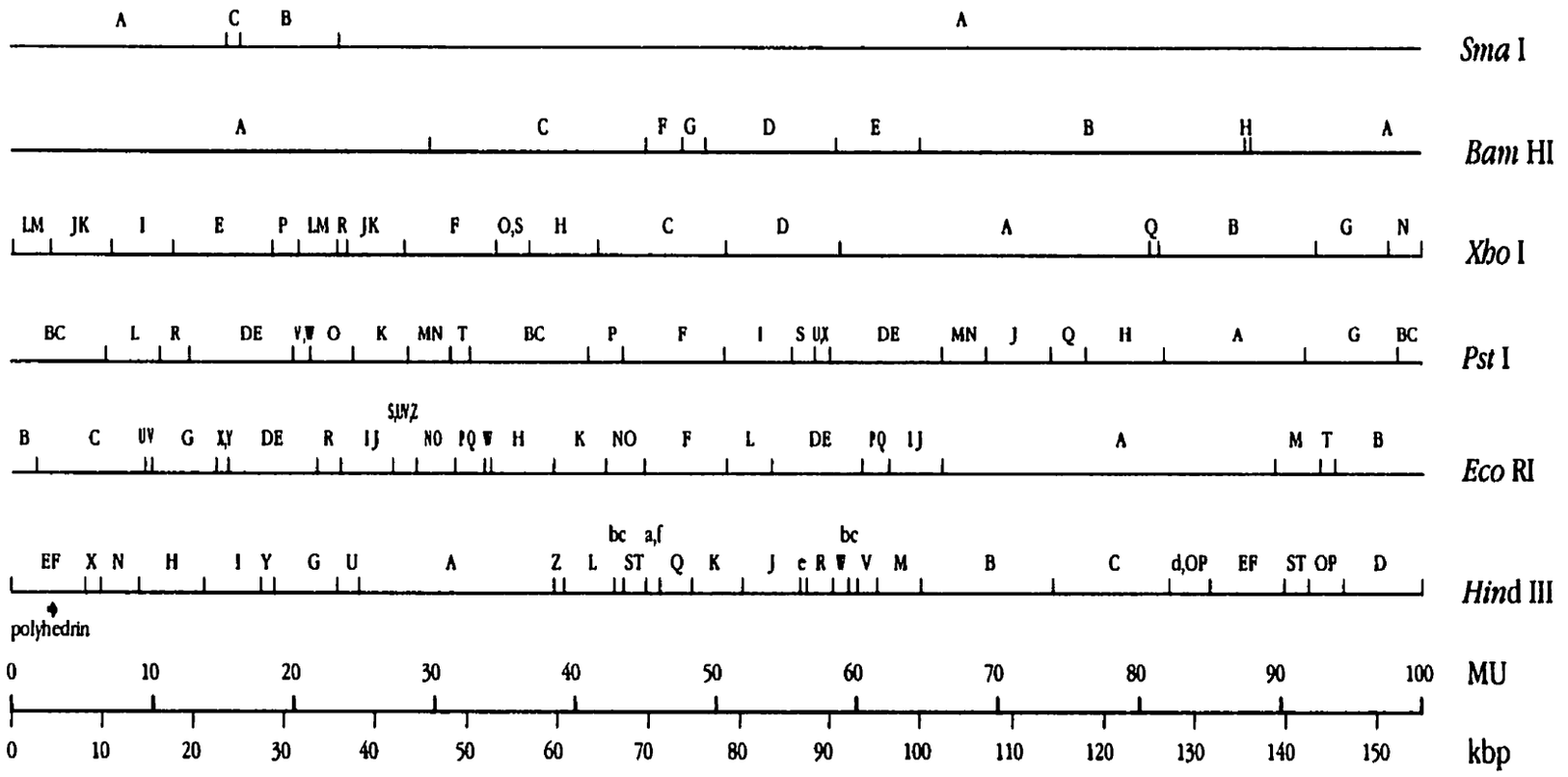
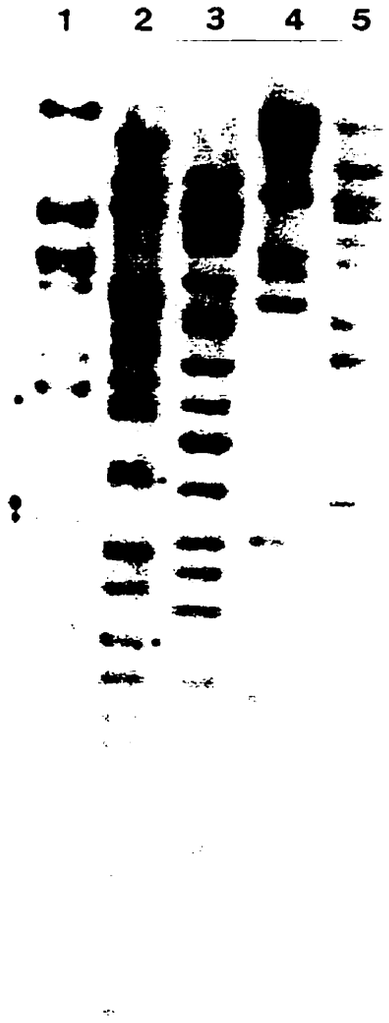
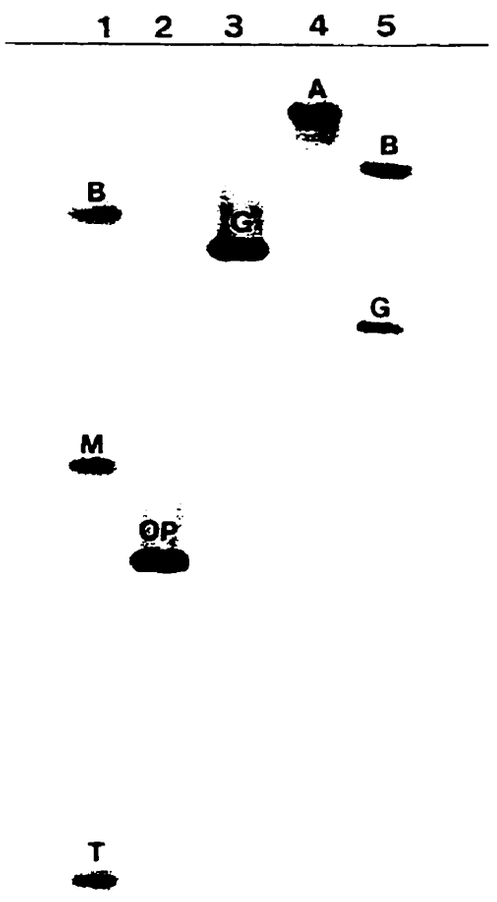

A C B A
Sma I
A C F G D E B H A
Bam HI
LM JK I E P LM R JK F O,S H C D A Q B G N
Xho I
BC L R DE V,W O K MN T BC P F I S U,X DE MN J Q H A G BC
Pst I
S,U,Z
B C UV G X,Y DE R I J NO P Q W H K NO F L DE P Q I J A M T B
Eco RI
bc a,f bc
EF X N H I Y G U A Z L ST Q K J e R W V M B C d,OP EF ST OP D
Hind III
polyhedrin
0 10 20 30 40 50 60 70 80 90 100
MU
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
kbp

Figure 4.9. Physical map of MacoNPV-90/2. The map shows the positions of the cleavage sites of six different REN (*Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sma*I, and *Xho*I) on a linear map of MacoNPV. A scale in kbp and one in map units are presented at the bottom. The entire genome is 156 kbp, and 1 map unit is 1.56 kbp. The zero point for the physical map was determined by hybridizing the AcMNPV *Hin*dIII-V fragment clone, containing a portion of the AcMNPV polyhedrin gene, with the genomic blots of MacoNPV DNA.

The relative order of the MacoNPV DNA restriction fragments on the linearized physical map was determined from the Southern blot hybridization results using the cloned MacoNPV DNA fragments. Each restriction site is indicated with a vertical line.



A



B

Table 4.2. Southern blot hybridization to MacoMNPV-90/2 restriction fragments using cloned *Hin*dIII fragments as probes .

		Restriction fragment						
Probe fragment	clone#	*Bam*HI	*Eco*RI	*Hin*dIII	*Pst*I	*Sma*I	*Xho*I	*Bam*HI+*Sma*I
*Hin*dIII-EF	157	A	C,B	EF	BC	A	JK,LM,N	A
*Hin*dIII-D	230-6	A	B	D	BC,G	A	N,G	A
*Hin*dIII-OP	346-2	A	B,T,M	OP	G	A	G,B	A
*Hin*dIII-ST	117	A	M	ST	G,A	A	B	A
*Hin*dIII-EF	154	A,H,B	M,A	EF	A	A	B	A,K,B
*Hin*dIII-OP	133	B	A	OP	A	A	B	B
*Hin*dIII-d	345	B	A	d	A	A	B	B
*Hin*dIII-C	211	B	A	C	A,H,Q	A	B,Q,A	B
*Hin*dIII-B	150	B	A,IJ	B	Q,J,MN,DE	A	A	B
*Hin*dIII-M	151	B,E	IJ	M	DE	A	A	B,F
*Hin*dIII-V	212	E	PQ,DE	V	DE	A	A	F
*Hin*dIII-bc	132	E	DE	bc	DE	A	A	F
*Hin*dIII-W	244	E,D	DE	W	DE	A	A,D	F,D
*Hin*dIII-R	EH2	D	DE	R	DE,U,X,S	A	D	D
*Hin*dIII-e	404	D	DE	e	S	A	D	D
*Hin*dIII-J	144	D	DE,L	J	S,I	A	D	D
*Hin*dIII-K	125	D,G	L,F	K	I,F	A	D,C	D,I
*Hin*dIII-Q	149	G,F	F	Q	F	A	C	I,H
*Hin*dIII-a	161	F	F	a	F	A	C	H
*Hin*dIII-f	286-4	F	F	f	F	A	C	H
*Hin*dIII-ST	142	F,C	F,NO	ST	F	A	C	H,C
*Hin*dIII-bc	140	C	NO	bc	F,P	A	C	C
*Hin*dIII-L	233-3	C	NO,K	L	P,BC	A	C,H	C
*Hin*dIII-Z	122	C	K	Z	BC	A	H	C
*Hin*dIII-A		C,A	K,H,W,PQ,NO,S,UV,Z,IJ	A	BC,T,MN,K	A	H,O,S,F,JK	C,G
*Hin*dIII-U	224	A	IJ,R	U	K,O	A	JK,R	G
*Hin*dIII-G	145	A	R,DE	G	O,V,W,DE	A,B	R,LM,P,E	G,E
*Hin*dIII-Y	162	A	DE	Y	DE	B	E	E
*Hin*dIII-I	152	A	DE,X,Y,G	I	DE	B,C,A	E	E,J,A
*Hin*dIII-H	128	A	G,UV	H	DE,R,L	A	E,I	A
*Hin*dIII-N	134	A	C	N	L,BC	A	I,JK	A
*Hin*dIII-X	130	A	C	X	BC	A	JK	A

Table 4.3. Southern hybridization to MacoNPV-90/2 restriction fragments using other cloned fragments as probes.

	Restriction fragment						
Probe fragment	*Bam*HI	*Eco*RI	*Hin*dIII	*Pst*I	*Sma*I	*Xho*I	*Bam*HI+*Sma*I
*Eco*RI-DE	E,D	DE	V,bc,W,R,e,J	DE,U, X,S,I	A	A,D	F,D
*Eco*RI-K	C	K	L,Z,A	P,BC	A	C,H	C
*Eco*RI-H	C	H	A	BC	A	H,O,S	C
*Eco*RI-W	C	W	A	BC	A	F	C
*Eco*RI-PQ	C	PQ	A	BC,T	A	F	C
*Eco*RI-NO	C	NO	A	T,MN	A	F	C
*Eco*RI-UV	A	UV	A	K	A	JK	G
*Eco*RI-Z	A	Z	A	K	A	JK	G
*Eco*RI-IJ	A	IJ	A,U	K,O	A	JK,R	G
*Eco*RI-X	A	XY	I	DE	C	E	J
*Eco*RI-Y	A	XY	I	DE	C	E	J
*Eco*RI-UV		UV	H				
*Pst*I-J	B	A	B	J	A	A	B
*Pst*I-DE	B,E,D	IJ,PQ ,DE	B,M,V,bc,W,R	DE	A	A,D	B,F,D
*Pst*I-X	D	DE	R	X	A	D	D
*Pst*I-T	C	PQ,NO	A	T	A	F	C
*Pst*I-VW	A	DE	G	VW	B	LM	E
*Pst*I-VW	A	DE	G	VW	B	LM	E
*Pst*I-DE	A	DE,X,Y,G	G,Y,I,H	DE	B,C,A	P,E	E,J,A
*Xho*I-Q	B	A	C	H	A	Q	B
*Xho*I-O	C	H	A	BC	A	O	C
*Xho*I-S	C	H	A	BC	A	S	C
*Xho*I-JK	A	IJ	A,U	K	A	JK	G
*Xho*I-R	A	IJ,R	U,G	O	A	R	G
pAcMNPV-*Hin*dV	A	C	EF	BC	A	JK	A

lesser degree to *Xho*I-N. The MacoNPV-90/2 *Hin*dIII-D cloned fragment also hybridized to *Xho*I-N as well as to *Xho*I-G. Thus, it was determined that *Hin*dIII-D was next to *Hin*dIII-EF (clone 157). In this fashion the alignment of the majority of REN fragments from the various enzyme digests of MacoNPV genomic DNA was determined. A physical map of the viral genome was constructed based on the above hybridization results (Figure 4.9).

For regions of the genome in which *Hin*dIII clones were not sufficient to define the order of REN fragments, cloned REN fragments from other enzyme digests were used as hybridization probes to determine REN alignments in detail (Table 4.3).

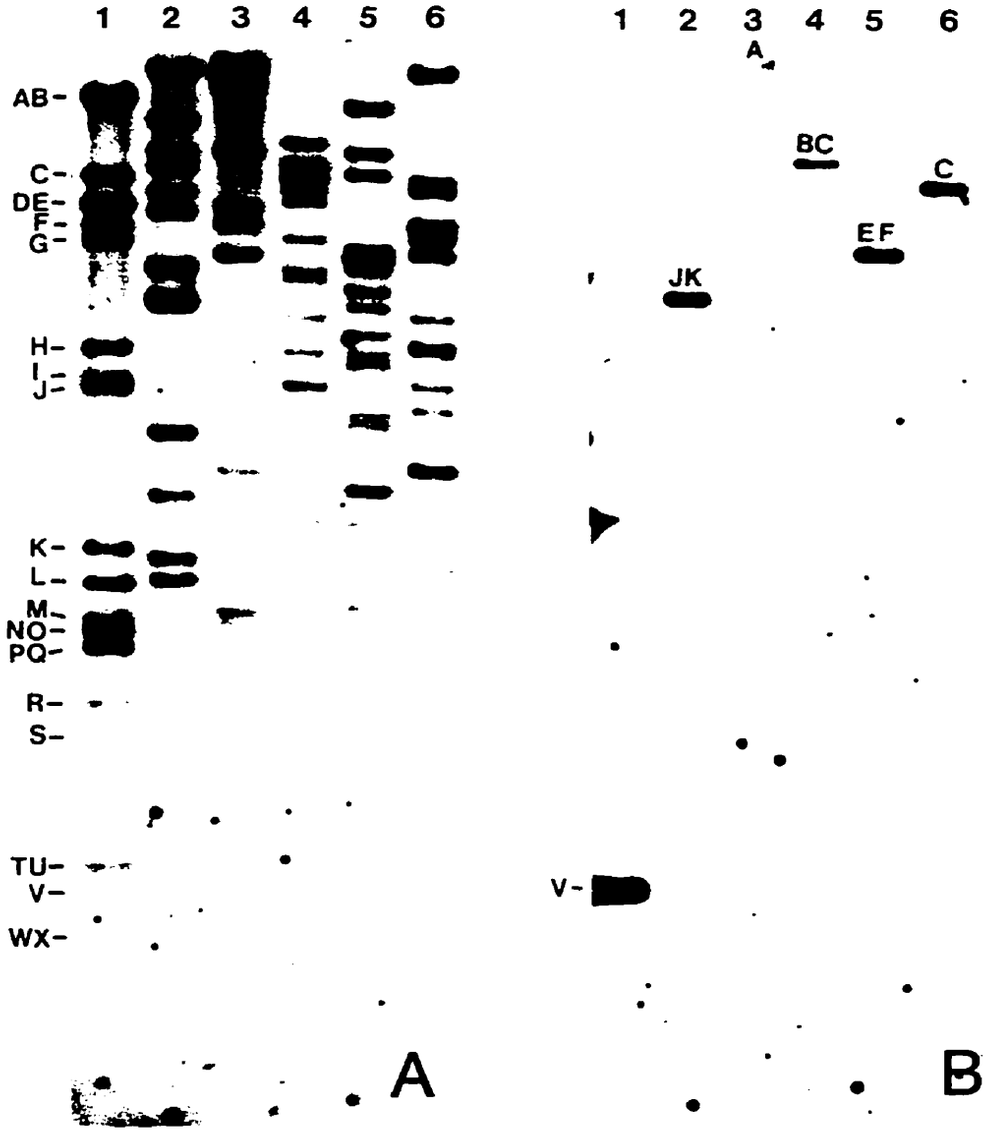
The exact order of certain small restriction enzyme fragments could not be determined from the above hybridization data. For example, both *Hin*dIII-a and *Hin*dIII-f hybridized to *Bam*HI-F, *Eco*RI-F, *Pst*I-F, *Sma*I-A, and *Xho*I-C (Table 4.2), and their relative position could not be determined from the results. As well, clones of both *Pst*I-VW fragments (clones pMc-Pst4 and pMc-Pst22) hybridized to *Bam*HI-A, *Eco*RI-DE, *Hin*dIII-G, *Sma*I-B and *Xho*I-E (Table 4.3). As discussed earlier, although their relative position could not be determined, reciprocal hybridization results demonstrated that both fragments had been cloned. Those fragments for which the exact order could not be determined were positioned on the physical map using the convention of Vlak and Smith (1982) using multiple letters separated by commas (e.g., *Hin*dIII a,f).

4.2.3.2. Zero Point of the Physical Map

Southern blot hybridization using the pAc*Hin*dIII-V clone, which contains the middle and the 3' end of the AcMNPV polyhedrin gene, as a probe under low stringency hybridization conditions (25% formamide) indicated that sequences homologous to the AcMNPV polyhedrin gene were located on the MacoNPV-90/2 *Xho*I-JK, *Bam*HI+*Sma*I-A, *Pst*I-BC, *HindIII*-EF, and *Eco*RI-C fragments, respectively (Figure 4.10). Thus, the junction of *Hin*dIII fragments D and EF (clone 157) was designed as the zero point of the linearized physical map. The clockwise orientation of the other *Hin*dIII fragments was set as -X, -N, -H, etc., with polyhedrin transcription from left to right (Figure 4.9).

A portion of the cloned McMNPV *Hin*dIII-EF fragment (clone 157) identified as containing the polyhedrin gene based on Southern blot hybridization was subcloned and sequenced. Figure 4.11 shows the subcloning and sequencing strategies (nucleotide sequencing was done in Plant Biotechnology Institute, National Research Council Canada). An ORF coding for a 246 aa protein was identified. The highly conserved 12-nt consensus sequence shared by baculovirus very late gene promoters was identified from the 5' flanking sequences of this ORF, followed by an AT-rich (75% A+T) sequence (Figure 4.12). The ORF showed greater than 95% and 85% DNA sequence identity with polyhedrin genes from MNPV isolated from two insects closely related to *M. configurata* - *P. flammea* and *M. brassicae*, respectively. The putative MacoNPV polyhedrin protein showed 98%, 97%, and 87% aa sequence homology

Figure 4.10. Hybridization of AcMNPV *HindIII*-V fragment with genomic blot of MacoNPV. AcMNPV genomic DNA digested with *Hin*dIII (lane 1) and MacoNPV DNA digested with *Xho*I (lane 2), *Bam*HI+*Sma*I (lane 3), *Pst*I (lane 4), *Hin*dIII (lane 5) or *Eco*RI (lane 6), electrophoresed on 0.6% agarose gels, and the separated DNA fragments were blotted onto nylon membranes (PhotoGene™, GIBCO-BRL). The Southern blot membranes were hybridized either with a mixed MacoNPV+AcMNPV DNA probe (panel A) or with pAc*Hin*dIII-V, a recombinant clone containing the 3' end of the AcMNPV polyhedrin gene (panel B). Panel B hybridization was done under low stringency conditions. The AcMNPV polyhedrin clone hybridized to MacoNPV DNA fragments *Xho*I-JK, *Bam*HI+*Sma*I-A, *Pst*I-BC, *Hin*dIII-EF and *Eco*RI-C.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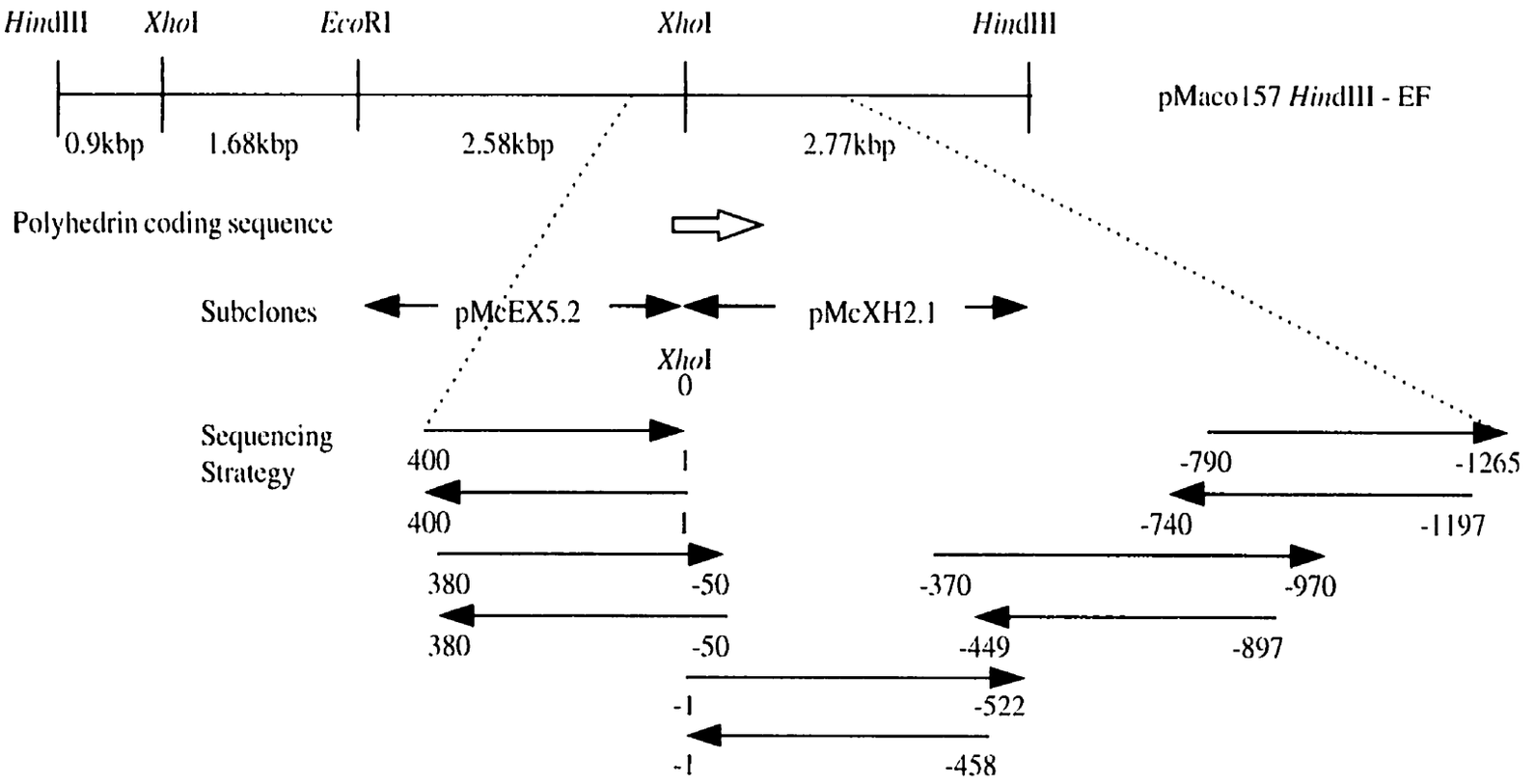


Figure 4.11. The strategies for subcloning and sequencing of MacoNPV polyhedrin gene region.

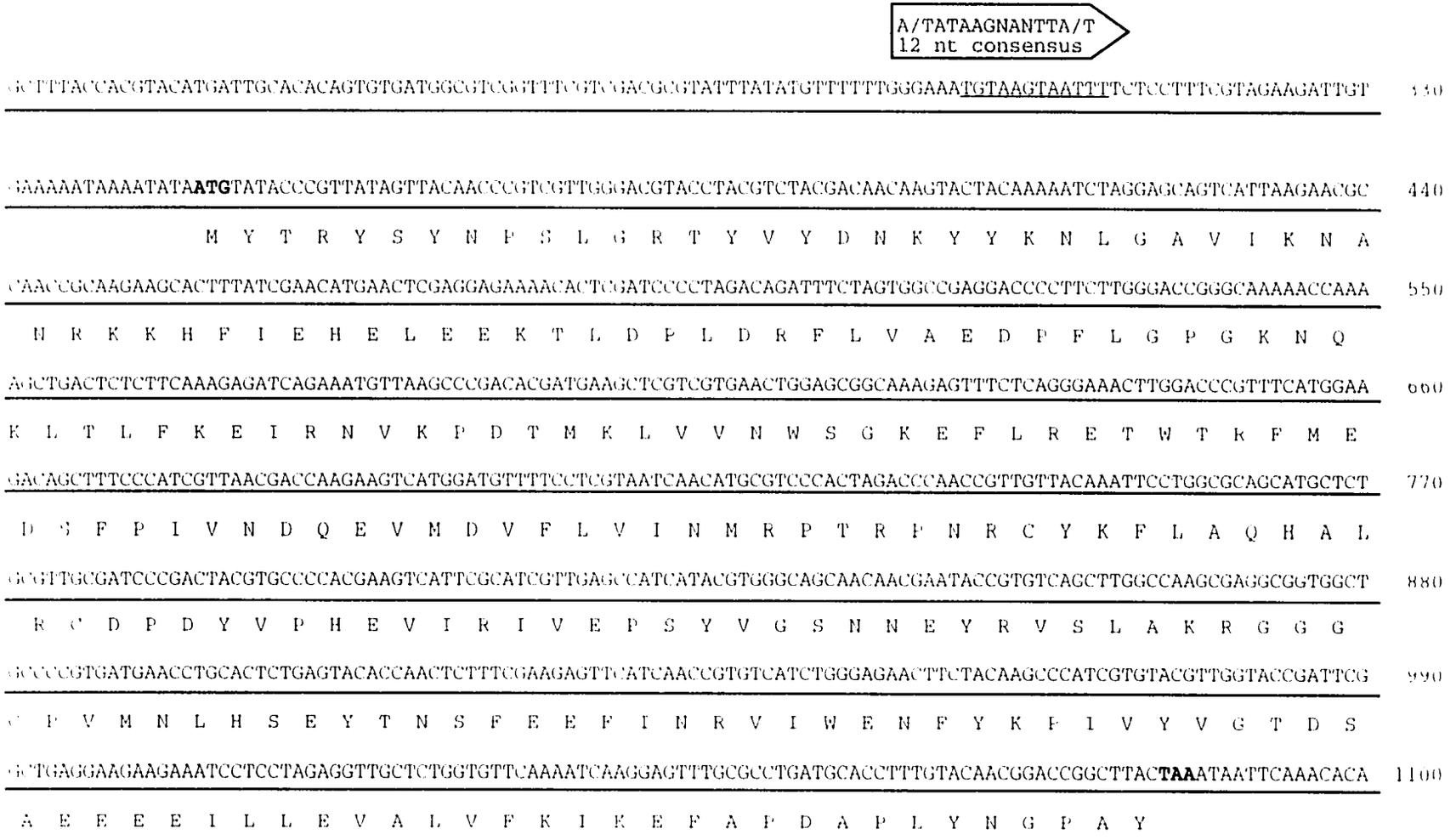


Figure 4.12. Nucleotide and putative amino acid sequence of MacoNPV polyhedrin gene. The highly conserved 12 nt consensus shared by baculovirus very late gene promoters was underlined, and the transcription start and termination codons are bolded.

with the polyhedrin protein of PaflNPV, MbMNPV, and AcMNPV, respectively (Figure 4.13). Up stream and down stream from MacoNPV polyhedrin gene, ORF sharing homology with MbMNPV and AcMNPV have also been identified.

```
MacoNPV         (   1)   MYTRYSYNPSLGRTYVYDNKYYTNLGAVIKNANRKNHFIEHVLEEKTLDP
PaflNPV         (   1)   ....................K...........K.....E........
MbMNPV          (   1)   ....................K...S........R.Y...E........
AcMNPV          (   1)   .-PD...R.TI..........K.........K..K..A..EI..A....

MacoNPV         (  51)   LDRFLVAEDPFLGPGKNQKLTLFKEIRNVKPDTMKLVVNWSGKEFLRETW
PaflNPV         (  51)   ...Y.....................................................
MbMNPV          (  51)   ...Y.....................................................
AcMNPV          (  50)   ..NY................................G.K....Y....

MacoNPV         ( 101)   TRFMEDSFPIVNDQEVMDVFLVINMRPTRPNRCYKFLAQHALRCDPDYVP
PaflNPV         ( 101)   ..........................................E...
MbMNPV          ( 101)   ..............................F..................
AcMNPV          ( 100)   ..S..................V.........................

MacoNPV         ( 151)   HEVIRIVEPSYVGSNNEYRVSLAKRGGGCPVMNLHSEYTNSFEEFINRVI
PaflNPV         ( 151)   ..................................................
MbMNPV          ( 151)   ..................................................
AcMNPV          ( 150)   .D........W........I....K.....I...........Q..D...

MacoNPV         ( 201)   WENFYKPIVYVGTDSAEEEEILLEVALVFKIKEFAPDAPLYNGPAY
PaflNPV         ( 201)   ........................S...................
MbMNPV          ( 201)   ........................S...................
AcMNPV          ( 200)   ..........I.............S....V.........FT....
```

Figure 4.13. Amino acid comparison of the putative MacoNPV polyhedrin protein with that of PaflNPV, MbMNPV, and AcMNPV. Identical sequence is indicated by a ".", absent sequence is indicated by a "-".

5. Description of the Geographic Isolates and Pick Plaque Isolates.

MacoNPV isolates collected from various geographic areas differ in terms of their virulence to bertha armyworm, with the LD_{50} for neonate bertha armyworm ranging from 11.9 to 73.5 OB/larva (Erlandson, unpublished data). These MacoNPV geographic isolates were preliminarily characterized by REN analysis.

The isolate MacoNPV-90/4, which contains the most variation in REN bands and theoretically provides the best chance for selection of viral strains with different REN patterns, was used for an investigation of genomic variation and for pick plaque purification.

Additional fragments compared to MacoNPV-90/2 were designated by numbers following the nearest larger REN fragment that appeared in MacoNPV-90/2 isolate REN patterns. For example, the extra fragments between *Eco*RI-A and *Eco*RI-B were named *Eco*RI-A1, -A2, and -A3, respectively. The additional fragment which is bigger than the biggest fragment of -90/2 isolate was designated as -A* (e.g. *Pst*I-A* in -90/4 isolate). It must be noted that in this system, extra fragments with the same designation but from different isolates (e.g., A1 from MacoNPV-90/3 and -90/4) are not considered identical.

To further study the possible relationship between genotypic variants and the infectivity and/or virulence of viruses, the MacoNPV-90/4 isolate was put through 2 rounds of plaque

purification, and the genomic DNA of plaque-purified strains characterized by REN analysis. These plaque isolates were divided into different categories according to the REN pattern variations compared with those of MacoNPV-90/2 and parental MacoNPV-90/4.

5.1. Materials and Methods

5.1.1. Infection of Cultured Insect Cells with ECV from Hemolymph of Virus-Infected Bertha Armyworm Larvae

A *Mamestra brassicae* (Mbr) cell line was used in the present study. The Mbr cells were kindly provided by Dr. Tom Roberts, and were maintained in Grace's tissue culture medium supplemented with 10% FBS and gentamycin at 10 µg/ml. The cells were incubated at 28°C.

Late 3rd- or early 4th-instar bertha armyworm larvae were inoculated with 5.0×10^4 OB/individual. Three days post inoculation, the larvae were surface sterilized with 70% ethanol and bled by cutting off a proleg. The hemolymph was collected directly into ice-cold Grace's tissue culture medium with gentamycin at 10 µg/ml and cysteine at 1 mM to delay melanization. The hemocytes were pelleted by centrifugation for 2 min at 14,000 xg. The supernatant of hemolymph-medium mixture containing ECV was filter-sterilized through a 0.45 µm filter.

The Mbr cells were set up at a density of 4×10^4 cell/ml, 1 ml/well in 12-well tissue culture plates. The cells were allowed to attach for 2 hr at 28°C. All but 0.1 ml of tissue culture medium in each well was removed, and 0.1 ml of filter-sterilized

hemolymph-medium mixture was added immediately. The tissue culture plate was rocked on a platform rocker for 2 hr at 28°C. A 0.8 ml aliquot of fresh tissue culture medium was then added to each well. The plate was incubated in a Tupperware container with moist filter paper for 7 days at 28°C. The cells were examined microscopically for signs of infection and were harvested into 1.5 ml microcentrifuge tubes. The tubes were spun for 3 min, and the supernatants containing the ECV were transferred to sterile 1.5 ml microcentrifuge tubes.

5.1.2. Pick Plaque Purification

For pick plaque purification, Mbr cells were seeded at 8.75 x 10^5 cells per 60 mm x 15 mm plate. The cells were incubated overnight at 28°C to form a monolayer of growing cells.

The medium was removed, and a 1.0 ml aliquot of diluted viral inoculum was immediately added to the monolayer and was incubated with intermittent rocking for 80 min at 28°C. The inoculum was then removed and the cell monolayer was washed twice with 1.5 ml of medium. Finally, 4.0 ml of overlay medium and agarose (2 volumes Grace's medium with 15% FBS and 2x concentrations gentamycin + 1 volume sterile 3% sea plaque agarose in Grace's medium without FBS) was layered on top of the cell monolayer. When the overlay solidified, the plates were incubated at 28°C in humid conditions for 5-6 days. When separate plaques were visible on the plates, they were marked and harvested with a sterile pasture pipet.

For the present study, 2 rounds of plaque purification were

performed for each pick plaque (pp) strain.

Pick plaques were replicated once in cultured cells (25 cm^2 flask of Mbr cells). The OB harvested from infected cell pellets and the ECV from infected supernatant were collected and frozen for further use.

5.1.3. REN Pattern Analysis of Viral Geographic Isolates and Pick Plaque Strains

Pass 2 pick plaque-purified viruses from insect cell culture were first used to extract DNA for REN analysis. Cell-culture-derived virus titres were too low to give sufficient DNA for extensive REN analysis. To get sufficient viral DNA for analysis, these pp strains were then used to infect late 3rd-instar and early 4th-instar bertha armyworm larvae by contamination of the diet surface with $1.4x10^4$ OB/cm^2 for virus production. Larvae dying from virus infection were pooled, and viral DNA was purified as described before.

Four REN (*Eco*RI, *Hin*dIII, *Pst*I, and *Xho*I) were used in the analysis of geographic isolates and pp strains of MacoNPV. The REN patterns of geographic isolates and pp strains were compared with MacoNPV-90/2 isolate as a standard. The genomic variants were defined as pp strains with REN fragments that differed (i.e., REN fragments with altered mobility on agarose gel or which were additional or missing) from that of MacoNPV-90/2 geographic isolate.

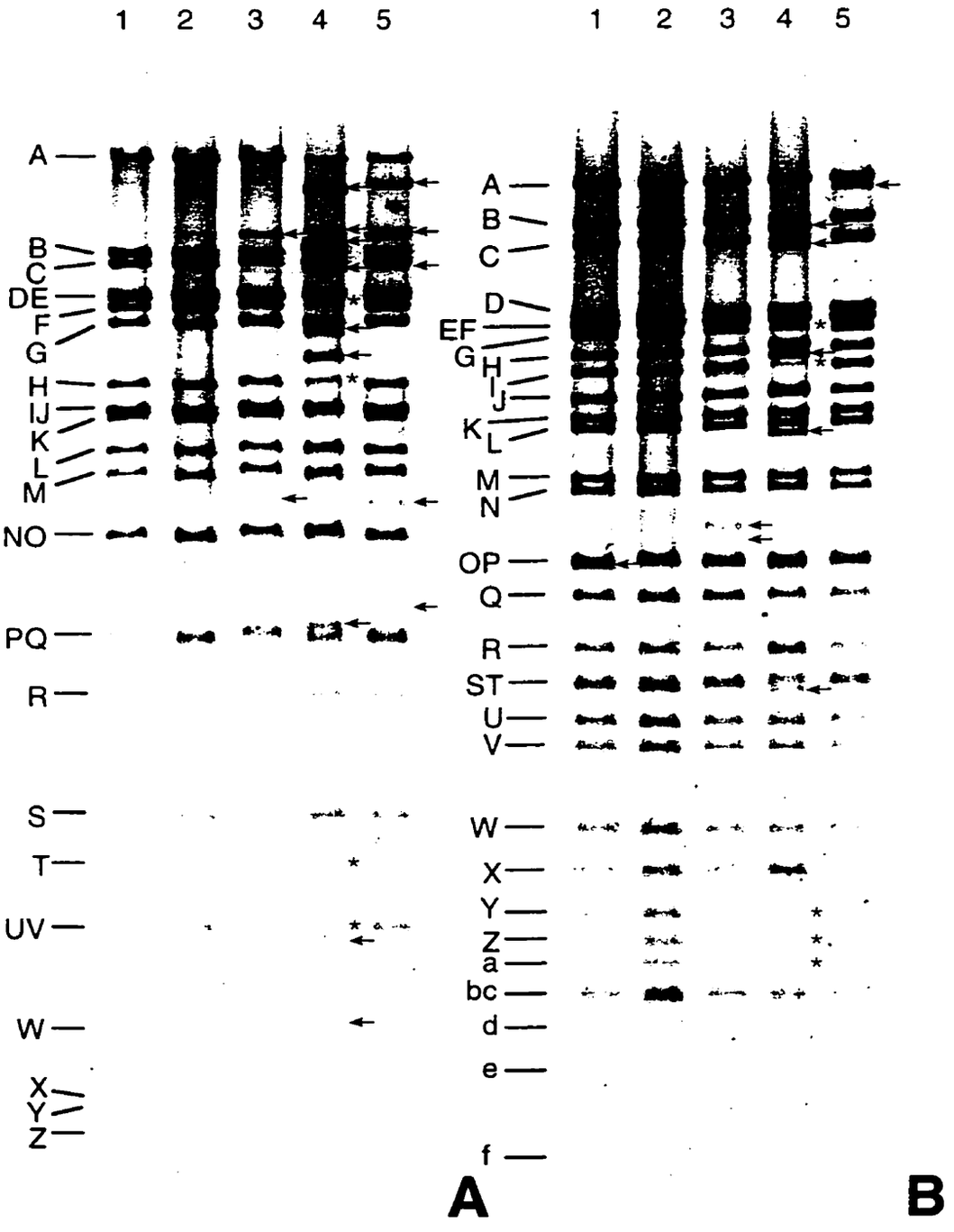
5.1.4. Analysis of Geographic Isolates

The sources of most of the variable fragments (in terms of sequence homology to specific regions of the MacoNPV-90/2 genomic map) were determined by Southern blot hybridization of MacoNPV-86/1, -90/1, -90/3, and -90/4 genomic blots with MacoNPV-90/2 fragment clones which represented about 50% of the viral genome.

5.2. Results

Figure 5.1 shows the REN restriction patterns of the five MacoNPV geographic isolates digested with *Eco*RI, *Hin*dIII, *Pst*I, and *Xho*I. Fragments in the geographic isolates which were not present in MacoNPV-90/2 (some of which are submolar bands) were designated as additional fragments. Though all the geographic isolates had similar REN restriction patterns, they differ from 90/2, in having isolate-specific additional fragments (many present in submolar ratio), or in having submolar ratios REN fragments. Additional fragments are indicated by an arrow and submolar fragments indicated by an asterisk (Figure 5.1). Two, nine, and five additional fragments were detected in the *Eco*RI digest patterns for MacoNPV-90/3, -90/4, and -86/1 geographic isolates, respectively. Similarly, one, two, and five additional fragments were detected in the *Hin*dIII REN fragment patterns of MacoNPV-90/1, 90/3 and 90/4 geographic isolates, respectively. It is possible that there are additional variations in submolar

Figure 5.1. REN restriction patterns of five MacoNPV geographic isolates. A negative image of ethidium bromide stained agarose gel of the restriction patterns of the five MacoNPV geographic isolates digested with *Eco*RI (panel A), *Hin*dIII (panel B), *Pst*I (panel C), and *Xho*I (panel D). For each REN digest, from left to right: MacoNPV-90/1 (lane 1), -90/2 (lane 2), -90/3 (lane 3), -90/4 (lane 4), and -86/1 (lane 5). Additional fragments not detected in -90/2 are indicated by an arrow. Fragments present in molar ratio in MacoNPV-90/2 but present in submolar ratio in other geographic isolates are indicated by an asterisk.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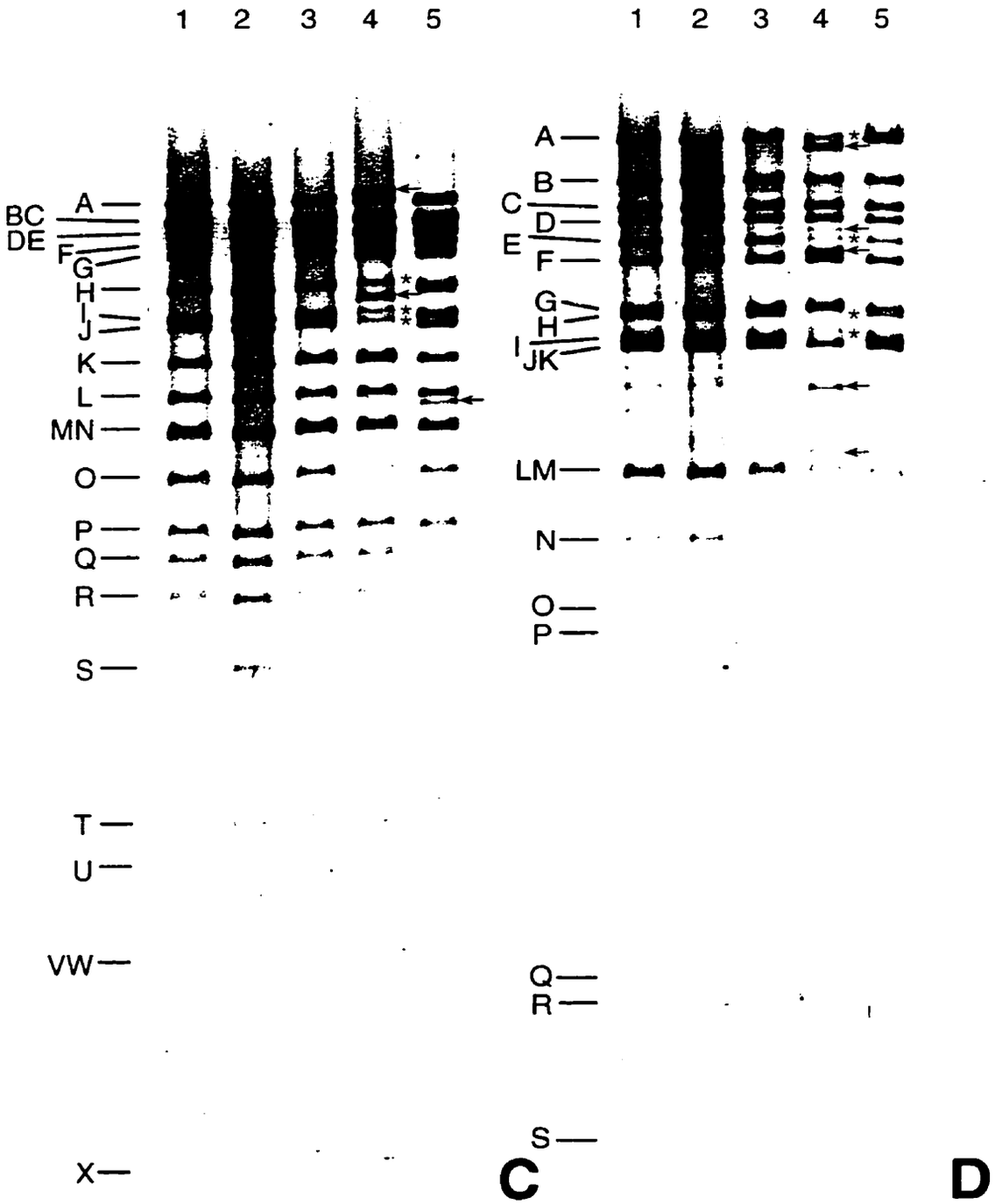
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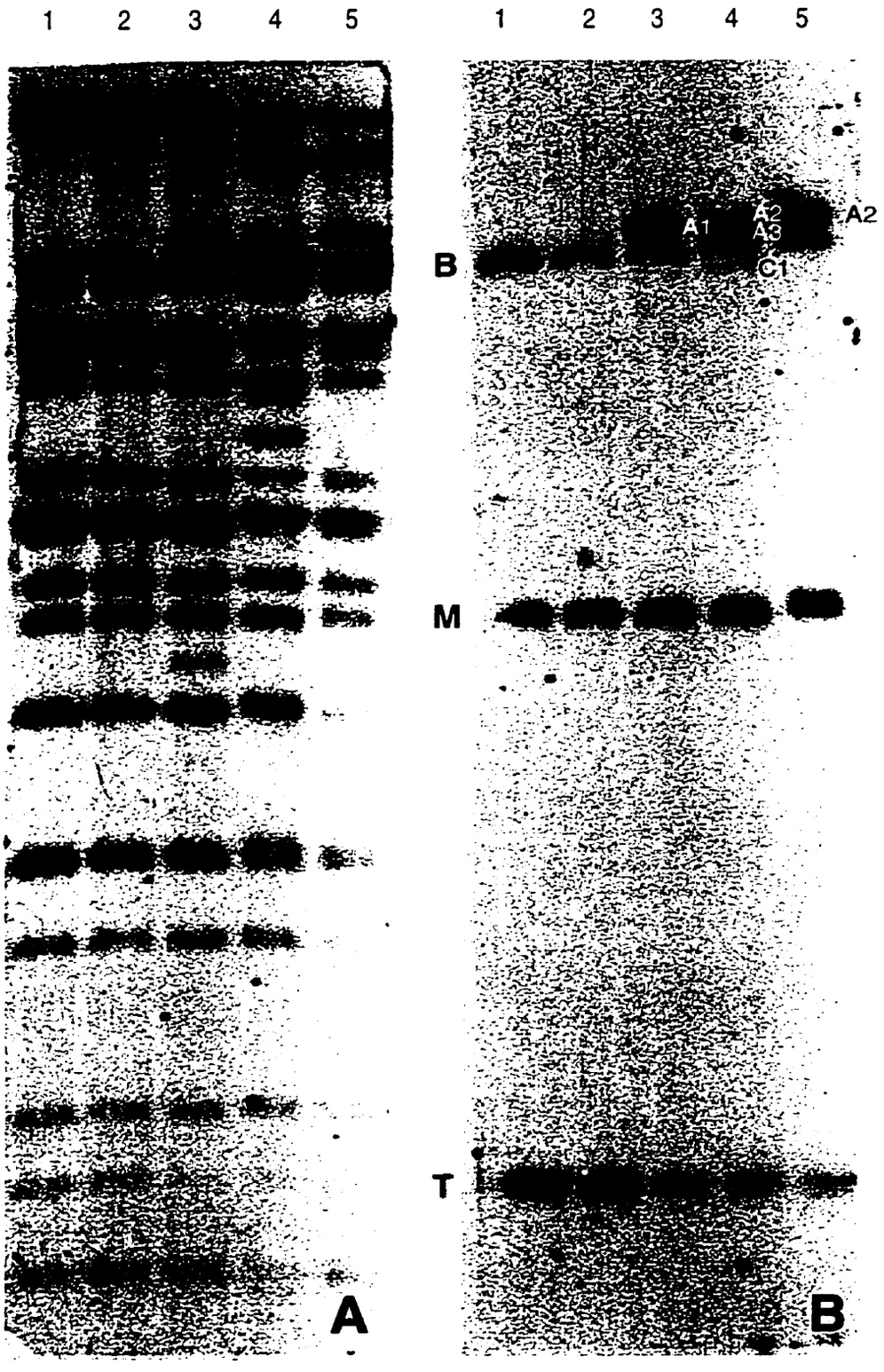
fragments which were not detectable because their representation in specific virus populations were too low, or the differences in their molar ratio were not significant enough to be detected by their ethidium bromide stain intensity. These variations became obvious when viral genomes representing these genotypes were plaque purified.

The sequence homology of most of the detectable *Eco*RI variable fragments was determined by Southern blot hybridization of MacoNPV-86/1, -90/1, -90/3, and -90/4 genomic DNA using MacoNPV-90/2 DNA REN fragment clones as probes. For example, Southern blot hybridizations demonstrated that MacoNPV-90/2 *Hin*dIII-O hybridized to the *EcoRI*-B, -T, and -M fragments in all five geographic isolates. In addition, it also hybridized to the *Eco*RI-A2 fragment of isolate -86/1, the *Eco*RI-A1 fragment of isolate -90/3, and the *Eco*RI-A3, -A2, -C1 fragments of isolate -90/4 (Figure 5.2). As the size of *Eco*RI-B + *Eco*RI-T is similar to that of *Eco*RI-A3 from isolate -90/4, it is possible that the *Eco*RI-A3 fragment from isolate -90/4 was generated by the loss of the *Eco*RI restriction site between *EcoRI*-B and *Eco*RI-T. Hybridization results indicated that *Eco*RI-A1 from isolate -90/3 and *Eco*RI-A2 from isolate -86/1 may be produced in the similar way.

Some of the variable bands in the REN patterns of MacoNPV geographic isolates may actually include more than one comigrating REN fragment. For example, the *Eco*RI-C1 band in the REN pattern of isolates -86/1 and -90/4 hybridized to cloned probes which represent a region of the viral genome that is much larger than that which the *Eco*RI-C1 fragment could cover based on fragment size estimates (data not shown). Another possible

Figure 5.2. Southern blot-hybridization analysis of MacoNPV geographic isolates. MacoNPV geographic isolate DNAs MacoNPV-90/1 (lane 1), -90/2 (lane 2), -90/3 (lane 3), -90/4 (lane 4), and -86/1 (lane 5) were digested with *Eco*RI and electrophoresed on 0.6% agarose gels, and the separated DNA fragments were blotted onto nylon membranes (PhotoGene™, GIBCO-BRL). The Southern blot membranes were hybridized either with MacoNPV genomic DNA probe (panel A) or with pMcHind-O fragment clone (panel B).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A1
A2
A3
A2
C1
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B

explanation for this result is that the *Eco*RI-C1 fragment contains significantly re-arranged sequences which come from a more extensive region of the genome. Table 5.1 shows the Southern blot hybridization results using MacoNPV-90/2 *Hin*dIII fragments as probes to detect the variable fragments in MacoNPV geographic isolates.

Twenty pick plaques were derived from the MacoNPV-90/4 isolate. OB from pp strains were used to infect late third-instar bertha armyworm larvae for DNA extraction because the amount of virus derived from infected-cell cultures was too low to provide sufficient purified DNA for extensive REN analysis. When purified pp strain DNA derived from infected larvae was analyzed by REN digestion, different REN patterns were detected in these plaque isolates, compared to those seen in the REN pattern of the original MacoNPV-90/4 isolate. Comparison of these new REN patterns confirmed the hypothesis that additional genotypes would be detected when geographic isolates were plaque purified. Some of the REN fragments which appeared to be in molar ratio in the geographic isolates are not present in the viral REN DNA patterns of some pp strains. For example, the *Eco*RI-F fragment was absent from the 90/4-pp8 and -pp11 strains, *Eco*RI-G is absent from the 90/4-pp2 strain, and *Eco*RI-H is absent from the 90/4-pp8, -pp11 and -pp18 strains (Figure 5.3). Some of these fragments may have been present as submolar fragments at very high molar ratios in the population of -90/4 geographic isolate (e.g. *Eco*RI-G). These differences became clear when the plaque purified strains were analyzed.

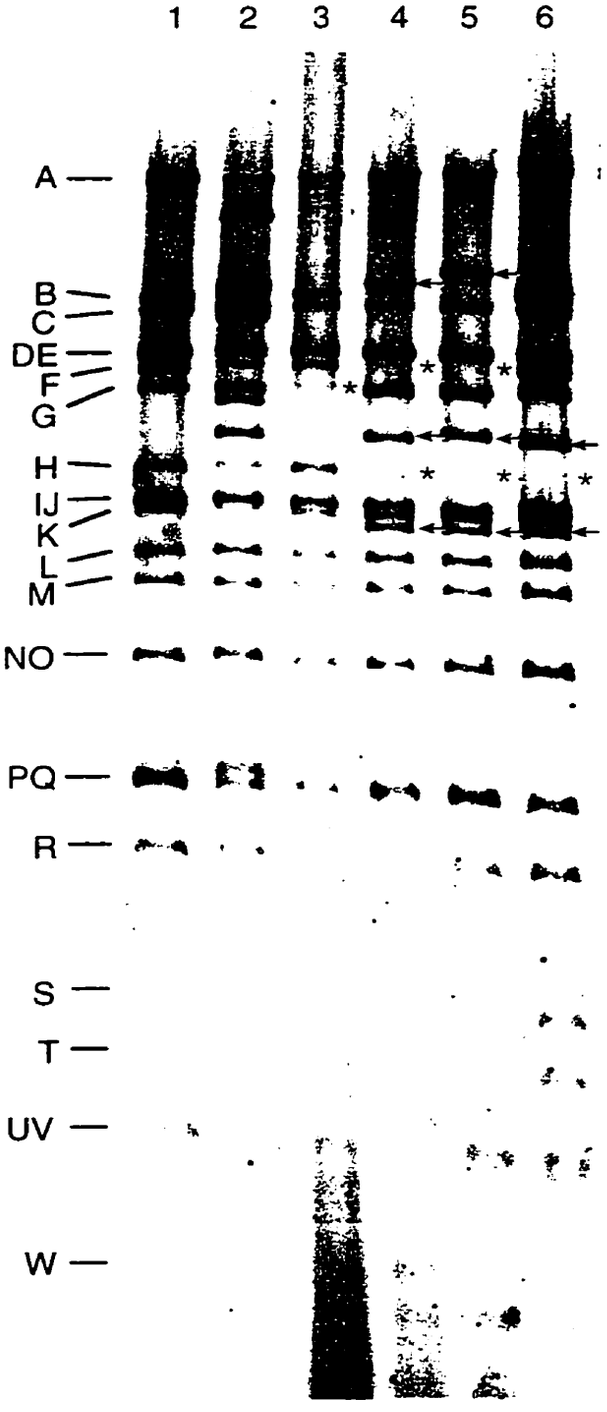
Table 5.1. Detection of variable fragments in MacoNPV geographic isolates.

		Restriction fragment				
Probe fragment	clone#	90/1	90/2	90/3	90/4	86/1
*Hin*dIII-EF	157	C,B	C,B	C,B, **A1**	C,B, **A3,A2**	C,B, **A2**
*Hin*dIII-D	230-6	B	B	B, **A1**	B, **A3,A2,C1**	B, **A2**
*Hin*dIII-OP	346-2	B,T,M	B,T,M	B,T,M, **A1**	B,T,M, **A3,A2,C1**	B,T,M, **A2**
*Hin*dIII-EF	154	M,A	M,A	M,A	M,A, **A2,O1,C1**	M,A, **A2,O1,C1**
*Hin*dIII-OP	133	A	A	A	A, **C1**	A, **A2,C1**
*Hin*dIII-C	211	A,	A,	A	A, **A2,C1,A1**	A, **A2,C1,A1**
*Hin*dIII-B	150	A,IJ	A,IJ	A,IJ	A,IJ, **A1**	A, **IJ,A1**
*Hin*dIII-M	151	IJ	IJ	IJ	IJ	IJ
*Hin*dIII-V	212	PQ	PQ	PQ	PQ, **A3**	PQ
.						
.						
.						
.						
*Hin*dIII-N	134	C	C	C	C	C
*Hin*dIII-X	130	C	C	C	C	C

* MacoNPV fragment clones were used as probes to hybridize genomic blots of EcoRI digests of MacoNPV geographic isolates.

** Variable fragments were bolded.

Figure 5.3. *Eco*RI restriction patterns of MacoNPV strains showing genomic variation between MacoNPV-90/4 pp strains and the parental MacoNPV-90/4 isolate. MacoNPV-90/2 (lane 1), MacoNPV-90/4 (lane 2), MacoNPV-90/4 -pp2 (lane 3), -pp8 (lane 4), -pp11 (lane 5), and -pp18 (lane 6) genomic DNA were digested with *Eco*RI, and separated on 0.6% at 50V for 16hr (negative image). REN fragments present in MacoNPV-90/2 but not detectable in the -90/4 pp strains are indicated by an asterisk. REN fragments present in MacoNPV-90/4 pp strains but not detectable in -90/2 are indicted by an arrow.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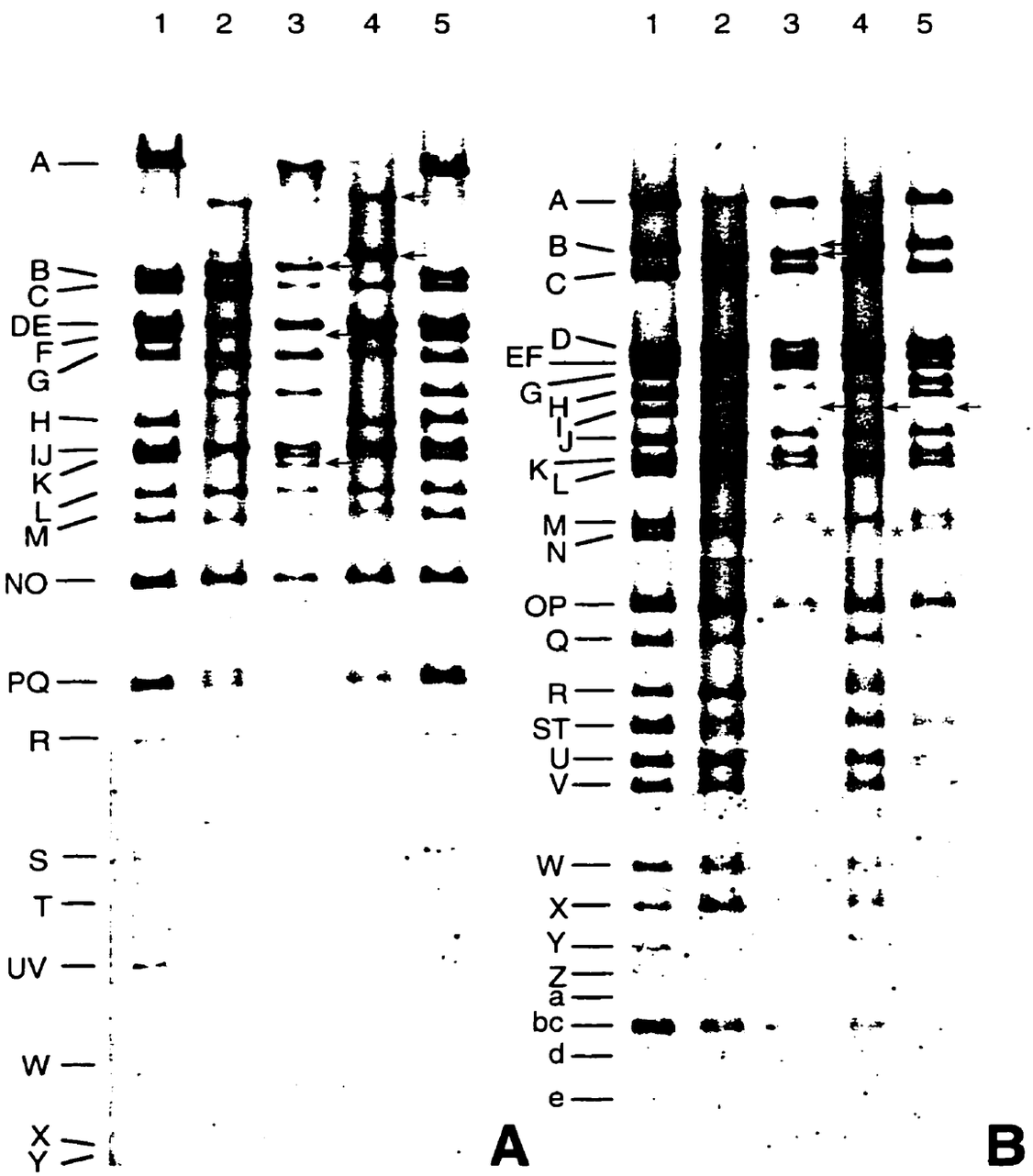
In other cases, there were additional REN fragment bands in the pp strains which were not detected in the geographic isolates. An example was the -K1 band, which was not detectable in the *Eco*RI restriction pattern of the MacoNPV-90/4 geographic isolate, but was present at a molar ratio in the *Eco*RI restriction pattern of the MacoNPV-90/4-pp8, -pp11, and -pp18 strains (Figure 5.3). These REN fragments might be present at very low ratios in the population of MacoNPV-90/4 isolate and were not detectable. When the genotypes containing the variant fragment(s) are plaque purified, these fragment(s) become detectable at molar ratios.

Submolar fragments were detected in the REN patterns of some of the MacoNPV-90/4 pp strains following several replication passages in bertha armyworm larvae. For example, submolar fragment bands were detected from *Pst*I restriction pattern of MacoNPV-90/4 -pp8 and *Hin*dIII pattern of -pp9 (Figure 5.4). These strains were originally considered not to contain submolar bands in REN patterns of DNA extracted from virus produced in cultured cells.

Data on the presence or absence of specific REN restriction fragments for the pp strains digested with *Eco*RI are presented in Table 5.2. Submolar bands are still detectable in some pp strains and more rounds of plaque purification would be required to purify these genotypes (e.g. the MacoNPV-90/4-pp16, Table 5.2). Some geographic isolates (e.g. MacoNPV-90/4) are heterogeneous mixture of genotypic variants which were more complex than was

Figure 5.4. *Eco*RI restriction patterns of MacoNPV strains -90/2, MacoNPV-90/4, MacoNPV-90/4 -pp8, -pp9, and -pp15. MacoNPV-90/2 (lane 1), MacoNPV-90/4 (lane 2), MacoNPV-90/4 -pp8 (lane 3), -pp9 (lane 4), and -pp15 (lane 5) genomic DNA were digested with *Eco*RI (panel A), *Hin*dIII (panel B), *Pst*I (panel C) and *Xho*I (panel D), and separated on 0.6% at 50V for 16hr (negative image). Additional or missing fragments in the pp strains compared to MacoNPV-90/2 were indicated by an arrow. Submolar fragments are indicated by an asterisk.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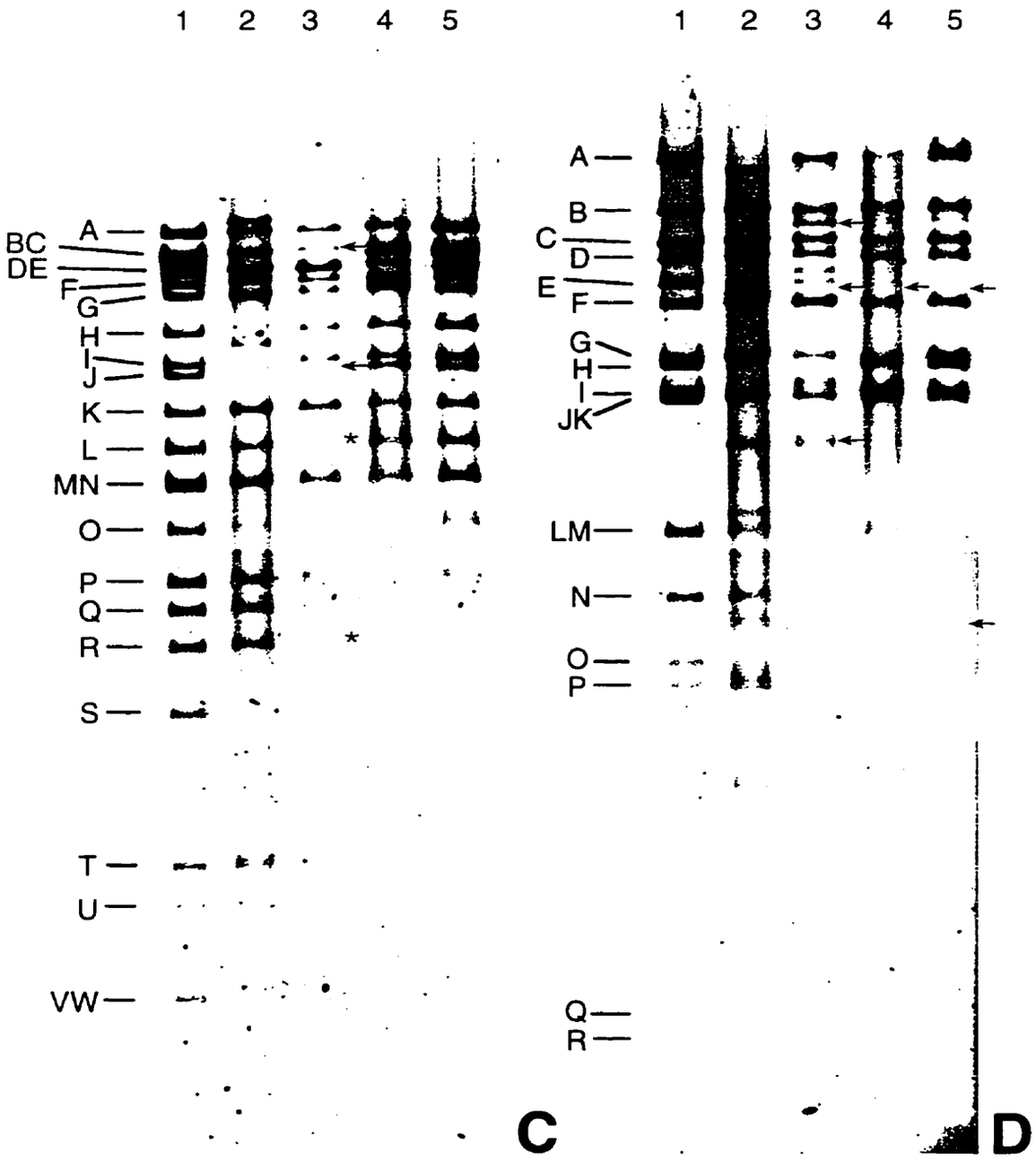
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Table 5.2. MacoNPV-90/4 pp strains *Eco*RI restriction patterns.

pp#	3	10	13	15	1	4	7	16	20	14	2	12	6	5	19	17	9	18	8	11
A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
A1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-
A2	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+
A3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
B	+	+	+	+	+	+	+	+	+	s	+	+	+	+	+	+	-	+	-	-
C	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+
C1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
DE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*	+	+
F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-
G	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	=	+	+	=	=
G2	+	+	+	+	s	s	s	s	s	s	-	+	-	+	+	+	-	+	+	+
H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
IJ	+	+	+	+	+	+	+	+	+	+	+	+	+	*	+	+	+	*	*	*
K	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
L	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M	+	+	-	+	+	-	+	-	+	+	+	+	+	-	-	+	+	+	-	+
NO	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PQ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-
UV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
V1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
V2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
W																				?
X																				?
Y																				?
Z																				?

Bold: with alteration, +: Plus, -: Minus, s: Submolar,
=:Double band, *: single, ?: data not available

originally anticipated. The pp strains from MacoNPV-90/4 fell into 10 different categories defined by their REN restriction patterns. Several pp strains representing different categories, including MacoNPV-90/4 -pp2, -pp8, -pp9, -pp13, -pp15, and -pp17 in which no submolar REN bands were detected, were used for further studies as described in the next chapter.

6. Bioassays in Larvae and Cultured Cell Line

6.1. Insect Bioassays

6.1.1. Materials and Methods

6.1.1.1. Insects

Neonates from *M. configurata* maintained as described above were used for bioassays. Bioassays were conducted as previously described by Erlandson (1990) and outlined below.

6.1.1.2. Viruses

MacoNPV-90/2 geographic isolate and pp strains MacoNPV-90/4 -pp2,-pp8, -pp9, -pp13, -pp15, and -pp17 were used for the investigation of ST_{50}. MacoNPV-90/4 pp9 and pp15 were used in further dose-mortality bioassays to estimate 50% and 95% lethal doses (LD_{50} and LD_{95}, respectively) values.

6.1.1.3. Method for Inoculation

The droplet-feeding inoculation method of Hughes and Wood (1981) with minor modification was used to inoculate the neonate bertha armyworm larvae. Basically, a suspension with a known concentration of virus OB was diluted 1:1 with a solution of 5% blue food colouring and 1% sucrose, and then pipetted onto a sheet of Parafilm in 4-µl droplets. A glass funnel was placed over the droplets and the Parafilm was sealed to the funnel. Sixty-70 neonate larvae, between 12 and 24 hr old, were then introduced into the funnel and the funnel was inverted. The larvae were left to feed on the droplets for 20 min and those larvae that had fed (as indicated by the blue coloration of the gut) were transferred to fresh diet cups at five larvae per cup.

The average dose level ingested by an individual was determined by the mean weight difference of larvae before and after ingestion. Larvae that died within the first 48 hr were considered to have been injured during manipulation and were removed from the assays.

6.1.1.4. Data Collection and Statistics

Two kinds of assays were conducted with neonate larvae. Pure genotype pp strains were first investigated to determine ST_{50} as an initial screen of the viral strains. Then, for those strains with obviously different ST_{50} values, dose-mortality assays were done to determine LD_{50} and LD_{95} values. For the initial ST_{50} investigations, insects were inoculated only at medium and high dosages (100 or 500 OB/larva). Mortality was recorded daily to 14 days post treatment. The cadavers and survivors were examined by phase-contrast microscopy for the presence of viral OB. Three individual assays were conducted for ST_{50} estimations. The time-mortality response data was analyzed using the computer program ViStat which is based on the time-mortality response analysis of Bliss (1937), with modification by Dr. Doug Robson at Cornell University (Dr. Patrick R. Hughes, personal communication).

For dose-mortality bioassays (LD_{50}/LD_{95} investigations), insects were inoculated with 6 doses from 1 to 1250 OB/larva. LD_{50} and LD_{95} values were estimated based on the mortality data from day 10 post- inoculation. Day 10 post-inoculation data was chosen because it is the standard time used for MacoNPV bioassays and gives the most representative dose-mortality response curves. The

individual dose-mortality response data sets were analyzed using a multiline quantal bioassay computer program (S108, developed by the Statistical Research Section, Engineering and Statistical Research Centre, Research Branch, Agriculture Canada) based on a probit analysis method described by Finney (1971). The final LD_{50}/LD_{95} values are means of 3 individual assays.

The mean and standard error for the LD_{50} and LD_{95} values from a series of bioassays were determined using STATISTIX statistical software in order to compare the MacoNPV-90/4 pp strains with MacoNPV-90/1 and -90/2 as standards. In addition, the potency ratios of individual virus strains were determined for each dose-mortality bioassay using the S108 program. This gives an additional estimate for comparison of the relative virulence of the various virus isolates.

6.1.2. Results

The MacoNPV-90/2 isolate and pp strains MacoNPV-90/4 -pp2, -pp8, -pp9, -pp13, -pp15, and -pp17 were used for the investigation of ST_{50}. The mean ST_{50} values for the MacoNPV isolates were determined from three replicate assays (Table 6.1). At 500 OB/larva, the ST_{50} values ranged from 7.2 days for MacoNPV-90/4 pp15 to 8.94 days for pp8, but were not statistically significant. The pp9 and pp15 isolates along with MacoNPV-90/1 and -90/2 geographic isolates as standards were

Table 6.1. The mean estimstes of the surviral time in days (±SE) for 50% first instar *M. configurata* larvae (ST_{50}) infected with MacoNPV pp strains (500 OB/larva).

Viral Strain	Mean ± SE of ST_{50}
MacoNPV90/2	8.119 (± 0.344)
MacoNPV 90/4 pp2	7.697 (± 0.367)
MacoNPV 90/4 pp8	8.938 (± 0.712)
MacoNPV 90/4 pp9	8.666 (± 0.405)
MacoNPV 90/4 pp13	8.219 (± 0.687)
MacoNPV 90/4 pp15	7.197 (± 0.659)
MacoNPV 90/4 pp17	8.643 (± 0.856)

further assessed in replicate dose-mortality assays. An analysis of the means of LD_{50} estimates from the three replicate assays indicated that there were no significant differences between the replicates so the data was pooled for a single probit analysis using S108 program. The probit analysis data and LD_{50}/LD_{95} estimates from the pooled data is shown in Table 6.2. The LD_{50} and LD_{95} estimates for MacoNPV-90/4 pp9 (53.8 and 1040.6 OB/individual, respectively) were significantly greater than the other isolates tested, based on exclusively of the 95% confidence intervals (Hughes and Wood, 1986) (Table 6.2 and 6.3). Estimates of potency rates were also undertaken using S108 program. The X^2 value (6.00, 3 df) for the test of parallelism of the probit lines for each isolate was not significant, indicating a common slope of 1.279 for the probit lines (Table 6.3). The relative potency which compared the LD_{50} values for the MacoNPV-90/1, -90/4 pp9 and pp15 to that of MacoNPV-90/2 showed that -90/4 pp9 was significantly less potent (0.32 X) than -90/2, while 90/1 and -90/4 pp15 were 1.33 and 1.46 X more potent (virulent), respectively (Table 6.3), but not statistically significant.

Table 6.2. LD_{50} and LD_{95} estimations (95% confidential interval) in OB per larva for MacoNPV strains.

Viral Strain	LD_{50} (95% CI)	LD_{95} (95% CI)	b	X^2
MacoNPV 90/1	12.81 (9.62-17.04)	247.60 (182.79- 338.58)	1.2976	2.3835
MacoNPV 90/2	17.02 (12.80-22.62)	329.06 (243.05- 449.82)	1.3821	2.2637
MacoNPV 90/4 pp9	53.82 (40.47-71.62)	1040.59 (764.99-1430.45)	1.0988	9.5291
MacoNPV 90/4 pp15	11.61 (8.73-15.43)	224.50 (165.94- 306.57)	1.4266	4.0667

*: CI = confidence interval

Table 6.3. Potency estimation using pooled data on S108 using MacoNPV-90/2 as standard.

Viral strains	Relative potency (95% CI)
MacoNPV 90/1	1.3290 (0.8880 - 1.9890)
MacoNPV 90/4 PP9	0.3162 (0.2112 - 0.4730)
MacoNPV 90/4 PP15	1.4660 (0.9802 - 2.1920)

* X^2 for parallelism = 5.9995, 3 d.f. Not significant. Therefore probit lines are parallel.

** Estimate of slope of parallel-line analysis = 1.28.

*** The X^2 for heterogeneity 13.5067, 7 d.f. Not significant. Therefore data fit probit model.

6.2. Virus Replication in Cultured Cells

6.2.1. Materials and Methods

6.2.1.1. Insect Cell Line and Viruses

The *M. brassicae* cell-line IZD-MB-0503 (American Type Culture Collection designation CRL 8003) (Mbr) was used to investigate the replication of MacoNPV-90/4-pp9 and -pp15.

6.2.1.2. Virus Replication

To produce MacoNPV ECV virus stock for virus growth-curve assays, Mbr cells were set up at $2x10^6$ cell/75 cm^2 flask in 15 ml of medium. After the cells had been incubated overnight at 28°C to allow for attachment, all but 4 ml of tissue culture medium was removed, and 250 µl of ECV viral stock was added to each flask as inoculum. The flask was rocked on a platform rocker for 2 hr at 28°C, 10 ml of fresh medium was added, then the cells incubated for 7 days at 28°C. The cells and medium were harvested, transferred to 15 ml centrifuge tubes and the cells were pelleted by centrifugation at 350x g for 10 min. The supernatant containing the ECV was transferred to a sterile 15 ml centrifuge tube for storage at 4°C until further use.

6.2.1.3. Determination of Viral Titers

The titer of virus was determined using an end-point-dilution assay in 60-well microtest plates and median tissue culture infection doses ($TCID_{50}$) (the dose that produces positive

responses in 50% of the inoculated wells) were estimated using the Spearman-Karber Method (Hughes and Wood, 1986). Briefly, Mbr cells were diluted to a density of 1.5 x 10^5 cell/ml. Cells were set up in 60-well microtest plates at 5 µl/well (i.e. 750 cell/well). After the cells had attached for 2 hr at 28°C, a series of 10-fold virus dilutions were used to inoculate the wells. A 5 µl aliquot of virus dilution was added to each well in a column of wells except the well in the first row which served as a negative virus control. A 5 µl aliquot of medium was added to each well in the first row for mock-infected control. Starting on day 4 post-infection, the plates were scored daily for positive response (OB present in nucleus of at least one cell in a well) until no further responses were noted (about day 9).

The $TCID_{50}$ was calculated using the following formula:

$$\text{Log } TCID_{50} = X_{p-1} + (1/2\ d) - d\Sigma p$$

where: X_{p-1} is the highest log dilution giving all positive responses, d is the log of the dilution factor, p is the proportion of positive response at a given dose, and Σp is the sum of values of p for X_{p-1} and all higher dilutions.

The standard error (SE) of the log of the $TCID_{50}$ was calculated from the formula

$$SE = [d^2\sum(p.(1-p)/(n-1))]^{1/2}$$

where n is the number of inoculated samples in a given dose (Finney, 1978).

6.2.1.4. Viral Growth Curves in Insect Cell Lines

Two replicates of viral growth curve assays were conducted

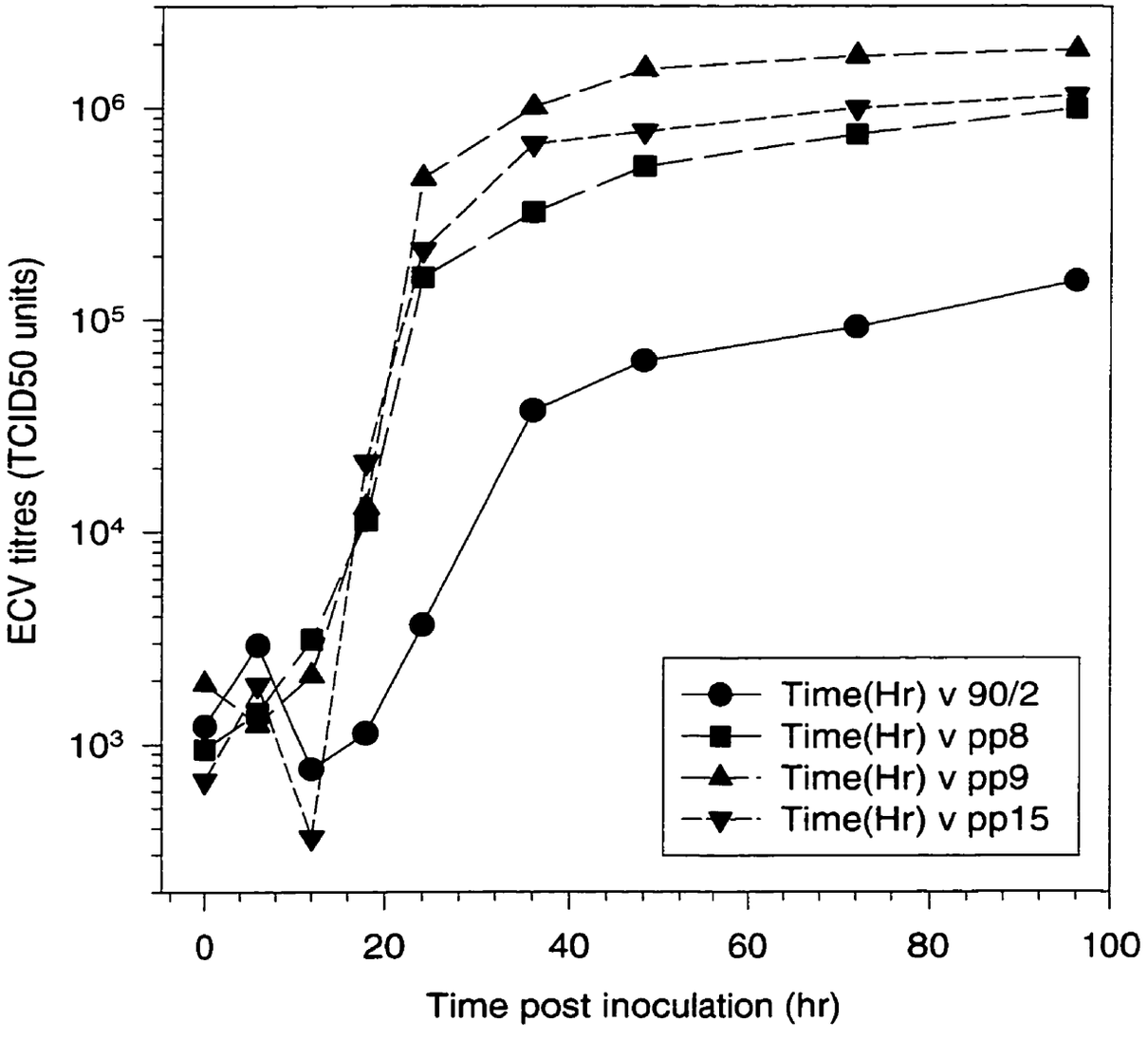
in 24-well tissue culture plates (FALCON®, 16 mm in diameter). The plates were set up with 2.5 x 10^5 cell/well in 1 ml of Grace's tissue culture medium supplemented with 10% FBS and gentamycin at 10 µg/ml. The cells were allowed to attach for 2 hr at 28°C. The medium was then removed and 1 ml of virus inoculum diluted to 1.9 x 10^6 $TCID_{50}$ unit/ml was immediately added to each well (equivalent to an m.o.i of 7.6 $TCID_{50}$ unit/cell). The tissue culture plates were incubated at 28°C for 4 hr with constant rocking on a platform rocker. The medium was then removed, and each well rinsed with 0.5 ml of fresh medium. Fresh medium (0.5 ml) was added to each well (This was designated as time point 0 hr after inoculation). At 0, 6, 12, 18, 24, 36, 48, 72, and 96 hr post-inoculation, cells were dislodged by pipetting medium across the cell monolayer several times. The cells and medium were then harvested and the cells pelleted in a sterile 1.5 ml centrifuge tube. Cells were pelleted in a sterile 1.5 ml centrifuge tube. The supernatant containing the ECV was transferred to a sterile tube, while the pellet containing the cell debris with viral OB was kept in the original tubes. The supernatants collected at different time points post-inoculation were serially diluted, and the titre of ECV was determined by $TCID_{50}$ assays as described above.

6.2.2. Results

The virus growth curves as represented by the titre of ECV at different time points post inoculation are shown in Figure 6.1. During the first 12 hr, there was typically a drop in the virus titre in the tissue culture supernatant. The log growth phase started between 12-18 hr post inoculation, and reached the plateau phase at 36-48 hr post inoculation. The MacoNPV isolates with higher virulence to the host insects did not necessarily replicate to higher titres in insect cell lines. For example, MacoNPV-90/4 pp9, which had the lowest virulence to the insect host (53.82 OB/larva), had the highest ECV titre (more than 10 times as high as that of MacoNPV-90/2), and reached a titre of 1.89×10^6 $TCID_{50}$ unit/ml at 96 hr post inoculation, MacoNPV 90/2 only reached a titre of 1.53×10^5 $TCID_{50}$ unit/ml at the same time. MacoNPV-90/4 pp15, which had the highest virulence to the insect host (11.61 OB/larva), had a titre between MacoNPV-90/2 and MacoNPV-90/4 pp9. The ECV titre for MacoNPV-90/4 pp15 was 1.15×10^6 $TCID_{50}$ unit/ml. Although the absolute titres of ECV were lower in the virus-growth curve assays (probably due to too high inoculum) compared to the stock titres of the viral strains used in the assays ($1.9\text{-}4.57 \times 10^6$ $TCID_{50}$ unit/ml), the final ECV titre of MacoNPV-90/2 was always the lowest one. The number of OB from MacoNPV -pp9 and -pp15 produced between 36 and 96 hr post inoculation was determined (data not shown). The results showed that these two pp strains produced similar number of OB at each specific time point.

Figure 6.1. MacoNPV growth curves on Mbr cell line. The ECV titres ($TCID_{50}$ units) of MacoNPV strains -90/2, 90/4-pp8, -pp9, and pp15 were plotted against the time point post inoculation (hr).

MacoNPV growth curves on Mbr cells (mean of 2 replicates)



Viral DNA synthesis over the time course of infection was determined by means of dot blot hybridization (data not shown). The hybridization results showed that the accumulation of viral DNA as reflected in the intensity of the dot blot hybridization detected by X-ray films was coincident with the titres of ECV (pp9>pp15>pp8>90/2).

7. Discussion

In the present study, the genomic DNA of a baculovirus from bertha armyworm, *Mamestra configurata*, MacoNPV-90/2 was digested with six common REN for the construction of a physical map. Type II REN cleave DNA at specific nucleotide sequences and consistently produce the same profile of specific size fragments from a specific DNA sequence. Thus the REN fragment profile will be consistent for a specific viral genome for a particular REN. A physical map reflects the locations of these restriction sites on the viral genome. Thus, the REN fragment patterns and the physical map of a viral genome serve as essential standard references for a specific virus, which can facilitate the direct comparison of information in the study of genetic organization, gene transcription and regulation, and genome replication (Cochran et al., 1986).

The MacoNPV-90/2 genome was digested with *Eco*RI, *Hin*dIII, *Pst*I, *Bam*HI+*Sma*I, *Xho*I, *Bam*HI, and *Sma*I, and 26, 32, 24, 11, 19, 8, and 3 fragments, respectively, were generated (Figure 4.1). The number of REN sites on the viral genome depends on the DNA sequences of the viral genome and the recognition sequence of a specific REN. The G+C ratio can often be used to predict the probable frequency of REN sites. The G+C ratio of baculoviruses varies from 28-59% (Francki, 1991). The extremely small number of restriction sites for *Sma*I (recognition sequence: 5'-CCCGGG-3'), and large number of restriction sites for *Eco*RI and *Hin*dIII (recognition sequence: 5'-GAATTC-3' and 5'-AAGCTT-3',

respectively) may indicate a low frequency of G+C rich regions of sequences in the MacoNPV genome. Sequence data from a limited region of MacoNPV genome (Li et al., submitted for publication) showed that the G+C ratio of the MacoNPV polyhedrin coding sequence is 49.8%. The number of *Sma*I (3 and 5) and *Hin*dIII (29 and 30) sites for PaflMNPV and MbMNPV, respectively, (Possee & Kelly, 1988) are very similar to that detected in MacoNPV-90/2. The AcMNPV genome, which has a G+C ratio of 41% (Ayres et al., 1994), also has low number of *Sma*I REN sites (4), and considerably more for *Eco*RI (24) and *Hin*dIII (24) sites (Cochran et al., 1982). It is interesting to note that the number of *Bam*HI sites (8, 7, 9, and 6, respectively) on MacoNPV, AcMNPV, MbMNPV, and PfMNPV genomes are consistently fewer than for *Xho*I (19, 14, 30, and 20 sites, respectively) (Figure 4.1; Cochran et al., 1982; Possee & Kelly, 1988). The recognition sequences of *Bam*HI (5'-GGATCC-3') and *Xho*I (5'-CTCGAG-3') both contain 2/3 G+C nucleotides.

The sum of the sizes of the REN fragments from each of *Eco*RI, *Hin*dIII, *Pst*I, *Xho*I, and *Bam*HI+*Sma*I digests of MacoNPV-90/2 DNA were used to estimate the total genome size. These estimates ranged from 155.9 kbp for the *Hin*dIII digest to 159.4 kbp for the *Bam*HI+*Sma*I digest, with a mean of 156.9 kbp. This estimate is in the size range of other known baculovirus genomes (80-200 kbp - King and Possee, 1992 and Rohrmann, 1992; 90-160 kbp - Volkman et al., 1995). The size estimate of MacoNPV-90/2 is larger than certain of the better studied NPV including AcMNPV (including the HR3 strain [128 kbp] and the C6 strain [134 kbp])

(Cochran et al., 1982; Ayres et al., 1994) and an NPV from a closely related insect, *P. flammea* (144.34-149.83 kbp) (Possee & Kelly, 1988). However, it is in line with the size estimates for the MbMNPV genome of 148.49-154.56 kbp (Possee & Kelly, 1988).

The 156.9 kbp estimate for the MacoNPV-90/2 genome is considerably larger than the initial estimate of 144 kbp for the MacoNPV-86/1 geographic isolate (Erlandson, 1990). However, in comparison to the initial analysis of MacoNPV-86/1, a number of additional small REN fragments (e.g., *Eco*RI-U to -Z, *Hin*dIII-Z to -f, *Pst*I-V to -X, and *Xho*I-Q to S) have been identified in the present study, which may partly represent the increased total genomic size. Another possible explanation is that some REN bands which were originally considered single bands in the analysis of MacoNPV-86/1 DNA were confirmed to be double bands (e.g., PstI-DE and -MN) in the present study. In addition, the use of high molecular weight standard (GIBCO BRL) in the present study made the size estimates of large fragments, which are out of the range of lambda/*Hin*dIII fragment size standard, more precise. For example, *Eco*RI-A was previously estimated to be 25.49 kbp for MacoNPV-86/1 isolate. In the present study, it was estimated to be 38.20 kbp using the high molecular standards. The estimates for high molecular weight fragments such as *Eco*RI-A using high molecular standards coincided with the summation of REN fragments which hybridized to *Eco*RI-A. In this case, *Eco*RI-A covers 6.81 kbp of *Hin*dIII-EF + *Hin*dIII-OP (3.64 kbp) + *Hin*dIII-d (0.76 kbp) + *Hin*dIII-C (13.66 kbp) + 13.75 kbp of *Hin*dIII-B, which totalled 38.62 kbp. Another example is the estimate of *Xho*I-A. The

previous size estimate for MacoNPV-86/1 *Xho*I-A was 23.8 kbp, while the estimates using high molecular standards and summation of REN fragments which hybridized to *Xho*I-A for MacoNPV-90/2 in this study were 36.15 kbp, and 36.52 kbp, respectively.

More than 70 MacoNPV REN fragments covering almost the entire viral genome have been cloned in plasmid vectors. DNA of these cloned fragments have been used as probes in Southern blot hybridization for the construction of the MacoNPV-90/2 physical map and the mapping of variable fragments in the MacoNPV geographic isolates. These clones will also be useful for further studies including gene isolation, mapping, and sequencing. For example, the *Hin*dIII-EF fragment clone, which contains the polyhedrin gene region, has been subcloned and partially sequenced.

A physical map of MacoNPV-90/2 with restriction sites for *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sma*I, and *Xho*I was constructed in this study (Figure 4.9). Smith & Crook (1988) suggested that the polyhedrin gene be used as the zero point of NPV REN maps for greater precision. However, because the exact position of the polyhedrin gene had not been determined for MacoNPV-90/2 when construction of the physical map of MacoNPV-90/2 was begun and because it is convenient to begin a physical map at an REN restriction site, we used the convention of Vlak & Smith (1982). The physical map of MacoNPV-90/2 was linearized at the junction of *Hin*dIII-D and -EF (Figure 4.9), because *Hin*dIII-EF hybridized with the AcMNPV polyhedrin sequences. The physical map of MacoNPV-90/2 genome will serve as a framework for further studies

of MacoNPV, including mapping of biological functions involved in host range specificity and virulence, transcriptional and translational maps, mapping of origins of DNA replication, mapping of regions of variations among genotypic variants, and potentially in genetic engineering efforts to produce a virus with enhanced potential as a biological control agent.

A small region of MacoNPV-90/2 genome including the polyhedrin gene has been sequenced (Li et al., submitted for publication). The sequence data shows high homology with polyhedrin genes from PaflNPV and MbMNPV respectively. The region of the MacoNPV genome surrounding the polyhedrin gene appears to be co-linear in organization when compared to the polyhedrin region from AcMNPV and MbMNPV (Figure 7.1). For example, downstream from the MacoNPV polyhedrin gene, two ORF [ORF2 (ORF1629?) and ORF3 (PK1?)] were identified which showed a limited degree of sequence homology with the ORF1629 and PK1 (protein kinase) gene of AcMNPV (Figure 7.1). These genes thus occupy a similar position in relation to polyhedrin as occurs in AcMNPV. MacoNPV ORF2 (ORF1629?) and AcMNPV ORF1629 shared a 100% homology in a short stretch of aa sequence (PPPPPPPPPPA). This aa sequence stretch was also identified from MbMNPV ORF2 (Cameron and Possee, 1989). The 3' end 189 aa sequence (276-464 aa) from

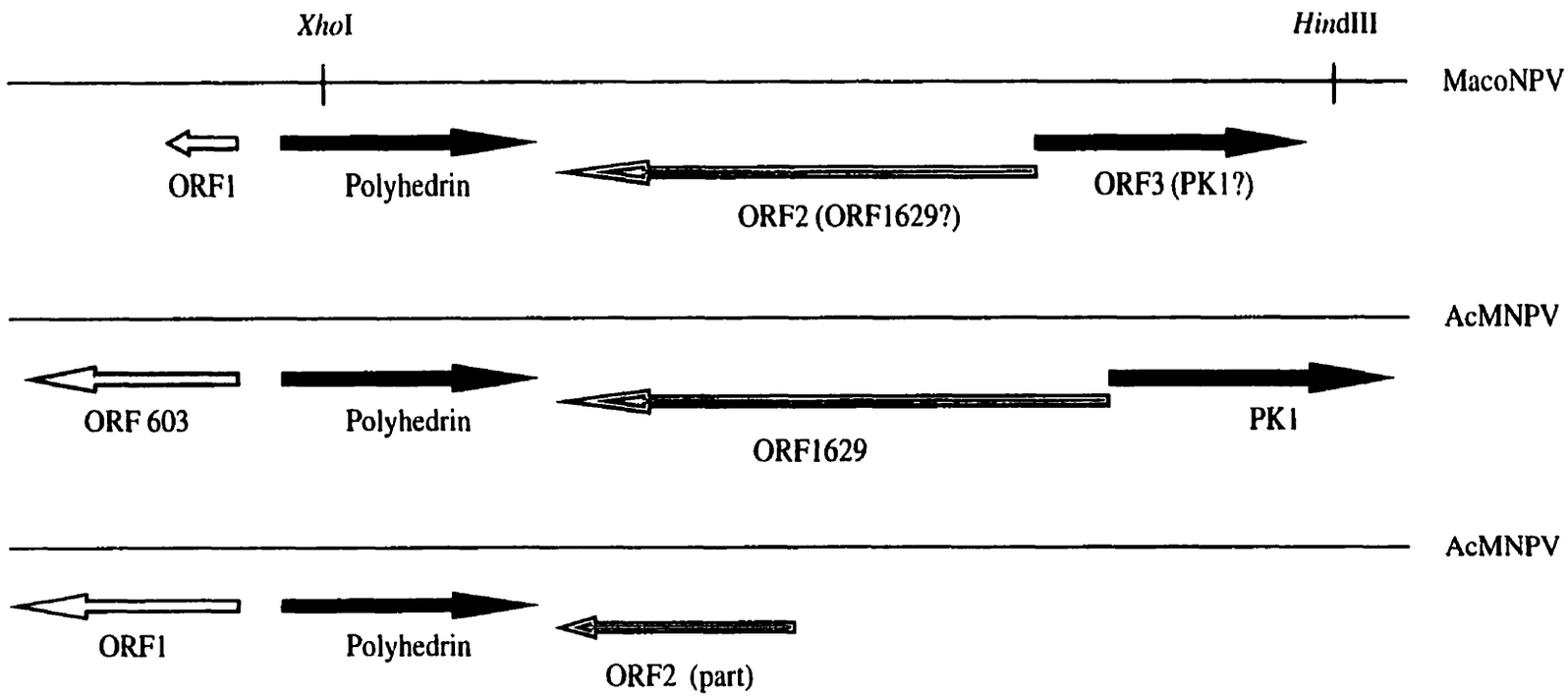


Figure 7.1. Alignment of the MacoNPV polyhedrin gene region with the cross-hybridizing region of AcMNPV and MbMNPV. Homologous ORF are indicated using same kind of arrows.

MacoNPV ORF2 (ORF1629?) share 88% homology with that of the published 3' end aa sequence from MbMNPV ORF2 (130-318 aa) (Figure 7.1). Upstream of the MacoNPV polyhedrin gene, an ORF (ORF1) transcribed in the opposite orientation to the polyhedrin gene showed significant level of homology to the MbMNPV ORF1 (Figure 7.1). These ORF were found not to have significant homology to the ORF upstream of polyhedrin in AcMNPV (Cameron and Possee, 1989). Although the MacoNPV ORF1 (72 aa) is much smaller than that of MbMNPV (216 aa), the 64 aa sequences at the 5' end of both ORF shared 92% homology. The co-linear organization of baculovirus genomes has been reported frequently. Using five OpMNPV cosmid clones as hybridization probes to Southern blots of AcMNPV genomic DNA, Leisy et al. (1984) showed that the genomes of these two MNPV were similarly organized, although the transcription direction of the two polyhedrin genes were in opposite orientations for the two viruses (Figure 2.5). Possee and Kelly (1988) carried out cross-blot hybridization studies between the genomes of PaflNPV and MbMNPV in order to align the two restriction maps. They showed that the two genomes shared overall similarity in gene organization. Jehle et al. (1992) showed that at least three regions of homologous sequences were located at similar positions on the physical maps of two GV, *Cryptophlebia leucotreta* (Crle) GV and *Cydia pomonella* (Cp) GV. Experimental data showed that regional co-linear organization among baculoviruses is also evident. For example, although the p10 protein of SeMNPV showed only 39% and 26% amino acid homology to that of OpMNPV and AcMNPV, respectively, the gene organization

in the p10 region of these three MNPV are similar. The putative amino acid sequence from a partial ORF detected upstream of p10 in SeMNPV shared 55% and 53% amino acid homology with the OpMNPV and AcMNPV p26 protein, respectively. The amino acid sequence of a putative protein from an ORF downstream of SeMNPV p10 gene shared 71% and 74% homology with the p74 protein of OpMNPV and AcMNPV, respectively (Zuidema et al., 1993). Extensive study of MacoNPV genomic structure will likely reveal more regions of relatedness between MacoNPV and other NPV.

The REN patterns of the five geographic isolates of MacoNPV were compared in this study. The presence of obvious genotypic variants (genotypes), baculoviruses with very similar DNA genomes that can be distinguished only by small changes in their DNA REN profiles (as defined by McIntosh et al., 1987), was clearly demonstrated (Figure 5.1). The existence of genotypic variants is a common phenomenon in baculoviruses (reviewed by McIntosh et al., 1987; Erlandson, 1990). Geographic isolates of a particular virus can fall into three categories: (1) A mixture of heterogeneous genotypes. For example, at least five genotypes of AcMNPV (S1, E2, M3, S3, and R9), were distinguished from a wild type AcMNPV isolate through plaque-purification by Smith and Summers (1978). The majority of the pp strains (32 out of 41) had *Eco*RI restriction patterns of the E2 genotype DNA (Smith and Summers, 1979). (2) Similar but not identical homogeneous genotypes from different geographic areas. (3) Homogeneous strains from two or more geographic areas which can not be distinguished by REN patterns.

Baculoviruses isolated from different host insects have also been reported to be closely related and considered to be genotypic variants of the same virus. For example, the SeMNPV-25 plaque-purified strain has been considered to be a genotype of AcMNPV (Brown et al., 1984). By the comparison of AcMNPV and SeMNPV genotypes, Brown et al. (1985) concluded that at least three separate mechanisms have been involved in the generation of genotypic variants of SeMNPV: the duplication of viral sequences, the insertion of cellular sequences and point mutation. In addition, recombination between baculoviruses have also been considered a possible source of genotypic variants (Summers et al., 1980).

Cory and Bishop (1995) predicted that any wild-type isolate of a baculovirus might be a mixture of several genotypic variants. Each of the five geographic isolates of MacoNPV was unique, showing distinct differences in mobilities of specific REN fragments, although the REN patterns for these isolates were basically similar. Most of the geographic isolates were mixtures of genotypic variants, as confirmed by plaque purification. MacoNPV-90/3, -90/4 and -86/1 contain some co-migrating variable fragments (Figure 5.1). These variable fragments might have been generated in the same way. For example, the *Eco*RI-M1 fragments from -90/3 and -86/1 co-migrated (Figure 5.1). Also, the *Eco*RI-A1 fragment from -90/3 and -A2 fragment from -90/4 and -86/1 co-migrated (Figure 5.1, panel A), and these fragments hybridized to the same region of the MacoNPV-90/2 genome (Table 5.1). The co-migrating EcoRI-C1 fragments from -90/4 and -86/1 also hybridized

to the same region on the MacoNPV-90/2 genome (Table 5.1). It is possible that these geographic isolates contain similar genotypic variants with alterations in REN fragments mapping to same region of the genome. However, too few pp strains have been isolated from the various geographic isolates to determine whether identical genotypic variants exist in each isolate.

The REN restriction patterns of these isolates did not show obvious relatedness to geographic locations of isolation. For example, MacoNPV-90/1 (Lamont, Alberta) and -90/2 (Wilkie, Saskatchewan) have identical REN patterns for *Eco*RI, *Pst*I, and *Xho*I digests, and they only showed minor differences in the size of *Hin*dIII-OP (Figure 5.1), though they were collected from widely separated locations. In addition, MacoNPV-90/4 (Lamont, Alberta), which was collected from an area close to where -90/1 was isolated, showed the highest proportion of submolar fragments among the five geographic isolates, indicating it contained the greatest diversity of genotypes.

Andrews et al. (1980) reported that plaque purification of AcMNPV was neither deleterious nor advantageous with respect to the isolation of strains with increased virulence for *T. ni* larvae. However, Vail et al. (1982) did show a significant difference in virulence between plaque-purified AcMNPV (9 times less virulent to *T. ni* larvae), GmMNPV (2.5 times less virulent to *T. ni* larvae), and TnMNPV variants (4 times more virulent to *T. ni* larvae and 3 times less virulent to *Heliothis virescens* larvae), as compared to wild-type virus. This indicates the possibility to derive viral strains with different levels of

virulences by plaque purification. In the case of MacoNPV, the differences in virulence to the host insects already existed between the parental MacoNPV-90/4 and -90/2 isolates. Thus it is of interest to see if plaque-purification would be useful in deriving strains with different levels of virulence and distinct differences in REN profiles.

Theoretically, geographic isolate MacoNPV-90/4 will provide the best opportunity for deriving the most genotypic variants with distinct REN patterns. Twenty pp strains were derived from this isolate through plaque purification. All but two (pp16 and pp20) of these pp strains showed distinct REN patterns. Most REN fragments in the pp strains were of similar, if not identical, size compared to fragments in the MacoNPV-90/2 geographic isolate or the parental -90/4 geographic isolate. However, additional REN fragments, which had not been detected in the parental MacoNPV-90/4 isolate, were detected. It is possible that these variable fragments were present at very low ratios in the parental -90/4 genome populations. Brown et al. (1985) reported that 12 out of 14 genotypic variants of SeMNPV distinguished by *Eco*RI and *Hin*dIII REN profiles were at low frequency (<3%, calculated by the number of times the variant pattern was observed out of a total of 71 plaque purified stocks that have been screened). If the low frequency does represent the ratio of the genotypes in the wild-type molecule populations, most of the low molecular weight bands for those genotypes would not be detectable on ethidium bromide-stained agarose gel due to low intensity of staining. Another possible explanation for the presence of

additional fragments might be the amplification of a specific region of viral DNA or incorporation of small pieces of host insect-cell DNA into the viral genome when the virus replicated in cultured insect cell lines. Burand and Summers (1982) reported the alteration of AcMNPV strains upon serial passage in TN-368 cells. After 30 generations of serial passage of pp strain E2 and re-plaque purification, 7 out of 20 pp strains contained additional *Eco*RI fragments mapping in approximately the same region of the viral genome. They suggested that viral DNA molecules containing the additional *Eco*RI DNA fragments have a selective advantage in cell culture. It is now known that these additional *Eco*RI DNA fragments contain the homologous repeat (hr) sequences and thus contain the additional origins of DNA replication. Burand and Summers (1982) predicted that about 30% of the serial passage strains of many-polyhedra (MP) phenotype contained additional DNA, although these strains are phenotypically indistinguishable from wild-type virus.

Acquisition of host insect-cell DNA sequences (up to 2.8 kbp) by baculoviruses has also been reported during the evolution of AcMNPV FP strains in TN-368 cells (Fraser et al., 1983) and of SeMNPV genotypes in SF-21 and TN-386 cell lines (Brown et al., 1985). Hybridization of MacoNPV genomic DNA with labelled Mbr cell DNA as a probe or *vice versa* may confirm if the insertion of Mbr cellular DNA into MacoNPV pp strains occurred.

Submolar fragments in the REN patterns of MacoNPV-90/4 -pp8 and -pp9 were detected following several replication passages in bertha armyworm larvae. These strains were originally considered

not to contain submolar bands in REN patterns of DNA extracted from virus produced in cultured cells. In these pp strains, the fragments which are present in submolar ratios (e.g., *Pst*I -L and -R in -pp8, *Hin*dIII-N in -pp9, figure 5.4) all mapped to 6.0 - 13.3% map unit region of the MacoNPV genome. One possible explanation for this may be that during replication in the insect host specific regions of the viral genome (*Hin*dIII-N to -H) were deleted. Successive passage of MacoNPV in insect and/or cell culture with subsequent plaque purification of progeny virus may confirm the alteration of virus genome upon replication in insect versus insect cell culture.

One of my long-term objectives is to select pure genotypes by plaque purification from geographic isolates and to investigate their differences in virulence to the host insects. Differences in infectivity or virulence to the host insects exist between the natural baculovirus isolates and genotypes (Shapiro et al., 1984; Ebling and Kaupp, 1995). By comparing the natural variability in the median lethal concentration (LC_{50}) of *Lymantria dispar* (Ld) MNPV geographic isolates to gypsy moth, Shapiro and Robertson (1984) showed up to 2900-fold virulence difference between LdMNPV geographic isolates, indicating the probability of deriving high virulence viral strains from a broad geographic area. Up to 102-fold difference in virulence between random samples within an LdMNPV geographic isolate (plaque purification was not performed) has also been reported (Shapiro and Robertson, 1991), showing that heterogeneity within geographic isolates is a common phenomenon. These data indicates that it is possible to

derive viral strains with different virulence for host insects by plaque purification. The pp strains might have differences in REN patterns which make it easier to begin mapping genes related to virulence.

The pp strains from MacoNPV-90/4 were divided into 10 categories according to the REN patterns. Some of these were used for bioassays both in neonate bertha armyworm larvae and in cultured Mbr cell line. Bioassay data showed that at 500 OB/larva, the ST_{50} values for neonate bertha armyworm larvae ranged from 7.2 days to 8.94 days. Although the difference in ST_{50} between the tested strains are not statistically significant, the LD_{50} value of MacoNPV-90/4 pp9 for neonate bertha armyworm larvae was significantly higher than that of -90/1, -90/2, and -90/4 pp15. It was originally anticipated that it might be possible to analyze the relationships between the genome structure and virulence of MacoNPV using some of the pp strains, though it does not necessarily mean that the variation in REN patterns directly reflects the differences in infectivity or virulence. However, because no extreme high (or low) virulence pp strains have been derived, the assessment of these relationships would be very difficult.

The reasons the virulence of MacoNPV strains to insect host did not coincide with the final ECV titres in the viral growth curves are yet to be investigated. However, the OB numbers/per cell for MacoNPV-90/4 pp9 and pp15 determined between 36-96 hr in the viral growth curve assays were similar, indicating that these pp strains were not distinct phenotypes in terms of number of OB

produced per cell.

As a conclusion, plaque-purification did derive some MacoNPV genotypic variants with distinct differences in REN profiles. However, there is no big difference in their levels of virulence to the host insect, indicating that no distinct phenotypes in terms of virulence have been derived.

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