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Hyposoter fugitivus Polydnavirus (HfPV) infection *in vitro*: Cytopathology and viral genome sequence maintenance in a tissue culture cell line

by

Min Kyung Kim

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

at

Dalhousie University

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by Min Kyung Kim

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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For my mother

Table of contents

List of Figures

List of Tables

Abstract

Polydnaviruses replicate only in the ovaries of certain species of endoparasitic wasp. Polydnavirus is a difficult virus to study since there is no tissue culture system that could support viral infection and replication. Developing *in vitro* models for polydnavirus infection is important in order to find some of the factors that are involved in successful parasitism *in vivo*. *Hyposoter fugitivus* polydnavirus (HfPV) is one of the ichnoviruses that is involved in the parasitization of *Malacosoma disstria* by the wasp, *Hyposoter fugitivus*. To understand polydnavirus infection *in vitro*, three different lepidopteran cell lines were exposed to HfPV, and one of the cell lines, Ld 652Y, a cell line derived from the gypsy moth, *Lymantria dispar*, was found to exhibit a variety of cytopathic effects resulting from the viral infection.

Early signs of cytopathology in Ld 652Y cells were similar to the responses of haemocytes to viral infection *in vivo*: transient inhibition of cell growth, rounding up, aggregation, and apoptosis. In addition, changes in protein synthesis in two different cell lines were observed following introduction of viral DNA into Ld 652Y cells either by infection or transfection. The persistence of viral genomic DNA, another aspect of viral infection *in vivo* was also studied in Ld 652Y cells. After virus-infected cells recovered from an initial wave of cytopathic effects, certain viral genome segments were found to be maintained in the HfPV-infected cells.

However, the mechanism of how some viral genome segments persist in Ld 652Y cells appears to be different from that of parasitized *Malacosoma disstria*. While viral DNA appears to be maintained in episomal form *in vivo*, certain viral genome segments are maintained in an integrated form in the chromosomal DNA of Ld 652Y cells. Further investigation identified two HfPV genome segments I and υ which were integrated in the chromosomal DNA of Ld 652Y cells. In addition, the integrative property of genome segment I was demonstrated by transfection.

Abbreviations

HfPV	*Hyposoter fugitivus* polydnavirus
CcPDV	*Cotesia congregata* polydnavirus
EP1	early-induced protein 1
CsV	*Campoletis sonorensis* polydnavirus
ECM	extracellular matrix
MdPD	*Microplitis demolitor* polydnavirus
CrV	*Cotesia rubecula* polydnavirus
RFLP	restriction fragment length polymorphism
dsDNA	double-stranded DNA
VSV	varicella-zoster virus
HSV I	herpes simplex virus type I
HPV	human papillomavirus
EBV	Epstein-Barr virus
NK	natural killer
T_{CTL}	cytotoxic T lymphocytes
CMV	cytomegalovirus
HIV-1	human immunodeficiency virus type 1
SV40	simian virus 40
BPV	Bovine papillomavirus
T Ag	large T antigen
R1	recovered cell line 1
R2	recovered cell line 2
TGF-beta 1	transforming growth factor-beta 1
Fas L	Fas Ligand
TNF	tumor necrosis factor
PCR	Polymerase chain reaction
rc	Relaxed circle genome segment
sh	Superhelical genome segment
ch	Chromosomal DNA
min	minute
Kb	Kilobase
bp	base pairs
kDa	kilodalton

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I. Introduction

Overview

This is a study of *Hyposoter fugitivus* polydnavirus (HfPV), a polydnavirus that is involved in the life cycle of a parasitic wasp *Hyposoter fugitivus.* Developing an *in vitro* system for studying polydnaviruses was a primary goal of this study, since there has been no tissue culture system available to study polydnaviruses.

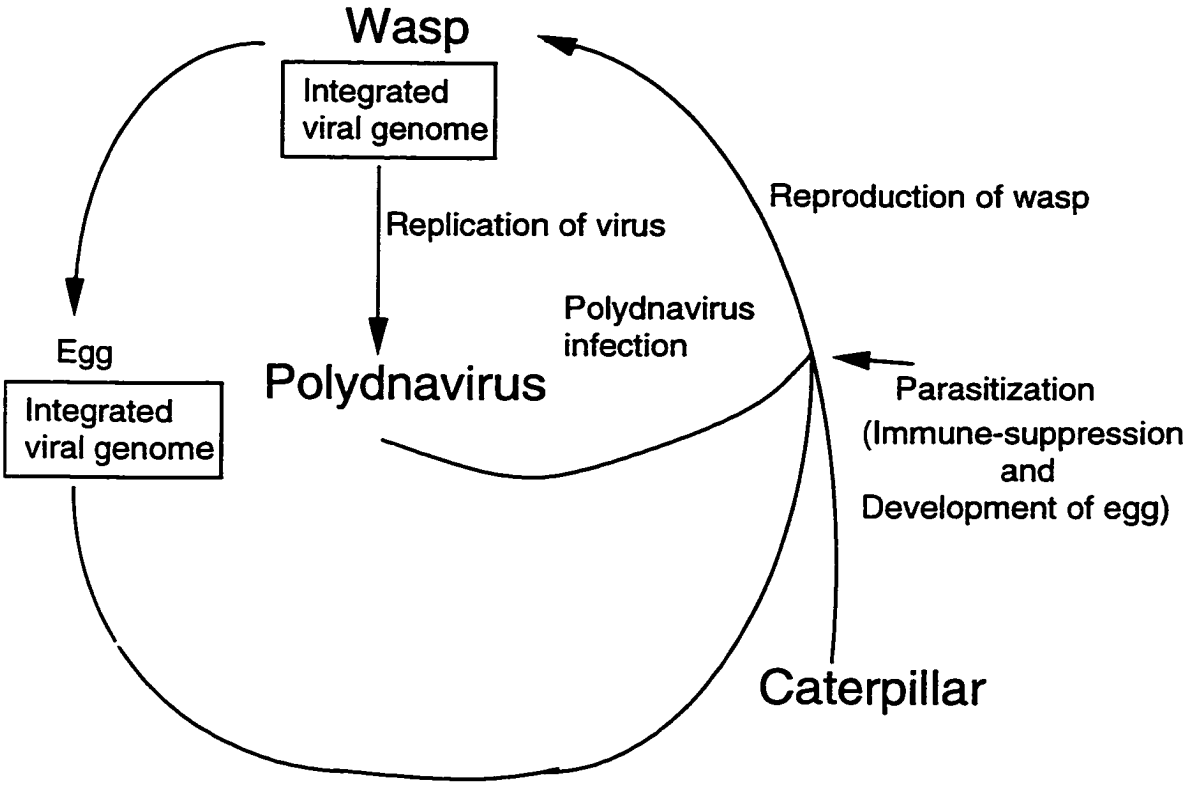
The Polydnaviridae, a family of insect viruses, consists of two groups: ichnoviruses, found in wasps of the family Ichneumonidae, and bracoviruses, found in wasps of the family Braconidae (Stoltz and Whitfield, 1992). These two genera of polydnaviruses differ in their morphology and host range, but they are similar in having an obligatory symbiotic relationship with some parasitic wasps, and in having polydisperse segmented double-stranded circular DNA as their genetic material (Stoltz et al.,1995; Fleming, 1992).

The replication of polydnaviruses only occurs in the ovaries of some parasitic wasps, and polydnaviruses are essential for successful reproduction of the wasps. Polydnaviruses are injected along with wasp eggs into a host, a caterpillar, by parasitic wasps such as *Hyposoter fugitivus.* Once injected into the caterpillars, polydnaviruses play a major role in suppression of the host immune response by inducing cytopathology in haemocytes of the caterpillar (Edson et al., 1981; Stoltz and Guzo, 1987; Tanaka, 1987). The interaction between wasps, caterpillars and polydnaviruses is shown in Figure 1.

Figure 1. The interaction between wasps, caterpillars and polydnaviruses.

Polydnaviruses replicate only in the ovaries of some parasitic wasps. Wasps inject their eggs along with polydnaviruses into caterpillars (parasitization), which results in the infection of caterpillars by polydnaviruses (polydnavirus infection). After infecting caterpillars, polydnaviruses induce cytopathology in caterpillar haemocytes (resulting in immune-suppression) without virus replication. As a result, developing eggs of the wasps, which contain integrated polydnavirus genome can avoid the caterpillar immune system, and mature to become an adult wasp (reproduction).

Figure 1


Wasp
Integrated viral genome
Replication of virus
Polydnavirus
Polydnavirus infection
Reproduction of wasp
Egg
Integrated viral genome
Parasitization
(Immune-suppression and Development of egg)
Caterpillar

Polydnavirus genomic DNA is integrated into the chromosomal DNA of wasps in a "proviral" form, and replicates only in the reproductive tract of the female wasp, in the calyx epithelium. The replicated virus particles, along with wasp eggs and venom, are then injected into caterpillars during parasitization (Stoltz, 1993). The injected viruses are essential for successful parasitization of the host caterpillar by the developing wasp eggs (Edson et al., 1981; Stoltz and Guzo, 1987; Tanaka, 1987). The viral DNA persists in the caterpillars throughout the development of the parasitoid without the apparent replication of the viral DNA (Theilmann and Summers, 1986; Stoltz and Guzo., 1986; Strand, 1992). The viral DNA is detected as late as 15 days post-parasitization when the eggs of the wasp have developed to a larval stage which will feed on haemolymph of the caterpillars. Because the complexity of the polydnaviral life cycle makes *in vivo* study of the virus difficult, developing an *in vitro* system would be useful in understanding how polydnaviruses function in a host.

Although some studies have been carried out using the haemocytes of caterpillars *in vitro* (Stoltz and Guzo, 1986; Strand, 1994), the difficulty of isolating individual haemocyte cell types and of long term maintenance of such cells have been major obstacles in the development of a useful *in vitro* system. Even though lepidopteran cell lines were not expected to support viral replication (since the infection of polydnavirus does not result in the replication of virus *in vivo*), lepidopteran cell lines might provide insight into the events occurring *in vivo*. To develop an *in vitro* system, three different lepidopteran cell lines were tested for their responses to HfPV infection.

This *in vitro* system was used i) to characterize polydnavirus-induced

cytopathology in the different lepidopteran cell lines; ii) to examine effects on host polypeptide synthesis; iii) to demonstrate HfPV genome segment integration and iv) to identify the viral genome segments that are maintained as an integrated form in one of the cell lines. Before discussing the results of these studies of polydnavirus infection *in vitro*, rationale for this study and a general review on i) classification and morphology of polydnaviruses, ii) their life cycle, iii) the host immune response in insects, and iv) the infection of some other viruses and persistence of the viral DNA are provided. This background will provide a framework to establish the rationale behind my current study.

1. Rationale for this study

Developing a tissue culture system may facilitate the identification of viral sequences or gene products that are involved in the induction of haemocyte cytopathology and the persistence of polydnavirus DNA in parasitized caterpillars. Since it is difficult to separate viral products from host products *in vivo,* developing a tissue culture system is an important first step for the study of polydnaviruses at the molecular level. Using a tissue culture system, the complexity of an experimental system is reduced and experimental parameters can be better controlled.

Additional information can be obtained from studying polydnaviruses *in vitro*. First, one can gain biological insight into a mutually beneficial relationship between polydnaviruses and their hosts. Also, the understanding of how foreign DNA can persist in a host can lead to strategies for controlling

foreign DNA in biological systems in general. The maintenance and expression of foreign DNA in a host is important in a number of different situations, such as in gene expression from a plasmid, or in gene therapy. Because polydnaviruses require two different hosts for their replication, these viruses are a convenient system to identify factors for persistence of foreign DNA and for foreign gene expression in different biological systems. The replication of the viral DNA is assumed to be a necessary part of foreign DNA maintenance in a host. Understanding which components mediate viral DNA persistence in caterpillars in the absence of virus replication will help us to understand the maintenance of viral DNA in a host.

In this study, some aspects of polydnavirus-induced cytopathology *in vitro* were characterized, and attempts were made to identify components involved in viral DNA persistence using an *in vitro* system. The results suggest that one insect cell line, Ld 652Y, can be used to study some of the cytopathologies observed in parasitized caterpillars. Using this *in vitro* system, a HfPV genome segment was found to integrate into the chromosomal DNA of Ld 652Y cells.

2. Background

A. Classification of Polydnaviruses

The two genera of polydnaviruses, ichnoviruses and bracoviruses, differ in their replicative host and in their morphological characteristics such as shape, size and number of unit membranes (Stoltz and Vinson, 1979). The

nucleocapsids of bracoviruses are cylindrically shaped, while the nucleocapsids of ichnoviruses are quasicylindrically shaped. The nucleocapsids of ichnoviruses tend to be larger than those of bracoviruses (85 X 330 nm vs. 40 X between 15 and 250 nm) (Stoltz and Vinson, 1979). Also, for the ichnoviruses, a single nucleocapsid is enveloped in two unit membranes, while for the bracoviruses, one nucleocapsid (for example, *Chelonus nigriceps* polydnavirus) or more than one nucleocapsid (for example, *Cotesia melanoscela* polydnavirus) is contained within a single unit membrane (Stoltz and Whitfield, 1992).

B. Life cycle of polydnaviruses

Despite their differences, both bracoviruses and ichnoviruses induce similar cytopathologies in the haemocytes of parasitized caterpillars, and their viral DNA persists in the caterpillars throughout parasitization (Stoltz, 1993; Davies et al., 1987; Strand, 1992). There are two very different stages of any polydnavirus life cycle corresponding to the two hosts: one stage is virus transmission and replication in the ovaries of parasitic wasps, and the other stage is immune suppression and persistence of the viral genome in the parasitized caterpillars.

The genomic DNA of polydnaviruses is integrated into the chromosomal DNA of host wasps, and the viruses are transmitted vertically as endogenous proviruses (Xu and Stoltz., 1991; Fleming and Summers, 1991). The replication of virus particles is observed only in the ovaries of parasitic wasps, and occurs without any apparent damage to the wasps. The replication of the circular polydnavirus DNA may be under the control of the wasp hormone, 20-

hydroxyecdysone that regulates the developmental expression of many insect genes (Webb and Summers, 1992). Viral DNA replication is first detected at a specific stage in pupal development (Norton and Vinson, 1983). Following viral replication, the mature viral particles are released from calyx cells into the oviduct lumen, and the fluid in the lumen (calyx fluid) contains a high concentration of polydnaviruses, which are injected along with eggs and venom during oviposition.

The second stage of the virus life cycle involves suppression of immune responses and persistence of viral DNA in the parasitized caterpillars. Polydnavirus infection of parasitized caterpillars is an important aspect of the symbiotic life cycle of the wasps and the virus. Successful parasitization depends on suppressing the immune response of host caterpillars against foreign objects such as the eggs of wasps. The virus can continue its own life cycle only by ensuring the successful development of wasps in the parasitized caterpillars, and in so doing it can survive and replicate within the ovaries of female wasps.

However, in contrast to conventional viruses, the infection of caterpillar haemocytes by polydnavirus does not directly result in the replication of the viral particles in the "permissive" host, suggesting that the permissive host in polydnavirus infection should be defined differently from the studies of other viruses (such as induction of cytopathologies). The permissive host in the study of viruses usually supports not only infection but also replication of the viruses (Roizman and Palese, 1996). So far in the study of polydnaviruses, a host that supports both infection and replication has not been found.

The virus-induced immune suppression in the parasitized caterpillars appears to be due, in part, to cytopathological changes induced in certain types of haemocyte populations (Stoltz and Guzo, 1986; Davies et al., 1987; Strand 1994). Aggregation of granular haemocytes, rounding up of plasmatocytes (Stoltz and Guzo, 1986, Strand, 1994) and apoptosis (Strand and Pech, 1995) are alterations in haemocyte morphology induced by polydnavirus infection. Functional viral DNA is essential for immune suppression, since inactivation of viral DNA by psoralen cross-linking abolished the virus-induced effects (Stoltz and Guzo, 1986; Beckage et al., 1993; Strand and Noda, 1991).

In polydnavirus-infected caterpillars, several proteins were found to be induced by polydnavirus infection (Cook et al., 1984; Harwood et al., 1994). These proteins were shown to be present in a glycosylated form, and in an amount that could be detected by Coomassie blue staining. One such protein, early-induced protein 1 (EP1), from the haemolymph of *Manduca sexta* larvae parasitized by *Cotesia congregata* polydnavirus (CcPDV), was detectable from 2 to 144 hrs following parasitization (Harwood and Beckage, 1994). In addition, a cysteine-rich polydnavirus protein, VHv1.1, from *Campoletis sonorensis* polydnavirus (CsV)-infected cells was secreted into the haemolymph (Li and Webb, 1994). The secreted VHv1.1 protein bound to haemocytes and reduced the encapsulation response of the host against washed eggs of the wasps, suggesting that this protein is associated with the inhibition of the host cellular immune response (Li and Webb, 1994).

Furthermore, the persistence of the viral DNA in the parasitized caterpillar during the parasitism is observed with both bracoviruses and

ichnoviruses (Stoltz et al., 1986; Strand and Noda, 1991). The persistence of polydnavirus DNA in the parasitized caterpillars was observed not only in a permissive host, which allows reproduction of wasps, but also in a non-permissive host that does not allow the reproduction of wasps (Stoltz et al., 1986). However, the relationship between polydnavirus DNA persistence and virus-induced cytopathology in the parasitized caterpillars is not yet understood.

C. Host immune responses

The host immune response during polydnavirus infection is part of the interaction between the parasitoid and the host immune system, reviewed by Strand and Pech (1995). Host defense against parasitoids depends primarily on encapsulation (the formation of a multilayered cellular envelope) by haemocytes around the parasitoid (Salt, 1968; Lackie, 1988). The encapsulation process appears to involve two major types of haemocytes, granular cells and plasmatocytes (Ratcliffe, 1993). The studies by Ratcliffe and Gagen (1977) and Schmit and Ratcliffe (1977) suggest that the granular cells recognize foreign bodies and release non-self recognition molecules onto the surface of the foreign object. Following this process, the plasmatocytes arrive at the location of the foreign object and form a multicellular sheath around the central core of degranulated and lysed granular cells, thus encapsulating the foreign object. Encapsulation of parasitoids is thought to be carried out by i) recognition of non-self, ii) strong adhesion between haemocytes, and iii) killing of the parasitoid (Strand and Pech, 1995).

Recognition of non-self in the insect immune system appears to involve

basement membrane, which is composed primarily of the extracellular adhesion molecules laminin, collagen IV, and proteoglycans (Strand and Pech, 1995; Pech et al., 1995; Fesseler and Fesseler, 1989; Hortsch and Goodman, 1991). For instance, allogeneic transplants of fat body or nerve chord with intact basement membrane are not encapsulated when transplanted into another insect, while transplants of mechanically disrupted or collagenase-treated fat body or nerve chord are encapsulated (Rizki and Rizki, 1980; Scott, 1971).

Following non-self recognition, formerly non-adhesive haemocytes become adhesive and bind strongly to foreign surfaces and other haemocytes. How do haemocytes change from non-adhesive to strongly adhesive cells in order to encapsulate? The changes of haemocytes from non-adhesive to adhesive during encapsulation might involve extracellular matrix (ECM) and its cell-surface receptors, such as integrins (Strand and Pech, 1995). Integrins are involved in cell-cell aggregation in mammalian systems, and also appear to be involved in the insect immune system (Hynes, 1992; Strand and Pech, 1995). Cell adhesion molecules (laminin, collagen IV and fibronectin) contain the cell adhesion recognition sequence (Arg-Gly-Asp), which is recognized by cell receptors that belong to a subset of the integrin family of receptors (Hynes, 1992). The inhibition of cell adhesion or attachment can be achieved by addition of soluble peptides containing the cell adhesion recognition sequence to the adhesive cells (Bunch and Brower, 1992). It is possible that the encapsulation process starts when haemocytes of insects bind to the cell adhesion molecules present on the surface of foreign objects.

The exact killing mechanism of encapsulated parasitoids is not clear at this point. Following non-self recognition and encapsulation by the host

immune response, the parasitoid could die from asphyxiation (Salt, 1970), toxic quinones or hydroxyquinones produced from melanization (Sugumaran and Kanost, 1993), or reactive oxygen intermediates such as superoxide and hydrogen peroxide (Adema et al., 1993; Toru, 1994).

The role of polydnavirus in parasitism is to inhibit the host immune responses as described above. It appears that polydnavirus infection induces the alteration of certain types of haemocyte morphology to inhibit adhesion and spreading of haemocytes in the lepidopteran host. For example, the inhibition of adhesion and spreading of host plasmatocytes from *M. disstria* by *Hyposoter fugitivus* polydnavirus (HfPV) infection *in vitro* (Stoltz and Guzo, 1986) was observed. Infection by another ichnovirus, *Campoletis sonorensis* polydnavirus (CsV), resulted in destruction of the majority (75%) of plasmatocytes in *Heliothis virescens* (Davies et al., 1987). In addition, the bracovirus *Microplitis demolitor* polydnavirus (MdPDV) infected the majority of haemocytes in the circulation of *Pseudoplusia includens*, which resulted in the suppression of haemocyte spreading of the host (Strand et al., 1994; 1995). The induction of apoptosis by a polydnavirus in purified haemocytes has only been observed in MdPDV infection of *P. includens* (Strand, 1995).

D. *Hyposoter fugitivus* Polydnavirus (HfPV)

The polydnavirus that is the focus of my interest is found in one of the ichneumonid parasitic wasps, *Hyposoter fugitivus*. HfPV replicates only in the ovaries of *Hyposoter fugitivus* whose natural host is the tent caterpillar *Malacosoma disstria* (Stoltz et al., 1986). HfPV shares similarity to many other

polydnavirus systems. For instance, HfPV is replicated in the ovaries of a parasitic wasp, *Hyposoter fugitivus,* and when the wasps parasitize tent caterpillars, HfPV is injected along with the eggs of *H. fugitivus*. After the viruses are injected into the caterpillars, the host immune response to foreign objects is suppressed and the viral DNA persists throughout the parasitization (Stoltz and Guzo, 1986; Stoltz et al., 1986). However, different from infections by CsV and CcPDV that show addtional polypeptide bands in the haemolymph of the polydnavirus-infected caterpillars (Cook et al., 1984; Harwood et al., 1994), no changes were observed in the haemolymph of HfPV-infected *M. disstria* when an initial attempt to find altered polypeptide synthesis was made (Stoltz, unpublished data).

The HfPV genome is located within the chromosomal DNA of the wasp, *H. fugitivus* as observed for other polydnaviruses (Xu, 1991). The chromosomally located viral genome appears to be responsible for the transmission of HfPV from generation to generation in the wasp (Stoltz and Xu, 1990). As many as 30 HfPV genome segments have been cloned, and some of the cloned genome segments have been characterized for their cross-hybridization to other genome segments, and for restriction fragment length polymorphism (RFLP) (Xu, 1991). Similar to the other ichnovirus CsV, HfPV genome segments are also named alphabetically by the size of the genome segments from the lowest molecular weight segments to the largest (A, ~ 1.0 Kb to Z, ~ 6.6 Kb) (Xu, 1991). Although it is difficult to determine the precise size of the viral genome due to the difficulty of determining the exact number of the viral genome segments, the estimated size of the ichnovirus genome size is

approximately 300 Kb (CsV genome size is estimated to be 280 Kb; Krell et al., 1982).

The viral genome segments are present in non-equimolar amounts with some genome segments exhibiting the same molecular weights (Xu, 1991). Two different types of genome segments are present in HfPV: the genome segments with unique sequences and the genome segments that cross-hybridize with other genome segments. The genome segments with unique sequences do not cross-hybridize to other viral genome segments and are present at a single site in the chromosomal DNA of the wasp (Xu and Stoltz, 1991). Many HfPV genome segments, however, cross-hybridize with other genome segments, and they are referred to as genome segment families. The genome segment families of HfPV consists of at least two member genome segments for which the smaller size genome segment contains sequences that are a part of the larger size genome segment (Xu and Stoltz, 1993). Also, it appears that more than one genome segment could be derived from a common chromosomal locus as shown in the study of genome segment families (Xu and Stoltz, 1993). Several genome segment families in HfPV genome were identified such as G, S, R, U, and Z (Xu and Stoltz, 1993; Xu, 1991).

In addition to the complexity of having unique genome segments and genome segment families, HfPV also contains a high degree of allelic polymorphisms in the viral genome (Xu and Stoltz, 1990). Allelic polymorphisms were identified in genome segments I , G and S (Xu, 1991). For example, there are two types of genome segment I with one type containing an *EcoR* I endonuclease site that is absent in the other type, and the

polymorphic genome segments are known to segregate in Mendelian fashion (Xu, 1991). These studies of polydnavirus genomes suggest that polydnaviruses contain the most complex viral genomes known, and the exact mechanism of polydnavirus replication is not clear at this point.

3. Infection by other viruses

The infection of polydnaviruses results in cytopathology and persistence of the viral DNA in the host, as is observed in infection of many other double-stranded DNA (dsDNA) viruses. Thus, studies of viral DNA persistence in other viruses might be helpful in understanding persistence of polydnavirus DNA in the parasitized caterpillars. In addition, the suppression of host immune responses due to virus-induced cytopathology is also observed during infection by human immunodeficiency virus (Chirmule and Pahwa, 1996). Some of the similarities between infections by other viruses and infections by polydnaviruses are reviewed here.

A. Virus-induced cytopathology

Different viruses induce cytopathology in their hosts through many different mechanisms, and HfPV might use one or more of these mechanisms to induce cytopathology in parasitized caterpillars. Virus-induced cytopathology is produced or influenced by: i) inhibiting host immune responses; ii) modulating the activity of growth factors, cytokines, and transmitters; iii) disrupting intracellular calcium homeostasis; iv) altering deoxynucleoside triphosphate pools; and v) either induction or inhibition of apoptosis (Tyler and Fields, 1996).

Among other double-stranded DNA viruses, virally-encoded inhibitors of the host immune responses are found in adenoviruses and herpesviruses. An adenovirus E3 protein, gp 19K, localizes to the membrane of the target cells endoplasmic reticulum and binds to newly synthesized MHC class I antigens, which prevents transport of the antigen to the cell surface (Anderson et al., 1985). Two other adenovirus E3 proteins (14.7 K, and 10.4 K) and the E1B 19 K protein can also protect the virus-infected cells from cytolysis induced by tumour necrosis factor (Gooding et al., 1991 a, 1991 b; Ranheim et al., 1993). Varicella-zoster virus (VSV) and herpes simplex virus type I (HSV I) encode Fc receptors that bind to immunoglobulins and, therefore, interfere with antibody-mediated immune responses (Bell et al., 1990; Litwin, et al., 1990). HSV I also encodes gC, which functions as a receptor for complement component C3b to inhibit complement-mediated cell lysis and virus neutralization (Friedman et al., 1984; Harris et al., 1990).

The induction of cytopathology by modulating host growth factors, cytokines, or transmitters has been documented for many other viruses. The BCRF1 protein encoded by Epstein-Barr virus (EBV) is one of the examples among double-stranded DNA viruses. BCRF1 is a homolog of IL-10, with greater than 90 % amino acid sequence identity (Hsu et al., 1990; Moore et al., 1990). Host immune function can be altered by modulating a cytokine such as IL-10 that is secreted by T helper cells. IL-10 inhibits the synthesis of other cytokines such as IL-2 and interferon-γ (Moore et al., 1990). The expression of BCRF1 was suggested to facilitate viral infection by inhibiting natural killer (NK) and cytotoxic T lymphocytes (T_{CTL}) response against EBV (Kurilla et al., 1993).

Cytopathology can also result from disruption of intracellular calcium homeostasis as shown in the study of cytomegalovirus (CMV)-infected fibroblasts (Nokta et al., 1987). Another mechanism for virus-induced cytopathology is the alteration of deoxynucleoside triphosphate pools in the infected cells. For example, herpesviruses encode dUTPases that catalyze the hydrolysis of dUTP to dUMP and PP (Pyles et al., 1992). HSV-1 mutants deficient in dUTPase activity are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency (Pyles et al., 1992).

In addition, cytopathology in the host may be caused by either induction or inhibition of the apoptotic response in virus-infected cells (Shen and Shenk, 1995). Induction of apoptosis produces cytopathology by inducing cell death in the host cells, while inhibition of apoptosis produces cytopathology by allowing virus replication in the host. The suppression of the immune response in human immunodeficiency virus type 1 (HIV-1) infection is produced by a progressive loss of CD4+ T cells through virus-induced apoptosis (Meyaard et al., 1992). HIV-induced apoptosis is produced by the interaction of the envelope glycoprotein, gp120 with its receptor, CD4 (Capon and Ward, 1991).

The E1A protein of adenovirus induces apoptosis that is dependent on the p53 tumor suppressor (Hinds and Weinberg, 1994). The p53 tumor suppressor protein is known to be the cellular gatekeeper for cell growth and division, and p53 functions as a transcription activator of genes that are involved in cell cycle arrest, DNA repair, and apoptosis (Levine, 1997). Expression of E1A stabilizes p53, resulting in an elevated p53 level, which in turn signals the apoptoptic response among virus-infected cells (Lowe and

Ruley, 1993). Apoptosis induced by the expression of E1A is counteracted by the E1B proteins. The 19K E1B protein inhibits apoptosis by binding to Bax, a protein that accelerates apoptosis (Han et al., 1996), while the 55 K E1B protein inhibits apoptosis by direct binding to p53 and inactivating p53 (Yew and Berk, 1992).

Other viral proteins, such as simian virus 40 (SV40) T antigen and human papillomavirus E6, are also known to inhibit apoptosis by inactivating p53 (MaCarthy et al., 1994; Pan and Griep, 1994). Inhibition of apoptosis also occurs by modulation of other proteins involved in the apoptotic response. For instance, the LMP1 protein encoded by EBV induces the expression of Bcl-2 (Henderson et al., 1991). In addition, EBV and African swine fever virus encode BHRF1 and LMW5-HL proteins that are similar to Bcl-2 (Henderson et al., 1993; Nielan et al., 1993). Although baculovirus p35 and iap genes are not similar to Bcl-2, they block apoptosis in virus-infected cells, indicating that factors other than the Bcl-2 regulators are involved in the apoptotic process (White, 1996). It appears that P35 blocks apoptosis by inhibition of ICE family proteases, caspases (cysteine proteases) that induce apoptosis (Bump et al., 1995; Enari et al., 1996). Inhibition of apotosis during polydnavirus infection might be important in order to protect the virus-infected cells that express viral genes which are involved in immune suppression.

B. Persistence of the viral genome

i) General conditions for viral genome persistence

The persistence of HfPV DNA is observed as in the infection of many

other double-stranded DNA viruses. For a viral DNA to persist in the host, the deleterious effects of the virus in the host must be attenuated (so that infected cells can survive), the virus must avoid detection and elimination by the immune system of the host, and the viral genome must be maintained (Ahmed et al., 1996). Since inhibition of apoptosis is important in virus genome maintenance, many other double-stranded-DNA viruses, such as SV40 (large T antigen), HPV (E6), adenovirus (E1B), EBV (LMP1) and baculovirus (p35) encode genes that inhibit the apoptotic responses of virus-infected cells (Conzen et al., 1997; Pan and Griep, 1995; Querido et al., 1997; Teodoro and Branton, 1997; Henderson, et al., 1991; Clem et al., 1991).

Attenuation of cytopathology can be achieved in several ways. First, attenuated viral effects on the host could be achieved by infection of non- or semi-permissive cells, as shown in infection of sensory neurons by HSV (Lillycrop et al., 1994) and infection of B lymphocytes by EBV (Khanna et al., 1995). Herpes simplex virus (HSV) is capable of producing lesions by lytic replication in epithelial cells. In addition, the virus establishes asymptomatic latent infections in sensory neurons where viral replication is not observed (Lillycrop et al., 1994). Similar to HSV, Epstein-Barr virus (EBV) can infect permissive epithelium in the oropharynx where it replicates, but it can also maintain a lifelong latent infection in B lymphocytes (Khanna et al., 1995). Attenuated viral effects on the host could also be achieved by infecting only a small proportion of the available permissive cells (as observed in the persistent infection of adenoviruses), or by generating attenuated viral variants to reduce deleterious viral effects (Ahmed, 1994).

To evade host immunity, viruses use many different strategies: i) restricted expression of viral genes (expression of late membrane proteins (LMPs) in latent infection of B-cells by EBV) (Rawlins et al., 1985); ii) infection of immunologically privileged sites (infection of kidney by CMV) (Ahmed, et al., 1996); iii) emergence of antigenic variation (Holland et al., 1982); iv) suppression of cell-surface molecules required for T-cell recognition (inhibition of MHC class I antigen expression by CMV and HSV) (Lehner et al., 1997 and Beinert et al., 1997); v) interference with cytokine functions (inhibition of interferon-α/β by the adenovirus VA RNA and the EBV EBER RNA) (Moore et al., 1993); and vi) immunologic tolerance produced by the antigenic peptide of simian virus 40 large T antigen (Soldevila et al., 1995). Because polydnavirus infection appears to result in cytopathology leading to immune-suppression in parasitized caterpillars, the strategies used by other DNA viruses for persistence are probably not the most important aspect in understanding polydnavirus-infection. In polydnavirus infection, the persistence of viral DNA may be required just long enough for the wasp embryo to develop since viral replication does not occur in the caterpillars. The subject of viral genome maintenance in persistently infected cells is reviewed in detail below, due to its importance in this study.

ii) Persistence of other double-stranded DNA viruse genomes

The viral genome can be maintained in either an integrated form or an episomal form. In many studies, Southern blot analysis has been used to determine how viral dsDNA persists in a host. Viral DNA could persist in a

variety of forms. For instance, viral DNA could persist as an extra-chromosomal monomer, a non-integrated concatemer, an integrated concatemer and/or as monomers that are integrated at multiple sites. The expected Southern blot results for these different forms of foreign DNA maintenance are shown in Figure 2. In addition to Southern blot analysis, an established *in vitro* system has been used to study the persistence of viral genomes in host cells.

Persistence of viral DNA as an integrated form is the focus of this review since some similarities are found between the infection of other dsDNA viruses and the infection of polydnavirus *in vitro*. However, the significance of viral genome integration in the life cycle of dsDNA viruses is not clearly understood. Studies of viral DNA integration described in this section suggest that viral DNA may integrate at more than a single specific site in the host chromosomal DNA, that the form of the integrated viral DNA may vary, and that the integration of the viral genome often occurs when the virus is grown in a non-permissive host. Some studies indicate virus-induced cytopathology might help viral genome integration. For example, due to virus-induced cytopathology increased frequency of hepadnavirus DNA integration was observed (Petersen et al., 1997). The expression of E6 and E7 of the human papillomavirus (HPV) genome is known to help the integration of the viral genome (Kessis et al, 1996).

On the other hand, instability of the host chromosomal DNA may result from integration of viral DNA. Instability of the host chromosomal DNA is often associated with cells under crisis that give rise to immortal or tumorigenic cells (Ray et al., 1992; Bravard et al., 1992) suggesting that the integration of HfPV

Figure 2. Possible forms in which viral DNA can be maintained in a host. Viral DNA can persist in a host in the form of episomal DNA (A), as integrated DNA (B) or both. Chromosomal DNA is indicated as a thick line while viral DNA is indicated as a thin line. Expected Southern blot results for the different forms of viral DNA are shown at the bottom, when total viral genomic DNA is used as a probe. Letter D (double) indicates lanes containing chromosomal DNA digested with a restriction enzyme which has two restriction sites in the viral genome while letter S (single) indicates lanes containing chromosomal DNA digested with a restriction enzyme that has a single site in the viral genome. Expected intensity of the bands are indicated by their thickness.

Figure 2

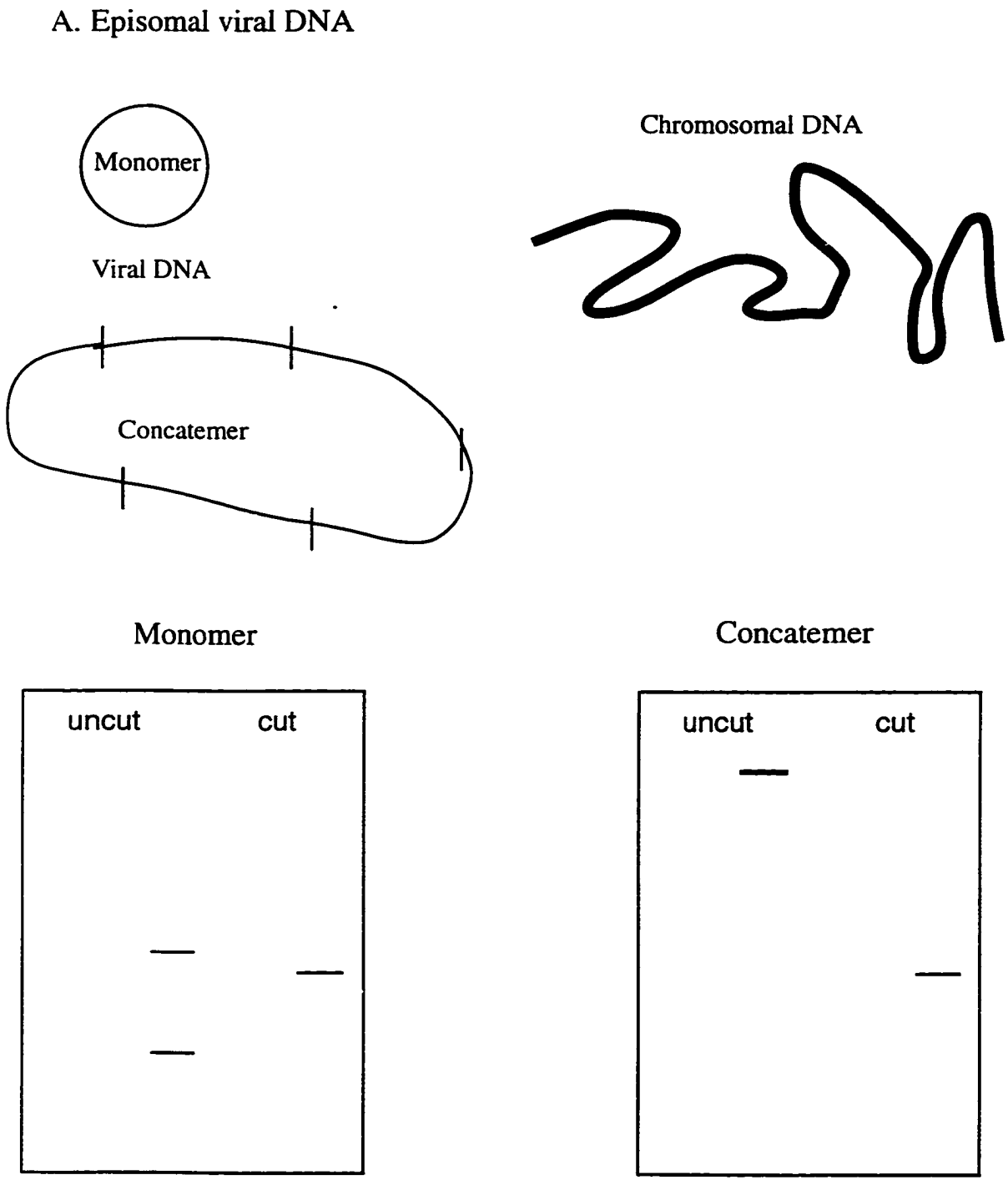
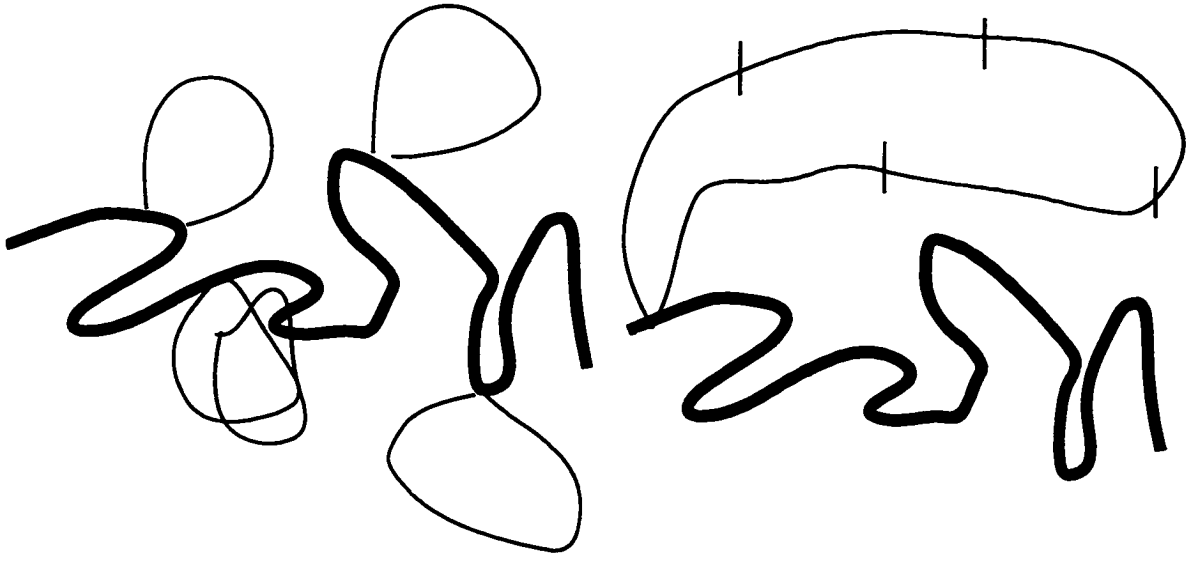
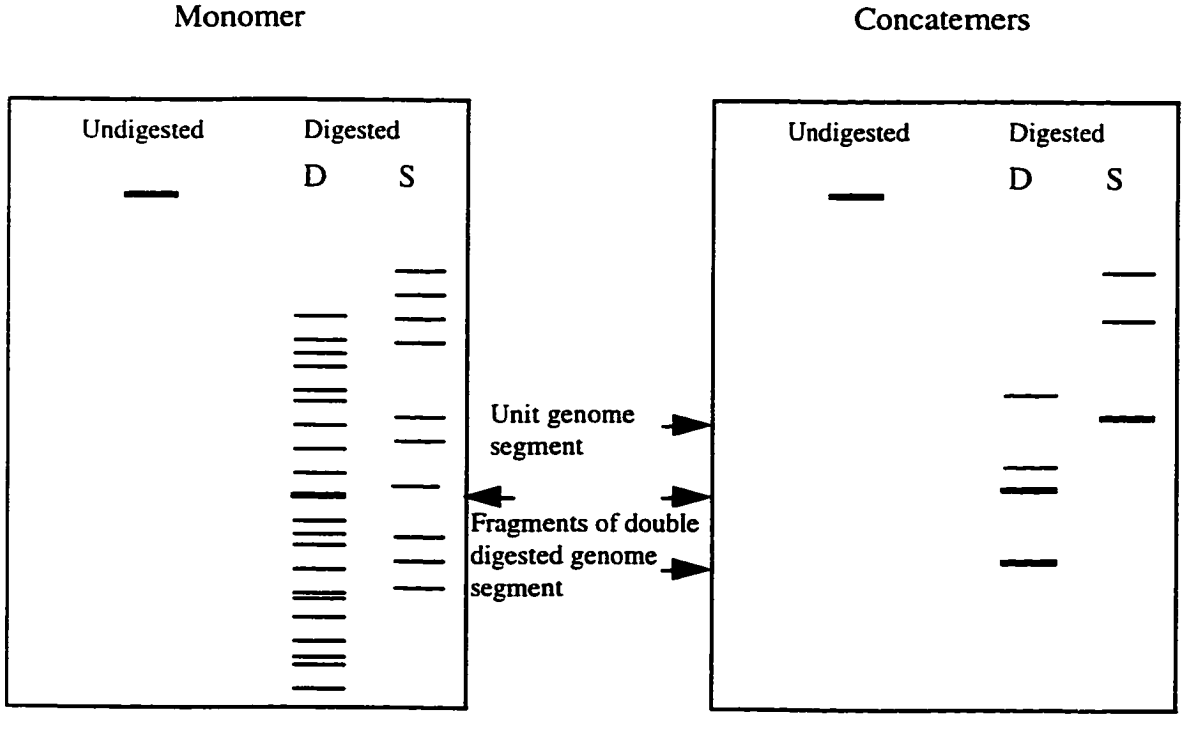

A. Episomal viral DNA
Monomer
Viral DNA
Concatemer
Chromosomal DNA
Monomer
uncut
cut
Concatemer
uncut
cut

Figure 2

B. Integrated viral DNA





DNA may trigger the virus-induced cytopathology. Integration of HPV-18 DNA induced a complex genomic rearrangement in a cervical carcinoma cell line (Gallego et al., 1997), and the integration of the EBV genome produced a fragile breakage site (Wolf et al., 1995). Also, the integration of SV40 DNA resulted in spontaneous rearrangement of chromosomal DNA (Gurney and Gurney, 1989).

When Bovine papillomavirus (BPV) is grown in a mouse cell line, the viral genome is often found to integrate as a large concatemer in the chromosomal DNA (Allshire and Bostock, 1986). Both monomeric extra-chromosomal replication and chromosomal integration of the BPV genome have been observed in the same host cell line (Allshire and Bostock, 1986; Hagen et al., 1995). Further studies of subcloned BPV-transformed cell lines have indicated that there is cell to cell variation. The extent and location of BPV-specific cytogenetic staining varies from cell to cell, as does the Southern blot hybridization pattern observed when total cellular DNA is probed with the BPV genome (Ravnan et al., 1992). Further study on subclones of BPV-infected cells has indicated that BPV persists in cells as long head-to-tail tandem arrays of unit-length viral genomes integrated into the chromosomal DNA of host cells (Hagen et al., 1995).

Genomic DNA of another dsDNA virus, SV40, also integrates into the chromosomal DNA of a non-permissive host cell line, which results in instability of the DNA (Bender and Brockman, 1981; Gurney and Gurney, 1989; Mogneau et al., 1980), and the instability of chromosomal DNA is associated with development and progression of tumors (Bishop, 1987; Klein, 1988). There are two possibilities to explain the SV40 genome rearrangements. First, SV40

large T antigen (T Ag) could bind to the SV40 origin of replication and initiate unscheduled DNA replication, resulting in the over-replication of the origin and flanking sequences, and then recombination of the over-replicated DNA into the chromosomal DNA (Botchan et al., 1978). Second, the viral DNA excised from the chromosomal DNA, could replicate as an extrachromosomal element, and reintegrate into the host chromosomal DNA at a new or the same site (Carroll et al., 1987; Daya-Grojean and Monier, 1978). It has been shown that the origin of replication and early control region of the SV40 genome are responsible for the genome instability (Hunter and Gurney, 1994).

Resting B-cells infected by Epstein-Barr Virus (EBV) *in vitro* exhibit persistence of the viral genome in an integrated form as well as an episomal form (Hurley et al., 1991). Upon infection, some of the resting B-cells become activated and a single linear EBV genome closes to generate a covalently closed circular DNA. However, a substantial fraction of the B-cells (~ 40 %) fail to contain a circularized viral genome (Hurley and Thorley-Lawson, 1988) and express only a limited set of EBV latent genes (Hurley and Thorley-Lawson, 1988; Azim et al., 1990). Nevertheless the EBV genomic DNA persists in these cells (Calender et al., 1987; Murray et al., 1988). Further studies on these EBV transformed cells indicate that the viral genome persists as an integrated copy (Hurley et al., 1991).

Cytopathology and persistence in the infection of other viruses reveiwed here suggest that i) cytopathologies observed in HfPV-infected cells could be induced by one or more of the mechanisms used by other viruses, and ii) the persistence of HfPV DNA might occur by one of the mechanisms used by other

dsDNA viruses for persistence of viral DNA.

II. Materials and methods

1. Insects.

The ichneumonid parasitoid (*H. fugitivus*) was reared from larvae of the forest tent caterpillar (*Malacosoma disstria*). Female parasitoids were allowed to mate and mature. One week to 10 days after mating they were placed in a plastic tub that contained 3rd to 4th instar *M. disstria* larvae for parasitization. When the parasitoids emerged from the host, the wasps were separated by their sex and placed into 6 ounce cups. The wasps were fed by placing a drop of honey and water inside the petri dish lid used as to cover the cup.

The host, the forest tent caterpillar (*M.disstria*) was reared on an artificial medium (Bell et al., 1981).

2. Tissue culture.

The Ld-652 Y cell line used was derived from ovaries of the gypsy moth, *Lymantria dispar* (Goodwin et al., 1978). The Md 66 cells were derived from haemocytes of *Malacosoma disstria* larvae (Sohi, 1971). Another cell line, Ea 1174A, was derived from haemocytes of the salt marsh caterpillar, *Estigmene acrea* (Granados and Naughton, 1976). All cell lines were grown at 25°C in modified Grace's medium (JRH) supplemented with 10% fetal bovine serum, pH 6.5. The cell lines that had been infected and recovered from the cytopathology induced by HfPV were designated as recovered cell lines using the following nomenclature; recovered cell line 1 (Ld/R 1), recovered cell line 2 (Ld/R 2) and

so on depending on the order of independent infection.

3. Virus isolation and infection.

HfPV was used in the form of calyx fluid freshly dissected from wasp ovaries since experiments using purified virus yielded identical viral infection results. The reproductive tracts from adult female *H. fugitivus* wasps were removed by pulling out the ovipositor using jeweller's forceps. The reproductive tract was placed in either phosphate buffered saline (PBS, pH 7.0, Dulbecco and Vogt, 1954) or Grace's medium depending on the nature of the experiment. The calyces were opened by severing the calyx and common oviduct. A standard infection experiment procedure was carried out by using calyx fluid from a single female parasitoid isolated in 0.5 ml Grace's medium. 1 X 10^6 cells were incubated with the virus in a 25 cm^2 tissue culture flask for 1 hour. 2.5 ml of additional Grace's medium supplemented with 10% fetal calf serum was added after the 1 hour infection period.

4. SDS-PAGE.

Ld 652Y cells were harvested by being rinsed twice with PBS. Cell extracts were prepared by resuspending cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM ethylenediamine tetraacetate [EDTA] , 150 mM NaCl, 0.5 mM dithiothreitol [DTT], 0.5% Triton X-100 and 1.0 mM phenylmethylsulfonyl fluoride [PMSF] followed by high speed centrifugation (14,000xg, 30 min., 0-4°C). The supernatant was saved as a cell extract. Samples were analyzed using standard SDS-PAGE procedures (Laemmli, 1970). SDS-PAGE was

performed using either 12.5% or 10% polyacrylamide.

For crude fractionation, cytoplasmic membranes, organelles, and cytosolic fractions, were prepared as described (Chen-Levy et al., 1989). Cells ($1x10^7$) were rinsed twice in PBS, and then resuspended in cold hypotonic buffer A (10 mM-HEPES buffer pH 7.8, 2 mM $MgCl_2$ and 0.1 mM EDTA). Cells were frozen in liquid nitrogen and thawed three times, and centrifuged for 6 min. at 1000xg, providing a pellet of nuclei. The supernatant was further centrifuged for 1 hour at 150,000xg. The pellet at this point contained cytoplasmic membranes and organelles, while the supernatant represented the cytosolic fraction.

5. ^{35}S-methionine labeling.

Virus-infected and transfected cells were also labeled with ^{35}S-methionine in order to determine whether protein synthesis was altered by viral infection. At various time points, cells were washed twice with Grace's medium lacking methionine and were incubated further in the medium for two hours before labeling. After the starvation period for methionine, the cells were incubated in Grace's medium containing ^{35}S-methionine (final concentration of 0.1 to 0.2 mCi/ml) for one hour. The ^{35}S-methionine labeled samples were then analyzed using SDS-PAGE and autoradiography.

To analyze secreted proteins, the proteins in the medium were concentrated by the addition of 10% trichloroacetic acid (TCA) to 1% fianl concentration . The samples were centrifuged for 10 min. at 14,000 x g. The precipitate was washed twice with 100% ethanol and dried before the samples

were resuspended in 50 µl of 1xSDS/sample buffer (0.5% SDS, 0.016 M Tris-HCl, pH 6.8, 1.25% 2-β-mercaptoethanol, 2.5% glycerol, 2.5 µg/ml bromophenol blue). The samples (25 µl each) were boiled (95 ^{0}C, 4 minutes) and then loaded on to 10 or 12% SDS-PAGE gels at 200 volts for 1 hour for analysis. Prestained high-molecular weight markers from Gibco-BRL were used as molecular weight standards. SDS-PAGE gels were stained with Coomassie blue stain (1/2 hour with 0.1% Coomassie blue in 40% methanol and 10% acetic acid) and destained with destaining solution (40% methanol and 10% acetic acid) for 1 to 3 hours. The gels were then dried on a gel drier (Model 583, Bio-Rad) for 30 min. or dried in between gel drying film (Promega) over night. ^{35}S-methionine labeled protein bands were detected by exposure to Kodak X-ray film.

6. Apoptosis.

DNA was extracted from Ld 652Y cells as described (MacCabe et al., 1993). 1X10^7 cells were harvested and washed twice with PBS, and the cells were resuspended in 0.25 ml of ice-cold TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8). 0.25 ml of ice-cold lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% (V/V) Triton X-100, pH 8.0) was then added to the samples and incubated on ice for 15 minutes. The cell lysates were centrifuged at 14,000xg for 15 minutes. The supernatant was collected, NaCl (0.16 M final concentration) and 2 volumes of 95% ethanol were added, and the samples were stored at -70^{0}C for 30 minutes. The precipitated samples were then pelleted by centrifugation at 14,000xg for 15 minutes, the pellets were washed twice with 70% ethanol, and dried.

The dried samples were resuspended in 0.5 ml TE containing 0.5% (V/V)

sodium dodecyl sulfate (SDS), and extracted with phenol, chloroform and isoamylalcohol (25:24:1). The aqueous phase was separated by centrifugation at 14,000xg for 10 minutes, and the DNA in the aqueous phase was ethanol precipitated as before. The pelleted DNA samples were resuspended in TE buffer (20 µl) and treated with proteinase K (0.5 µg/ml) for one hour at 37°C. Samples were re-extracted with phenol-chloroform, precipitated with ethanol, and electrophoresed in a 1.8% agarose gel using TEA buffer.

7. Growth curves.

The proliferation of *HfPV* infected adherent Ld 652Y cells was measured by counting the number of cells per 0.1 mm^2 area in the cultures using a 0.1 mm^2 square grid in optical lenses (Ld 652Y cells) of a phase contrast microscope (Nikon phase contrast-2 ELWD 0.3). Growth of suspending Ea 1174A cells was monitored by counting cell numbers in a culture using a hemacytometer.

8. Cell viability.

0.1 ml of 0.4% Trypan Blue stock in PBS, pH 7.2-7.3 was added to 1 ml of cells to monitor the viability of cells in cultures. Non-viable (Trypan Blue stained) and viable (non-stained) cell numbers were counted under a phase contrast microscope using a hemacytometer following the procedure of Summers and Smith (1987).

9. Light microscopy.

A Nikon phase contrast-2 ELWD 0.3 microscope equipped with Nikon F-601u camera was used to photograph alterations in cell morphology induced by HfPV infection and transfection.

10. Transfection.

Lipofectin was purchased from Gibco-BRL, and the "lipofection" procedure was followed according to the manufacturer's instructions. 2.5 μg of DNA in 100 μl Grace's medium was used for each lipofection of 1-2 x 10^5 cells. 10 μg of Lipofectin reagent in 100 μl was used per lipofection. The cells were washed twice with Grace's medium, and 200 μl of the the DNA and lipofectin mixture that had been incubated at room temperature for 30 min. was mixed with 0.8 ml of additional Grace's medium and overlayed for four hours on the washed cells. 2.5 ml of fresh Grace's medium containing 10% fetal calf serum was added after four hours. Depending on the experiment, cells were harvested at different time points.

11. Digitonin treatment of the virus particles.

Pelleted virus particles from 3 female wasps were resuspended in 100 μl of 10% digitonin dissolved in dimethylsulfoxide (DMSO) and incubated at room temperature for 10 minutes. The volume of the sample was then increased by adding 3 ml Grace's medium, and placed in a 1.91 ml/cm volume dialysis tube (BioDesign, Inc.). The samples were dialyzed in 2 changes of 100 ml Grace's medium for four hours each. After dialysis, the sample was filter sterilized by

passage through a 0.45 μm filter unit (Millipore). The sterile viral sample was used for the study of virus infection.

12. Nucleic acid isolation and manipulations.

HfPV genomic DNA was purified from crude calyx fluid. Calyx fluid was collected by dissecting the reproductive tract of the female wasps in 1X SSCE (0.15 M NaCl, 0.015 M sodium citrate, 1mM EDTA). After brief centrifugation (1000xg, 1 min.) to pellet any parasitoid eggs and tissue debris, the supernatant was removed, and a final concentration of 4% sodium sarcosinate was added from a 20% stock solution. The samples were then incubated at 60^{0}C for 1 hr before proteinase K (100 μg/ml final) was added. The proteinase K digestion was carried out at 50^{0}C for 2 hours.

Nucleic acids from the sample were then extracted with two volumes of TE buffer-saturated phenol:chloroform:iso-amyl alcohol (50:48:2). The aqueous and the organic solvent phases were separated by centrifugation at 14,000xg for 10 minutes. The aqueous phase was re-extracted using the same volume of phenol followed by the same volume of chloroform:iso-amyl alcohol (24:1). The viral genomic DNA in the aqueous phase was then precipitated by adding NaCl to a final concentration of 0.25 M and 2 volumes of 95% ethanol. The sample was incubated at -70^{0}C for one hour, and the precipitated DNA was recovered by centrifugation at 14,000xg for 15 minutes. The DNA pellet was then washed twice with 1 ml of 70% ethanol and dried, and the dried DNA pellet was resuspended in ddH_2O.

Chromosomal DNA from Ld 652Y cells was purified following the

protocol described in Short Protocols in Molecular Biology (Ausubel et al., 1992). Standard methods were used for DNA digestion, ligation, and agarose gel electrophoresis (Maniatis et al., 1988). PUC19 (Gibco-BRL) and pBLUESCRIPT SK + (Stratagene) were used as cloning vectors.

Plasmid DNA for cloning and sequencing was purified by the method of Lee and Rasfeed (1990). Cloned genome segments (I, M, U, υ) were also purified by the same method (Lee and Rasfeed, 1990) from *E. coli* strain DH5α. *E. coli* strain DH5α was transformed with plasmid DNA by $CaCl_2$ heat shock treatment (Hanahan, 1985).

DNA fragments were purified from agarose gel slices using the GeneClean kit (Bio 101, La Jolla, Calif.). A minimal portion of agarose gel containing the DNA fragment to be purified was excised using a razor blade on a UV transilluminator (Bio-Rad) before the DNA purification procedure provided by the manufacturer was followed.

13. Polymerase Chain Reaction (PCR).

PCR reaction mixtures were prepared and amplification was performed in 1x PCR buffer (50 mM KCl, 20 mM Tris- HCl, 2.5 mM $MgCl_2$, 0.1μg/ml BSA) containing 0.2 mM dNTPs, 2.5 ng/μl or 3.0 ng/μl of each primer, 0.05 U/μl Taq DNA polymerase (Gibco-BRL), and 1 μg of chromosomal DNA or 50 ng HfPV DNA in a total volume of 50 μl. Each PCR reaction was carried out in a DNA thermal cycler (MJ Research). DNA was denatured at 94 °C for 1 min, annealed at 65 °C for 1 min., and extended at 72 °C for 1 min. This cycle was repeated 35 times. Primer melting temperatures were calculated and the lowest melting temperatures for the primers were used as the annealing temperature. PCR

products were then analyzed on 1.5% agarose gels. Genome segment I-specific primers had the following sequences: 5'TGTAAGCGAAGTGTCAACT-CCGT3' and 5'ATCGGTCTAGAGGACTGACGGTG3' (2.5 ng/µl). Genome segment U-specific primers had the sequences 5'ACGACGTCCAGCAGCA-CAT3' and 5'GGAGCCAAACTGCAGTT3' (3.0 ng/µl).

14. Inverse PCR.

Inverse PCR (Ochman et al., 1990) was carried out using the same conditions as described for PCR except for the primer sequences and the amount of the templates used. The primers were designed from genome segment I sequences at the area shown in Figure 2. Two sets of primers were designed and used for inverse PCR, and the sequences for the primers are; one set of primers, IN PCR I, 5'GCAGGGTGACGATGCAATGACCT3' and 5'CACTGACTGTATGCCGTTAGGTG3' and the other set of primers, IN PCR II, 5'CAGGATCCTTGTTCCGGACGGAGTT3' and 5'CAGGATCCACGTGATCA-GTACTCGT3'. Inverse PCR differs from standard PCR because it involves restriction enzyme digestion and ligation steps for the templates.

Chromosomal DNA from recovered cell lines was digested with *Hind*III and then ligated in order to circularize the linear DNA fragments prior to inverse PCR. Following the ligation reaction, 1 µl of the ligation reaction was used for agarose gel electrophoresis on 0.7% agarose gels to confirm the circularization. 5 µg of ligated chromosomal DNA was used as template for each inverse PCR reaction. For a positive control for the inverse PCR experiment, HfPV DNA (100 ng) was used to generate an expected 1.4 Kb DNA fragment following inverse

PCR procedure (Figure 3).

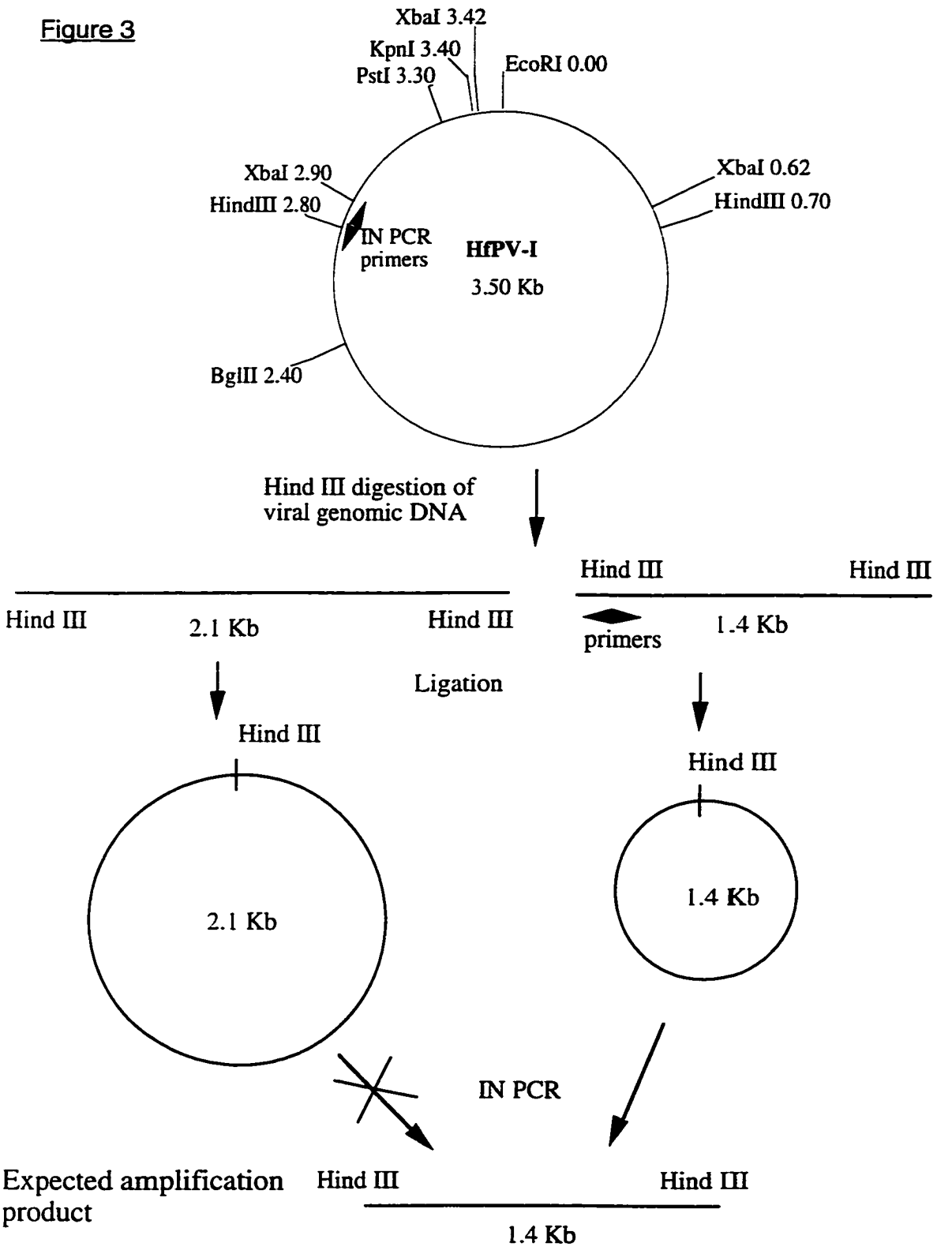
15. Nucleic acid hybridization.

For Southern blot analysis, chromosomal DNA (10 µg) was separated on 0.7 % agarose gels, blotted on to neutral nylon membranes (Boehringer Mannheim), and immobilized by UV cross linking. HfPV DNA and other probes used for Southern blot analysis or colony screening were labeled using non-radioactive labeling kits provided by Du pont/New England Nuclear or Boehringer Mannheim. Random primers with fluorescein-12-d or digoxigenin labeled UTP (Du pont/New England Nuclear or Boehringer Mannheim) were used for the primers for the random labeling reaction according to instructions provided by the manufacturer. For both systems the incorporation of non-radioactive label to the probe was measured by comparing the probe to the standard provided in the kit.

To reduce background problems with the Boehringer Mannheim nonradioactive kit, hybridization and detection solutions were modified as described in Blum et al. (1993); prehybridization solution (0.25 M Na_2HPO_4, pH 7.2, 1 mM EDTA, 20% SDS, 0.5% blocking reagent), hybridization solution (prehybridization solution with 2.5 ng DNA probe/ml), washing buffer (20 mM Na_2HPO_4, 1 mM EDTA, 1% SDS), detection washing buffer 1 (0.1 M maleic acid, 3 M NaCl, 0.3% Tween 20, pH 8), detection blocking buffer 2 (washing buffer with 0.5% blocking reagent), detection conjugate buffer 3 (blocking buffer with anti-Dig-AP conjugate, 1:15,000 dilution), detection substrate buffer 4 (0.1M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) and detection substrate

Figure 3. Inverse PCR. The restriction map of the HfPV DNA is shown indicating the region complementry to the primers designed for inverse PCR. The predicted inverse PCR result of HfPV DNA is shown with one set of primers. Amplification of 1.4 Kb DNA fragment of genome segment I is expected from the viral DNA.

Figure 3


XbaI 3.42
KpnI 3.40
PstI 3.30
EcoRI 0.00
XbaI 2.90
HindIII 2.80
IN PCR primers
HfPV-I
3.50 Kb
XbaI 0.62
HindIII 0.70
BglII 2.40
Hind III digestion of viral genomic DNA
Hind III
Hind III
2.1 Kb
Hind III
Hind III
primers
1.4 Kb
Ligation
Hind III
Hind III
2.1 Kb
1.4 Kb
IN PCR
Expected amplification product
Hind III
Hind III
1.4 Kb

solution 5 (substrate buffer 4 with 0.24 M CSPD). Hybridization was carried out at 68^{0}C over night., and three 15 min. washing was carried out also at 68^{0}C.

Other steps in Southern blot hybridization were carried out also by the manufacturer's instructions. Hybridization signals were recorded using NEN Reflection or Kodak film.

For dot blotting, 6 female wasps were dissected for the isolation of HfPV in the form of calyx fluid. The viral sample was divided into two equal volumes, and one half of the sample was filtered through 0.45 µm filter unit for HfPV infection study. Another half of the sample was centrifuged at 14,000xg for 10 min. and treated with digitonin as described earlier. After infection of three different cell lines by HfPV, a modified Hirt procedure (Clark and Cross, 1987) was followed to separate small molecular weight viral genomic DNA from large size cellular chromosomal DNA.

Cells (1 X 10^7) were harvested and resuspended in 1 ml of TE10 (10 mM Tris-HCl, 10 mM EDTA, pH 8.0). 10% SDS and then NaCl (5 M) were added to the sample dropwise with gentle swirling to a final concentration of 1% and 1 M. The chromosomal DNA was precipitated by leaving the sample overnight at 4^{0}C and then pelleting at 6,000xg for 45 min. The pellet was saved and stored at -20^{0}C. The supernatant was digested with proteinase K (0.5 mg/ml) at 37^{0}C for 2 hours, and then gently extracted twice with phenol, chloroform, and iso-amylalcohol (50:48:2). The DNA in the samples was precipitated by adding two volumes of 95% ethanol. When the chromosomal fraction was required, the pellet was resuspended in 500 µl of TE10 for the same proteinase K digestion and phenol, chloroform, and iso-amylalcohol (50:48:2) extraction. The samples

were then precipitated by adding 0.25 M NaCl and two volumes of 95% ethanol. The precipitated DNA was pelleted at 14,000xg for 10 min., and the pellets were washed twice with 70% ethanol. The pellets were dried and resuspended in TE.

The concentration of purified DNA was measured, and 0.5 μg of DNA was loaded in each well of a Dot blot apparatus (BRL). The blot was probed with genome segment L using a nonradioactive labeling kit from Boehringer Mannheim and DNA dot blotting was carried out according to the Manufacturer's instructions.

16. Construction and screening of a size-selected genomic libraries. Chromosomal DNA isolated from one of the cell lines that recovered from virus induced cytopathology (recovered cell line 2) was used to construct a size-selected genomic library. The chromosomal DNA from the cell line was digested with *EcoR* I or *Hind* III and separated by 0.7% agarose gel electrophoresis. 3-4 Kb (*EcoR* I) or 2-4 Kb (*Hind* III) sized DNA was cut out from the agarose gel, and DNA fragments from the gel were isolated using the GeneClean kit for ligation into appropriately treated pUC19 vector DNA. The library was constructed from the ligation of *Hind*III digested DNA since only 45 colonies were produced from the ligation of *EcoR*I digested DNA. The recombinant colonies were screened using total viral DNA as a probe.

17. Construction and screening of viral genomic libraries. The restriction enzymes that had a single site on the genome segments

of interest were identified by Southern blot analysis. After the identification of the single site restriction enzymes on genome segment I and genome segment υ, *Pst*I for genome segment I and *Hind* III for genome segment υ were chosen to construct viral genomic libraries to identify a clone which contains the genome segments. Total viral genomic DNA was digested with *Pst*I or *Hind* III, and ligated into a *Pst*I or *Hind* III digested pBLUESCRIPT SK+ vector. *Pst*I and the *Hind* III viral genomic libraries were constructed by transforming *E. coli* with the ligation mix.

The *Pst*I library was screened using a clone isolated from screening the *EcoR* I recombinant clones of cellular genomic DNA, pMK300, as a probe, and *Hind*III library was screened using part of genome segment W_{1-1}, a genome segment previously cloned by Deming Xu and characterized in this study, as a probe.

18. Nucleotide sequencing and nucleotide sequence analysis

Part of the nucleotide sequence of pMK 300 and pMK 500 was obtained by double-stranded DNA sequencing of pMK 300 and pMK 500 subclones cloned into pBluescript. Some of the subclones were sent to Mobix (sequencing facility at McMaster University) while some of subclones were sequenced in the lab using a Sequenase version 2.0 kit (United States Biochemical Company, Cleveland, OH) according to the manufacturer's instructions.

Plasmid DNA was prepared by the method of Lee and Rasfeed (1990) from *E. coli* strain DH5α. The purified plasmid (2 μg) DNA was denatured by

incubation of the DNA in denaturation buffer (0.1 M NaOH, 0.05 M EDTA) at 85^{0}C for 5 min. and neutralized by addition of 0.3 M sodium acetate (pH 4.8). Denatured DNA was precipitated by adding two volumes of 95% ethanol, and pelleted by centrifugation. The pelleted DNA was dried and resuspended in 5 µl of ddH_2O. 5X reaction buffer (2 µl) from the Sequenase kit and T3 or T7 primers (25 ng) from Gibco/BRL were added to the DNA. The annealing was achieved by heating 2 min. at 65^{0}C and then slowly cooling the samples to 37^{0}C for 30 min. followed by 10 min. incubation at room temperature. ^{35}S-dATP was used for all sequencing reactions, and the reaction mixtures were resolved by denaturing polyacrylamide (6%) gel electrophoresis. The sequencing gel was fixed in fixer solution (10% methanol and 10% acetic acid) for 30 min. to remove urea, and then the gel was dried on a gel drier (Model 583, Bio-Rad) for 2 hours. The sequence ladders from the dried gel were detected by exposure to Kodak X-ray film.

Nucleotide sequences generated from the sequencing reactions were recorded and analyzed using a computer software program, DNA Strider 1.2 (Marck, 1992) that allowed identification of full restriction maps, open reading frames and repeat sequences. Sequences were also compared to the entries in the Genbank database by using the BLAST search program (Altshul et al., 1990; Warren and States, 1993).

19. Preparation of non-photographic images.

Non-photographic images were prepared by using commercially available computer software, Ofoto and Adobe Photoshop for scanning and image preparation, and Aldus Pagemaker for labeling.

III. Results

1. Alteration of cell morphology and behaviour

A. Introduction

The polydnavirus-induced cytopathology observed in HfPV-infected lepidopteran cell lines are reported in this chapter. Although the infection of lepidopteran cell lines by HfPV was not expected to result in virus replication, three different lepidopteran cell lines, Ld 652Y, Ea 1174A, and Md 66 were infected with HfPV to observe any virus-induced cytopathology *in vitro*. A variety of cytopathic effects were produced by HfPV infection, and the cytopathology that was observed was similar to that occurring in HfPV-infected haemocytes of parasitized caterpillars. In addition, some of the cytopathic effects observed in HfPV-infected Ld 652Y cells were duplicated by transfection of purified viral DNA. These transfection results suggested that the viral DNA itself is important in polydnavirus-induced cytopathology.

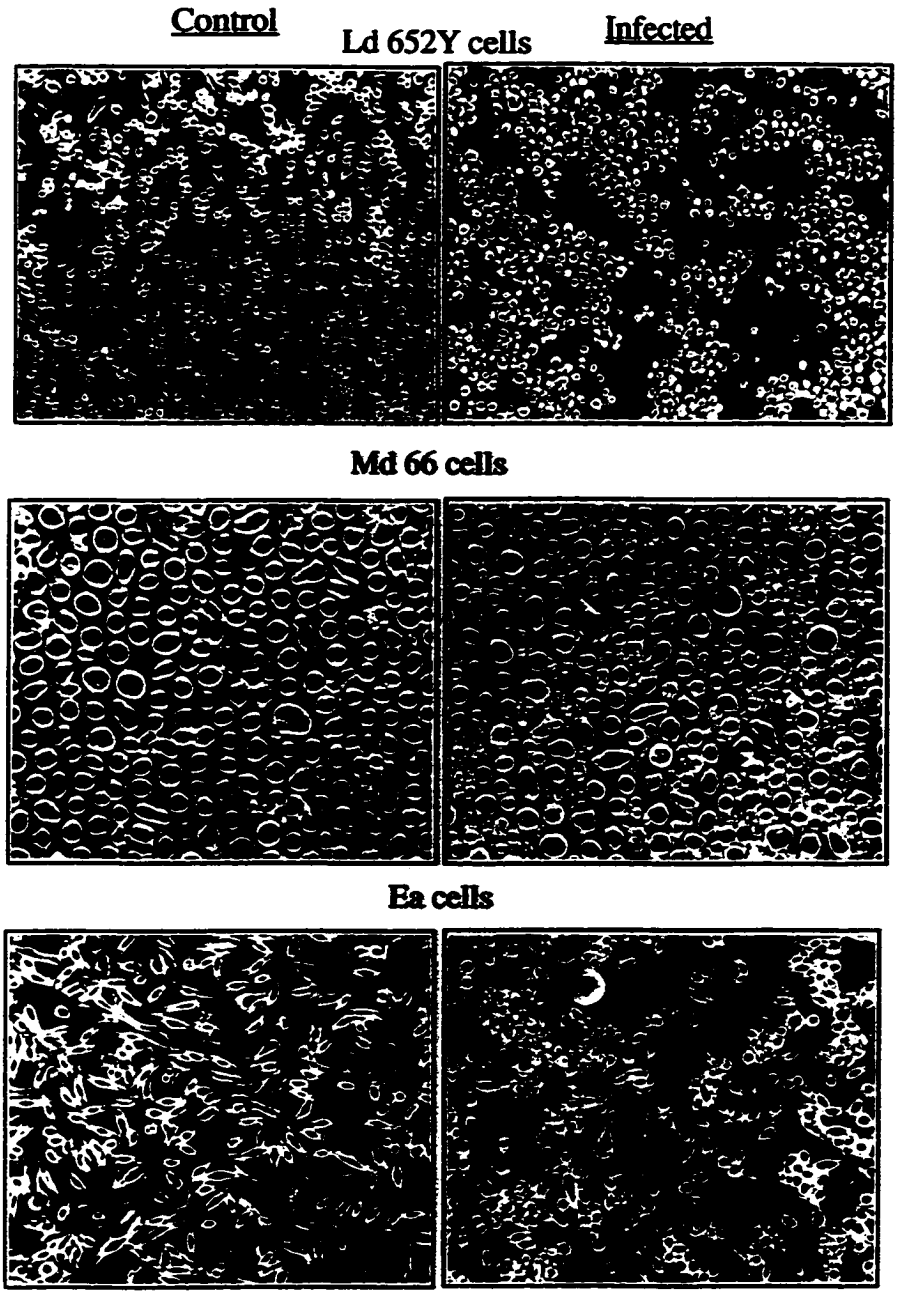
B. Alteration of Ld 652Y cell morphology and behaviour

When Ld 652Y cells were infected with HfPV, early responses of adherent Ld 652Y cells were rounding up and aggregation of the spindle-shaped cells present in normal Ld 652Y cell populations (Figure 4). Altered cell morphology, such as rounding up and aggregation of haemocytes, has previously been observed in parasitized caterpillars (Stoltz and Guzo, 1986; Strand, 1994; Kim et al., 1996). The rounding up of cells in virus-infected

Figure 4. Alteration of cell morphology induced by HfPV infection.

Three different lepidopteran cell lines showed altered cell morphology when they were infected with HfPV: Ld 652Y (two days post-infection), Md 66 (four days post-infection), and Ea 1174A (two days post-infection) were observed, as shown in this figure. Uninfected control cells are shown for comparison.

Figure 4



cultures was clearly observed at 16 hours post-infection (Figure 6). When trypan blue staining was carried out at 24 hours post-infection, most of the cells excluded the dye, indicating that they were viable (Figure 5), and that the alteration of cell morphology was not due to cells being killed by viral infection. For comparison, non-viable cells stained by trypan blue are shown in HfPV-infected Ea 1174A cells (Figure 10).

Following rounding up, cells began to be less adherent to the substrate, and aggregated in three dimensions to grow as clumps instead of as a monolayer (Figure 6, at 33 and 59 hours post-infection). A small population of cells showing cell membrane blebbing was observed from approximately 33 hours post-infection. Even after 6 months of culture, many cells continued to grow as clumps although spindle-shaped cells reappeared (Figure 6, 6 months post-infection). The Ld 652Y cells cultured continuously for six months post-HfPV-infection were named as a recovered cell line and were assigned numbers depending on the order of independent infection (R1, R2, R3, etc.). A small number of cells in the recovered cell lines appeared to undergo apoptosis. The altered cell morphology observed in recovered cells suggests that polydnavirus-infection has produced long term effects.

C. Apoptosis and Inhibition of Ld 652Y cell proliferation

As indicated by membrane blebbing and DNA fragmentation in Figure 8, a small fraction (1-5%) of the HfPV-infected Ld 652Y cells appeared to undergo apoptosis beginning at approximately 33 hours post-infection, and apoptosis

Figure 5. HfPV does not induce extensive cell death in Ld 652Y cells.

Due to the inhibition of cell growth by HfPV infection, the HfPV-infected culture showed fewer cells, although the same number of cells were used for both HfPV-infected and the uninfected control cultures (see the growth curve shown in Figure 7). At 24 hours post-infection there were very few cells stained with trypan blue, although the alteration of cell morphology was very obvious at this stage of viral infection (for comparison, see Figure 9).

Figure 5

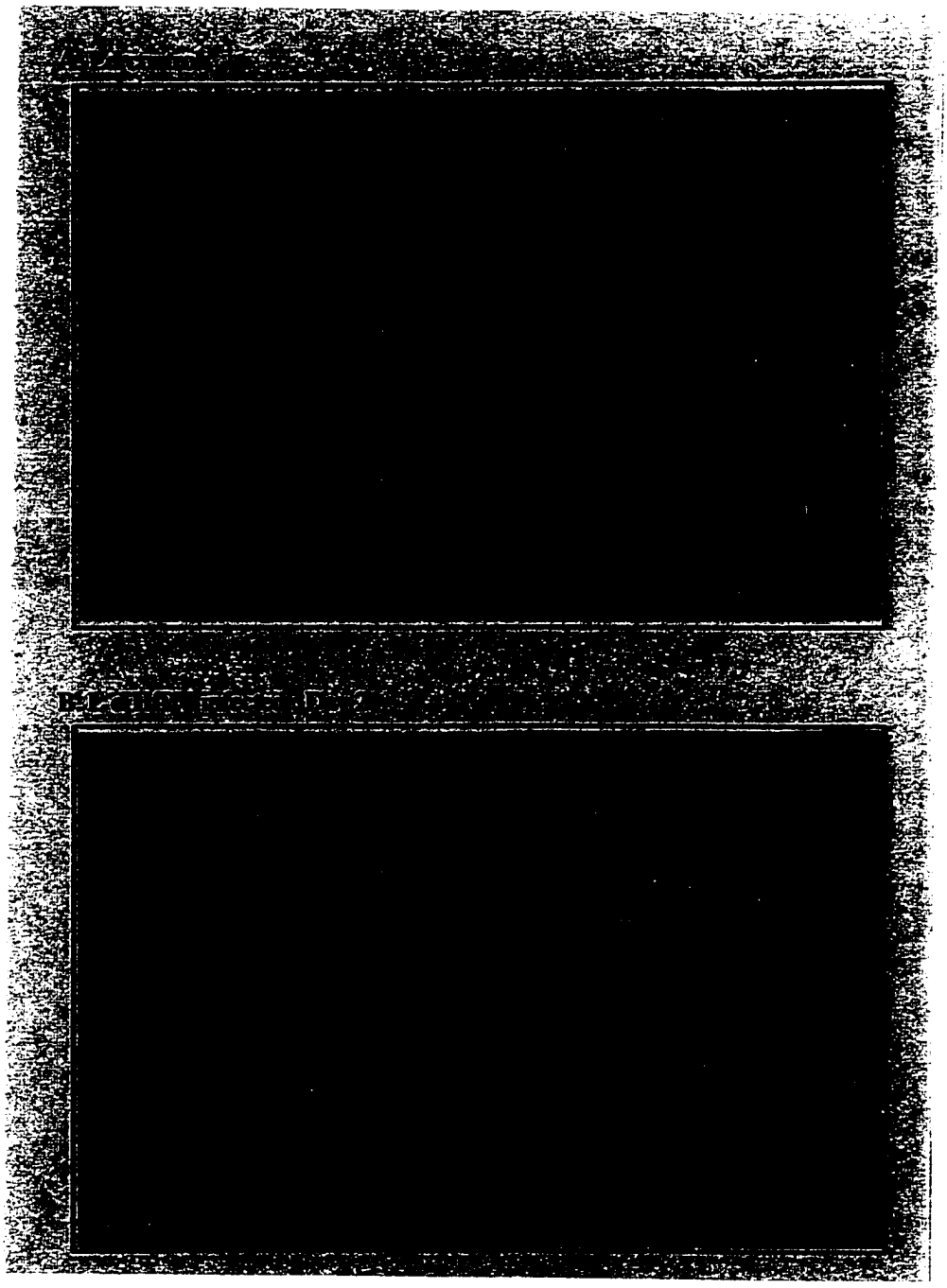
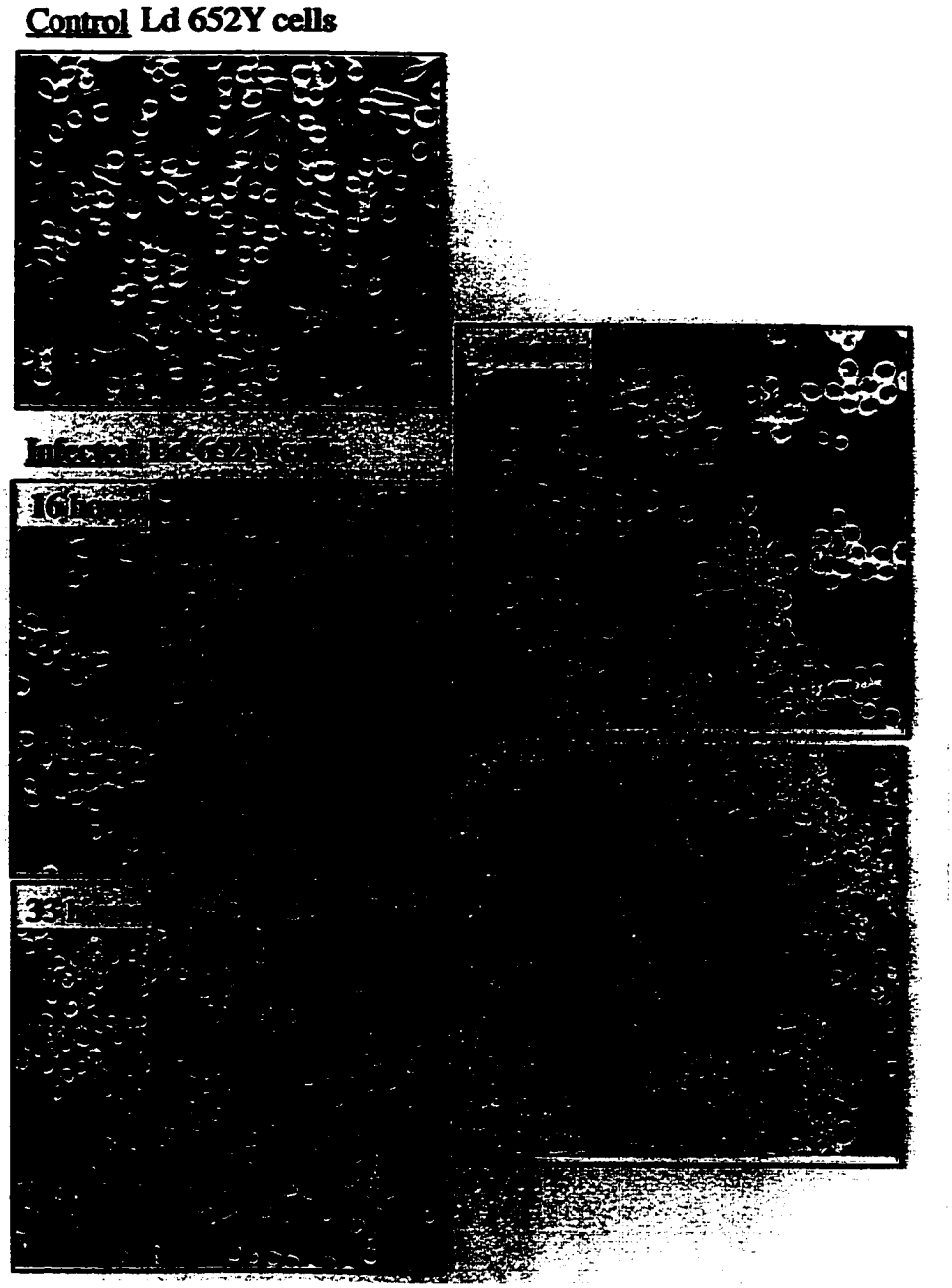


Figure 6. Alteration of Ld 652Y cell morphology by HfPV infection.

Infection of Ld 652Y cells by HfPV resulted in cytopathology, as shown in this figure. Uninfected control cells are shown in the top panel. Alteration of cell morphology, especially the disappearance of the elongated cell type (starting 16 hours post-infection) and cell aggregation (starting 33 hours post-infection) can be noted.

Figure 6



was also observed in cell populations which had essentially recovered from most of the cytopathology after 6 months of culture. Although recovered cells exhibited different growth morphology from the control (see Figure 6, control and 6 month panels), both proliferated at a similar rate (Figure 7, B).

Trypan blue staining of the cells at 24 hours post-infection indicated that most of the cells excluded the dye even after the alteration of cell morphology had occurred (Figure 5), indicating that most of the virus-infected cells are viable. However, significant inhibition of cell proliferation was observed for HfPV-infected Ld 652Y cells (Figure 7, A). Beginning as early as 10 hours and continuing as late as 200 hours post-infection, HfPV-infected Ld 652Y cells did not proliferate (Figure 7, A). These results suggest that the inhibition of cell proliferation may be due to a cell division arrest or induction of apoptosis, the consequences of which can not be detected by trypan blue staining.

Ld 652Y cells undergoing apoptosis were identified by cell membrane blebbing (Figure 8, A) and nuclear DNA fragmentation (Figure 8, B). Since apoptotic morphology was not observed until much later than the time at which one could detect the inhibition of cell proliferation (Figure 7), apoptosis might not be the only mechanism by which the inhibition of cell proliferation occurs in HfPV-infected Ld 652Y cells. However, it was clear that apoptosis contributes in some degree to HfPV-induced inhibition of Ld 652Y cell proliferation.

When Ld 652Y cells were exposed to conditioned medium or U.V.-treated conditioned medium, the progression of altered cell morphology was not

Figure 7. Ld 652Y cell proliferation by HfPV infection.

A. Inhibition of cell proliferation by early HfPV infection. Open circles represent duplicate control cultures and triangles represent duplicate HfPV-infected cultures. Cells were sub-cultured at 75 hours post-infection.

B. Proliferation of recovered cell lines. The comparison of cell proliferation between uninfected Ld 652Y cells (filled circles Ld) and two recovered Ld 652Y cell lines, 1 (open squares LdR1) and 2 (open triangles LdR2).

Figure 7

A.

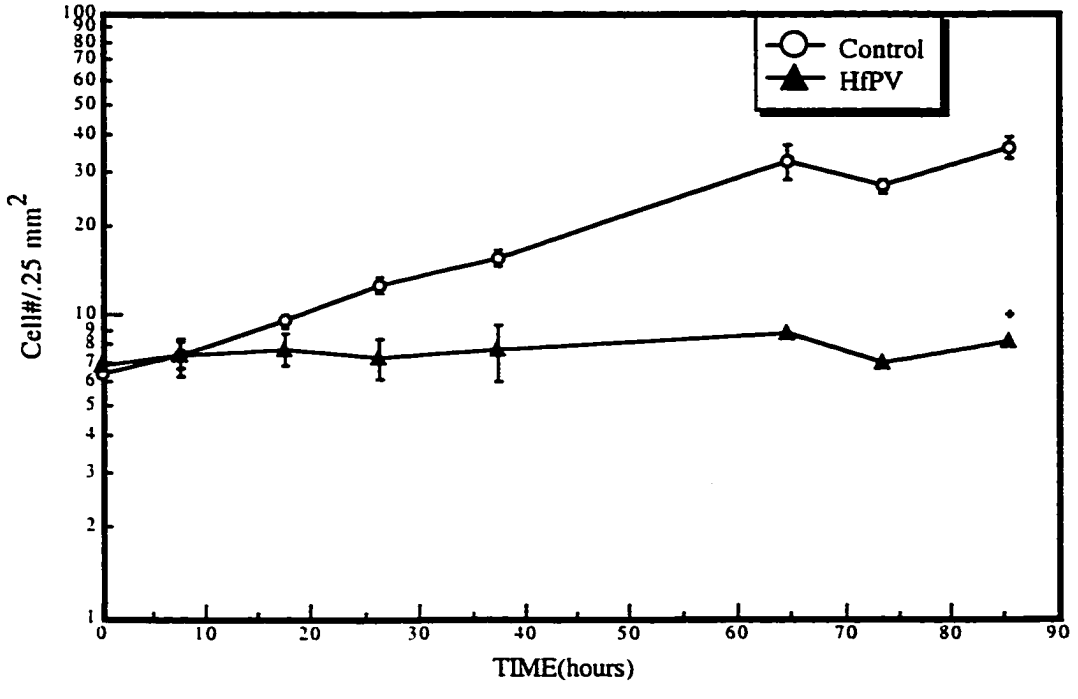

Control
HfPV
Cell#/.25 mm2
TIME(hours)
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0
10
20
30
40
50
60
70
80
90

Figure 7

B.

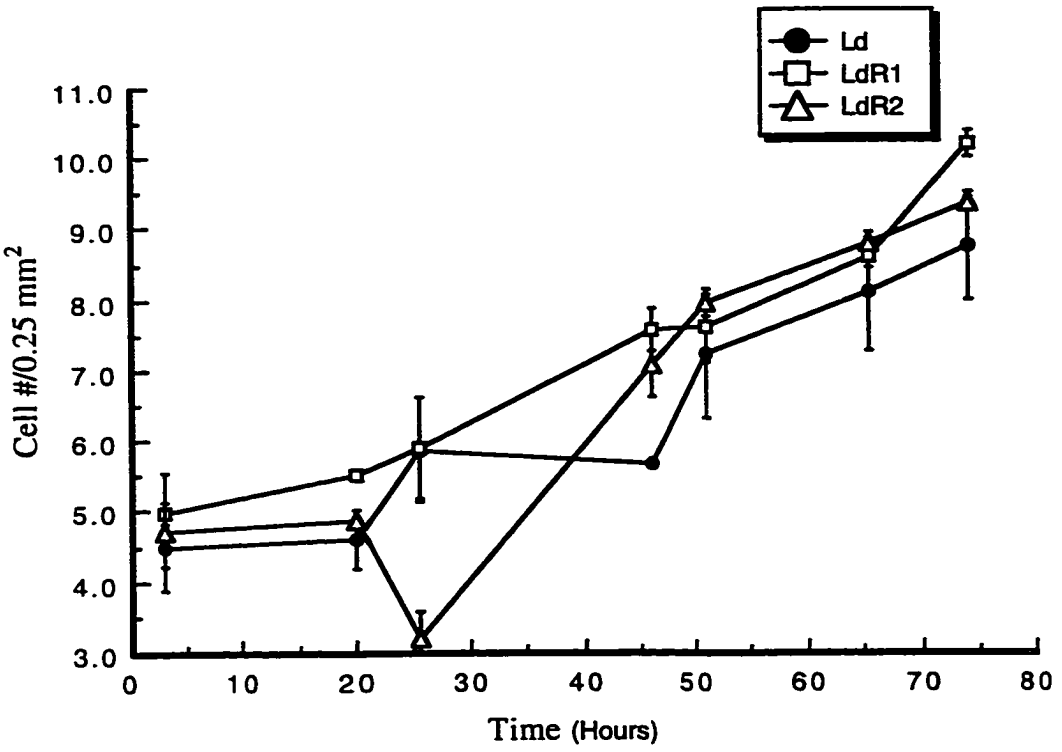

Ld
LdR1
LdR2
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
Cell #/0.25 mm2
0
10
20
30
40
50
60
70
80
Time (Hours)

Figure 8. Cell membrane blebbing and DNA fragmentation.
HfPV-infected Ld 652Y cells exhibited apoptotic morphology (cell membrane blebbing and an apoptotic DNA ladder) at 48 hours post-infection.

A. Some of the HfPV-infected cells showed cytoplasmic membrane blebbing. Also, unlike the control, there were no elongated shaped cells present in the virus-infected culture.

B. "Apoptosis ladder" observed from HfPV-infected Ld 652Y cells. Lanes are; M, 100 bp molecular weight standard marker; C, DNA from control cells; V, DNA from HfPV-infected cells at 48 hours post-infection; S, DNA from cells exposed for 4 hours to conditioned medium from HfPV-infected cells, and harvested 48 hours after the treatment.

Figure 8

A.

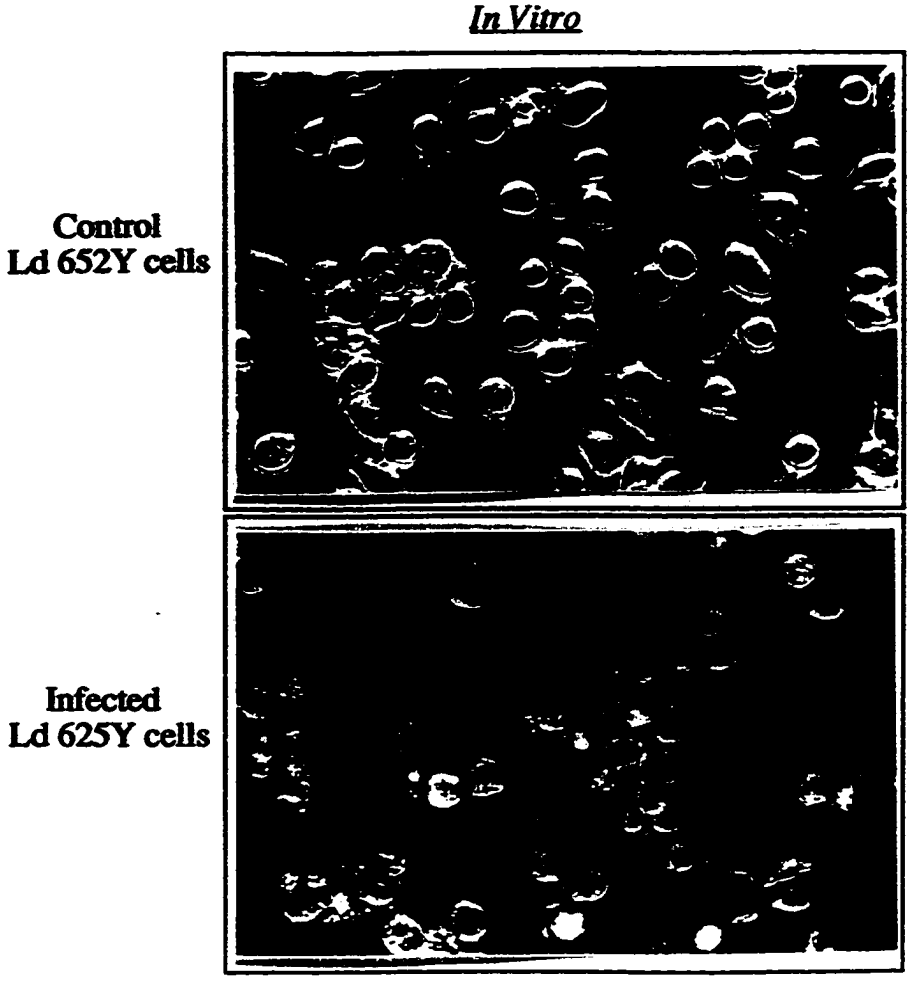
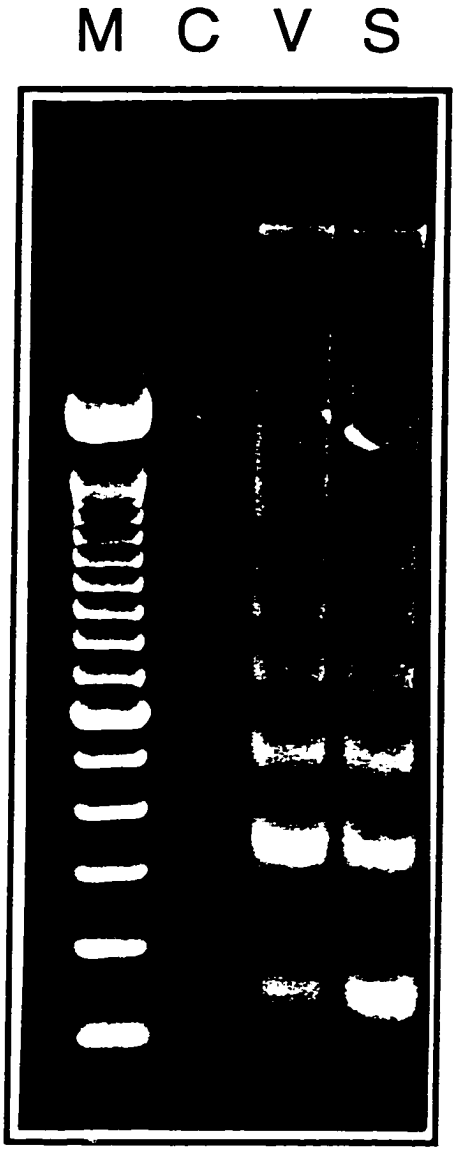


Figure 8

B.



as distinctive as in infection (data not shown). However, a higher proportion of cells in the culture was observed to undergo apoptosis. This result indicated that HfPV-infected cells probably secrete a molecule into the medium that can induce apoptosis in uninfected Ld 652Y cells.

D. Alteration of cell morphology in other lepidopteran cell lines.

Other lepidopteran cell lines were infected with HfPV to see whether any other HfPV-induced cytopathology could be observed. Alteration of cell morphology was observed in Ea 1174A cells grown in suspension. HfPV-infected cells rounded up 6 hours post-infection (data not shown) and later grew as aggregated forms while uninfected control cells did not show these cytopathic effects (Figure 9). Extensive cell debris was observed from virus-infected cultures at 26 hours post-infection (Figure 10). Approximately 20% of the Ea 1174A cell population infected with HfPV was stained by Trypan blue. Cell death and the generation of extensive cell debris was different from the apoptotic response observed in HfPV-infected Ld 652Y cells (see figure 5), indicating that these two cell lines show somewhat dissimilar responses to HfPV infection.

Different dilutions were made from the viral stock to see whether the cytopathology could be correlated to the amount of virus used in the infection. Since attempts to measure the absorbance of virus particles in a sample by spectrophotometry were unreliable, an HfPV sample from a known number of female wasps was serially diluted to determine the correlation between the amount of virus in the infection and the progression of the virus-induced

Figure 9. HfPV-induced altered cell morphology in Ea 1174A cells. Alteration of cell morphology due to HfPV infection was observed in Ea 1174 A cells at 48 hours post-infection. Uninfected control cells are shown in the top panel, and HfPV-infected cells are shown in the bottom panel.

Figure 9

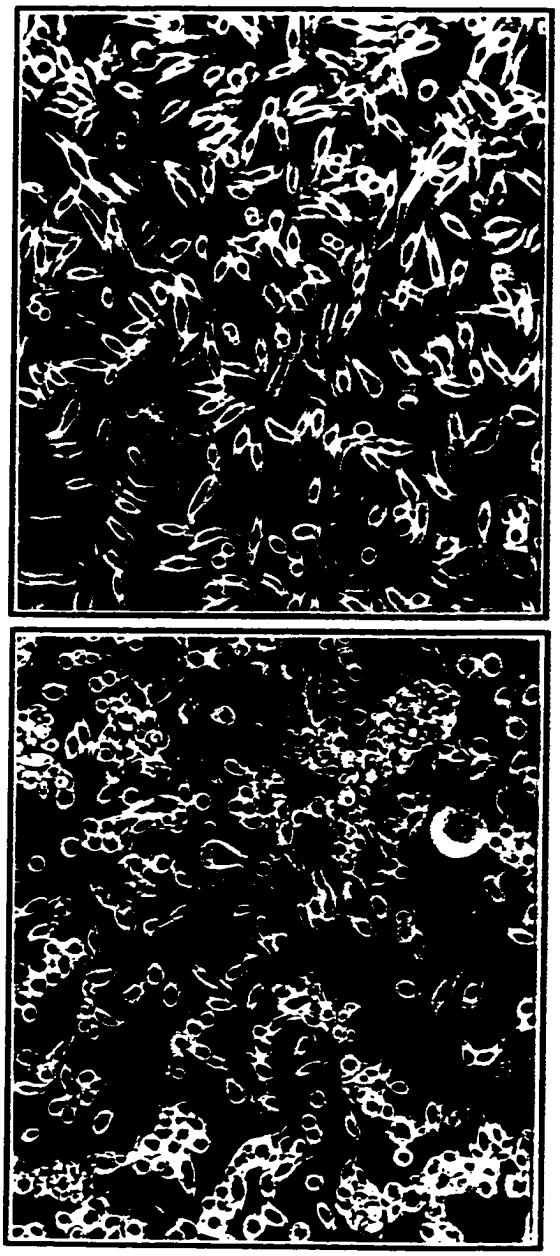
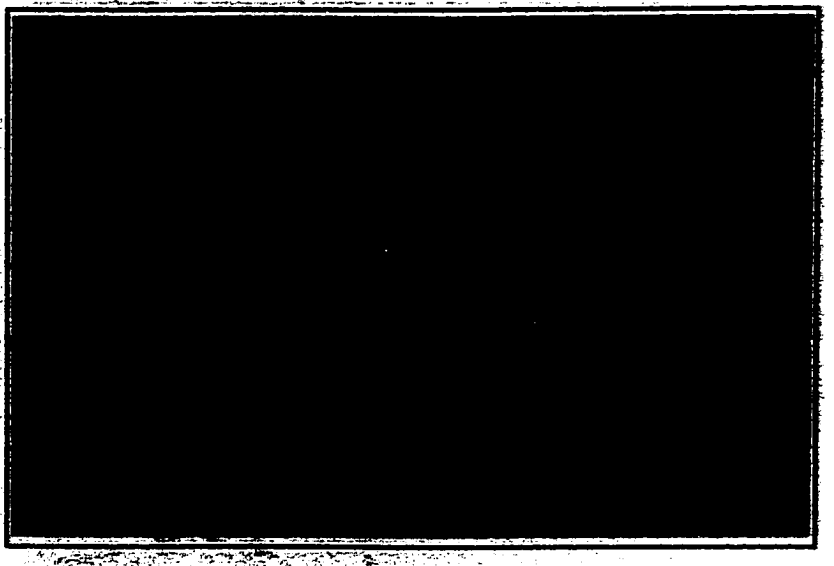


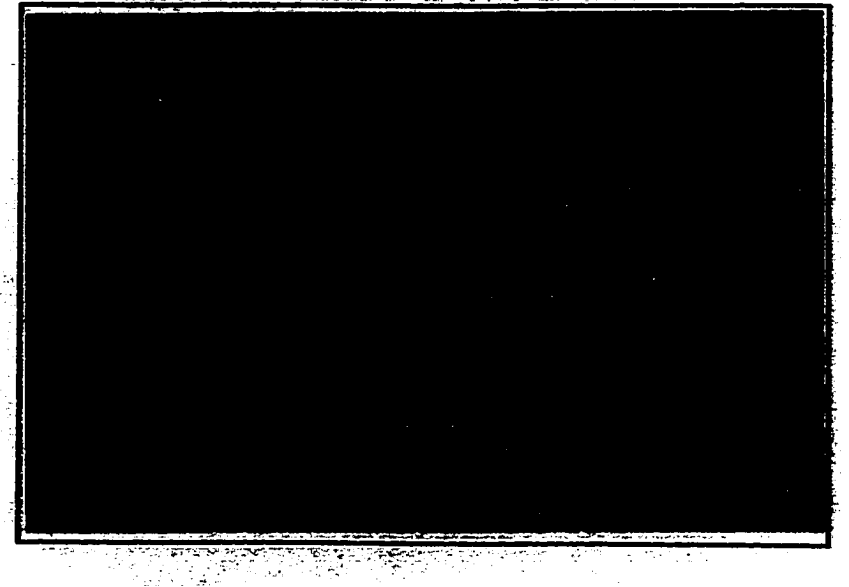
Figure 10. HfPV infection induces cell death in Ea 1174A cells. Approximately 20% of the infected Ea 1174A cells stained with trypan blue at 26 hours post-infection. Uninfected control cells are shown in the top panel while HfPV-infected cells at 26 hours post-infection are shown in the bottom panel.

Figure 10

A. Ea Control



B. Ea HfPV Infected



cytopathology. When HfPV isolated from a known number of female wasps is pooled as one sample, and the pooled viral sample is used for serial dilution for infection, one can obtain an estimate of the average amount of virus required for a single infection. In addition, if the virus-induced cytopathology is dependent on the infection efficiency, the more concentrated viral sample would induce the cytopathology at an earlier time point post-infection. HfPV isolated from 3 female wasps was pooled, and a portion of the viral sample was diluted 1:1,1:100 and 1:1000. The undiluted and diluted viral samples were used to infect the same number of Ea 1174A cells.

The development of virus-induced cytopathology depended on the number of virus particles added. For example, infections using a higher concentration of the viral sample produced the HfPV-induced cytopathology at an earlier time point than infections using a lower concentration of the viral sample. Infections carried out using undiluted virus samples resulted in rounding up of the cells as early as 6 hours post-infection, while the same effect was not observed until 48 hours and 96 hours post-infection for the cultures infected with 1:100 and 1:1000 dilutions of the viral sample (data not shown).

Contrary to the results from the infection of Ea 1174A and Ld 652Y cells, no distinctive cytopathic effects were observed in the infection of Md 66 cells at 48 hours post-infection, at a time when both Ld 652Y cells and Ea 1174A cells exhibited distinctive cytopathology. However, Md 66 cells underwent apoptosis at 4 days (96 hours) post-infection (Figure 11) which indicated that the response of Md 66 cells to HfPV-infection is different from the responses of Ea 1174A and Ld 652Y cells.

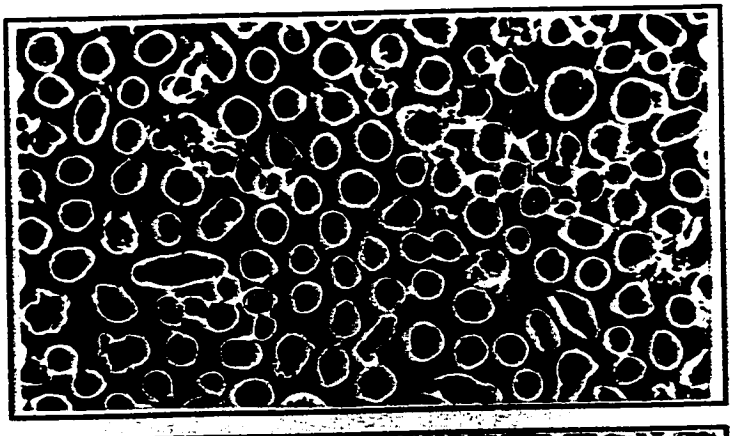
Figure 11. HfPV infection of Md 66 cells results in apoptosis.

Uninfected control cells are shown in the top panel while virus-infected cells (96 hours post-infection) are shown in the bottom. Cell membrane blebbing typical of apoptotic morphology was observed from a large number of cells in the culture.

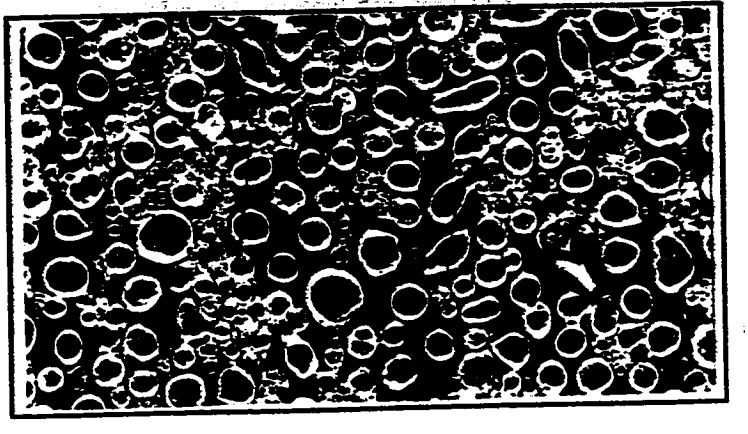
Figure 11

In vitro

Control
Md 66 cells



Virus infected
Md 66 cells



E. Infection and inhibition of cell proliferation in other cell lines

Similar to the results from the infection of Ld 652Y cells, inhibition of Ea 1174A cell proliferation was also observed following HfPV infection (Figure 12). When Trypan blue staining was carried out at 26 hours post-infection, about 20% of cells in the culture were not viable compared to the control flask (Figure 10). Although the inhibition of Ea 1174A cell proliferation was not as dramatic as that observed in the case of Ld 652Y cells (Figure 12 and Figure 7), HfPV-infection of Ea 1174A cells resulted in a greater decrease in cell viability than that observed in HfPV-infected Ld 652Y cultures.

HfPV infection of Md 66 cells, the lepidopteran cell line derived from haemocytes of the tent caterpillar, produced apoptosis. Induction of apoptosis was the only distinctive cytopathology observed in the HfPV-infected Md 66 cells (Figure 11). The different responses between the three insect cell lines may suggest that *in vivo,* different cell types may respond in different ways to polydnavirus infection.

F. Transfection of HfPV DNA into Ld 652Y cells

The importance of HfPV DNA for the induction of HfPV-induced cytopathologies was tested by introducing purified HfPV DNA into Ld 652Y cells by transfection. The results illustrated that the altered cell morphology and the inhibition of cell proliferation observed in HfPV infection could also be induced by some transfection experiments (Figure 13). With the exception of one other independent transfection experiment, most other transfection experiments showed less distinctive cytopathic effects in transfected cells. There are several

Figure 12. Inhibition of Ea 1174A cell proliferation by HfPV infection.
Open circles represent duplicate control cultures and diamonds represent duplicate HfPV-infected cultures. The cell cultures were subcultured at 65 hours post-infection.

Figure 12

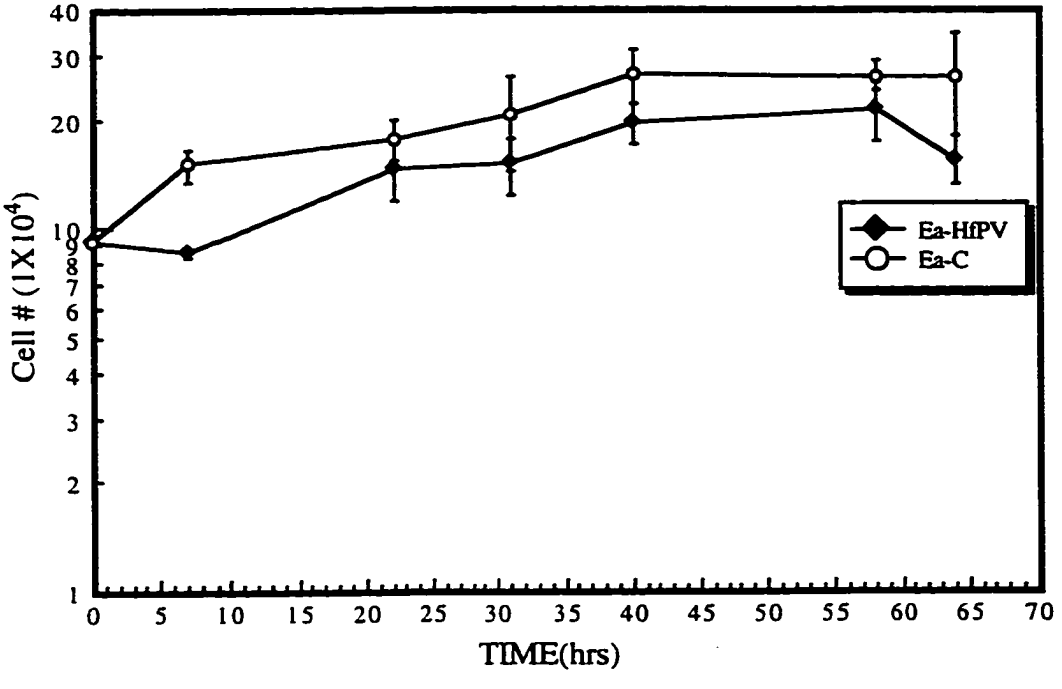

40
30
20
10
9
8
7
6
5
4
3
2
1
Cell # (1X10^4)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
TIME(hrs)
Ea-HfPV
Ea-C

Figure 13. Transfection of Ld 652Y cells.

Total HfPV genomic DNA was transfected into Ld 652Y cells, and as a control, Ld 652Y cells were also transfected with superhelical DNA molecular weight standards (Gibco-BRL). Transfection of the viral DNA produced similar results to those observed in the infection assay in Figure 7 and 8.

A. 4 hours post-transfection. Cells were observed under 20 x magnification. Ld 652Y cells transfected with control DNA did not show any aspect of the cytopathology observed in the cells transfected with virus DNA.

B. 19 hours post-transfection. Cells were observed under 10 x magnification. The reappearance of the spindle-shaped cells and the cells showing cytoplasmic membrane blebbing was observed.

C. Ld 652Y cell proliferation following transfection. Open circles indicate the cells transfected with control DNA, while diamonds indicate HfPV-DNA-transfected cells. A reduction in cell number was observed in the virus-DNA-transfected culture.

Figure 13

A.

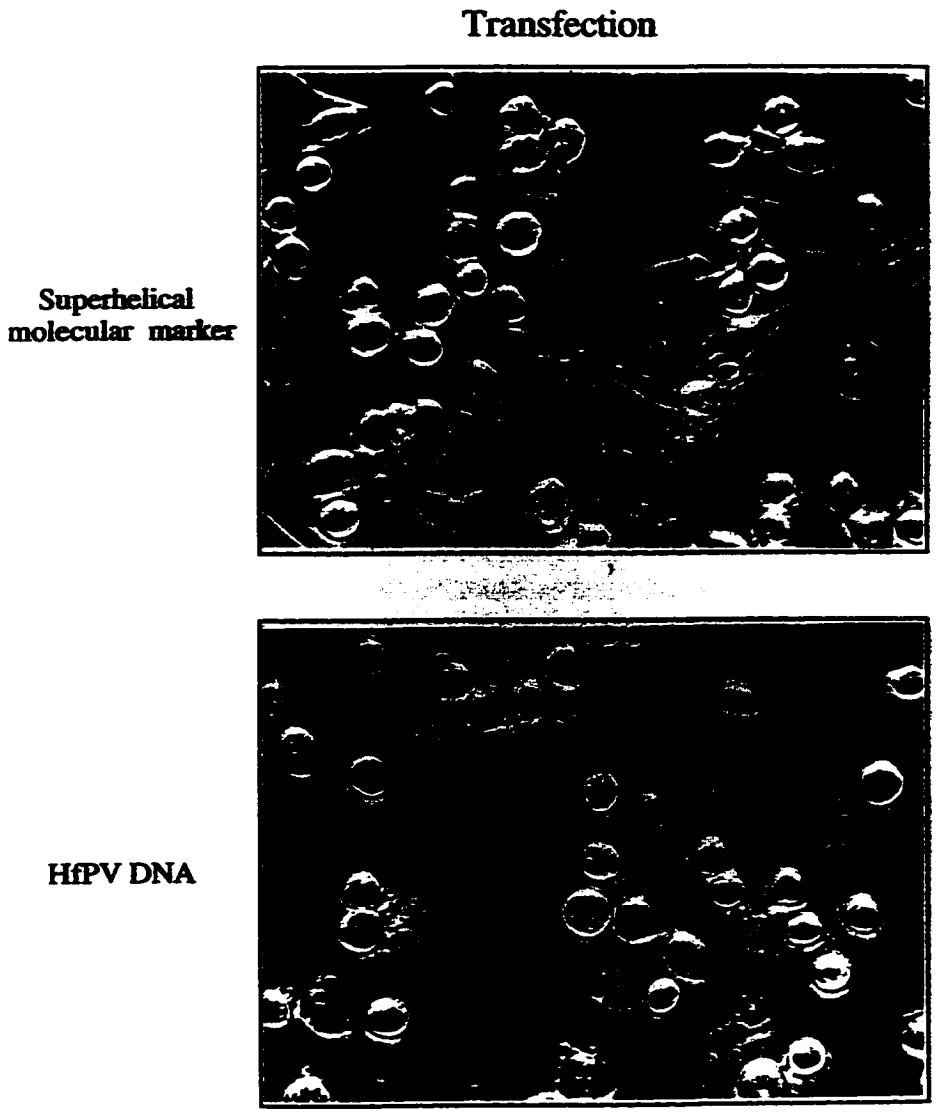


Figure 13

B.

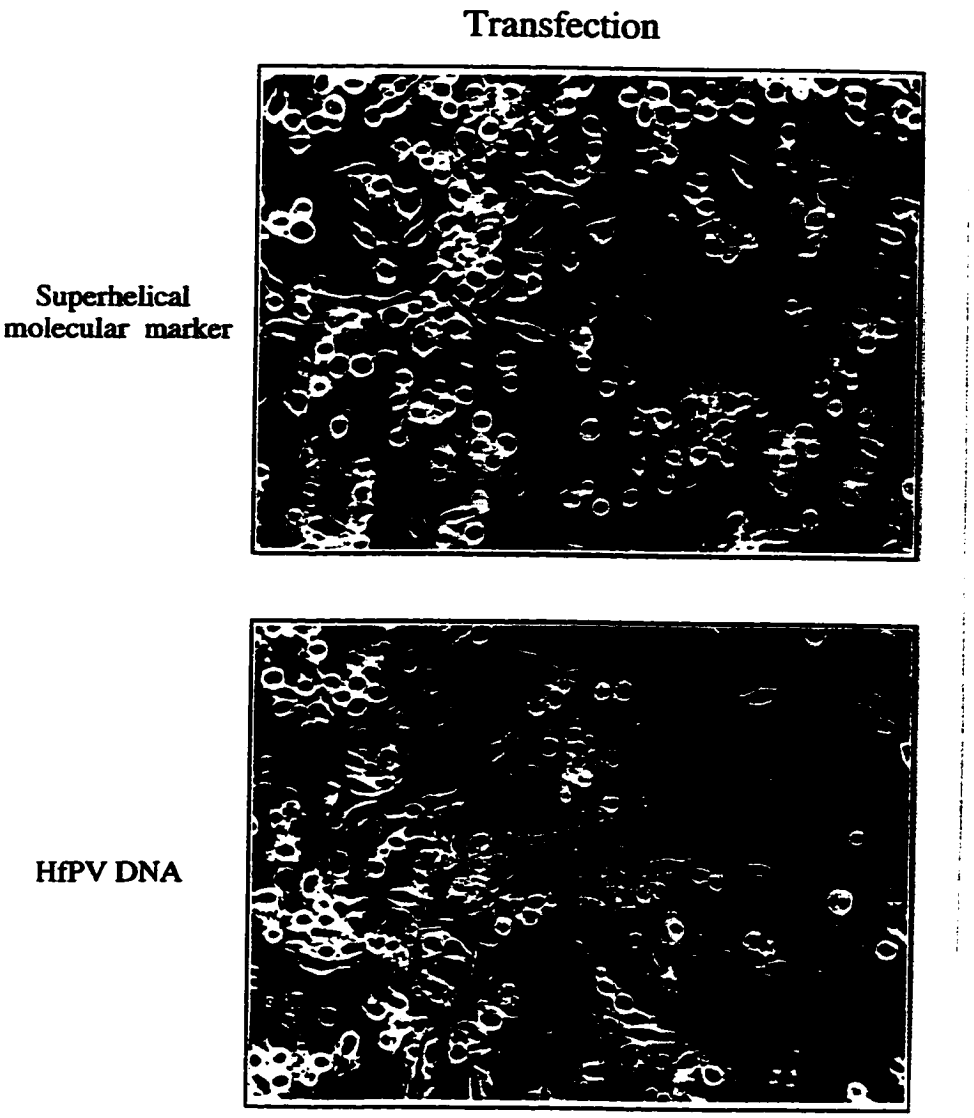
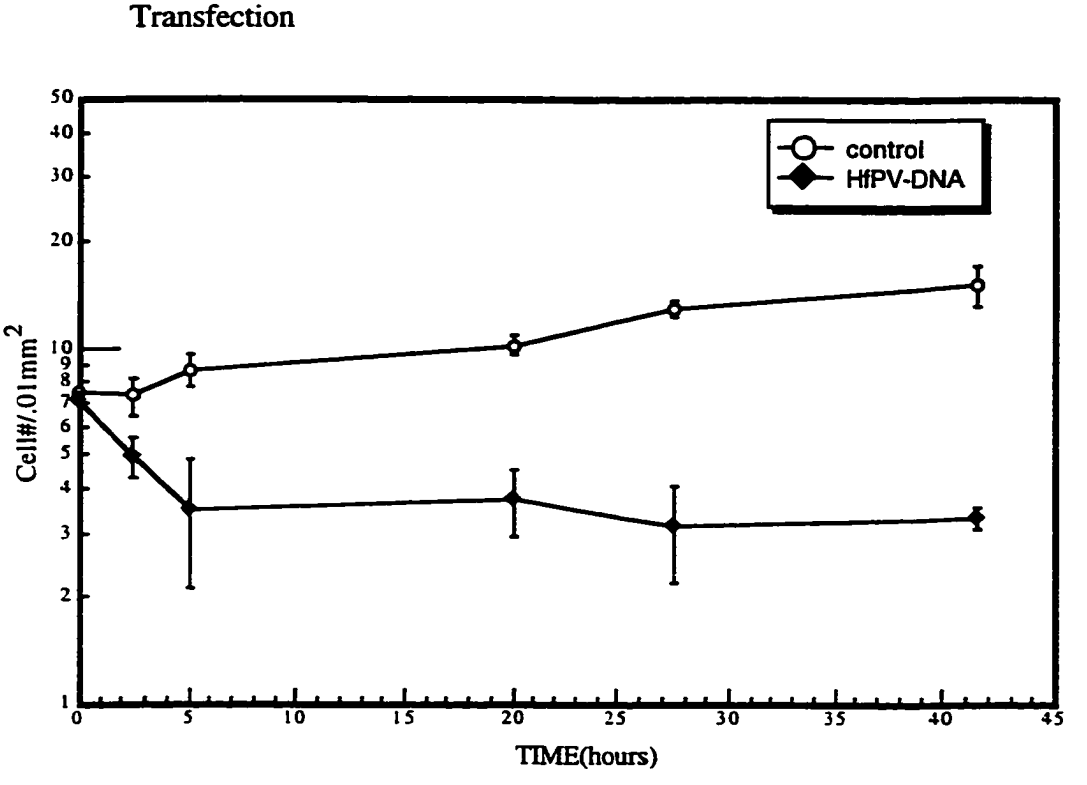


Figure 13

C.


Transfection
control
HfPV-DNA
Cell#/.01mm2
TIME(hours)
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0
5
10
15
20
25
30
35
40
45

problems with transfecting total viral genomic DNA into a single cell. For instance, tools to monitor transfection efficiency and delivery of the complete set of viral genome segments into a transfected cell were not available. Thus, the data from these transfection experiments may not reproducibly reflect the events occurring during viral infection.

However, there are some observations from the transfection experiments worth noting. Ld 652Y cells responded much more rapidly to the transfection than to the infection. The induction of apoptosis in response to the introduction of HfPV DNA could be observed as early as 1 hour post-transfection (data not shown), and a significant number of Ld 652Y cells undergoing apoptosis were observed by 4 hours post-transfection (Figure 13-A). Also, similar to the HfPV infection study (Figure 7 and 8), the virus DNA-transfected cells exhibited cytopathology as shown by an alteration of cell morphology (Figure 13-B), apoptosis (Figure 13-A) and inhibition of cell growth (Figure 13-C). Although fewer numbers of cells were observed in the HfPV DNA-transfected cultures than in superhelical molecular weight standard DNA-transfected control cultures (Figure 13-B and -C), initially the same number of cells were used for both (see Figure 13-C). The transfection of control DNA indicated that the lipofectin was not the cause of cell death in the cells transfected with HfPV DNA.

In addition, although some of the viral DNA transfections did not produce very distinctive cytopathology at 24 hours post-transfection, the transfected cells produced unusual paracrystalline structures (Stoltz, unpublished results) and an altered pattern of polypeptide synthesis (Figure 14) identical to that observed in cells infected with HfPV (Kim et al., 1996).

2. Altered pattern of polypeptide synthesis

A. Introduction

The altered pattern of polypeptide synthesis in HfPV-infected cells is another aspect of HfPV-infection that was studied using the *in vitro* system developed. Because virus infection often induces changes in the pattern of polypeptide synthesis in cells, either by expressing virus-encoded genes or by altering host protein synthesis, patterns of polypeptide synthesis in HfPV-infected cells were examined. HfPV-infected cells were labeled with ^{35}S-methionine, and total cell extracts (as well as the medium from the virus-infected cells) were analysed by SDS-PAGE to determine whether changes in the pattern of polypeptide synthesis occurred. The same altered pattern in polypeptide synthesis was observed after both HfPV-infection and viral DNA transfection, suggesting that viral gene expression has probably occurred in haemocytes of HfPV-infected caterpillars. Because any changes in cell morphology probably require changes in macromolecule synthesis, the altered pattern of polypeptide synthesis was studied in this chapter.

B. Altered pattern of polypeptide synthesis in Ld 652Y cells

To detect any altered pattern of polypeptide synthesis caused by HfPV-infection, cell extracts and media from HfPV-infected Ld 652Y cell cultures were analyzed by ^{35}S-methionine labeling followed by SDS-PAGE analysis (Figure 14). The comparison between the medium of virus-infected and the medium of control cells identified an infection-specific polypeptide at 24 hours post-

infection. The infection-specific polypeptide had a molecular weight of 55-kDa, and the same size polypeptide could also be detected in the medium of cells transfected with viral DNA (Figure 14, A). However, when the same gel was stained with Coomassie Blue, the polypeptide band was not detected (data not shown), suggesting that the 55-kDa polypeptide was present in relatively low amounts and was secreted into the medium.

When total cell extracts of HfPV-infected and HfPV DNA-transfected Ld-652Y cells were examined, bands migrating at approximate molecular weights of 23-kDa and 8-kDa were observed by Coomassie Blue staining (Figure 14, B and Figure 17, A). The induction of the 23-kDa polypeptide was observed following exposure to viral-infection or after transfection of viral DNA suggesting that the 23-kDa protein is specific to the presence of viral DNA in the cells. On the other hand, an 8-kDa polypeptide was observed following exposure to conditioned medium (Figure 15), or plasma derived from the parasitized larvae (Kim et al., 1996). The detection of an 8-kDa polypeptide in cells exposed to conditioned medium and other treatments suggests that the polypeptide might be a host protein whose production is induced by viral infection.

The 23-kDa polypeptide was detected by both Coomassie Blue staining and ^{35}S-methionine labeling and was observed to be transiently expressed (Figure 14, C). The 8-kDa polypeptide was better detected by Coomassie Blue staining, as shown in the transfection experiment (Figure 17, A and B). Both polypeptides were detected at similar levels from 24 to 120 hours post-infection by Coomassie Blue staining. However, reduced intensity of the 23-kDa

Figure 14. Altered pattern of polypeptide synthesis induced by HfPV infection. The mobility of prestained broad-range molecular weight markers (Bio-Rad) is shown as lines on the right (A) and left (B and C) of the gels (the sizes in kDa are, top to bottom: 116, 97.4, 66, 45, 31, and 21.5 kDa).

A. Autoradiogram of a 10% SDS-PAGE on which supernatant (medium) from Ld 652Y cells labeled with ^{35}S-methionine for one hour at 24 hours post-infection or post-transfection was separated. Lanes are: medium from cells transfected with superhelical DNA molecular weight standard markers (Gibco-BRL) (S); medium from cells transfected with HfPV DNA (VD); control, medium from, uninfected cells (C); medium from cells infected with HfPV (V).

B. Total cell extracts from HfPV-infected Ld 652Y cells at different time points analyzed by 12% SDS-PAGE followed by Coomassie blue staining. Lanes represent 0, 3, 24, 48, 72 and 120 hours post-infection. A polypeptide (indicated by the arrow) whose molecular weight was later determined to be 8-kDa and a 23-kDa polypeptide were observed from 24 hours post-infection.

C. Autoradiogram of 10% SDS-PAGE was used to analyze total cell extracts from HfPV-infected Ld 652Y cells labeled with ^{35}S-methionine at different time points. Lanes represent 0, 3, 24, 48, 72, and 120 hours post-infection. Bands observed in cell extracts from infected cells that were not observed in the control were indicated by their molecular weights. Note that the 25.5- and 27-kDa polypeptides disappear after 48 hours.

Figure 14

A.

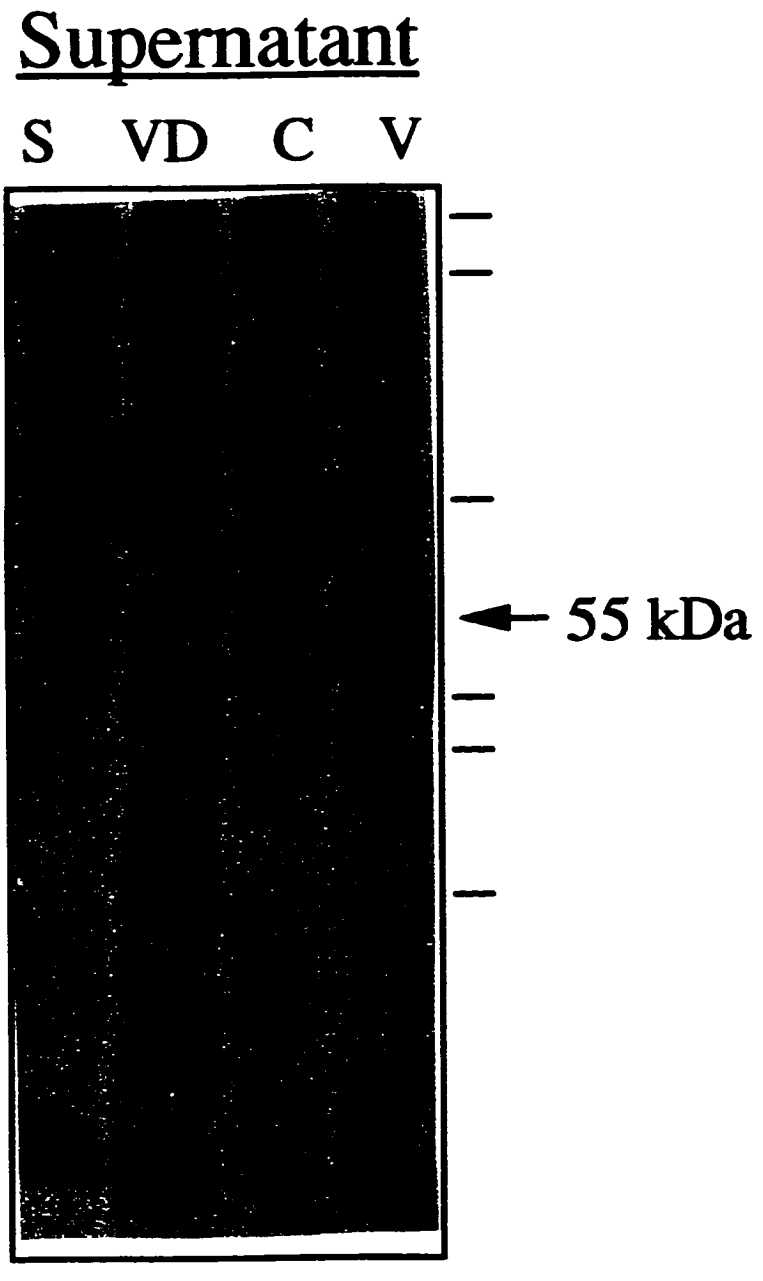


Figure 14

B. Coomassie Blue stained gel

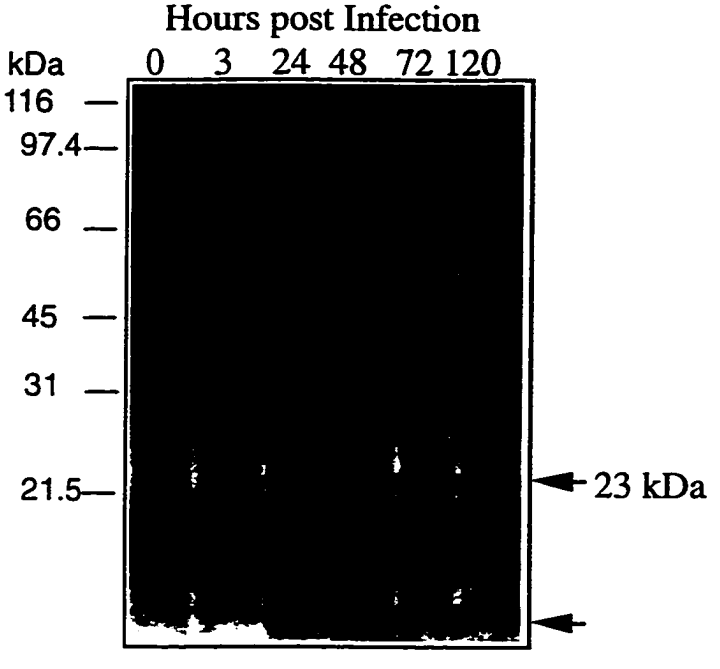


Figure 14

C. ^{35}S Methionine Time course

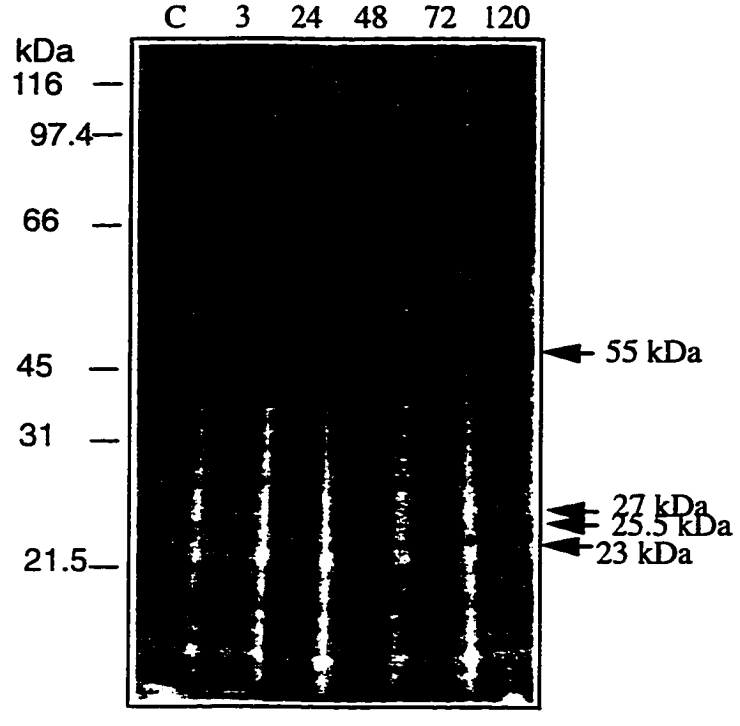
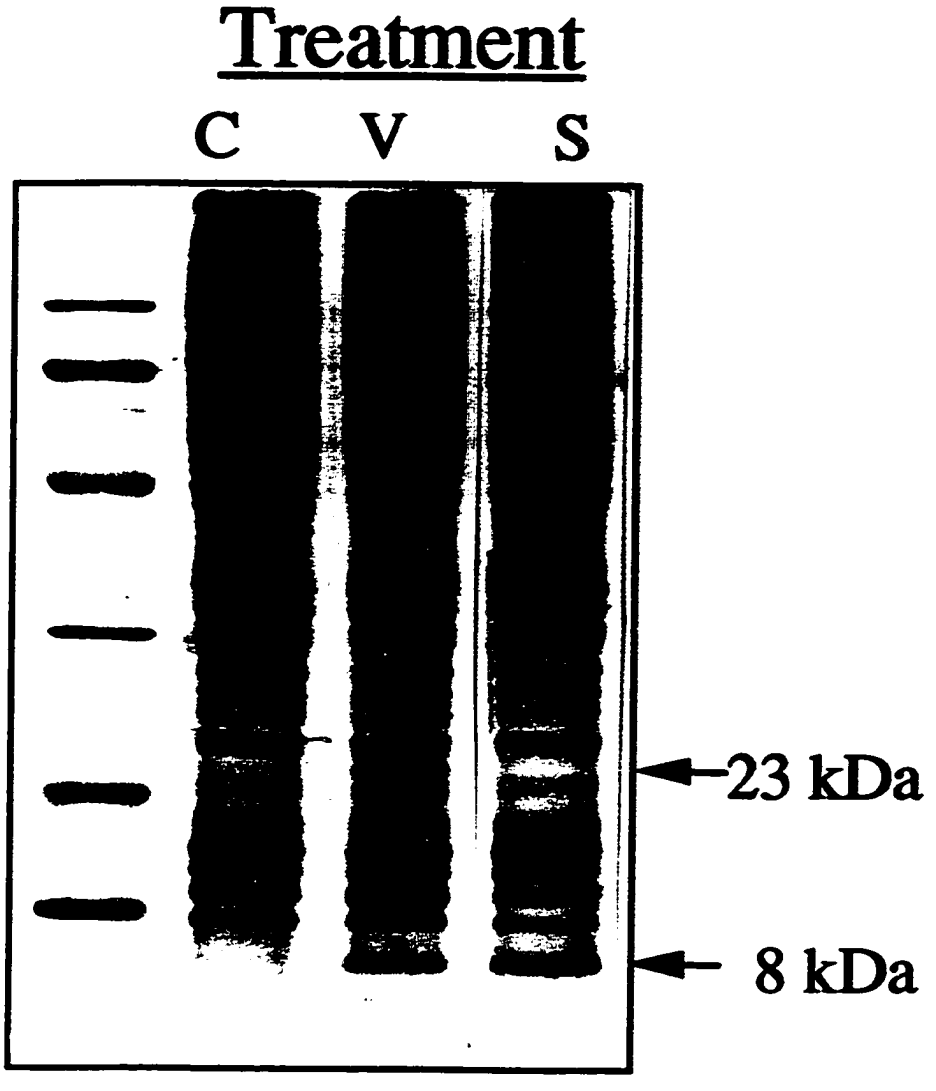


Figure 15. Detection of 8- and 23-kDa polypeptides at 48 hours post-treatment. 11% SDS-PAGE followed by Coomassie blue staining was used to analyze total cell extracts from Ld 652Y cells exposed to different treatments. Lanes are: control, uninfected cells (C); HfPV infected cells (V); cells exposed to conditioned medium from HfPV-infected cells (S). The lane on the far left shows prestained high-range molecular weight markers (Gibco-BRL): 97.4, 68, 43, 29, 18.4 and 14.3-kDa from top to bottom.

Figure 15



polypeptide band was detected by ^{35}S-methionine labeling at 120 hours post-infection, suggesting this polypeptide is not synthesized in late HfPV-infection (Figure 14, C, lane 120).

The location of the 8- and 23-kDa polypeptides in the Ld 652Y cells was determined by fractionation of cell extracts from HfPV-infected cells according to the procedure given by Chen-Levy et al. (1989). Following fractionation of the cell extracts, the fractionated samples were analyzed by SDS-PAGE followed by Coomassie Blue staining (Figure 16). The results indicate that the 23-kDa polypeptide may be located in the membrane fraction, while the 8-kDa polypeptide may be located in a cytosolic fraction.

In addition to the 23-kDa and the 8-kDa polypeptides, three other polypeptides specific to HfPV-infection were detected only by ^{35}S-methionine labeling (25.5-kDa, 27-kDa, and 55-kDa polypeptides). These three polypeptides were observed to be expressed transiently and were only detected by ^{35}S-methionine labeling, suggesting that these polypeptides are expressed at relatively low levels (Figure 14, C, lanes 24 and 48). Two of the polypeptides, at molecular weights of 25.5-kDa and 27-kDa were detected between 24 hours and 48 hours post-infection, and the 55-kDa polypeptide was detected later, at 72 and 120 hours post-infection.

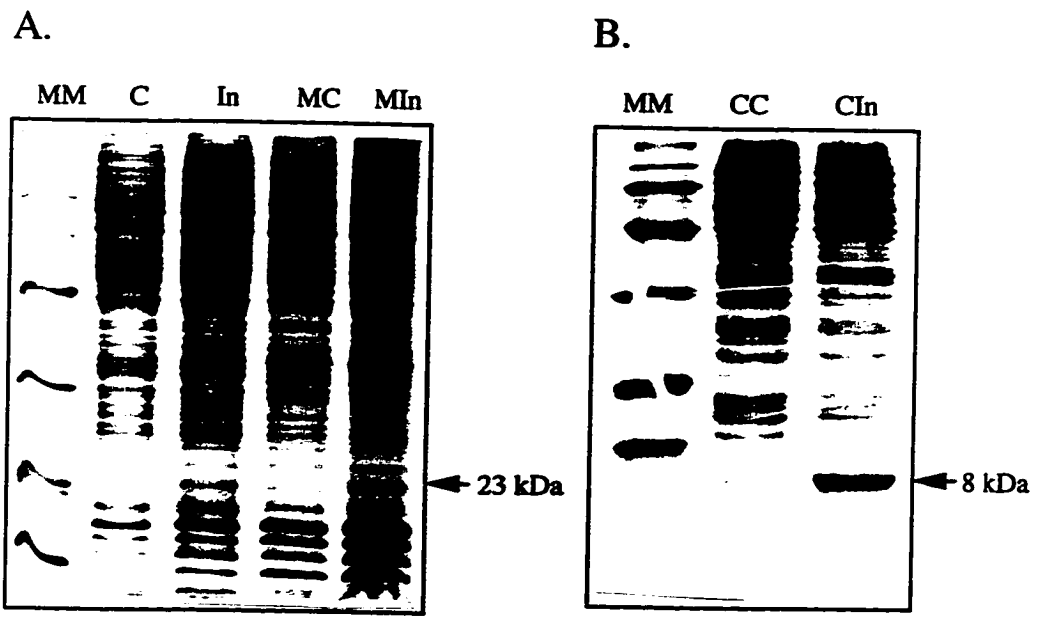
In summary, HfPV infection induces an altered pattern of polypeptide synthesis in cells. Some of the polypeptides in HfPV-infected cells were present transiently. In addition, HfPV infection induces the production of an 8-kDa polypeptide that is probably a host protein.

Figure 16. Cellular location of polypeptides. Cellular location of polypeptides whose production was induced by HfPV infection. Ld 652Y cell extracts were fractionated and analyzed by 12% SDS-PAGE followed by Coomassie blue staining. The 8-kDa polypeptide was identified in the cytosolic fraction and the 23.5-kDa polypeptide in the membrane fraction.

A. Lanes are: prestained high molecular weight markers (Gibco-BRL) which represent sizes of 97.4, 68, 43, 29, 18.4 and 14.3-kDa (from top to bottom) (MM); control, total cell extract from uninfected cells (C); total cell extract from HfPV-infected cells (In); membrane fraction from control cell extract (MC); membrane fraction from HfPV-infected cell extract (MIn).

B. Lanes are: prestained high molecular weight markers (MM) which represent sizes of 97.4, 68, 43, 29, 18.4 and 14.3-kDa (from top to bottom); control, cytosolic fraction from control cells (CC); cytosolic fraction from HfPV-infected cells (CIn).

Figure 16



C. Transfection of the viral DNA

To understand the role of viral DNA in the altered pattern of polypeptide synthesis in HfPV-infected cells, purified viral DNA was transfected into Ld 652Y cells. The introduction of purified viral DNA into Ld 652Y cells by transfection induced similar changes in polypeptide production to that observed in HfPV-infected Ld 652Y cells. When cells transfected with HfPV DNA were harvested at 48 hours post-transfection, an altered pattern of polypeptide synthesis was observed, as well as the alteration of cell morphology shown in Figure 13. The 23-kDa, 8-kDa, 25.5-kDa and 27-kDa polypeptides whose induction was observed in HfPV infection were also observed in cells transfected with HfPV DNA (Figure 17). The results from the transfection experiments suggested that HfPV DNA in the viral infection was responsible for the induction of the altered pattern in polypeptide synthesis, along with the cytopathic effects.

D. Polypeptide synthesis in cells recovered from HfPV infection

Cell line R2, one of HfPV-infected Ld 652Y cell lines that had recovered from most HfPV-induced cytopathology, was analyzed by SDS-PAGE to study any long term effects of HfPV infection on polypeptide synthesis. Although recovered cells exhibited different growth morphology from the control (see Figure 6, control and 6 month panels), the cells proliferated at a similar rate (Figure 7, B). When the patterns of polypeptide synthesis were compared, no additional bands were observed for the recovered cell lines (Figure 18).

Figure 17. Transfection of viral DNA into Ld 652Y cells.

Transfection of Ld 652Y cells with total viral genomic DNA resulted in an altered pattern of polypeptide synthesis, similar to the alteration observed following virus-infection.

A. Coomassie Blue-stained 12% SDS-PAGE analysis of a transfected sample (48 hours post-infection). Lanes are: control, uninfected cells (C); Ld 652Y cells transfected with HfPV DNA (HfPV). The mobility of prestained low molecular weight markers (Gibco-BRL) is shown as lines on the left of the gel.

B. Autoradiogram of 12% SDS-PAGE on which total cell extracts from Ld 652Y cells was separated. The cells transfected with viral DNA were labeled with ^{35}S-methionine at 48 hours post-transfection. Lanes are: control, lipofectin treated cells (C); Ld 652Y cells transfected with HfPV DNA (HfPV). The mobility of prestained low molecular weight markers (Gibco-BRL) is shown as lines on the left and the lines represent 43, 29, 18.4, 14.3 and 6.2-kDa from top to bottom.

Figure 17

A. Coomassie blue staining

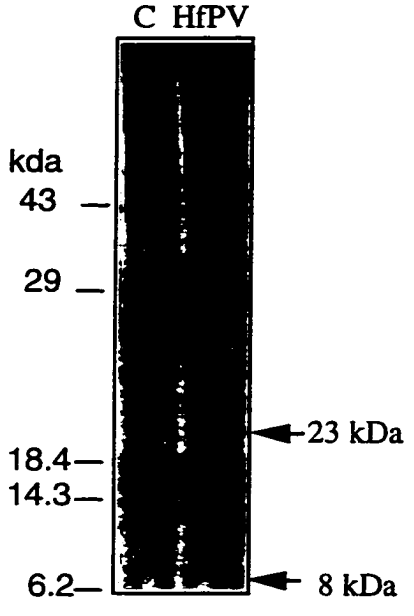


Figure 17

B. ^{35}S-Methionine labeling

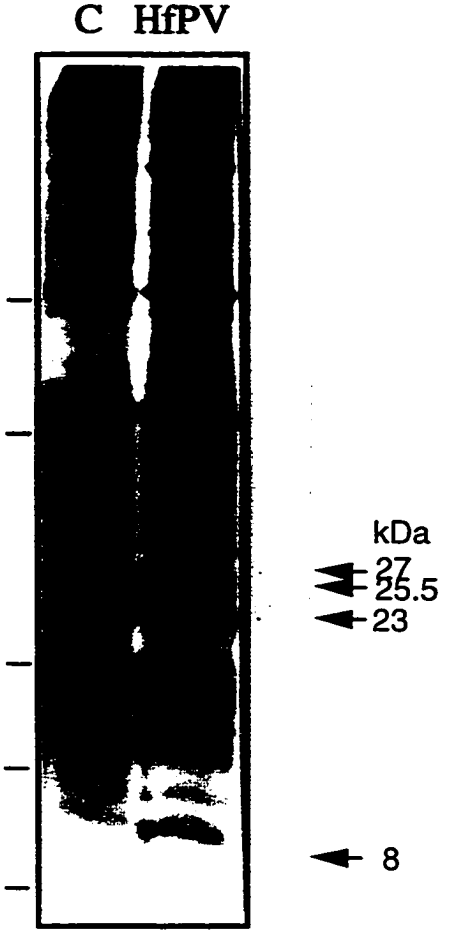
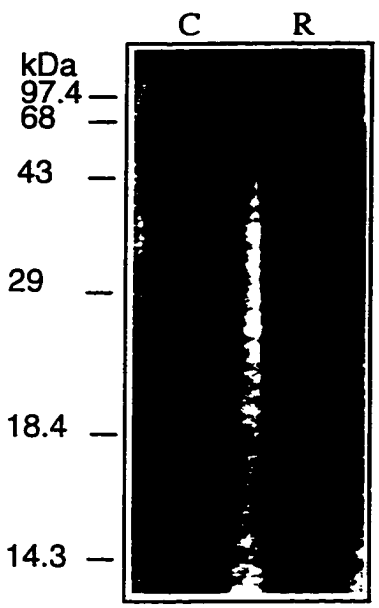


Figure 18. Polypeptide synthesis of a recovered cell line.

10% SDS-PAGE followed by Coomassie Blue staining of cell extracts. Lanes are; control, uninfected cells (C); recovered cell line 2 (R). The mobility of prestained high molecular weight markers (Gibco-BRL) are shown on the left of the gel.

Figure 18



E. Polypeptides synthesis in other cell lines infected with HfPV.

In order to find any commonly produced polypeptide in HfPV-infected cells, other lepidopteran cell lines such as Ea 1174A and Md 66 cells were also infected with the virus, and polypeptide synthesis was examined. HfPV-infected Ea 1174A cells showed an altered pattern of polypeptide synthesis at 48 hours post-infection (Figure 19) as well as the alteration of cell morphology discussed in the previous chapter. A 17.5-kDa polypeptide was present in HfPV-infected Ea 1174A cells, and the polypeptide was detected by both ^{35}S-methionine labeling (Figure 19) and Coomassie Blue staining (data not shown).

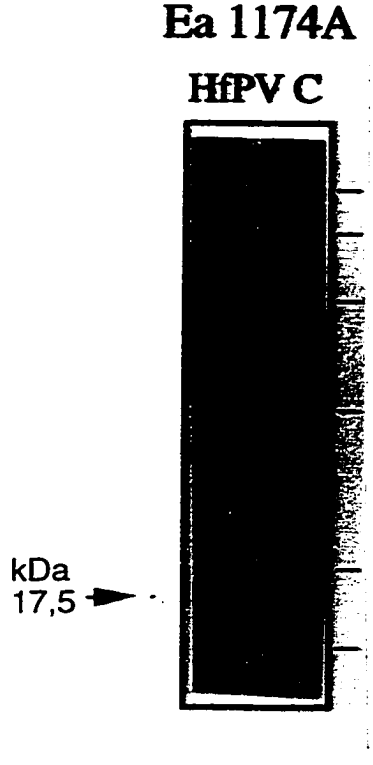
However, when HfPV-infected Md 66 cells were harvested at 48 hours post-infection and analyzed, no altered pattern of polypeptide synthesis was observed by HfPV-infection, either by ^{35}S-methionine pulse labeling or by Coomassie Blue staining (data not shown).

In summary, three different lepidopteran cell lines were infected with HfPV, and the patterns of polypeptide synthesis were studied. Virus-infection induced an altered pattern of polypeptide synthesis in Ld 652Y and Ea 1174A cells, as well as inducing changes in cell morphology in all the cell lines infected. Altered patterns of polypeptide synthesis were observed in HfPV-infected Ld 652Y and Ea 1174A cells. Some polypeptide bands detected in HfPV-infected Ld 652Y cells may be host polypeptides whose production was induced by HfPV-infection, while others may be encoded by the viral genome.

Figure 19. HfPV-induced an altered pattern of polypeptide synthesis in Ea 1174A cells.

Autoradiogram of a 10% SDS-PAGE gel on which total cell extracts from Ea 1174A cells labeled with ^{35}S-methionine at 48 hours post-infection have been electrophoresed. Lanes are: control, uninfected cells (C); HfPV-infected Ea 1174A cells (HfPV). The mobility of prestained high molecular weight markers shown as lines on the right of the gel represent 97.5, 68, 43, 29, 18.4 and 14.3-kDa from top to bottom.

Figure 19



3. Viral genome persistence

A. Introduction

Because the persistence of HfPV DNA is observed in parasitized caterpillars, *in vivo*, the persistence of the viral genome in HfPV-infected cells, *in vitro*, was investigated. The study of HfPV genome persistence in virus-infected cells is reported in this chapter.

During the study of HfPV DNA persistence, one of the cell lines, Ld 652Y, was found to maintain some of the viral sequences over a long period of time (2-3 years). To monitor the presence of HfPV DNA in the virus-infected cells, the uptake of virus particles was compared between the three cell lines by a dot blot analysis. Also, persistence of the viral DNA introduced by transfection was monitored in Md 66 cells.

Some viral sequences maintained by Ld 652Y cells were identified and characterized from one of the recovered cell lines (R2) by constructing and then screening a size-selected genomic library. Following identification of the viral sequences maintained by the recovered cell line, an HfPV genome segment whose sequences appeared to be integrated into the chromosomal DNA of Ld 652Y cells was isolated. The particular viral genome segment that hybridized to the fragment of HfPV sequences isolated from the recovered cell line was then characterized. The importance of the genome segment in viral DNA persistence was studied by introducing the individual genome segment into Ld 652Y cells by transfection.

Section 3.1 Persistence of HfPV DNA.

A. The uptake of viral particles by lepidopteran cell lines.

The uptake of viral particles into different lepidopteran cell lines was tested by dot blot analysis to test whether any correlations existed between the uptake of viral particles and the virus-induced cytopathology in the HfPV-infected cells *in vitro*. Three different cell lines, Ea 1174A, Ld 652Y and Md 66 were infected by HfPV (Figure 14), and the cells were harvested 48 hours post-infection. HfPV genome segments whose sizes are much smaller than chromosomal DNA of host cells were selectively purified for a dot blot analysis using the modified Hirt procedure developed by Clark and Cross (1987). For Ea 1174A cells a high background is seen for DNA from control cells, while DNA isolated from Md 66 cells did not show much hybridization signal to the probes for either the control and the virus-infected cells (Figure 20). The best contrast of the hybridization signals between virus-infected and -uninfected samples was observed from Ld 652Y cells, indicating that Ld 652Y cells might be the best cell line to use in virus infection studies (Figure 20). Correlation between the uptake of viral particles and the physiological responses to HfPV infection by different cell lines could not be determined since even Md 66 cell lines that showed a very weak hybridization signal also exhibited virus-induced cytopathology.

To test whether better virus uptake could be achieved, purified HfPV was treated with digitonin, a non-ionic detergent, to partially remove the outer membrane of the virus. Infection with digitonin-treated virus yielded results

Figure 20. Dot blot analysis of HfPV uptake by different lepidopteran cell lines. Equal amounts of DNA (5 μg) were loaded in each well of the dot blot apparatus, and the blots were probed with a clone of HfPV genome segment U and pUC 19 vector DNA.

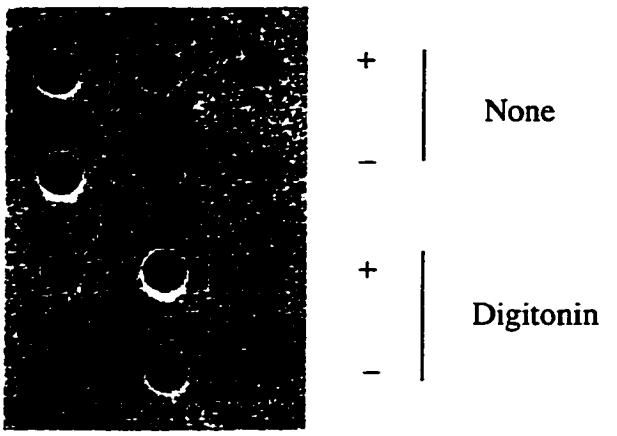
A. DNA isolated from cells harvested at 24 hours post-infection was tested for indication of cellular uptake of the viral particles. Three different cell lines Ea (Ea 1174A), Ld (Ld 652Y) and Md (Md 66) were infected with HfPV (virus +) or not infected (virus -). DNA from cell lines infected with digitonin-treated virus (virus +) or not infected (virus -) are also indicated (digitonin treatment).

B. DNA isolated from Md 66 cells transfected with purified viral DNA (HfPV) and with superhelical molecular markers (sup). Cells were harvested at 2, 4, and 7 days post-transfection.

Figure 20

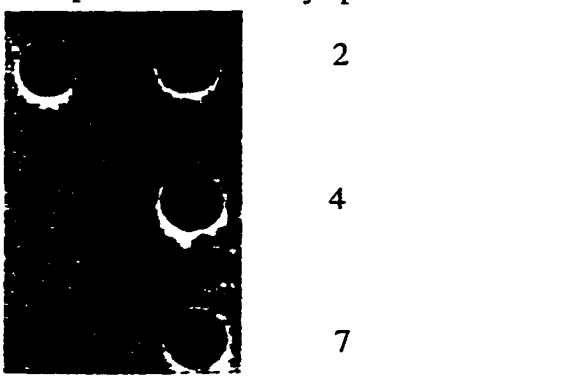
A.

HfPV Infection of different cell lines



B.

HfPV DNA transfection of Md



similar to infection with untreated HfPV (Figure 20). Although the background signal in control cells was much higher, again the strongest hybridization signals were detected from HfPV-infected Ld 652Y cells. In addition, the differences in the hybridization signals between uninfected control and virus-infected cells were most distinctive in Ld 652Y cells. Digitonin-treated virus seemed to be less efficient in mediating cellular uptake of viral particles (Figure 20). These results suggest that the untreated HfPV particle can be used to deliver the viral genome into Ld 652Y cells.

Since the uptake of HfPV seemed very low in Md 66 cells (as indicated by a weak hybridization signal), viral genomic DNA was introduced into Md 66 cells by a transfection method, using a superhelical DNA molecular weight standard marker (derived from pUC-plasmid-based sequences) as a control. The results suggested that although Md 66 cells did not show obvious uptake of the viral particles by the dot-blot assay (Figure 20, A), when total HfPV DNA was transfected into the Md 66 cells, the viral DNA persisted in the cells longer than the control DNA. When both viral genomic DNA and a control were transfected into Md 66 cells, the viral DNA was detected as late as 7 days post-transfection, while superhelical molecular marker was detected only until 2 days post-transfection (Figure 20, B). The experiment suggests that there may also be a cis-acting (such as origin of replication) or a gene that encodes a trans-acting (such as transcription factor) element in the viral DNA that allows the persistence of the viral DNA in the Md 66 cells.

B. Persistence of HfPV DNA in Ld 652Y cells

As mentioned earlier, because the persistence of HfPV DNA and virus-induced cytopathology are observed in the parasitized caterpillars, the persistence of HfPV DNA as well as virus-induced cytopathology in Ld 652Y cells was studied. The persistence of HfPV sequences was detected in HfPV-infected Ld 652Y cells by a Southern blot analysis. Virus sequences could be detected from a recovered cell line that was continuously cultured for two years after infection (Figure 21). The possible modes of viral DNA maintenance in recovered cell lines are illustrated in Figure 2.

If the viral genome segments are present as episomes, they will be separated from chromosomal DNA by agarose gel electrophoresis, and the episomal genome segments can be detected by Southern blot analysis (Figure 2). By contrast, if the viral genome segments are integrated into the host chromosome, the viral genome sequences will comigrate with the unrestricted chromosomal DNA from virus-infected cells (Figure 2). A Southern blot analysis of unrestricted chromosomal DNA from control cells, uninfected Ld 652Y cells, and 4 recovered cell lines from independent virus infection (the recovered cell lines) indicated that the viral DNA persisted in a high molecular weight form (Figure 21).

Although the results of Figure 21 suggested that viral genome segments may have integrated, it is also possible that unintegrated viral genome segments form high molecular weight concatemers by tandem duplication. These two different possibilities can be distinguished by a restriction enzyme digestion followed by a Southern blot analysis as shown in Figure 2. A

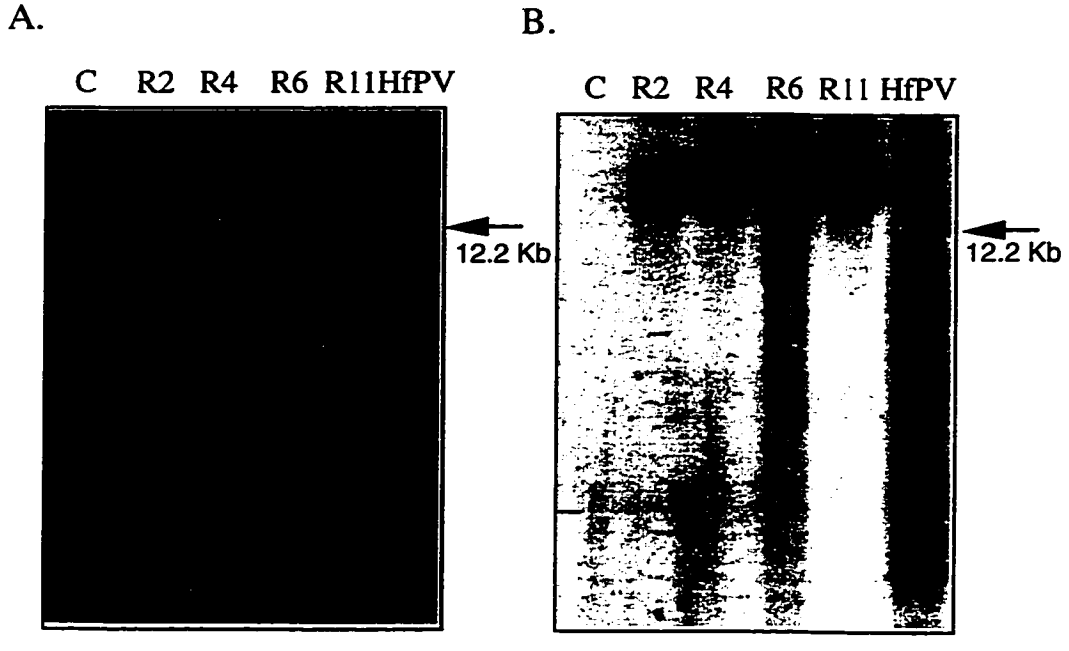
Figure 21. Persistence of HfPV genome sequences in Ld 652Y cells.

A. A photograph of an ethidium-bromide-stained 0.7% agarose gel on which unrestricted genomic DNA isolated from an uninfected control and four recovered cell lines has been analyzed. Lanes are: control genomic DNA from uninfected cells (C); chromosomal DNAs from recovered cell lines 2, 4, 6 and 11 (R2, R4, R6, and R11); HfPV genomic DNA (HfPV).

B. Autoradiogram of a Southern blot of the gel shown in Figure 21 A. Total viral genomic DNA was used as the probe.

The arrows indicate the location of the 12.2-Kb band of the 1-Kb ladder.

Figure 21



Southern blot analysis was carried out on restriction enzyme digested chromosomal DNA from the recovered cell lines using total viral genomic DNA as a probe (Figure 22). When hybridization signals were compared from the chromosomal DNA extracted from cells in early infection (Figure 22, lane D 10) to the chromosomal DNA from cells that had recovered from the viral infection (cells were harvested at least 60 days post-infection), the patterns of the hybridization signals were different. It appears that cells maintain episomal viral genome segments early after HfPV infection since both HfPV and D10 lanes showed some of the the same hybridization signals (Figure 22, lane HfPV and D10). However, only a subset of HfPV genome segments is present at 10 days post-infection. In contrast, DNA from recovered cell lines did not show this subset of viral *EcoR* I fragments. Instead a broad molecular weight size range of bands, with a distinctive *EcoR* I fragment of approximately 3.5 Kb was observed (Figure 22, lanes R1, R2, R4, and R6).

If the high-molecular-weight band was derived from a genome segment that formed a concatemer, the concatemer would have been resolved into a single unit genome segment when the concatemer was digested with an enzyme that digests the genome segment only once. However, if the restriction enzyme did not cut the genome segment, the concatemer would stay as a high-molecular-weight band. Since an extrachromosomal concatemer would not produce the pattern shown in Figure 22, the results suggested that some HfPV genome segments persisted in a form that is integrated into the chromosomal DNA of Ld 652Y cells.

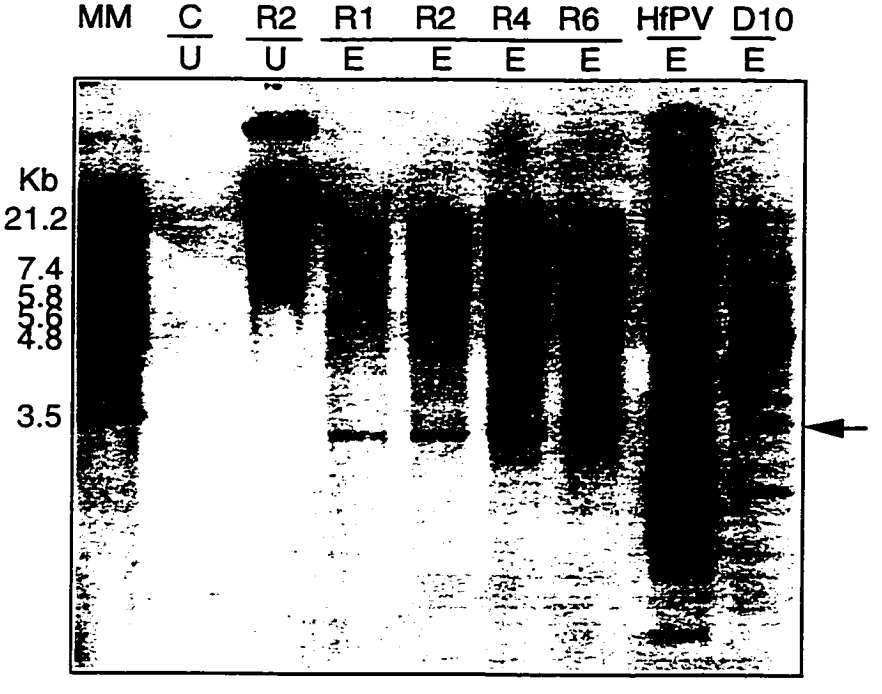
Several restriction enzymes (*BamH* I, *EcoR*I, *Hind* III, *Kpn* I, and *Pst* I)

Figure 22. The integration of HfPV DNA.

An autoradiogram of a Southern blot of a 0.7% agarose gel on which chromosomal DNA isolated from four different recovered cell lines (R1, R2, R3 and R6) was separated. The blot was probed with total HfPV DNA. Lanes: molecular weight standard markers, λ DNA restricted with *EcoR* I (MM); unrestricted control chromosomal DNA from uninfected cells (C/U); unrestricted chromosomal DNA from R2 (R2/U); chromosomal DNA from R1, R2, R4, and R6 restricted with *EcoR* I (R1/E, R2/E, R4/E, and R6/E); *EcoR*I-restricted HfPV DNA (HfPV/E); chromosomal DNA from cells at 10 days post-infection (D10/E).

The arrow indicates a distinctive band around 3.5 Kb generated by *EcoR* I digestion.

Figure 22



were used in an attempt to find a restriction enzyme that would resolve the putative high-molecular weight concatemer into a linear genome segment. The Southern blot analysis was carried out on restriction enzyme digested chromosomal DNA from one of the recovered cell lines, Ld/R2. The results showed a pattern similar to the hybridization pattern generated from *EcoR* I digestion (Figure 22) (data not shown). Restriction enzyme digestions with all the restriction enzymes showed a broad size range of bands with some distinctive bands at different molecular weight sizes. These results showed long term persistence of HfPV genome sequences in Ld 652Y cells and suggested that genome segments were integrated at multiple sites in the host chromosomal DNA.

Section 3.2. HfPV sequences in chromosomal DNA of Ld 652Y cells

A. HfPV sequences in chromosomal DNA of Ld 652Y cells.

i). Construction and screening of a partial genomic library

In order to identify HfPV sequences maintained by Ld 652Y cells, a size-selected partial genomic library of recovered cell line 2 was constructed. Chromosomal DNA from Ld R/2 was completely digested with *Hind* III or *EcoR* I, and appropriate size fractions were isolated for *Hind* IIII-digested DNA (2 Kb to 4 Kb) and for *EcoR* I digested DNA (3 Kb to 4 Kb). The size-fractionated DNA was ligated into pUC19 vector that was prepared by appropriate restriction

enzyme digestion and dephosphorylation.

Transformation of *E. coli* with the ligation mix of *Hind* III size-selected DNA produced 8964 insert-containing clones (white colonies) while ligation from *EcoR* I size-selected DNA produced only 45 white colonies. The partial genomic library generated from *Hind* III restriction enzyme digestion and the small number of *EcoR* I colonies were screened by colony hybridization using total viral DNA as a probe. PMK300, a 3.5 Kb *EcoR* I fragment that hybridized to the viral probe was isolated from colonies derived from the *EcoR* I ligation mix. No recombinant colony that contained HfPV sequences was isolated from the *Hind* III partial genomic library.

ii). Viral sequences from a recovered cell line.

Following the isolation of pMK300, a restriction map of the clone was generated (Figure 23). To confirm that the pMK300 subclones represents the integrated virus sequences in the recovered cell lines, chromosomal DNAs from recovered cell lines #2 and #4, were digested with *EcoR* I or *Hind* III and analyzed by Southern blot hybridization using pMK300 as a probe (Figure 24). The probe, pMK300, hybridized to a single band at 2.1 Kb of the *Hind* III-digested chromosomal DNA (Figure 24, lanes R2/H and R4/H). On the other hand, the probe hybridized to many broad high-molecular-weight bands in the *EcoR* I-digested R2 and R4 chromosomal DNA (Figure 24, lanes R2/E and R4/E). The result shows that there is a common internal *Hind* III fragment present in most of the high-molecular-weight *EcoR* I bands in the R2 and R4 chromosomal DNA.

Figure 23. Restriction map of pMK300 insert.

Figure 23

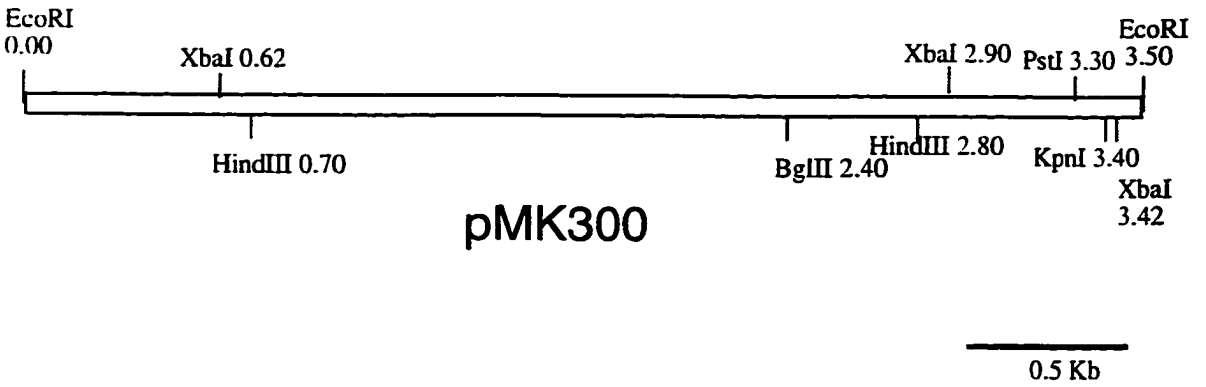
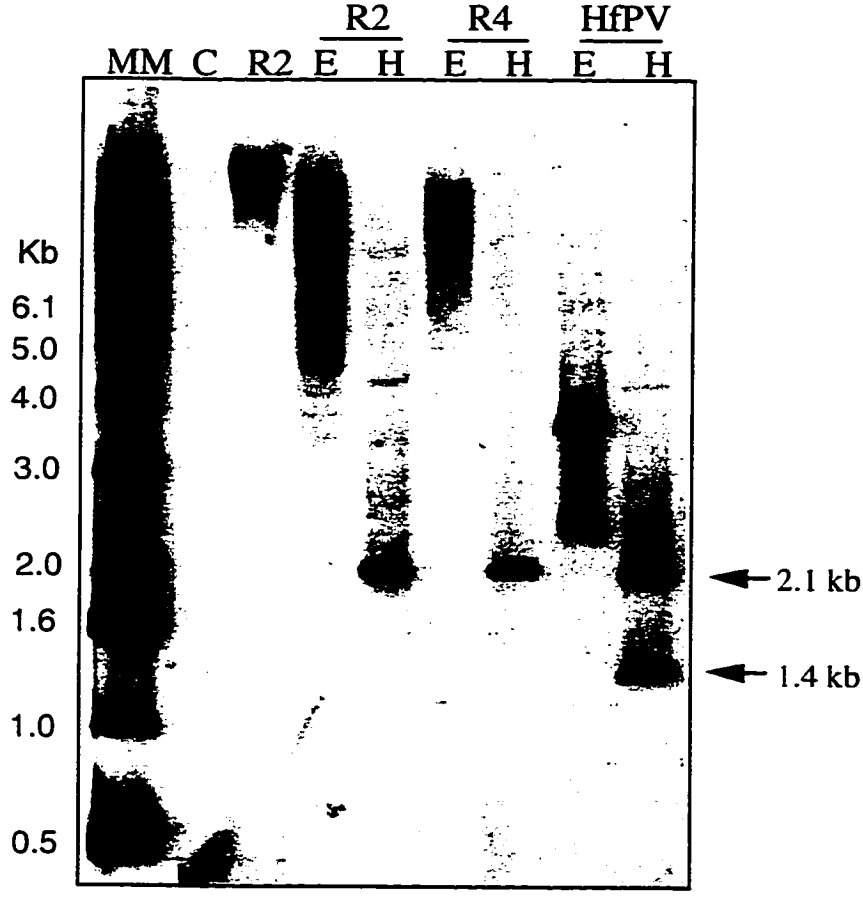

EcoRI
0.00
XbaI 0.62
XbaI 2.90
PstI 3.30
EcoRI
3.50
HindIII 0.70
BglII 2.40
HindIII 2.80
KpnI 3.40
XbaI
3.42
pMK300
0.5 Kb

Figure 24. Integration of pMK300 insert sequences.

An autoradiogram of a Southern blot of a 0.7% agarose gel on which chromosomal DNA from two recovered cell lines was separated The blot was probed with the insert of pMK300. Lanes are: 1-Kb molecular weight standard markers (MM); control, unrestricted chromosomal DNA from cells that not infected (C); chromosomal DNA from R2, unrestricted (R2); chromosomal DNA from R2, digested with *EcoR* I (R2/E) and *Hind* III (R2/H); chromosomal DNA from R4, digested with *EcoR* I (R4/E) and *Hind* III (R4/H); HfPV DNA, digested with *EcoR* I (HfPV/E) and *Hind* III (HfPV/H).

Figure 24



Two *Hind* III fragments, 2.1 Kb and 1.4 Kb in size were detected in the HfPV genome by Southern hybridization using pMK300 as a probe (Figure 24, HfPV/H) indicating the integration of the genome segment occur in the 1.4 Kb *Hind* III fragment. The two *EcoR* I bands in the *EcoR* I digested HfPV DNA was later determined to be circular and superhelical forms of the genome segment (Figure 25).

iii). Origin of pMK300

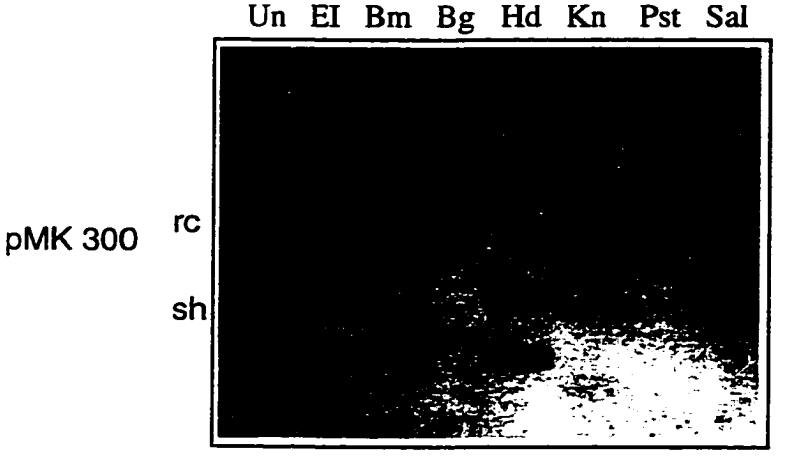
In order to study the integration of an HfPV genome segment in more detail, the genome segment from which pMK300 is derived was identified, and cloned. This HfPV genome segment was compared to pMK300. Cloning a genome segment that persists is the first step in finding any important component in the persistence of HfPV genome segments in Ld 652Y cells.

The viral genome segment corresponding to pMK300 was identified in total viral DNA by a Southern blot analysis using pMK300 as a probe. Then, a second Southern blot analysis was carried out on the viral DNA digested with several different restriction endonucleases to identify a restriction enzyme that digested the viral genome segment only once (Figure 25). The restriction enzyme *Pst* I was found to have only a single recognition site in the viral genome segment that hybridized to pMK300, therefore it was used to construct a viral genomic library. The genomic library was screened using pMK300 as a probe. The clone, pMK500, was isolated from this library.

Figure 25. Southern blot analysis of HfPV genomic DNA.

An autoradiogram of a Southern blot in which restriction endonuclease digested HfPV genomic DNA was probed with pMK300 insert. Relaxed circle (rc), superhelical (sh) and linear (l) forms of the viral genome segment are indicated. HfPV genomic DNA was digested with the following restriction enzymes: *EcoR*I, *BamH* I, *Bgl* II, *Hind* III, *Kpn* I, *Pst* I, and *Sal* I. The lanes are: Unrestricted (Un), *EcoR* I (EI), *BamH* I (Bm); *Bgl* II (Bg) *Hind* III (Hd) *Kpn* I (Kn), *Pst* I (Pst), *Sal* I (Sal) digested HfPV genomic DNA.

Figure 25



B. Characterization of an HfPV genome segment.

A restriction map of pMK500 was constructed (Figure 26). A Southern blot analysis using pMK500 as a probe indicated that the genome segment is integrated into the chromosomal DNA of recovered cell lines (Figure 27). Further Southern blot analysis using pMK500 as a probe against restriction-enzyme-digested chromosomal DNA of the recovered cell lines (R2 and R4) produced the identical hybridization pattern to that observed when pMK300 is used as a probe (Figure 24). The size of the viral genome segment was 3.5 Kb, and the restriction map of the viral genome segment showed strong similarity to pMK300 (Figure 26).

C. Comparison of pMK300, pMK500 and genome segment I.

i). Restriction map comparison

A restriction map of pMK500 was constructed (Figure 26), and a Southern blot analysis indicated that the genome segment cloned is integrated into the chromosomal DNA of Ld 652Y cells (Figure 27). Because no *EcoR* I site was present in the viral genome segment (Figure 25, *EcoR* I; Figure 24, HfPV/E), both circular and superhelical forms of the genome segment are detected in *EcoR* I-digested viral DNA. However, when restriction maps of the clones, pMK300 and pMK500, were compared (Figure 26), the only difference in the restriction maps of the two clones appears to be the *EcoR* I site on pMK300, which is not present in pMK500. The size and restriction map of the pMK300 insert match previously characterized HfPV genome segment I (Xu,

Figure 26. Comparison of the restriction maps of pMK300 (A) and pMK500 (B) inserts. The *EcoR* I site present in pMK300 is missing in pMK500.

Figure 26

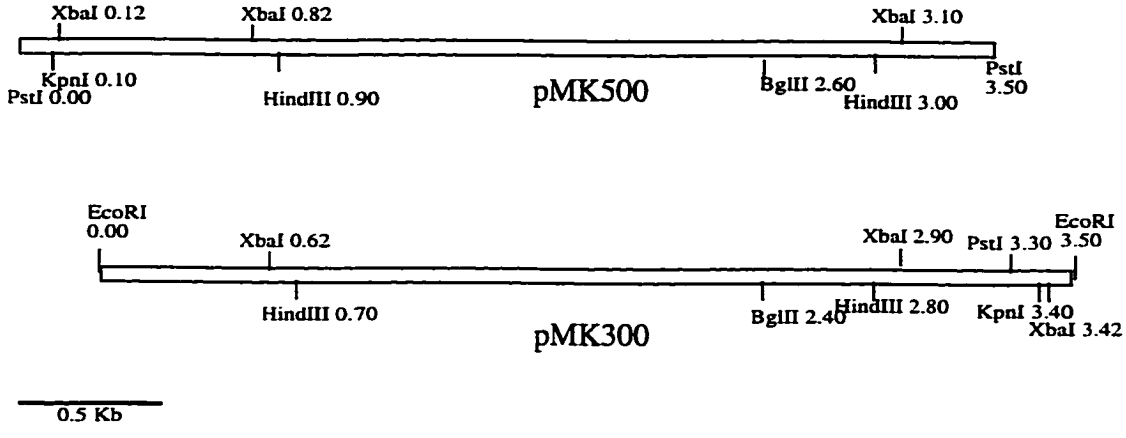
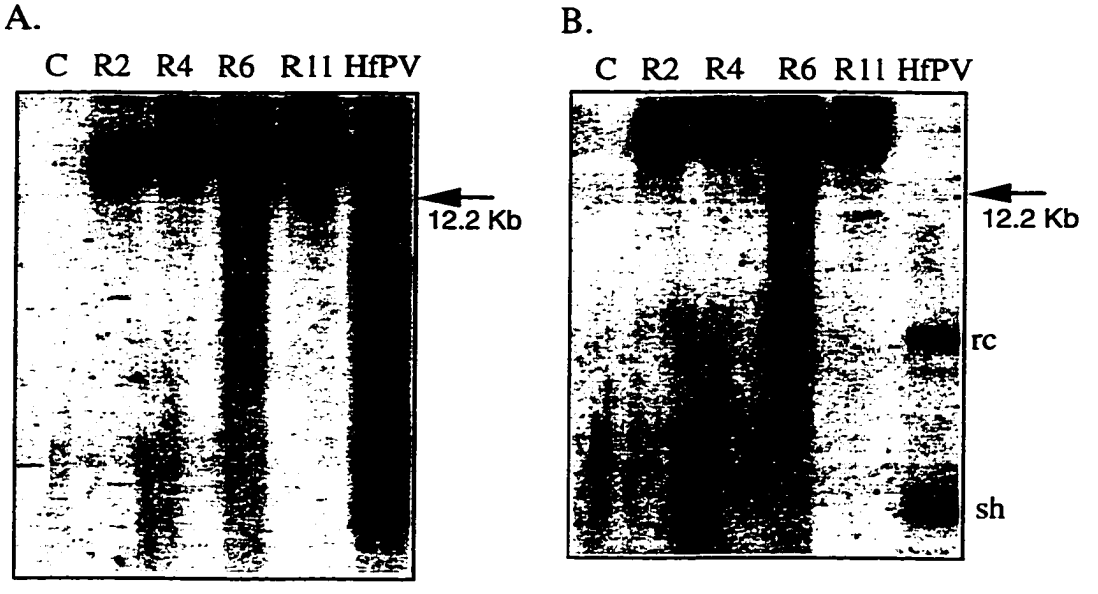

XbaI 0.12
XbaI 0.82
XbaI 3.10
KpnI 0.10
PstI 0.00
HindIII 0.90
pMK500
BglII 2.60
HindIII 3.00
PstI 3.50
EcoRI 0.00
XbaI 0.62
XbaI 2.90
PstI 3.30
EcoRI 3.50
HindIII 0.70
BglII 2.40
HindIII 2.80
KpnI 3.40
XbaI 3.42
pMK300
0.5 Kb

Figure 27. Persistence of pMK500 insert.

Autoradiograms of Southern blots from duplicate 0.7% agarose gels were probed with HfPV genomic DNA (A) or the cloned genome segment, pMK500 (B). Relaxed circle (rc) and superhelical (sh) forms of the viral genome segment were identified. Lanes are: control genomic DNA from uninfected cell lines (C); chromosomal DNAs from recovered cell lines 2, 4, 6 and 11 (R2, R4, R6, and R11); HfPV genomic DNA (HfPV). The arrows indicate the location of the 12.2 Kb band of the 1-Kb ladder.

Figure 27



1991), and genome segment I was shown to exhibit polymorphism for the *EcoR* I site (Xu, 1991).

Although a previous study indicated that genome segment I had a polymorphism at the *EcoR* I site (genome segment I *EcoR* I/+ or I *EcoR* I/-), we did not find the genome segment I *Eco*R I/+ in the virus stock in the lab (data not shown). As a result, more detailed comparison between pMK300 and pMK500 was required to assess the sequence variation at the region where the *EcoR* I site is in pMK300.

ii). Subcloning and comparison of the pMK300 and pMK500

Although the restriction maps of these two clones appeared similar except for the *EcoR* I site, more detailed comparison between the clones was necessary to understand whether pMK300 contains any host sequences. Subcloning of pMK300 and pMK500 was carried out to allow a more detailed comparison of the two clones. The subclones are described in Table 1.

The subclones pMK301, pMK302, pMK501 and pMK502 were sequenced on one strand. The comparison of the sequences indicates that *EcoR* I site of pMK300 was produced from the genome segment I *EcoR* I/- by a single nucleotide insertion and a single nucleotide exchange of A to T to create the *EcoR* I restriction enzyme site on genome segment I *EcoR* I /- (Figure 28). The HfPV sequences from pMK304 and pMK504 (internal *Hind* III fragments of pMK300 and pMK500) were also compared to determine whether any polymorphism for recognition sites for frequently cutting restriction

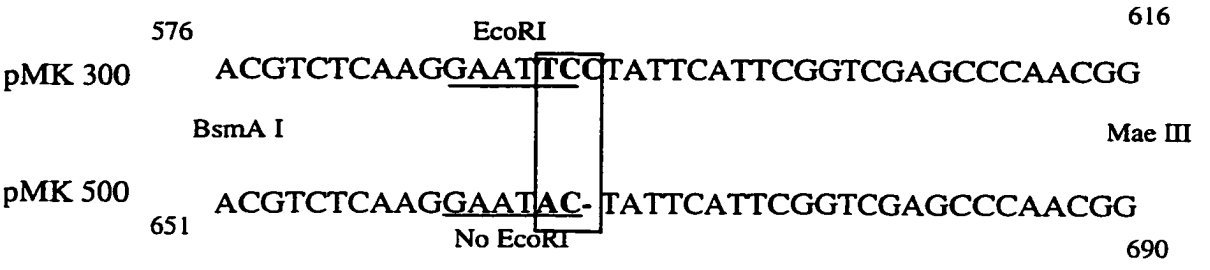
Table 1 . Subclones of pMK300 and pMK500

Name of plasmid	Cloning vector	Cloning site	Size of insert and origin
pMK300	pUC19	EcoRI	Chromosomal DNA
pMK500	pBluescript SK+	PstI	Viral genomic DNA
pMK301	pBluescript SK+	EcoRI/Hind III	0.8 Kb, pMK300
pMK302	pBluescript SK+	EcoRI/HindIII	0.6 Kb, pMK300
pMK304	pUC19	Hind III	2.1 Kb, pMK300
pMK305	pUC19	EcoRI/BglII	2.3 Kb, pMK300
pMK306	pUC19	BglII/ EcoRI	1.2 Kb, pMK300
pMK501	pBluescript SK+	PstI/ Hind III	0.9 Kb, pMK500
pMK502	pBluescript SK+	PstI/Hind III	0.5 Kb, pMK500
pMK504	pBluescript SK+	Hind III	2.1 Kb, pMK500
pMK505	pUC19	BglII/PstI	2.3 Kb, pMK500
pMK506	pUC19	PstI/BglII	1.2 Kb, pMK500

Figure 28. Comparison of the *EcoR* I region.

The sequence of pMK300 was compared to the sequence of the pMK500 in the region around the *EcoR* I site. The replacement of an **A** with a **T** nucleotide, and insertion of a single C nucleotide at position 718 of the pMK500 insert can account for the generation of the *EcoR* I site in the pMK300 sequences.

Figure 28


576
EcoRI
616
pMK 300
ACGTCTCAAGGAATTCCTATTCATTCGGTCGAGCCCAACGG
BsmA I
Mae III
pMK 500
ACGTCTCAAGGAATAC-TATTCATTCGGTCGAGCCCAACGG
651
No EcoRI
690

endonucleases, such as *Sau* 3A and *Taq* I, could be observed (Figure 29). The internal *Hind* III fragments were isolated from the vectors, and the purified DNA fragments were digested with *Sau*3A or *Taq* I. The comparison of the DNA fragments from the restriction enzyme digestions illustrated that they were identical in molecular weight, suggesting that the inserts probably contain the same sequences.

iii). Comparison between pMK300 and genome segment I.

Because the size and restriction map of pMK300 matched the previously cloned genome segment I, pMK300 was compared in more detail to the genome segment. Since both plasmids have inserts cloned in the *EcoR* I site of pUC19, the plasmids were digested with triple restriction enzyme digestion (*EcoR* I, *Hind* III and *Xba* I), and the result suggested that pMK300 and genome segment I are identical (Figure 30). Not only was the restriction map of pMK300 identical to genome segment I *EcoR* I/+ cloned and characterized by Deming Xu (1991), but they also cross-hybridized (data not shown), indicating that the insert of pMK300 is probably identical to genome segment I. As a result, the inserts of the plasmids were renamed from pMK300 to genome segment I *EcoR* I /+ and from PMK500 to genome segment I *EcoR* I/ -.

D. The identification of a second HfPV genome segment integration

The comparison of Southern blots probed with total viral genome and blots probed with genome segment I suggested that other viral genome segments are also integrated into the chromosomal DNA of Ld 652Y cells

Figure 29. Comparison of pMK304 and pMK504 inserts.

A photograph of a ethidium-bromide-stained 1.5% agarose gel. The inserts from pMK304 and pMK504 were restriction enzyme digested and the restirction enzyme digested DNA fragments were separated by an agarose gel electrophoresis. Lanes: 1, 100 bp molecular weight ladder; 2 and 5, *Sau* 3A digested inserts of pMK304 and pMK504; 3 and 6, *Taq* I digested inserts of pMK304 and pMK504; 4 and 7, uncut inserts from pMK304 and pMK504; 8, 1-Kb molecular weight ladder.

Figure 29

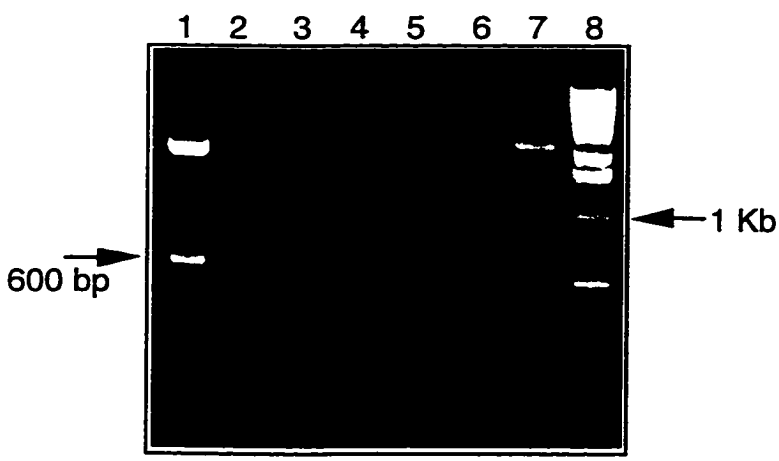
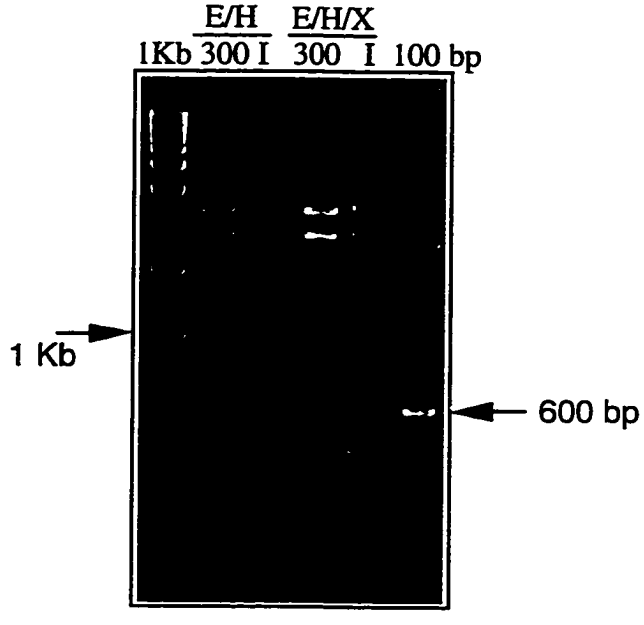


Figure 30. Comparison of pMK300 insert and genome segment I.

A photograph of edthidium-bromide-stained-1.5% agareose gel. Restriction enzyme digestion patterns generated from pMK300 and genome segment I. Both ciones are digested with *EcoR* I and *Hind* III or *EcoR* I, *Hind* III and *Xba* I to compare their restriction enzyme digestion sites. Lanes are: 1-Kb molecular weight marker (1Kb); *EcoR* I and *Hind* III digested pMK300 (E/H/300) and genome segment I (E/H/ I); *EcoR* I, *Hind* III and *Xba* I digested pMK300 (E/H/X/300) and genome segment I (E/H/X/I); 100-bp molecular standard marker (100 bp).

Figure 30



(Figure 31). When chromosomal DNA from one of the recovered cell lines was digested with *Hind* III and probed with genome segment I, the 2.1 Kb *Hind* III band was readily detected on Southern blots. However, when viral genomic DNA was used as a probe for the Southern blot analysis, several other *Hind* III bands in addition to the 2.1 Kb *Hind* III fragment appeared (Figure 31, HfPV probed blot, lanes R2/H and R4/H). These results suggested that genome segment I is not the only HfPV genome segment which may be integrated into the chromosomal DNA of Ld 652Y cells.

Another HfPV genome segment that had integrated into the chromosomal DNA of Ld 652Y cells was identified by probing the chromosomal DNA of recovered cell lines with some of HfPV clones that were previously cloned by Deming Xu (1991) (Figure 32). A previously cloned but uncharacterized genome segment was found to be integrated into the chromosomal DNA of the recovered cell line 2. By restriction enzyme digestion followed by a Southern blot analysis of HfPV genomic DNA using the insert of the clone as a probe, *Hind* III was identified as a restriction enzyme that cut at a single site within the genome segment. Following the identification of the single site restriction enzyme, another viral genomic library was constructed using *Hind* III restriction enzyme to clone a full length genome segment (data not shown). The uncharacterized genome segment (unpublished data) was used as the probe to screen the genomic library, and a clone, pMK1000, was found to hybridize to the probe.

The insert of the clone, pMK1000, was mapped (Figure 32), and the viral genome segment was later named genome segment υ. The genome segment

Figure 31. Integration of other HfPV genome segments.

Autoradiograms of Southern blots of duplicate gels (0.7 % agarose) probed with total HfPV genomic DNA (A) or genome segment I (B). Lanes are: M, 1-Kb molecular weight markers; control, chromosomal DNA from uninfected cells (C); chromosomal DNA from recovered cell line 2, digested with *EcoR* I and *Hind* III (R2/E and R2/H); chromosomal DNA from recovered cell line 4 digested with *EcoR* I and *Hind* III (R4/E and R4/H); viral genomic DNA digested with *EcoR* I and *Hind* III (HfPV/E and HfPV/H).

Figure 31

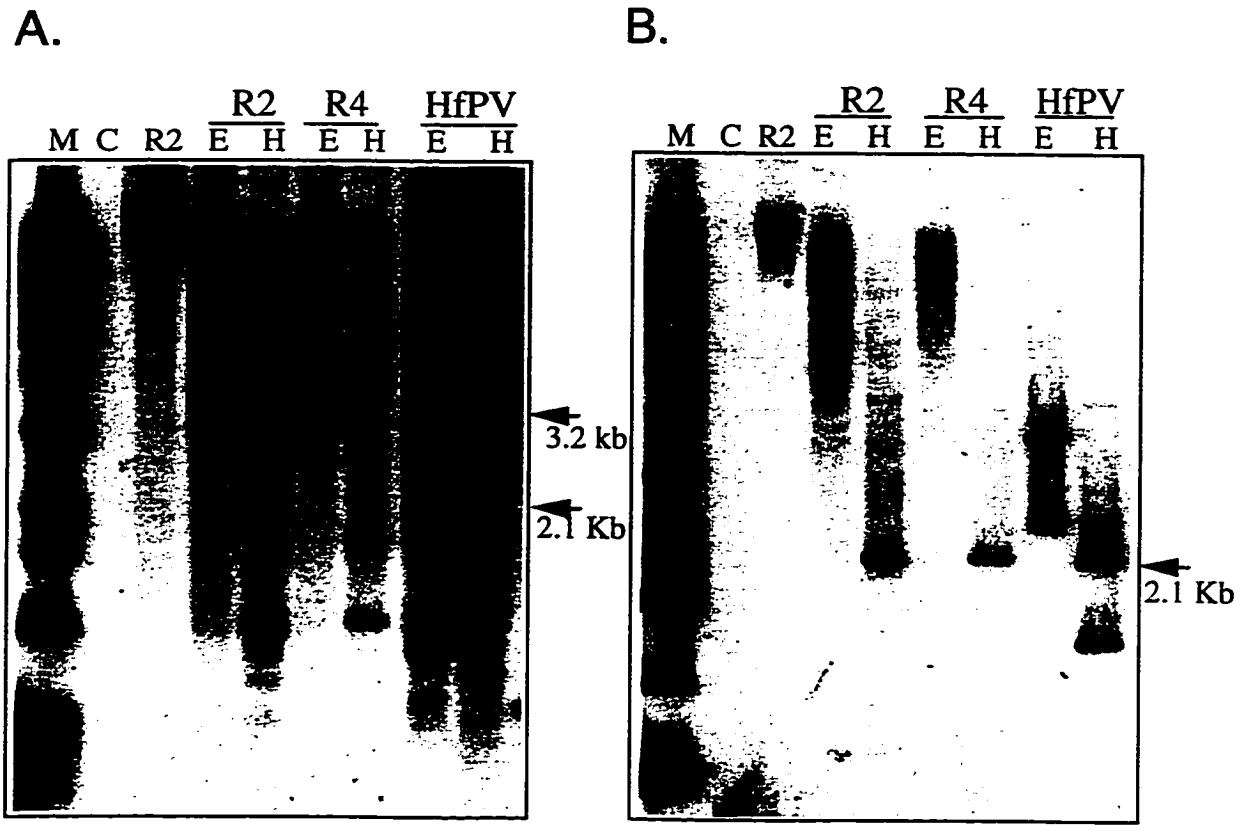

A.
B.
M C R2 E H E H E H
R2 R4 HfPV
3.2 kb
2.1 Kb
M C R2 E H E H E H
R2 R4 HfPV
2.1 Kb

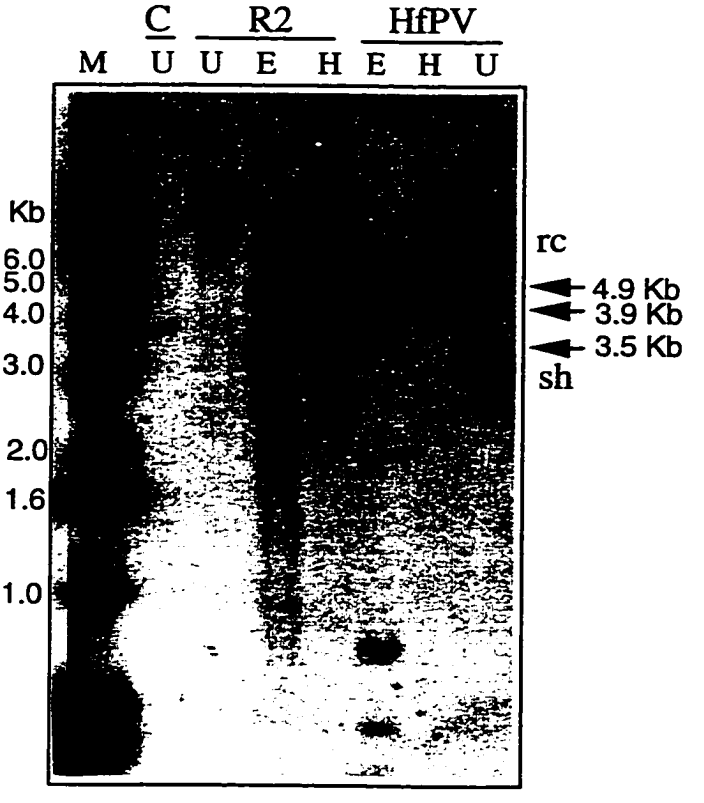
Figure 32. Integration of a second viral genome segment.

A. An autoradiogram of a Southern blot using the insert of pMK1000 (genome segment υ) as a probe against chromosomal DNA from recovered cell line 2. Relaxed circle (rc) and superhelical (sh) forms of the viral genome segment are indicated. Lanes are: 1-Kb molecular weight markers (M); control, unrestricted chromosomal DNA from uninfected cells (C/U); chromosomal DNA from recovered cell line 2 (R2) and HfPV genomic DNA (HfPV) either unrestricted (U) or restricted with *EcoR* I (E) or restriced with *Hind* III (H).

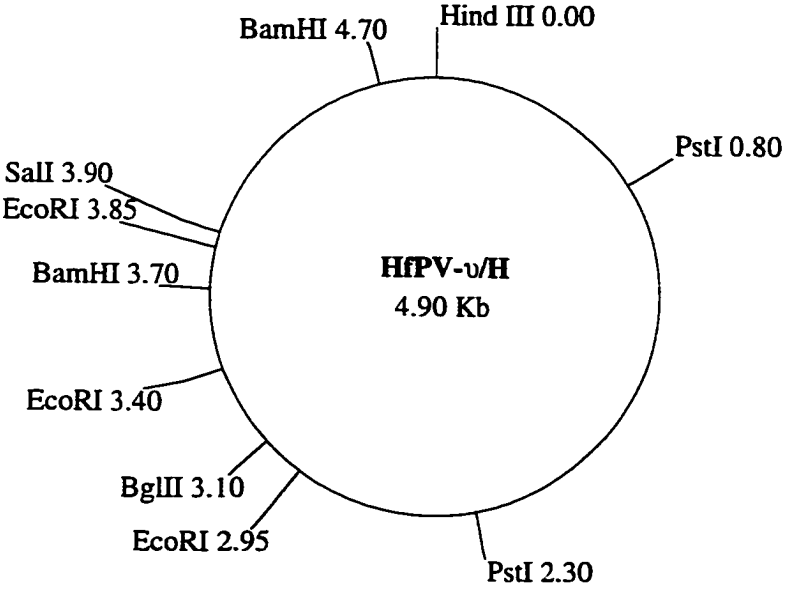
B. Restriction map of genome segment υ.

Figure 32

A.


C
R2
HfPV
M U U E H E H U
Kb
6.0
5.0
4.0
3.0
2.0
1.6
1.0
rc
4.9 Kb
3.9 Kb
3.5 Kb
sh

B.


BamHI 4.70
Hind III 0.00
PstI 0.80
SalI 3.90
EcoRI 3.85
BamHI 3.70
HfPV-υ/H
4.90 Kb
EcoRI 3.40
BglII 3.10
EcoRI 2.95
PstI 2.30

shows a distinctive band at 3.5 Kb by *EcoR* I digestion of the chromosomal DNA of R2 while a broad range of *Hind* III bands were hybridized to the genome segment υ sequences. In addition, the genome segment cross-hybridizes to another HfPV genome segment whose size is 1 Kb smaller than genome segment υ (Figure 32, lanes HfPV/H and HfPV/U).

Some other previously cloned and characterized genome segments such as genome segment U and M (Xu and Stoltz, 1993; 1991) did not hybridize to the chromosomal DNA of the recovered cell lines (data not shown) indicating that these genome segments are not maintained in the host.

Section 3.3. Integration of genome segment I

A. Integration of genome segment I.

i). Time course of genome segment I integration

The time course of genome segment I integration was analyzed by a Southern blot analysis using genome segment I as a probe (Figure 33). The integration of the genome segment into the chromosomal DNA can be detected as early as 3 days post-infection (Figure 33). One can detect the non-integrated genome segment I as well as the high-molecular-weight chromosomal DNA as long as 21 days post-infection (Figure 34).

To confirm that the detection of the viral sequences at the molecular weight of chromosomal DNA in early infection samples is not due to the trapping of the viral DNA, another experiment was carried out. Chromosomal

Figure 33. Time course of viral genome segment I integration.

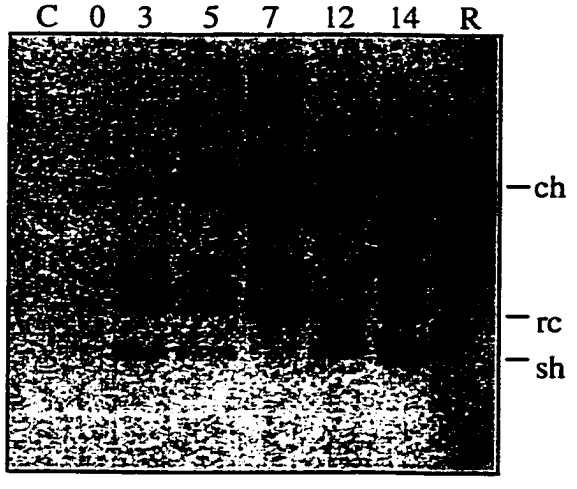
An autoradiogram of Southern blot, (A), and a photograph of an ethidum-bromide-stained 1.8% agarose gel, (B).

A. Genome segment I was used as a probe against a Southern blot of a gel on which chromosomal DNA samples from Ld 652Y cells at 0, 3, 5, 7, 12 and 14 days post-infection had been electrophoresed. Lanes: control, chromosomal DNA from uninfected cells (C); chromosomal DNA from one of recovered cell lines, R2 (R). Relaxed circle (rc) and superhelical (sh) forms of the viral genome segment as well as the location of chromosomal DNA (ch) are indicated.

B. PCR was carried out on the chromosomal DNA from cells at 10, 22 and 34 days post-infection using two sets of PCR primers. Both genome segment I and U specific primers were used in the same reaction in order to contrast the persistence of genome segment I (expected size is 600 bp) with a genome segment U (expected size is 550 bp) that does not persist. Lanes C to V contains both sets of primers while V/I and V/U contain only I and U primers. Lanes: control, chromosomal DNA from control cells (C); genomic DNA from HfPV (V); 100-bp molecular weight marker ladder (MM).

Figure 33

A.



B.

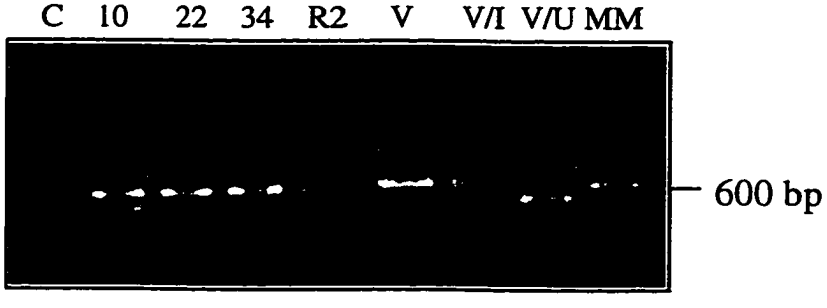
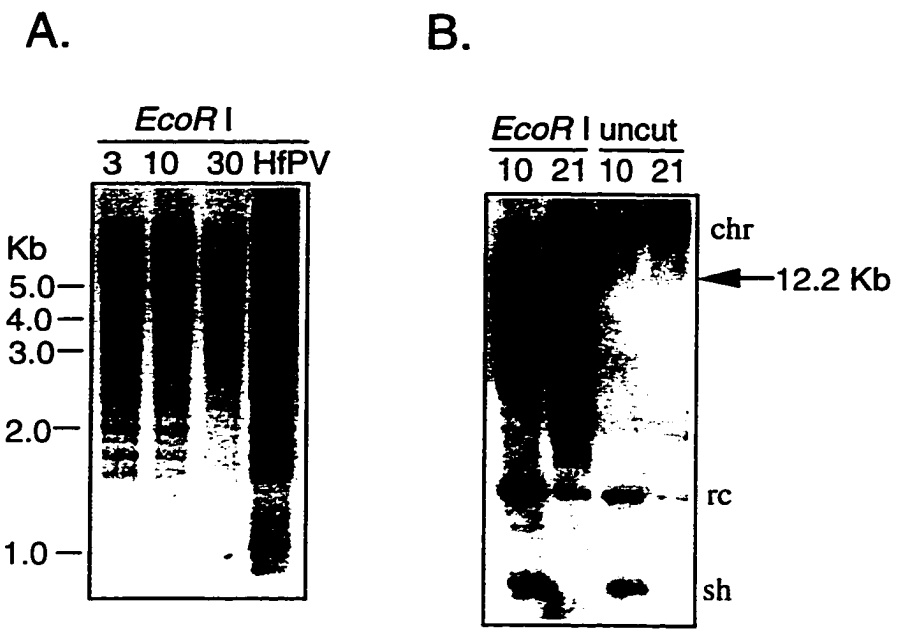


Figure 34. Detection of episomal and integrated genome segment I.

A. An autoradiogram of a Southern blot of a 0.7% agarose gel on which chromosomal DNAs isolated from Ld 652Y cells at different times post-infection were separated. The blot was probed with total HfPV genomic DNA. Lanes are: 3, 10, and 30 day post-infection chromosomal DNA digested with *EcoR* I (3/*EcoR* I, 10/*EcoR* I, and 30/*EcoR* I are); HfPV genomic DNA digested with *EcoR* I (HfPV).

B. An autoradiogram of a Southern blot of 0.7% agarose gel on which chromosomal DNAs isolated from HfPV-infected Ld 652Y cells at different time points were separated. The blot was probed with genome segment I. Lanes are: chromosomal DNA from day 10 and 21 post infected cells are digested with *EcoR* I (10/*EcoR* I and 21/*EcoR* I); undigested chromosomal DNA from cells 10 and 21 days post-infection (10/uncut and 21/uncut). Relaxed circle (rc) and superhelical (sh) forms of the viral genome segment as well as the location of chromosomal DNA (chr) are indicated. The location of the 12.2-Kb band in the 1-Kb molecular weight standard markers is indicated by an arrow.

Figure 34


A.
B.
EcoR I
3 10 30 HfPV
Kb
5.0
4.0
3.0
2.0
1.0
EcoR I uncut
10 21 10 21
chr
12.2 Kb
rc
sh

DNA was mixed with viral genomic DNA, and then they were separated by agarose gel electrophoresis for a Southern blot analysis. The results showed that viral DNA was separated from the chromosomal DNA indicating the high-molecular-weight DNA shown at the molecular weight of chromosomal DNA at early infection is not due to trapped viral DNA in the chromosomal DNA of Ld 652Y cells (data not shown).

Although the episomal genome segments were no longer detected by Southern blot analysis at 21 days post-infection (Figure 34), PCR carried out using primers from genome segment U and genome segment I sequences suggested that genome segment I might still be present in the cells as an episome even after 21 days post-infection since the sequences of unintegrated genome segment U could also be amplified as late as 34 days post-infection (Figure 34 and 33, B).

ii). Genome segment I integration site

To further our understanding of HfPV DNA persistence in recovered cell lines, the integration site or sites in the host DNA needed to be determined. When *Hind* III-digested chromosomal DNAs from different recovered cell lines were probed with genome segment I, the 2.1 Kb internal *Hind* III fragment but not the 1.4 Kb *Hind* III fragment of the genome segment was detected (Figure 24). This suggested that an integration site on genome segment I is probably located somewhere within the 1.4 Kb *Hind* III fragment of the genome segment (Figure 23). If intact 1.4 Kb *Hind* III fragment of the genome segment I was present in the recovered cell lines, 0.9 Kb and 0.5 Kb *Hind* III/*Pst* I sub-

fragments of the 1.4 Kb *Hind* III fragment used separately as probes for a Southern blot analysis would give the same hybridization pattern. Also, if some sequences of the 1.4 Kb *Hind* III fragment had been joined to the chromosomal DNA of Ld 652Y cells at a specific location in both genome segment I and the chromosomal DNA, a single distinctive band would be observed. The hybridization patterns observed in Southern blot analysis using the 0.9-Kb or the 0.5-Kb *Hind* III/*Pst* I DNA fragments as probes are very similar, indicating that most of this 1.4 Kb region is intact in the chromosomal DNA. The probes hybridize to multiple bands of the restricted chromosomal DNA. The multiple bands in each of the recovered cell lines shown in the Southern blot analysis (Figure 35) suggest that there is more than a single chromosomal site for integration of viral genome segment I.

In addition, integration of genome segment I probably does not involve a "hot spot" for its integration, since the hybridization pattern from these two probes on recovered cell lines varies from one infection to another. Although integration of the genome segment I repeatedly occurs in 1.4 Kb *Hind* III fragment of the genome segment, multiple sites of host chromosomal DNA are involved in the integration of the genome segment since the patterns of hybridization signals in different recovered cell lines are not identical.

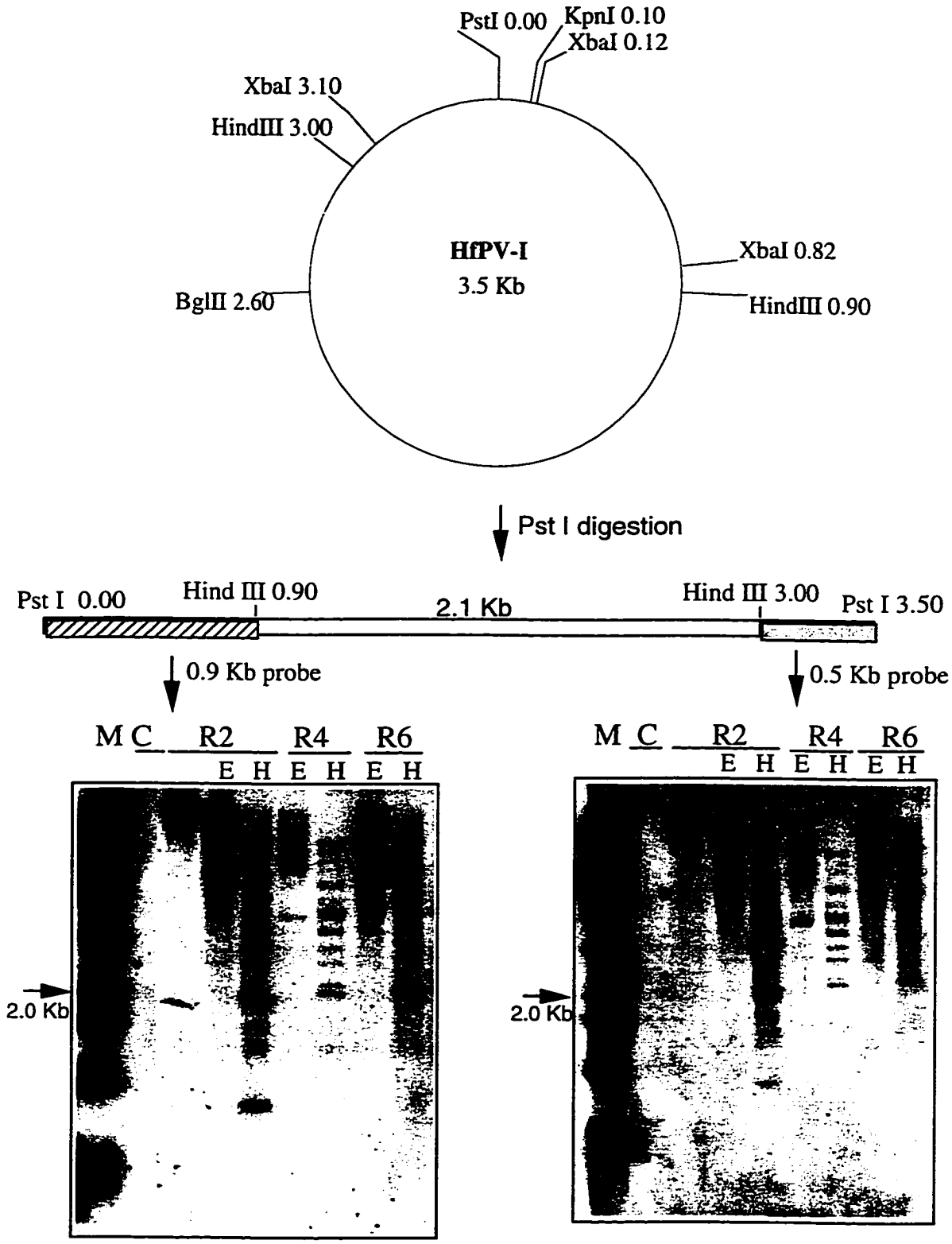
iii) Cloning of a integration site

Several methods were used unsuccessfully in an attempt to clone a chromosomal integration site for genome segment I. First, two sets of primers were designed from the 1.4 Kb region of genome segment I that was lost by

Figure 35. Analysis of genome segment I integration.

An autoradiogram of a Southern blot of 0.7% agarose gel on which chromosomal DNA from three recovered cell lines independently infected with HfPV, Ld R2, Ld R4 and Ld R6, was separated. The blots were probed with 0.9 Kb or 0.5 Kb *Hind* III/*Pst* I fragments from the genome segment I. Lanes are: 1 Kb molecular weight markers (M); control, chromosomal DNA from uninfected Ld 652Y cells (C); chromosomal DNA from recovered cell line 2 (R2), 4 (R4), and 6 (R6) digested with *EcoR* I (E) and with *Hind* III (H).

Figure 35


PstI 0.00
KpnI 0.10
XbaI 0.12
XbaI 3.10
HindIII 3.00
HfPV-I
3.5 Kb
XbaI 0.82
BglII 2.60
HindIII 0.90
Pst I digestion
Pst I 0.00
Hind III 0.90
2.1 Kb
Hind III 3.00
Pst I 3.50
0.9 Kb probe
0.5 Kb probe
M C R2 R4 R6
E H E H E H
2.0 Kb
M C R2 R4 R6
E H E H E H
2.0 Kb

integration event (Figure 36, A). Inverse PCR was carried out to amplify a flanking region of chromosomal DNA that was adjacent to the putative integrated genome segment I sequences. HfPV genomic DNA was used as a positive control for the experiment (Figure 36, B, lane V/E and V/H).

Since both sets of primers were designed from the 1.4 Kb *Hind* III fragment of genome segment I, a 1.4 Kb size amplification product is expected from inverse PCR when *Hind* III-digested and ligated viral DNA is amplified (Figure 36, B, lane V/H). On the other hand, when viral DNA is digested with *EcoR* I, the full length genome segment, 3.5 Kb should be amplified. As shown in Figure 37, the expected size products were amplified by the inverse PCR of the viral samples.

However, no distinctive band was amplified from HfPV-transformed cell lines, although a broad size range of DNA was amplified from the PCR, confirming that the genome segment I might have been integrated in many different sites of chromosomal DNA (Figure 36, B). Repeated attempts to clone the PCR products following the inverse PCR amplification failed.

As another approach to obtain genome segment I junction sites, the partial genomic library constructed using *Hind* III restriction enzyme (see construction and screening of a partial genomic library) was also screened using the 0.9 Kb and 0.5 Kb *Hind* III/*Pst* I fragments from genome segment I as probes. Again, no clone was identified containing genome segment I sequences.

Figure 36. Inverse PCR.

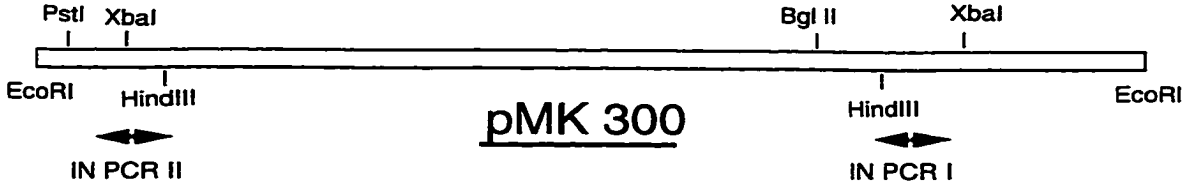
The location of inverse PCR primers on genome segment I (A) and a photograph of ethidium-bromide-stained 1.8% agarose gel (B).

A. Two sets of primers, INPCR I and INPCR II, for inverse PCR were designed from genome segment I to amplify chromosomal DNA flanking the integrated genome segment I. The direction of arrows point 5' (start) to 3' (end).

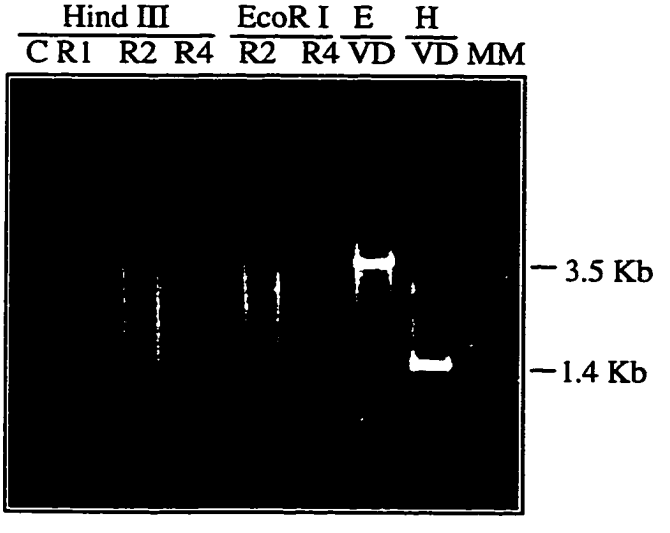
B. Inverse PCR. All samples were digested with a restriction enzyme as indicated and self-ligated before the PCR amplification. Lanes are: chromosomal DNA from uninfected cells (C), HfPV DNA (HfPV) and recovered cell lines 1 (R1), 2 (R2), and 4 (R4) restricted with *Hind* III (H) and with *EcoR* I (E); 1-Kb molecular weight markers (M).

Figure 36

A.


PstI
XbaI
Bgl II
XbaI
EcoRI
HindIII
pMK 300
HindIII
EcoRI
IN PCR II
IN PCR I

B.


Hind III
EcoR I
E
H
C R1 R2 R4 R2 R4 VD VD MM
3.5 Kb
1.4 Kb

B. Transfection of genome segments.

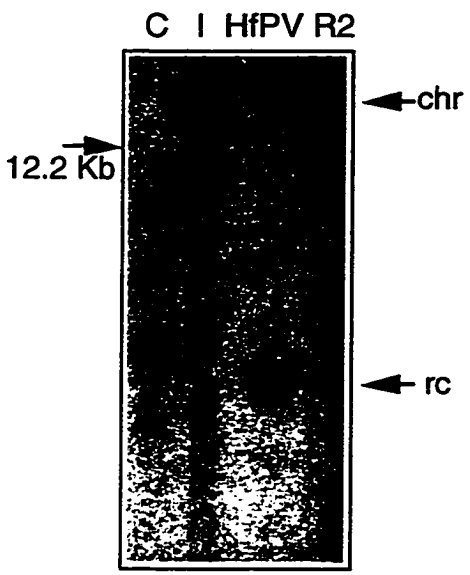
To test the ability of genome segments I and υ to integrate in the absence of other HfPV genome segments, the viral sequences were cut away from the vector sequences and self-ligated before transfection. Transfection was carried out with the self-ligated genome segments I and υ, and the integration of the genome segments was monitored by Southern blot analysis.

The cells transfected with genome segment I or υ did not show any of the cytopathologies observed in virus-infected cells or cells transfected with HfPV genomic DNA (data not shown). However, the persistence and the integration of genome segment I was observed as late as 30 days post-transfection (Figure 37). While a control, pBluescript, and genome segment υ were detected at an earlier time point (7 days post-transfection) in transfected cells, the persistence of the control and genome segment υ was not observed (data not shown).

Figure 37. Integration of purified genome segment I by transfection.

An autoradiogram of a Southern blot analysis of chromosomal DNAs isolated from genome segment I transfected cells. Two probes, genome segment I and 1 Kb ladder (containing pBluescript sequences), were used to detect genome segment I and the control, pBluescript sequences. Two different forms of genome segment I, integrated (chr) and relaxed circle (rc) are indicated. Lanes are: unrestricted chromosomal DNAs from Ld 652Y cells transfected with pBluescript, genome segment I, and HfPV DNA at 30 days post-transfection (C, I, and HfPV); unrestricted chromosomal DNA from the recovered cell line 2 (R2). The location of the 12.2-Kb band in the1-Kb molecular weight standard markers is indicated by an arrow.

Figure 37



IV. Discussion

HfPV is a polydnavirus (ichnovirus) whose natural host is the tent caterpillar, *Malacosoma disstria.* Previous work has shown that polydnaviruses are involved in the successful parasitization of caterpillars by many different parasitic wasps, and the role of the virus during parasitism is linked to cytopathology of caterpillar haemocytes (Stoltz, 1993). Polydnavirus-induced cytopathology includes inhibition of host development (Dover et al, 1987; Hayakawa and Yasuhara, 1993) and removal and altered morphology of certain haemocyte types (Davies et al., 1987; Strand, 1994) that are believed to play a role in immune suppression of the host (Stoltz and Guzo, 1986; Strand and Noda, 1991). These virus-induced cytopathologies in the parasitized caterpillars require viral gene expression without the replication of the viral particles (Theilmann and Summers, 1988; Strand, 1992). In addition to the cytopathology observed in the parasitized host haemocytes, the viral genome persists in the parasitized host throughout the course of parasitization, although replication of viral DNA has not been detected in the parasitized host (Fleming and Krell, 1993; Strand, 1992).

Since studying the virus *in vivo* involves many complexities such as observing responses of many different cell types as well as several host factors that are induced upon viral infection, an *in vitro* system was developed in order to study HfPV in a well controlled environment. In the present study of developing an *in vitro* model, some of the previously characterized HfPV-induced cytopathology reported *in vivo* was also observed in insect cell lines

infected with HfPV. The effects of polydnavirus infection *in vitro* were examined in three different lepidopteran cell lines. Furthermore, HfPV-induced cytopathology and polypeptide synthesis as well as the persistence of viral DNA in Ld 652Y cells were observed in the study of HfPV infection *in vitro*. This study presents an investigation of three aspects of polydnavirus infection *in vitro*: i) alteration of cell morphology and proliferation; ii) changes in the patterns of polypeptide synthesis; and iii) persistence of the viral DNA in virus-infected cells. The implications of this work and possible future directions will be discussed in this chapter.

1. Alterations in cell morphology and proliferation.

Altered cell morphology was observed when Ld 652Y, Ea 1174A, or Md 66 cells were infected with HfPV. The cell lines were found to differ in some aspects of their responses to HfPV infection. Among the cell lines tested, Ld 652Y cells were the most affected by HfPV infection. For this reason, the response of Ld 652Y cells to HfPV infection was studied most extensively.

HfPV-induced cytopathology in Ld 652Y cells included alteration of cell morphology, inhibition of cell proliferation, and induction of apoptosis. Similar, but not identical, changes were also observed in the other lepidopteran cell lines, Ea 1174A and Md 66. These observations suggest that: 1) the different cytopathologies observed in the different cell lines might be due to differences in viral gene expression; 2) apoptosis and the other cytopathologies observed in virus-infected cells probably contribute to the inhibition of cell proliferation;

and 3) HfPV infection of Ld 652Y cells might be a good *in vitro* system to study the apoptotic response to polydnavirus infection.

A. HfPV infection *in vitro*

The cell line Ld 652Y showed rounding up of spindle-shaped cells, aggregation, detachment from the substrate, inhibition of cell proliferation, and apoptosis occurring between 12 and 48 hours post-infection. Another cell line, Ea 1174A, showed altered cell morphology similar to Ld 652Y cells and some degree of inhibition in cell proliferation between 12 and 48 hours post-infection. However, the only distinctive virus-induced effect observed in the case of Md 66 cells was apoptosis, which occurred at a later time in viral infection (96 hours post-infection).

Why do these insect cells show different responses upon HfPV infection? Different responses among HfPV-infected cell lines might be due to differences in the utilization of cell-type-specific transcription factors by HfPV. Different HfPV promoter and enhancer elements might utilize cell-type-specific transcription factors in different cell types of caterpillars. Many cell-type-specific transcription factors such as BLyF and Early B-cell factor (EBF) are known to regulate cell-type-specific transcription, and a cell-type-specific, transcription factor signaling pathway for inducing apoptosis was suggested in the infection of Sindbis virus (SV) (Lin and Grosschedl, 1995; Lin et al., 1995; Feldhaus, et al., 1992; Scholer and Gruss, 1984). Cell-type-specific transcription factors in granulocytes and plasmatocytes may recognize certain HfPV promoters to produce viral gene products which are directly involved in HfPV-induced

cytopathology or which induce the synthesis of additional viral or cellular factors that influence cytopathology. Cell-specific transcription of certain viral gene products or of viral gene products specifically activating cellular promoters are observed in the infection of many other viruses. For instance, The EBV ED-L2 promoter, an early lytic cycle promoter, is expressed in a tissue-specific manner in the tongue, esophagus, forestomach, and skin, all sharing stratifying squamous epithelia (keratinocytes). (Nakagawa et al., 1997). Similarly in fibroblast cell lines HPV 16 oncoprotein E6 significantly (sixfold) induced promoter activity of the transforming growth factor-beta 1 (TGF-beta 1), a cytokine (Dey et al., 1997). As a result, it is possible that different HfPV genes could be expressed from different haemocyte cell types, or cell lines, which in turn could produce different responses in virus-infected cells.

Although polydnavirus gene expression by caterpillar haemocytes does occur, haemocyte morphotype-specific (granulocytes, spherulocytes or plasmatocytes) expression of polydnavirus genes has not been identified. However, haemocyte morphotype-specific responses were observed in the study of *Microplitis demolitor* polydnavirus by Strand (1994; Strand and Pech, 1995). In his study, Strand found that even among haemocyte populations in caterpillars, some cell types (granulocytes and plasmatocytes) are affected by polydnavirus infection in a cell type-specific manner. While 95% of granulocytes underwent apoptosis, plasmatocytes lost the ability to adhere to foreign surfaces upon viral infection. This type of effect was also found by Davies et al. (1987), who reported a 75% reduction in the plasmatocyte population in *Heliothis virescens* parasitized by *Campoletis sonorensis* carrying CsV.

Some of the altered cell morphology and behaviour observed in HfPV-infected cells *in vitro* is similar to that observed for haemocytes isolated from polydnavirus-injected or polydnavirus-parasitized caterpillars, suggesting Ld 652Y and Ea 117A cell lines could probably be used as an *in vitro* model to study polydnavirus infection. For example, haemocyte aggregation, inhibition of haemocyte spreading, and inhibition of binding to a substrate are observed in haemocytes from caterpillars infected by either an ichnovirus or a bracovirus (Stoltz and Guzo, 1986; Strand, 1995). When purified haemocytes from *P. includens* are infected with *Microplitis demolitor* bracovirus (MdPDV) *in vitro*, alteration of cell morphology is observed 12-18 hours post-infection for granulocytes and 18-36 hours post-infection for plasmatocytes (Strand, 1994; 1995). Also, the virus-induced changes in these two types of haemocytes are not identical, since granulocytes go through apoptosis while plasmatocytes do not.

Observations similar but not identical to these were made for ichnovirus-infected haemocytes, also indicating that different cell types behave differently upon polydnavirus infection *in vivo*. For example, alterations of haemocyte morphology are observed in granulocytes and plasmatocytes in CsV- and HfPV-injected caterpillars (Davies et al., 1987; Stoltz and Guzo, 1986; Kim et al., 1996). The viral gene and/or genome segment that induced these changes could be identified by comparing viral gene expression in HfPV-infected cells to that in uninfected control cells, both *in vivo* and *in vitro*. However, since Ld 652Y and Ea 1174A cell lines can also be used to study viral gene expression, it would be advantageous to utilize an *in vitro* system.

One way to explain the differences between *in vivo* and *in vitro* results might be by defining a permissive infection for polydnavirus. The replication of the polydnavirus has only been found to occur in the ovaries of the parasitic wasps, and the replication of the virus occurs only from the provirus genome (Stoltz, 1993). As a result, the definition of permissive host in polydnavirus infection is limited to the entry of the virus and to the induction of cytopathology in a new host that results in the successful development and reproduction of wasps in the parasitized caterpillar (Stoltz, 1993). The "permissive host" in polydnavirus infection does not necessarily support the replication of the virus although the basis for defining a permissive viral infection is the replication of viral particles in most virus-host systems (Roizman and Palese, 1996). Therefore, the permissive cell line for HfPV infection in this study is defined by induction of cytopathology associated with an alteration of cell morphology, rather than by the replication of the virus particles.

Not all tissues or cell types of parasitized caterpillars that are "permissive" for polydnavirus infection respond identically to HfPV infection. A cell line that is found to respond similarly to one of the specific tissues or cell types in the permissive natural host could be used to study some of the host responses to polydnavirus infection. One of the lepidopteran cell lines investigated in the present study (Ld 652Y) responds similarly to particular haemocyte (granulocytes) in the parasitized caterpillars, although the gypsy moth from which the Ld 652Y cell line is derived is not a permissive host for *Hyposoter fugitivus*.

In addition, the cytopathology induced by HfPV infection of Ld 652Y cells

is probably due to the expression of viral genes, since transfection of naked viral DNA produced similar results to virus infection by intact virus. However, virus-induced cytopathology was not observed in all transfection experiments, perhaps due to limitations in the transfection experiments. One such limitation was the variability in the efficiency of DNA uptake. Developing a system that can monitor viral DNA uptake during transfection experiments is important, since the efficiency of DNA uptake may differ from one transfection to another. Transfection of a reporter plasmid along with naked viral genomic DNA could be used to indicate transfection efficiency which is critical to understand the results derived from transfection of viral DNA.

Another obstacle in the interpretation of the transfection experiments obtained in this study is the question of whether a complete set of HfPV genome segments is delivered by transfection. Unlike virus particles, delivering a complete set of viral genome segments into a cell by transfection is unlikely to occur with any great degree of efficiency. When the viral capsid that contains the HfPV genome segments is removed by viral DNA purification procedure, the 28-32 HfPV genome segments that represent the complete HfPV genome will act like 28-32 individual plasmids. Thus, it is more likely that individual cells transfected with the viral genome will contain less than the total complement of HfPV genomic DNA, producing more variable results than the HfPV infection study.

In summary, since transfection of the HfPV genome induces most of the cytopathology observed in virus infection, it is likely the virus-induced cytopathology is produced by HfPV gene expression. Furthermore, because

different haemocyte cell types *in vivo* may express different viral genes upon viral infection, the same could be true for cell lines *in vitro* which might explain the varied responses from HfPV-infected cell lines.

B. Apoptosis and cell proliferation

Apoptosis is a newly discovered aspect of polydnavirus-induced cytopathology (Strand and Pech, 1995; Kim et al., 1996). Apoptosis, or programmed cell death, is a form of cell death that involves specifically regulated morphological and biochemical changes in cells (Kornbluth, 1994) and plays major roles in development and homeostasis, as well as in diseases such as cancer and in viral infections (Steller, 1995).

Since Md 66 and Ld 652Y cells treated with the conditioned medium from HfPV-infected cells produced higher proportions of cells undergoing an apoptotic response than the HfPV-infected cells, the apoptotic response might be induced by a soluble protein like Fas Ligand (Fas L). Among several components that are involved in apoptosis, Fas L can induce apoptosis as a soluble protein (Nagata and Golstein, 1995). Fas L is a cytokine that belongs to a family of proteins known to regulate cellular proliferation and differentiation, the tumor necrosis factor (TNF) family (Nagata and Golstein, 1995). Some viruses, such as HIV appear to involve the Fas L signalling pathway in their induction of apoptosis (Finkel et al., 1995). Other viruses, such as baculovirus (p35) and cowpoxvirus (CrmA), encode proteins that inhibit the Fas L signaling pathway for apoptosis (Fraser and Evan, 1996). These virus-encoded proteins inhibit apoptosis by forming stable complexes with caspases, cysteine proteases that induce apoptosis (Enari et al., 1996; 1995).

Infection of Ld 652Y cells by HfPV indicated that viral infection results in an apoptotic response in a small fraction of the cell population. Testing the viability of cells by dye exclusion (trypan blue staining) revealed that most of the cells in the HfPV-infected Ld 652Y culture appeared to be viable at the time when an apoptotic response was observed. This result suggests that measuring cell viability by a standard dye exclusion assay such as trypan blue staining may not indicate the fate of the cells that are committed to undergo apoptosis. A similar pattern has been reported when purified haemocytes are infected by MdPDV (Strand,1994). In Strand's study, purified granulocytes from *P. includens* were infected with MdPDV *in vitro* and found to display an apoptotic response upon viral infection. Strand (1994) has reported that although a large proportion of granulocytes infected with polydnavirus undergo apoptosis at 18-48 hours post-infection, cells appear to be viable according to the dye exclusion assay through 30 hours post-infection.

My investigation of different lepidopteran cell lines infected with HfPV is the first study to find an apoptotic response induced by an ichnovirus infection. The *in vitro* results suggest that an apoptotic response might also occur in an ichnovirus infection *in vivo*. The observations of apoptosis in HfPV-infected Ld 652Y cells suggest that the inhibition of cell proliferation is in part due to apoptosis. However, if cell death is the only cause for the slower cell proliferation subsequent to viral infection, one would expect to see a slower cell proliferation in virus-infected Ea 1174A cells than in Ld 652Y cells, since a higher proportion of dead cells were observed in virus-infected Ea 1174A culture than in Ld 652Y culture. Instead, Ld 652Y cells showed a greater

inhibition of cell proliferation upon HfPV infection than Ea 1174A cells. This result suggests that there are other factors involved in the inhibition of cell proliferation rather than just host cells being killed by viral infection.

One technical explanation for the higher inhibition of cell proliferation in Ld 652Y cells is that one can not detect non-viable cells with the dye exclusion method. There are indications that apoptotic bodies exclude vital dyes such as trypan blue and nigrosine (Bowen and Bowen, 1990). Another possibility is that Ld 652Y cells stop proliferating while Ea 1174A cells continuously proliferate although both cell types are being killed by viral infection. For instance, HfPV infection of Ld 652Y cells might induce the expression of a protein like p53 that signals cells to stop proliferating and activates the apoptotic response to viral infection. On the other hand, Ea 1174A cells might go through necrosis that is independent from cell cycle. Cell-cycle-independent virus replication is known to occur in Hepatitis B virus replication (Guidotti et al., 1997).

In contrast to programmed cell death (apoptosis), which occurs by genetic signalling, necrosis is usually brought about by lethal environmental stimuli. Necrosis is characterized by plasma membrane changes resulting in a loss of the calcium and sodium ion balance, acidosis, osmotic shock and clumping of chromatin. These events lead to irreversible damage to cells by swelling of the lysosomes, dilation and vesiculation of the endoplasmic reticulum, leakage of enzymes and proteins, and resultant loss of cellular compartmentalization (Trump et al., 1981). Events such as exposure to toxin or viral infection that increase the permeability of the plasma membrane either by

structural changes affecting membrane pores and channels, or by inhibiting membrane ion pumps are known to induce necrotic cell death (Trump et al., 1981). Neuronal damage brought on by HIV-1 infection (Lipton et al, 1996) and p53-independent necrotic cell death of human A549 cells by adenovirus infection (Subramanian et al., 1995) are some examples of virus-induced necrotic cell death.

In conclusion, the inhibition of cell proliferation observed from HfPV-infected Ld 652Y cells is probably due partly to an apoptotic response of the cells. However, inhibition of Ea 1174A cell proliferation by HfPV infection suggests that HfPV infection can also induce cell death by some other mechanism, possibly necrosis.

C. Apoptosis and Polydnaviruses.

Studying the induction of apoptosis by HfPV *in vitro* would be even more interesting if the apoptotic response were also observed *in vivo*, since a direct correlation might exist between *in vitro* and *in vivo* results. HfPV-induced apoptosis could be a host defense mechanism against viral infection, and possibly there are HfPV gene encoded viral products that counteract apoptosis, as has been shown in studies of other viruses.

Another possibility is that HfPV induces apoptosis in only a subset of haemocyte populations or tissue culture cell types that it infects. Cell-type-specific killing by polydnaviruses has been reported in both ichnovirus and bracovirus infections (Davies et al, 1987; Strand, 1994). By examining the HfPV-infected cell population for viral uptake, viral gene expression, and

induction of the apoptotic response, one can determine whether HfPV-infected cells or bystander cells go through apoptosis. Alternatively, polydnavirus-induced apoptosis could be studied using an *in vitro* system, allowing identification of a polydnavirus genome segment, or a gene that is involved in the apoptotic response. In HIV infection, HIV-producing cells rarely undergo apoptosis (Frankel et al., 1995) while HIV infection induces apoptosis in T-cells (Meyaard et al., 1992) suggesting HIV may kill uninfected bystander cells (Finkel and Banda, 1994). Other double-stranded-DNA viruses, such as SV40 (large T antigen), HPV (E6), adenovirus (E1B), EBV (LMP1) and baculovirus (p35), are known to carry genes that inhibit the apoptotic responses of virus-infected cells (Conzen et al., 1997; Pan and Griep, 1995; Querido et al., 1997; Teodoro and Branton, 1997; Henderson, et al., 1991; Clem et al., 1991).

One possible explanation of how polydnavirus suppresses the immune system of the caterpillar is by removing haemocytes through induction of apoptosis either by direct or indirect infection. Interestingly, the infection of *H. virescens* by another ichnovirus, *C. sonorensis* polydnavirus (CsV), resulted in the removal of approximately 75% of the circulating plasmatocytes (Davies et al., 1987). When granulocytes purified from *P. includens* were infected with the bracovirus MdPDV an apoptotic response was observed in over 95 % of granulocytes (Strand, 1995). Since most haemocytes of insects are granulocytes and/or plasmatocytes (Ratcliffe, 1993; Pech et al., 1994), it is important for polydnaviruses to target these cells to suppress host immune responses.

Even if polydnaviruses infect only a proportion of haemocytes, polydnavirus could induce significant loss of haemocyte population by inducing

apoptosis in non-infected cells. For instance, if virus-infected cells secreted an apoptotic signaling molecule like Fas L, polydnaviruses could induce an apoptotic response in cells that are not infected with the virus.

Induction of apoptosis in cells that are not infected with virus is observed during HIV infection. T cells from HIV-1 infected patients are known to undergo apoptosis *in vitro* (Meyaard et al., 1992), and the interaction of virus-encoded glycoproteins gp120 and gp160 with CD4 molecules are known to be responsible for the induction of apoptosis (Capon and Ward, 1991). Gp160 is the envelope glycoprotein encoded by the HIV *env* gene and the precursor of external gp120 and transmembrane gp41. Gp120 is known to associate noncovalently with gp41, as well as to shed readily from the cell surface to the culture medium (Schneider et al., 1986). The observation that cross-linking of gp120 to CD4 can induce apoptosis of T-cells (Banda et al., 1992), and that HIV-producing cells rarely undergo apoptosis (Frankel et al., 1995) suggest that HIV may kill uninfected bystander cells (Finkel and Banda, 1994). Cross-linking of gp120 to CD4 molecules also increases the expression of Fas L in T cells (Westendorf et al., 1995), suggesting involvement of the Fas L signalling pathway in HIV infection (Finkel et al., 1995).

Studying the role of apoptosis in viral genome persistence and immune suppression may be important in understanding HfPV infection. If HfPV-infected cells are destroyed by apoptosis, the persistence of viral DNA would not be observed *in vivo* in Ld 652Y cells recovered from virus-induced cytopathology. Insect cell lines may provide more convenient systems than the *in vivo* system to study the effects of polydnavirus on apoptosis. Furthermore, findings from the

in vitro study could be used to guide *in vivo* studies in HfPV-infected caterpillars. For example, when a viral gene that is responsible for the induction of apoptosis in HfPV infection *in vitro* is found, the gene could be introduced into the caterpillars to see its effect *in vivo*.

In comparison to some studies of polydnavirus infection *in vivo*, the results from HfPV infection *in vitro* suggest that it might be possible to observe an apoptotic response *in vivo*. Thus, Ld 652Y cells may be used to study the apoptotic response, as well as some of the other cytopathic effects induced by polydnavirus infection.

2. Alteration of polypeptide profile

Changes in the pattern of polypeptides synthesized in HfPV-infected cells were detected by both SDS-PAGE and ^{35}S-methionine labelling followed by SDS-PAGE analysis. Several additional polypeptide bands were observed from HfPV-infected and HfPV DNA-transfected cells. A 55-kDa polypeptide band was detected in the culture medium of virus-infected and viral-DNA-transfected Ld 652Y cells. In total cell extracts, 8-kDa and 23-kDa polypeptide bands were stably expressed, and transiently expressed 25.5-kDa and 27-kDa polypeptides were also observed from HfPV-infected and HfPV-DNA-transfected cells.

Although alteration of polypeptide profile by HfPV infection of *M. disstria* was not detected *in vivo* using standard SDS-PAGE analysis (Stoltz, unpublished result), alterations in polypeptide synthesis due to polydnavirus

infection *in vivo* were observed in caterpillars infected with other polydnaviruses. For example, infection with the ichnovirus CsV (Cook et al., 1984) or the bracovirus CcPDV (Beckage and Kanost, 1993) results in additional polypeptides in virus-infected caterpillars. In addition, transient transcription of polydnavirus genes was reported in parasitized caterpillars (Asgai, 1996), suggesting the possibility of transient polypeptide expression *in vivo*.

Contrary to HfPV infection *in vivo*, additional polypeptides were identified from HfPV-infected Ld 652Y and Ea 117A cells. It is possible that a transient and low level of polydnavirus gene expression *in vivo* that can not be detected by SDS-PAGE analysis might occur in the parasitized caterpillars. More extensive studies will be required to detect any additional polypeptides in HfPV-infected tent caterpillars or any other habitual host, in order to make a comparison between *in vitro* and *in vivo* results. The comparison between *in vitro* and *in vivo* changes in the pattern of polypeptides in cells by HfPV-infection could identify any polypeptides expressed in common and give more insight into HfPV gene expression and its role in cytopathology *in vivo*.

A. Alteration of polypeptide profile *in vitro*

The response of different cell types to HfPV infection varied in regard to changes in the pattern of polypeptides in HfPV-infected cells as well as in their morphology and behaviour, indicating that alterations in cell morphology and behaviour are probably due to changes in the pattern of polypeptides in virus-infected cells. As a result, alterations in polypeptide profile due to HfPV infection *in vitro* were investigated in three different cell lines, Ld 652Y, Ea

1174A and Md 66. Although HfPV-induced changes in polypeptide profile in Ld 652Y and Ea 1174A cell lines were not identical (Figure 11, C and Figure 13, A), both cell lines showed additional bands that were not observed in uninfected control cells at 24 hours post-infection. No additional bands were found from Md 66 cells at 24 hours post-infection. In addition, several additional polypeptides were observed in Ld 652Y cells by pulse-labeling with ^{35}S-methionine and SDS-PAGE analysis after HfPV infection. Such changes must reflect either virus-encoded polypeptides expressed by the host cells, or host-encoded polypeptides expressed in response to the viral infection.

A 55-kDa polypeptide detected in the culture medium from virus-infected and virus-DNA-transfected cells is probably secreted, since this polypeptide was not detected in the culture medium from control cells. Because conditioned medium induced apoptosis from Ld 652Y cells, it is conceivable that the secreted 55-kDa polypeptide is responsible for inducing the apoptotic response from haemocytes.

The 55-kDa polypeptide detected in the culture medium of both virus-infected and virus-DNA-transfected cells would be interesting to study, although its low level of expression makes purification for microsequencing or other biochemical studies difficult. Because the 55-kDa polypeptide band could not be detected by Coomassie Blue staining, its level of expression is estimated to be less than 2.4 μg (25 pmol) per $1X10^{6}$ cells (by the estimation method of Matsudaira, 1987). If the 55-kDa polypeptide could be produced in sufficient amounts, the N-terminal amino acid sequence of the purified polypeptide could be determined by microsequencing, and the deduced DNA sequence could be

used to design degenerate oligonucleotide probes. Such probes could be used to identify and isolate the gene that encodes the 55-kDa polypeptide in order to assess the function of the polypeptide by introducing the gene into Ld 652Y cells.

Fractionation of a cell extract of HfPV-infected Ld 652Y cells suggests that one polypeptide, 8-kDa in size, is located in the cytoplasmic fraction, while another polypeptide of 23-kDa, is located in the membrane fraction (Figure, 17). It appears that the synthesis of the 23-kDa polypeptide is strongly correlated with the introduction of viral DNA into the cells, either by infection or by transfection. The 8-kDa polypeptide is likely to be a host gene product because an 8-kDa polypeptide is induced by many different factors, such as exposure to the conditioned culture medium from HfPV-infected cells, exposure to plasma from parasitized caterpillars, depletion of nutrients in the medium, and heat-shock treatment. However, since a Western blot analysis was not carried out, it is not clear whether the 8-kDa polypeptide induced under these different conditions is the same polypeptide or not.

Introduction of purified HfPV DNA into Ld 652Y cells by transfection induced the synthesis of the 8 and 23-kDa polypeptides, as well as the 25-kDa and 27-kDa polypeptides that were observed in cells infected with HfPV. This result suggests that the induction of cytopathology, as well as the synthesis of some specific polypeptides, are the result of viral DNA expression in the host cells. In addition, since HfPV-induced alteration of cell morphology and behaviour differs between Ld 652Y cells and Ea 1174A cells, it might be expected that the patterns of polypeptide synthesis in these cell lines might also

differ, suggesting differential HfPV gene expression in different cell types or cell lines.

B. HfPV gene expression?

The question of whether these polypeptides are also expressed *in vivo* will require more detailed and controlled experiments on HfPV-infected tent caterpillars and gypsy moth caterpillars. Although alteration of host protein synthesis by polydnavirus infection was observed in infection of other polydnaviruses, such as CsV (Cook et al., 1986) and *Cotesia congregata* polydnavirus (CcPDV) *in vivo* (Beckage and Kanost, 1993; Harwood and Beckage, 1994), it was not observed in infection of HfPV *in vivo*. It might be possible to detect newly synthesized polypeptides in purified tent caterpillar haemocytes infected with HfPV by labeling with ^{35}S-methionine. In Ld 652Y cells, the transfection of viral DNA results in a similar alteration of polypeptide synthesis as observed following infection, therefore it is likely that polypeptide synthesis results from viral gene expression. However, whether the 8-kDa and the 23.5-kDa polypeptides as well as other polypeptides induced by viral infection are virus-encoded or host-encoded remains to be determined.

How HfPV gene expression is involved in the persistence of viral DNA as well as in immune suppression of parasitized caterpillar is one of the very interesting aspects of studying polydnaviruses. Viral gene expression is important for the persistence in the infection of other viruses. For example, expression of EBNA1 in EBV-infected cells is required for maintenance of an episomal copy of the viral genome (Robertson et al., 1996), while the

expression of E6 and E7 helps in the integration of the HPV genome (Kessis et al, 1996). Studying alterations in polypeptide synthesis by polydnavirus infection *in vitro* may help to understand how viral gene products are involved in the persistence of polydnavirus DNA.

A recent study by Asgari et al. (1996) indicates that a *Cotesia rubecula* polydnavirus (CrV) gene transcript is transiently synthesized in the parasitized caterpillars of *Pieris rapae*. Although it is possible that transient viral gene expression observed *in vitro* might reflect the transient expression of the viral genes *in vivo*, it is difficult to compare these results since an altered pattern of polypeptide synthesis in HfPV-infected *Malacosoma disstria* was not observed using SDS-PAGE analysis followed by Coomassie blue staining. Without developing a system to detect low amounts of polypeptide synthesis *in vivo* (for instance, ^{35}S-methionine labeling *in vivo*) it would not be easy to compare the alteration of polypeptide synthesis *in vitro* and *in vivo*.

Transient viral gene expression following polydnavirus infection raises the question of the functional significance of such gene expression in the infection of polydnavirus. If polydnavirus infection of haemocytes results in the reduction of haemocyte populations, which in turn results in immune suppression leading to successful development of parasitic wasps, what more is there for the virus to do in the parasitized caterpillar? Is it possible that much of what we see in polydnavirus-infected caterpillars represents the unsuccessful attempt of the virus to replicate that would require transient gene expression? It might be possible that polydnavirus infection in caterpillars represents the unsuccessful attempts of the virus to replicate in its infected host, in which case immune suppression might be a side effect from the abortive viral infection.

Cytopathologies induced by abortive virus infection are observed for herpes simplex type 2, Human cytomegalovirus (HCMV), polyomavirus and simian virus 40 (Oliveira et al., 1993; Maciejewski et al., 1993; Chen and Fluck, 1993; Khandjian, 1992).

3. HfPV DNA persistence

The role of viral genome persistence in the process of parasitism is one aspect of the polydnavirus life cycle that might be studied using an *in vitro* system, and this was the rationale for the work discussed in this section. Polydnavirus infection *in vitro* results in cytopathology similar to that observed in haemocytes of parasitized caterpillars, including alteration of the cell morphology and behaviour (Stoltz and Guzo, 1986; Strand, 1995), apoptosis (Strand, 1995), and transient inhibition of host growth (Beckage, 1993). In addition, cytopathology and alteration of polypeptide synthesis accompanied long-term persistence of some viral genome sequences in the virus-infected cells.

Analysis of the recovered cell lines for the persistence of viral genome sequences suggested that sequences from some viral genome segments persist as an integrated form. Therefore, Ld 652Y cells were used to assess the biological importance of HfPV DNA persistence in polydnavirus infection. Furthermore, the integrative ability of one of the genome segments was also studied using transfection experiments.

A. Integration of HfPV DNA

Integration of HfPV DNA might be understood by examining results from other viral systems. Several other double-stranded DNA (dsDNA) viruses such as papillomavirus, SV40 and Epstein-Barr Virus, when studied *in vitro*, show persistence of viral DNA in the host cells in both episomal and integrated forms (Hagen et al., 1995; Hunter and Gurney, 1994; Hurley, 1991). Also, some DNA viruses that are known to integrate their genome into chromosomal DNA *in vitro* such as HPV (Barbosa, 1996) and EBV (Ohshima et al., 1997) are also found as integrated forms *in vivo*.

When bovine papillomavirus (BPV) is grown in a non-permissive cell line (a cell line that does not support viral replication) the viral genome often integrates as a large concatemer (Allshire and Bostock, 1986). In some infections with BPV, both monomeric extra-chromosomal replication and chromosomal integration of the viral genome have been observed in the same host cell line (Allshire and Bostock, 1986; Hagen et al., 1995).

SV40, another dsDNA virus, also integrates its genomic DNA into the chromosomal DNA of a non-permissive host cell line, and the integration of the viral DNA causes host genome instability (Bender and Brockman, 1981; Gurney and Gurney, 1989; Mogneau et al., 1980). Both duplication and deletion of SV40 DNA and flanking chromosomal DNA have been reported. Once integrated into the chromosomal DNA of the host, the SV40 genome also can be excised from the chromosome by homologous recombination (Dora et al., 1989).

As another example, infection *in vitro* with Epstein-Barr Virus (EBV),

another dsDNA virus also results in persistence of the viral genome in an integrated form as well as an episomal form (Hurley et al., 1991). Although it was once believed that EBV-transformed cells only contain a low copy number of episomal viral genomes, it has been reported that some EBV-transformed cells often contain an integrated viral genome (Hurley et al., 1991; Robertson et al., 1996; Wuu et al., 1997).

These studies of viral DNA integration indicate that i) viral DNA may integrate at more than a single specific site in the host chromosomal DNA (Gurney and Gurney, 1989; Passananti et al., 1987; Ravnan et al., 1992; Lestou et al., 1993), ii) the form of the integrated viral DNA may vary (although the mechanisms responsible for the integration of different forms of viral DNA are not understood), and iii) integration of the viral genome often occurs when the virus is grown in a non-permissive cell line. Viral infection of non-permissive cells does not result in the replication of the viral particles, as seen in the study of HSV (Lillycrop et al., 1994). However, it is shown in the infection of HSV that viral infection of non-permissive cells does occur *in vivo,* and sometimes results in the persistence of the viral genome in the host (Lillycrop et al., 1994).

Integration of the viral genome is known to accompany cytopathology for the infection of SV 40 (Orend et al., 1994), HPV (Gallego et al., 1997) and EBV (Ohshima et al., 1996). Other DNA viruses such as HPV (Barbosa, 1996) and EBV (Ohshima et al., 1997) that are known to integrate their genome into chromosomal DNA *in vitro,* are also found as integrated forms *in vivo*, and the association of viral genome integration with liver cancer and chronic active EBV infection (CAEBV) indicates that the integration of the viral genome might be involved in the development of pathogenesis *in vivo.*

This raises the question of whether the cytopathology observed from the insect cell lines is the cause or the result of HfPV DNA integration. In SV40 and hepadnavirus infection, it has been reported that cytopathology upon viral infection resulted in the integration of the viral DNA (Orend et al., 1994; Petersen et al., 1997). It is possible that cytopathology upon HfPV infection induces DNA repair mechanisms, which in turn results in the illegitimate recombination of HfPV DNA. In hepadnavirus infection, DNA damage sites can serve as sites for hepadnaviral DNA integration, suggesting that the DNA repair mechanism might be involved in the integration of hepadnaviral DNA (Petersen et al., 1997; Butel et al., 1996). Increased frequency of hepadnavirus DNA integration due to virus-induced cytopathology has been reported (Petersen et al., 1997). Furthermore, expression of E6 and E7 of the HPV genome helps the integration of the viral genome (Kessis et al, 1996).

It appears that HfPV genome integration into the chromosomal DNA of Ld 652Y cells may also share some of these characteristics, although more evidence is necessary to confirm the details. When other families of dsDNA viruses infect a cell line that does not support viral replication, infection usually results in the integration of the viral DNA into the chromosomal DNA of the host cells. As we know, even the permissive host caterpillars in polydnavirus infection do not support the replication of the virus, and would be regarded as a non-permissive host in the studies of other DNA viruses. If polydnavirus infection of permissive host caterpillars is similar in its mechanism to the infection of non-permissive cells by other viruses, HfPV DNA can persist in a cell in episomal or integrated forms or both. Furthermore, the persistence of HfPV DNA sequences in Ld 652Y cells may result in the integration of some HfPV genome segment sequences into the host chromosomal DNA.

How could viral DNA integrate into the chromosomal DNA of the host? Viral DNA can integrate by homologous recombination or by illegitimate (non-homologous) recombination. It is possible that HfPV DNA integrates into the host chromosomal DNA by illegitimate recombination carried out by one of the topoisomerases (Bodley et al., 1993; Aratani et al., 1996; Zhu and Schiestl, 1996). Topoisomerases type I and type II are both known to promote non-homologous recombination *in vitro* and *in vivo* (Zhu and Schiestl, 1996; Hiasa and Marians, 1996; Zechiedrich et al., 1989). Once the HfPV genome has been sequenced, the viral sequences could be examined for the presence of the consensus sequences of both type I (Thomsen et al., 1989; Zhu and Schiestl, 1996) and type II (Sander and Hsieh, 1985) topoisomerases. To determine which mechanism HfPV uses to integrate its DNA, one will need sequences from regions flanking the integration sites. The sequences of the flanking region will reveal the presence of any homologous region between viral and host DNA or of type I or II topoisomerase consensus sequences.

Is the integration of HfPV DNA mediated by host mechanisms or virus-encoded mechanisms? If a host mechanism such as topoisomerase is involved in the integration of HfPV DNA, sequencing of the polydnavirus genome might reveal that the virus genome does not encode any specific genes involved in integration. On the other hand, if the viral DNA is integrated by a virus-specific mechanism as in transposons and retroviruses, it is likely that the viral genome will encode a protein that is involved in the integration of the viral DNA, as is the case for transposons and retroviruses (Craig, 1995). At this point, there is not enough information to determine which mechanism HfPV uses for its genome integration.

Because studying persistence of HfPV DNA in an insect cell line can provide many advantages (as seen in the *in vitro* studies of other dsDNA viruses such as EBV, HPV and SV40), Ld 652Y cells were infected with HfPV, and persistence of HfPV DNA was investigated. Understanding the features of HfPV genome segments I and υ that are neccessary for their persistence in Ld 652Y cells might provide insight into how other viral genome segments persist in the parasitized host. Also, comparison between the genome segments that do not persist (such as genome segments M and U) and any common element found in genome segments that persist might reveal any important component in the integration of HfPV genome segments. However, it is critical to develop a more sensitive and simple method such as inverse PCR to study the integration of HfPV DNA. Inverse PCR could provide a more efficient way to distinguish an integrated copy from an episomal copy of viral DNA. In contrast to Southern blot analysis, which can not be used to detect low copy number episomal DNA, inverse PCR is sensitive enough to detect low copy number episomal DNA.

B. Integration of genome segment I

Previous studies by Deming Xu (Xu, 1991) showed that genome segment I, from which pMK300 insert sequences were derived, exhibits polymorphism for the *EcoR* I site (genome segment I with the *EcoR* I site and genome segment I without it). Most of the genome segment I sequences appear to be present in the insert of the clone pMK300, which was isolated from chromosomal DNA of a recovered cell line; however, the exact source of the insert sequences is not clear since virus stocks in the lab did not seem to contain genome segment I with the *EcoR* I site. The insert of pMK300 could

have been derived from either the virus or the integrated genome segment. As a result, it is not possible to conclude at this point that the insert of pMK300 is the genome segment I (with the *EcoR* I site).

There are two possibilities to explain how the insert of pMK300 could have been found in the recovered cell lines. The simplest explanation is that the HfPV used for the infection study contained genome segment I with an *EcoR* I site. Although some experiments using viral genomic DNA did not provide evidence for the presence of the *EcoR* I site on genome segment I in the lab viral sample, it could have been present in the viral samples in low amounts that could not be detected by Southern blot analysis. Alternatively, the *EcoR* I-site-containing genome segment I could have been generated from the excision or replication of an integrated copy of the genome segment that introduced two base pair changes necessary to get the *EcoR* I site in genome segment I (Figure 29). At present, it is not clear which of these two mechanisms produced the insert of pMK300.

When the 2.1 Kb internal *Hind* III fragments from both pMK300 and pMK500 were analyzed using 4 bp recognition restriction enzymes, identical results were produced (Figure, 30). Since comparison of pMK300 and pMK500 by sequencing and by restriction mapping indicated that they are identical except for the *EcoR* I restriction enzyme site, they were determined to be genome segment I. Thus, the clones were renamed to genome segment I *EcoR* I/ + (pMK300) and genome segment I *EcoR* I /- (pMK500). This is to minimize the confusion of nomenclature, although the possibility still exists that the insert of pMK300 was generated from integrated genome segment I. Cloning and sequencing the flanking chromosomal region of an integration site

is essential to understand how genome segment I integrates.

Since it is possible that genome segment I integrates by illegitimate recombination, available sequences of the genome segment were searched for the possible presence of the consensus sequences of both type I (Thomsen et al., 1989; Zhu and Schiestl, 1996) and type II (Sander and Hsieh, 1985) topoisomerases. The consensus sequences of the topoisomerases are required for the DNA and enzyme to form a complex for catalysis of DNA breakage and rejoining events (Jaxel et al., 1991; Svejstrup et al., 1990; Christiansen et al., 1993: Fisher et al., 1986; Peng and Marians, 1995).

One hot spot sequence [(G/C)(A/T)T] for type I topoisomerase was identified in the genome segment I sequence, indicating type I topoisomerase might be involved in integration. However, a genome segment I integration site is required to determine whether the genome segment did integrate by illegitimate non-homologous recombination or not. If the recombination occurred through illegitimate recombination, the sequences of the integration site will reveal topoisomerase binding sites in both chromosomal DNA and viral DNA, as well as the absence of homologous sequences between the viral and chromosomal DNA sequences.

Two obstacles were encountered in attempts to clone the regions flanking putative viral integration sites. Multiple bands observed in the Southern blot analysis using genome segment I as a probe indicated heterogeneous integration sites in the chromosomal DNA of recovered cells (Figure 37). In order to reduce the complexity of heterogeneous integration sites, attempts were made to grow a clone from a single recovered cell, to remove the possiblity of varying integration sites from one cell to another aithough each recovered cell line is from a single infection, However, it is also possible that the multiple integration sites are present in a single recovered

cell. Because the attempts to clone an individual recovered cell failed, it is not clear whether the recovered cells contain multiple integration sites.

The second obstacle was to determine which region of the 1.4 Kb *Hind* III fragment should be used for designing inverse PCR primers. It is important to use different sets of primers that anneal at different regions of the viral sequences, because the Southern blot analysis to estimate the size of the restriction fragments containing integrated genome segment I shows many different sizes of bands, including high-molecular-weight bands that might be difficult to amplify by PCR.

Because screening a size-selected genomic library of the transformed cells produced a fragment of DNA almost identical to the genome segment I, and another attempt to isolate a clone with the integration site did not produce any clone, an attempt was made to clone the genome segment integration site using inverse PCR. Inverse PCR allows one to discriminate against the amplification of any episomal viral genome segment by designing primers specific for a selected region. Although attempts to clone the integration sites failed with the primers that were used, it may be possible to find a set of primers that will amplify less broad-sized bands from inverse PCR.

C. Integration of other genome segments

Another HfPV genome segment, υ, was also found to integrate into the chromosomal DNA of Ld 652Y cells. Genome segment υ probably belongs to a family of genome segments (Figure 33). When a Southern blot hybridization was carried out using genome segment υ as a probe, not only the 4.9 Kb genome segment υ, but also another genome segment, which is 3.9 Kb in size, hybridized to the probe (Figure 33). Thus, finding the 3.9 Kb genome segment

would be the next step to understand which of the two genome segments is integrated into the chromosomal DNA of Ld 652Y cells, and how similar the two genome segments are.

Also, the integration of the genome segment υ could be studied using a strategy similar to that used in studying genome segment I. However, studying the integration site of genome segment υ would be more complicated, since there is another genome segment that cross-hybridizes with genome segment υ (Figure 33). It is not yet clear which of the two genome segments integrates into the chromosomal DNA of Ld 652Y cells.

When a Southern blot probed with total viral genomic DNA was compared to the blots probed with genome segments I and υ, it was clear that these are not the only HfPV genome segments in the recovered cell lines (Figures 32 and 33). The Southern blot results that combine the hybridization signal of the two genome segments do not fully explain the results of the Southern blot using total HfPV genomic DNA as a probe (Figure 32 and 33). Thus, it is concluded that genome segments I and υ are not the only HfPV genome segments integrated into the chromosomal DNA of Ld 652Y cells. Southern blots with *Hind* III-digested chromosomal DNA of recovered cell line 2 probed with total viral DNA illustrated that there were two distinctive bands, 1.6 and 1.0 Kb in size, (Figure 33, A, lane R2/H and lane R4/H) that were not due to hybridization with genome segment I (Figure 32, B), nor genome segment υ (Figure 33), indicating other HfPV genome segments were also integrated into the chromosomal DNA of Ld 652Y cells. However, not all HfPV genome segments are integrated into the chromosomal DNA of Ld 652Y cells. For example, genome segments M and U were not found in an integrated form. This suggests that these genome segments do not carry components that are

involved in the integration of HfPV genome segments.

D. Integration of viral DNA and cytopathology

What is responsible for the selective integration of certain HfPV genome segments? Identifying the remaining genome segments that are integrated into the chromosomal DNA of Ld 652Y cells, and analysing the sequences of the genome segments for common elements, might give some clue as to what component is important in the persistence of the HfPV genome segments. Also, comparing the sequences of integrated genome segments to the sequences of non-integrating genome segments may give an answer to the question of what is responsible for the selective integration of certain genome segments.

Some of the available sequences from genome segment I were analyzed by a computer program, DNA Strider 1.2 (Marck, 1992), in order to identify any open reading frames present in the sequences. Several open reading frames (ORFs) were found, with lengths varying between 75 and 294 nucleotides, representing potential 2.7-kDa to 11-kDa polypeptides. The amino acid sequences derived from the DNA sequences were also compared to the Genbank protein database (http://WWW.ncbi.nlm.nih.gov/BLAST/). Two small stretches of amino acid sequences (13 and 24 amino acids) show 40 to 50 % similarity to hirudin. Hirudin is a specific inhibitor of thrombin, a blood clotting factor, isolated from the leech *Hirudo medicinalis* (Markwardt, 1970). Hirudin is known to inactivate thrombin, a serine protease, by binding into its active site (Malley, 1996). The stretch of 24 amino acid residues matches 5 out of 6 cysteine residues that are important for formation of disulfide bonds present in the N-terminal region of hirudin (Chang et al., 1995). The other stretch of 13 amino acid residues shows similarity to the C-terminal end of hirudin, involved

in the inhibition of thrombin-induced chemotaxis (Rowand, et al., 1992). However, when the DNA sequences were compared to nucleotide sequences in the Genbank databases, no significant matches were found. If genome segment I does encode a hirudin-like molecule, it might suggest that the small stretch of amino acids (13 amino acids) might be involved in the function of the polypeptide.

Nevertheless, because genome segments of polydnaviruses are known to contain introns (Dib-Hajj et al., 1993; Cui and Webb, 1996), it is difficult to interpret the results of genome segment I sequence analysis. Thus, the sequences were analysed by another computer program that predicts exon sequences from given sequences, the BCM gene finder (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html). The 13 amino acid residues were found in a predicted exon sequence while the 24 amino acid residues were not located in any of the predicted exons. Although the importance of this sequence analysis for explaining the persistence of the genome segment is not clear, it is possible that a hirudin-like molecule might play a role in the persistence of genome segment I.

Possibly, HfPV DNA insertion in the chromosomal DNA of Ld 652Y cells could have resulted in the cytopathology. Although the integration of genome segment I by transfection did not induce any cytopathology, integration of other genome segments (perhaps in combination with genome segment I) might induce some of the cytopathologies observed in the infection with the virus. Instability of the host chromosomal DNA has been observed from integration of viral DNA in several cases. For instance, integration of HPV-18 DNA induced a complex genomic rearrangement in a cervical carcinoma cell line (Gallego et al., 1997). Also, integration of the EBV genome produced a fragile site in a chromosomal region prone to breakage events (Wolf et al., 1995). Also, SV40

DNA integration resulted in spontaneous rearrangement of chromosomal DNA (Bender and Brockman, 1981; Gurney and Gurney, 1989; Mogneau et al., 1980). Thus, studies of other dsDNA viruses suggest that the cytopathology observed in HfPV infected Ld 652Y cells might be either the cause or the result of viral DNA integration.

Future studies

There are a number of questions still to be addressed in the future. What is a polydnavirus? Is it a part of the wasp genome being used to reproduce wasps, or a virus that uses two independent hosts for its life cycle? The life cycle of another dsDNA virus, HSV, is divided into the replication of the virus in permissive epithelial cells and the persistence of the viral genome in non-permissive sensory neurons (Lillycrop et al., 1994). The life cycle of polydnaviruses might be similar to that of HSV, except that polydnaviruses use two hosts to complete their life cycle. A number of parasites use more than a single host to complete their life cycle. Some of the examples are as follows: The swimbladder parasite *Anguillicola crassus* (Nematoda: Dracunculoidea) uses a crustacean intermediate host as well as a fish host (the eel) where they suck blood from the swimbladder wall and lay eggs (Szekely, 1996). The eggs and second larvae (newly hatched larvae) in the fish then pass through into the digestive tract of the eel and are expelled with the feces and, once excreted into environment are consumed by small freshwater crustaceans. The freshwater crustaceans are, in turn, eaten by eels and the last larval stage of the parasite bores through the intestinal wall of the eel into the swimbladder (Peters and Hartmann, 1986). *Trypanosoma cruzi* has life cycle stages in mammal as an

intracellular form where it replicates, and in insect develops as an extracellular form that disseminates and invades a new host (Kahn et al., 1995). There are many examples of parasites having more than a single host for their life cycle; possibly, polydnaviruses are viruses that require two hosts to complete their life cycle.

What could the relationship be between virus-induced cytopathology and alteration of polypeptide synthesis? Is the virus-induced cytopathology, including apoptosis, the cause or the result of the viral DNA integration? Where do genome segments I and υ integrate into the chromosomal DNA of Ld 652Y cells? Although some HfPV genome segments appear to integrate into a number of different sites in chromosomal DNA, virus-infected cells proliferate at the same rate as the uninfected control cells after they recover from initial cytopathology.

Would the integrated genome segments in Ld 652Y cells behave like the provirus in the chromosomal DNA of the wasps? How are the viral genome segments integrated into multiple sites of host chromosomal DNA? Is it a single event that produces the integration of the viral genome segment in multiple sites, or do the integrated genome segments excise out and then reintegrate?

Although it is too early to conclude how polydnavirus DNA integrates, the studies of other dsDNA viruses suggest that it probably occurs by an illegitimate recombination event in the host cell. HfPV contains multiple genome segments, only some of which are shown to integrate into the chromosomal DNA of host cells; as a result, studying the mechanism of polydnavirus DNA integration will tell us which component is important in the integration of viral DNA or foreign DNA. *Agrobacterium tumefaciens* can transfer its T-DNA not only to its plant host but also to *Saccharomyces cerevisiae*, resulting in the integration of T-

DNA in the host chromosomal DNA (Risseeuw et al., 1996). This suggests that the integration of foreign DNA into the chromosomal DNA of a host can occur in many different situations.

Conclusion

Infection of an insect cell line, Ld 652Y, by a polydnavirus, HfPV, results in cytopathology and persistence of the viral DNA. Some of the cytopathology observed *in vitro* reflects the cytopathology of *in vivo* infection, suggesting that the system described in this study is a valuable model for studying polydnavirus infection *in vitro*. In addition, the persistence of the viral DNA in the cells indicates that the cell line can also be used to study one of the most interesting questions in biology, how a foreign DNA can be maintained in a host cell.

V. References

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IMAGE EVALUATION
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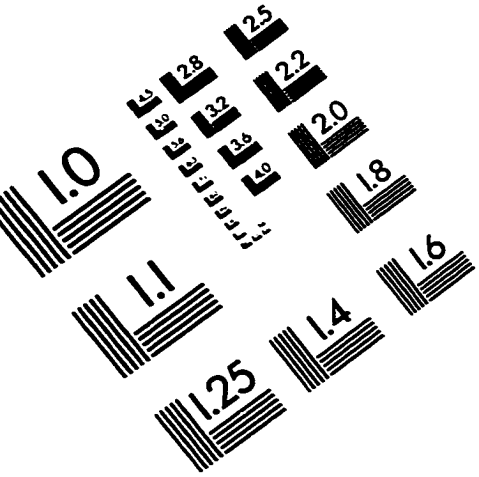

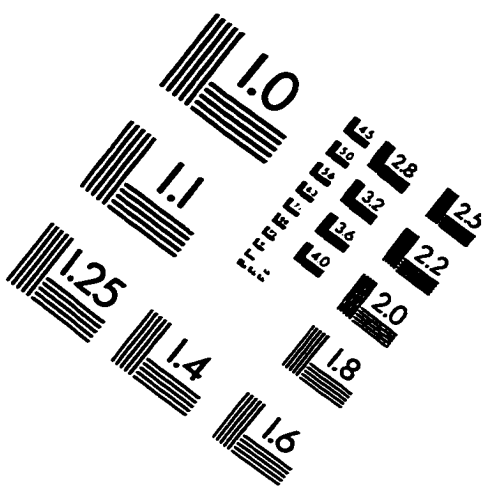

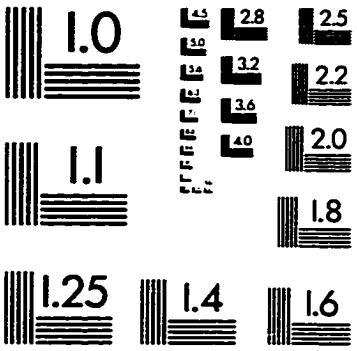

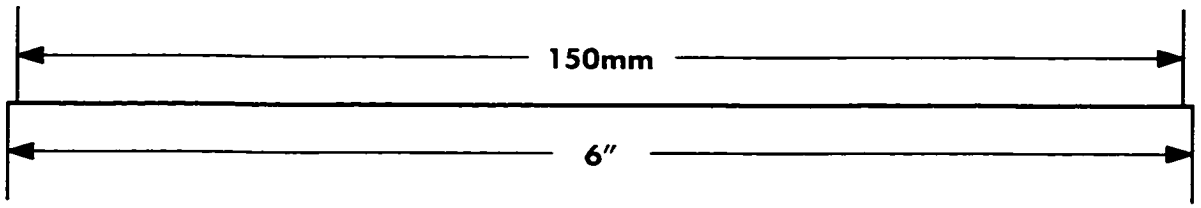

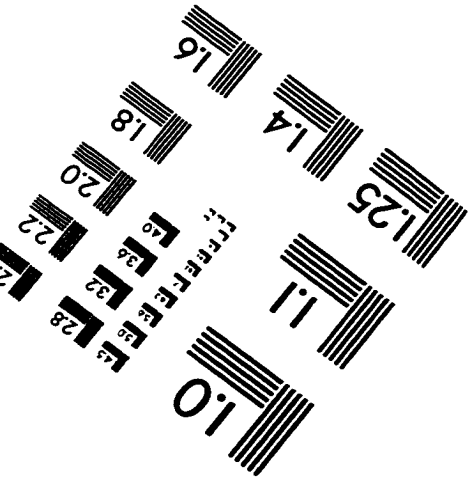

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