Cotesia rubecula Polydnavirus-specific Gene Expression in the Host *Pieris rapae*

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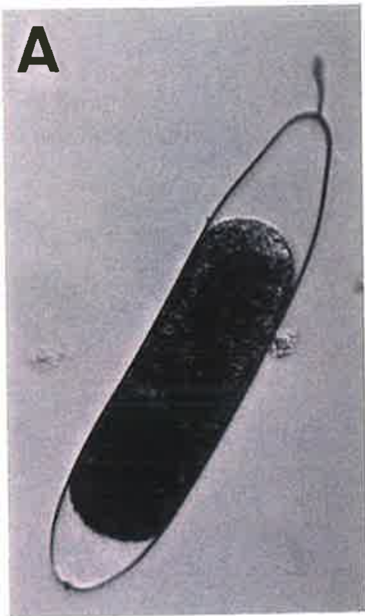
To My Parents

Figure

Life stages of *Cotesia rubecula* (Hym: Braconidae). **A)** Egg; **B)** first instar larva; **C)** pupa inside the coccon; and **D)** female adult wasp.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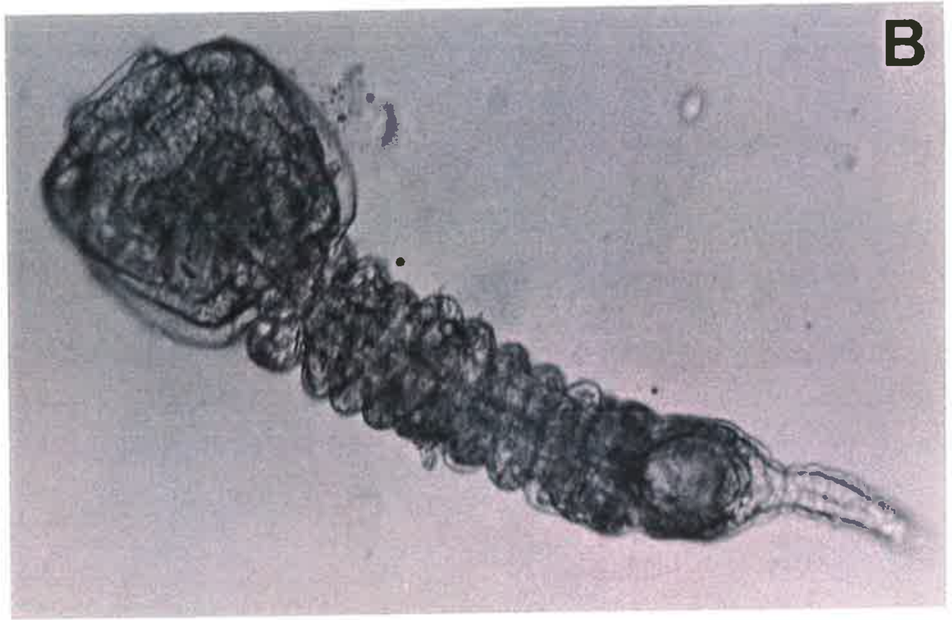
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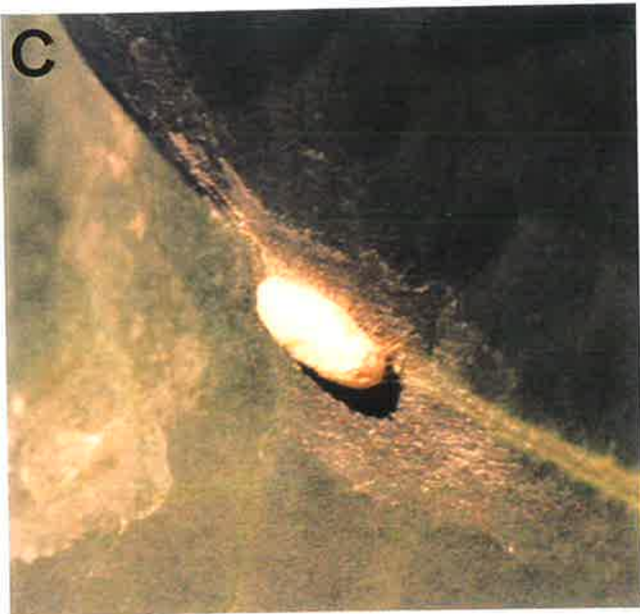
C

D

Table of contents

Summary vi

Statement viii

Acknowledgements ix

Abbreviations x

Chapter 1 Literature review 1

1.1. Introduction 1

1.2. Insect immune reactions 2

1.2.1. Humoral or cell-free immunity 2

1.2.2. Cellular immunity 4

1.2.2.1. Phagocytosis 5

1.2.2.2. Encapsulation 5

1.2.2.3. Nodule formation 7

1.3. Abolition of the host immune system by parasitoids 7

1.3.1. Calyx fluid proteins 8

1.3.1.1. Virus-like protein particles (VLPs) 8

1.3.1.2. Protein secretions 10

1.3.2. Polydnaviruses (PVs) 10

1.3.2.1. Structure and morphology 11

1.3.2.2. Genomic organisation 12

1.3.2.3. Distribution 13

1.3.2.4. Parasitoid-polydnavirus interaction 14

1.3.2.5. Host-polydnavirus interaction 15

1.3.2.5.1. Polydnavirus expression in the host 15

1.3.2.5.2. Developmental and physiological alterations 18

1.3.2.6. Synergistic factors in PV efficacy 21

1.3.3. Other associated virus-like particles 23

1.4. Concluding remarks 24

Chapter 2 General materials and methods.......26

2.1. Introduction.......26

2.2. Insect cultures and parasitisation.......26

2.3. Isolation of fat body, hemocytes and hemolymph.......26

2.4. F-Actin staining of hemocytes with Phalloidin.......26

2.5. Lectin binding to hemocytes in vitro.......27

2.6. Isolation of polydnaviruses.......27

2.7. Extraction of DNA from polydnaviruses.......28

2.8. Extraction of DNA from wasps.......28

2.9. Extraction of total RNA.......28

2.10. Isolation of mRNA from total RNA.......29

2.11. Radiolabelling of DNA.......30

2.12. Southern blot analysis.......30

2.13. Northern blot analysis.......31

2.14. Cloning DNA fragments.......32

2.15. Cloning PCR amplified DNA fragments.......35

2.16. Spot test (concentration check).......37

2.17. Extraction of DNA from agarose gel.......37

2.18. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).......37

2.19. Staining and destaining of polyacrylamide gel.......37

2.20. Western blotting.......38

2.21. Buffers and solutions.......39

Chapter 3 Host hemocyte inactivation and expression of *C. rubecula* polydnavirus genes.......44

3.1. Introduction.......44

3.2. Materials and Methods.......45

3.3. Results.......46

3.3.1. Host hemocyte alterations following parasitisation.......46

3.3.2. Temporal expression of CrV genes in the host.......48

3.4. Discussion 49

Chapter 4 **Isolation and molecular characterisation of a *C. rubecula* PV gene expressed in the host** 52

4.1. Introduction 52

4.2. Materials and Methods 53

4.2.1. Construction of a cDNA library 53

4.2.2. PCR amplification of cDNA fragments 59

4.2.3. Cloning of PCR amplified cDNA fragments and sequencing 60

4.2.4. Sequence analysis 60

4.2.5. *In situ* hybridisation of tissue sections 60

4.2.6. Northern and Southern blotting 62

4.3. Results and discussion 63

4.3.1. Construction and screening of a cDNA library 63

4.3.2. Expression of CrV1 in host tissues 64

4.3.3. CrV1 is integrated in the wasp genome 64

4.3.4. DNA sequence and putative protein sequence 65

Chapter 5 **Expression of a *C. rubecula* polydnavirus gene in baculovirus expression systems and its functional analysis** 67

5.1. Introduction 67

5.2. Materials and Methods 68

5.2.1. *In vitro* transcription/translation of CrV1 68

5.2.2. Expression of CrV1 in a baculovirus system 69

5.2.3. Affinity chromatography 77

5.2.4. Protein purification and immunisation 79

5.2.5. Dot blotting 79

5.2.6. Plasmid transformation of Sf21 cells 79

5.2.7. Injection of the recombinant protein and cytochalasin D 82

5.2.8. Indirect immunofluorescence 82

5.2.9. General methods 83

5.3. Results 83
5.3.1. Hemocyte immune activation 83
5.3.2. Recombinant CrV1 84
5.3.3. CrV1-mediated hemocyte inactivation 85
5.3.4. Hemolymph-mediated alteration of CrV1 86
5.4. Discussion 87
Chapter 6 Inhibition of phagocytosis by a polydnavirus encoded protein with an M-like protein homologous region 90
6.1. Introduction 90
6.2. Materials and Methods 91
6.2.1. Labelling of the yeast cells 91
6.2.2. Phagocytosis assay 91
6.2.3. Recombinant 45kDa/CrV1 protein 92
6.2.4. Electrophoresis techniques 92
6.3. Results 92
6.3.1. Inhibition of phagocytic activity 92
6.3.2. 45kDa/CrV1 protein with a homologous region to M-like proteins 93
6.4. Discussion 93
Chapter 7 Molecular cloning and characterisation of a protein involved in passive protection of *C. rubecula* eggs in the host 95
7.1. Introduction 95
7.2. Materials and Methods 96
7.2.1. Construction of a *C. rubecula* cDNA expression library 96
7.2.2. Screening cDNA library 96
7.2.3. Phage lysate preparation 97
7.2.4. Isolation of phage DNA 97
7.2.5. Preparation of fusion protein 97
7.2.6. Elution of antibodies from nitrocellulose blots 98
7.2.7. Cloning and sequencing 98

7.2.8. Peptide microsequencing....99

7.2.9. Elution of proteins from eggs....100

7.2.10. General methods....100

7.3. Results and discussion....101

7.3.1. Calyx fluid proteins on the virus and egg surface....101

7.3.2. Molecular cloning of the 32 kDa coding cDNA....101

7.3.3. Deduced protein sequence....102

Chapter 8 General discussion....104

References....109

Summary

Polydnaviruses of hymenopteran endoparasitoids are evolutionary adaptations enabling the developing parasitoid to escape the cellular defence reactions of its habitual host caterpillar. The viruses, which have a segmented, circular and double-stranded DNA genome, are produced in the calyx region of the female reproductive organ and injected into the host upon oviposition. Inside the host, polydnaviruses enter cells of various host tissues where their genomes are transcribed to produce a number of gene products. The result of parasitisation by many hymenopteran parasitoids are cellular alterations of host hemocytes, that correlate with a failure to aggregate or spread on a foreign surface. Although experimental evidence suggests that polydnaviruses are causing the immune suppression in parasitised caterpillars, the exact mechanism of virus-mediated inactivation of host immune defences is difficult to investigate due to the paucity of molecular knowledge of insect cellular immunity. Moreover, some endoparasitoid species use a complex combination of different wasp components to circumvent the host defence reactions.

In the braconid *Cotesia rubecula* the newly deposited egg is protected against the defence reactions of the host *Pieris rapae* through a layer of calyx fluid proteins that provide a passive protection of the developing wasp during embryogenesis. This layer consists of two major proteins of 65 and 32 kDa in size. DNA coding for the latter was isolated by screening an expression cDNA library using an anti-virus antiserum. However, in the absence of polydnavirus (CrV), the developing parasitoid is presumably only protected until the larva emerges from the chorion, whereas in the presence of CrVs, the newly hatched larva is protected by a virus-mediated inactivation of host hemocytes. The CrVs infect host hemocytes and fat body cells, which subsequently express the virus genome during a short time period between six and twelve hours after parasitisation. This causes cellular changes in hemocytes including breakdown of actin cytoskeleton, alterations in surface molecules and microparticle formation and lack of spreading upon contact to a foreign surface.

A single polydnavirus-encoded glycoprotein (CrV1) is expressed in infected *Pieris* hemocytes and the corresponding coding DNA was isolated by screening a cDNA library. The protein is produced by infected hemocytes and fat body cells and secreted into the hemolymph where it presumably interacts with the surface of hemocytes. The result of this interaction is a breakdown of actin-filaments causing a transient inhibition of immune-related hemocyte-specific surface changes, involving the exposure of a lectin-binding protein and microparticle formation. Inhibition of hemocyte activation precludes cellular encapsulation reactions during a critical time when the parasitoid larva emerges from the chorion. In order to show that CrV1 is required and sufficient for this immune suppression, baculovirus and plasmid expression systems were employed to express the recombinant CrV1. The protein was partially purified using a lectin column and used to produce a specific antiserum. Injection of the recombinant protein resulted in cellular effects on host hemocytes that are indistinguishable from that of naturally parasitised caterpillars.

Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Sassan Asgari

March 1997

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Abbreviations

AcMNPV	*Autographa californica* multiple nuclear polyhedrosis virus
Amp	Ampicilline
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	Base pair(s)
BSA	Bovin serum albumin
CcV	*Chelonus* near *curvimaculatus*
CmV2	*Cotesia melanoscela* virus 2
CsV	*Compoletis sonorensis* virus
dATP	Deoxyadenine triphophate
dCTP	Deoxycytosine triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanine triphosphate
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
DOPA	Dihydroxy phenylalanine
dTTP	Deoxythyamine triphosphate
EDTA	Ethylendiamine tetra acetic acid
EMBL	European molecular biology laboratory
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GalNAc	N-acetylgalactoseamine
GIM	Grace's insect medium
h	Hour(s)
*H.p.*lectin	*Helix pomatia* lectin
HeV	*Hyposoter exigue* virus
HfV	*Hyposoter fugitivus* virus
HPLC	High performance liquid chromatography
IgG	Immunoglubine G

IPTG	Isopropyl β-D-thiogalactopyranoside
kb	Kilobase pair(s), 1000 base pair
kDa	KiloDalton(s)
LB	Luria-Bertani
lit	Liter(s)
M	Molar
mA	Miliamper(s)
mCi	Mili Curie
MdPV	*Microplitis demolitor* PV
mg	Miligram(s)
min	Minute(s)
ml	Mililiter(s)
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	Messenger RNA
N	Normal
NBT	Nitro blue tetrazolium chloride
ng	Nonogram(s)
nm	Nanometre(s)
ObV	*Olesicampe benefactor* virus
p.i.	Post infection
p.p.	Post parasitisation
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	plaque forming unit
PS	Phosphatidyle serine
PTTH	Prothoracicotropic hormone
PVs	Polydnaviruses

r.p.m	Round per minute
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
sec	Second(s)
Sf21	*Spodoptera frugiperda* cell line 21
SH DNA	Superhelical DNA
TAE	Tris-acetate/EDTA
TBE	Tris-borate/EDTA
TEMED	N, N, N´, N´, Tetra methyl ethylene diamide
u	Unit (enzyme)
UV	Ultraviolet
V	Volt(s)
v	Volume(s)
VLFPs	Virus-like filamentous particles
VLPs	Virus-like particles
X-Gal	5-Bromo-4-chloro-3-indolyl- β-D-galactopyranoside
μg	Microgram(s)
μl	Microliter(s)
μM	Micromolar

CHAPTER 1

Literature review

Insect immune mechanisms and its evasion by insect parasitoids

Chapter 1

Literature review

Insect immune mechanisms and its evasion by insect parasitoids

1.1. Introduction

Insects are the largest group of organisms in the animal kingdom with a great capacity to adapt to a variety of conditions and occupy almost every ecological niche. One major reason for this biological prosperity may be their considerable competence to defend and respond to the invasion of other organisms. Insect defences involve a number of behavioural and physiological adaptations that reduce the chance of the organism becoming a host (Vinson, 1990a). In other words, insect defences might be defined as the external and internal adaptations dealing with a number of structural, behavioural and physiological reactions that increase their chance of surviving invasion and attack by other organisms.

The external defence systems of insects are basically concerned with the avoidance of attack, and include: 1) the exoskeleton as a structural barrier, preventing the penetration of invading organisms. In addition, the peritrophic membrane and the cuticle layer of the alimentary canal (intima) prevent the penetration of microorganisms into the hemocoel; 2) behavioural adaptations, including camouflage, mimicry, passive defence (play dead or motionlessness), escape, locating shelter and fighting; and 3) chemical defences, such as the release of deterrents, distasteful body fluids or the injection of poisons.

The internal defence system of insects may be regarded as the immune system. It is well known that the insect immune system is different from that of vertebrates in that there are no adaptive response interactions involving immunoglobulins or T-cell receptors in insects. However, like vertebrates, the insect immunological system involves cellular and humoral defence reactions. Cellular immune reactions are mediated by hemocytes, and consist of phagocytosis, encapsulation and nodule formation. Microorganisms (such as bacteria-like endocytobionts) are cleared from the hemolymph by phagocytosis, while metazoan endoparasites (such as fungi,

nematodes and the eggs of parasitoids) are encapsulated. Nodules are formed around aggregations of bacteria.

In spite of the highly effective immune responses of insects, parasitic organisms are able to overcome these impediments in different ways. Parasitic interactions impose selective pressure on both sides, either for the host to protect self against the intruding parasite or for the parasite to escape from immunological host reactions. Certain hymenopteran endoparasites avoid the host immune system by coating surfaces to avoid recognition by the host (molecular mimicry) or by suppressing the immune system in association with so-called polydnaviruses replicated in their reproductive organs. In this review, various aspects of insect immune reactions, their avoidance and suppression by invading organisms are discussed.

1.2. Insect immune reactions

Similar to other organisms, insects are subject to the invasion of other living organisms. Apart from external (behavioural and structural) strategies, insects adopt certain internal immune reactions against potential invaders. However, insects, due to their short life span and their small size, have not evolved the complex and adaptive (antibody-based) immune system of higher animals. Therefore, they present a range of responses which share fundamental characteristics with the innate immune response of vertebrates.

The immune defence in insects consists of three closely associated reactions (Hoffmann *et al.*, 1996): 1) wound healing and the induction of proteolytic reactions which leads to coagulation and activation of prophenoloxidase cascade; 2) cellular defence reactions including phagocytosis, nodule formation and encapsulation of invading organisms; 3) induction of a suite of antimicrobial peptides by the fat body and hemocytes. To provide an effective immune response, humoral and cellular components of the hemolymph tightly interact with each other (Boman and Hultmark, 1987; Dunn, 1986; Vinson, 1990a).

1.2.1. Humoral or cell-free immunity

Humoral factors involved in insect immune reactions consist of proteins that are either normally present or are inducible (Berg *et al.*, 1988; Boman and Hultmark, 1987). These are presumably

able to recognise and interact with foreign objects, like lectins, antibacterial factors, complement-like factors, prophenoloxidase activating system and detoxification biochemicals (Gupta, 1991).

Upon microbial infection, an array of antibacterial and antifungal peptides/polypeptides are induced in insects (Boman, 1991) that can be divided into two groups (Hoffmann *et al.*, 1996): 1) The cyclic peptides formed by disulfide bridges such as insect defensins and drosomycin and, 2) linear peptides/polypeptides which lack cysteines. Linear peptides/polypeptides can be further grouped into the cecropins, the proline-rich peptides such as apidaecin and the glycine-rich polypeptides such as the attacins.

The cecropins were the first antibacterial peptides to be described. They were isolated from diapausing pupae of *Cecropia hyalophora* (Lep.) (Boman, 1991) and then from other insects belonging to Lepidoptera and Diptera (Hoffmann *et al.*, 1996; Hultmark *et al.*, 1982; Hultmark *et al.*, 1980). Cecropins are 4 kDa in size and strongly cationic peptides active against gram-positive and gram-negative bacteria presumably by forming voltage-dependant anion channels which leads to disintegration of cytoplasmic membranes (Hetru *et al.*, 1994).

Insect defensins were first isolated from bacteria-infected larvae of *Phormia terranovae* (Hetru *et al.*, 1994). Later on, their presence was reported in other dipterans (Dimarcq *et al.*, 1994), Coleoptera (Moon *et al.*, 1994), Hymenoptera (Casteels-Josson *et al.*, 1994), and Hemiptera (Chernysh *et al.*, 1996). They are active against gram-positive and less active against gram-negative bacteria by disrupting permeability of the plasma membrane, and causing loss of cytoplasmic potassium, partial depolarisation of inner membranes, reductions in cytoplasmic ATP and inhibition of respiration (Hetru *et al.*, 1994). Drosomycin is a 5 kDa antifungal peptide isolated from *Drosophila melanogaster* which also belongs to the class of cyclic antimicrobial peptides (Fehlbaum *et al.*, 1994).

Several small proline-rich peptides have been isolated from insects (Hymenoptera, Diptera and Hemiptera) which are bactericidal against gram-negative bacteria (Hetru *et al.*, 1994) such as apidaecin isolated from the hemolymph of bacterial challenged honey bees (Hetru *et al.*, 1994) and metalnikowins from *Palomena prasina* (Hem.) (Chernysh *et al.*, 1996). Although the mode of action of these peptides is not well-understood, they exhibit an immediate lethal activity to a

broad range of microorganisms (Hoffmann *et al.*, 1996). Glycine-rich polypeptides are another group of antibacterial peptides which range from 9 to 30 kDa of size such as attacins and sarcotoxins II isolated from *S. peregrina.* Although their bactericidal spectrum seems rather narrow, it is assumed that they also facilitate the action of the cecropins and lysozyme (Boman and Hultmark, 1987).

Although each group of antibacterial proteins may have a specific antimicrobial mode of action, it appears that bacterial infection of insects causes the immune system to produce an array of antibacterial proteins (Dunn, 1986). However, recent investigations indicate that the induction of antifungal and antibacterial peptides are controlled by different mechanisms (Lemaitre *et al.*, 1996).

Hemolin, which was previously called P4, is a soluble protein that is present in low concentration in the hemolymph and belongs to the immunoglobulin superfamily (Sun *et al.*, 1990). However, its concentration may increase 18-fold upon bacterial infection. It was found that hemolin was localised on the surfaces of the fat body and certain hemocytes (Sun *et al.*, 1990). *In vitro* experiments indicated that hemolin binds to bacterial surface components and attaches to hemocytes (Schmidt *et al.*, 1993). Accordingly, it was suggested that hemolin could be a non-self recognition factor and might have opsonic properties. It also blocks the aggregation of hemocytes suggestive that it acts as a negative modulator of hemocyte adhesion (Zhao and Kanost, 1996).

Another main type of factor which is normally present in the hemolymph in an inactive form is prophenoloxidase which is activated by a cascade of proteolytic reactions (Seybold *et al.*, 1975). Upon activation, the enzyme oxidises tyrosine and melanisation occurs. It seems that the activation of the prophenoloxidase system plays an important role in the non-self recognition process (Ratcliffe *et al.*, 1984).

1.2.2. Cellular immunity

Another aspect of the insect immune response involves the participation of hemocytes. The three main cellular reactions towards invading organisms or implanted objects are phagocytosis, encapsulation and nodule formation (Vinson, 1990a). Hemocytes are also indirectly engaged in

immunological responses by participating in the secretion of immunologic factors involved in coagulation, prophenoloxidase activation and poison detoxification (Gupta, 1991).

1.2.2.1. Phagocytosis

Small objects such as bacteria, viruses, protozoa, fungi spores and other minute implanted materials are cleared from insect hemolymph by means of phagocytosis. Its effectiveness depends on the number of phagocytosing immunocytes (phagocytic index), the nature of the microorganisms, the frequency of attack and other factors (Gupta, 1991). Recognition, attachment, endocytosis, ingestion and clearance are recognised as subsequent stages of phagocytosis (Götz and Boman, 1985; Gupta, 1991). The major hemocytes involved in phagocytosis are granulocytes and plasmatocytes, which are also called immunocytes.

1.2.2.2. Encapsulation

Encapsulation is a hemocytic reaction directed against metazoan parasites or abiotic objects when these objects are too large to be phagocytosed by individual immunocytes. It leads to the formation of a multicellular envelope surrounding the foreign object (Götz and Boman, 1985; Gupta, 1991; Whitcomb *et al.*, 1974). As in phagocytosis, granulocytes and plasmatocytes are the two main hemocytes that take part in encapsulation (Ratcliffe *et al.*, 1984). Ultrastructural examinations revealed that typical capsules consist of three different zones (Ratner and Vinson, 1983). The innermost layers are appressed closely to the surface of the foreign object and the corresponding cells show necrotic and autolytic signs. The next region is composed of tightly packed hemocytes which are extremely flattened. Cells of the outer layer were also found to be tightly packed but retained their rounded shape (Ratner and Vinson, 1983). Encapsulation reaction occurs through a series of steps as evidenced in various species. The first stage is the recognition of non-self, which is found to be accomplished by granulocytes (Ratcliffe, 1993). It appears that before cell attachment a deposition of a thin layer on the parasitoid eggs of *Leptoplinia boulardi* occurs, as has been ultrastructurally documented in *D. melanogaster* (Russo *et al.*, 1996). This layer is composed of electron-dense material and cell debris which might be involved in recruitment of other hemocytes. The next phase (layer formation) is primarily mediated by plasmatocytes; these respond to coated surfaces formed during the first phase

(Ratcliffe, 1993). Similarly, three steps in capsule formation were recently described in detail (Pech and Strand, 1996) using monoclonal antibodies against plasmatocytes and granulocytes of *Pseudoplusia includens* (Strand and Johnson, 1996). Accordingly, encapsulation initiates when granulocytes recognise and attach to the foreign target which is followed by attachment of multi-layers of plasmatocytes. Capsule formation is terminated when granulocytes form a monolayer around the capsule. Neither of the aforementioned hemocyte subtypes alone are capable of capsule formation independently. In addition, it has also been shown that the role of granulocytes in this process involves an RDG-dependent cell adhesion mechanism (Pech and Strand, 1996) since the addition of RGDS prevents capsule formation (Pech and Strand, 1995). Integrins, one major class of adhesion molecule receptors, recognise a binding site defined by the sequence Arg-Gly-Asp (RGD) and therefore addition of the soluble peptide can inhibit cell adhesion (Humphries *et al.*, 1993). This implies an interaction between adhesion molecules of the extracellular matrix and granulocytes surface receptors similar to the situation in the mammalian immune system.

Using more susceptible and resistant strains of *D. melanogaster* to the parasitoid, *Leptopilina boulardi*, it has been shown that encapsulation and the production of antibacterial peptides are activated by different mechanisms. However, a correlation in the genetic regulation of cellular and humoral components exists (Lemaitre *et al.*, 1995). Nevertheless, the parasitised larvae which are immune suppressed are quite capable of conducting humoral immune reactions by producing antimicrobial peptides (Nicolas *et al.*, 1996). This might even be an advantage for the developing parasitoid since the host, and therefore the parasite, will be protected against any possible microbial infection.

In species that have low numbers of hemocytes, mainly certain dipterans, it appears that humoral encapsulation, involving the formation of a melanotic layer, plays a major role in host immune defence (Vey, 1993). In *Drosophila simulans*, the capacity of cellular capsule formation which is superior to *D. melanogaster* larvae parasitised by *Asobara tabida* (Hym: Braconidae) was partially attributed to the higher concentration of circulating hemocytes in the former species (Eslin and Prevost, 1996). It seems that a threshold of hemocyte numbers is required to allow cellular capsule formation.

1.2.2.3. Nodule formation

Nodules can be formed around aggregates of bacteria, fungal spores, protozoa or small particles. The hemocytes involved and the mechanism of nodule formation are similar to encapsulation. From the literature, it seems rather likely that both phenomena are similar, since there is no obvious difference between them apart from the size.

1.3. Abolition of the host immune system by parasitoids

Insect parasitoids and their hosts co-exist in a close and complex physiological interaction. In some systems, the nature of host-parasitoid relationships is dominated by the structural features, metabolic, and biochemical milieu of the host whereas in others, it appears to be influenced by regulatory signals from the parasite. A basic requirement for an endoparasitic lifestyle is to avoid the defensive and immune responses of their hosts, namely encapsulation. Therefore, parasitoids have been under selective pressure to overcome these reactions.

Some insects oviposit in host eggs or young larvae. Since the host eggs are not able to respond to the presence of a foreign body they are frequently selected as a target for parasitisation. In young larvae, the smaller volume of blood, the lower density of hemocytes and the smaller proportion of immune-reactive cells may mean that the immune reaction is not strong enough to endanger the parasitoid's survival (Salt, 1968). Other insects oviposit into certain tissues of their host, so that the egg can finish its embryonic development without being in direct contact with the hemolymph (Salt, 1968).

Parasitoids, however, which choose to oviposit in late instar larval stages, are in direct contact with their host hemolymph and, therefore, exposed to the encapsulation reaction. They have adopted mechanisms to suppress the immune reaction of their hosts. Habitual parasites avert encapsulation by actively suppressing the host reactions. In another scenario suggested by Salt (1965, 1966) the parasite avoids recognition by the host as a foreign object (Whitcomb *et al.*, 1974). It was also suggested that a substance or substances might be secreted by the parasitoid eggs into the hemolymph of the host to protect the eggs from the encapsulation reaction (Kitano and Nakatsuji, 1978). The egg surface properties of *Cardiochiles nigriceps* passively protect the

egg as it does not elicit an encapsulation reaction from the host hemocytes (Davies and Vinson, 1986). Cleptoparasites, which exploit primary parasitoids to access the host, probably escape from the host immune system because it has been previously impaired by the primary parasitoid.

Some braconid and ichneumonids employ a mutualistic association with certain unusual viruses called polydnaviruses (PVs). The role of PVs in host-parasitoid interaction has been a major subject of interest in insect immunity. PVs are secreted by epithelial cells of the calyx region of the female reproductive organ (Fig. 1-2) and injected simultaneously with eggs into the host body cavity which cause several physiological and developmental changes including host immune suppression (Edson *et al.*, 1981; Stoltz and Faulkner, 1978; Stoltz and Vinson, 1979b). Also, protein secretions from the calyx region were found to provide passive protection of parasitoid eggs. In *Venturia canescens*, the protein secretions are assembled as particles and cover the eggs passing through the lumen of calyx region (Feddersen *et al.*, 1986). Protein secretions from the calyx region of the wasp, *Cotesia rubecula*, were found on the egg surface that provide passive protection of the parasitoid eggs in the host, *Pieris rapae* (Asgari and Schmidt, 1994). The role of PVs and calyx fluid proteins is the major topic of this review.

1.3.1. Calyx fluid proteins

1.3.1.1. Virus-like protein particles (VLPs)

VLPs described mainly in the ichneumonid wasp, *Venturia canescens*, are electron-dense protein particles which are assembled in the nuclei of the calyx cells (Feddersen *et al.*, 1986). These protein particles are called virus-like particles as they lack any detectable nucleic acid molecules, but they do have certain morphological resemblance to viruses (Feddersen *et al.*, 1986; Stoltz, 1992). The VLP was subsequently referred to as a "defective polydnavirus, the genome of which resides within the parasitoid genome, but fails to become packed into virus particles" (Stoltz, 1992). The particle production starts from the pupal stage and visible particles are subsequently formed inside the nuclei of calyx cells and become coated with an envelope (Schmidt and Feddersen, 1989). They ultimately bud into the lumen of the calyx region of the ovary and, as the eggs pass through the gland, the particles cover their surface. At a molecular level, electrophoresis of the particles revealed that they consist of four main protein components

of 60, 52, 40 and 35 kDa in size (Feddersen *et al.*, 1986). Immunohistochemistry, using antibodies raised against VLPs from *V. canescens,* indicated that the particles are localised only in the calyx region of the reproductive organ (Schmidt and Feddersen, 1989). VLPs were also found in the ovary in *Bathyplectes* spp. (Ichneumonidae) (Hess *et al.*, 1980; Stoltz, 1981), but little work has been performed to elucidate their mode of action in host-parasitoid interaction.

Since DNA molecules are not present in VLPs of *V. canescens*, they might not be able to play an active role in the suppression of the host immune system, by infecting hemocytes or tissues involved in the immune reaction (Schmidt *et al.*, 1990). Masking particle proteins with anti-VLP-antibodies or removal of particles from the egg surface by using denaturing solutions leads to encapsulation of the eggs. Furthermore, Sephadex beads were encapsulated when injected into previously parasitised or VLP-injected larvae (Feddersen *et al.*, 1986). This implies that the immune system of the host is still intact and able to react against other foreign objects, even in the presence of particle proteins. These findings led to the speculation that protein particles might play a passive role in protecting parasitoid eggs. Separate injection of particles simultaneously with the denuded eggs did not prevent encapsulation. Therefore, the presence of particles on the surface of the eggs is necessary to protect eggs from the immune response. It was found that VLPs share antigenic determinants with a host component, which are localised specifically in the fat body of the host larvae (Feddersen *et al.*, 1986). Using an approach based on immunofluorescence, it was shown that components at the basal lamellae surrounding the host's fat body cells which were exposed to the hemolymph were labelled. Correspondingly, the 52 and 60-kDa components of the VLPs shared antigenic determinants with a 42 kDa host protein (Schmidt and Feddersen, 1989). Therefore, VLPs contain host-like protein component(s) and coating the surface of the egg, they may protect parasitoid eggs by avoiding recognition as a foreign object. This kind of passive protection might be evolutionarily advantageous for the parasitoid since the intact immune system of the host provides a microbe-free environment for the endoparasitoid.

1.3.1.2. Protein secretions

In *Cotesia rubecula* (Hym: Braconidae), a habitual parasitoid of the white cabbage butterfly, *Pieris rapae*, it has been shown that the parasitoid eggs are protected from encapsulation reaction of the host by a passive mechanism (Asgari and Schmidt, 1994). When parasitoid eggs were injected into the host without PVs, they were found to be protected. Masking the egg surface by anti-virus antibodies or washing them with detergents led to the encapsulation of the eggs. Parasitoid eggs were found to be covered with a protein coating that cross-reacts with anti-virus antibodies (Asgari and Schmidt, 1994). Ovarian proteins (29-36 kDa) secreted from the calyx region of *Campoletis sonorensis* injected into the host upon parasitisation were found to cause an early immunosuppression of the host immune system by interacting with the host hemocytes and disruption of actin cytoskeleton (Luckhart and Webb, 1996; Webb and Luckhart, 1994). This early protection by non-viral components seems to be crucial for prevention of encapsulation of the parasitoid egg since *Heliothis virescens* larvae are capable of mounting an effective encapsulation response to denuded *C. sonorensis* eggs within 3 hours (Webb and Luckhart, 1996). Furthermore, within a short time after parasitisation, the polydnaviral gene products are not at an effective concentration in the hemolymph. Therefore, it was proposed that the immune-resistant nature of the parasitoid egg delays the rate of encapsulation until ovarian immune-suppressive proteins come into action and change the properties of hemocytes which are then followed by a long-term protection provided by polydnavirus-encoded products (Webb and Luckhart, 1996).

1.3.2. Polydnaviruses (PVs)

Early work indicated that particles produced in the calyx region of some parasitoid hymenoptera and co-injected as calyx fluid during oviposition protected the egg from the immune system of the host (Rotheram, 1967; Salt, 1955; Vinson and Scott, 1975). Subsequently, virus particles were described that replicated in the calyx region of the reproductive tract of female parasitoids (Stoltz and Vinson, 1979b) which were recognised as baculoviruses and virus-like particles in braconid and ichneumonid wasps, respectively. Thereafter, the term "Polydnaviridae" was introduced as a proposed family of viruses associated with the reproductive tract of braconid and

ichneumonid parasitoid wasps (Stoltz *et al.*, 1984). The authors suggested four PV-types: type 1, *Hyposoter exiguae* (HeV) as type species; type 2, *Campoletis sonorensis* (CsV); type 3, *Hyposoter fugitivis* (HfV) and type 4, *Olesicampe benefactor* (ObV). However, the International Committee on Taxonomy of Viruses held in Sendai (1986) proposed that the former subgroup D of the Baculoviridae be removed from that family. Consequently, the viruses belonging to that subgroup were classified into a new family with the name Polydnaviridae consisting of one genus, *Polydnavirus* (Brown, 1986).

1.3.2.1. Structure and morphology

PVs can be divided into two distinct subgroups on the basis of their morphology and host range. "Ichnoviruses" are associated with wasps belong to the Ichneumonidae, while "bracoviruses" are associated with members of the family Braconidae (Stoltz, 1990; Stoltz and Vinson, 1979b). Ichnoviruses generally have fusiform or quasicylindrical nucleocapsids which are approximately 85×330 nm and surrounded by two envelopes (Fig. 1-2a) (Fleming, 1992; Stoltz *et al.*, 1984; Stoltz and Vinson, 1979b). The virion is very large and ovoid, with a molecular weight ranging from approximately 2×10^6 to 20×10^6. Once they pass through the nuclear membrane of infected calyx cells, they lose one of their unit membrane envelopes.

Bracoviruses possess a cylindrical nucleocapsid enclosed by a unit membrane envelope (Fig. 1-2b). Protrusions or tail-like structures were observed which probably play a role in the penetration of viruses through the nuclear pores of host cells (Stoltz and Vinson, 1979b). The nucleocapsid is uniform in width but variable in length (~25-250 nm). Sometimes, several nucleocapsids are observed per envelope (Fleming, 1992; Stoltz and Vinson, 1979b). It is not known whether the multipartite genome of PVs (see below) is encapsidated in single virions or exist as a multicomponent virus. The smaller DNA circles could be encapsidated in the shorter nucleocapsids and the longer ones could be in larger nucleocapsids (Krell *et al.*, 1982).

Despite the morphological differences of ichnoviruses and bracoviruses, they have a number of common features that are relevant to classify them in a family: 1) their site of replication in the nucleus of calyx cells; 2) having multipartite and segmented double stranded DNA; 3) integration of the viral genome in the wasp genome; and 4) their mode of action in the host.

1.3.2.2. Genomic organisation

PVs are a unique family of viruses as they contain a segmented (polydisperse) circular double-stranded DNA (Stoltz *et al.*, 1984; Stoltz *et al.*, 1981). The genome of *C. sonorensis* PV (CsV), which has been studied in more detail, consists of at least 30 superhelical DNA (SH DNA) segments ranging from 6 to 28 Kb. However, the number of segments and the sizes of the DNAs are different in PVs from different wasp species. For instance, *Hyposoter exiguae* PV (HeV) consists of about 16 different segments with a size range of 2-8 kb (Krell, 1991). The distribution of DNA segments in virus particles is not well-understood. However, it has been shown that in *Chelonus inanitus* (Braconidae) each nucleocapsid contains only one DNA molecule (Albrecht *et al.*, 1994) whereas it seems that ichnoviruses contain all the DNA molecules together in each nucleocapside (Krell *et al.*, 1982). Therefore, the presence of a diverse population of virions with different DNA molecules was suggested in *C. inanitus* (Albrecht *et al.*, 1994).

In addition to having a complex and polydisperse nucleic acid structure, PVs acquired an intricate polypeptide composition. Studies of the polypeptide composition of three types of ichnoviruses (HeV, CsV and HfV) indicated that each is composed of at least 20 different sized polypeptides. However, the location of genes coding for the polypeptides is not well understood (Krell, 1991). From the observation that any one DNA circle has an insufficient coding capacity for all of the virus structural polypeptides, it was assumed that several superhelical SH DNAs might collectively code for these polypeptides (Krell *et al.*, 1982). Moreover, it is probable that the virus genome codes for the additional non-structural polypeptides required for virus replication or polypeptides which could be involved in altering host physiology and affecting the encapsulation response of the host.

The observed cross-hybridisation within several sets of SH DNAs led to the hypothesis that the CsV genome may consist of families of related SH DNAs (Blissard *et al.*, 1987). In addition, some of the CsV transcripts expressed in the parasitised host are homologous among each other. In this system, the abundantly expressed 1.6 kb mRNA was shown to be at least partially homologous to a less abundant 1.0 kb mRNA. The genes encoding the two related CsV mRNAs

(1.6 and 1.0 kb) are located on a single SH DNA segment (W) of the multipartite CsV genome (Blissard *et al.*, 1987). Although some hybridisation probes hybridise to numerous CsV SH DNA, not all probes hybridise to a single large SH DNA, indicating that there is no large SH DNA containing all the CsV sequences.

The interindividual genome of PVs is highly polymorphic (Stoltz, 1990) which might have been caused by mutations, duplication events or homologous recombination (Blissard *et al.*, 1987). This variation in genome segments may provide an evolutionary advantage for the parasitoid, as it may enable it to respond to new immune strategies of the host or to increase its host range.

1.3.2.3. Distribution

PVs have only been found in two families of parasitoid Hymenoptera, the Ichneumonidae and the Braconidae (Fleming, 1992; Stoltz, 1992). All the infected parasitoids found thus far are endoparasites of lepidopteran larvae. Most of the ichneumonid species that contain PVs belong to the subfamily Campopleginae and some to Bachiniae (Table 1-1). Braconid species in which the presence of PVs have been recorded are classified in the subfamily Microgastrinae, Cardiochilinae and Cheloninae. However, there is the possibility that they exist in other subfamilies of braconid and ichneumonid families since a number of subfamilies have not been examined for the presence of viruses.

From experience, if one species is found to be infected by the virus, all individuals of that species can be assumed to carry the virus (Stoltz, 1990). It is worthwhile to mention that electrophoresis of PVs has indicated that each species carries a variant PV electrotype which is characteristic of that species (Stoltz, 1990; Stoltz, 1992). Even at the population level PVs appear not to be identical, but similar (Stoltz, 1992).

The distribution of PVs and their evolutionary origin in parasitoid wasps is of great interest. From where and when did they arise in parasitoid hymenoptera? Did all the various types of PVs originate from a common ancestral virus or did they evolve from different lineages of ancestry? Why do PVs exist only in two families of parasitoid hymenoptera? There are many speculations concerning the origin of PVs. One suggests that PVs are an evolved form of passively

Table 1-1

Reported parasitoid species contain polydnaviruses

(Adopted from Stoltz & Whitfield, 1992)

Braconidae	Ichneumonidae
Cheloninae	**Bachinae**
Ascogaster argentifrons (Provancher)	*Glypta fumiferanae*
Ascogaster quadridentata Wesmael	*Glypta* sp.
Chelonus blackburni Cameron	*Lissonota* sp.
Chelonus altitudinis Viereck	**Campopleginae**
Chelonus nr. curvimaculatus Cameron	*Campoletis aprilis* (Viereck)
Phanerotoma flavitestacea Fischer	*Campoletis sonorensis*(Cameron)
Cardiochilinae	*Campoletis* sp.
Cardiochiles nigriceps Vierech	*Casinaria forcipata* Walley
Microgastrinae	*Casinaria* sp.
Apanteles crassicornis (Provancher)	*Diadegma acronycta* (Ashmead)
Apanteles fumiferanae Viereck	*Diadegma interruptum* (Holmgren)
Cotesia congregata (Say)	*Dusona* sp.
Cotesia flavipes (Cameron)	*Eriborus tenebrans* (Gravenhorst)
Cotesia glomerata (Linnaeus)	*Hyposoter annulipes* (Cresson)
Cotesia hyphantriae (Riley)	*Hyposoter exiguae* (Viereck)
Cotesia kariyai Watanabe	*Hyposoter fugitivus* (Say)
Cotesia marginiventris (Cresson)	*Hyposoter lymantriae* (Cushman)
Cotesia melanoscela (Ratzeburg)	*Hyposoter rivalis* (Cresson)
Cotesia rubecula (Marshall)	*Olesicampe benefactor* Hinz
Cotesia schaeferi (Marsh)	*Olesicampe gebiculatae* Quednau & Lim
Diolcogaster facetosa (Weed)	*Synetaeris tenuifemur* (Walley)
Glyptapanteles flavicoxis (Marsh)	*Tranosema* sp.
Glyptapanteles indiensis (Marsh)	
Glyptapanteles liparidis (Bouche)	
Hypomicrogaster ecdytolophae (Muesebeck)	
Microgaster canadensis Musesbeck	
Microplitis croceipes (Cresson)	
Microplitis demolitor Wilkinson	
Pholetesor ornigis (Weed)	
Protapanteles paleacritae (Riley)	

transmitted baculoviruses from lepidopteran hosts, as there are similarities between bracoviruses and baculoviruses. Another assumption is that PVs are a derived form of a previously pathogenic virus which infected the wasps themselves. However, whatever the origin, there is only one reported case of the occurrence of a baculovirus pathogenic to a hymenopteran parasitoid, *Mesoleius tenthredinis* (Ichneumonidae) (Hamm *et al.*, 1988). A third postulation is that PVs are not of viral origin, but are packaged wasp-generated virulence genes which are delivered to the host (Whitfield, 1990). However, these presumptions entail a complex tripartite (host-parasitoid-virus) evolutionary association. Further studies are needed to determine the origin of these unusual viruses.

1.3.2.4. Parasitoid-polydnavirus interaction

PVs only replicate in the nuclei of those calyx epithelial cells situated between the ovarioles and lateral oviducts of the female reproductive organ (Krell and Stoltz, 1980; Stoltz and Vinson, 1979b). However, they have no pathogenic association with the wasps (Blissard *et al.*, 1986b). There are cells in the calyx region in which PVs do not replicate, but there is no difference between infected and non-infected cells (Fleming, 1992). Investigations indicated that the replication and morphogenesis of PVs in *C. sonorensis* initiates during a specific phase of pupal development (Norton and Vinson, 1983). The replication of *Cotesia inanitus* PV, a bracovirus, begins in the pupa when eyes, ocelli, mandibles and thorax are pigmented (Albrecht *et al.*, 1994). The replication of PVs in the ovaries of *C. sonorensis* has been demonstrated to be regulated by the ecdysteroid titre (Webb and Summer, 1992). Ligation between the thorax and the abdomen of 48h pupae of the wasp prevented the initiation of viral DNA replication.

Significantly two forms of CsV-specific DNA appear to be present in male and female wasps: i) a form which is identical to that isolated from purified virions (extrachromosomal), and ii) a second type which represent integrated viral sequences (Fleming and Summers, 1986). The discovery of putative integrated forms of viral DNA in the male and female wasp genome suggests that CsV is transmitted vertically through the germline (Stoltz *et al.*, 1986). Whether the possible observation of extrachromosomal viral DNA in male wasps is episomal or present in virions is unclear. Although extrachromosomal PVs have been detected in some parasitoids

(Fleming and Summers, 1986), using a genetic approach it was shown that extrachromosomal PV DNA present in every parasitoid species that has been examined does not participate in the productive transmission of PV genomes (Stoltz, 1990). A simple explanation would be that extrachromosomal DNA is, in fact, incapable of participating in viral replication, thus extrachromosomal DNA could well be transmitted within germ cells, but still not play a productive role in determining the physical structure of the encapsidated PV genome (Stoltz, 1990). The possibility of horizontal transmission of PV DNA *per os* to the parasitoid larva that eventually become incorporated into germ line tissue is ruled out (Stoltz *et al.*, 1986).

Using a PCR approach, it was recently shown that a *C. inanitus* polydnavirus 12 kb segment is integrated in the wasp genome and is excised after the pupal stage 3 (Gruber *et al.*, 1996). The genomic DNA is then rejoined after excision of virus DNA by recombination of both termini of the integrated copy that contain short imperfect direct repeats. This shows that both groups of polydnaviruses, bracoviruses and ichnoviruses, have a similar mode of replication.

1.3.2.5. Host-polydnavirus interaction

1.3.2.5.1. Polydnavirus expression in the host

During the course of oviposition, female parasitoids simultaneously inject egg(s) and calyx fluid, which usually contains high concentrations of PVs, into the host body. Quantitative studies indicate that *Microplitis demolitor* injects an average of 2.4 ng of PV DNA into a *Pseudoplusia includens* larva at oviposition. Based on the assumption that the females contain 150 ng of PV DNA per reproductive tract, a female wasp injects approximately 0.02 wasp equivalents of MdPV into a host at oviposition (Harwood and Beckage, 1994).

Virions were observed to be mainly associated with host basement membranes, fat body, muscle cells, tracheal epithelia (Stoltz and Vinson, 1979a) and hemocytes (Davies *et al.*, 1987; Prevost *et al.*, 1990). The tail-like protrusion of bracoviruses is assumed to be involved in the penetration of virions and DNA through the plasma membrane and nuclear pores, respectively.

Consequently, the DNA penetrates the nucleus where expression of the virus is observed (Stoltz and Vinson, 1979a).

There is no evidence for replication of polydnaviral DNA in the host (Theilmann and Summers, 1986), but it has been shown that *C. sonorensis* PV (CsV) DNA is expressed in the host larvae, *H. virescens*, 2h after parasitisation, and persists for 9 to 10 days (Fleming *et al.*, 1983; Theilmann and Summers, 1986). At least 10 CsV specific mRNAs can be detected 2h following parasitisation, with a 1.6-Kb mRNA being the most abundant (Blissard *et al.*, 1986b). In another case in which a bracovirus, *M. demolitor* PV, is involved, the virus gene expression starts at about 4 hours after parasitisation and persists for 6 days in the host, *P. includens* (Strand *et al.*, 1992). Since PVs do not replicate in the host body, it can be inferred that the virus infection is abortive, in other words either the host cells are susceptible to infection but non-permissive, allowing only a few viral genes to be expressed or the virus is defective and lacks a full complement of viral genes (Roizman and Palese, 1996).

Extensive molecular studies performed so far on most CsV genes expressed in *H. virescens* indicate that they belong to two gene families: repeat and cysteine-rich gene families which are located on different superhelical DNA segments (reviewed in (Summers and Dibhajj, 1995)). Accordingly, the three abundantly expressed genes VHv1.1, WHv1.0 and WHv1.6 (named after segment:host:RNA size) comprise a cysteine-rich family which have a similar gene structure (Dib-Hajj *et al.*, 1993). They contain cysteine motifs characteristic of ω-conotoxins which are snail ion-channel ligands that target ion-channels and receptors in the neuromuscular system (Olivera *et al.*, 1990). Using a recombinant baculovirus, expressing VHv1.1 protein *in vivo*, it was demonstrated that the baculovirus reduced the encapsulation response to washed parasitoid eggs of *C. sonorensis* relative to control (Li and Webb, 1994). The secretion of a 30 kDa VHv1.1 protein from PV-infected cells into the hemolymph was suggested. The protein binds to hemocytes and subsequently inhibits the cellular immune responses in the absence of other factors from the parasitic wasp. In this case, the encapsulation response is reduced by 50% relative to control. Recently, a new member of the cysteine-rich gene family from CsV was isolated and characterised (Cui and Webb, 1996). The gene encodes a 1.4 kb mRNA mapped to viral segment V by Southern blotting. Similar to other members of the gene family, the VHv1.4

motifs are characterised by six cysteines at conserved positions and variable inter-cysteine amino acids. From a functional point of view, it has been suggested that the cysteine-rich genes might provide a versatile mean for the virus to deal with various types of hemocyte receptors to circumvent the immunity of a range of host species (Cui and Webb, 1996).

Members of the repeat gene family contain one or more of a 540-bp repeat elements which are present on several CsV DNA segments (Reviewed in (Summers and Dibhajj, 1995)). However, they do not show any significant similarity to the cysteine-rich gene family and are not as highly expressed as the cysteine-rich ones. It has been suggested that they might play a role in the virus life cycle distinct from the other gene family, and perform other functions such as integration/excision events.

There are several reports on the appearance of novel proteins in parasitised hosts which are virus related. Injection of *Cotesia congregata* PVs into the host, *Manduca sexta*, induces the production of several early-induced proteins (EPs) (Harwood and Beckage, 1994). Among four host tissues sampled, the primary sites of EP1 (190 kDa, following denaturation and deglycosylation the molecular weight was 33 kDa) synthesis was found to be the fat body and hemocytes. Later, it was shown that this glycoprotein is PV encoded (Harwood *et al.*, 1994). A high molecular weight protein (185 kDa) was found to be expressed in the host, *Trichoplusia ni,* following parasitisation by a wasp, *Chelonus* near *curvimaculatus* (Soldevila and Jones, 1994). The protein is synthesised by the developing parasitoid during the terminal stage of parasitisation and secreted into the hemocoel of the host. Antibodies against CcVs specifically cross-react with this protein.

The penetration of PVs into various host tissues has been shown (Stoltz and Vinson, 1979a). In addition, the expression of viral genes in hemocytes, gut, nervous system, fat body and malphigian tubules has been documented (Harwood and Beckage, 1994; Strand *et al.*, 1992). However, there are variations in the level and duration of expression which reflects tissue specific variation regarding the level of expression of different PV transcripts. This might be due to specific alterations in the physiology of the host with regard to the immune system and development (see below).

1.3.2.5.2. Developmental and physiological alterations

Polydnaviruses cause a number of physiological, developmental and behavioural alterations in parasitised hosts (Table 1-2). PVs are of crucial importance for survival of the parasitoid larva as they play a role in suppression of the host immune system, although the exact mechanisms by which PVs affect the immune system is not known. In a classical paper PV-encoded products have been reported as the source of protection for the egg (Edson *et al.*, 1981). However, recent observations suggest that the initial protection is provided by maternal components found in the calyx fluid. The immediate suppression of the host immune system is then replaced by PV-encoded products (Webb and Luckhart, 1994). This effect might be accomplished either by interfering with the host recognition system or by direct inactivation of hemocytes.

Occurrence of apoptosis in *P. includens* hemocytes parasitised by *M. demolitor* has been reported (Strand and Pech, 1995b). Apoptosis is a programmed cell death that can be induced by several factors including virus infection (Cohen, 1993) during which apoptotic cells undergo condensation and fragmentation of the nucleus, blebbing and eventually die (King and Cidlowski, 1995). Apoptosis of *P. includens* hemocytes was shown to start *in vivo* and *in vitro* approximately 12h following infection and continues through to 48h post-infection (Strand and Pech, 1995b). However, the effects of apoptosis are transient in this system and new granulocytes are generated from undifferentiated cells in the circulation which do not contain transcriptionally active MdPV and behave normally towards foreign objects (Strand and Noda, 1991).

Artificial injection of calyx fluid causes changes in both hemocytes and hemolymph composition. Injection of CsV into *H. virescens* 5th instar larvae leads to an increase in the volume of the hemolymph compared to control larvae (Davies *et al.*, 1987). The hemolymph has a lower viscosity and a reduced total hemocyte count. In addition, there is a significant change in differential hemocyte count; the proportion of plasmatocytes diminishes 8-24 hours after injection of calyx fluid. An *in vitro* study revealed that the plasmatocytes of PV-injected larvae spread significantly more slowly than normal ones. However, both the abnormal behaviour and the differential hemocyte count of plasmatocytes return to normal 16 days after injection. These

Table 1-2

Major physiological and developmental effects of polydnaviruses on host

Effect	References
Changes in hemocyte count and behaviour	Davies *et al*. (1987); Prevost *et al*. (1990); Stoltz & Guzo (1986); Guzo & Stoltz (1987); Wago & Tanaka (1985)
Developmental arrest	Dahlmann & Vinson (1977); Tanaka (1987); Thompson (1983); Vinson *et al*. (1979); Dover & Vinson (1990); Hayakawa & Yasuhara (1993); Beckage *et al*. (1990), Hayakawa (1991a,b), Tanaka (1992)
Changes in hemolymph protein patterns	Greene & Dahlmann (1973); Frokovich *et al*. (1983); Cook *et al*. (1984); Beckage *et al*. (1987); Beckage & Kanost (1993)
Inhibition of hemolyph phenoloxidase activity and melanization	Beckage *et al*. (1990); Stoltz & Cook (1983); Stoltz & Guzo (1986)
Reduction of ecdystroid titre	Dahlmann *et al*. (1990); Dover *et al*. (1987); Dover *et al*. (1988); Dover & Vinson (1990); Tanaka & Vinson (1991); Tanaka (1987)
Changes in trehalose level	Dahlmann & Vinson (1976, 1977)
Suppression of the cellular immune response	Edson *et al*. (1981); Guzo & Stoltz (1985, 1987); Stoltz & Guzo (1986); Stoltz & Vinson (1977, 1979a)
Impairment of food consumption and utilization	Guillet & Vinson (1973)
Inhibition of protein storage in fat body	Tanaka (1986)
Reduction in hemolymph viscosity	Davies *et al*. (1987)
Degeneration of hemopoietic tissue	Guzo & Stoltz (1987)

alterations presumably result in failure of the host to encapsulate the parasitoid eggs, possibly due to the insufficiency and altered spreading behaviour of plasmatocytes. Infection of hemopoietic tissues is suggested to result in the observed depletion of adherent plasmatocytes (Guzo and Stoltz, 1987).

Similar observations were made in *Malocosoma disstria* parasitised by *H. fugitivus* or injected by PVs (Stoltz and Guzo, 1986). In addition, a suppression in melanization reaction and associated changes in oenocytoids were discernible. Whereas, oenocytoids from unparasitised larvae lysed very rapidly compared to parasitised larvae. The apparent stabilisation of oenocytoids in parasitised or PV-injected caterpillars results in the lack of melanization in hemolymph cultures and the subsequent failure of immune responses. Since oenocytoids are found to be involved in melanization, their alteration due to parasitisation can affect the immune reaction of the host.

Encapsulation which is the primary immune reaction toward parasites is usually accompanied by melanization in which tyrosine and DOPA are converted to melanin via toxic quinone intermediates (Nappi *et al.*, 1995). The monophenoloxidase activating system has been shown to be involved in melanization and non-self recognition (Ratcliffe *et al.*, 1984). Suppression of this activating system might, therefore, have been one of the primary evolutionary achievements of parasitoids. In this regard, an inhibition of monophenoloxidase activity of the hemolymph was shown after the injection of purified *Cotesia congregata* PVs into *Manduca sexta* (Beckage *et al.*, 1990). Although the mechanism(s) by which the prophenoloxidase activation is repressed is not known, it is assumed that parasitism might alter levels of prophenoloxidase inhibitors or activating factors, which would in turn affect the rates of tyrosine conversion to DOPA and hemolymph melanization (Beckage and Templeton, 1986). A complete inhibition of phenoloxidase activity was also noticed in *Trichoplusia ni* injected with *H. exiguae* PVs (Stoltz and Cook, 1983).

Koinobiont parasitoids, which allow the host to continue its growth and development post-parasitisation until it has reached its maximum size (Naumann, 1991), have acquired mechanisms to retard their host development until the parasitoid has completed larval

development. Several lines of evidence indicate that PVs reduce ecdysteroid titres in parasitised larvae (Dahlman *et al.*, 1990; Lawrence and Lanzrein, 1993). This could be achieved by direct cellular infection and deterioration of the prothoracic gland (Dover *et al.*, 1988) or affecting fat body cells, which produce stimulating factors that affect the responsiveness of the prothoracic gland (Dahlman *et al.*, 1990; Dover *et al.*, 1987). Injection of *C. sonorensis* calyx fluid into the late 5th instar larvae of *H. virescens* causes a significant reduction in ecdysteroid titre and developmental arrest by prevention of pupation (Dover *et al.*, 1987). Ecdysone injection of retarded larvae provokes pupation.

Alternatively, inhibition of prothoracicotropic hormone (PTTH) synthesis and/or secretion by parasitisation might be responsible for the reduction in ecdysteroid titre (Tanaka, 1987). Implantation of brains from naive *H. virescens* larvae, or injection of PTTH, activates the prothoracic gland of parasitised larvae (Dahlman *et al.*, 1990). These observations were supported by the finding that when prothoracic glands from parasitised larvae were incubated with PTTH fractions, they produced ecdysteroid levels comparable to controls.

It is known that juvenile hormone controls the release of PTTH (Hammock, 1985). Therefore, its absence in early last instar larvae is necessary for the secretion of PTTH and subsequent pupation. The clearance of juvenile hormone from the hemolymph is due to juvenile hormone esterase activity in the last instar larvae (Hammock, 1985). There are several reports indicating that parasitised or PV-injected larvae fail to metamorphose (Beckage, 1990; Dahlman and Vinson, 1977; Dover and Vinson, 1990) implying a hormonal disruption in the host. In *Pseudoletia separata* larvae parasitised by *Apanteles kariyai*, an inhibitor which is induced after parasitisation, was found to be responsible for the inhibition of juvenile hormone esterase activity (Hayakawa, 1991). Injection of the purified blocking factor leads to reduced plasma juvenile hormone esterase activity by 80% in armyworm larvae. Also, injection of purified PVs and venom fluid caused the induction of the growth-blocking peptide similar to naturally parasitised larvae. Therefore, it was proposed that this factor is produced directly or indirectly by PV infection (Hayakawa and Yasuhara, 1993). Recently, it was shown that the growth-blocking peptide also induces an increase in dopamine levels in the hemolymph of the parasitised larvae released by the integument (Noguchi and Hayakawa, 1996). This was demonstrated to be

due to an increase in dopa decarboxylase activity in the integument which is involved in dopamine metabolism. Since dopamine retards larval development (Noguchi *et al.*, 1995), it was proposed that the increase of dopamine levels in the hemolymph is partly responsible for a retardation of larval development (Noguchi and Hayakawa, 1996). The juvenile hormone (JH) titre also increases 20-fold in *P. includens* parasitised by *M.demolitor* compared to unparasitised control presumably due to a decreased metabolism of the hormone (Balgopal *et al.*, 1996). The effect was simulated by the injection of MdPV plus venom, however, the increase in JH titre was not as high as natural parasitisation. In this case, the possibility of JH release by the parasitoid larva or associated teratocytes (see below) was ruled out (Balgopal *et al.*, 1996).

The occurrence of precocious metamorphosis during the penultimate larval stadium was shown in *T. ni* parasitised by *Chelonus* sp. caused by material from female wasps (Jones, 1987). However, it was later shown that from the two effects of natural parasitisation of *Spodoptera littoralis* larvae by *C. inanitus*, namely induction of precocious metamorphosis and developmental arrest of the prepupal stage, only the latter is caused by calyx fluid and venom (Soller and Lanzrein, 1996). Injection of antibody against the purified PVs prevented developmental arrest of the host indicating their active role in this process. Therefore, it seems that in *S. littoralis-C. inaintus* other factors from the parasitoid are responsible for untimely metamorphosis such as direct secretions by the parasitoid.

An increase in trehalose level in the hemolymph of parasitised hosts has been described (Dahlman and Vinson, 1976). Injection of calyx fluid from *C. sonorensis* into *H. virescens* larvae revealed its functional role in the elevation of trehalose level in the host (Dahlman and Vinson, 1977). There is also evidence of PV effects on host weight gain, growth and food consumption in parasitised or PV-injected larvae (Guillot and Vinson, 1973; Vinson *et al.*, 1979). However, this could be due to the general observation that parasitism often lessens total food consumption or that selective uptake by the parasitoid larvae creates a depletion of hemolymph components.

1.3.2.6. Synergistic factors in PV efficacy

In certain host-parasite interactions, PVs are only efficient when they are accompanied by venom gland fluid. This is mainly the case in braconid parasitoids, as ichnoviruses are solely able to abolish host immune reactions (Stoltz and Guzo, 1986). In *Cotesia melanoscela*, it was reported that venom facilitates the uncoating of PVs *in vitro* and persistence *in vivo* (Stoltz *et al.*, 1988a). In the absence of venom, viruses could not enter host cells or attach to nuclear pores.

In *Apanteles glomeratus* (=*Cotesia glomerata*) (Kitano, 1986) and *A. kariyai* (Tanaka, 1987), venom fluid is an absolute requirement for successful parasitism. Although, the calyx fluid from parasitoid *Chardiochiles nigriceps* was able to prevent egg encapsulation (Guillot and Vinson, 1972), however, some first instar larvae were encapsulated or their development was retarded. The authors suggested that the presence of venom was essential for proper development of the larval parasitoid, even though the mechanism is not clear. It was proposed that in the period between oviposition and the expression of polydnaviral DNA, venom might protect the egg from the host immune reaction (Webb and Summers, 1990). Nonetheless, venom is unable to prevent encapsulation by itself (Wago and Kitano, 1985).

Evidence obtained from a variety of experiments indicate that the degree of dependence of calyx fluid on venom is variable among the virus-affected parasitoids. It ranges from complete independence in most ichneumonid wasps to different levels of dependence in braconids. These results can be associated with the variation in PVs and their host species-specificity which culminates in these assorted functional roles.

Teratocytes, cells of the embryonic serosal membrane of certain endoparasitoid hymenopteran embryos, are assumed to play a synergistic role in the attrition of the host immune system (Vinson, 1990b) as well as having nutritional values to the developing parasitoid larva (Okuda and Kadono-Okuda, 1995). They are released into the host hemolymph at hatching by dissociation of serosa into individual cells, which persist during parasitoid's larval development and their size increases several-fold (Fig. 1-3) (Strand and Wong, 1991). Teratocytes were found to be essential for prolongation of the *Pseudaletia separata* larval stages, together with calyx and venom fluid from *A. kariyai* (Wani *et al.*, 1990). It was suggested that they might

secrete substances which were responsible for larval-larval and larval-pupal ecdysis since the injection of calyx and venom fluid alone did not cause developmental prolongation. However, an active suppression of the host immune system by PVs is perhaps crucial for the survival of these cells since artificial injection of teratocytes obtained from *in vitro* culture of *M. demolitor* eggs into *P. includens* led to the encapsulation of most of the cells within 24 hours (Strand and Wong, 1991). Also, involvement of teratocytes in host regulation by interfering with physiological processes of the host has been documented (Pennacchio *et al.*, 1992; Zhang *et al.*, 1992).

1.3.3. Other associated virus-like particles

There are several reports showing the existence of other virus-like entities, which are present in the calyx or accessory glands of the parasitoids and injected into the host upon parasitisation. Virus-like filamentous particles (VLFPs) were characterised in the braconid wasp *Cotesia congregata,* which replicates in the nucleus of calyx cells (Buron and Beckage, 1992). It is important to note that they do not replicate in the same cells that support PV replication. However, the number of cells in which VLFPs are replicated are few. VLFPs acquire two envelopes per virion, whereas the known bracoviruses acquire only one envelope. Since any detailed study of these particles are lacking, it is not known whether they belong to the family Polydnaviridae or are novel viral entities.

Similar VLFPs have been described from *Cotesia marginiventris* that replicate in the hypodermal and the tracheal matrix cells of parasitised host larvae (Styer *et al.*, 1987). Comparison of five colonies of *C. marginiventris* with regard to the presence of VLFPs revealed that only two colonies had them (Hamm *et al.*, 1990). As PVs are present in every individual of the affected species, it can be presumed that the transmission of VLFPs might not be through the wasp germline. Further studies at the molecular and ultrastuctural levels are needed to clarify their association with PVs, as well as their occurrence in parasitoid wasps.

Unusual virus-like particles were described in *C. melanoscela* which apparently replicate in both parasitised and host tissues (Stoltz and Faulkner, 1978). Further investigations indicated that the former described particles do not belong to Polydnaviridae since they lack polydisperse DNA

(Stoltz *et al.*, 1988b). The authors designated the virus-like particles as CmV2. It appears that CmV2 also replicates in non-reproductive tissues of the female and male parasitoid. However, their pathogenicity toward parasitoids is not clear. These observations are consistent with the theory that PVs might have been derived from a pathogenic virus associated with an ancestral parasitoid. Thus, CmV2 might be an evolutionary, transitional phase between an absolute pathogen and a mutualistic PV.

Virus-like particles (VLPs) were described from a cynipid parasitoid hymenoptera, *Leptoplinia heterotoma*, that replicate in the accessory gland of the female reproductive organ and are injected into the host, *D. melanogaster* (Rizki and Rizki, 1990). The VLPs specifically attack lamellocytes of the host larvae and prevent egg encapsulation. The intriguing question is whether there is any relationship between cynipid VLPs and PVs. However, Cynipidae belong to a different lineage, Proctotrupomorpha, which is not a sister group of Ichneumonomorpha (Whitfield, 1992). Studies have shown that these VLPs appear as different morphotypes with unequal efficiency to disrupt the host immune system (Dupas *et al.*, 1996).

1.4. Concluding remarks

Insects possess peculiar ways of defending themselves externally and internally. Internal defence mechanisms, known collectively as the immune system, involve humoral and cellular reactions toward invading micro- and macroorganisms. Insect humoral immunity mainly involves antimicrobial factors such as cecropins, attacins and lysozymes. Cellular immunity involves phagocytosis, encapsulation and nodule formation toward microorganisms and metazoans. Parasitoid eggs encounter the capsule formation reaction of their host caterpillar and wasp larvae adopted strategies to overcome the immune reaction of their habitual hosts. PVs and other VLPs, which are produced in the calyx region of certain parasitoid hymenoptera, are found to play a functional role in the suppression of the host immune system. Studies which have been conducted so far are focusing on the effects of PVs and VLPs on the host immune system, physiology and development, the evolutionary relationships among PVs, and the molecular aspects of genome and gene expression of the viruses. Since the immune system in insects is not well-understood, the influence of PVs on the host cannot be expected to be documented before a

molecular mechanism is available. Although an overall picture of the role of PVs in the host-parasitoid relationship has been obtained, many questions still remain. The exact mechanisms by which the cellular immune system of the host is impaired, the association of PVs with venom and teratocytes, the presence of other virus entities along with PVs and their role in the suppression of the host immune system are major questions that remain to be solved in host-parasitoid interactions. Furthermore, the transmission, replication and complex genome of PVs and the evolutionary relationship among different PVs are not fully understood. Further studies are required to better understand PVs and VLPs and their mode of action in order to design more efficient biological control systems. It might be possible to identify and exploit parasitoid species to improve biological control agents or define and/or change their host range to use it as efficient tools against agricultural pests.

The literature was reviewed prior to March 1997.

Figure 1-1

Female reproductive organ of a braconid endoparasitoid, *Cotesia rubecula*. *ca*, calyx gland; *ov*, ovary; *vg*, venom gland; *vs*, venom reservoir.

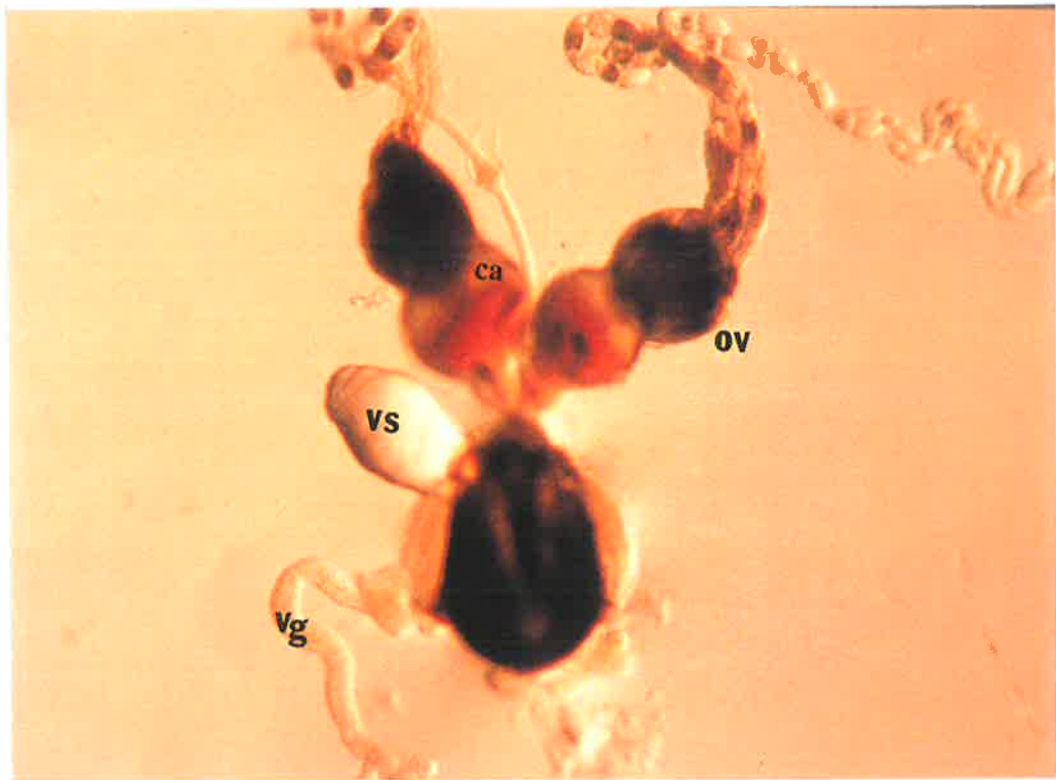
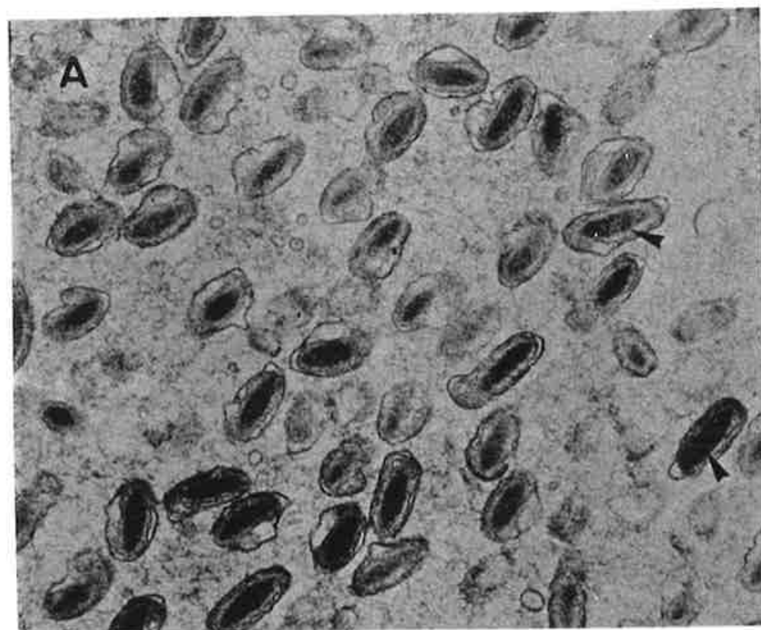

ca
ov
vs
vg

Figure 1-2

Transmission electron micrograph of polydnaviruses. **A)** Ichnoviruses from *Campoletis sonorensis* with lenticular nucleocapsids (adopted from Krell, 1991); **B)** a bracovirus from *Cotesia rubecula* with a cylindrical nucleocapsid (arrow) and a tail-like structure (arrowhead), bar indicates 29.5 nm.


A

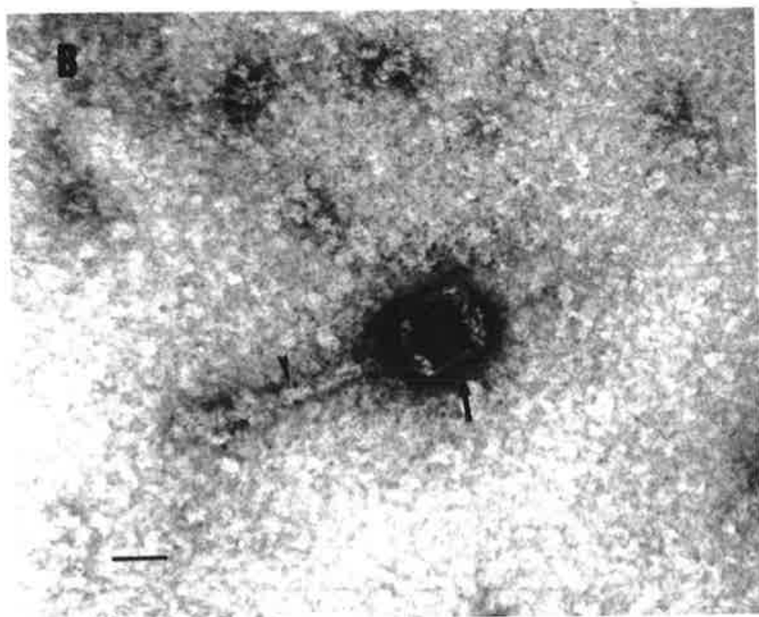
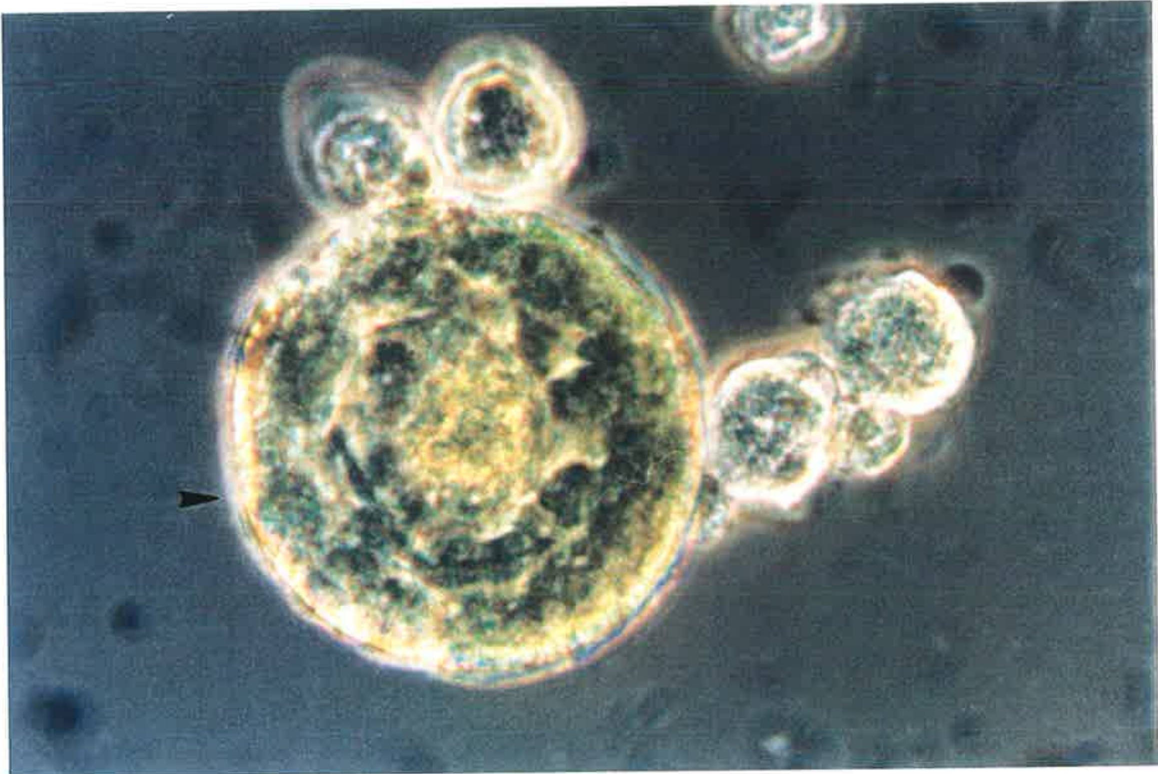
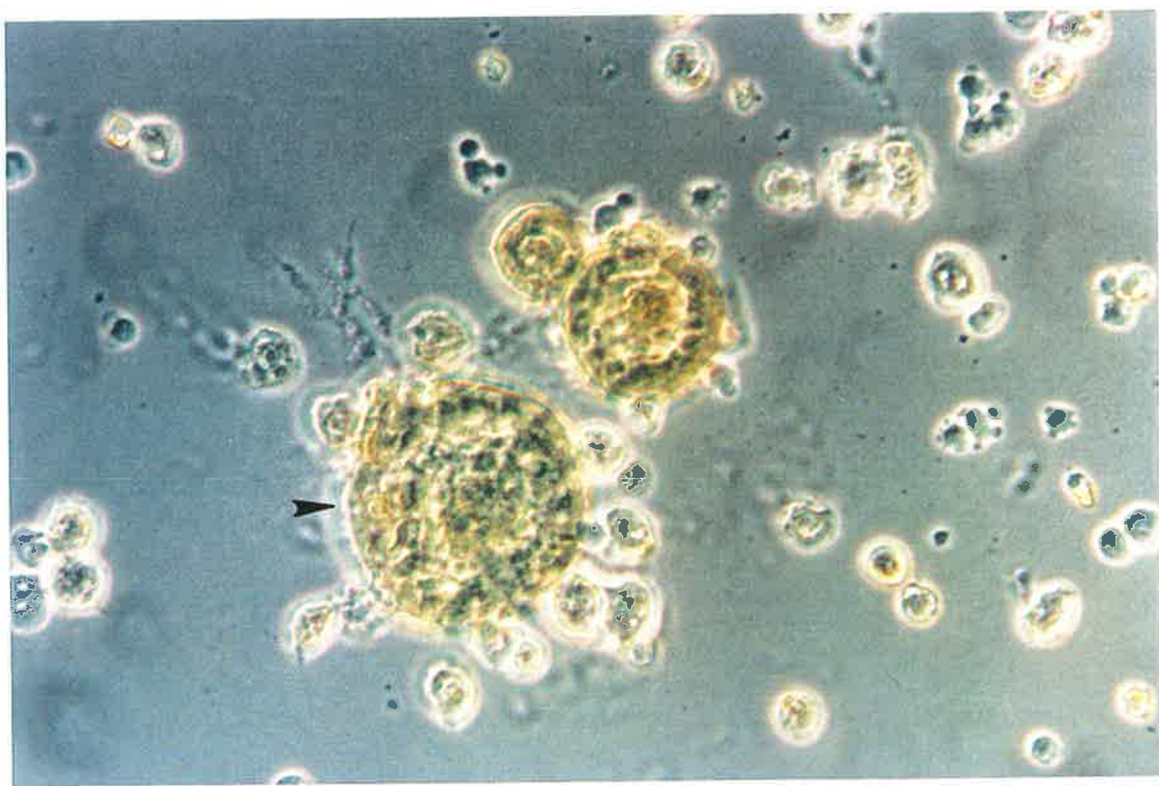

B

Figure 1-3

Teratocytes from a 5 days parasitised *Pieris rapae* caterpillar by *Cotesia rubecula* (arrowheads). Teratocytes are embryonic serosal cells released into the host hemolymph after hatching of the parasitoid larva. The size of these cells increases several-fold over time. The smaller cells are the host hemocytes.





Chapter 2

General materials and methods

Chapter 2

General materials and methods

2.1. Introduction

General materials and methods which were often used in various experiments are described in this chapter. Also, a general list of solutions and buffers utilised in the described protocols and experiments are listed at the end. Specific materials and methods are described in their corresponding chapters.

2.2. Insect cultures and parasitisation

The parasitoid wasp, *Cotesia rubecula*, was reared in the laboratory on the host caterpillars *Pieris rapae*, grown on cabbage plants. Insects were maintained under 14h light: 10h dark photoperiodic regime at 25°C. Adult wasps and butterflies were fed with 100% and 25% honey solutions, respectively.

To obtain parasitised larvae, *P. rapae* caterpillars were placed in small containers along with female wasps. The caterpillars were collected at certain times after parasitisation as required.

2.3. Isolation of fat body, hemocytes and hemolymph

To isolate fat body, *P. rapae* caterpillars were surface sterilised in 70% ethanol and dissected in PBS with fine forceps under a stereo-microscope. Fat bodies were collected in PBS and used either for protein analysis or RNA extraction.

Hemolymph was collected in PBS from surface sterilised caterpillars by cutting off one of the prolegs and bleeding. Then, it was centrifuged at 750× g for 5 min to pellet the hemocytes. The supernatant was recovered as cell-free hemolymph.

2.4. F-Actin staining of hemocytes with Phalloidin

The procedure was adopted from (Webb and Luckhart, 1994) with minor modifications. Caterpillars were bled into 20 µl PBS on a multiwell slide (ICN Biomedicals) as described

above. Hemocytes were allowed to settle for 10 min and then buffer-hemolymph was replaced with fresh buffer to prevent melanisation. Afterwards, the slide was incubated at room temperature in a humid box for about an hour to allow spreading of hemocytes. Buffer was removed and replaced by 20 µl 4% paraformaldehyde in PBS to fix the cells. After 10 min incubation, cells were gently washed three times with PBS and subsequently 50 µl of 0.165 µM FITC-labelled Phalloidin in PBS (Sigma) was applied onto each well and incubated in dark for 1h. Hemocytes were washed with PBS, overlaid with glycerol:PBS (1:1) and a glass coverslip. Hemocytes were examined under indirect UV illumination using FT510 filter in a Zeiss microscope.

2.5. Lectin binding to hemocytes *in vitro*

P. rapae caterpillars were bled into Cecropia Ringer on a multi-well slide as described above. Hemocytes were allowed to attach for 10 min and fixed by applying 4% paraformaldehyde in PBS for 15 min at room temperature. The slide was rinsed with PBS several times and blocked by applying 0.25% bovine serum albumin (BSA) in PBS for 10 min at room temperature. Blocking solution was removed and FITC-conjugated *Helix pomatia* lectin (*H.p.* lectin, Sigma) in 0.25% BSA-PBS (10 mg/ml) was added to hemocyte monolayers and incubated for 10 min in dark. Hemocytes were washed and observed under indirect UV illumination and phase contrast microscope.

In cases where the caterpillars were bled into EDTA containing buffer, the Cecropia Ringer was prepared to a final concentration of 20 mM EDTA.

2.6. Isolation of polydnaviruses

About 30-40 wasps were anaesthetised on ice. Ovaries were dissected in PBS and cut into pieces with microscissors until an even whitish suspension was achieved. The suspension was centrifuged twice at 720× g for 5 min to pellet tissues, eggs and debris. Supernatant was centrifuged twice at 15,800× g for 15 minutes to pellet virus particles and resuspended in 100 µl PBS and stored at -20°C.

2.7. Extraction of DNA from polydnaviruses

Viral DNA was extracted as described by (Stoltz *et al.*, 1986) with minor modifications. Accordingly, the virus suspension in PBS was made to 4% N-lauroyl sarcosine (sodium salt) and heated at 60°C for an hour. To digest the protein contents of the virus particles, 300 µg/ml proteinase K from a stock solution at 10 mg/ml in 1.0 M Tris (pH 7.4), 0.05 M EDTA, 0.5 M NaCl, was added. Digestion was allowed to proceed for 2h at 37°C. For nucleic acid extraction, one volume of the sample of phenol:chloroform (1:1) was added and mixed gently. The mixture was centrifuged at 15,800× g for a minute to separate the phases. The upper phase was retained and extracted again with phenol:chloroform.

DNA was precipitated by adding two volumes of ethanol and incubation at -20°C for 2h. Then, it was centrifuged at 15,800× g for 20 min to pellet the DNA. The pellet was washed with 70% ethanol and centrifuged at 15,800× g for 15 min. The pellet was dried and dissolved in water.

2.8. Extraction of DNA from wasps

Five wasps were thoroughly homogenised in homogenisation buffer using a pestle in an eppendorf tube. 5 µl of Proteinase K (20µg/µl) was added and incubated at 40°C for 2h. 0.8 µl RNaseA (10mg/ml) was added and incubated at 37°C for 30 min. Then, phenol:chloroform extraction was performed followed by ethanol precipitation. The pellet was dried, resuspended in sterile water and stored at 4°C.

2.9. Extraction of total RNA

The RNA extraction method was adopted from (Chomczynski and Sacchi, 1987).

SOLUTIONS AND GLASSWARE TREATMENT

To prevent RNase activity, the solutions and glassware were treated. All the solutions were made using diethylpyrocarbonate (DEPC)-treated water (except Tris containing solutions). The glassware was washed with DEPC-water and autoclaved.

PROCEDURE

1. About 100 mg of the tissue ground in liquid nitrogen using a mortar and pestle was added to 500 μl of denaturing solution or homogenised in the solution by a homogenizer and mixed by inversion.

2. The following were added:

 50 μl 2 M NaAc, pH 4, mixed by inversion
 0.5 ml phenol (water-saturated), mixed by inversion.
 100 μl chloroform

3. The mixture was shaken vigorously for 10 sec on a vortex and cooled on ice for 15 min.

4. The mixture was centrifuged at 10,000× g for 20 min at 4°C. The upper phase was transferred into a new tube.

5. One volume of isopropanol was added and the RNA precipitated at -20°C for at least half an hour. Then, it was centrifuged at 10,000× g for 20 min at 4°C.

6. The pellet was dissolved in 300 μl of denaturing solution, one volume of isopropanol was added and precipitation carried out at -20°C for half an hour, then, centrifuged at 10,000× g for 20 min at 4°C.

7. The pellet was washed with 75% ethanol and centrifuged at 10,000× g for 15 min at 4°C.

8. The pellet was afterwards dissolved in 50 μl DEPC-treated water and stored at -80°C.

2.10. Isolation of mRNA from total RNA

A protocol for small scale mRNA isolation was used by applying PolyATract® mRNA Isolation System IV (Promega, Technical Manual). In this system, a biotinylated oligo(dT) primer is used to hybridise to the 3' poly(A) region present in most mature mRNA species. The hybrids coupled with Streptavidin MagneSphere® particles are then captured with a magnetic stand. The mRNA is eluted from the solid phase by adding ribonuclease-free deionised water. The concentration and purity of the isolated mRNA was determined by spectrophotometry. The isolated mRNA was stored at -80°C until use.

2.11. Radiolabelling of DNA

To label DNA for hybridisation analyses, Megaprime DNA labelling system (RPN 1604, Amersham) was used for a primer extension purposes. α-^{32}P-dCTP was used as a radioactive nucleotide in the labelling reactions. The following reaction was prepared according to the manufacturer's protocol:

1. 25 ng DNA was mixed with 5 µl primer solution (random nanomers) and heated in a boiling water bath for 5 min. Then, the following were added:

4 µl	dATP
4 µl	dTTP
4 µl	dGTP
5 µl	Reaction buffer
2 µl	DNA polymerase I (Klenow fragment)
16 µl	H_2O
5 µl	^{32}P-dCTP (50 µCi)

2. The reaction was incubated at 37°C for 10 min, then heated at 100°C for 5 min and chilled on ice. The labelled probe was added to the pre-hybridisation solution for hybridisation analyses.

2.12. Southern blot analysis

DNA samples were run on a 1% TBE agarose gel stained with ethidium bromide as described (Sambrook *et al.*, 1989) and photographed. To denature DNA molecules, the gel was incubated in 1.5 M NaCl, 0.5 N NaOH for 45 min and then rinsed several times with deionised water and soaked in 1 M Tris (pH 7.4), 1.5 M NaCl for 30 min to neutralise.

A. Transfer of DNA from gel to membrane

DNA molecules were transferred onto a nylon membrane (Amersham) as described in (Sambrook *et al.*, 1989). Briefly, the gel was placed on two pieces of 3 mm Whatman paper on a glass stand soaked and in contact with a tray containing 20×SSC. A nylon membrane of the gel size was placed above the gel and 3 pieces of Whatman paper on the top. A stack of tissue papers and a weight were put on the top to allow the suction of the buffer from the gel through the membrane on which the DNA molecules are trapped. The blots were usually carried out

overnight. The transferred DNA was then cross-linked to the membrane by exposing to UV illumination for 50 sec (BioRad UV cross-linker).

B. Hybridisation analysis

The membrane was placed in a hybridisation tube along with 1 ml prehybridisation solution per 10 cm^2 of the membrane and incubated 2h at 65°C with rotation. Thereafter, radiolabelled probe was added to the prehybridisation solution and the incubation was continued overnight with rotation at 65°C.

C. Washing and autoradiography

The hybridisation solution was transferred into another tube and kept at -20°C for further use. The membrane was washed twice in 2×SSC/0.1% SDS and 0.2×SSC/0.1%SDS, with rotation for 20 min at 65°C. The final wash solution was poured off and the membrane was exposed to a sensitive film (Kodak) which was then developed using an automatic film developer (Agfa).

2.13. Northern blot analysis

RNA samples were run on a denaturing paraformaldehyde containing agarose gel, transferred onto a nylon membrane and hybridised with a probe. The methods were adopted from (Ausubel *et al.*, 1993; Sambrook *et al.*, 1989).

A. Electrophoresis of RNA

1. A 1.2% agarose solution was prepared in sterile water and to 150 ml agarose solution 17 ml of 10 × MOPS and 5.1 ml of 37% paraformaldehyde were added:

2. The samples were prepared as following:

5 µl	10 × MOPS
11.25 µl	RNA (2 µg)
8.75 µl	37% paraformaldehyde
25 µl	formamide

3. The samples were heated for 15 min at 65°C and 10 µl RNA loading buffer was added to each sample and loaded onto the gel. The gel was run at 6 V/cm in 1×MOPS until the first blue dye reached about 3/4 of the gel.

B. RNA transfer from gel to membrane

RNA samples run on the gel were transferred onto a nylon membrane and then cross-linked by UV exposure as described in Southern blot analysis (see above).

D. Prehybridisation

The UV cross-linked membrane was incubated in prehybridisation solution (1 ml/10 cm^2 of the membrane) at 65°C for 2h using rotary hybridisation machine to block unspecific sites on the membrane.

E. Hybridisation

Radiolabelled probe (see above) was added to the prehybridisation solution and incubated at 65°C overnight.

F. Washing and autoradiography

Following hybridisation, the membrane was washed twice in 2×SSC/0.1% SDS and 0.2×SSC/0.1%SDS, 20 min each.

The membrane was dried, wrapped between plastic and exposed to a Kodak diagnostic film in a cassette at -70°C. The duration of exposure was determined by radioactivity bound to the membrane assessed by a Geiger counter. The film was then developed in an automatic film developer (Agfa).

2.14. Cloning DNA fragments

DNA fragment to be cloned and the selected plasmid vector were cut with proper restriction enzymes. An aliquot of the digestion reaction was analysed on a 1% agarose gel to confirm completeness of the digestion. Samples were run on a 1% low-melting agarose gel (Sigma) and

the desired DNA fragments were isolated as described below. The concentration of DNA was estimated by spot test (see below).

A. Ligation of DNA fragment into the vector

The following reaction was assembled:

0.5 µl	10×T4 ligase buffer	
0.5 µl	Vector (25 ng)	
0.5 µl	T4 ligase	
X* µl	insert (DNA fragment)	
Y µl	H_2O	Σ 5 µl

* The amount of insert used in the reaction was calculated by the following formula:

$$X\ \text{ng} = \frac{\text{ng vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}}$$

To obtain optimal ligation, a 3:1 molar ratio of the insert to vector was used. The reaction was incubated at 15°C for 3 h.

B. Transformation of recombinant plasmids

To transform constructed recombinant plasmids to bacteria, highly competent cells of *E. coli*, JM109 (Promega), were used as following:

1. Highly competent JM109 cells were thawed on ice and a 50 µl aliquot was added to the ligation reaction and mixed.

2. The mixture was kept at 4°C for 20 min, then, heat shocked at 42°C for 50 seconds and then kept at 4°C for 2 min.

3. 1 ml LB was added to the tube and incubated at 37°C for an hour with shaking.

4. Aliquots of cells (50, 200 and 700 µl) were spread onto prewarmed LB plates supplemented with ampicilline and incubated at 37°C overnight. In cases in which the vector contained *lac*Z promoter, X-gal and IPTG were added to the plates (see buffers and solutions below).

C. Screening the clones

The promoter of the *lac*Z gene in the pBluescriptII KS$^{(+)}$ plasmid provides a means for blue/white colour selection of recombinant plasmids. An induced lac promoter upstream from the *lac*Z gene with IPTG permits fusion protein expression with the β-galactosidase gene product which leads to the appearance of blue colonies in the plates. The plasmid has a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertion inactivation of the α-peptide allows recombinant clones to be directly identified by colour screening. The colonies containing an insert appear as white.

A few white colonies were selected from the plates and grown individually in 3 ml LB containing ampicillin (100 ng/ml) at 37°C overnight. Plasmids were isolated from the cells by the minipreparation procedure (see above).

C1. Cracking method

In cases where other plasmids which lack the *lac*Z promoter were used as a vector, clones were screened by a so-called "cracking method". Simply, a fraction of a selected clone was transferred to a microfuge tube containing 25 µl water and vortexed. To each tube 25 µl of 2×cracking solution (see buffers and solutions) was added and vortexed. The samples were run on a 1% agarose gel along with the undigested vector as a control size marker. Samples containing plasmids, larger in size than the control, were reselected for minipreparation (see below) to confirm the presence of the right insert.

D. Minipreparation of plasmids

Minipreparation of plasmids is based on lysis of bacterial cells, precipitating proteins, chromosomal DNA and high molecular weight RNA molecules and isolation of plasmids from the aqueous phase by phenol extraction and ethanol precipitation (Applied Biosystems, Inc., model 373A).

1. A colony of bacteria was grown overnight in 3 ml LB/Amp. Then, the bacteria were pelleted at 2900× g for 5 min.

2. The supernatant was removed and the pellet resuspended in 200 µl of solution I.

3. 300 µl of solution II was added, mixed gently and kept on ice for 5 min.

4. The solution was neutralised by adding 300 µl of precooled solution III, mixed gently and incubated 5 min on ice. The precipitated protein-dodecyl sulphate complexes and chromosomal DNA were removed by centrifugation at 13,000× g for 10 min.

5. RNase A (DNase free) was added to a final concentration of 20 µg/ml and incubated at 37°C for 20 min which was followed by a phenol:chloroform (1:1) extraction.

6. An equal volume of 100% isopropanol was added to the supernatant, mixed well, and centrifuged immediately at 13,000× g for 10 min. The pellet was washed with 70% ethanol and vacuum dried.

Note: To prepare plasmids for sequencing analysis the following additional steps were carried out.

7. The plasmid DNA was dissolved in 32 µl of deionised water and precipitated by adding 8 µl of 4 M NaCl, and 40 µl of autoclaved 13% PEG_{8000} (polyethylene glycol).

8. After thorough mixing, the sample was incubated on ice for 20 min, and then centrifuged for 15 min at 4°C to pellet the DNA.

9. The supernatant was carefully removed and the pellet was rinsed with 500 µl of 70% ethanol. The pellet was dried under vacuum for 5 min, resuspended in 20 µl of deionised water and stored at -20°C.

F. Restriction digestion of the plasmids

In order to confirm the presence of the desired insert in the isolated plasmids, they were digested with proper restriction endonucleases.

2.15. Cloning PCR amplified DNA fragments

pBluescript II $KS^{(+)}$ (Stratagene, 2.96 kb) was used as a vector to clone PCR products. The vector was prepared by cutting $pBKS^{(+)}$ with *Eco*RV and adding a 3' terminal thymidine to both

ends. These single 3'-T overhangs at the insertion site ligate to the non-template dependent addition of a single deoxyadenosine to the 3'-end of PCR products by *Taq* polymerase.

A. Construction of a T-tailed vector using pBluescript®II KS(+)

A.1. Digestion

The vector was purified using Promega's Wizard™ Clean-up DNA System. The following digestion reaction was assembled:

10 µl	pBKS(+) (~10-20 µg)	
4 µl	10× restriction buffer	
4 µl	*Eco*RV (40 u, a blunt-end cutter)	
22 µl	H_2O	Σ: 40 µl

The mixture was incubated at 37°C for 3.5h which was followed by DNA purification using Promega's Clean-up system. The DNA concentration was estimated by spot-test (see below).

A.2. T-tailing

In order to add a T tail to the blunt ends of the vector, the following reaction was set up by providing dTTP and *Taq* polymerase:

5 µl	pBKS(+) (~1 µg)	
2 µl	10×Taq polymerase buffer	
0.2 µl	*Taq* polymerase (5.5 u/µl)	
0.4 µl	100 mM dTTP	
1.2 µl	25 mM $MgCl_2$	
11.2 µl	H_2O	Σ: 20 µl plus 20 µl mineral oil

The mixture was incubated at 70°C for 2h. 100 µl H_2O and twice the volume of phenol was added, mixed and centrifuged at 13,000× g for 3 min. The supernatant was transferred to a fresh tube to which NaAc (200 mM) and two volumes ethanol were added, mixed and DNA precipitated at -20°C overnight. DNA was pelleted by centrifugation at 13,000× g at 4°C for 15 min. The pellet was vacuum dried and dissolved in 20 µl H_2O and stored at -20°C.

PCR amplified DNA fragments were then ligated into the vector as described in cloning DNA fragments (see above).

2.16. Spot test (concentration check)

The concentration of small amounts of DNA was estimated by this method. A 1% agarose gel containing ethidium bromide (4.3 μg/ml) was cast. When the agarose had polymerised, 1μl of the diluted DNA solution (1×, 5× and 10×) was spotted on the gel. Also, 1 μl of various concentrations of a marker (molecular weight marker IV, Boehringer Mannheim, 40, 20, 10, 5, 2.5 and 1.25 ng/μl) was spotted on the gel. After the drops were dried, the gel was observed over UV illumination and photographed. The concentration of the DNA was estimated by comparing the illumination intensity of the samples with that of the markers.

2.17. Extraction of DNA from agarose gel

DNA samples were run on a 1% low-melting agarose gel in TAE containing ethidium bromide (4.3 μg/ml). The desired DNA fragment was cut out by visualising the DNA in the gel on a UV illumination box. The gel piece was transferred into a fresh microfuge tube and incubated in a waterbath at 65-70°C for 15 min to melt the agarose. The DNA was extracted from agarose using Wizard™ PCR Preps (Promega). The concentration of the extracted DNA was measured by a spot test.

2.18. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed to separate proteins on the basis of their size and charge under denaturing conditions. Mini-Protean®II Dual Slab Cell system (BioRad) was used to run the proteins on 12% polyacrylamide gels at 20 mA per gel. The reagents and buffers were made as described (Laemmli, 1970; Sambrook *et al.*, 1989). The gels were either stained or the proteins were transferred onto a nitrocellulose membrane by Western blotting for further analysis (see below).

2.19. Staining and destaining of polyacrylamide gel

To stain the gel, it was immersed in Commassie stain (0.25 g Coomassie Brilliant Blue R-250 in 90 ml of 50% methanol and 10 ml glacial acetic acid) for 30 min at room temperature on an

orbital shaker and then destained by incubating the gel in destain solution (10% glacial acetic acid, 30% methanol) and shaking until the desired destaining was obtained (Sambrook *et al.*, 1989).

The gels, were dried using a gel drier (BioRad).

2.20. Western blotting

In order to analyse the proteins separated on a denaturing polyacrylamide gel, they were transferred onto a nitrocellulose membrane (Amersham) by Western blotting as described in (Sambrook *et al.*, 1989). The basis is to transfer negatively charged proteins from the gel to a solid support (membrane) by applying an electric field.

A. Probing Western blots with lectin

1. The nitrocellulose filter containing the transferred proteins was washed twice for 15 min in TBST.

2. The membrane was incubated in peroxidase-conjugated *H.p.* lectin (Sigma, dilution in 1:10000 TBST) for 3h to overnight at room temperature.

3. Washing the membrane was carried out four times by shaking it in TBST for 10 min each.

4. The membrane was then immersed in staining solution (6 mg 3,3'-diaminobenzidine, 20 µl of 30% H_2O_2 in 10 ml 50 mM Tris-HCl, pH 7.0) until the bands appeared. Then, the membrane was washed in deionised water and dried.

B. Probing Western blots with antibodies

1. The nitrocellulose membrane was washed in PBS for 10 min and then blocked by the first blocking solution for 1h with shaking.

2. The antiserum (first antibody) was added to the blocking solution (1:10,000) and incubated for 2h to overnight with shaking.

3. The membrane was washed three times in 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 10 min each.

4. The membrane was then incubated in the second blocking solution containing secondary antibody (1:10,000; anti-rabbit IgG, Sigma) conjugated with alkaline phosphatase for 1 h.

5. The membrane was washed twice in 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 15 min each and immersed in the staining solution consisting of alkaline phosphatase buffer containing NBT and BCIP until the desired bands were appeared. The reaction was stopped by rinsing the membrane with water several times.

2.21. Buffers and solutions

PBS (1 l)

8 g	NaCl
0.2 g	KH_2PO_4
2.9 g	Na_2HPO_4
0.2 g	KCl

Cecropia Ringer

100 mM	Tris-HCl, pH 6.8
100 mM	NaCl
40 mM	KCl
15 mM	$MgCl_2$
4 mM	$CaCl_2$

Homogenisation buffer

4 µl	Tris-HCl, pH 8.0
8 µl	0.5 M EDTA, pH 8.0
40 µl	10% SDS
343 µl	H_2O

10×TBE (1 l)

108 g	Tris-base
55 g	boric acid
20 ml	0.5 M EDTA (pH 8.0)

50×TAE (1 l)

242 g	Tris base
57.1 ml	glacial acetic acid
100 ml	0.5 M EDTA (pH 8.0)

DEPC (Diethylpyrocarbonate)-water

0.2 ml DEPC was added to 100 ml of water, shaken vigorously to get the DEPC into solution. The solution was autoclaved to inactivate the remaining DEPC.

Denaturing solution

25 g guanidium thiocyanate was dissolved in 29.3 ml DEPC-treated water. 1.7 ml 0.75 M sodium citrate, pH 7.0, and 2.6 ml 10% sarcosyl was added at 65°C. The solution was kept as a stock solution up to three months at room temperature. For every application, 5 ml of the stock solution was mixed with 36 µl 2-mercaptoethanol.

2 M NaAc, pH 4.0

16.42 g of sodium acetate was added to 40 ml water and 35 ml glacial acetic acid. The pH was adjusted to 4.0 with glacial acetic acid and the final volume was made to 100 ml with water.

Phenol (water-saturated)

100 g phenol crystals were dissolved in water at 60°C. The upper water phase was aspirated and the phenol was stored at 4°C.

10×MOPS

0.4 M	MOPS, pH 7.0
0.1 M	sodium acetate
0.01 M	EDTA

20×SSC

175.3 g NaCl and 88.2 g of sodium citrate were dissolved in 800 ml water. The pH was adjusted to 7.0, the volume to 1 l and autoclaved.

RNA loading buffer

50%	glycerol
1 mM	EDTA (pH 8.0)
0.25%	bromophenol blue
0.25%	xylene cyanol FF

DNA loading buffer (6×)

0.25%	bromophenol blue
40% (w/v)	sucrose in water

Prehybridisation solution

6× SSC
5×Denhardt's reagent
0.5% SDS
100 μg/ml Herring sperm DNA (boiled before adding to the solution)

50× Denhardt's reagent (0.5 l, stored at -20°C)

5 g	Ficoll (Type 400)
5 g	polyvinylpyrrolidone
5 g	bovine serum albumin
500 ml	water

LB medium (1 l)

10 g	bacto-agar
5 g	yeast extract
5 g	NaCl
0.2 ml	5M NaCl

Adjusted to 1 lit and sterilised by autoclaving.

LA-plates

15 g agar was added to 1 l LB, autoclaved and cooled to 50°C. 100μg/ml ampicillin was added before pouring into petridishes.

When IPTG/X-Gal containing plates were required, 200 μl IPTG (20 mg/ml) and 20 μl X-Gal (50 mg/ml) were added per plate by spreading them with a sterile spreader.

Solution I

50 mM	glucose
25 mM	Tris-HCl (pH 8.0)
10 mM	EDTA (pH 8.0)

Solution II

0.2 N	NaOH
1%	SDS

Solution III

3 M	potassium acetate
5 M	glacial acetic acid

2×Cracking solution (50 ml)

5.0 ml	1 M NaOH
1.0 ml	0.5 M EDTA
0.5 g	SDS
0.025 g	bromocresol green
5 ml	glycerol

Transfer Buffer (pH 8.3)

2.9 g	Glycine
5.8 g	Tris base
200 ml	Methanol
0.37 g	SDS

TBST

10 mM	Tris-HCl, pH 8.0
150 mM	NaCl
0.05%	Tween 20

First blocking solution

8 g	non-fat dry milk
0.02%	sodium azide
100 ml	PBS

Second blocking solution

5 g	non-fat dried milk
15 ml	1M NaCl
5 ml	1M Tris-Cl (pH 7.5)
80 ml	distilled water

Alkaline phosphate buffer

100 mM	NaCl
5 mM	$MgCl_2$
100 mM	Tris-Cl (pH 9.5)

50 µl NBT and 50 µl BCIP were added to 10 ml alkaline phosphate buffer to make the staining solution.

NBT

0.5 g NBT (Nitro Blue Tetrazolium chloride)
10 ml 70% Dimethylformamide

BCIP

0.5 g BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate), disodium salt
10 ml 100% Dimethylformamide

CHAPTER 3

Host hemocyte inactivation and expression of *C. rubecula* polydnavirus genes

Chapter 3

Host hemocyte inactivation and expression of *C. rubecula* polydnavirus genes

3.1. Introduction

Polydnaviruses (PVs) have been isolated and characterised from several hymenopteran wasps belonging to a number of subfamilies (Fleming, 1992; Krell, 1991; Stoltz and Vinson, 1979b). PVs are produced in the calyx region of the female reproductive organ. The virus particles are injected into the host caterpillar along with the egg(s) upon parasitisation. Several studies have shown their functional role in host regulation and they invade tissues of the host such as hemocytes, fat bodies, and prothoracic gland as well as the nervous system (Edson *et al.*, 1981; Krell, 1987; Strand *et al.*, 1992). As a result of virus infection, the parasitoid is able to survive detrimental defence reactions of its host. PVs also bring about a number of physiological and developmental changes in the host to provide a suitable environment for the development of the parasitoid (Dahlman *et al.*, 1990; Davies *et al.*, 1987; Tanaka and Vinson, 1991).

Infection of host hemocytes by PVs causes major changes in cell shape and structure leading to failure to mount a cellular defence. One of the most prominent syndromes is the loss of spreading behaviour (Davies *et al.*, 1987; Prevost *et al.*, 1990) which is normally exhibited by normal hemocytes upon attachment to a foreign surface such as a glass slide. Reduction in the total hemocyte count has also been reported (Davies *et al.*, 1987) which could be due to cell death and subsequent removal from the circulation. Apoptosis of *Pseudoplusia includens* granulocytes following infection by *Microplitis demolitor* PVs has been described as a possible cause for the reduction in hemocyte numbers (Strand and Pech, 1995b). Apoptosis involves programmed cell death where the cell undergoes blebbing, fragmentation of nuclear material and eventually disintegration of chromosome into apoptotic bodies (Cohen, 1993). Granulocytes are the first blood cell types to be involved in encapsulation (Pech and Strand, 1996), therefore, by removing them from circulation via PV-mediated apoptosis, the immune system of the host is basically inactivated and cannot launch effective cellular defence measures against the parasitoid egg.

Besides using cellular criteria for immunocompetency that were previously applied to hemocytes such as the detection of spreading, the staining of actin and the detection of apoptosis, two novel assays were developed in order to examine hemocytes from *P. rapae* caterpillars: 1) the staining of hemocyte surfaces with lectins which is based on earlier observation that imply a redistribution of specific surface glycoproteins in the cellular immune response (Rizki and Rizki, 1986); and 2) the detection of membrane rearrangements that lead to the formation of microparticles and can be detected by the exposure of aminophospholipids on the surface of hemocytes (Theopold and Schmidt, 1997).

Here, the alterations observed in hemocytes of *P. rapae* caterpillars parasitised by *C. rubecula* are described with regard to the integrity of their cell cytoskeleton and expression of surface molecules. In addition, the timing and tissue-specific expression of *C. rubecula* PV (CrV) genes in the parasitised host is documented on Northern blots and tissue sections.

3.2. Materials and Methods

The methods used in the experiments reported in this chapter are described in chapter 2 (general materials and methods):

1. Isolation of fat body, hemocytes and hemolymph

2. F-Actin staining of hemocytes with phalloidin

3. Lectin binding to hemocytes *in vitro*

4. Isolation of polydnaviruses

5. DNA extraction of polydnaviruses

6. Extraction of total RNA and isolation of mRNA from total RNA

7. Radiolabelling of DNA

8. Northern blot analysis

3.3. Results

3.3.1. Host hemocyte alterations following parasitisation

One of the most prominent changes observed in hemocytes from parasitised *P. rapae* caterpillars is the apparent loss of spreading behaviour on a glass surface. Normal hemocytes from unparasitised caterpillars attach and flatten extensively upon contact with a foreign surface such as a glass surface (Fig. 3-1a) while hemocytes isolated from parasitised caterpillars do not spread (Fig. 3-1b) and appear round shaped making it difficult to distinguish morphotypes. Since changes in cell shape are mediated via alterations of actin filaments (Carlier, 1993), hemocytes were examined with regard to their F-actin filaments status. Using FITC-conjugated phalloidin as a specific indicator of F-actin, hemocytes were examined under indirect UV illumination. Cells from unparasitised caterpillars showed extensive spreading of plasmatocytes with abundant stress actin fibres (Fig. 3-2a) and granulocytes remained somewhat round shaped with protruding lamellopodia. In contrast, hemocytes from parasitised caterpillars where all round shaped without stress fibres and lamellopodia. In cases where attachment to the glass surface was detected, the cells showed a "fried egg" appearance with a faint staining as a thin ring around the cell membrane and the nucleus, indicating breakdown of cytoskeleton (Fig. 3-2b).

To test whether cell surface properties are changed in parasitised hemocytes, cells were allowed to attach to a glass surface in a calcium containing buffer and treated with FITC-conjugated *Helix pomatia* lectin (*H.p.* lectin). *H.p.* lectin specifically recognises a mucin-like glycoprotein on *D. melanogaster* hemocytes, which is involved in immune induction (Theopold *et al.*, 1996). In hemocytes from unparasitised caterpillars a large proportion of hemocytes, mainly granulocytes, were heavily labelled by the lectin (Fig. 3-3B), whereas in hemocytes from parasitised caterpillars changes in lectin-binding became visible 6h after parasitisation when the lectin-label on the hemocyte surface starts to decrease and by 18-24h had completely disappeared (Fig. 3-3D). In addition, when caterpillars were bled into an EDTA-containing or calcium-free buffer, the labelling was not observed in both parasitised and unparasitised hemocytes. This indicates that in normal hemocytes the mucin-like glycoprotein appears on the surface upon

immune activation, given that the binding of lectins to sugar moieties is calcium-independent (Bayne, 1990). Loss of exposure of lectin-binding molecules on their surface in parasitised caterpillars may imply a concomitant loss of immune competency. However, when TRITC-conjugated wheat germ agglutinin was applied to hemocytes no difference was observed in staining of hemocytes from parasitised and unparasitised caterpillars (data not shown). This suggests that *H.p.* lectin may recognise specific glycoproteins that are part of the cellular alterations occurring in immune-activated hemocytes.

Observations at different time points following parasitisation showed that the immune-related alterations in the surface properties of hemocytes are temporary. After 2-3 days the cells recover and manifest normal characteristics indistinguishable from uninfected hemocytes. Using Hoechst staining on hemocytes from parasitised caterpillars, no evidence for apoptosis was found (Fig. 3-4). This is in contrast to the observations in a related braconid system (Strand and Pech, 1995b) where most of the granulocytes are removed from circulation by PV-mediated cell death. Apoptotic cells which are programmed to die show blebbing, fragmentation of nuclear material and eventually disintegrate and are subsequently scavenged by other hemocytes. The system recovers after several days due to increased mitotic activities of remaining precursor cells. In parasitised *P. rapae*, the recovery of hemocytes occurs in the absence of newly induced mitotic figures, as indicated by Hoechst staining. This further supports the assumption of a hemocyte recovery rather than a replacement by new populations of hemocytes.

To test whether the cellular process of hemocyte activation is inhibited in parasitised caterpillars, microparticle formation was analysed in isolated hemocytes from parasitised and non-parasitised caterpillars. When hemocytes are isolated in calcium-containing buffer and exposed to glass surfaces, blebbing and microparticle formation occurs (Theopold and Schmidt, 1997). A specific property of microparticle formation is the exposure of phosphatidylserine (PS) on the outer leaflet of cellular and particle membranes, which is specifically recognised by annexin V (Pasquet *et al.*, 1996). Annexin V is a member of the calcium and phospholipid binding proteins with vascular anticoagulant activity that is largely found on the cytosolic face of plasma membranes (Thiagarajan and Tait, 1990). In the presence of calcium, annexin V has a high affinity for negatively charged phospholipids which only occur at the outer leaflet under

conditions when cell death is imminent or when membrane vesicles are formed (Theopold and Schmidt, 1997). Therefore, the presence of exposed PS in *P. rapae* hemocyte preparations was investigated using annexin V as a probe. It was found that microparticle formation and PS exposure are a feature of normal hemocytes (Fig. 3-5), but are virtually absent in hemocyte preparations from parasitised caterpillars (Fig. 3-6) which suggests that the hemocytes are in effect immune incompetent.

3.3.2. Temporal expression of CrV genes in the host

To test whether virus expression and hemocyte inactivation correlate, Northern blots were prepared and analysed using labelled probes. Total RNA extracted from *P. rapae* caterpillars at various times after parasitisation was analysed on blots using radioactively labelled *C. rubecula* viral DNA (CrV-DNA), comprising the complete virus genome. Hybridisation results suggest that the virus RNA is expressed in host tissues within a distinct time period, starting from four hours after parasitisation to eight hours post-parasitisation (Fig. 3-7a). Analyses of the RNA on a Northern blot revealed that two major RNAs were detected in caterpillars and the peak of gene expression in both RNAs was observed six hours after parasitisation (Fig. 3-7b). RNA from unparasitised caterpillars did not cross-hybridise to the probe. To establish whether the expressed RNA constitutes protein-coding RNA, a polyA^{+}-RNA fraction from caterpillars was tested on a Northern blot (Fig. 3-8, lane 1). The hybridisation experiments suggest that the labelled virus DNA hybridised to two transcripts with an estimated size of 1.4 kb (CrV1) and 1.1 kb (CrV2).

To establish whether there is a tissue-specific expression of virus DNA in the host caterpillar, RNA from the fat body and hemocytes was tested on Northern blots using total CrV-DNA as a probe. Under these conditions only a single transcript (CrV1) was detected in hemocytes (Fig. 3-8, lane 5), even under overexposed conditions, whereas in the fat body both CrV1 and CrV2 were detected similar to the signals in total RNA (Fig. 3-8, lane 3). The two transcripts, CrV1 and CrV2, are probably from unrelated virus genes (see chapter 4).

3.4. Discussion

Transcription of *C. rubecula* polydnavirus (CrV) genome in the host caterpillar, *P. rapae,* occurs from one or two genes in a transient fashion, in contrast to other known host-parasitoid systems, where viral multigene families are transcribed in host tissues and transcripts persist over most of the parasitoid's development inside the host caterpillar (Strand *et al.*, 1992; Theilmann and Summers, 1986). Hybridisation experiments indicate that CrV-DNA is expressed within a distinct time period in the host tissues, starting from 4h post-parasitisation (p.p.) to approximately 12h p.p. suggestive of a high turn-over of transcripts. In contrast, *C. sonorensis* (Hym: Ichneumonidae) PV transcripts were found 2h to 9 days p.p. in *H. virescens* larvae (Fleming *et al.*, 1983). Similarly, the expression of *M. demolitor* (Hym: Braconidae) polydnaviral DNA in *P. includens* larvae initiates 4h p.p. and continues at least six days following parasitisation (Strand *et al.*, 1992).

The exceptional mode of expression of CrV-DNA is not only manifested through its narrow peak of expression but also by its low number of viral transcripts. The hybridisation data presented here, demonstrate that only two viral poly(A)$^+$ containing RNAs are expressed in *P. rapae* caterpillars, namely CrV1 and CrV2, from which CrV1 is highly expressed. In all other parasitoid-virus-host interactions known at the molecular level, several PV transcripts are found in the host following parasitisation. In *H. virescens* larvae parasitised by *C. sonorensis* more than 12 viral mRNAs are detected between 12 to 48h p.p. (Blissard *et al.*, 1986a). Although the functional role of CrV genes is not known, the observation that only two of the genes are expressed in parasitised caterpillars suggests that immune suppression in this system may be achieved by the product of one or two genes and therefore facilitate the identification of the immune suppressor gene(s).

Similar to other known systems, *C. rubecula* PVs infect various host tissues including fat bodies and hemocytes which are the main tissues involved in intermediate metabolism and immune reactions, respectively. However, a unique and fascinating observation which was made in hybridisation experiments was that only CrV1 is expressed in infected hemocytes of the host. This suggests that CrV1 may be solely responsible for the immune suppression.

The disappearance of cell surface glycoproteins recognised by the *H. pomatia* lectin may constitute a cellular read out for changes in the adhesive properties, since other lectin-binding properties, like wheat germ agglutinin did not change after parasitisation. In *Drosophila*, the *H.p.* lectin specifically binds to a mucin-like glycoprotein which is involved in immune induction of hemocytes (Theopold *et al.*, 1996). In *P. rapae* hemocytes, the lectin binds predominantly to granulocytes, which in lepidopterans are known to initiate the immune response (Ratcliffe, 1993). In parasitised caterpillars the lectin-labelling on the surface of isolated granulocytes changes between 4 and 8h p.p. and has completely disappeared by 24h p.p. The disappearance of lectin-labelling could be due to the reduction in the amount of exposed glycoprotein on hemocytes or underglycosylation of the surface molecule (see below). The changes of lectin-labelling coincide with changes in adhesive properties of the cells. A reduction in glycosylation of proteins has been reported in insect cell lines infected with baculoviruses (Vandie *et al.*, 1996). Also, reduction of glycosylation has been shown to increase susceptibility of human cell lines to human immunodeficiency virus (HIV) and papovavirus infection (Keppler *et al.*, 1994; Talbot *et al.*, 1995).

A significant observation is that both granulocytes and plasmatocytes from parasitised caterpillars differ from non-parasitised hemocytes in the cytoplasmic structure of F-actin filaments, suggestive of a disruption of cell surface and cytoplasmic connections. This could explain the incapacity of virus infected hemocytes to expose mucin-like molecules on the surface. The virus-related hemocyte inactivation in parasitised caterpillars is only temporary and disappears after 2-3 days p.p. suggestive that actin connections to the cell cortex are restored by a slow process.

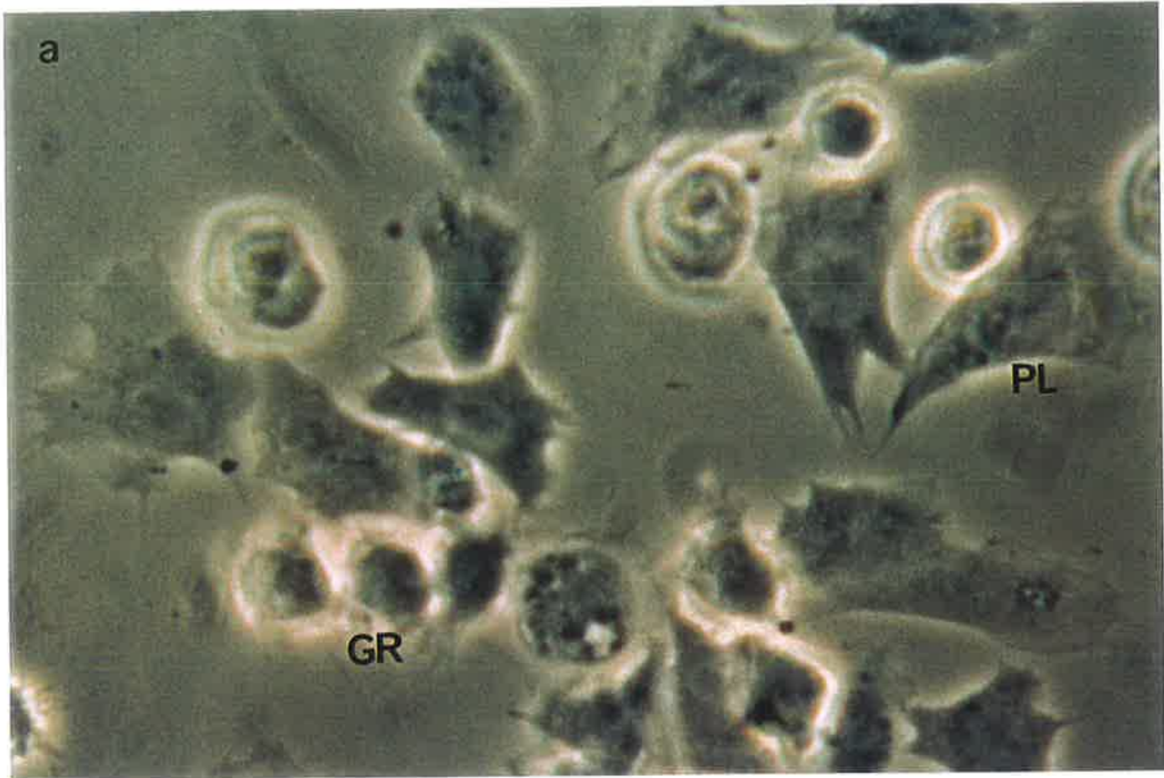
The discharge and exposure of adhesive components on the cell surface probably enables hemocytes to attach to foreign surfaces. In addition, the formation of microparticles has been recently shown to be part of insect hemocyte activation (Theopold and Schmidt, 1997). Activated hemocytes form blebs and cellular extensions leading to vesicle formation, which are similar to microparticles in activated mammalian platelets (Yano *et al.*, 1994). A characteristic feature of particle formation is the exposure of phosphatidylserine on the outer leaflet of the cellular and particle membrane, which can be identified using annexin V as a diagnostic marker.

The presence of PS on microparticles is known to enhance the coagulation process in vertebrate systems. Since both degranulation and microparticle formation involve a rearrangement of the cytoskeleton (Rosales *et al*., 1994; Yano *et al*., 1994), the absence of functional actin-filaments in CrV infected hemocytes is probably the primary cause of hemocyte inactivation. Since CrV1 was found to be the only CrV gene expressed in host hemocytes, the changes observed are presumably caused exclusively by this gene (see chapter 5 for experimental proof).

Previous results suggest that *C. rubecula* eggs are passively protected against the host's immune recognition by a calyx fluid protein(s) covering the egg surface (Asgari and Schmidt, 1994) precluding immune recognition and inactivation of the egg immediately after parasitisation. Hemocyte inactivation, starting 4h p.p., may then provide protection for the emerging parasitoid larva at a later stage of wasp development inside the caterpillar.

Figure 3-1

Phase contrast micrographs of hemocytes from *P. rapae* caterpillars after contact with a glass slide. **a)** Hemocytes from an unparasitised caterpillar, plasmatocytes (PL) showing normal spreading and granulocytes (GR) forming lamellopodia; **b)** hemocytes from an 18h parasitised caterpillar, the hemocytes do not spread and appear as round shaped.


a
PL
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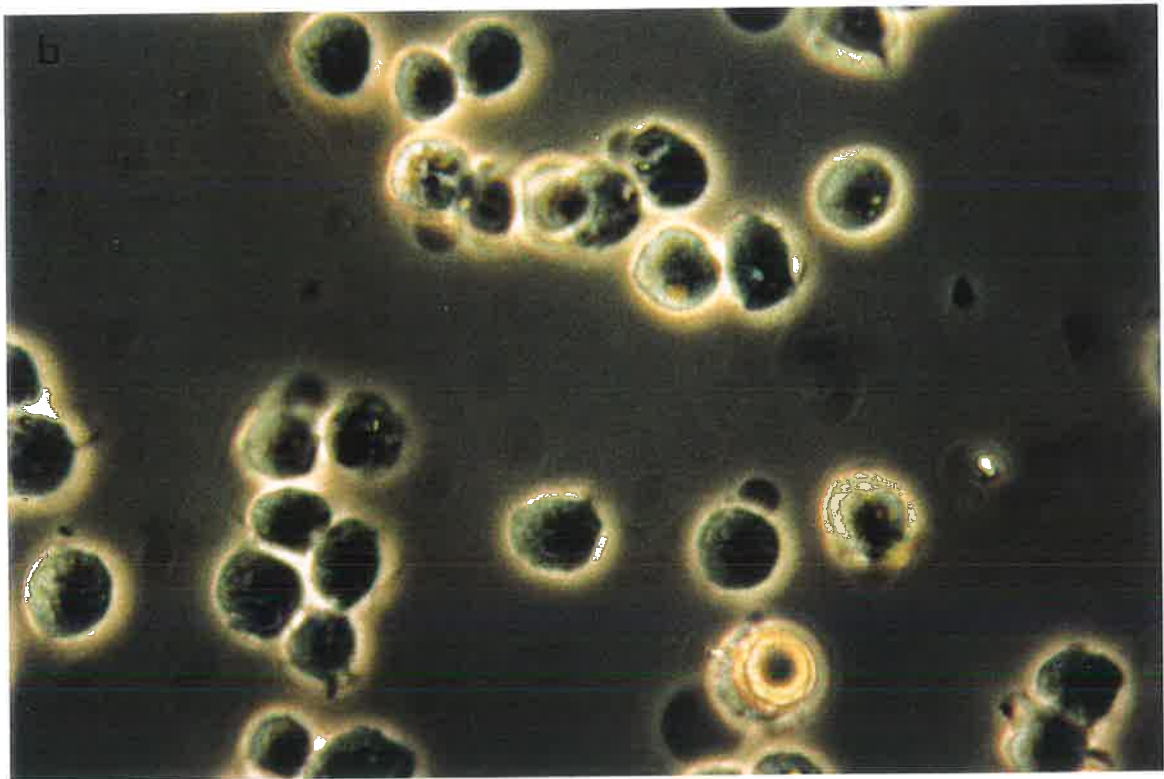
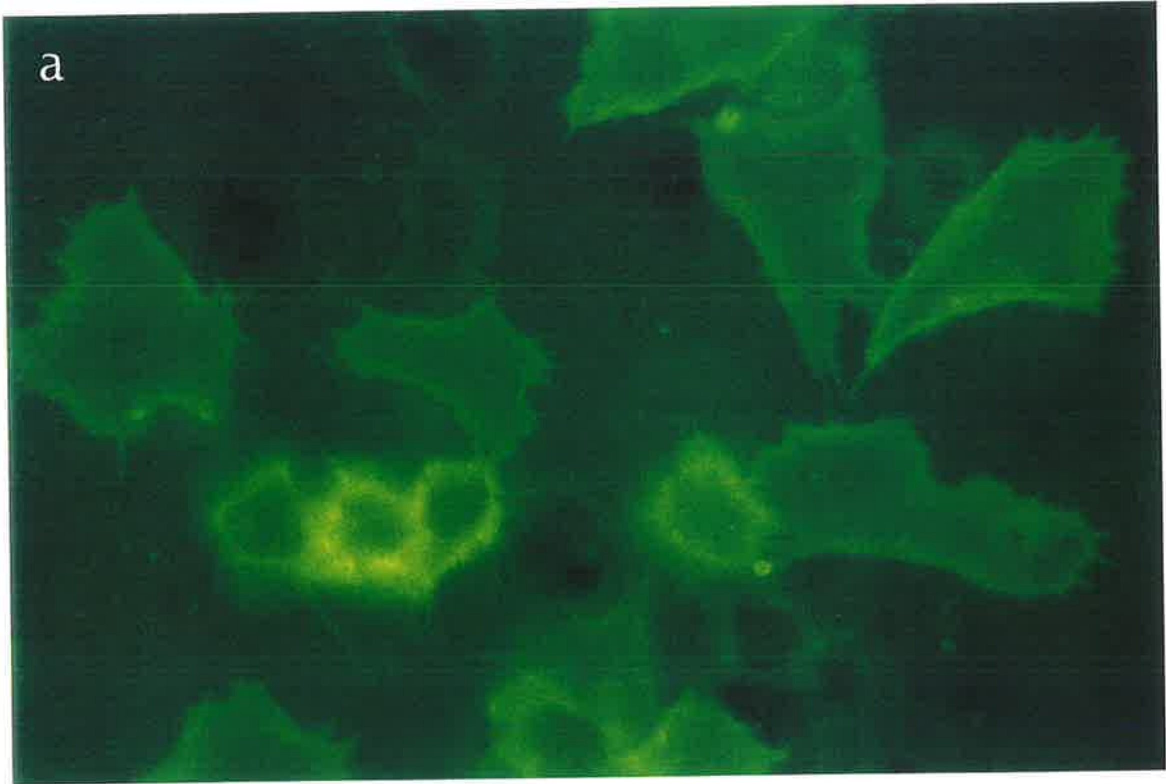

b

Figure 3-2

F-actin staining in *P. rapae* hemocytes. **a)** Hemocytes form an unparasitised caterpillar stained with FITC-conjugated phalloidin under indirect UV light showing normal spreading of plasmatocytes with abundant stress actin fibres and granulocytes with lamellopodia; **b)** hemocytes from a caterpillar parasitised 18h previously. Actin staining is present as a thin ring around the cell membrane and nucleus, indicating the breakdown of cytoskeleton.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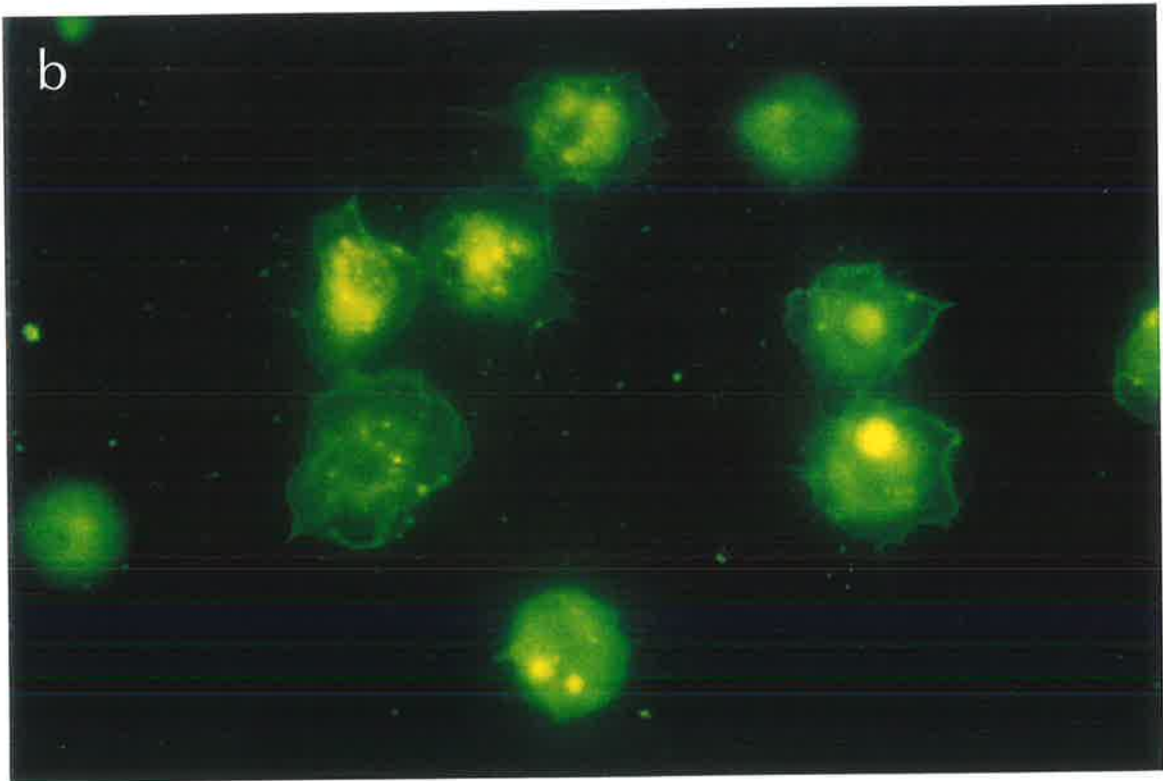
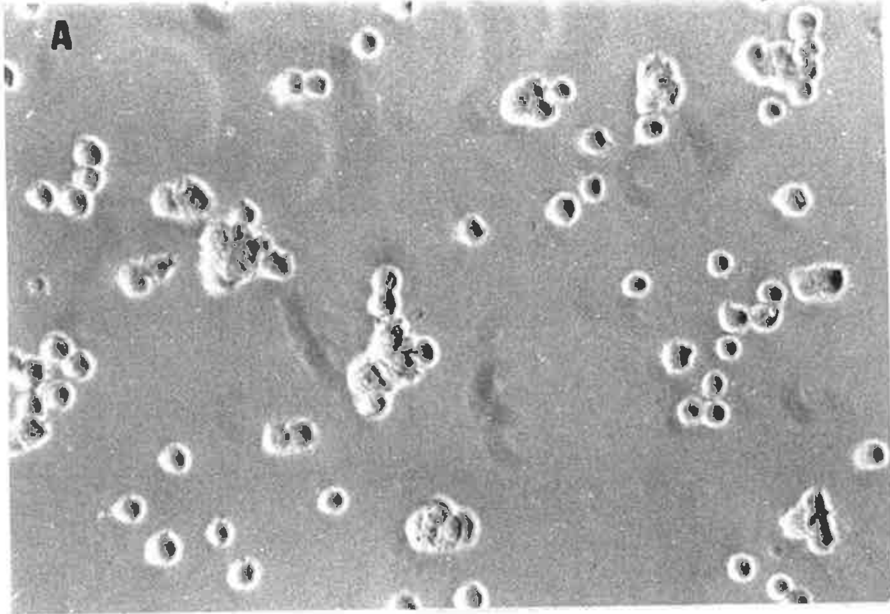

b

Figure 3-3

Lectin-staining of *P. rapae* hemocytes. **A)** Hemocytes from an unparasitised caterpillar under phase contrast; **B)** hemocytes stained with FITC-conjugated *Helix pomatia* lectin under indirect UV light; **C)** hemocytes from a caterpillar parasitised 24h previously under phase contrast; **D)** hemocytes from a parasitised caterpillar stained with FITC-conjugated *H. pomatia* lectin under UV light.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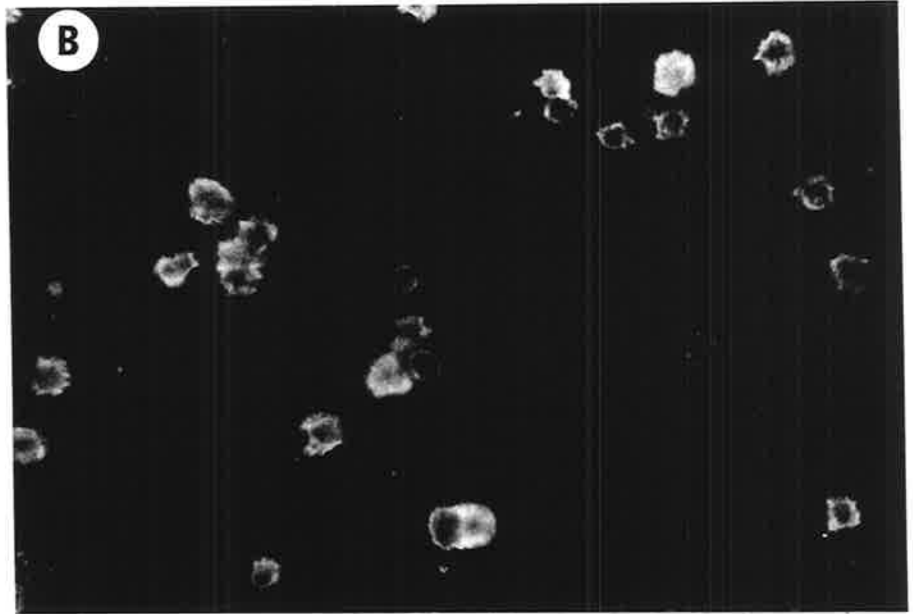
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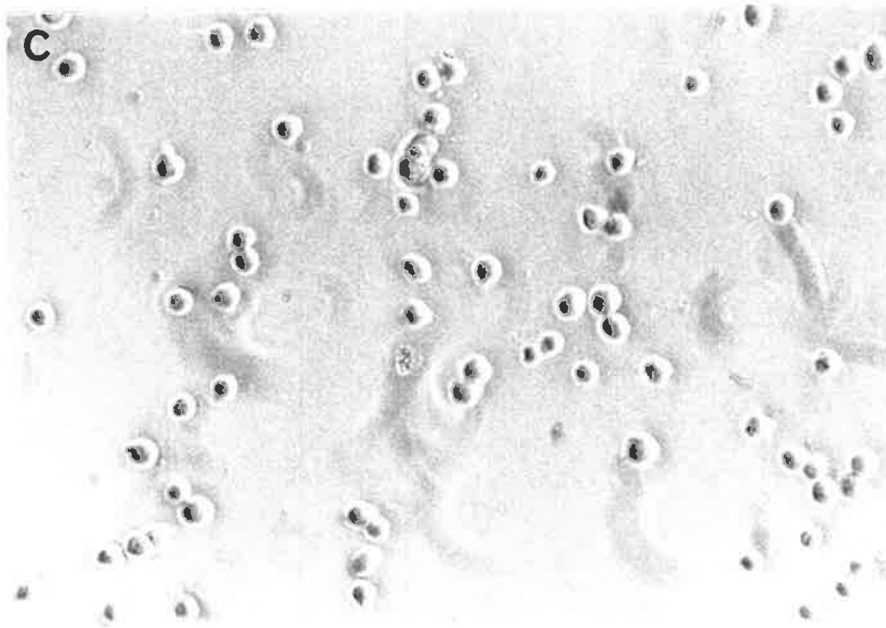
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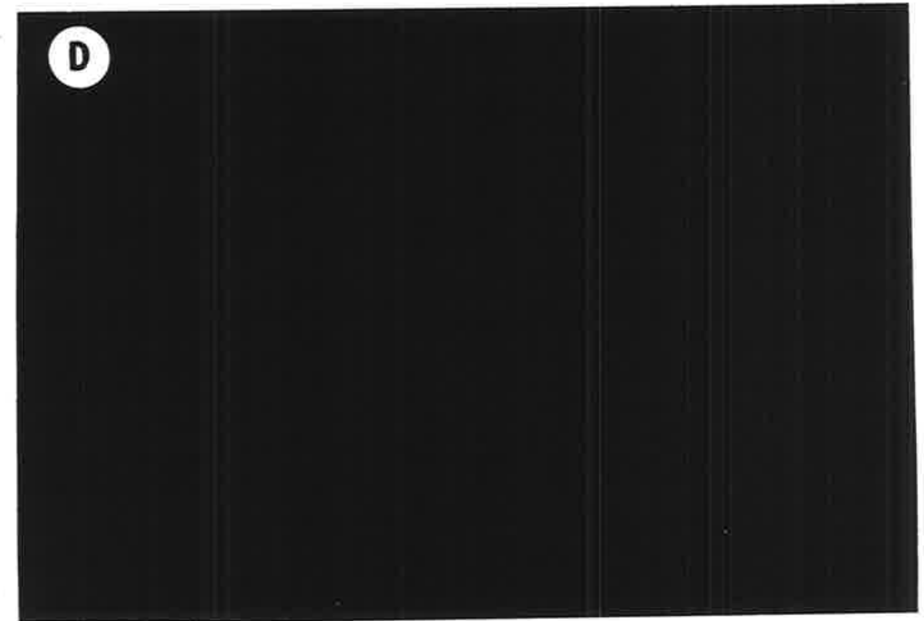

D

Figure 3-4

Hoechst staining in hemocytes from a 24h parasitised *P. rapae* caterpillar. The nuclei were found intact and no sign of apoptosis (fragmentation of nucleus) was observed.

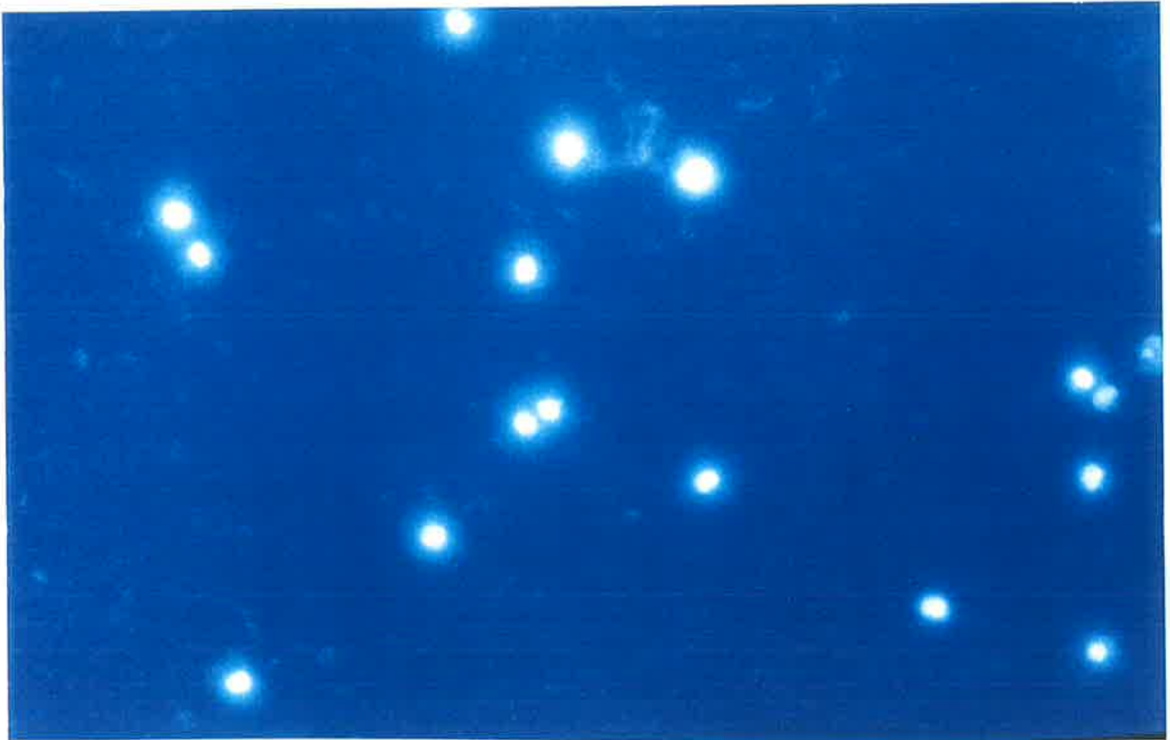
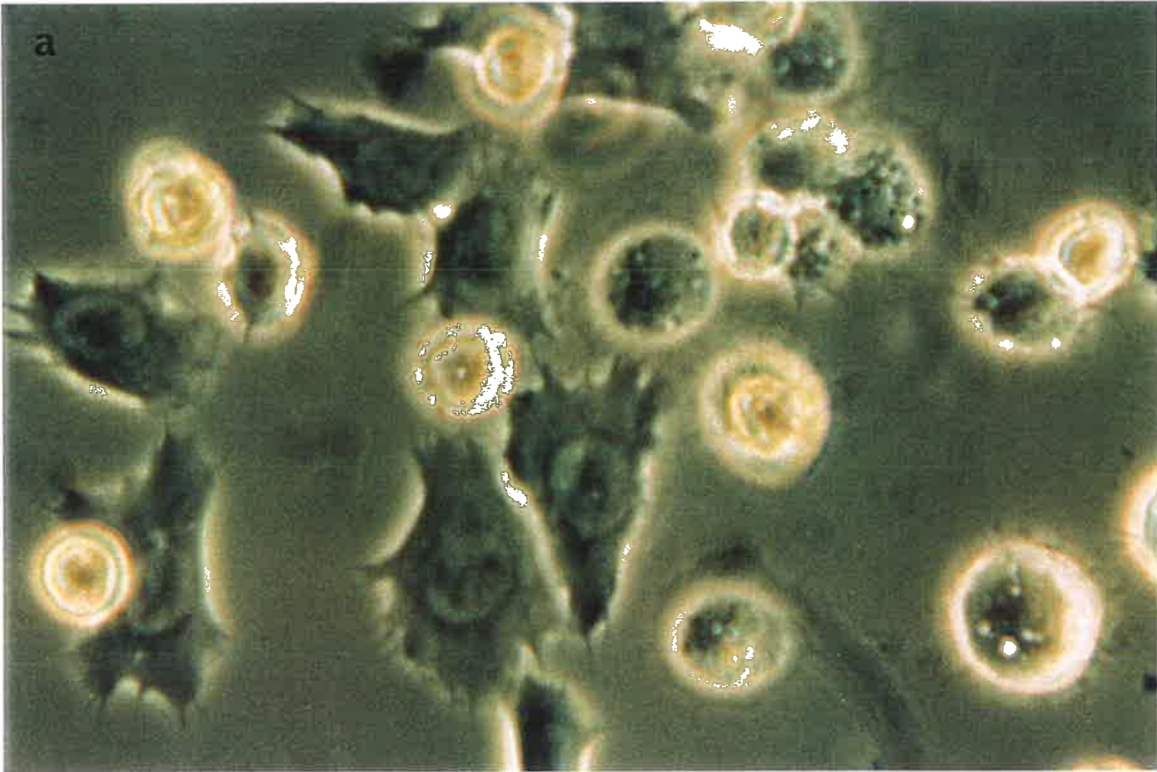


Figure 3-5

Microparticle formation in activated hemocytes of an unparasitised *P. rapae* caterpillar. **a)** Hemocytes under phase contrast; **b)** FITC-conjugated annexin V staining of the corresponding hemocytes. Annexin V specifically recognises phosphatidylserine exposed on the microparticles.


a

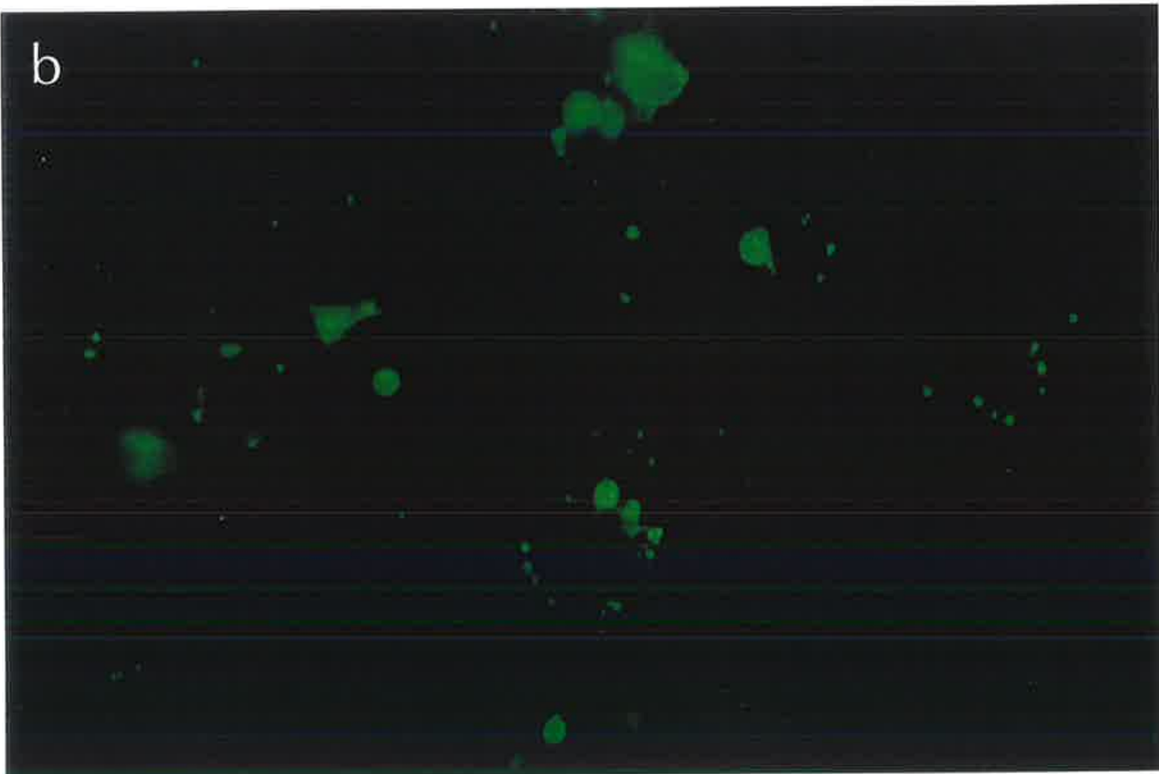
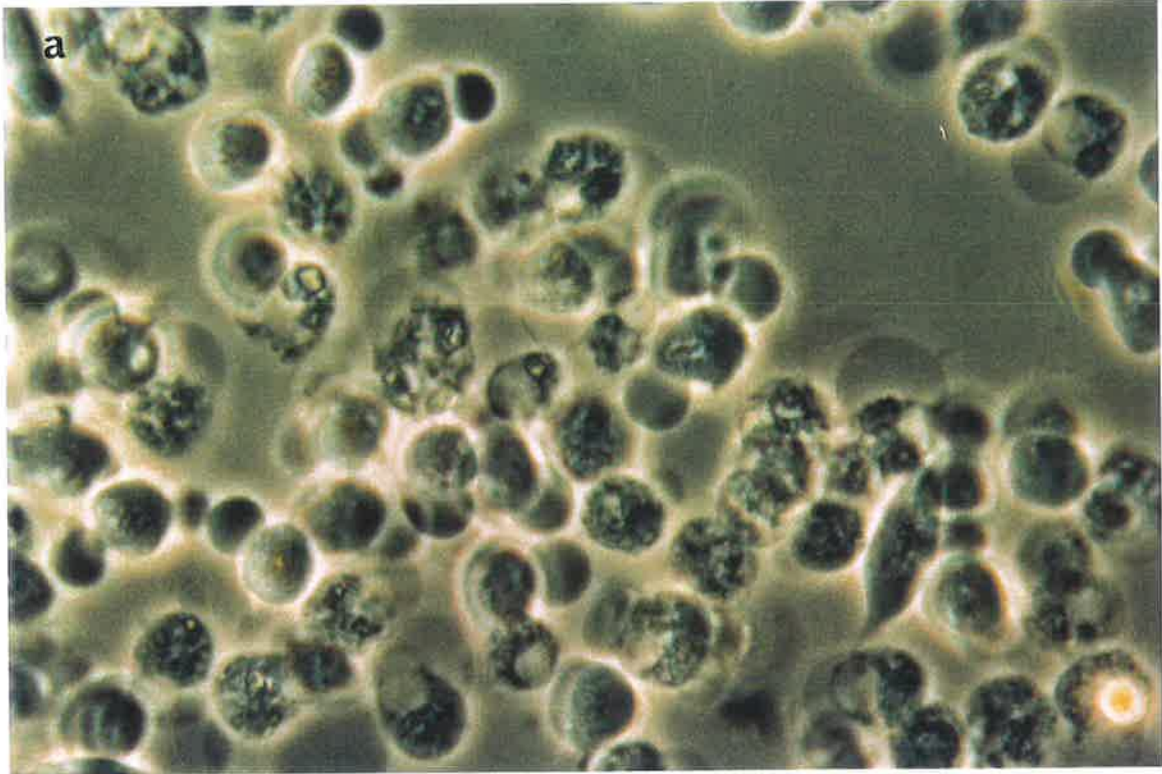

b

Figure 3-6

Microparticle formation in hemocytes from an 18h parasitised *P. rapae* caterpillar. **a)** Hemocytes under phase contrast, the cells do not spread; **b)** FITC-conjugated annexin V staining of the corresponding hemocytes, no microparticle formation was observed.


a

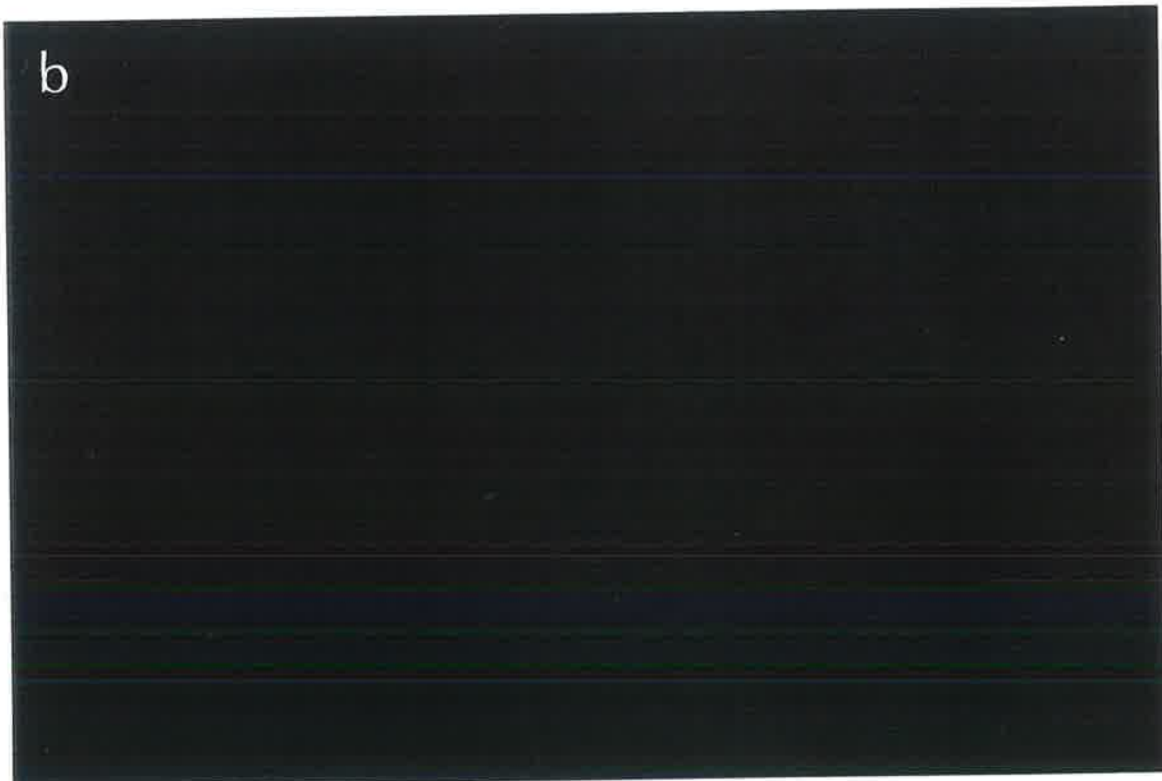
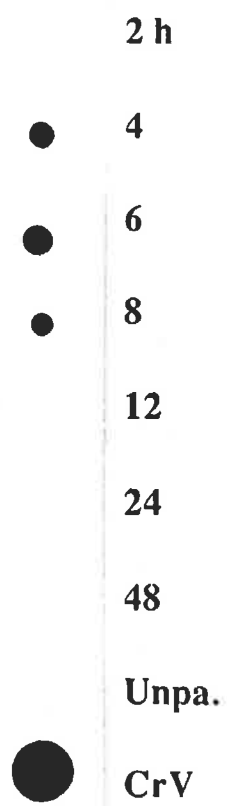

b

Figure 3-7

Hybridisation to blots containing total RNA isolated from *P. rapae* caterpillars at 2, 4, 6, 8, 12, 24 and 48h after parasitisation by *C. rubecula*. The filters were probed with ^{32}P-labelled *C. rubecula* viral DNA comprising the complete polydnavirus genome. **A)** Dot blot analysis; RNA from unparasitised caterpillars as a negative control (Unpa.); CrV, polydnaviral DNA as a positive control; **B)** Northern blot analysis, each RNA fraction was spectroscopically measured and equal aliquots of RNA (2 μg) were applied to each slot; RNA from unparasitised caterpillars was used as a control (lane C).

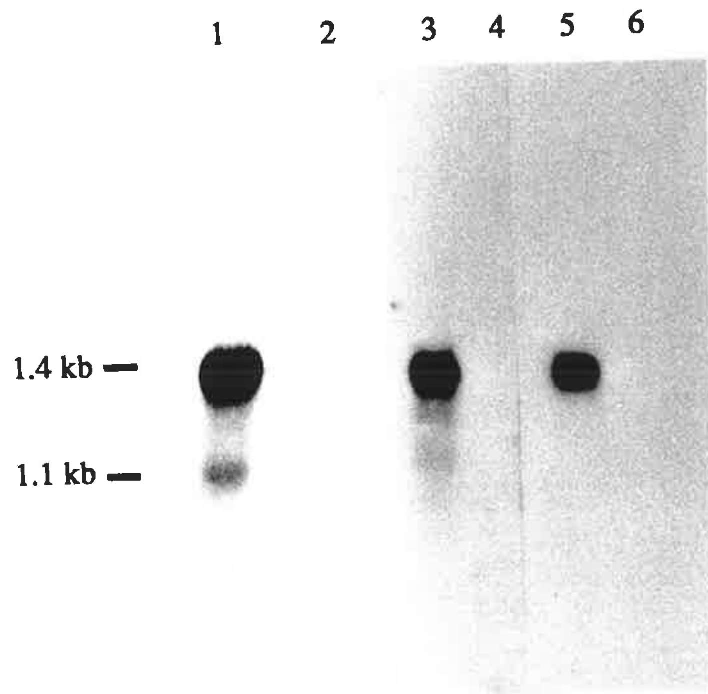
(A)



(B)

Figure 3-8

Hybridisation to a Northern blot containing **lane 1)** mRNA isolated from 6h parasitised *P. rapae* caterpillars (2µg per lane); **lane 2)** mRNA isolated from unparasitised caterpillars; **lane 3)** total RNA from the fat body of 6h parasitised caterpillars; **lane 4)** total RNA from the fat body of unparasitised caterpillars; **lane 5)** total RNA isolated from hemocytes of 6h parasitised caterpillars and **lane 6)** total RNA isolated from hemocytes of unparasitised caterpillars. The filter was probed with ^{32}P-labelled total virus genomic DNA exposed to X-ray for 30 min for hemocytes and 5h for the fat body. CrV1 was the major virus gene expressed in the fat body and hemocytes. Minor expression of CrV2 was detected in the fat body but not in hemocytes even under overexposed conditions.


1
2
3
4
5
6
1.4 kb
1.1 kb

CHAPTER 4

Isolation and molecular characterisation of a *C. rubecula* PV gene expressed in the host

Chapter 4

Isolation and molecular characterisation of a *C. rubecula* PV gene expressed in the host

4.1. Introduction

To understand the mechanisms by which PVs debilitate the host immune system in hymenopteran parasitoids, it is essential to isolate the coding DNA for the immune suppressor gene(s). However, this attempt has been hampered by the complexity of viral gene expression. *Campoletis sonorensis* PV (CsV) transcripts are found in *Heliothis virescence* larvae as early as 2h following parasitisation until 9 days (Blissard *et al.*, 1986a), while *Microplitis demolitor* PV genes start to show expression 4h after parasitisation of *Pseudoplusia includens* larvae and persist to 6 days (Strand *et al.*, 1992). In both cases, several viral transcripts have been detected but with no obvious criteria available to decide which is coding for the immune suppressor molecule, characterisation of immune suppression was hampered.

To date, the well-characterised PV genes from *C. sonorensis* in *H. virescens* are VHv1.1, VHv1.4, WHv1.0 and WHv1.6. These virus genes have a similar structure, forming the so-called cysteine-rich gene family (Blissard *et al.*, 1986b). They contain cysteine motifs characteristic of ω-conotoxins which are snail ion-channel ligands that are known to target ion channels and receptors in the neuromuscular system (Olivera *et al.*, 1990).

As shown previously (chapter 3), *C. rubecula* PV genes are expressed over a short period between 4 to 8h following parasitisation, and only two viral mRNA transcripts are detected in parasitised *P. rapae* caterpillars, CrV1 and CrV2. Interestingly, only CrV1 was found to be highly expressed in the hemocytes isolated from parasitised caterpillars. The cloning and sequencing of the coding DNA described in this section provides an experimental basis for the study of virus-mediated hemocyte changes in parasitised host insects using recombinant proteins.

4.2. Materials and Methods

4.2.1. Construction of a cDNA library

In order to isolate CrV genes expressed in the host, a cDNA library was constructed from 6h parasitised *P. rapae* caterpillars since it was found that the highest level of the virus gene expression occurs at 6h after parasitisation (see chapter 3).

A. Isolation of mRNA

Total RNA and subsequent mRNA molecules were extracted from parasitised caterpillars as described in chapter 2.

B. cDNA synthesis

Complementary DNA molecules were synthesised from the mRNA extracted from 6h parasitised *P. rapae* caterpillars. The RiboClone® cDNA Synthesis System (Promega) was used for this purpose and α-^{32}P-dCTP as a tracer to follow the quality of cDNA synthesis in first and second strand reactions as described in manufacturer's instructions.

B.1. cDNA synthesis analysis on alkaline agarose gel

The size distribution of cDNA molecules synthesised (usually 350-6,000 bases) in the first and second strand reactions were checked by electrophoresis of tracer reactions 1 and 2 on a 1.4% alkaline agarose gel to assess the quality of cDNA synthesis. The methods were obtained from Promega's technical manual and (Sambrook *et al.*, 1989).

I. Labelling of size markers

Molecular marker II (λDNA cut with *Hin*dIII, Boehringer Mannheim) was labelled with ^{32}P-dCTP in a fill-in reaction with klenow DNA polymerase. The following reaction was assembled:

*Hind*III 10× buffer	2.5 µl	
dATP	0.5 mM	
dGTP	0.5 mM	
^{32}P-dCTP	0.3 µl	
MW II (0.25 µg/µl)	4 µl	
Klenow	1 µl	
H_2O	16.2	Σ: 25 µl

The reaction was incubated at room temperature for 10 min. To stop the reaction, 2.5 µl of 200 mM EDTA was added. The sample was mixed with 2×sample buffer (20 mM NaOH, 20% glycerol, 0.025% bromophenol blue) and used for gel analysis.

II. Gel analysis

1. A 1.4% agarose solution was prepared in H_2O and cooled down to 60°C. Then, 7.5 ml of 1 M NaCl and 300µl of 0.5 M EDTA were added.

2. The gel was cast and equilibrated for at least 30 min in alkaline gel running buffer (30 mM NaOH, 1 mM EDTA).

3. 15 µl of the sample was mixed with an equal volume of 2× sample buffer.

4. The samples were loaded and the gel was run at 80 V until the dye run to 2/3 of the gel.

5. The DNA contents were transferred to a nylon membrane as described in Southern blotting (see chapter 2).

6. The filter was exposed to a Kodak diagnostic film and autoradiography was performed.

C. Adaptor ligation and phosphorylation reaction

In order to provide sticky ends at the blunt-ended cDNA molecules, RiboClone® *Eco*RI Adaptor Ligation System (Promega) was applied which was followed by phosphorylation (kinasing) of cDNA molecules according to manufacturer's instructions .

D. Test ligation

To test the efficiency of cDNA ligation to lambda arms, test ligations were performed. The following reactions were set up and incubated at 4°C overnight.

	A	B	C	D	E
λgt 10 *Eco*RI (0.5 µg/µl)	1µl	1	1	1	1
T4 DNA ligase 10× buffer	0.5µl	0.5	0.5	0.5	0.5
cDNA (10 ng/µl)	2µl	1.5	1	0.5	1*
H_2O	1µl	1.5	2	2.5	2
T4 DNA ligase	0.5µl	0.5	0.5	0.5	0.5
cDNA concentration	20 ng	15 ng	10 ng	5 ng	* Control DNA

E. Packaging and titration of ligated DNA

Packaging *in vitro* refers to the use of a phage-infected *E. coli* cell extract to supply the mixture of proteins and precursors required for encapsidating lambda DNA (Promega).

1. A colony of *E. coli* C600 was grown in 3 ml LB-MMg (LB, containing 30 µl 20% Maltose, 30 µl 1M $MgSO_4$) overnight at 37°C with shaking.

2. 600 µl of the overnight C600 bacteria was added to 50 ml LB-MMg (LB containing 0.5 ml $MgSO_4$, 0.5 ml 20% Maltose) and the bacteria were grown until the OD_{600} was about 0.6-0.8. The culture was kept at 4°C until use.

3. Ten LA-plates (90 mm) were incubated with the lid open at 37°C until use.

4. Packaging extracts were thawed on ice and aliquoted into 5 tubes (25 µl/tube) and mixed. The tubes were incubated for 3h at 22°C.

5. 220 µl dilution buffer (Dil) plus 12.5 µl $CHCl_2$ were added to each tube.

Dilution buffer (Dil)	
20 mM	Tris-HCl, pH 7.5
100 mM	NaCl
10 mM	$MgSO_4.7H_2O$

6. Dilutions were prepared (10^4, 10^5) of each tube in Dil.

7. About 50 ml of TB top agar was melted and 0.5 ml 1 M $MgSO_4$ was added. 3 ml top agar was aliquoted into 10 tubes and kept in a water bath at 48°C until use.

TB top agar (1lit)	
10 g	tryptone
5 g	NaCl
8 g	agar

8. 150 µl from each λ dilution was mixed with 150 µl prepared C600 cells and incubated at 37°C for 30 min. Then, the content of each tube was mixed with 3 ml top agar kept at 48°C,

poured onto warm LA-plates. The plates were allowed to solidify for 5 min and then incubated at 37°C overnight.

9. The titre of the library was calculated by counting the number of clear and turbid plaques.

Tube	titre	µg DNA/ml	lig λ/µg DNA	expected
A	4.6×10^6	2.08	2.2×10^6*	$>10^6$
B	2×10^6	2.06	9.7×10^5	$>10^6$
C	2.2×10^6	2.04	1.6×10^6	$>10^6$
D	3×10^5	2.02	1.4×10^6	$>10^6$
E	5.1×10^6	2.0	2.5×10^6	5×10^6-2×10^7

* Tube A gave the expected result.

F. Large-scale ligation of cDNA into λgt10

1. Based on the test ligation results, 200ng cDNA was ligated into 5 µg λgt10 for construction of the library. Accordingly, ten replicates of reaction A were set up as in test ligation and incubated at 4°C overnight.

2. An overnight culture of *E. coli* strain C600*hfl* was prepared in 3 ml LB-MMg.

3. Another culture of C600*hfl* was prepared by inoculating 50 ml LB-MMG with the overnight culture until OD_{600} of about 0.6-0.8 was obtained.

4. Four LA plates were dried at 37°C and kept until use.

5. Six Packagene extract tubes (Promega) were thawed on ice. Then, 25 µl of the extract was added to each tube and incubated for 3h at 22°C.

6. About 20 ml TB top agar was melted, mixed with $MgSO_4$ (1 ml/100 ml), aliquoted 3 ml in 4 tubes and kept at 48°C until use.

7. After 3h packaging, 220 µl Dil was added to each tube. The contents of all 10 tubes were mixed.

8. Two dilution series of 10^4 and 10^5 were prepared by diluting the library with Dil for test ligation.

9. 125 µl of chloroform was added to the library and stored at 4°C until use.

10. 150 µl from the dilutions were mixed with 150 µl C600*hfl* culture and incubated at 37°C for 30 min. 200 µl of each dilution was added to tubes containing 3 ml top agar, plated and incubated at 37°C overnight.

G. Amplification of the library

Based on the titre of the library, it was plated onto 11 plates (150 mm) having 2×10^5 plaques per plate. Accordingly, the library was mixed with C600*hfl* (OD= 0.6-0.8), incubated at 37°C for 30 min and plated. The plates were removed from 37°C as soon as the plaques were at pin point size. The plaques must not overgrow confluence.

Ten ml suspension medium (SM) was added per plate and the plaques were eluted at 4°C overnight. The eluted plaques were collected in 50 ml Falcon tubes, spun at 3000 r.p.m for 5 min. The supernatant was taken without disturbing the pellet and collected in a 500 ml bottle. 1.5 ml chloroform was added to the amplified library, mixed gently and aliquots were kept at 4°C, -20°C and -70°C. The library was also titrated as described above.

Suspension Medium (SM)	
100mM	NaCl
8 mM	$MgSO_4.7H_2O$
50 mM	1M Tris-HCl, pH 7.0
0.01%	gelatine

H. Screening the cDNA library

In order to isolate CrV genes that are expressed in the host, *P. rapae*, the cDNA library constructed from 6h parasitised caterpillars in λgt10 was screened by using total CrV genomic DNA as a probe. A recombinant DNA library consists of a large number of recombinant DNA clones and the usual procedure for isolating the desired clones involves a positive selection for a particular nucleic acid sequence. The procedure involves the identification of the recombinant

DNA clones in the library and the purification of the clone. To screen the library, a large number of clones (bacteriophage plaques) are plated, transferred to filter membranes and then the clones are hybridised by a particular probe.

H.1. Probe preparation

CrV genomic DNA was digested with three restriction enzymes (*Eco*RI, *Hin*dIII and *Alu*I) and labelled as described in chapter 2.

H.2. Plating and transferring bacteriophage library

A proper amount of the library was mixed with C600*hfl* and plated so as to produce about 500 pfu per plate. The library was screened on 20 plates. Plates were removed from 37°C before the plaques become confluent and kept at 4°C for an hour. Nylon membranes were cut to the size of the plates were marked for alignment and then placed on the cold plates, left for 1-10 min during which plates were marked and numbered according to filters marks. Filters were lifted and dried on Whatman paper for 10-15 min.

To denature the capsids, filters were placed on a 3 mm paper saturated with denaturation solution (0.2 M NaOH, 1.5 M NaCl) for 1-2 min with phage side facing up. This releases phage DNA from capsids. Thereafter, filters were transferred onto a 3 mm paper saturated with neutralisation solution (0.4 M Tris-Cl pH 7.6, 2×SSC) for 1-2 min and then transferred onto a 3 mm paper saturated with 2×SSC for 1-2 min (phage side facing up).

Filters were dried completely at room temperature and exposed to UV illumination for 50 seconds to fix the DNA (BioRad, Gene linker).

H.3. Hybridisation and washing

1. Filters were incubated in 50 ml prehybridisation solution (see Southern blotting in chapter 2) for 1h at 65°C with shaking.

2. ^{32}P-labelled probe (total virus genomic DNA) was added to prehybridisation solution and hybridisation was carried out overnight at 65°C.

3. Filters were washed with washing solutions I (2×SSC/0.1% SDS) and II (0.2×SSC/0.1%SDS) twice for 20 min, respectively.

4. Filters were wrapped with plastic and autoradiography was performed.

5. Positive signals were aligned with the λ-plates and the corresponding plaques were picked up with a Pasteur pipet and incubated in 1 ml SM plus chloroform at 4°C to elute phages from pieces of agarose.

6. The isolated clones were titrated and replated for rescreening. Once the positive signal was confirmed by second screening, the clone was amplified as above.

4.2.2. PCR amplification of cDNA fragments

Specific primers on the sides of the unique *Eco*RI sites in λgt10 (Promega, see below) were used to amplify cDNA fragments from positive plaques obtained from screening of the cDNA library. A phage plaque was cored out from the plate and transferred into a tube containing 100μl suspension medium (SM) and incubated at 25°C for 30 min to allow phage particles to diffuse into the buffer. Five μl of the phage sample was boiled for 10 min and chilled on ice. A standard PCR reaction was set up as following:

Template DNA	5 μl
dNTP's	4 μl
Forward primer (0.1 μg/ml)	1 μl
Reverse primer (0.1 μg/ml)	1 μl
10×Reaction buffer	3 μl
H_2O	16 μl
Taq polymerase (5u/μl)	0.2 μl

λgt 10 forward primer: 5'-GCA AGT TCA GCC TGG TTA AG-3'
λgt 10 reverse primer: 5'-TGA GTA TTT CTT CCA GGG TA-3'

The following thermal cycling program was applied in PTC-100^{TM} Programmable thermal controller:

Step 1 95°C 4 min (denaturation of DNA)
Step 2 94°C 1 min (denaturation of DNA)

Step 3 55°C 2 min (primer annealing)
Step 4 74°C 2 min (chain extension)
Step 5 34 cycles from step 2 to 4 (amplification)
Step 6 74°C 5 min (final chain extension)

The PCR product was run on a 1% TAE low-melting agarose gel, isolated from the gel and purified using Wizard PCR preps (Promega).

4.2.3. Cloning of PCR amplified cDNA fragments and sequencing

In order to sequence the cDNA fragment isolated from screening the cDNA library, the PCR amplified fragment was cloned into pBluescript II KS$^{(+)}$-T as described in chapter 2 and sequenced in an automative sequencer (Applied Biosystems Sequencer). Primers were designed on the basis of initial sequence data and used for producing PCR fragments and subcloning the gene. The individual subclones were sequenced to cover the whole cDNA fragment.

4.2.4. Sequence analysis

The sequence data were analysed using SeqEd. (version 1.0.3), MacMolly, and Lasergene computer programs. For DNA and protein sequence similarity Blast data base from the National Centre for Biotechnology Information was searched using NetScape program on the network.

The putative protein structures and properties were obtained from Expasy protein search on the network using PSORT for predicting protein localisation sites (Nakai and Kanehisa, 1992), NetOglyc for O-glycosylation sites (Hansen *et al.*, 1995), and TMprd for predicting transmembrane domains (Hofmann and Stoffel, 1993).

4.2.5. *In situ* hybridisation of tissue sections

In order to detect the location of CrV1 expression in the host tissues, a PCR DIG-labelled probe was applied on tissue section for *in situ* hybridisation.

A. Preparation of DIG-labelled probe

A PCR DIG labelling kit (Boehringer Mannheim) was used to generate a probe labelled with digoxigenin (DIG)-dUTP in a PCR reaction. Digoxigenin is a non-radioactive substance which

is used for labelling. Using a lambda gt10 specific reverse primer and a CrV1-specific primer, a 330 bp DIG-labelled fragment was produced. The components and thermocycling conditions of the PCR reaction was carried out according to the manufacturer's protocol as following:

Reaction

Template DNA (100 ng)	0.48 µl
PCR 10×buffer	5 µl
$MgCl_2$	8 µl
PCR Dig mix	5 µl
Primer 1 (λ Rev., 0.1 µg/µl)	5 µl
Primer 2 (R1, 0.1 µg/µl)	5 µl
H_2O	25.6 µl
Taq polymerase	0.5 µl

Thermocycling

1. 95°C 7 min (denaturation)
2. 95°C 45 sec (denaturation)
3. 60°C 1 min (annealing)
4. 72°C 2 min (elongation)
5. 29 cycles from 2 to 4.

B. Frozen tissue sectioning

P. rapae caterpillars were surface sterilised in 70% ethanol and embedded in O.C.T compound (Tissue-Tek®) using cubic moulds and freezed at -20°C overnight. The specimens were sectioned at -15°C in 10 nm thickness using Cryocut 1800 (Leica) and mounted on Silane-prep™ slides (Sigma). The slides were left on a 42°C heating block for 1-2 min or for 1-2h at room temperature. The specimens were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. The slides were washed twice in PBS for 5 min at room temperature and dehydrated in ethanol dilution series (35, 50, 70 and 100%) 5 min each.

C. Protein digestion

To digest the proteins, about 50 µl proteinase K (1 µg/ml in 100 mM Tris-HCl, pH 7.5; 50 mM EDTA) was applied onto each slide, covered with coverslips and kept at 37°C for 15 min. The slides were washed twice in 2×SSC for 5 min and then in H_2O for 5 min. Subsequently, the slides were dried on a 65°C plate.

D. Acetylation

About 50 µl 100 mM triethanolamine (pH 8) was put onto each slide, covered with coverslip and incubated for 5 min at room temperature. The slides were immersed in 0.25% acetic

anhydride for 10 min, washed twice in 2×SSC for 5 min and in H_2O for 5 min. The sections were dehydrated in ethanol dilution series 5 min for each dilution.

E. Pre- and hybridisation

The slides were incubated at 70°C for 5 min to denature RNA molecules and then cooled on ice for 5 min. The slides were prehybridised by applying 50 µl prehybridisation solution onto each slide covered with coverslip at 45°C for 2h. For hybridisation, 5 µl PCR DIG labelled probe (see above) was mixed with 50 µl prehybridisation solution, denatured at 70°C for 5 min, cooled on ice for 5 min and applied onto each slide and covered with coverslip. The coverslips were sealed by rubber cement and incubated at 45°C overnight.

F. Washing

The slides were washed in 2×SSC at 42°C for 1h with gentle shaking, in 1×SSC at 42°C for 1h, in 0.5×SSC at room temperature for 30 min and in 0.2×SSC at room temperature for 30 min.

G. Detection

Slides were incubated in PBS for 5 min and then in PBS, 2% BSA, 0.3% Triton X100 for 1h. For detection, 50 µl anti-DIG antibody solution (1:1000 in PBS with 2% BSA, 0.3% Triton X100) was applied onto each slide and incubated for 1h at room temperature. Then, slides were washed twice in PBS with gentle shaking and 2 min in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 100 mM $MgCl_2$. To detect hybridised probe-antibody complexes, 50 µl colour solution (50 µl Nitro Blue Tetrazolium and 2.5 µl 5-Bromo-4-chloro-3-indolyl phosphate per 1 ml 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 100 mM $MgCl_2$) was applied onto each slide, covered with coverslip and incubated at room temperature in the dark overnight.

The slides were washed in TE (pH 8.0) for 10 min and in H_2O for 2 min, observed under the microscope and photographed.

4.2.6. Northern and Southern blotting

As described in chapter 2.

4.3. Results and discussion

4.3.1. Construction and screening of a cDNA library

As shown previously, the peak of *C. rubecula* (CrV) gene expression occurs at 6h following parasitisation (see chapter 3) and only one viral gene (CrV1) was found to be highly expressed in host hemocytes in contrast to other known parasitoid systems, where a number of viral genes are transcribed in host cells and tissues (Summers and Dibhajj, 1995). This suggests that CrV1 is probably the immune suppressor gene. The transient expression of this gene would agree with the reversible mode of inactivation of hemocytes which recover 2-3 days after parasitisation. In order to isolate CrV1 coding DNA, a cDNA library was constructed from *P. rapae* caterpillars that were parasitised 6 hours previously. The size distribution of synthesised cDNA molecules was analysed on an alkaline agarose gel and found to be 0.9 to 5.3 bases (Fig. 4-1). The highest titre obtained in a test ligation was found to be 1.9×10^6 pfu/ml by mixing 20ng of the constructed cDNA molecules with 0.5ng lambda gt10 *Eco*RI arms. After a large scale ligation based on the test ligation and amplification of the library, the titre was determined as 4.4×10^{10} pfu/ml.

Radioactively labelled CrV-DNA comprising the complete virus genome was used as a probe to screen the library under high stringency conditions. Positive recombinant plaques were analysed by PCR using specific primers adjacent to the unique *Eco*RI sites in λgt10 arms. A 1.4 kb DNA fragment amplified in the reaction (Fig. 4-2A) was chosen for subcloning into a plasmid vector.

To establish the identity of the cDNA fragment, the insert was used as a probe to test a Northern blot of total RNA from parasitised and unparasitised caterpillars. The probe hybridised to the CrV1 band indicative that the cloned cDNA fragment represented the expressed CrV transcript (Fig. 4-2B). The hybridisation pattern to caterpillar RNA from various time-periods after parasitisation coincided with the CrV1 band labelled with total CrV-DNA as a probe (Fig. 3-7B), and a similar short time window of expression with virtually no transcript visible until 4h following parasitisation. No transcripts are detected beyond 8h after parasitisation. In addition, the two viral transcripts CrV1 and CrV2 are probably from unrelated virus genes since no cross-

hybridisation of CrV1 was found to CrV2 when it was used as a probe in a Northern blot analysis containing total RNA from parasitised caterpillars (Fig. 4-2B).

4.3.2. Expression of CrV1 in host tissues

Hybridisation experiments suggest that CrV1 is expressed in hemocytes and fat body of parasitised *P. rapae* caterpillars using the isolated cDNA fragment as a probe (Fig. 4-2C). *In situ* hybridisation to tissue sections showed that the CrV1 transcript was localised to small patches of cells within the fat body of parasitised caterpillars (Fig. 4-3). No attempts were made to establish possible structural and functional changes in infected fat body cells from parasitised caterpillars. Infection of various host tissues by PVs has also been reported in other host-parasitoid interactions (Stoltz and Vinson, 1979a; Strand *et al.*, 1992). Viral transcripts have been detected in hemocytes, gut, nervous system, fat body and malphigian tubules (Strand *et al.*, 1992; Harwood and Beckage, 1994).

4.3.3. CrV1 is integrated in the wasp genome

To further verify the virus origin of the isolated cDNA fragment, the amplified cDNA fragment was used as a probe in a Southern blot containing digested DNA from purified virus and male wasp DNA. The isolated fragment hybridised to the virus genomic DNA digested with the restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII (Fig. 4-4). In addition, there was no hybridisation of the probe to the genomic DNA of unparasitised *P. rapae* caterpillars (Fig. 4-4) indicating that the isolated cDNA fragment is CrV specific. When the pattern of hybridisation between the male wasp genomic DNA and virus DNA was compared, differences in the restriction pattern were observed. *Eco*RI and *Hin*dIII fragments are similar in virus and wasp genomic DNA, whereas one of the two *Bam*HI fragments differ. This supports the notion that PV DNA is transmitted from one wasp generation to the next in the chromosomes and is packaged into virus particles as circularised DNA molecules in the female ovarian tract. It has previously been shown that viral DNA is integrated in the genome of parasitoid wasps and transmitted vertically through the germline (Stoltz *et al.*, 1986). The replication of viral genome starts at pupal stage of the wasp in specialised calyx cells (Albrecht *et al.*, 1994; Gruber *et al.*,

1996), where it is excised from the genome and recircularised by recombination (Gruber *et al.*, 1996). The recircularised DNA segments are then packaged in nucleocapsides.

4.3.4. DNA sequence and putative protein sequence

DNA sequencing of the cDNA fragment coding for the CrV1[1] protein confirmed the transcript to be a polyA$^+$-containing RNA with an open reading frame of 912 bp coding for a deduced protein of 304 amino acids (Fig. 4-5). A methionine codon (AUG) at the beginning of the open reading frame was identified as the only possible codon with the nucleotide sequence environment predicted for functional initiation codons (Cavener and Ray, 1991). Several termination codons starting at nucleotide position 912 and the polyA signal site deliminate the open reading frame at the 3'-end. The theoretical molecular weight of the protein sequence is 33.6 kDa with an isoelectric point of 4.97 (Table 4-1). The restriction enzyme sites of the gene found by computer analysis are shown in Table 4-2.

The deduced protein sequence contains a hydrophobic peptide at the N-terminus which has the characteristic features of a signal peptide (Fig. 4-5) also confirmed by hydrophobicity computer analysis (Fig. 4-6). According to computer prediction (TMpred), the preferred model for the deduced protein is a strong transmembrane domain from amino acid 1 to 23 with the N-terminus localised towards the inside of the cellular membrane. Furthermore, there is a possible cleavable N-terminus signal sequence at position 20 (Fig. 4-5) which suggests that the protein might be secreted and released into the extracellular environment (see Chapter 5). A putative N-glycosylation site is present at nucleotide 789 (Fig. 4-5). N-glycosylated proteins contain a common pentasaccharide core consisting of three mannose and two N-acetylglucosamine linked to an asparagine residue which is part of an N-X-S or N-X-T sequence (N: Asparagine, S: Serine, T: Threonine) (Stryer, 1988). The presence of O-glycosylation sites on CrV1 protein are also predicted using NetOglyc program available on the network (Fig. 4-5). O-glycosylated proteins contain oligosaccharide units linked to serine and threonine side chains (Stryer, 1988). Accordingly, 13 O-glycosylation sites were found on CrV1, six of which are on serine residues and eight on threonine residues (Fig. 4-5).

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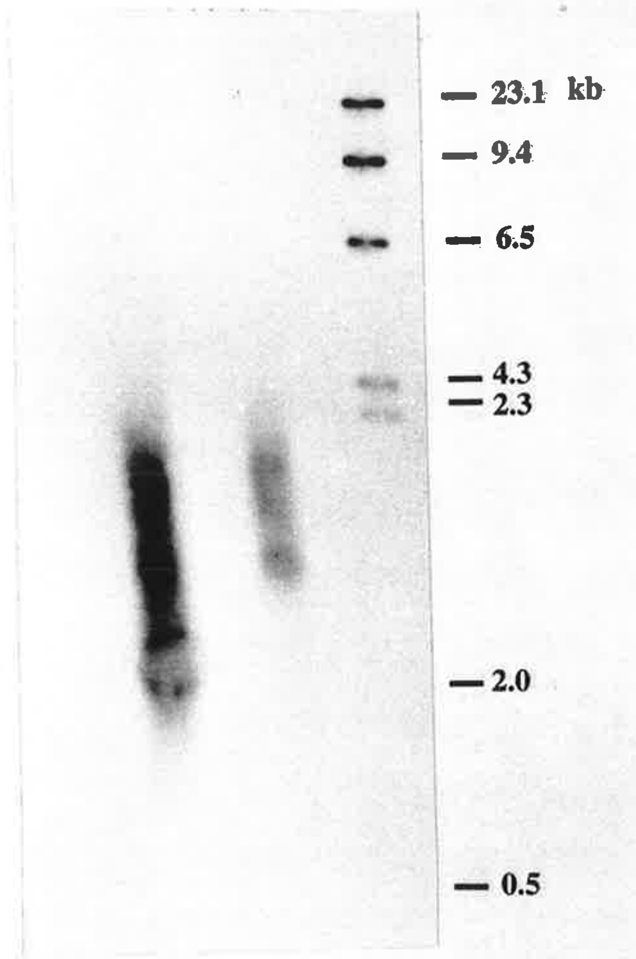
[1]The GenBank/EMBL accession number of the DNA sequence reported here is U55279.

Protein sequence comparisons were performed using National Centre for Biotechnology Information data bank. Among the genes which represented a certain degree of homology to CrV1, the most significant and interesting similarity was with the M-like protein from *Pseudomonas pyogenes* (M80) which will be discussed in more detail in chapter 6.

Figure 4-1

cDNA synthesis analysis. The first and second tracer reactions of cDNA synthesis were run on an alkaline agarose gel, transferred onto a membrane and exposed to a diagnostic film. The smear indicates cDNA synthesis in both reactions.



2nd
1st
23.1 kb
9.4
6.5
4.3
2.3
2.0
0.5

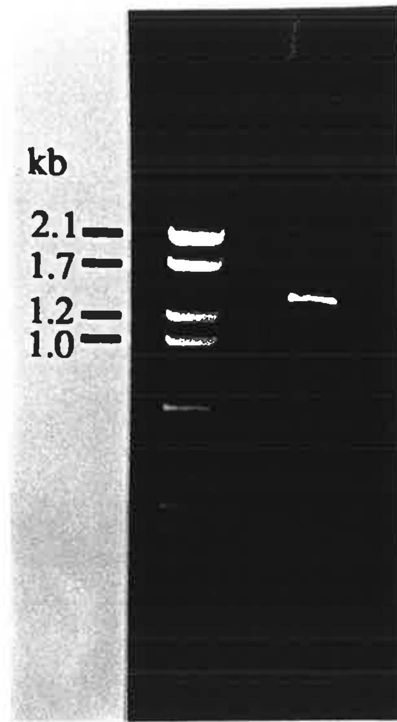
Figure 4-2

A) PCR amplification of a cDNA fragment isolated from a cDNA library constructed from 6h parasitised *P. rapae* caterpillars by *C. rubecula.* Lambda gt10 specific primers next to the unique *Eco*RI sites were used in the reaction which resulted in a fragment of about 1300 bp. (Left lane: DNA molecular markers).

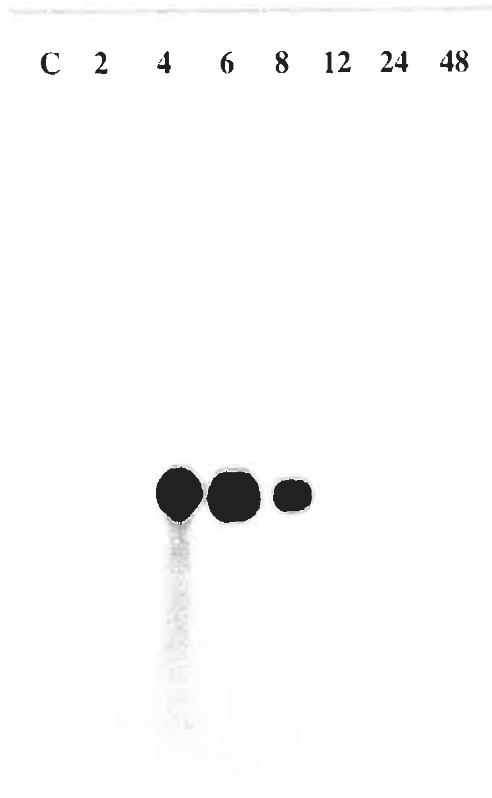
B) Hybridisation to a Northern blot containing total RNA isolated from *P. rapae* caterpillars at 2, 4, 6, 8, 12, 24 and 48h after parasitisation by *C. rubecula.* RNA from unparasitised caterpillars was used as a control (lane C). Each RNA fraction was spectroscopically measured and equal aliquots of RNA (2 µg) were applied to each slot. The filter was probed with ^{32}P-labelled isolated cDNA fragment. The probe hybridised to a 1.4 kb RNA fragment indicating that the isolated cDNA fragment is CrV1 and its temporal expression coincides with that of total viral DNA as described in chapter 3 (Fig. 3-7B).

C) Hybridisation to a Northern blot containing total RNA isolated from **lane 1)** fat body of unparasitised caterpillars; **lane 2)** fat body of 6h parasitised caterpillars; **lane 3)** hemocytes of unparasitised caterpillars; and **lane 4)** hemocytes from 6h parasitised caterpillars. The filter was probed with ^{32}P-labelled CrV1-cDNA fragment.

(A)



(B)



(C)

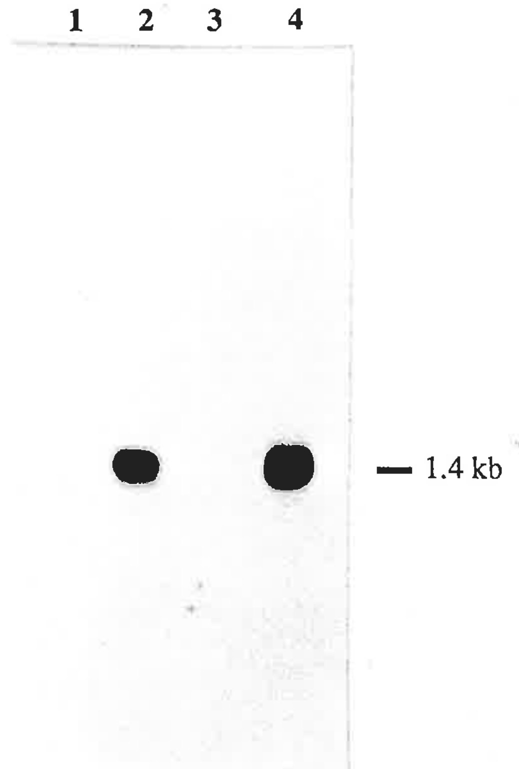


Figure 4-3

Expression of polydnavirus transcript in fat body sections of parasitised *P. rapae* caterpillars using DIG-labelled CrV1-cDNA as a probe. After *in situ* hybridisation the localisation of labelled probe on tissue sections was visualised using alkaline phosphatase-conjugated antibodies as prescribed in Materials and Methods. Examples of stained cells are indicated by arrowheads.

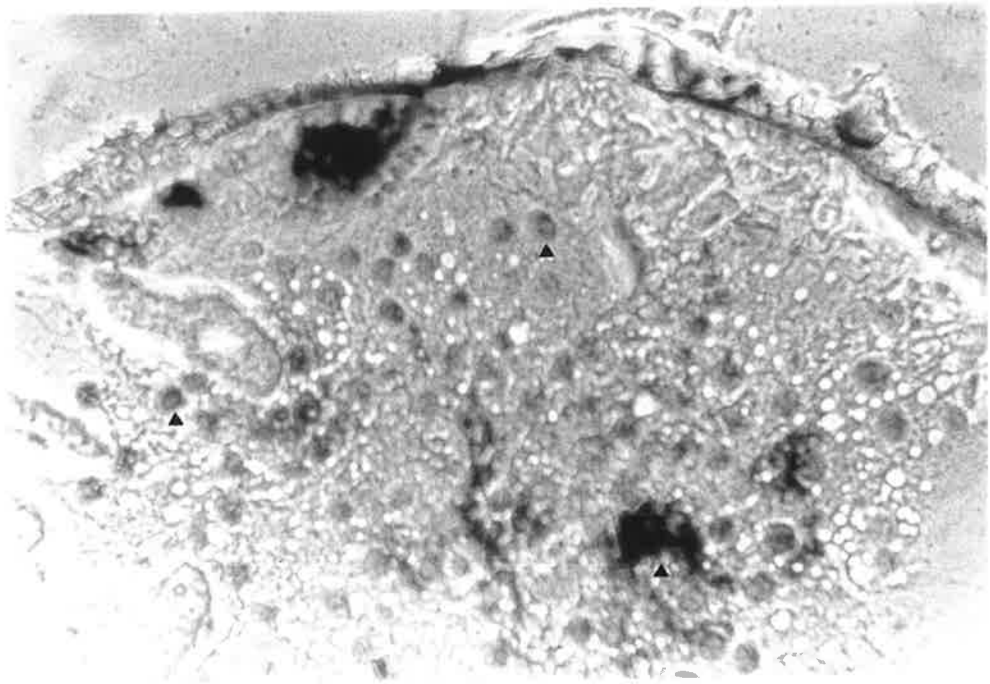


Figure 4-4

Hybridisation to a Southern blot containing restriction enzyme digested DNA from *P. rapae* caterpillars and from *C. rubecula* male wasps probed with ^{32}P-labelled CrV1 fragment. **Lane 1)** *P. rapae* genomic DNA digested with *Eco*RI; **Lane 2)** CrV-DNA digested with *Eco*RI; **Lane 3)** CrV-DNA digested with *Hin*dIII; **Lane 4)** CrV-DNA digested with *Bam*HI; **Lane 5)** *C. rubecula* male genomic DNA digested with *Eco*RI; **Lane 6)** *C. rubecula* male genomic DNA digested with *Hin*dIII; **Lane 7)** *C. rubecula* male genomic DNA digested with *Bam*HI. Note the differences between the banding pattern in the virus DNA and the male genome of the virus, which contains the virus DNA as a provirus. No cross-hybridisation was observed in the unparasitised host *P. rapae* suggestive that the CrV1 gene is unique to the wasp.

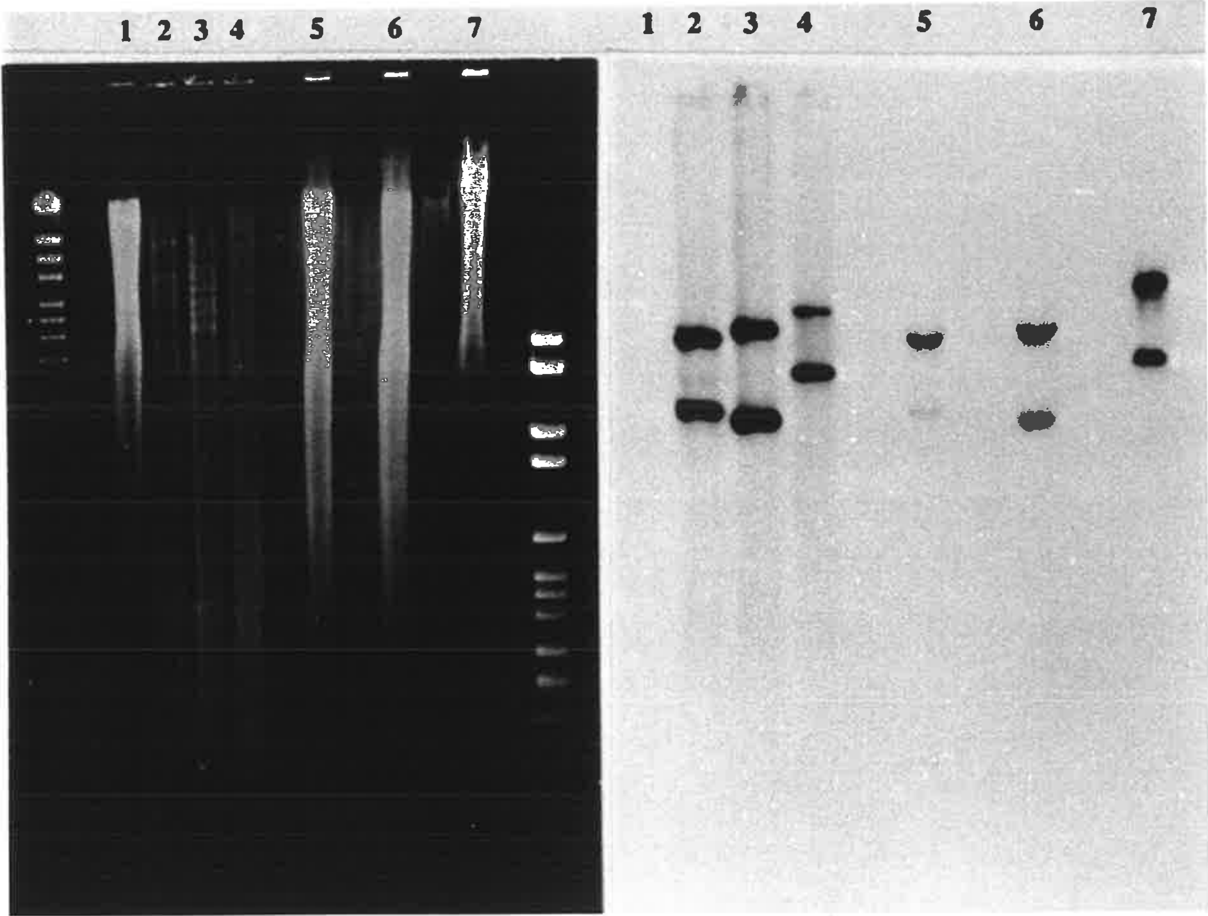

1 2 3 4 5 6 7
1 2 3 4 5 6 7

Figure 4-5

DNA nucleotide sequence and deduced amino acid sequence of the cDNA fragment in pCrV1. The hydrophobic region at the N-terminus, representing a putative signal peptide is boxed. A possible proteolytic site for signal peptide is indicated by an arrowhead. A putative N-glycosylation site is underlined. Predicted O-glycosylated serine and threonine residues are indicated by asterisks. The putative polyadenylation signal is underlined by a dotted line.

```
                                                                 CG TTG CTG TCG ATC TTG AAC
                 +                                                         ▽
 M   S   L   V   K  [S   A   S   V   L   L   S   A   L   L   S   Q   S   C   T   Q   A   Y   P]
ATG TCA CTC GTC AAA AGT GCG TCT GTG CTG CTC TCA GCT CTC CTG AGT CAA TCA TGT ACG CAA GCT TAT CCA
          9           18          27          36          45          54          63          72

 S   E   Y   Q   W   E   R   I   Q   N   I   P   F   A   D   V   A   S   L   S   P   L   T   D
TCC GAA TAC CAA TGG GAA AGA ATT CAA AAT ATT CCA TTT GCT GAC GTT GCT TCC CTT AGT CCG CTG ACC GAT
          81          90          99          108         117         126         135         144

 Y   F   S   P   K   S   D   S   T   A   E   R   L   A   P   S   T   N   D   F   D   E   S   D
TAT TTC TCT CCA AAA TCA GAT TCT ACA GCC GAA AGA CTT GCG CCA TCA ACA AAC GAC TTC GAT GAA TCT GAT
          153         162         171         180         189         198         207         216

 M   N   N   R   E   F   L   M   I   P   K   S   Y   S   E   A   P   R   K   H   F   D   V   E
ATG AAC AAT AGA GAA TTT TTG ATG ATT CCG AAG TCT TAT TCT GAG GCA CCT CGG AAG CAT TTT GAT GTT GAA
          225         234         243         252         261         270         279         288

 Y   F   L   K   H   H   P   H   I   K   P   G   H   S   T   R   H   R   P   A   F   E   I   R
TAT TTT CTT AAG CAT CAC CCT CAT ATC AAG CCT GGA CAC AGT ACA AGA CAT AGA CCT GCG TTC GAA ATA AGA
          297         306         315         324         333         342         351         360

                                                 *
 P   F   V   G   P   I   S   E   L   S   S   G   S   S   G   I   G   Q   L   G   S   S   V   A
CCT TTC GTA GGA CCT ATT AGT GAA TTA TCC AGT GGA AGT AGC GGT ATC GGT CAA CTT GGA TCG AGC GTT GCA
          369         378         387         396         405         414         423         432

                                                                                            *
 D   E   I   F   S   G   L   K   N   F   I   G   N   V   K   F   T   Q   P   E   L   F   D   T
GAT GAA ATA TTT TCC GGA CTT AAA AAT TTC ATT GGA AAT GTG AAA TTT ACT CAA CCT GAA TTA TTT GAC ACA
          441         450         459         568         477         486         495         504

 S   K   T   H   Q   Y   E   Y   E   S   E   T   S   E   K   R   H   I   T   R   E   D   L   L
TCC AAA ACC CAC CAG TAT GAA TAT GAA TCT GAA ACA TCT GAA AAA CGC CAC ATT ACC CGT GAA GAT TTG CTT
          513         522         531         540         549         558         567         576

 S   E   L   H   A   I   K   E   A   L   Q   N   L   K   S   A   V   I   R   V   E   N   E   I
TCT GAA CTA CAT GCA ATA AAA GAG GCA CTT CAA AAT CTA AAA TCT GCT GTC ATT CGG GTA GAA AAT GAG ATC
          585         594         603         612         621         630         639         648

                                         *
 S   F   N   K   E   G   V   T   Y   P   S   V   A   I   S   P   Q'  P   L   V   L   I   N   P
AGC TTT AAC AAA GAA GGA GTT ACT TAC CCC TCT GTG GCA ATC TCA CCG CAA CCT CTA GTT CTG ATT AAT CCT
          657         666         675         684         693         702         711         720

                                             *               *           *       *
 N   G   A   S   E   Q   G   P   I   E   A   I   T   E   E   A   V   T   H   P   T   V   T   N
AAC GGA GCA TCT GAA CAG GGA CCC ATA GAA GCT ATT ACC GAA GAA GCA GTT ACT CAT CCC ACT GTG ACA AAC
          729         738         747         756         765         774         783         792

 *   *
 S   S   Q   P   P   V   L   I   N   P   N   G   A   S   E   Q   G   P   I   E   A   N   N   E
TCA TCG CAA CCT CCA GTT CTG ATT AAT CCT AAC GGA GCA TCT GAA CAG GGA CCC ATA GAA GCT AAT AAC GAA
          801         810         819         828         837         846         855         864

                         *       *   *               *
 E   G   V   T   Y   P   S   D   T   T   P   S   Q   T   F   F   .
GAA GGA GTT ACT TAT CCC AGT GAT ACA ACC CCA TCG CAA ACT TTT TTT TGA TCC CTA TAT GTA GCA TCT GAA
          873         882         891         900         909         918         927         936

CTT GAA CTC AGT AGA GTC TAC TTA AGA AAG TAT GTA TAA AAA TAC TGC TAC AAA TGA AGC CAT ATG ATA ATC
          945         954         963         972         981         990         999         1008

GAC CCC TAG AGA ACA CAC TAT CTA AAA CTT ATT TCC CAT AAT TTT TTT AGG AGA AAG AAC AAG CGT TCT TAA
          1017        1026        1035        1044        1053        1062        1071        1080

AGT ACT GAA CAT ATC GAA TTT CTT GTC ATC GTC AAA TAT GTA TAT AAT AAC TTA GAA AAC CTT GCA ATC TAA
          1089        1098        1107        1116        1125        1134        1143        1152

ATG TTC TAG CTT TAA GTA AGT TTT GAT CGA AGA AAA ACG CCA ATT TTT ATA GTG TAA ACA GTT TTG TAT TTT
          1161        1170        1179        1188        1197        1206        1215        1224

GAT GAA TAA ATG AAT ATA TTT TCC AAT TTA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AA
          1233        1242        1251        1260
```

Figure 4-6

Hydrophobicity prediction curve of CrV1. A hydrophobic region at position 0 to 23 is evident.

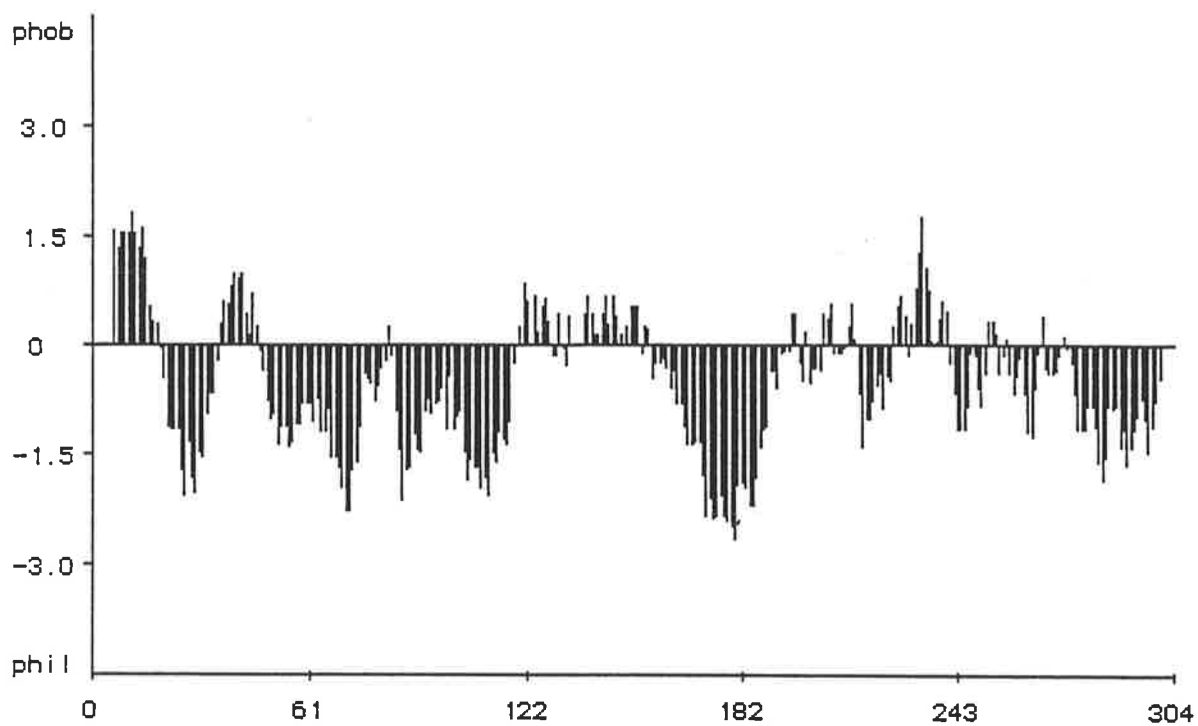

phob
3.0
1.5
0
-1.5
-3.0
phil
0
61
122
182
243
304

Table 4-1

Predicted characteristics and protein composition of CrV1 based on the dedued amino acid sequence. The data was created by Lasergene software.

Analysis	Whole Protein
Molecular Weight	33600.70 m.w.
Length	304
1 microgram =	29.761 pMoles
Molar Extinction coefficient	17330±5%
1 A(280) =	1.94 mg/ml
Isoelectric Point	4.93
Charge at pH 7	-15.40

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged(RKHYCDE)	83	33.28	27.30
Acidic (DE)	40	14.91	13.16
Basic (KR)	23	9.61	7.57
Polar(NCQSTY)	97	30.89	31.91
Hydrophobic (AILFWV)	92	29.68	30.26
A Ala	19	4.02	6.25
C Cys	1	0.31	0.33
D Asp	11	3.77	3.62
E Glu	29	11.14	9.54
F Phe	15	6.57	4.93
G Gly	14	2.38	4.61
H His	10	4.08	3.29
I Ile	19	6.40	6.25
K Lys	13	4.96	4.28
L Leu	22	7.41	7.24
M Met	3	1.17	0.99
N Asn	16	5.43	5.26
P Pro	25	7.22	8.22
Q Gln	13	4.96	4.28
R Arg	10	4.65	3.29
S Ser	39	10.11	12.83
T Thr	19	5.72	6.25
V Val	16	4.72	5.26
W Trp	1	0.55	0.33
Y Tyr	9	4.37	2.96
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	0	0.00	0.00

Table 4-2

Restriction sites of CrV1 cDNA fragment

Enzyme	Site	position
Acc I	GT'MKAC	953
Acc III	T'CCGGA	445
Afl II	C'TTAAG	295, 957
Aha III	TTT'AAA	1253
Alu I	AG'CT	37, 64 650, 751 853, 1161
Alw I	GGATC(N 4)'	426
Apy I	CC'WGG	320
Ase I	AT'TAAT	713, 815
Asp 700	GAANN'NNTTC	92, 290
Asu II	TT'CGAA	350
Ava II	G'GWCC	370, 739 841
Ban I	G'GYRCC	261
Bme 216	GG'WCC	371, 740 842
Bsp MI	ACCTGC(N 4)'	351
Bsp MII	T'CCGGA	445
Bst BI	TT'CGAA	350
Bst NI	CC'WGG	320
Cfo I	GCG'C	186
Dde I	C'TNAG	33, 43 127, 257 943, 1131
Dpn I	GA'TC	420, 646 915, 1178
Dra I	TTT'AAA	1253
Dra II	RG'GNCCY	370, 739 841
Eco NI	CCTNN'NNNAGG	365
Eco O 109I	RG'GNCCY	370, 739 841
Eco RI	G'AATTC	92
Eco RI*	'AATT	92, 229 382, 456 475, 490 1047, 1096 1193, 1248
Eco RII	'CCWGG	318
Fnu4 HI	GC'NGC	28
Hha I	GCG'C	186
Hinc II	GTY'RAC	412
Hind II	GTY'RAC	412
Hind III	A'AGCTT	62
Hinf I	G'ANTC	45, 163 208, 240 529, 950
Hin PI	G'CGC	184
Hpa II	C'CGG	446
Hsu I	A'AGCTT	62
Mae I	C'TAG	703, 1014 1158

Table 4-2.continued

Mae II	A'CGT	116
Mae III	'GTNAC	2, 666 768, 783 870
Mbo I	'GATC	418, 644 913, 1176
Mbo II	GAAGA(N 8)'	577, 772 874, 1193
Mnl I	CCTC(N 7)'	275, 317 687, 710 812
Mse I	T'TAA	296, 452 653, 713 815, 958 1077, 1164 1252
Msp I	C'CGG	446
Nde I	CA'TATG	998
Nla III	CATG'	56, 589
Nla IV	GGN'NCC	263, 740 741, 842 843
Nsp 7524 I	RCATG'Y	589
NspB II	CMG'CKG	135
Ple I	GAGTC(N 4)'	53, 958
Ppu MI	RG'GWCCY	370, 739 841
Pss I	RGGNCC'Y	374, 743 845
Rsa I	GT'AC	57, 330 1083
Sau 3AI	'GATC	418, 644. 913, 1176
Sau 96I	G'GNCC	370, 739 841
Sca I	AGT'ACT	1083
Scr FI	CC'NGG	320
Sfa NI	GCATC(N 5)'	309, 736 838, 937
Sso47 II	'CCNGG	318
Ssp I	AAT'ATT	102, 289 440
Taq I	T'CGA	203, 350 421, 1007 1094, 1179

Chapter 5

Expression of a *C. rubecula* polydnavirus gene in baculovirus expression systems and its functional analysis

Chapter 5

Expression of a *C. rubecula* polydnavirus gene in baculovirus expression systems and its functional analysis

5.1. Introduction

In the braconid *C. rubecula* the newly deposited egg is protected against the defence reactions of the host *P. rapae* through a layer of calyx fluid proteins that provide a passive protection of the developing parasitoid during embryogenesis (Asgari and Schmidt, 1994) (see also chapter 7). This protection is probably beneficial only to the egg but once the parasitoid larva emerges it is exposed to the host immune system. In previous sections, it was shown that CrVs enter host tissues where virus genes are transcribed transiently over a short period of time. Only a single polydnaviral gene (CrV1) was found to be expressed in host hemocytes which is accompanied by changes in the actin cytoskeleton, exposure of surface molecules and microparticle formation. This hemocyte inactivation is different from what has been described in another system where hemocytes die through apoptosis, a programmed cell death (Strand and Pech, 1995b). Disruption of the host cellular defence reactions via inactivation of hemocytes by polydnaviruses may therefore provide a long term protection for the emerging parasitoid larva.

Here, the aim is to show that a single polydnavirus-encoded glycoprotein (CrV1) is responsible for the inactivation of hemocytes. CrV1 is secreted from infected hemocytes and fat body cells into the hemolymph, where it interacts with the surface of hemocytes. The result of this interaction is a transient inhibition of immune-related hemocyte-specific surface changes, affecting the exposure of a lectin-binding protein and microparticle formation. Inhibition of hemocyte activation processes precludes cellular encapsulation reactions during a critical time when the parasitoid larva emerges from the chorion. Since the putative amino acid sequence of the protein indicates that it is O- and N-glycosylated (chapter 4), a baculovirus and a plasmid expression system in Sf21 cells were used to express the protein. Since the expression systems support post-translational modifications of foreign genes, we tested lectin-binding to the recombinant protein to identify CrV1 in the cell culture medium. As *H.p.* lectin was found to

bind to CrV1, a lectin-based purification system was established to purify the protein to produce specific antibodies and perform functional analyses.

5.2. Materials and Methods

5.2.1. *In vitro* transcription/translation of CrV1

In order to check the open reading frame, CrV1-cDNA cloned in bluescript downstream from T7 RNA polymerase promoter was transcribed and translated *in vitro* using TNT® Coupled Reticulocyte Lysate Systems (Promega). TNT® system requires plasmid constructs containing a prokaryotic phage RNA polymerase promoter (SP6, T3 or T7) for the initiation of transcription, but translation in this system is under eukaryotic control (using rabbit reticulocyte lysate). The protein product is identified by detecting incorporation of radio-labelled methionine or other labelled amino acids in the synthesised product. The reaction was carried out as following:

TNT® rabbit reticulocyte lysate	25 µl
TNT® reaction buffer	2 µl
T7 RNA polymerase	1 µl
Amino acid mixture minus methionine, 1 mM	1 µl
^{35}S-methionine (1,000 Ci.mmol) at 10 mCi/ml	4 µl
RNasin® ribonuclease inhibitor, 40u/µl	1 µl
DNA template (450 ng/µl)	2.2 µl
Nuclease-free H_2O	13.8 µl

The reaction was incubated at 30°C for 90 min. An aliquot of the reaction was run on a 12% SDS-PAGE and then electroblotted onto a nitrocellulose membrane. The membrane was washed several times with distilled water and the proteins were stained by incubating the membrane in 0.2% Ponceau S (3-Hydroxy-4-[2-sulfo-4-(4-sulfo-phenylazo)phenylazo]-2,7-naphthalenedisulfonic acid), 0.1% acetic acid for one min with shaking to label the molecular markers. The stain was drained and the membrane was washed with distilled water and exposed to a Kodak sensitive film for 2 days and developed afterward.

5.2.2. Expression of CrV1 in a baculovirus system

BacPAK6 baculovirus expression system (Clontech) was used to express CrV1 by constructing a recombinant virus. This system utilises BacPAK6 which is an engineered version of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV, Baculoviridae). BacPAK6 makes use of an essential gene for replication adjacent to the polyhedrin expression locus. Two restriction sites for *Bsu*36I has been introduced into the genes flanking the polyhedrin locus. Restriction digestion of the virus genomic DNA with *Bsu*36I releases two small fragments (3020 and 1004 bp) one of which carries part of the essential gene for replication. Thus, if the large fragment recircularises, the resulting viral DNA is not able to replicate due to the absence of the complete essential gene. However, if the large fragment recombines by double homologous recombination with a transfer vector (pBacPAK8) carrying the missing sequences along with the target gene (CrV1), the resulting circularised viral DNA can replicate and produce viable viruses (see the following figure). Therefore, using this system produces recombinant viruses at frequencies approaching 100%.

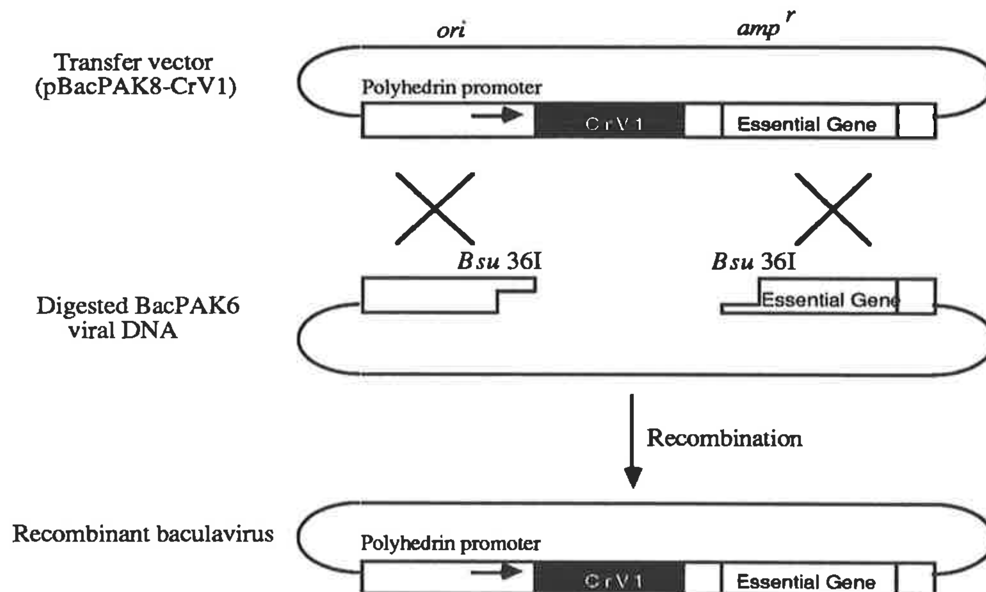


Figure. Construction of a recombinant baculovirus using BacPAK6 expression system. (Modified from Clontech catalogue).

Methods regarding this expression system were adopted from Clontech, catalogue no. K1601-1, (King and Possee, 1992) and (Summers and Smith, 1987).

1. Cell culture

Sf21 cells originally derived from *Spodoptera frugiperda* were used as an insect cell line for the baculovirus expression system. Cells were kindly provided by Dr. B. Hughes, University of South Australia. Sf21 cells were grown in TNM-FH (Gibco-BRL), also referred to as incomplete medium, which consists of Grace's insect cell medium supplemented with yeastolate (3.33 g/l) and lactalbumin hydrolysate (3.33 g/l). TNM-FH was also supplemented with 10% fetal bovine serum (FBS, Gibco-BRL) and gentamycin (50 µg/ml), TNM-FH/FBS. The media was stored protected from light at 4°C and the experiments were carried out under sterile condition.

1a. Subculturing Sf21 cells in monolayer

1. Medium was removed from a flask containing an already established monolayer of Sf21 cells (close to be confluent) and then 2 ml of prewarmed (27°C) TNM-FH/FBS was pipetted into the flask over the monolayer to resuspend a number of cells into the medium.

2. An aliquot of the cell suspension was transferred into a new flask containing 4 ml of prewarmed TNM-FH/FBS and stored at 27°C.

3. Subculturing was carried out almost twice a week up to 80 passages and then cells were replaced by an early passage.

1b. Subculturing Sf21 cells in suspension

An aliquot of cell suspension from a monolayer subculture was added to about 50 ml of TNM-FH/FBS in a spinner flask. Cells were grown in the spinner flask at 65 r.p.m. on a magnetic stirring platform at 27°C. Once the cell density reached to about $2\text{-}3\times10^6$ cells/ml, they were used to seed a fresh spinner flask at a density of $1\text{-}2\times10^5$.

2. Virus amplification and purification

BacPAK6 virus was a generous gift of Dr. B. Hughes, University of South Australia. The virus was amplified in large scale for DNA extraction as following:

1. Two suspension culture flasks were seeded at a density of 1-2×10^5 cells/ml (cells adapted to growth in suspension culture) in 150 ml TNM-FH/FBS and grown until the cell density reached 5×10^5 cells/ml.

2. The cells were inoculated with an aliquot of BacPAK6 virus stock and stirred for 6 days, until they were well infected (indicated by microscopic observations).

3. The suspension was transferred to sterile tubes and the cells were removed by centrifugation at 3000 r.p.m. (Beckman TJ-6) for 10 min at 4°C.

4. The supernatant was transferred to ultracentrifuge tubes and the virus particles were pelleted at 24,000 r.p.m. (Beckman Ti70 rotor) for 1h at 4°C. The supernatant was discarded into disinfectant and the virus pellets were soaked overnight in 1 ml TE buffer at 4°C.

5. The virus particles were resuspended by pipetting up and down and layered onto an ice-cold 10-50% one step sucrose gradient in TE (10 mM Tris-HCl, pH 7.8, 1 mM EDTA; 5 ml 10% (w/v) sucrose was layered over a similar volume of 50% (w/v) sucrose). The gradient was prepared immediately before the centrifugation.

6. The virus banded at the interface of the two sucrose solutions by centrifugation in a swing-out rotor at 24,000 r.p.m. (Beckman SW41 rotor) for 1h at 4°C.

7. The interface which appeared as a whitish band was harvested using a Pasteur pipet. The virus was diluted severalfold in TE buffer and concentrated by centrifugation, as in point 4 above to remove the sucrose.

8. The virus pellet was soaked overnight in 2 ml TE buffer at 4°C, resuspended and stored at 4°C.

3. Viral DNA extraction

Viral DNA was extracted from amplified and purified virus particles as following:

1. 100 µl 20% sarkosyl solution was added to 400 µl purified virus and incubated at 60°C for 30 min to lyse virions.

2. The lysed virus sample was immediately transferred onto a 5 ml cushion of caesium chloride (50% w/w in TE buffer) containing 12.5 µl ethidium bromide (10 mg/ml). The tube was topped-up with liquid paraffin.

3. The tube was centrifuged at 35,000 r.p.m. (Beckman SW41 rotor) for 18h at 20°C.

4. The virus DNA was visible as two orange bands (supercoiled DNA, lower band, and open circular DNA, upper band). UV light was used for better visualisation. The two bands were harvested since both forms are equally infectious. The bands were harvested by removing the liquid from the top and then collected using a sterile Pasteur pipet.

5. The ethidium bromide was extracted twice by adding an equal volume of butanol and shaking gently. The phases were allowed to separate on the bench (2 min) and then the upper phase containing butanol/ethidium bromide was discarded.

6. The DNA was placed into prepared dialysis tubing and dialysed against TE buffer in a sterile beaker with magnetic stirring at 4°C overnight. The dialysis tubing was prepared by boiling the tubing and the clips in TE buffer for 10 min and then rinsed with fresh TE buffer.

7. Following dialysis, the DNA was transferred into a microfuge tube and stored at 4°C.

4. Construction of a recombinant transfer vector

To construct a recombinant transfer vector, CrV1-cDNA was excised from pBluescript-CrV1 (see chapter 4) using *Pst*I and *Kpn*I restriction enzymes and ligated into these sites in a pBacPAK8 transfer vector (Gibco-BRL) using standard cloning techniques (see chapter 2). The AcMNPV sequences flanking the multicloning site of the vector enable homologous recombination with viral DNA to transfer the expression cassette to the polyhedrin locus of the baculovirus genome (see figure below).

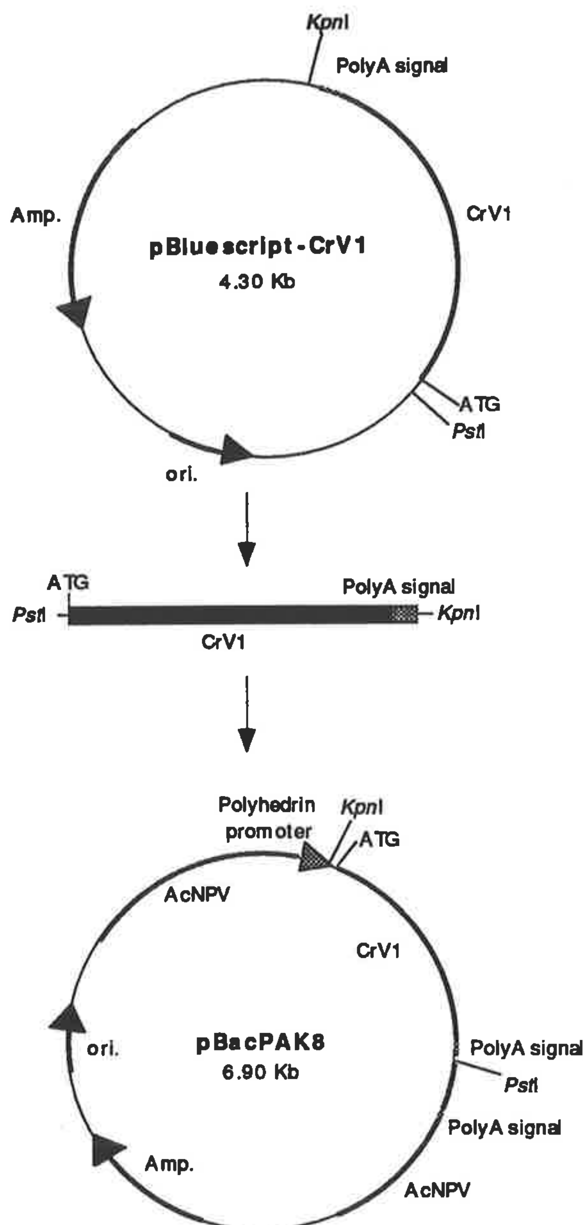


Figure. Construction of a recombinant plasmid transfer vector (pBacPAK8-CrV1). The size of plasmids are not to scale.

5. Construction of a recombinant viral expression vector

To construct a recombinant virus, the transfer vector, pBacPAK8-CrV1, was co-transfected with *Bsu*36I digested BacPAK6 viral DNA into Sf21 cells as following:

1. About 1.5 ml Sf21 cell suspension containing approximately 10^6 cells was added to two 35 mm tissue culture plates and incubated in a humid box (lunch box with pieces of wet tissue paper) at 27°C for 2h.

2. The medium was replaced by 2 ml incomplete medium (without serum and without antibodies), swirled gently and removed. Then, 2 ml incomplete medium was added and incubated at room temperature for 30 min.

3. The plasmid DNA (pBacPAK8-CrV1) was diluted to 100 ng/µl in TE.

4. The following was made in two sterile tubes:

	transfection	control
H_2O	33 µl	45 µl
plasmid DNA	5 µl	5 µl
BacPAK6 (*Bsu*36I)	12 µl	-

Note: For transfection, Lipofectin (Gibco-BRL) and Tfx™-50 (Promega) reagents were used in two different transfections. Both products are lipid based molecules which form lipid vesicles and carry the DNA into the cells by fusion with the plasma membrane.

5. To the mixture from 4, 1.5 ml incomplete medium was added plus 3 µl Tfx™-50 transfection reagent and incubated for 10-15 min at room temperature. In another trial, 50 µl of the Lipofectin reagent (0.1 mg/ml) was added to the DNA, mixed in a polystyrene tube and incubated for 15 min at room temperature.

6. The medium was removed from the plates and the above mixtures were added. In the case of Lipofectin, 2 ml incomplete medium was added to the plate and the DNA-Lipofectin mixture was added dropwise to the medium.

7. The plates were incubated at 27°C in a humid box for 5h.

8. About 1.5 ml complete medium (TNM-FH/FBS) was added to each dish and incubated at 27°C in a humid box.

9. About 60-72h later, the medium was transferred into a sterile tube and stored at 4°C.

10. To obtain more virus, a fresh aliquot (1.5 ml) of TNM-FH/FBS was added to each dish, incubated at 27°C for another 2 days and harvested as above.

6. Recombination evaluation

To find out whether any recombinant virus has been produced, an expression evaluation was carried out. Sf21 cells were infected with the virus mixture collected from cotransfection (see above). As a control, Sf21 cells were also infected with the parental virus (BacPAK6 virus).

The medium was removed and 100 µl of the virus suspension was added to 35 mm culture dishes containing a monolayer of Sf21 cells and incubated at room temperature for 1h. The inoculum was removed from the plates and 1.5 ml TNM-FH/FBS was added to each plate. After 24h (the inserted gene is usually expressed at this time), the cells were resuspended in the medium and collected in a microfuge tube. The cells were pelleted by centrifuging the suspension at 1000 r.p.m. for 2 min. The medium was removed and the cells were resuspended in 1 ml PBS, centrifuged and washed again. RNA was extracted from the cells as described in chapter 2 and a Northern blot analysis was performed using ^{32}P-labelled CrV1-cDNA as a probe to detect the expression of the gene.

7. Isolation of recombinant viruses

As indicated above, using *Bsu*36I-digested BacPAK6 viral DNA for cotransfection produces recombinant viruses at frequencies approaching 100%. However, plaque assays were carried out in order to isolate recombinant viruses.

7a. Plaque assay

1. Approximately 10^5 cells were seeded in 35 mm tissue culture dishes and rocked to distribute evenly. The cells were incubated at 27°C overnight in order to obtain required cell density (about 50% confluent).

2. Serial dilutions of the cotransfection supernatant was made to give final dilutions of 10^{-1}, 10^{-2} and 10^{-3}.

3. The medium was aspirated from the dishes and replaced with serial dilutions of the cotransfection supernatant by gently adding 100 µl of each dilution.

4. Incubation was carried out at room temperature for 1h to allow the virus to infect the cells.

5. During the incubation, 2% agarose in water was melted and cooled to 60°C. Also, an aliquot of TNM-FH/FBS was warmed to 60°C.

6. The virus inoculum was removed from the dishes. Prewarmed medium was mixed with 2% agarose solution 1:1 to make a final concentration of 1% agarose and then 1.5 ml of the mix was overlaid onto each dish.

7. When the agarose overlay had polymerised, 1.5 ml of TNM-FH/FBS was added to each dish and incubated in a humid box for 5-7 days at 27°C.

8. To stain for virus plaques, the medium was removed and 1 ml of 0.03% neutral red in PBS was added to each dish. The dishes were incubated at 27°C for 2-3h.

9. The stain was removed and the dishes were left inverted in the dark at room temperature overnight to allow the plaques to clear. Neutral red is taken up by healthy cells, but not by dead cells, therefore, the plaques appear as clear circles on a pinkish background.

10. Three well-isolated plaques were picked up using the tip of sterile Pasteur pipettes by sucking a plug of agarose into the pipet.

11. The agarose plugs were released into microfuge tubes containing 0.5 ml of TNM-FH/FBS, vortexed and stored at 4°C overnight to allow viruses to diffuse out of the agarose plug.

7b. Amplification of recombinant viruses

The viruses eluted from plaque-picks were amplified as following:

1. Culture dishes (35 mm) were seeded with 5×10^5 Sf21 cells and incubated at 27°C for 1-5h.

2. The medium was removed and 100 µl of a plaque-pick was added to the centre of the dish and incubated at room temperature for 1h.

3. Two ml TNM-FH/FBS were added to each dish and incubated at 28°C for 4 days, until the cells looked well infected.

4. The medium containing amplified virus was transferred to sterile tubes and centrifuged at 1000 r.p.m. for 1 min to remove cell debris.

5. The supernatant was removed to a fresh sterile tube and stored at 4°C (passage one virus stock).

6. The identity of recombinant viruses isolated from plaques was confirmed by infecting Sf21 cells with individual plaques and performing an expression evaluation as described in recombination evaluation (see above).

8. Large scale amplification of recombinant viruses

A large volume of the recombinant virus suspension was produced as described in virus amplification and purification (steps 1-3, see above).

9. Storage of recombinant viruses

For short-term storage, viruses were stored at 4°C and for long-term, aliquots were stored at -70°C.

5.2.3. Affinity chromatography

Since it was found that the 45kDa/CrV1 protein produced by the recombinant virus binds to *H.p.* lectin, affinity chromatography was performed to purify CrV1 protein from the medium collected from Sf21 cells infected with the recombinant virus.

A. Coupling *H.p.* lectin to Sepharose beads

The method was adopted from a Pharmacia booklet on Affinity Chromatography.

1. 0.2 g of freeze-dried CNBr-activated 4B Sepharose beads (Sigma) was swollen in 1 mM HCl for 15 min and then washed on a Poly-Prep® chromatography column using a total of 40 ml of the solution in several aliquots. The final volume after swelling was 1 ml.

2. *H.p.* lectin (2.5 mg) was dissolved in 2 ml coupling buffer (0.1 M Na-borate, pH 8.4, 0.5 M NaCl). A bead : buffer ratio of 1:2 gives a suitable suspension for coupling.

3. The swollen beads were washed with coupling buffer (5 ml per gram dry beads), the lectin-coupling buffer solution was added immediately and incubated for 2h at room temperature on a vertical orbital mixer.

4. The remaining active groups were blocked by transferring the beads into blocking buffer (0.2 M glycine, pH 8.0) and incubated 16h at 4°C.

5. The excess adsorbed and uncoupled lectins were removed by washing the beads four times by 0.1 M acetate buffer (pH 4.0) followed by coupling buffer (pH 8.3) both containing 0.5 M NaCl.

6. The blocking agent was washed away by coupling buffer and the lectin-Sepharose conjugate was stored at 4°C with 0.1% Na-azide.

B. Column purification

1. 10-50 ml of medium containing recombinant-CrV1 protein was mixed with 0.3 ml lectin-Sepharose conjugate over 1-2h at room temperature on a rotary shaker to allow the binding of CrV1 to the lectin.

2. The mix was passed through a Poly-Prep® chromatography column by gravity force.

3. The beads were washed on the column by 200 ml TBST buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20).

4. The bound proteins were eluted from the beads by adding 500 µl of 0.3 mM N-acetylgalactosamine (200 mM final concentration, GalNAc) and incubating for 1-2h at room temperature on a vertical orbital mixer.

5. The eluted proteins were collected from the column by gravity force. The volume was reduced by freeze-vacuum drying (Speedvac) and stored at -20°C.

6. The beads were washed with several volumes of TBST buffer and stored in 3 volumes of TBST containing 0.1% sodium azide for further use.

5.2.4. Protein purification and immunisation

Proteins eluted from *H.p.* lectin column (see above) were run on a 10% SDS-PAGE (see chapter 2) and the gel was then stained in Coomassie solution (0.05% Coomassie blue in water) for 20 min at room temperature. The gel was then destained by washing in deionised water. The protein band (containing 0.1-0.2 µg protein) of the expected size (45kDa/CrV1) was excised, rinsed several times with deionised water and then smashed in water using a homogeniser.

A rabbit was immunised to produce antiserum against 45kDa/CrV1. In each injection, about 1 µg of the protein was injected. The first injection was accomplished by mixing 1:1 of gel pieces suspension and complete Freund's adjuvant (1 ml total volume) followed by four boosters every two weeks using 1:1 mixture of the suspension with incomplete Freund's adjuvant.

The development of the antibody production was monitored by bleeding the animal every two weeks after the first booster and performing dot blots (see below). Once the titre reached a high level, the animal was bled by heart puncture. The blood was left at 4°C overnight to form a clot and the serum was then transferred to fresh tubes, centrifuged at 1000g for 10 min to remove blood cells and debris. The supernatant was mixed 1:1 with sterile glycerol, sodium azide was added to final concentration of 0.02% and stored at -20°C.

5.2.5. Dot blotting

One microliter aliquots of medium collected from Sf21 cells infected with the recombinant baculovirus (see above) containing 45kDa/CrV1 protein were spotted on nitrocellulose membranes. The membranes were treated as described in Western blotting (chapter 2), using the anti-CrV1 antiserum as the first antibody and alkaline phosphatase-conjugated antiserum against rabbit IgG as the secondary antibody. The preserum was used as a control.

5.2.6. Plasmid transformation of Sf21 cells

Insect cells infected with recombinant baculoviruses produce a suites of viral proteins in addition to the foreign gene. Baculoviral genes are expressed in four phases *in vitro* : immediate early, early, late and very late (Blissard and Rohrmann, 1990) from which immediate early genes do not require any viral gene products for their expression. In other words, the promoter of these

genes are transcriptionally active in the absence of other viral functions. IE1, an immediate early gene of AcMNPV contains such a promoter (Guarino and Summers, 1986). Plasmid vectors have been constructed containing the promoter and one of AcMNPV homologous regions (hrs) as an enhancer to produce transformed lepidopteran cells to express continuously a foreign gene in the absence of viral infection (Jarvis *et al.*, 1990). To produce CrV1 devoid of any baculoviral products, the gene was expressed in one of these vectors (pIE1HR4).

A. Construction of recombinant plasmid

A full length CrV1-cDNA fragment previously cloned in pBacPAK8 (see above) was excised by *Bam*HI and *Not*I restriction enzymes and cloned into the same sites in pIE1HR4 under IE1 promoter (see figure below) using standard cloning techniques (Chapter 2). The vector was a kind gift of Dr. Donald L. Jarvis, Texas A&M university.

B. Transformation

1. Sf21 cells were seeded at a density of about 1×10^6 in 35 mm culture dishes and allowed to attach for 3h.

2. Medium was removed and 2 ml incomplete medium (no serum, no antibodies) was added, swirled gently and replaced by 2 ml incomplete medium and incubated at room temperature for 30 min.

3. 2 µg plasmid DNA (pIE1HR4-CrV1) were added to 1.5 ml incomplete medium and vortexed. A similar transformation was set up by using the vector with no insert (pIE1HR4) as a control.

4. 30 µl Tfx™-50 (transformation reagent, Promega) were added to above and vortexed immediately. The mixture was left at room temperature for 15 min.

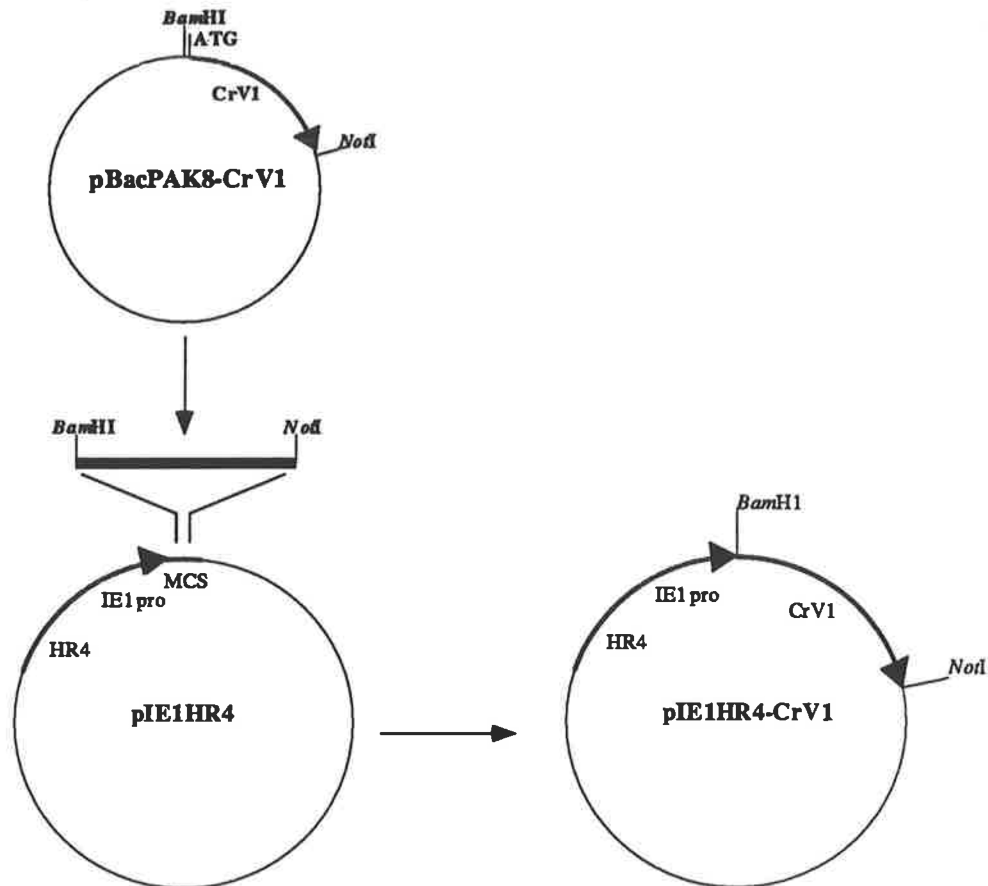


Figure. Cloning strategy of CrV1 into pIEHR4. MCS, multi-cloning site; HR4, homologous region 4. The size of plasmids are not to scale.

5. Medium was removed from the dishes and the mixture was added after a vortex. Cells were incubated at 27°C for 4h.

6. Approximately 1.5 ml complete medium was added to each dish and incubated at 27°C for 48h.

7. Medium was removed and the cells were transferred into a cell culture flask and left for another 48h.

C. Isolation and purification of CrV1

Medium was removed from transformed cells, replaced by fresh incomplete medium (without serum, without antibiotics) and incubated at 27°C for 5h. Medium was removed and CrV1 was purified by column chromatography using *H.p.* lectin as described above. The eluted proteins

were analysed on a Western blot using peroxidase-conjugated *H.p.* lectin and anti-CrV1 antiserum as probe.

5.2.7. Injection of the recombinant protein and cytochalasin D

Sf21 cells were infected with parental and recombinant viruses. After 18h following infection, the complete medium (TNM-FH/FBS) was removed and cells were washed with serum-free medium followed by incubating the cells with serum-free medium for 4-5h at 27°C. The culture medium was collected and cells were removed by centrifugation at 2000 r.p.m. for 2 min. The supernatant was centrifuged at 35,000 r.p.m. (Beckman TL-100) for 1h to pellet any possible virus particles. The supernatant was concentrated about ten times and desalted by using a Centricon-10 centrifugal concentrator (Amicon). Equal amounts of recovered supernatant (about 15 µl), representing similar amounts of proteins from recombinant and parental supernatants, were injected into *P. rapae* caterpillars using a flame extended pasteur pipet (the concentration of CrV1 in the recombinant fraction was between 0.1-0.5 µg/ml). The larvae were bled 24h later and hemocytes were examined. Also, column purified medium isolated from plasmid transformed Sf21 cells was injected into caterpillars (15 µl, 0.1-0.5 µg/ml) as above.

Cytochalasin D (10 µM in 5 µl) was injected into caterpillars as described above.

5.2.8. Indirect immunofluorescence

Naive *P. rapae* caterpillars were bled onto microscope slides and incubated for 10 min to allow the hemocytes to settle down. Hemolymph was removed and hemocyte monolayers were washed three times with PBS. Hemocytes were fixed in 4% paraformaldehyde in PBS for 20 min and washed as above. Culture media containing recombinant CrV1 and devoid of CrV1 were added to hemocyte monolayers and incubated for 2h at room temperature. Then, monolayers were washed with PBS and incubated with anti-CrV1 antiserum and preserum (1:100 PBS) for 45 min at 37°C. Monolayers were washed subsequently as above and incubated with FITC-conjugated anti-rabbit IgG (1:50 PBS) for 45 min at 37°C. After several washes, hemocytes were examined under indirect UV illumination.

5.2.9. General methods

Northern blotting, Western blotting, SDS-PAGE and actin, phosphatidyl serine and lectin staining in hemocytes as described in chapter 2.

5.3. Results

5.3.1. Hemocyte immune activation

As shown previously, cellular hemocyte properties are altered following parasitisation and expression of polydnavirus genes (see chapter 3). These include actin filaments rearrangement and suppression of cell spreading, loss of lectin binding sites on the surface and absence of microparticle formation. To test whether these changes were dependent on functional actin-filaments, cytochalasin D was injected into caterpillars and the hemocytes were isolated in the presence of calcium. Two hours after injection hemocytes failed to bind *H.p.* lectin on the surface (Fig. 5-1C). This suggests that intact actin-filaments are required for the exposure of the lectin-binding sites on the surface of hemocytes. Since lectin-binding proteins are involved in immune-related processes (Nappi and Silvers, 1984; Rizki and Rizki, 1986), the exposure of the *H.p.* lectin-binding sites in isolated hemocytes are probably part of the hemocyte activation process.

This suggests that polydnavirus infection of the host insect interferes with the cellular activation of hemocytes. The observation that cytochalasin D causes similar effects suggests that hemocyte activation, including exposure of *H.p.* lectin-binding sites on the hemocyte surface and microparticle formation, require a functional cytoskeleton. Similar observations have been reported in vertebrate blood cells (Rosales *et al.*, 1994; Yano *et al.*, 1994). Since cytochalasin D treated hemocytes (Fig. 5-1C) were morphologically and functionally similar to hemocytes from parasitised caterpillars (Fig. 5-1B), this implies that intact actin-filaments are required for hemocyte activation. This provided a diagnostic set of cellular criteria to test whether polydnavirus-mediated hemocyte inactivation is due to the single protein CrV1 or whether virus proteins or other virus-encoded proteins are required for the immune suppression.

5.3.2. Recombinant CrV1

The cDNA fragment coding for CrV1 protein (see chapter 4) was cloned into a Bluescript plasmid and transcribed and translated *in vitro* to test the open reading frame. In this system, a 45kDa protein was produced which was labelled with [^{35}S]methionine (Fig. 5-2A). The molecular weight of the produced protein was higher compared to the predicted molecular weight of isolated CrV1 (33.6 kDa) which is either due to slow migration of this protein on the SDS-PAGE or to post-translational modifications in this *in vitro* system such as glycosylation (Starr and Hanover, 1990).

To produce recombinant CrV1, the cDNA fragment coding for the protein was cloned into a baculovirus expression vector and expressed in Sf21 cells. Expression evaluation of viruses produced after transfection using Lipofectin and Tfx^{TM}-50 as transfection reagents showed that recombination has taken place in both cases although Tfx^{TM}-50 provided a better result (Fig. 5-2B). Two RNA transcripts of 1.6 and 1.4 kb in size were recognised by radioactively labelled CrV1 as a probe (Fig. 5-2B). This may be due to the presence of CrV1's own polyA signal which is probably added to the vector's polyA tail or due to the presence of a secondary initiation site upstream of CrV1's initiation site. No hybridisation was observed with RNA isolated from Sf21 cells infected with the parental baculovirus (Fig. 5-2B). Furthermore, expression evaluation of the viruses eluted from plaque-picks isolated from plaque assays in which Sf21 cells were infected with the transfection supernatant showed that they all express CrV1 (Fig. 5-3A). The expression of CrV1 was found to start as early as 18h post-infection (p.i.) of Sf21 cells by the recombinant virus and continued up to 72h p.i. with the peak of expression at 24 p.i. (Fig. 5-3B).

Since the deduced protein sequence predicted for the CrV1-protein to be secreted, with potential O- and N-glycosylation sites (see chapter 4), Sf21 cells and the culture medium from recombinant virus-infected Sf21 cells were analysed for the presence of recombinant CrV1, using lectins. On Western blots, the GalNAc-specific lectin (*H.p.* lectin) bound to a glycoprotein of the expected size (45kDa) according to *in vitro* translation (see above), which was not present in the lysate and the culture medium of cells infected with the parental baculovirus (Fig. 5-4A).

In addition, when the culture medium from [^{35}S]methionine pulse-labelled Sf21 cells infected with the recombinant virus was analysed, the 45kDa labelled protein was the only one that was absent in cells infected with the parental virus (Fig. 5-4B). *H.p.* lectin-affinity purified recombinant glycoproteins contained a major glycoprotein of the expected size (Fig. 5-5A).

To confirm the identity of the CrV1 protein, antibodies were raised against the affinity purified glycoprotein. The antiserum recognised the recombinant 45kDa protein (Fig. 5-6A) and tested on polydnavirus-infected hemocytes and cell-free hemolymph (Fig. 5-6B). Six hours after parasitisation, 45kDa/CrV1 was detected in hemocytes and in cell-free hemolymph from parasitised caterpillars on Western blots (Fig. 5-6B, lane 1,3), but not in preparations from non-parasitised caterpillars (Fig. 5-6B, lane 2,4). However, the protein isolated from hemolymph fraction always appeared slightly lower in size and more diffused compared to the recombinant protein (see below).

5.3.3. CrV1-mediated hemocyte inactivation

To efficiently express CrV1 *in vitro*, the cDNA fragment was cloned into a baculovirus expression vector and incubated with Sf21 cells. Since the deduced protein sequence predicted a secreted protein, the CrV1 was expected to accumulate in the cell culture medium. To test whether the recombinant glycoprotein (45kDa/CrV1) caused hemocyte inactivation, the virus-free culture media from recombinant and parental baculovirus-infected cells were each injected into caterpillars, and hemocytes were inspected at various times after injection. Whereas the effects of culture medium from parental baculovirus-infected cells were indistinguishable from normal caterpillars, the medium from recombinant baculovirus-infected cells caused changes in hemocyte behaviour with regard to spreading and *H.p.* lectin-binding that were identical to hemocyte alterations from polydnavirus-infected caterpillars. In this context, we analysed the actin-filaments of CrV1-treated hemocytes, using phalloidin as a diagnostic marker. In hemocytes from recombinant CrV1-injected caterpillars, the actin filaments were disrupted and unpolymerized actin was detected at the cell periphery and nucleus (Fig. 5-7B). *H.p.* lectin staining was also absent on infected hemocytes (Fig. 5-7D). The absence of *H.p.* lectin-binding to hemocytes from CrV1-injected caterpillars is particularly interesting, given that CrV1 is able

to bind to the lectin (see below). Although these effects were not observed in hemocytes from caterpillars that were injected with culture medium from parental baculovirus-infected cells (Fig. 5-7A,C), an indirect involvement of baculovirus proteins in the observed hemocyte alterations was not entirely excluded in these experiments.

To demonstrate that 45kDa/CrV1 alone is sufficient to cause hemocyte inactivation, the CrV1-coding DNA fragment was cloned into an expression plasmid under the control of an immediate early baculovirus promoter, capable of initiating transcription in lepidopteran cells (Jarvis *et al*., 1990). To identify the CrV1 gene product in the culture medium of transfected cells, the glycoprotein was enriched using a *H.p.* lectin column and analysed on a Western blot using *H.p.* lectin as a probe (Fig. 5-5B, lane 2). The identity of the protein eluted from the column was confirmed by the antibodies raised against the 45kDa/CrV1 protein (Fig. 5-6C). Injection of the plasmid-derived recombinant CrV1 glycoprotein into caterpillars caused hemocyte changes that were indistinguishable from those observed with the baculovirus-derived protein (Fig. 5-8, B,F), as well as polydnavirus-infected hemocytes (see Fig. 3-2,-5,-6). The inactivation of hemocytes can be detected in caterpillars for up to 30h after CrV1 injection. Hemocytes isolated after this time are normal in most caterpillars. Similarly, hemocytes isolated from caterpillars two days after parasitisation are indistinguishable from normal hemocytes. This suggests that CrV1 is probably responsible for the transient hemocyte inactivation caused by polydnavirus infection of caterpillars. Although CrV1 binds to the surface of hemocytes *in vitro* (Fig. 5-9), attempts to mimic the effects in isolated hemocytes by incubating the cells with the medium containing the recombinant CrV1 failed. It is quite likely that the protein is modified *in vivo* after being secreted into the hemolymph to be effective (see below).

5.3.4. Hemolymph-mediated alteration of CrV1

When hemocytes from parasitised *P. rapae* caterpillars were examined on a Western blot using *H.p.* lectin as a probe, a 45 kDa glycoprotein was detected representing CrV1 absent in hemocytes from unparasitised caterpillars (Fig. 5-10A). However, when cell-free hemolymph from parasitised caterpillars was examined, the 45 kDa protein was not discernible but instead a 40 kDa glycoprotein was detected by the lectin (Fig. 5-10B) which appeared 6h following

parasitisation (Fig. 5-10C). This suggested that the 40 kDa glycoprotein is either a modified form of 45kDa/CrV1 protein being processed after secretion into the hemolymph, the CrV2 product or a host protein induced following parasitisation. Since the parasitoid egg has not hatched by this time, the possibility of secretions from the parasitoid residing inside the host is unlikely.

To investigate the first possibility, the anti-45kDa/CrV1 antiserum was used as a probe on a Western blot containing hemolymph from 6h parasitised caterpillars. The antiserum did not recognise the 40 kDa protein but a 45 kDa representing CrV1 (Fig. 5-11, AB). This clearly shows that the 40 kDa glycoprotein which appears 6h after parasitisation in the hemolymph is not related to CrV1 but represents an induced host protein or possibly the CrV2 product. The recognition of 45kDa/CrV1 protein in the hemolymph by the antiserum and not by the lectin suggests that there might be modifications on the sugar determinants of the protein after being secreted into the hemolymph. To test this possibility, the recombinant 45kDa/CrV1 protein was mixed with hemolymph from unparasitised caterpillars and analysed on a Western blot using *H.p.* lectin as a probe. Under these conditions, the protein was not recognised by the lectin (Fig. 5-11A, CrV1+H) supporting the idea that the sugar determinants on the protein are modified in the hemolymph. In support of this notion, a slight reduction in size was notable in the CrV1 protein after incubation with cell-free hemolymph and visualisation with anti-CrV1 antiserum (Fig. 5-11B) probably due to removal of sugar residues. N-acetylgalactosamine (GalNAc) residues are the most likely candidates for removal since it is probably a terminal sugar and the protein mixed with the hemolymph is no longer recognised by *H.p.* lectin which specifically binds to GalNAc residues (Fig. 5-11A, CrV+H). It is worthwhile to mention that N-acetylgalactosaminidase activity has been reported in insects (Vandie *et al.*, 1996).

5.4. Discussion

The experimental data presented here provide evidence that the polydnavirus-mediated effects on hemocytes observed in parasitised *P. rapae* caterpillars are probably caused by a single glycoprotein, CrV1. Polydnavirus-infected caterpillars and caterpillars injected with CrV1

produced by recombinant baculoviruses revealed similar hemocyte alterations which are consistent with an inhibition of immune-related activation processes. In parasitised caterpillars, the CrV1 glycoprotein is secreted into the hemolymph by polydnavirus infected hemocytes and fat body cells, where it interacts with the hemocyte surface. Since CrV1 is recognised by *H.p.* lectin and is anticipated to bind to the surface of hemocytes, the observed absence of lectin-binding in hemocytes from polydnavirus-infected and CrV1-injected caterpillars was unexpected. A possible explanation for the loss of *H.p.* lectin binding which is provided by experiments indicate that the secreted CrV1 glycoprotein is exposed to glycosidase activities presumably present in the hemolymph. Moreover, CrV1 only shows its effects on hemocytes inside the caterpillar, but not when applied to isolated hemocytes which further supports the assumption that the protein has to be modified *in vivo*. It is possible that CrV1 interacts with a specific receptor on the non-activated hemocyte surface. CrV1 might act directly on the cell surface to alter hemocyte physiology by causing a breakdown of actin filaments, or is internalised to function in the cytoplasm. However, the exact mode of interaction with the hemocyte remains to be elucidated.

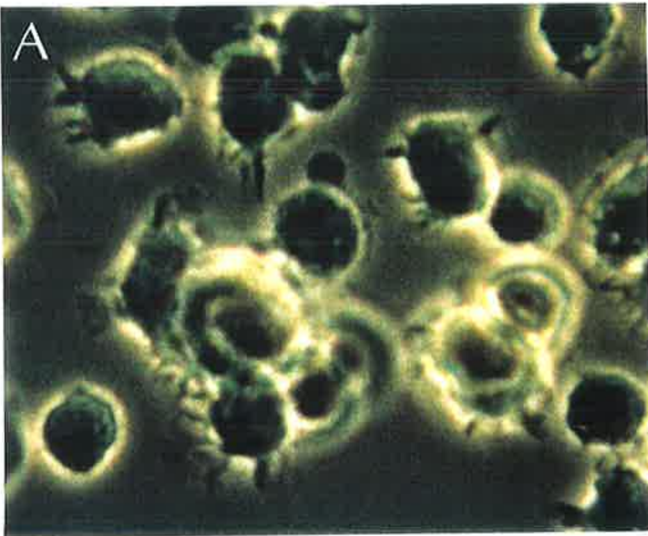
Although little is known about the molecular reactions leading to immune-related hemocyte activation, these events are essential for the attachment, aggregation (Gupta, 1991; Ratcliffe, 1993) and coagulation reactions (Bohn, 1986), that constitute the cellular defence response against bacteria and parasitoids. Exposure of hemocytes to foreign objects, or microorganisms, causes cell membrane rearrangements (Bohn, 1986; Nappi and Silvers, 1984; Strand and Pech, 1995a), which expose specific surface molecules and facilitate cell adhesion (Rizki and Rizki, 1983). These changes may also include the discharge of cortical granules containing components resembling extracellular matrix components (Strand and Pech, 1995a). The discharge and exposure of adhesive components probably enables hemocytes to attach to foreign surfaces. In addition, the formation of microparticles has been shown recently to be part of insect hemocyte activation (Theopold and Schmidt, 1997). Activated hemocytes form blebs and cellular extensions leading to vesicle formation, which are similar to microparticles in activated mammalian blood cells (Yano *et al.*, 1994). A characteristic feature of particle formation is the exposure of phosphatidylserine (PS) on the outer leaflet of the cellular and particle membrane,

which can be identified using annexin V as a diagnostic marker. The presence of PS is known to enhance coagulation processes in vertebrate systems (Franc *et al.*, 1996). Since both degranulation and microparticle formation involve a rearrangement of the cytoskeleton (Rosales *et al.*, 1994; Yano *et al.*, 1994), the absence of functional actin-filaments in CrV1-treated hemocytes is probably the primary cause of hemocyte inactivation considering that actin cytoskeleton plays a pivotal role in cell motility, cytokinesis and phagocytosis. More specifically, the inactivation of actin-filaments by CrV1 probably precludes cellular activation reactions of hemocytes similar to cytochalasin D inactivation in vertebrate blood cells (Bengtsson *et al.*, 1993; Mooney *et al.*, 1995).

It has been shown that GTPases of the Rho family are involved in regulation of the actin cytoskeleton in vertebrates and invertebrates (Eaton *et al.*, 1995; Nobes and Hall, 1995). Rho proteins transduce signals from plasma membrane receptors and control cell adhesion, motility and shape (Murphy *et al.*, 1996). Therefore, these proteins might be a potential target for CrV1 protein causing activation or inactivation of the relevant proteins. Recently, a new member of this family, RhoD, has been discovered that induces disassembly of actin stress fibres and focal adhesions from the cell periphery (Murphy *et al.*, 1996). Induction of this protein might result in disassembly of actin filaments in infected hemocytes although this remains to be shown experimentally.

Figure 5-1

Effects of parasitisation and cytochalasin D treatment on hemocytes. **A)** Exposure of *H.p.* lectin-binding protein on the surface of activated hemocytes after treatment with FITC-conjugated *H.p.* lectin. **B)** Exposure of *H.p.* lectin-binding protein on the surface of hemocytes from a parasitised caterpillar. The caterpillar was parasitised 24 h before hemocyte isolation. **C)** Exposure of *H.p.* lectin-binding protein on the surface of hemocytes from a caterpillar that was injected with cytochalasin D (10 μM) 2 h before hemocyte isolation. Phase contrast pictures are shown on the left side; the corresponding exposures with indirect UV-light are shown on the right side.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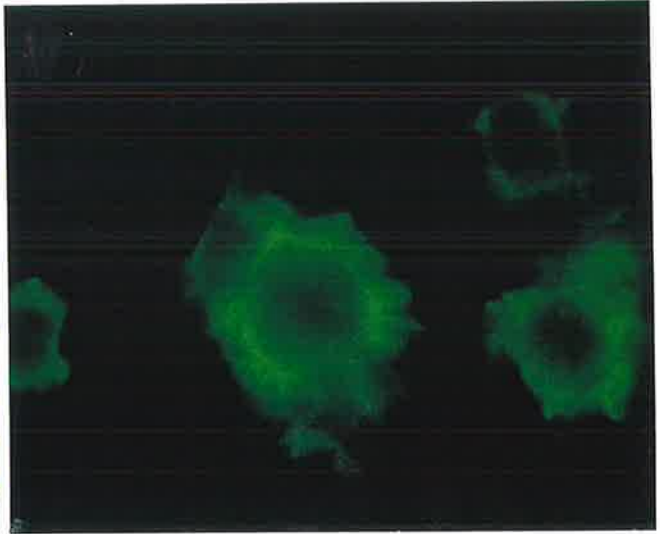
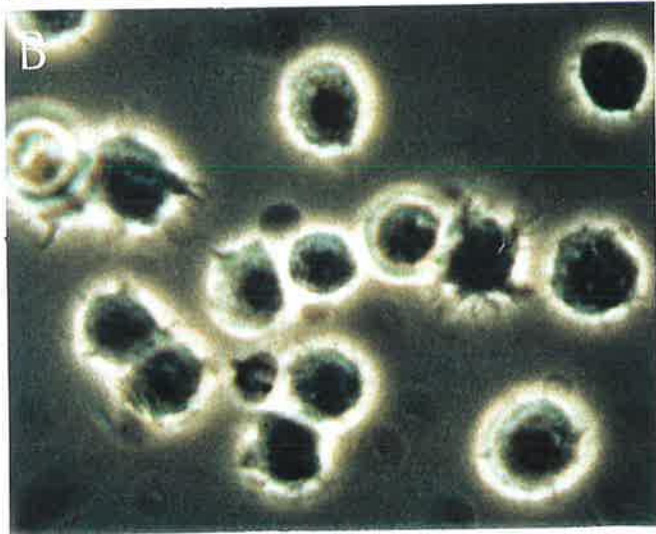
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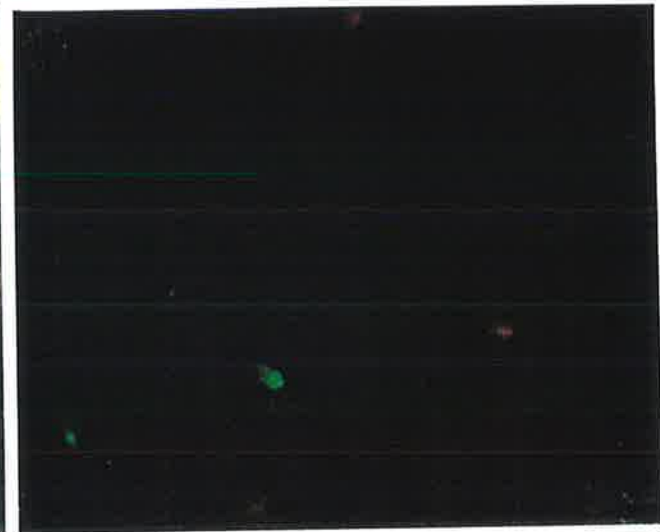
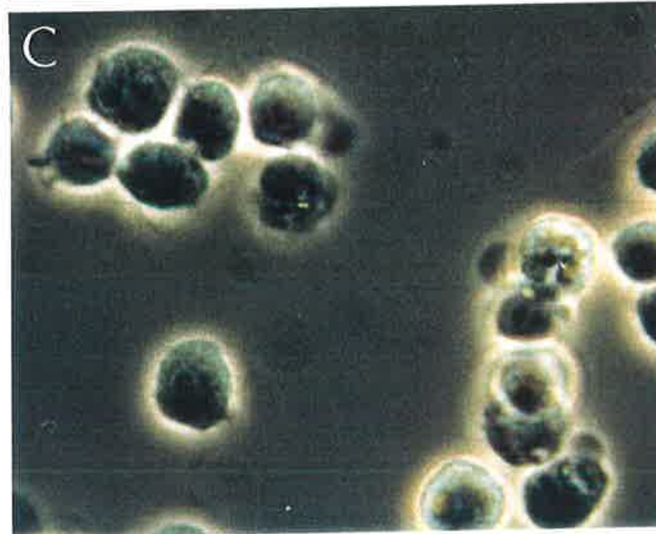
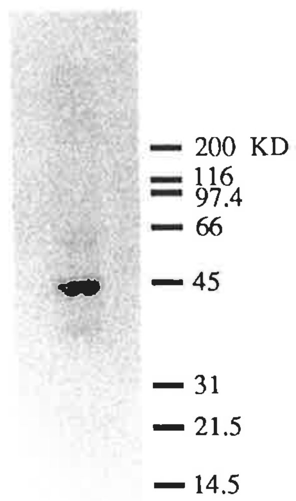


C

Figure 5-2

A) *In vitro* transcription/translation of CrV1-cDNA fragment using pBKS^{+}-CrV1 as a template and T7 promoter in rabbit reticulocyte lysate by incorporating [^{35}S]methionine. A major band of 45kDa is evident indicating the presence of a complete and functional open reading frame.

B) Evaluation of recombination and expression of CrV1 by Sf21 cells infected with recombinant viruses in Northern blot analysis. **Lane 1)** total RNA isolated from Sf21 cells infected with the virus mixture collected from cotransfection using Lipofectin; and **lane 2)** Tfx^{TM}-50 as transfection reagents. Two RNA transcripts of 1.6 and 1.4 kb in size were hybridised to radioactively labelled CrV1-cDNA fragment, indicating the expression of CrV1; **Lane 3)** RNA from Sf21 cells infected with the parental virus as a negative control.

A



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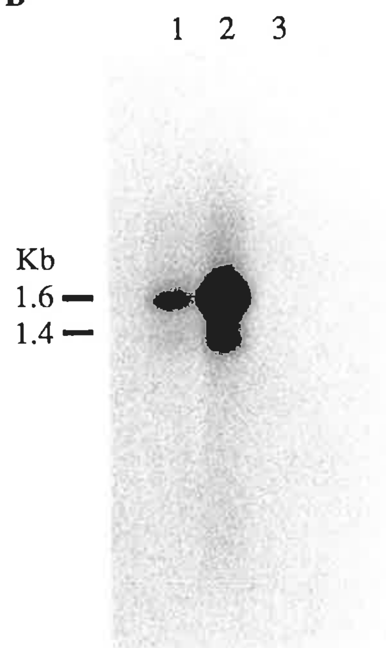


Figure 5-3

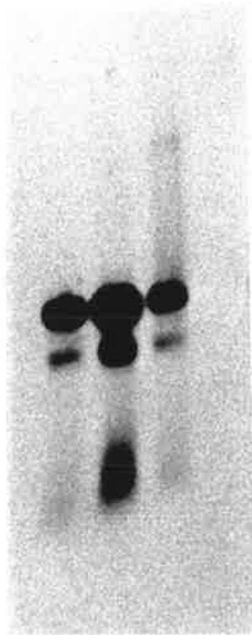
A) Evaluation of plaque-picked viruses with respect to expression of CrV1. Three plaques were picked from a plaque assay in which Sf21 cells were infected with the virus mixture collected from cotransfection. RNA was extracted from Sf21 cells infected with individual plaque-picked viruses and analysed on a Northern blot using radioactively labelled CrV1-cDNA fragment as a probe. **Lanes 1-3)** three plaque-picked viruses, all turned out to be recombinant and express CrV1; **Lane 4)** RNA from Sf21 cells infected with the parental virus as a negative control.

B) Hybridisation to a Northern blot containing total RNA isolated from Sf21 cells at 18, 24, 36, 48 and 72h after infection with CrV1-recombinant viruses. RNA from cells infected with the parental virus was used as a negative control (P). Each RNA fraction was spectroscopically measured and equal aliquots of RNA (2μg) were applied to each slot. The filter was probed with ^{32}P-labelled CrV1-cDNA fragment. The expression of CrV1 apparently starts as early as 18h after infection with a maximum expression at 24h post-infection and continues up to 72h post-infection.

A

1 2 3 4

1.8 kb—
1.4 kb—



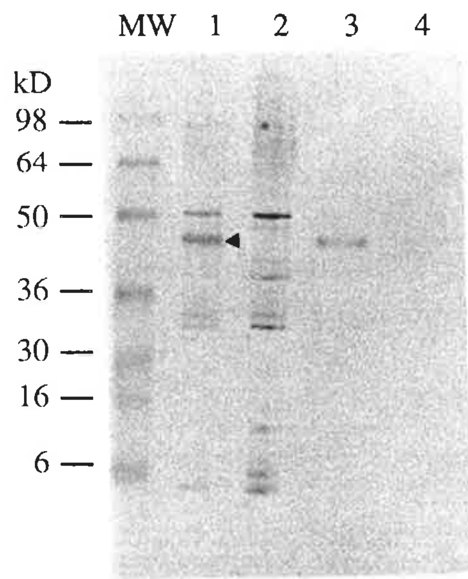
B

P 18 24 36 48 72

Figure 5-4

A) Western blot analysis of the proteins from Sf21 cells infected with recombinant and parental baculoviruses. **Lane 1)** cells infected with recombinant virus; and **lane 2)** parental virus; **lane 3)** corresponding cell culture media from recombinant; and **lane 4)** parental virus-infected cells. The membrane was probed with peroxidase-conjugated *H. pomatia* lectin. A 45kDa glycoprotein was found unique in cells and the corresponding medium infected with recombinant viruses (arrowhead).

B) Expression of 45kDa/CrV1 protein by recombinant virus-infected Sf21 cells and its secretion into the corresponding medium. Infected cells were pulse-labelled with [^{35}S]methionine for 2h at 20h following infection and the cell free medium was analysed on a 12% polyacrylamide gel with fluorography. **Lane 1)** cells infected with parental virus; and **lane 2)** recombinant virus.


A
MW
1
2
3
4
kD
98
64
50
36
30
16
6

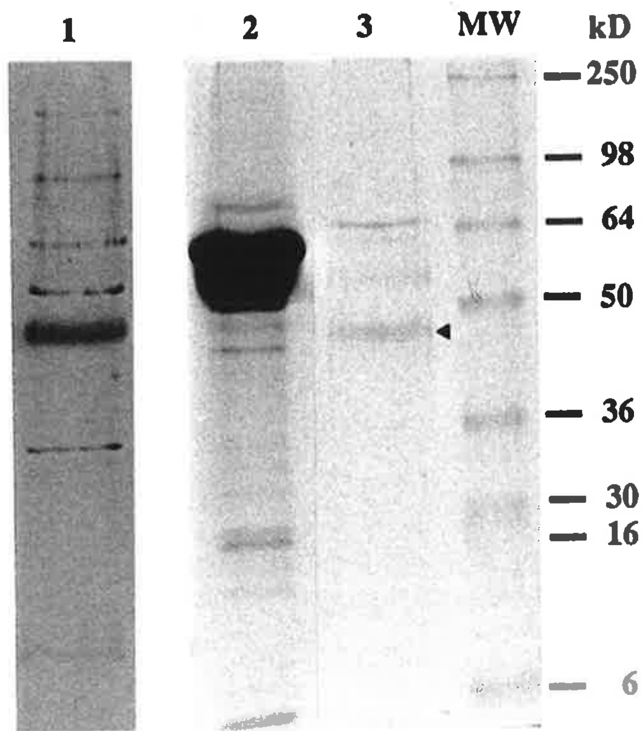
B
1
2
45 kD

Figure 5-5

A) Affinity purified CrV1. Culture medium from Sf21 cells was passed through a *H.p.* lectin-Sephadex column and the bound fraction was eluted with GalNAc solution. **Lane 1)** an aliquot of the eluted material analysed on a Western blot and probed with *H.p.* lectin.; a 45kDa glycoprotein corresponding to CrV1 is discernible; **lane 2)** medium containing CrV1 analysed on a 12% SDS-PAGE; **lane 3)** eluted glycoproteins from *H.p.* lectin column concentrated by vacuum drying and analysed on a 12% SDS-PAGE, the 45kDa/CrV1 protein is enriched in the eluted fraction (arrowhead) compared to lane 2 in which CrV1 is not visible. MW: molecular weight marker.

B) Affinity purified CrV1. Culture medium from Sf21 cells was passed through a *H.p.* lectin-Sephadex column and the bound fraction was eluted with GalNAc solution. Aliquots of the eluted material were analysed on a Western blot and probed with peroxidase-conjugated *H.p.* lectin. **Lane 1)** Culture medium from recombinant baculovirus-infected cells (15 µl concentrate, equivalent of 150 µl medium); **lane 2)** culture medium from recombinant plasmid-transfected cells (15 µl elute, equivalent of 0.7 ml medium; **lane 3)** culture medium from vector plasmid-transfected cells (15 µl elute, equivalent of 0.7 ml medium).

(A)



(B)

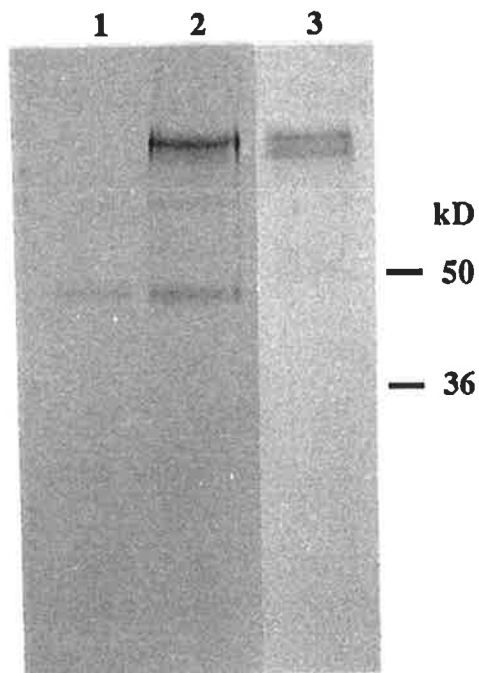


Figure 5-6

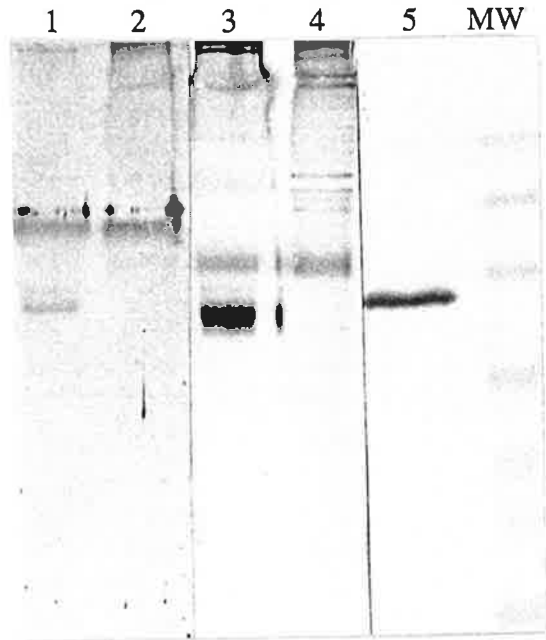
A) Testing the antiserum raised against affinity purified 45kDa/CrV1 protein on a Western blot. The antiserum recognised the protein in the medium containing CrV1 produced by recombinant virus-infected Sf21 cells **(AB)**, the antiserum was diluted 1:10,000. The preserum did not recognise the protein in the medium **(Pre)** using 1:5000 dilution of the serum. Molecular weight markers from the top to the bottom are 98, 64, 50, 36, 30, 16 and 6 kDa in size.

B) Antibody staining of CrV1. Antiserum raised against CrV1 was used to probe a Western blot containing **lane 1)** hemocytes from parasitised; and **lane 2)** non-parasitised caterpillars; **lane 3)** cell-free hemolymph from parasitised; and **lane 4)** non-parasitised caterpillars; **Lane 5)** protein extract from culture medium of CrV1-expressing baculovirus-infected Sf21 cells. Molecular weights from the top to the bottom are 98, 64, 50, 36, 30, 16 and 6 kDa in size.

C) Western blot analysis of CrV1 produced by recombinant plasmid-transformed Sf21 cells using anti-CrV1 antiserum as a probe. **Lane 1)** column purified medium from vector plasmid-transfected cells; **lane 2)** column purified medium from recombinant plasmid-transfected cells; **lane 3)** culture medium from recombinant baculovirus-infected cells. The molecular weights are in kDa.

(A)

(B)



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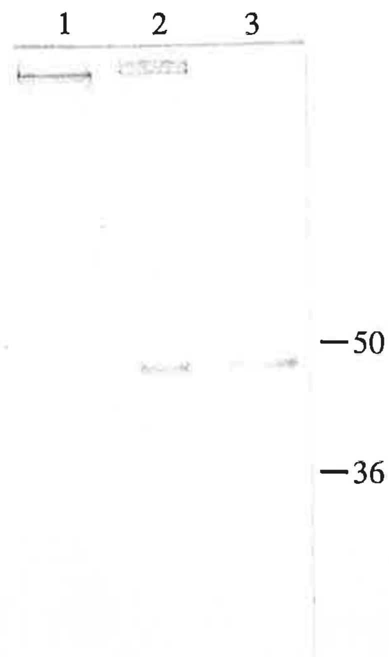
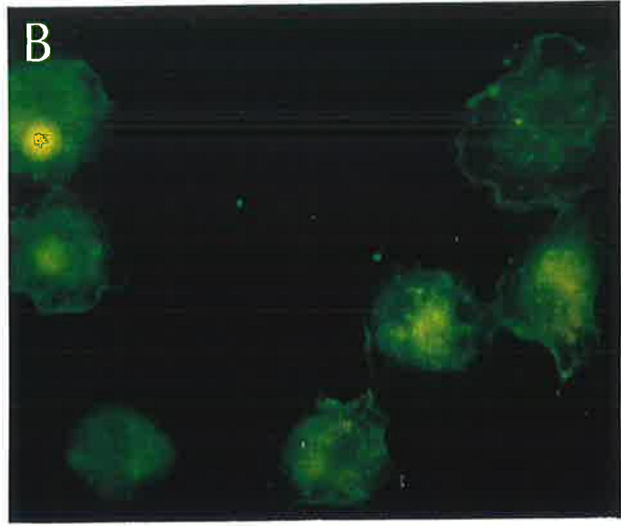


Figure 5-7

Hemocyte changes after injection of recombinant CrV1 into caterpillars. Hemocytes stained with FITC-conjugated phalloidin from **A)** a caterpillar that was injected 24 h previously with culture medium from cells infected with parental baculovirus, and **B)** a caterpillar which was injected with culture medium from cells infected with CrV1-expressing baculovirus. Hemocytes stained with FITC-conjugated *H.p.* lectin from **C)** a caterpillar which was injected with culture medium from cells infected with parental baculovirus, and **D)** a caterpillar which was injected with culture medium from cells infected with CrV1-expressing baculovirus.

A

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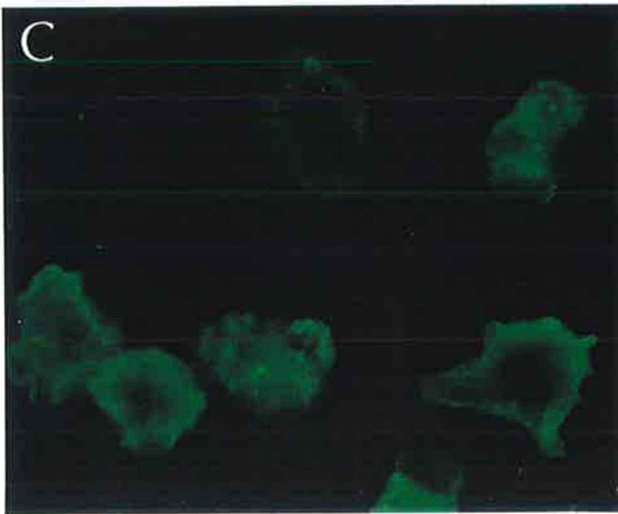
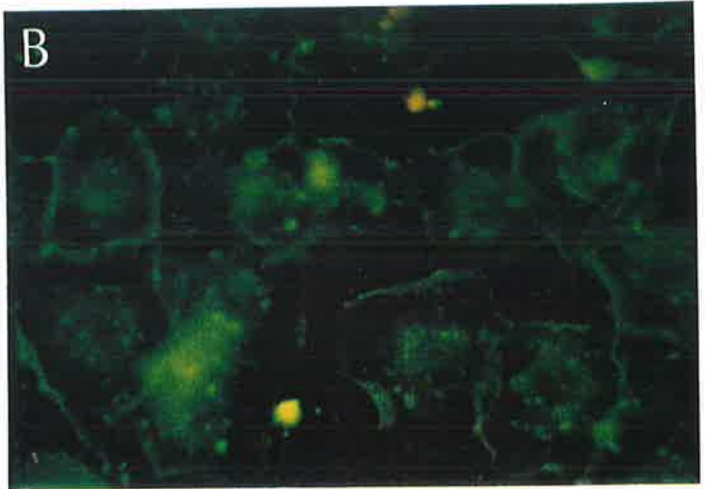

C

D

Figure 5-8

Hemocyte changes in caterpillars after injection with recombinant plasmid encoded CrV1 glycoprotein. **(A,B)** Hemocytes stained with FITC-conjugated phalloidin from caterpillars which were injected with **A)** culture medium from cells transfected with a plasmid vector, and **B)** culture medium from cells transfected with a CrV1-expressing plasmid. **(C-F)** Cells stained with FITC-conjugated annexin V from caterpillars injected with **D)** culture medium from cells transfected with a plasmid vector, and **F)** culture medium from cells transfected with a CrV1-expressing plasmid. **(C-E)** The same cells sections are shown in phase contrast.

A

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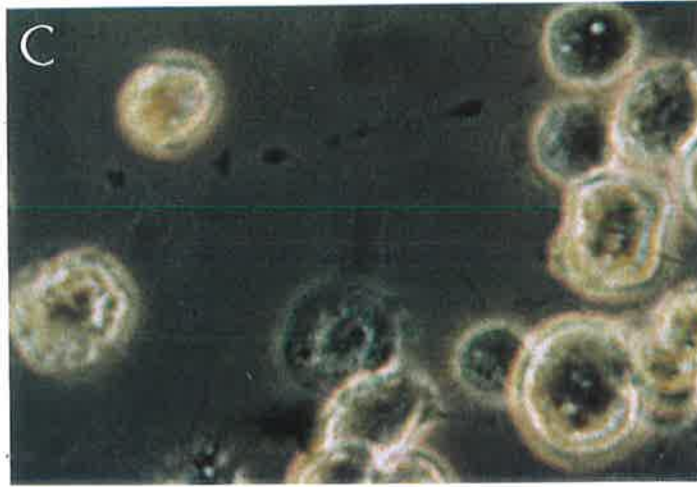
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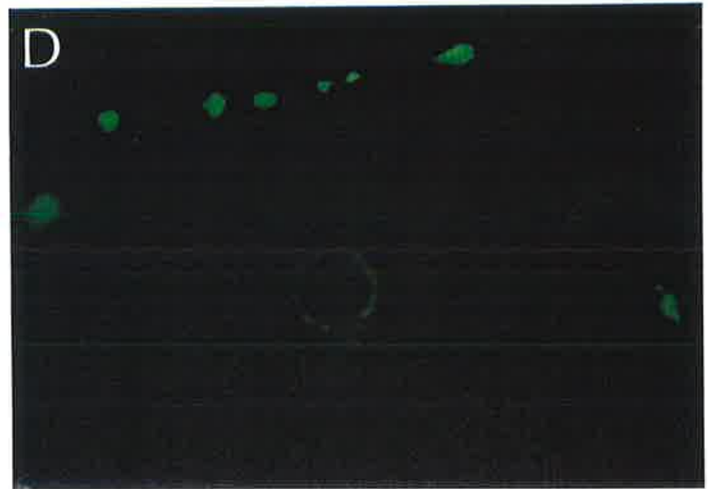
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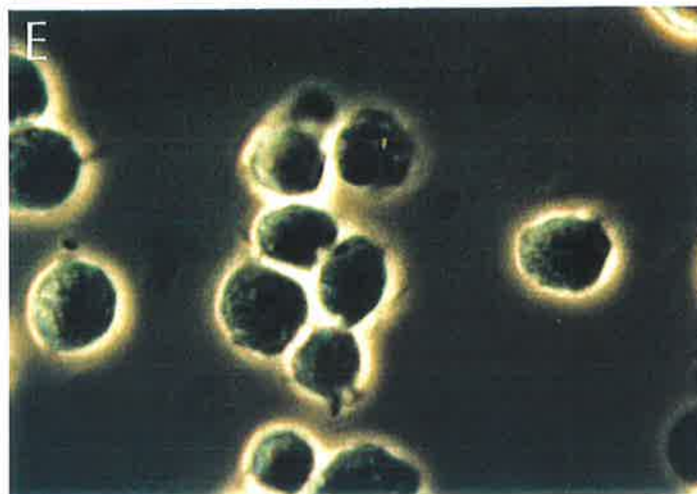
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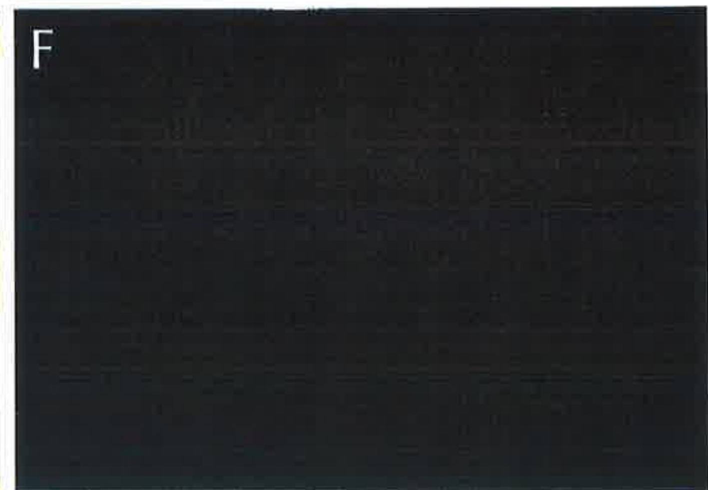
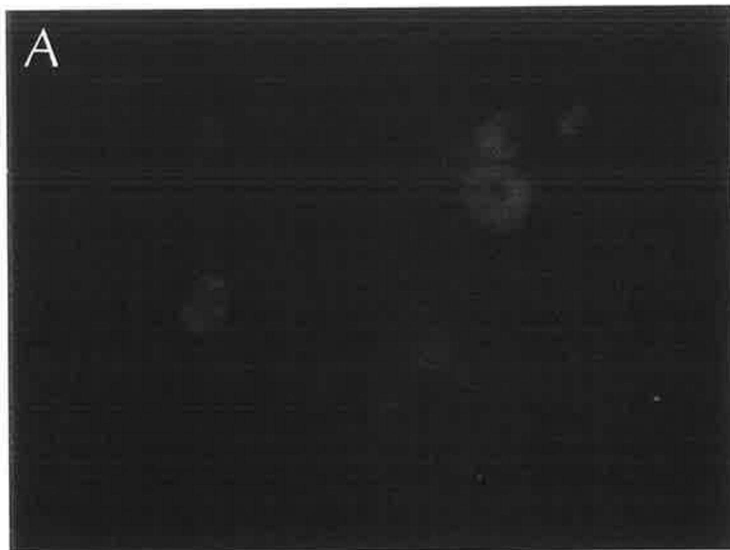

F

Figure 5-9

Immunofluorescence detection of 45kDa/CrV1 protein bound to *P. rapae* hemocytes. Hemocytes were fixed and incubated with culture medium containing recombinant CrV1 and subjected to indirect immunofluorescence labelling. **A)** Hemocytes incubated with culture medium devoid of CrV1 and subjected to anti-CrV1 antiserum. **B)** Hemocytes incubated with culture medium containing CrV1 and subjected to anti-CrV1 antiserum. **C)** Hemocytes incubated with culture medium containing CrV1 and subject to preserum.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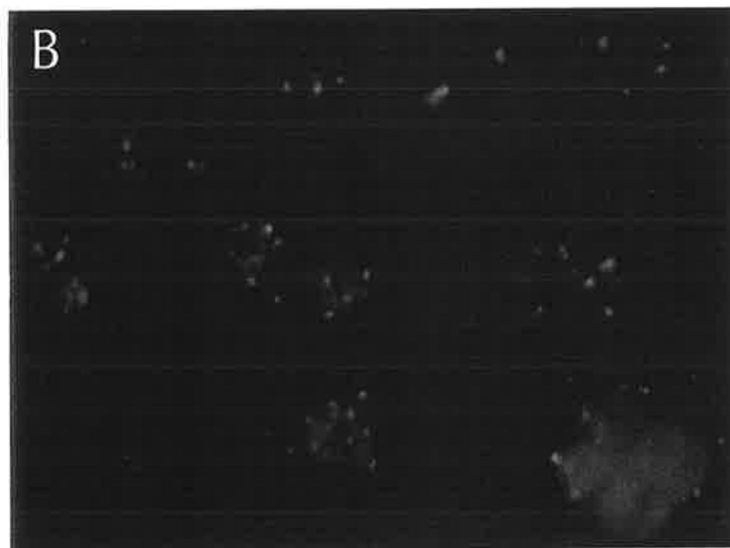
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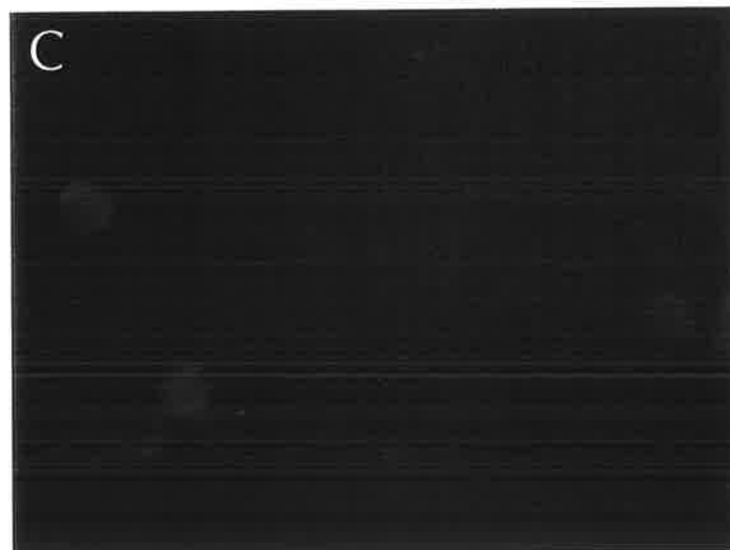

C

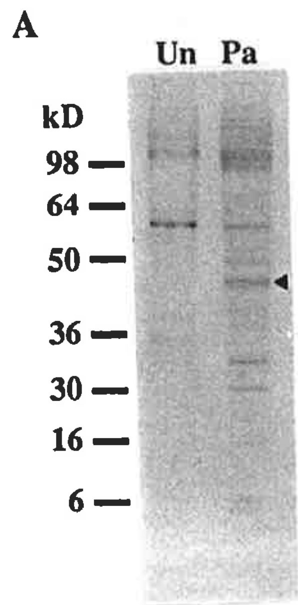
Figure 5-10

A) Western blot analysis of hemocytes from 18h parasitised caterpillars **(Pa)** and unparasitised caterpillars **(Un)** probed with peroxidase-conjugated *H.p.* lectin. A 45 kDa glycoprotein was only found in hemocytes isolated from parasitised caterpillars (arrowhead).

B) Western blot analysis of hemolymph from 18h parasitised caterpillars **(Pa)** and unparasitised caterpillars **(Un)** probed with peroxidase-conjugated *H.p.* lectin. A 40 kDa glycoprotein was detected in the hemolymph from parasitised caterpillars (arrowhead).

C) Western blot analysis of hemolymph at 0, 1, 2, and 6h following parasitisation using peroxidase-conjugated *H.p.* lectin as a probe. Hemolymph from unparasitised caterpillars was used as a negative control (Un). A 40 kDa glycoprotein was detected at 6h after parasitisation (arrowhead).

A



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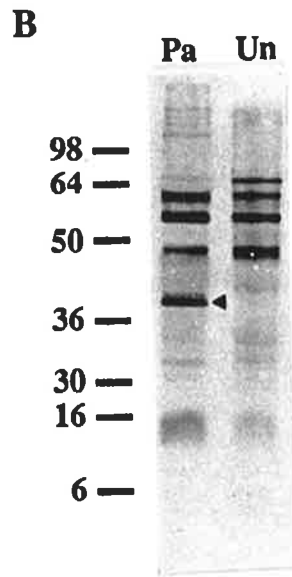

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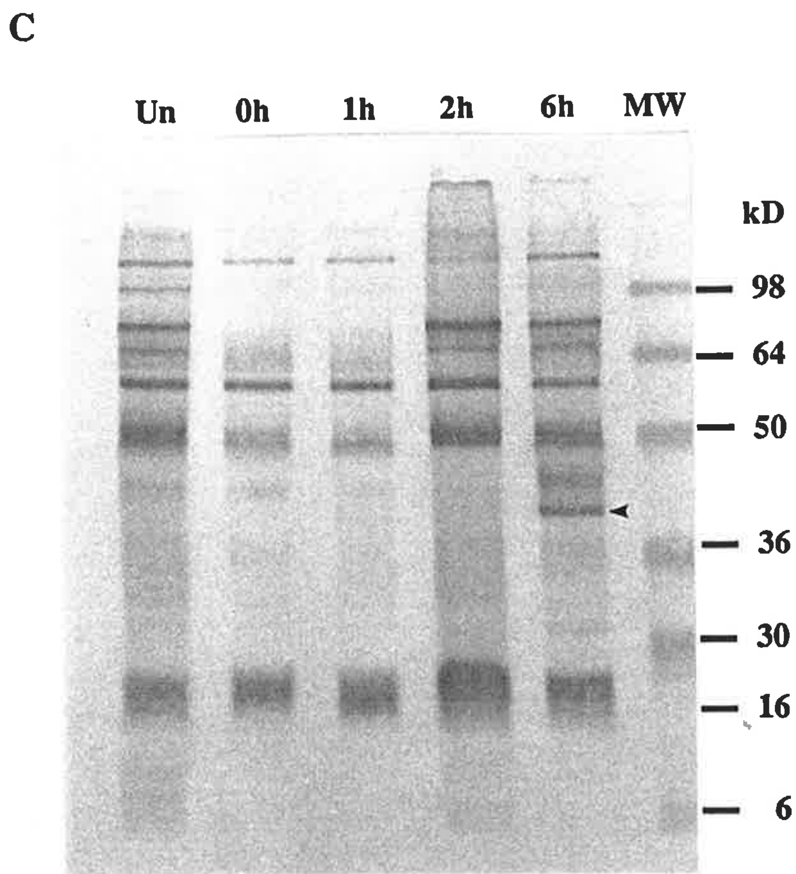
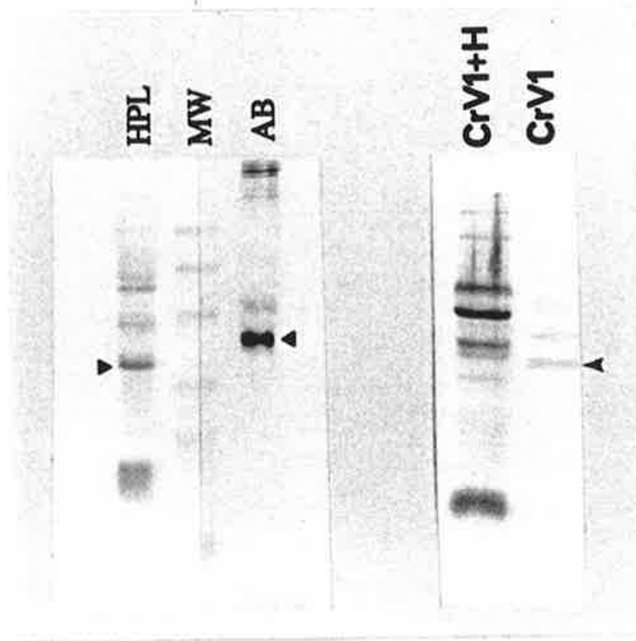


Figure 5-11

A) Western blot analysis of hemolymph from 6h parasitised caterpillars probed with peroxidase-conjugated *H.p.* lectin (**HPL**) and the anti-CrV1 antiserum (**AB**). The 40 kDa glycoprotein recognised in parasitised hemolymph by the lectin (left arrowhead) is not a CrV1 product since the antibody recognised a higher molecular weight protein corresponding to CrV1 (right arrowhead). When the medium containing the 45kDa/CrV1 protein (**CrV1**, arrowhead) was mixed with hemolymph from naive caterpillars, the protein was not recognised by the lectin (**CrV1+H**).

B) Western blot analysis of the medium from cells infected with the recombinant virus containing 45kDa/CrV1 protein (**Rec**) and the medium mixed with hemolymph from unparasitised caterpillars (**Rec+Hem**). A slight reduction in size of the protein may be evident after being exposed to hemolymph.

(A)



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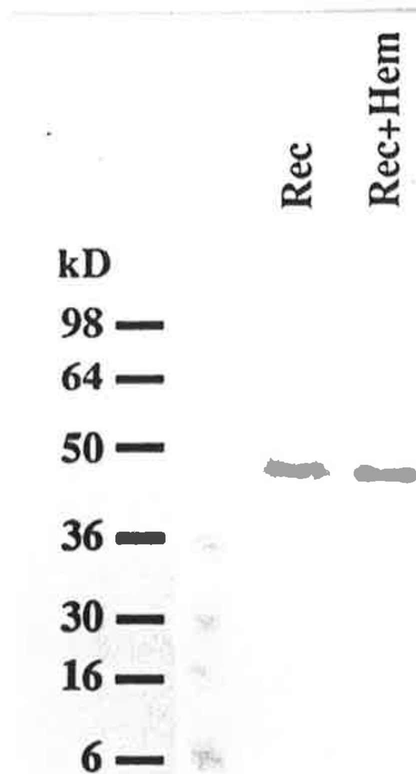


CHAPTER 6

Inhibition of phagocytosis by a polydnavirus encoded protein with an M-like protein homologous region

Chapter 6

Inhibition of phagocytosis by a polydnavirus encoded protein with an M-like protein homologous region

6.1. Introduction

Insect parasitoids employ a suite of strategies to ensure successful circumvention of their host immune responses, most importantly encapsulation. Factors introduced by the female wasp into the host hemocoel upon oviposition have been found to be the major source of components involved in the host immunosuppression. An elegant symbiotic association of certain hymenopterans with a group of viruses, known as polydnaviruses, which are produced in specialised calyx cells of the female reproductive organ have fascinating features in this regard (Stoltz and Vinson, 1979b). Polydnaviruses are injected into the host at oviposition where they enter various tissues and transcriptionally active, encoding a number of gene products. In the absence of the virus, the parasitoid egg is recognised as foreign, whereas in the presence of the virus the egg is protected from encapsulation (Edson *et al.*, 1981; Strand and Noda, 1991).

As shown previously, only one polydnavirus gene from *C. rubecula* (CrV1) is transiently expressed over 4-12h following parasitisation in hemocytes of *P. rapae* caterpillars (chapter 3 and 4). Actin microfilaments in virus-infected hemocytes were found to be disintegrated which was accompanied by the loss of normal spreading behaviour of the cells upon contact to a foreign surface. Infected hemocytes are also incapable of microparticle formation (chapter 3) under immune-related activation conditions (Theopold and Schmidt, 1997). In addition, using a recombinant 45kDa/CrV1 protein it was demonstrated that the hemocyte alterations following parasitisation are caused by CrV1 after the glycoprotein has been secreted into the hemolymph by polydnavirus-infected cells (chapter 5).

In parasitised caterpillars, the inactivation of cellular immune responses involve mainly the capability to encapsulate the egg of the parasitoid. However, one of the major hemocyte functions include phagocytosis of microorganisms. The question is therefore whether the immune suppression by the parasitoid is specific to encapsulation but leaves the capability of

hemocytes to undergo phagocytosis intact. Here, reduction of phagocytic activity in hemocytes from parasitised *P. rapae* caterpillars is described. A sequence homology of 45kDa/CrV1 protein with streptococcal M proteins which are known to inhibit phagocytosis of bacteria by human macrophages will be discussed as a possible analogy to the inhibition of phagocytosis in CrV1-affected hemocytes.

6.2. Materials and Methods

6.2.1. Labelling of the yeast cells

Yeast cells were labelled by FITC as described in the literature (Rohloff *et al.*, 1994). Briefly, *Saccharomyces cerevisiae* cells are grown 2-3 days at 25°C in 10 ml yeast culture medium (3 g yeast extract, 3 g malt extract, 10 g glucose, 5 g peptone, in 1 l total volume). One ml of this culture was centrifuged (200 g, 10 min) and the pellet was resuspended in 10 ml phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4, 0.2 g KCl dissolved in 1 l distilled water). The cells were washed three times with PBS (200 g, 10 min, 4°C), once with distilled water and autoclaved. The cells were centrifuged as above and resuspended in 10 ml carbonate-bicarbonate buffer, pH 9.4 (9.5 ml 0.2 M Na_2CO_3 mixed with 41.5 ml 0.2 M $NaHCO_3$). One mg FITC (Sigma) was added and shaken for 30 min at room temperature in the dark. The cells were washed again three times as described above. The pellet was resuspended in 2 ml Grace's insect medium (GIM, Gibco-BRL) and stored at -20°C.

6.2.2. Phagocytosis assay

P. rapae caterpillars were bled into 50 µl GIM on a microscope slide by removing one of the prolegs. Cells were allowed to settle for 10 min. The medium was removed and 50 µl containing 5×10^6 FITC-labelled yeast cells were added to each hemocyte monolayer. During phagocytosis, the slides were incubated at 31°C for 2h in a humid box. Then, the monolayers were rinsed three times with GIM to remove non-adherent yeast cells. Leaving 30 µl of medium, 70 µl of trypan blue (2mg/ml) was added to each monolayer and incubated for 10 min to quench uningested

yeast cells. The monolayers were rinsed with GIM, fixed with 4% formaldehyde in PBS and observed under indirect UV illumination.

6.2.3. Recombinant 45kDa/CrV1 protein

The recombinant protein was produced in a baculovirus expression system as described previously (Chapter 5).

6.2.4. Electrophoresis techniques

Protein samples were analysed on denaturing 12% SDS-polyacrylamide gels as described in chapter 2. SeeBlue™ pre-stained standard markers (Novex) were used to determine the molecular weights. The proteins were transferred onto a nitrocellulose membrane by Western blotting (chapter 2). An antiserum raised in goat against an oligopeptide from *S. pyogenes* serotype M80 M protein (KGETVPAHLWYYQKEENDKLK) was used as the first antibody (1:1000) and alkaline phosphatase-conjugated anti-goat IgG as the secondary antibody (1:5000, Sigma). The anti-M protein antibody was kindly provided by Dr. Goodfellow from Menzies School of Health Research, NT, Australia.

6.3. Results

6.3.1. Inhibition of phagocytic activity

When hemocyte monolayers of *P. rapae* were incubated with FITC-labelled yeast cells, a considerable number of cells were found to be engulfed by the hemocytes (Fig. 6-1B). In spite of using the whole population of hemocytes, comprising phagocytic and non-phagocytic cells, the number of hemocytes containing yeast cells always comprised about 40%. However, hemocytes from *P. rapae* caterpillars parasitised 18-24h previously were found to be incompetent to phagocytose yeast cells efficiently. Rarely, a few cells were found in monolayers from parasitised caterpillars containing a yeast cell comprising less than 1% (Fig. 6-1D).

6.3.2. 45kDa/CrV1 protein with a homologous region to M-like proteins

Using a computer based program for predicting the location of coiled-coil regions (Berger *et al.*, 1995), the deduced amino acid sequence of 45kDa/CrV1, which is transiently expressed in parasitised *Pieris* caterpillars encoded by a *C. rubecula* polydnavirus gene (CrV1) was analysed. The result showed that the protein contains a coiled-coil region from amino acid starting at about 170 to 230 (Fig. 6-2A). Sequence similarity analysis using National Centre for Biotechnology Information revealed that this region contains peptide sequences homologous to the M-like protein from *S. pyogenes* M80 (Fig. 6-2B) with 46% similarity and 29.6% identity. M proteins which are bacterial surface molecules have been found to confer the ability to group A streptococcal bacteria to resist phagocytosis by human phagocytes (Fischetti, 1991). In addition, amino acid sequence alignment of the coiled-coil region of CrV1 with other members of the so-called 525380 family of M proteins including M80 indicates conserved residues shared among different M protein sequences (Fig. 6-2C). This similarity is mainly to the hypervariable region and repeat regions A and B of the M-like proteins which constitute the antigenically variable region of the molecules.

To test whether 45kDa/CrV1 protein shares any antigenic similarity with M80, an antiserum raised against an oligopeptide synthesised on the basis of M80 amino acid sequence was used in a Western blot analysis. The result revealed that the anti-M80 antiserum recognises the 45kDa/CrV1 protein produced by recombinant baculoviruses indicating of antigenic similarity between the protein and M80 (Fig. 6-3).

6.4. Discussion

In vitro phagocytosis assays provided evidence indicating that hemocytes from *P. rapae* parasitised caterpillars fail to phagocytose yeast cells compared to unparasitised caterpillars which points to a disruption of the host cellular immune defence which includes the phagocytosis of microorganisms. As shown previously, a 45kDa/CrV1 protein encoded by *C. rubecula* polydnaviruses is responsible for the alterations observed in infected hemocytes

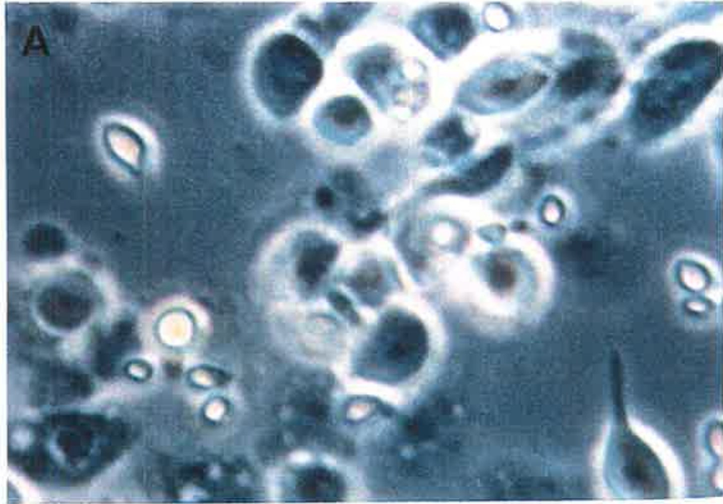
including breakdown of actin filaments and inhibition of immune-related surface changes (see chapter 5). This incapacitates hemocytes to form a capsule around the parasitoid egg. Our experiments demonstrate that this protein is probably also responsible for the inhibition of phagocytic activity in virus infected hemocytes.

Analysis of the deduced amino acid sequence of CrV1 revealed that the protein has a coiled coil region which shows sequence similarity to *S. pyogenes* M-like protein (M80). M and M-like proteins are cell surface molecules present on group-A streptococci with a fibrillar structure (Fischetti, 1991) extending from the bacterial cell membrane towards the outside with the N-terminus facing outside. The M proteins appear as dimers twisted about each other to form a coiled coil structure (Scott, 1990). This protein gives the bacteria the ability to resist ingestion by human phagocytes (Fischetti, 1991). There are three postulated mechanisms by which the M proteins may protect the streptococcal bacteria (Fischetti, 1991): 1) The N-terminus of the protein contains several negatively charged amino acids which results in a net negative charge for the region. The mammalian cells also exhibit a net negative charge on the surface. Therefore, the M proteins may prevent contact between the bacteria and the phagocytes by electrostatic repulsion. 2) The M proteins also exhibit a high antigenic variation in the N-terminus (hypervariable region), therefore, an antibody to one serotype would not prevent the other serotype. 3) Factor H which is a regulatory protein of the complement system in mammals, can bind specifically to the M protein. This prevents the binding of C3b, a component of the complement system involved in immune surveillance, to the bacteria which usually mark the pathogen as something to be killed and cleared from the body.

Serological experiments also indicates that the 45kDa/CrV1 protein has antigenic similarity with M80 implying a possible functional similarity. Whether the inhibition of phagocytic activity in infected hemocytes caused by CrV1 protein is related to its similarity to M proteins is not known and remains to be elucidated. One approach is to test whether CrV1 interacts with macrophages.

Figure 6-1

Micrograph of hemocyte monolayers after phagocytosis of FITC-labelled yeast cells. **A)** Hemocytes from unparasitised caterpillars under phase contrast; **B)** hemocytes from unparasitised caterpillars under indirect UV light showing phagocytic activity; **C)** hemocytes from 18h parasitised caterpillars under phase contrast; **D)** hemocytes from parasitised caterpillars under indirect UV illumination representing a dramatic decrease in phagocytic activity. The uningested yeast cells were quenched with trypan blue.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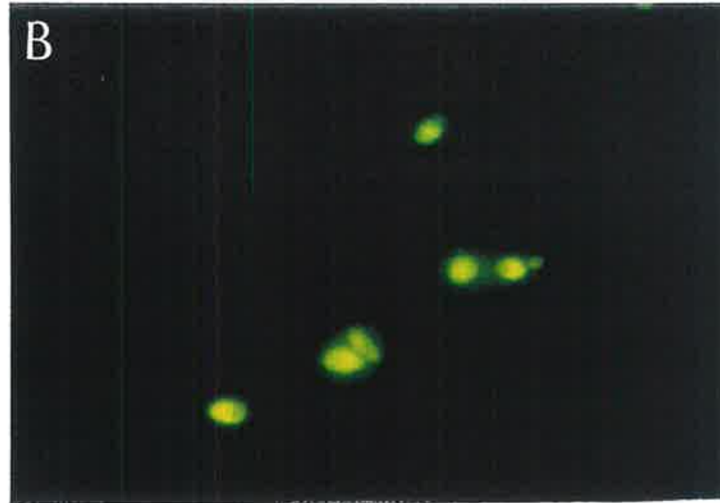
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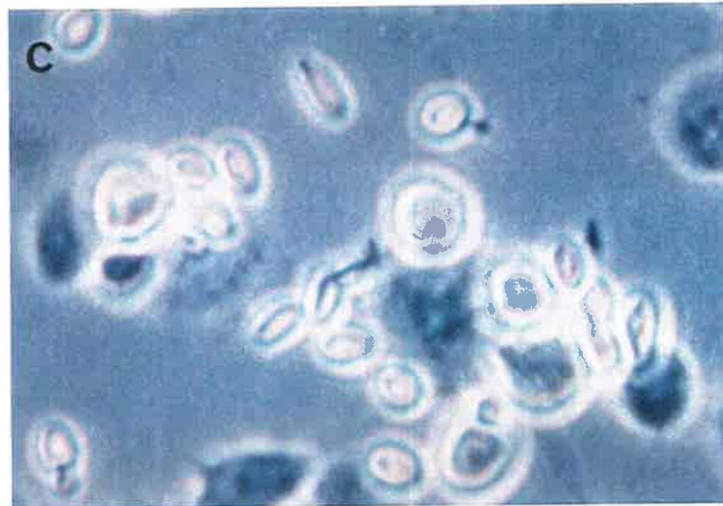
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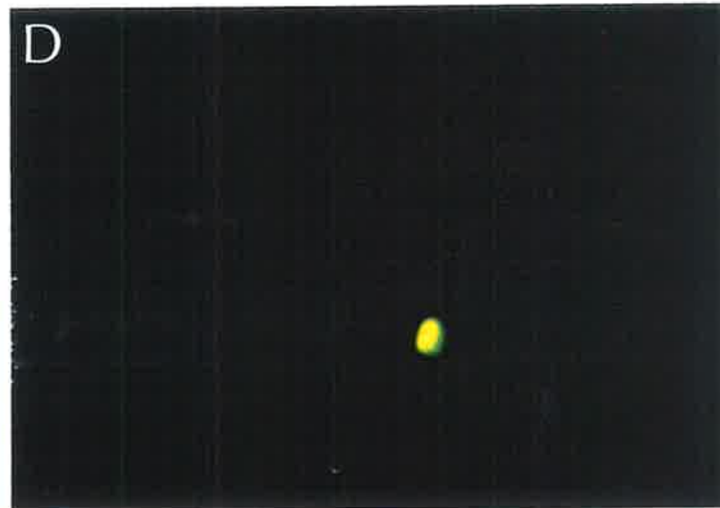
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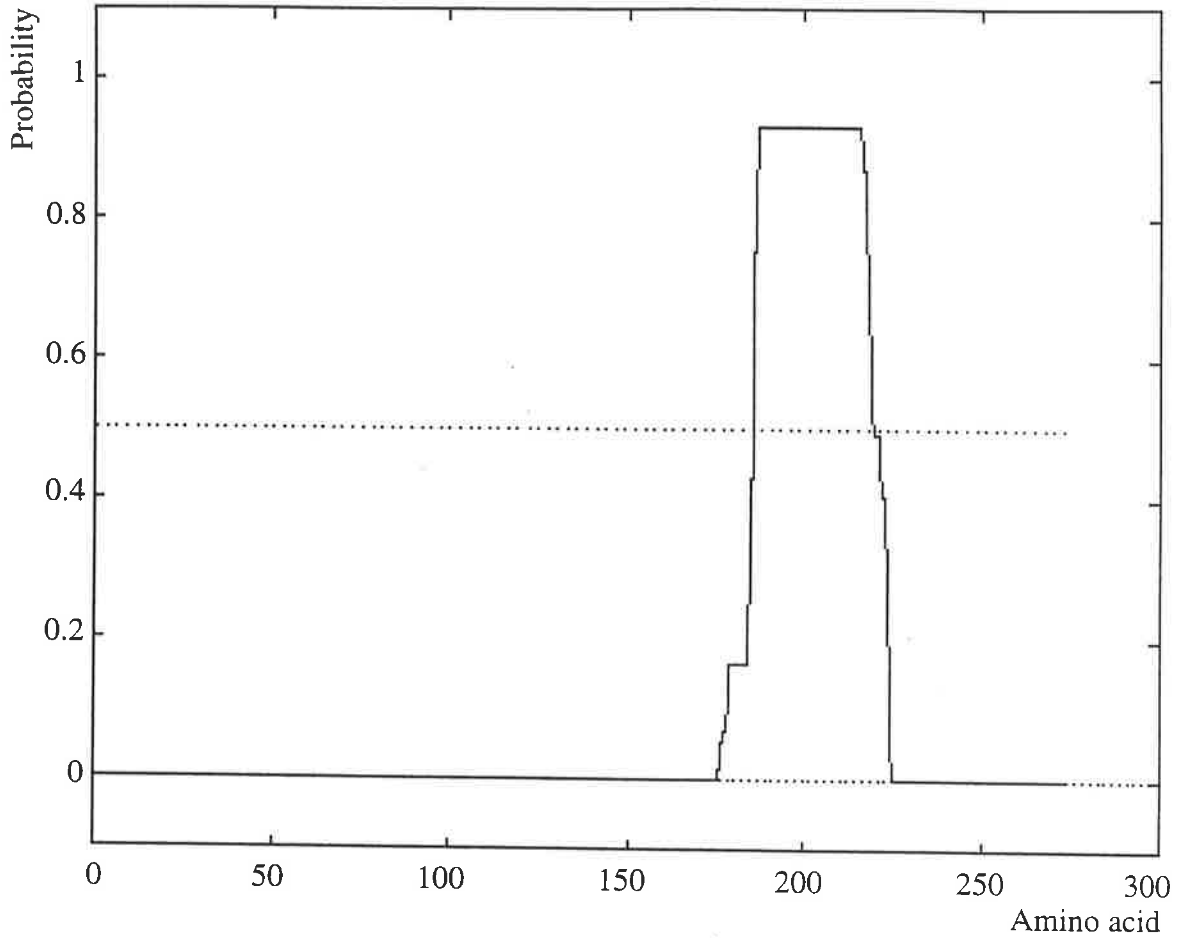
Figure 6-2

A) Prediction of the presence of a coiled-coil structure in CrV1 protein. A coiled-coil region from amino acid 160 to 230 is predicted.

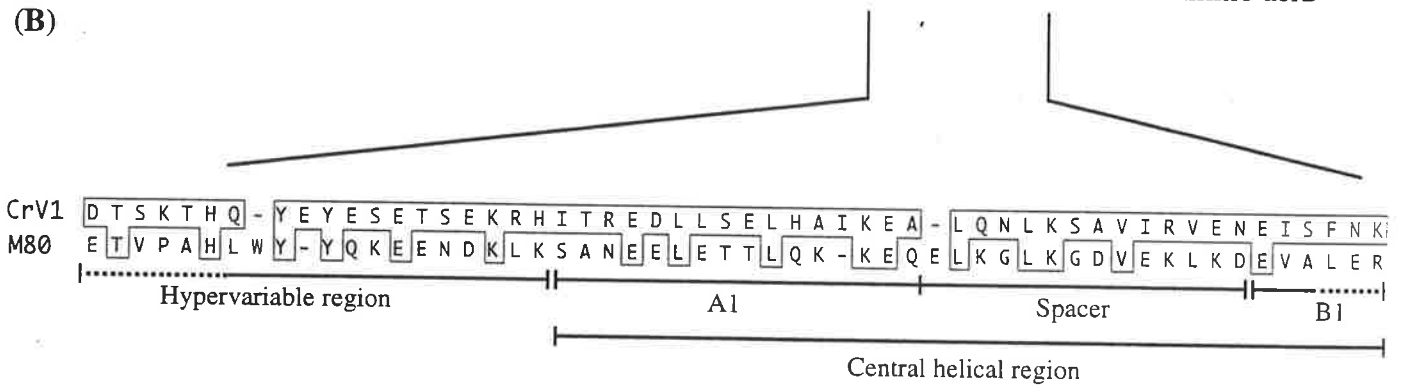
B) Alignment of the coiled-coil region of CrV1 protein with part of M-like protein from *S. pyogenes* (M80) that covers the N terminal hypervariable region and the central helical region. Identical residues are boxed. The dashes indicate gaps introduced in the sequence.

C) Alignment of the coiled-coil region of CrV1 with members of 525380 family of M-like proteins. The identical amino acids are boxed. The dashes indicate gaps introduced in the sequence. ML, M-like; ST, sequence type, M nontypeable serologically related to M53 and M52.

(A)



(B)



(C)

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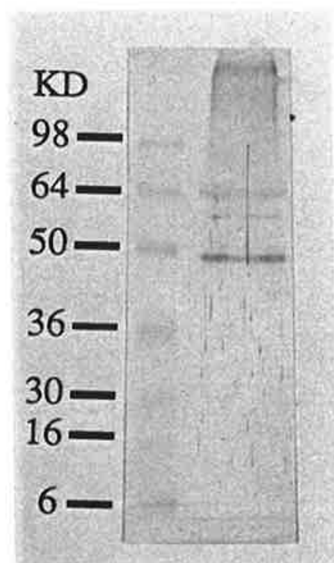
CrV1    D T S K T H Q Y E Y E S E T S E K R H I T R E D L L S E L H A I K E A - L Q N L K S A V I R V E N E I S F N K
ML80    E T V P A H L W Y Y Q K E E N D K L K S A N E E L E T T L Q K - K E Q E L K G L K G D V E K L K D E V A L E R
STNS5   F S V P G H V W A H E R E K N D K L S S E N E G L K A G L Q E - K E Q E L K N L K A D V E K L K D A A E L E R
STNS27  F S V P G N V W Y R Q H Q E I G K L K S E K E E L E T E L Q E - K E Q E L K N L K D N V E K L K D A A E L E R
ML52    R T V S A R A L L H E I N K N G Q L R S E N E E L K A D L Q K - K E Q E L K N L N D D V K K L N T E V E L E R
ML53    N L V R A E L W Y R Q I Q E N D Q L K L E N K G L K T D L R E - K E E E L Q G L K D D V E K L T A D P E L Q R
```

Figure 6-3

Western blotting of the recombinant 45kDa/CrV1 protein. The membrane was probed with an antibody raised against M80 peptide in goat. The antiserum recognised the protein indicating antigenic similarity between 45kDa/CrV1 protein and the M-like protein from *S. pyogenes* M80.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Chapter 7

Molecular cloning and characterisation of a protein involved in passive protection of *C. rubecula* eggs in the host

Chapter 7

Molecular cloning and characterisation of a protein involved in passive protection of *C. rubecula* eggs in the host

7.1. Introduction

It is known that most host insects react quickly to foreign objects that are introduced into the hemolymph (Götz and Boman, 1985; Ratcliffe, 1993). Thus, the time that is required for polydnaviral genes to be expressed inside host cells (Fleming *et al.*, 1983; Theilmann and Summers, 1986) and to change the immune status of the host might exceed the time it takes to encapsulate the egg of a parasitoid. It is therefore conceivable that some parasitic insects might have evolved alternative mechanisms which provide a short term protection at earlier stages of parasitisation while polydnaviruses provide a long term protection at later stages after parasitisation (Asgari and Schmidt, 1994; Davies and Vinson, 1986; Feddersen *et al.*, 1986).

As shown in previous chapters, polydnaviral genes from *C. rubecula* are expressed over a short period of time from 4 to 8 hours following parasitisation of *P. rapae* caterpillars from which a single gene (CrV1) seems to be responsible for most of the alterations in host hemocytes leading to their immune inactivation. This probably provides a protection at a subsequent stage when the parasitoid larva emerges. Hence, there should be a mechanism by which the parasitoid eggs are protected at a time period immediately following parasitisation. In this context, it was shown earlier that *C. rubecula* eggs are protected against the host defence reactions in the absence of polydnaviruses (Asgari and Schmidt, 1994). This was assumed to be due to a proteinaceous layer covering the egg surface. Here, the aim is to describe proteins which are likely to be involved in rendering the passive protection. Two calyx fluid proteins of 65 and 32 kDa in size are found on the surface of parasitic eggs and symbiotic polydnaviruses from which the 32 kDa protein was isolated and characterised at a molecular level. This work was done in collaboration with Dr. Ulrich Theopold and Mr. Craig Wellby. Peptide microsequencing was carried out by C. Wellby and the antibody screening of a bacterial expression library was accomplished in collaboration with U. Theopold.

7.2. Materials and Methods

7.2.1. Construction of a *C. rubecula* cDNA expression library

An expression cDNA library was constructed from female *C. rubecula* wasps by synthesising cDNA molecules from poly(A)+RNA isolated from the wasps and ligating into lambda gt11 arms. The procedure was as described in chapter 4 except the source of mRNA (*C. rubecula* wasp), the lambda vector (λgt11) and the host bacteria (Y1089 and Y1090).

7.2.2. Screening cDNA library

DNA sequences inserted into the *Eco*RI site can be expressed as fusion proteins under the control of the lac promoter. Therefore, recombinant DNA libraries in lambda gt11 can be screened with antibodies as well as with nucleic acid probes. Thus, the expression library was screened using anti-CrV antiserum.

1. Nitrocellulose filters were saturated with 10 mM IPTG and air dried.

2. An appropriate dilution of the library was made in phage buffer to produce 500 plaques per plate and added to an equal amount of Y1090 bacteria grown to OD_{600} of 0.6-0.8 and incubated at 37°C for 30 min.

3. Three ml molten (45°C) TB top agar (chapter 2) containing IPTG (7µl, 20 mg/ml) and X-Gal (7µl, 50 mg/ml) was added to above per plate, mixed quickly and poured onto LB plates prewarmed to 37°C. The agar was allowed to harden and the plates were incubated at 42°C for approximately 3.5h until plaques were pinpoint in size.

4. The filters were labelled and placed onto the plates and incubated at 37°C for at least 3.5h.

5. Plates were incubated at 4°C for 30 min to harden the agar and the orientation of the filters to the plates was recorded by a 20-gauge needle.

6. The filters were rinsed in TBST buffer (chapter 2) to remove any top agar and to block the filters.

7. The filters were screened using the anti-CrV antiserum (1:5000 in TBST) as a probe and detected by alkaline phosphatase-conjugated anti-rabbit IgG (1:10,000 in TBST) similar to developing a Western blot (chapter 2).

8. By aligning the filters with the plates, positive plaques were isolated using Pasteur pipets and released into phage buffer (SM, chapter 2) to allow the phages to diffuse out.

9. The phages were titrated and rescreened by the antiserum.

7.2.3. Phage lysate preparation

1. An overnight culture of Y1090 was prepared by inoculating 5ml LB medium containing 10 mM $MgSO_4$ and 0.2% maltose and shaking at 37°C.

2. From the overnight culture, 5 µl was added to a tube containing 20° µl of phage plug eluate (see above) and incubated at 37°C for 20 min.

3. The infected culture was transferred into a 250 ml flask containing 100 ml of prewarmed (37°C) LB medium supplemented with 1 ml of 1M $MgSO_4$ and shaken at 37°C until lysis occurred. After cell lysis, 500 µl chloroform was added.

4. The lysate was centrifuged at 8,000× g for 10 min to remove the cellular debris. The supernatant was transferred to a sterile tube and stored at 4°C for up to 6 months.

7.2.4. Isolation of phage DNA

Phage DNA was isolated from phage lysates using a kit for lambda DNA preparation (Qiagen) according to manufacturer's instructions.

7.2.5. Preparation of fusion protein

1. Y1089 cells were grown overnight at 37°C in LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$. The OD_{600} was read and cells were diluted to 1×10^8 cells/ml. An OD_{600} of 1.0 is equivalent to 8×10^8 cells/ml.

2. About 100 μl of the cells were infected with the phage elute (see above) at a multiplicity of infection of approximately 5 phage per cell and incubated at 32°C for 20 min.

3. The infected cells were diluted in LB medium supplemented with 10 mM $MgSO_4$ and plated on LB plates at a density of approximately 200 per plate and incubated at 32°C.

4. A few single colonies were tested as following. Using a sterile toothpick, a single colony was picked and streaked onto two LB plates. One plate was incubated at 32°C and the other at 42°C. Lysogens grew confluently at 32°C but spotty at 42°C.

5. About 100 μl of LB medium was inoculated with a single colony of the Y1089 recombinant lysogen and incubated at 32°C with good aeration until an OD_{600} of 0.5. The culture was transferred to 42°C and incubated for 20 min.

6. IPTG was added to a final concentration of 10 mM and incubated at 37°C for 60 min.

7. Cells were harvested immediately by centrifugation at 3,000× g for 5 min and frozen immediately.

9. Induced and uninduced cells were analysed on a Western blot for production of recombinant fusion protein.

7.2.6. Elution of antibodies from nitrocellulose blots

Antibodies were eluted from Western blots as previously described (Smith and Fisher, 1984). Briefly, the desired bands were excised from the blot after staining for alkaline phosphatase activity and transferred to tubes containing washing solution. Antibodies were eluted by three times washes with 0.5 mM glycine-HCl, pH 2.3, 1M NaCl, 0.5% (v/v) Tween 20, 100 μg/ml BSA. The elutes were immediately neutralised by adding Na_2PO_4 to a final concentration of 50 mM.

7.2.7. Cloning and sequencing

Lambda DNA isolated from selected recombinant phages was digested with *Kpn*I and *Sac*I restriction enzymes closest to the *Eco*RI sites flanking the insert and ligated into same sites in

pBluescript plasmid vector as described in chapter 2. The insert was sequenced by termination reaction in an automative sequencer (Applied Biosystems Sequencer) using λgt11 specific forward and reverse primers and six other primers which were designed on the basis of the preliminary sequence data to complete the sequencing.

7.2.8. Peptide microsequencing

Carried out by Craig Wellby as following:

1. Virus-free calyx fluid was run in aliquots on a 10% SDS-PAGE and the appropriate band (32kDa, Fig. 7-1B) was excised from the gel using a sharp, clean razor blade.

2. The gel slices containing the protein were transferred to an eppendorf tube containing 300µl of elution buffer comprising 50mM Tris-HCl/0.1% SDS, pH 8.0. The gel slices were thoroughly homogenised using a glass rod and the resulting mixture was incubated at 4°C overnight.

3. The gel matrix was spun down by centrifuging for 30 min at 2000× g and the supernatant containing eluted protein was collected. The remaining eluted protein was collected by washing the acrylamide pellet in 100µl of H_2O and adding the wash to the 300µl elution supernatant.

4. The 400µl extract was reduced to a volume of 50-200µl by lyphilisation on a speed-vac. The protein was then precipitated from solution by slowly adding ice-cold ethanol to a final concentration of 80% and centrifuging for 30 min at 13,000g.

5. The supernatant, containing salts and SDS, was removed until a volume of 10-100µl remains and the remaining solvent was removed by lyphilisation.

6. The protein was then redissolved in 50 µl of 50mM Tris-HCl/ 0.1%SDS.

7. The protein was reduced by adding β–mercaptoethanol to a concentration of 4mM and heating the solution at 60°C for 45 min. After the solution cooled to room temperature, reduced cysteine residues were alkylated by adding iodoacetamide in water to a final concentration of 50mM and incubating for 15 min in the dark.

8. To obtain optimal conditions for trypsin digestion, 50µl of solution comprising 50mM Tris-HCl pH7.2, 20% DMF and 4mM $CaCl_2$ was added to the protein solution.

9. Trypsin (TPCK) in water was added to attain a protease to protein ratio of 1:20. The digestion was carried out at 37°C for up to 44h and stopped by boiling for 5 min.

10. In preparation for HPLC, the sample was lyophilised, resuspended in 3M guanidine hydrochloride and incubated for 30 min at room temperature before centrifugation at 5000g for 10 min. The supernatant was collected without disturbing the pellet.

11. HPLC was carried out by loading 50µl of the sample onto HPLC column and the appropriate peptides were collected.

12. Peptides were sequenced on a Hewlett-Packard G1000A protein sequencer.

7.2.9. Elution of proteins from eggs

C. rubecula eggs collected from parasitised *P. rapae* caterpillars or from wasp ovaries were incubated in 1%SDS for 5 min. The supernatant containing the dissolved proteins from the eggs surface was collected.

7.2.10. General methods

The following methods were used as described in chapter 2:

1. Northern blotting

2. Western blotting

3. Isolation of polydnaviruses and viral DNA

4. Extraction of DNA from wasps

5. Southern blotting

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7.3. Results and discussion

7.3.1. Calyx fluid proteins on the virus and egg surface

It was shown previously that *C. rubecula* eggs are protected inside the host caterpillar, *P. rapae* in the absence of polydnaviruses (CrV) (Asgari and Schmidt, 1994). When parasitoid eggs were washed with a mild detergent and injected into *P. rapae* caterpillars, the eggs were found to be encapsulated. Although scanning electron microscopy showed no virus particles present on the egg surface, the proteinaceous protective layer was found to be antigenically related to virus proteins. When parasitoid eggs were incubated with the antiserum produced against CrVs and injected into naive caterpillars, they were found to be encapsulated by host hemocytes (Asgari and Schmidt, 1994). To explore whether virus-related proteins are attached to the parasitoid egg, surface eluted proteins from the egg surface were analysed on a Western blot using the anti-CrV antiserum as a probe. Interestingly, in addition to the 65 kDa which was previously identified in the calyx fluid and on CrVs, a 32 kDa protein was strongly recognised by the antiserum (Fig. 7-1A). The 32 kDa protein was also found abundantly on purified virus particles and in the virus-free calyx fluid (Fig. 7-1A,B). The 32 and 65 kDa proteins are possibly produced by calyx cells and are released into the lumen by secretion or cell lysis and cover the eggs which are passing through the lumen of the gland. This implies a possible role of the proteins in providing passive protection to the parasitoid eggs (Asgari and Schmidt, 1994) in conjunction with the 32 and 65 kDa proteins. However, the protective function needs further investigation. The presence of the protein on virus particles suggests that viruses may avoid the host immune system by a similar mechanism.

7.3.2. Molecular cloning of the 32 kDa coding cDNA

An expression cDNA library constructed from *C. rubecula* female wasps was screened using the anti-CrV antiserum as a probe. Two positive clones were selected and used as a probe in a Northern blot comprising RNA isolated from the wasp ovaries. Both probes strongly recognised a 1.5 kb RNA fragment and weakly a 1 kb fragment (Fig. 7-1C), indicating that the two clones are most likely identical. However, no hybridisation was detected in the RNA fraction isolated

from the carcass of the wasps devoid of ovaries (Fig. 7-1C). This suggests that the gene is specifically expressed in the ovaries and most likely in the calyx region.

To further investigate that the protein is produced in the ovaries, a fusion protein was produced by inducing a lambda lysogen containing the insert. The proteins were analysed on a Western blot using anti-CrV antiserum as a probe. A 32 kDa protein was only found in the induced fraction not present in uninduced fraction (Fig. 7-2A) similar in size to the protein on the egg surface (Fig. 7-1A). Antibodies specific to the 32 kDa protein eluted from a preparative Western blot containing several aliquots of the fusion protein was used as a probe on a Western blot comprising proteins from the wasp ovaries. The eluted antibodies recognised a 32 kDa protein (Fig. 7-2B) indicating that the protein is produced in the ovaries. In addition, the antibodies specific to the 32 kDa protein cross-react to a 34 kDa protein on the hemocytes isolated from naive *P. rapae* caterpillars (Fig. 7-2C) supporting the assumption that the 32 kDa protein covering the egg and CrVs is similar to a host protein. This suggests that a possible mechanism of protection is based on molecular mimicry.

To elucidate whether the gene which codes for the 32 kDa protein is incorporated in the CrV genome, a Southern blot analysis was performed in which the cDNA fragment coding for the protein was used as a probe. No hybridisation of the probe was detected with the viral DNA, however, the probe reacted with the genomic DNA from male wasps (Fig. 7-3). The result indicates that the 32 kDa protein specifically produced in calyx cells is not virus-encoded but rather a conserved protein that exists in the hemolymph of lepidopteran and hymenopteran species. In parasitoid wasps, it also appears to be synthesised as an ovarian product which is abundantly produced to confer protection to the eggs and possibly to polydnaviruses.

7.3.3. Deduced protein sequence

DNA sequencing of the cDNA fragment coding for the 32 kDa protein indicated that the transcript contains an open reading frame of 765 bp coding for a deduced protein of 253 amino acids (Fig. 7-4). A methionine codon at the beginning of the open reading frame was identified as a possible initiation codon (Cavener and Ray, 1991). However, sequence analysis of the cDNA clones obtained from screening the library revealed that another transcript of the gene

exists that has a different nucleotide at position -1, adenine instead of cytosine (Fig. 7-4). In invertebrates, cytosine is more conserved than adenine at position -1 and possibly provides a stronger initiation site (Cavener and Ray, 1991). The open reading frame was also confirmed by comparing the deduced protein sequence with amino acid sequences obtained from microsequencing of peptides after digestion of the protein (Fig. 7-4). The deduced protein sequence contains a transmembrane domain at the N-terminus with no cleavage site (Nakai and Kanehisa, 1992) (Fig. 7-4). This indicates that the 32 kDa protein is presumably a type II membrane protein (Heijne and Manoil, 1990) with a cytoplasmic tail from amino acid 1 to 8 (Fig. 7-4). The protein also contains several positively charged amino acids which provide an amphipathic α-helical structure for the protein (Fig. 7-5) characteristic of basic antibacterial peptides such as cecropin (Holak *et al.*, 1988). The deduced protein sequence also contains a common hyaluronan binding motif (Yang *et al.*, 1994) (Fig. 7-4). Hyaluronan is a glycosaminoglycan containing disaccharide units of N-acetyl glucosamine and glucuronic acid (Nelson *et al.*, 1995) which is present in the extracellular matrix and on cell surfaces and affect cell behaviour such as adhesion, motility and growth (Nelson *et al.*, 1995).

Using the data bank at National Centre for Biological Information, the deduced protein sequence shows similarity to surface proteins of microorganisms, such as lmp2 gene product from *Mycoplasma hominis* and M protein from *S. pyogenes* (M6). M and M-like proteins are known to confer protection to streptococcal bacteria against human phagocytes (see chapter 6).

In summary, the data indicate that the 32 kDa protein produced in the calyx region constitutes a major component coating the egg surface and is most probably involved in passive protection of the eggs. However, a possible active role for the protein cannot be excluded at this stage and needs further investigation.

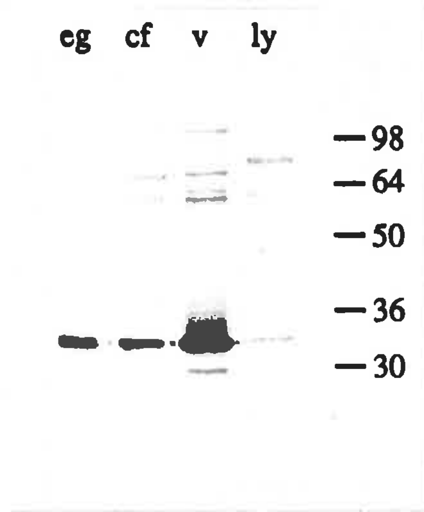
Figure 7-1

A) Western blot analysis of proteins from *C. rubecula* using anti-CrV antiserum as a probe. **eg)** Proteins eluted from eggs; **cf)** virus-free calyx fluid; **v)** polydnaviruses; and **ly)** a 32 kDa protein produced by recombinant phages. A 65 and a 32 kDa protein are discernible in eg, cf and v fractions. Molecular weight markers are indicated at right in kDa.

B) Purified CrVs analysed on a 10% SDS-PAGE. The 32 kDa protein (arrowhead) was excised from the gel and microsequenced. MW) molecular weight markers.

C) Northern blot analysis of RNA extracted from ovaries **(ov)** and the carcass **(car)** of *C. rubecula* female wasps. Similar amounts of RNA were loaded for each sample (2µg). The samples were probed with two cDNA clones (A and C) obtained from screening of the *C. rubecula* cDNA library. Both probes similarly hybridised to a 1.5 kb and a 1 kb RNA fragments in the ovary sample. MW) Molecular weight markers.

(A)



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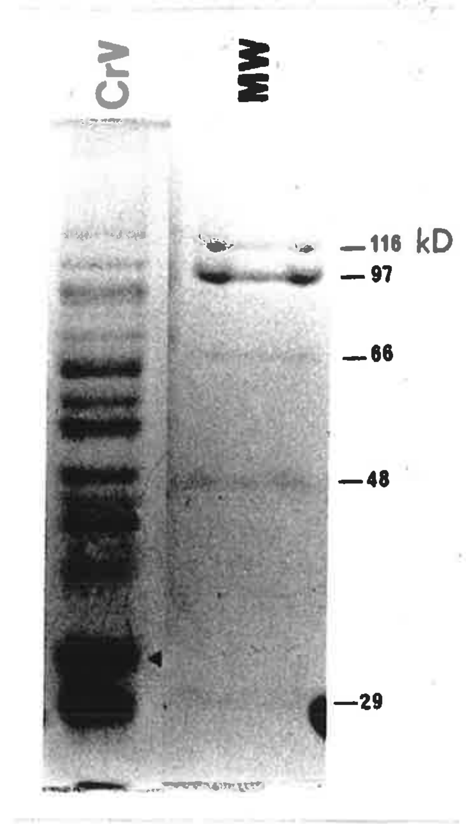


(C)

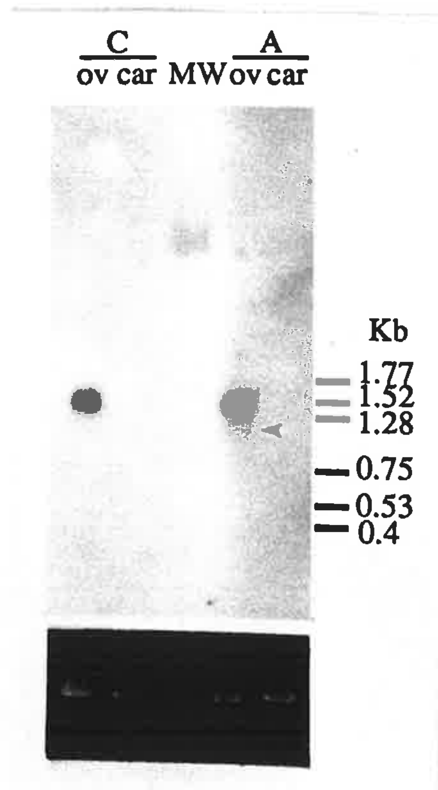


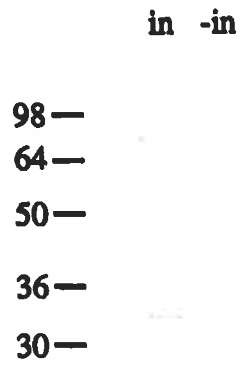
Figure 7-2

A) Western blot analysis of proteins from IPTG induced **(in)** and uninduced **(-in)** recombinant phages isolated from screening a *C. rubecula* expression cDNA library and probed with anti-CrV antiserum. A 32 kDa protein is only visible in the induced sample.

B) Western blot analysis of proteins from the ovaries of *C. rubecula* probed with antibodies eluted from the 32 kDa protein produced by recombinant phages (as in A, **Elu.AB**) and secondary antibodies **(2AB)**. A 32 kDa protein was recognised by the eluted specific antibody indicating that the isolated cDNA clone from the library codes for the 32 kDa protein detected on the eggs and in the calyx fluid (see figure 7-1A).

C) Western blot analysis of hemocytes **(Hc)** and hemolymph **(Hl)** from naive *P. rapae* caterpillars probed with antibodies specific to the 32 kDa protein **(elu)** and secondary antibodies **(2nd)**. A 34 kDa protein was recognised by the eluted antibodies in hemocytes.

(A)



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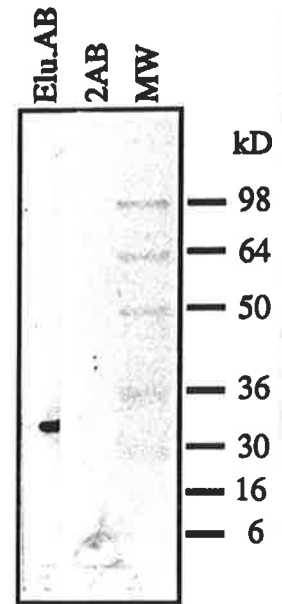

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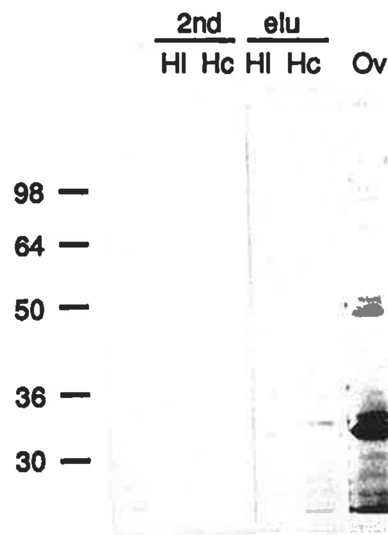
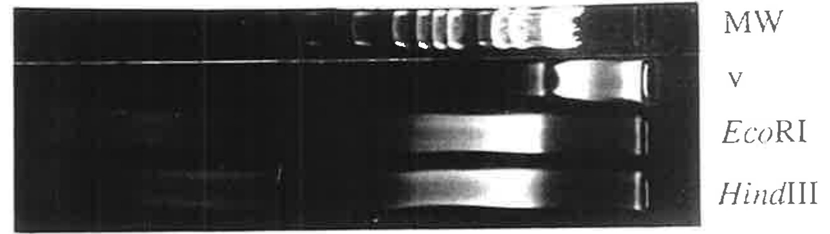


Figure 7-3

Southern blot analysis of genomic DNA isolated from CrVs (v) and *C. rubecula* male wasps digested with restriction enzymes (***Eco*RI**) and (***Hin*dIII**). The membrane was probed with ^{32}P-labelled cDNA fragment coding for the 32 kDa protein. The result indicates that the gene is not part of CrV genome but integrated in the wasp genome. The corresponding agarose gel micrograph is shown at left.


MW
v
*Eco*RI
*Hind*III

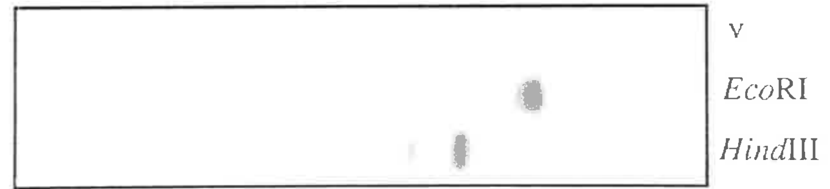

v
*Eco*RI
*Hind*III

Figure 7-4

DNA nucleotide sequence and deduced amino acid sequence of the cDNA fragment coding for the 32 kDa protein in the calyx glands of *C. rubecula*. The hydrophobic region at the N-terminus, representing a putative transmembrane region is boxed in grey and the cytoplasmic tail in blank. Amino acid sequences obtained from microsequencing of the protein are underlined. Two cDNA clones obtained from screening *C. rubecula* cDNA library were found different in the nucleotide at position -1 with respect to the initiation codon. The termination site at the end of the open reading frame is indicated by a dot. A putative hyaluronan binding motif is underlined by a dotted line.

ATT CCG TTG CTG TCG CAT ATT AAA TCA GAT GAG TTT ATT ATG ACC ACA TTA TAC TGC CGA CTT ATA CTA AAA
9 18 27 36 45 54 63 72

GGC CAA TTG CAT CTT CAC ATC TTT TTC CTG CCT CAT TTC CAT TTT CAT CAG CAT AAA TTG CGC AGT ATT CGG
81 90 99 108 117 126 135 144

TGA ATC GAT CAC AAT CGT CAT TAT CTT TAC ATG GAG CAT CAA TCA TTG CGT CCA CAG TAA CAA AAA TGA GCC
153 162 171 180 189 198 207 216

M D K K I I
CAC ACA GGA CGA TTA AAA AAA TAA ATA GTT CAT TGA AAC AAA CAT AAT TAA AAA/C ATG GAT AAG AAG ATA ATA
225 234 243 252 261 270 279 288

W S I I I A G V V V I A G A A I W G I W S L W K
TGG AGT ATA ATA ATT GCG GGC GTC GTC GTT ATA GCA GGC GCT GCA ATT TGG GGT ATA TGG TCT CTG TGG AAA
297 306 315 324 333 342 351 360

E F K D P K E E H K L K E E L N E A V E K A S A
GAA TTT AAA GAC CCA AAA GAG GAA CAC AAG CTG AAG GAG GAA CTT AAT GAG GCC GTT GAG AAA GCT TCA GCT
369 378 387 396 405 414 423 432

D A V D A F I T T K S D A F I G D K A K L D A V
GAT GCA GTA GAT GCA TTT ATT ACT ACA AAA TCA GAT GCG TTT ATA GGA GAC AAA GCA AAA TTA GAT GCA GTA
441 450 459 468 477 486 495 504

K T G A T I T I A E A D I T R R K T E L K N K Y
AAA ACT GGA GCA ACA ATA ACC ATT GCA GAA GCC GAC ATA ACC AGA AGA AAA ACA GAG TTA AAG AAT AAG TAT
513 522 531 540 549 558 567 576

D A G D Q A K I D N V D K A G G S K P T E A L D
GAC GCA GGG GAT CAA GCT AAA ATT GAT AAT GTT GAT AAA GCA GGC GGC AGT AAA CCA ACA GAA GCC CTA GAC
585 594 603 612 621 630 639 648

K A K A T A T T K I D E K A K E I A R K L I S Q
AAA GCT AAA GCC ACA GCT ACT ACC AAG ATA GAT GAA AAG GCG AAG GAG ATC GCG AGA AAA CTA ATA TCG CAA
657 666 675 684 693 702 711 720

K I K E K V S K K N E E A A K S A T A D D I S N
AAA ATT AAG GAG AAA GTA AGT AAG AAG AAT GAA GAA GCG GCA AAA TCT GCT ACA GCC GAT GAC ATT TCT AAT
729 738 747 756 765 774 783 792

L V K K G S S A D N A A K D K I I V K A K D A A
CTT GTT AAA AAA GGA TCA AGC GCT GAT AAC GCT GCC AAG GAT AAA ATC ATT GTC AAA GCT AAA GAT GCA GCT
801 810 819 828 837 846 955 864

G K K V N D I I S D T V W S E I E R A V Q T E G
GGA AAA AAA GTG AAT GAT ATT ATC AGT GAT ACA GTG TGG TCT GAG ATT GAG CGA GCC GTC CAA ACT GAA GGT
873 882 891 900 909 918 927 936

N E P L K S Q V A E I N K N K D T I I D D A I L
AAT GAA CCT CTA AAA AGC CAA GTG GCA GAA ATT AAT AAA AAT AAA GAC ACC ATT ATC GAC GAT GCT ATC CTA
945 954 963 972 981 990 999 1008

T V A G L P K K G .
ACT GTA GCC GGA CTG CCA AAA AAA GGC TAA TGC TTC GTG ATA TCG AAA AAA ATT AAA CAA AAT ATT GTA ATA
1017 1026 1035 1044 1053 1062 1071 1080

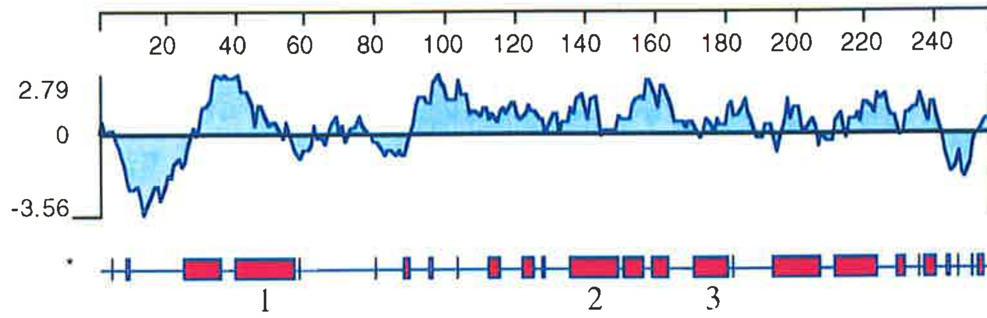
TTG ACA CGA CAT TTA TTA AAA TAG ACA TTG GTT ATA AAA CAG ATT TCA TTT AAT TTG ATA ATA TTT AAA AAC
1089 1098 1107 1116 1125 1134 1143 1152

GAT ACT TTT TAT CAG TTT GCA AAA AAC AAT CTC TTA ATG CTG GAA AGT CAT TGA TGA CCA TAA AAA TTA TCG
1161 1170 1179 1188 1197 1206 1215 1224

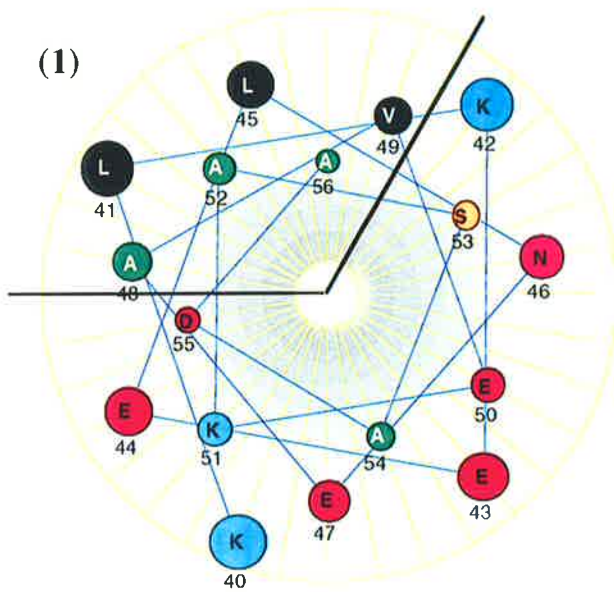
AGA AAT TAC AAT TTG ATA CCA TTA TTA AAA AAA AAA AAA AAA AAA AAA AA
1233 1242 1251 1260 1269

Figure 7-5

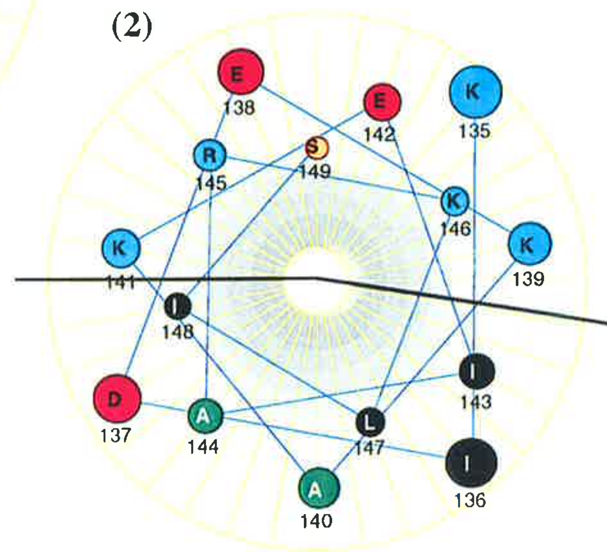
Hydrophobicity curve (top) and alpha amphipathic regions (below) of the coding region of the 32 kDa protein. The helical wheel of three selected helices (1, 2 and 3) are illustrated. The black line on helical wheels separates hydrophilic and hydrophobic sides. The curves and the helical wheels were produced by Lasergene software.



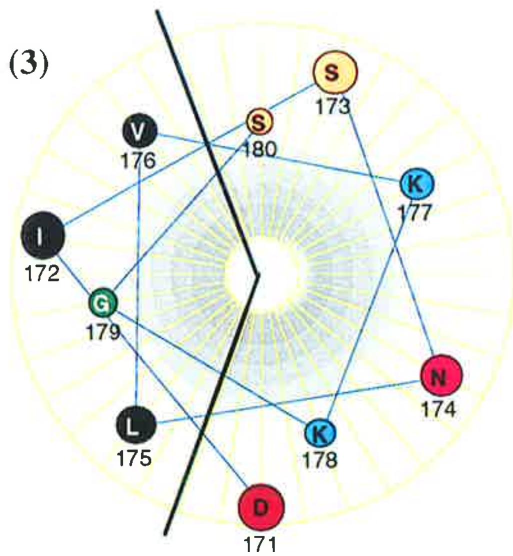
20
40
60
80
100
120
140
160
180
200
220
240
2.79
0
-3.56
1
2
3



(1)
L 45
V 49
K 42
L 41
A 52
A 56
S 53
A 48
N 46
D 55
E 50
E 44
K 51
A 54
E 43
E 47
K 40



(2)
E 138
E 142
K 135
R 145
S 149
K 146
K 141
K 139
I 148
I 143
D 137
A 144
L 147
I 136
A 140



(3)
S 173
V 176
S 180
K 177
I 172
G 179
N 174
L 175
K 178
D 171

CHAPTER 8

General discussion

Chapter 8

General discussion

To live inside another organism that is capable of launching a fatal immune attack, a parasite has to be competent to overcome its host defence reactions. Hymenopteran endoparasitoids which lay their eggs inside another insect and complete their larval stages in the host hemocoel have adopted effective mechanisms to counteract their host's immune response. Ovarian secretions introduced into the host upon oviposition are believed to be evolutionary adaptations involved in the manipulation of host physiology. The most interesting of all are polydnaviruses produced in the calyx region of the female reproductive organ and co-injected together with the egg into the host insect. Once inside the host, they enter various host tissues including hemocytes where specific parts of the segmented, circular DNA molecules of their genome are transcribed and the protein products are assumed to be responsible for a range of physiological alterations including immunesuppression. In some instances, polydnaviruses are accompanied by other factors produced by the female wasp such as venom and protein secretions from the calyx fluid which facilitate or accelerate their effects. The interaction between proteins that confer protection at different stages and the fact that in many cases multiple gene families of polydnaviruses are expressed in the host adds to the complexity which hampers attempts directed at understanding the mechanisms of host immune abrogation.

As demonstrated in several insects, the immune system is able to respond to the presence of a foreign object within minutes and take measures to destroy non-self objects. Therefore, a delay in protection caused by the expression of polydnavirus genes which usually occurs a few hours following parasitisation and the time it takes for the viral products to accumulate and reach the effective dose might endanger the survival of the developing embryo. In this regard, endoparasitoid wasps benefit from other maternal factors such as ovarian secretions accompanying polydnaviruses which are involved in early immunesuppression of the host (Luckhart and Webb, 1996; Webb and Luckhart, 1994) or ovarian materials covering the egg surface that provide passive protection (Davies and Vinson, 1986).

In the braconid parasitoid *C. rubecula*, passive protection of the egg surface against immune recognition (Asgari and Schmidt, 1994) is combined with an active abrogation of cell-mediated defence system of the host caterpillar, *P. rapae*. Parasitoid eggs devoid of virus particles (CrV) are protected when injected into the host, whereas eggs washed with a mild detergent or masked with anti-CrV antiserum are recognised as foreign and encapsulated (Asgari and Schmidt, 1994). Experimental data provided evidence indicating that the parasitoid eggs are covered with two proteins of 65 and 32 kDa in size. The 32kDa-specific antiserum cross-reacts with a protein of approximately 34 kDa on the hemocytes of naive *P. rapae* caterpillars indicating of a possible molecular mimicry. The 65 and 32 kDa proteins are abundantly produced in the calyx region and released into the lumen where they cover the egg surface while they pass the oviduct. Immunological studies show that the virus particles also contain both proteins on the surface which may give them the ability to escape the host immune reactions by passive means. A similar situation has been discovered in the ichneumonid *Venturia canescens* in which the parasitoid eggs are covered with protein particles that share antigenic similarities with a host component (Feddersen *et al.*, 1986). As a consequence, the parasitoid eggs escape the host immune defences by molecular mimicry.

Expression of the *C. rubecula* polydnavirus genome in hemocytes of parasitised *P. rapae* caterpillars appears to be restricted to a single RNA which is expressed in a highly transient fashion from 4 to 8 hours following parasitisation. This suggests that regulatory events initiated by the polydnavirus expression cause dramatic changes in the differentiation status leading to the temporary inactivation of these cells. This is in contrast to other known parasitoid systems, where a number of viral multigene families are transcribed in host cells and tissues (Summers and Dibhajj, 1995) and where most of the transcripts persist for a large portion of parasitoid development inside the host caterpillar (Strand *et al.*, 1992; Theilmann and Summers, 1986). After parasitisation of the host caterpillar, the initial protection of the *C. rubecula* egg against the host defence is achieved by a passive protection caused by a protein coating of the egg surface (Asgari and Schmidt, 1994). The hemocyte changes observed four hours post-parasitisation (p.p.) are probably caused exclusively by CrV encoded gene products, since hemocytes are not visibly altered. The virus-related hemocyte changes in parasitised caterpillars are only temporary

and disappear after 2-3 days p.p. At this time the emerged wasp larva appears to be protected against the recovered host defence by an unknown mechanism. This could indicate that the initial protective strategy of *C. rubecula* eggs and early larvae rely on two types of molecules, one providing passive protection against host immune recognition (Asgari and Schmidt, 1994), the other actively altering immune competence of host hemocytes in a transient fashion. Given that host hemocytes are probably changed by a short pulse of CrV1 gene activity, the *C. rubecula* endoparasitoid may constitute an ideal experimental system to study the transformation of hemocyte properties in the parasitised host.

Exposure of hemocytes to foreign objects, or microorganisms, causes cell membrane rearrangements (Gupta, 1991; Nappi and Silvers, 1984; Strand and Pech, 1995a), which exposes specific surface molecules and facilitate cell adhesion (Rizki and Rizki, 1983). The disappearance of cell surface glycoproteins recognised by the *H.p.* lectin was therefore used as a cellular read out for changes in the adhesive properties, since other lectin-binding properties, like wheat germ agglutinin did not change after parasitisation. In *D. melanogaster H.p.* lectin specifically binds to a mucin-like glycoprotein which is involved in immune induction of hemocytes (Theopold *et al.*, 1996). In *P. rapae* hemocytes, *H.p.* lectin binds predominantly to granulocytes, which in Lepidopterans are known to initiate the immune response (Ratcliffe, 1993). In granulocytes from parasitised caterpillars the lectin labelling changes between four and eight hours and has completely disappeared 24 hours p.p. The disappearance of lectin-labelling is probably due to inability of granulocytes to expose lectin binding molecules on the surface which coincides with changes in adhesive properties of the cells. Other hemocytes, like plasmatocytes, lack a mucin-like glycoprotein, but it is possible that surface properties in all hemocytes from parasitised caterpillars are changed, resulting in a dramatic shift of adhesive properties. Both granulocytes and plasmatocytes from parasitised caterpillars differ from non-parasitised hemocytes in the cytoplasmic structure of actin filaments, suggestive of a disruption of cell surface and cytoplasmic filament connections.

The presence of a signal peptide in the deduced amino acid sequence of CrV1 suggests that the protein is secreted. This was confirmed by expressing the protein in plasmid-transformed or recombinant baculovirus-infected Sf21 cells. However, the mode of interaction with cellular

proteins and the identity of possible cellular receptors for the CrV1 protein are not known at this stage.

Polydnavirus-infected caterpillars and caterpillars injected with recombinant CrV1 revealed similar hemocyte alterations which are consistent with an inhibition of immune-related activation processes. In parasitised caterpillars, the CrV1 glycoprotein is secreted into the hemolymph by polydnavirus infected hemocytes and fat body cells, where it interacts with the hemocyte surface. Moreover, CrV1 only shows its effects on hemocytes inside the caterpillar, but not when applied to isolated hemocytes implying the necessity of modifications in the protein. Removal of *H.p.* lectin binding sugars from the protein by hemolymph further supports this notion implying that the modification affects the terminal sugars of CrV1. A N-acetygalactosamindase activity which has been reported in insects (Vandie *et al.*, 1996) might be responsible for that modification. It is possible that CrV1 interacts with a specific receptor on the non-activated hemocyte surface or is internalised to function in the cytoplasm. The exact mode of interaction with the hemocyte remains to be elucidated.

Although nothing is known about the molecular reactions leading to hemocyte activation, these events are essential for the attachment, aggregation (Gupta, 1991; Ratcliffe, 1993) and coagulation reactions (Bohn, 1986), that constitute the cellular defence response against bacteria and parasitoids. Activation of hemocytes usually causes cell membrane rearrangements (Gupta, 1991; Nappi and Silvers, 1984; Theopold and Schmidt, 1997), which leads to exposure of specific surface molecules and facilitate cell adhesion (Rizki and Rizki, 1983). These changes may also include the discharge of cortical granules containing components resembling extracellular matrix components. The discharge and exposure of adhesive components probably enables hemocytes to attach to foreign surfaces. In addition, the formation of microparticles has been shown recently to be part of insect hemocyte activation (Theopold and Schmidt, 1997). Activated hemocytes form lamellopodia and cellular extensions leading to vesicle formation, which are similar to microparticles in activated mammalian blood cells (Yano *et al.*, 1994). A characteristic feature of particle formation is the exposure of phosphatidylserine (PS) on the outer leaflet of the cellular and particle membrane, which can be identified using annexin V as a diagnostic marker. The presence of PS is known to enhance coagulation processes in vertebrate

systems. Since both degranulation and microparticle formation involve a rearrangement of the cytoskeleton (Rosales *et al.*, 1994; Yano *et al.*, 1994), the absence of functional actin-filaments in CrV1-treated hemocytes is probably the primary cause of hemocyte inactivation. More specifically, the inactivation of actin-filaments by CrV1 probably precludes cellular activation reactions of hemocytes similar to cytochalasin D inactivation in vertebrate blood cells (Bengtsson *et al.*, 1993; Mooney *et al.*, 1995).

The CrV1-effects on hemocytes are reversible. After two to three days the hemocytes have virtually returned to normal activation behaviour, both in parasitised caterpillars as well as in caterpillars injected with recombinant CrV1-protein. In contrast to other systems, no evidence for cell-death (Rizki and Rizki, 1990) or apoptosis (Strand and Pech, 1995b) of hemocytes are observed in *P. rapae* caterpillars recovering from the PV and CrV1-mediated effects, as judged by Hoechst-staining and annexin V-labelling. This and direct observations of hemocytes at various time periods after CrV1-application indicate that hemocytes gradually recover from CrV1-mediated alterations, suggesting that the CrV1-induced hemocyte changes are reversible. Since CrV1-mediated inactivation of hemocytes is transient and probably affects specific immune functions, related to cell-mediated defence reactions, the protein has the potential to become a useful tool in basic and applied research. Since insects respond to viral infections such as nuclear polyhedrosis virus by recognising and destroying infected hemocytes (Washburn *et al.*, 1996), CrV1 or similar genes from polydnaviruses can be used for constructing genetically engineered entomopathogenic viruses to generate efficacious biological control agents.

Polydnavirus infected hemocytes do not attach to each other and no phagocytosis activity is discernible in these hemocytes. Sequence analysis of CrV1 protein indicates that the protein contains a coiled-coil region which shows similarity to M proteins. These proteins which are surface molecules of group A streptococcal bacteria have also a coiled-coil structure and protect the bacteria from being phagocytosed by human macrophages. Whether the inhibition of phagocytosis induced by CrV1 in parasitised *P. rapae* caterpillars is similar to the mechanism by which M proteins inhibit phagocytosis in macrophages remains to be elucidated.

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